KUVEMPU UNIVERSITY

INVESTIGATION OF NOVEL QUINOLINE HETEROCYCLES AND SOME SELECTED MEDICINAL PLANTS FOR VARIOUS PHARMACOLOGICAL ACTIVITIES

Thesis Submitted to the Faculty of Science, Kuvempu University
for the award of the Degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

Submitted By

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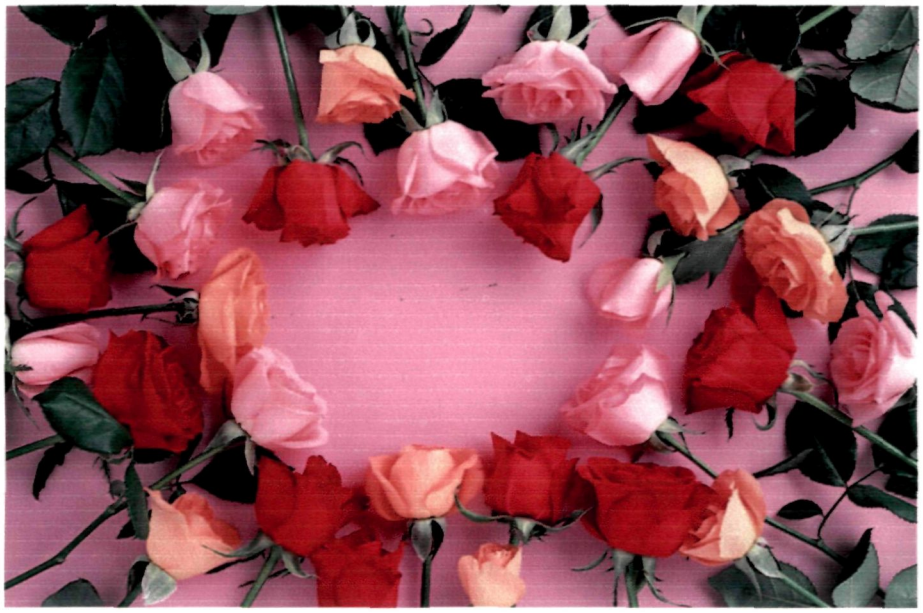
Dedicated to My Beloved

Parents and Well wishers



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Declaration

I hereby declare that the Ph.D., thesis entitled **"Investigation of Novel Quinoline Heterocycles and Some Selected Medicinal Plants for Various Pharmacological Activities"** embodies the results of my investigation and this has been composed by me under the supervision of **Dr. K.M. Mahadevan,** Sr. Lecturer, Department of Chemistry, Kuvempu University, Jnana Sahyadri, Shankaraghatta-577451, Shimoga.

I further declare that the results presented in the thesis or any part thereof has not been submitted elsewhere for any other degree, diploma of similar title of any other Universities.

Date: 12/5/2009

Place : **Shankaraghatta**

12/5/09

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Certificate

This is to certify that the work reported in this thesis entitled **"Investigation of Novel Quinoline Heterocycles and Some Selected Medicinal Plants for Various Pharmacological Activities"** submitted by **Mr. Prasanna V. Habbu** to the Faculty of Science, Kuvempu University, for the award of **Doctor of Philosophy in Chemistry** is a record of the bonafide and original research work carried out by him under my guidance and direct supervision. The work reported in this thesis has not formed the basis for the award of any degree or diploma or any other similar title.

Date: 12/05/2009

Place : **Shankaraghatta**

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Dr. K.M. MAHADEVAN

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

^{1}H NMR	Proton Nuclear Magnetic resonance spectroscopy
$BiCl_3$	Bismuth (III) chloride
TLC	Thin Layer Chromatography
Na_2SO_4	Sodium sulphate
MES	Maximal Electric Shock
PTZ	Pentelyntetrazol
ANOVA	Analysis of Variance
SGPT	Serum Glutamate Pyruvate Transaminase
SGOT	Serum Glutamate Oxaloacetate Transaminase
DNPH	Di-Nitro Phenyl Hydrazine
ALP	Alkaline Phasphatase
CCl_4	Carbon tetra chloride
IR	Infra Red spectrum
^{13}C NMR	Carbon Nuclear Magnetic resesonance
$CDCl_3$	Duterated Chloroform
KBr	Potassium Bromide
LCMS	Liquid chromatography Mass spectroscopy
NVP	with *N*-vinyl pyrrolidin-2-one
npa	4-nitro phthalic acid
TMS	Trimethyl Silane
GLC	Gas Liquid Chromatography
PEAS	Petroleum Ether fraction of *Argyreia speciosa*
CAS	Chloroform fraction of *Argyreia speciosa*
EAAS	Ethyl Acetate fraction of *Argyreia speciosa*
EtAS	Ethanol (95%) fraction of *Argyreia speciosa*
AQAS	Aqueous fraction of *Argyreia speciosa*
ROS	Reactive Oxygen Species
CYP2E1	Cytochrome 250 (2E1)
NBT	Niroblue Tetrazolonium

EDTA	Ethylene diamine tetra acetate
SOD	Super oxide dismutase
KCl	Potassium Chloride
H_2O_2	Hydrogen Peroxide
KI	Potassium Iodide
TL	Transfer Latency
ELT	Escape Latency Time
TSQT	Time spent in the Target Quadrants
EPM	Elevated plus maze
AChE	Acetylcholinesterase
DTNB	5, 5'-dinitrobenzoic acid
OD	Optical Density
AS	Acute immobilizaton Stress
CS	Chronic immobilization Stress
ALT	Alanine aminotransferase
AST	Asphartate aminotransferase
CK	creatinine kinase
WS	Standardized *Withania somnifera* extract
DPPH	1,1 diphenyl 2-picryzyl radical
TCA	Trichloro acetic acid
TBA	Thiobarbituric acid
LPO	Lipid Peroxidation assay
MI	Myocardial infraction
IHD	Ischemic Heart disease
ISO	Isoproterenol
Na-CMC	Sodium Carboxy methyl cellulose
ECG	Electrocardiogram
LDH	Lactate dehydrogenase
TG	Triglycerides
TC	Total Cholesterol

H & E stain	Haematoxyline and Eosin stain
IDDM	Insulin dependent diabetes mellitus
NIDDM	Non-insulin dependent diabetes mellitus
STZ	Streptozotocin
GLB	Glibenclamide
DC	Diabetic control
HDL-c	High density lipoproteins
HOMA	Homeostatic Model Assessment
VLDL	Very low density lipoprotein
$AUC_{glucose}$	Area under curve for glucose
OSTT	Oral Sucrose Tolerance Test
OGTT	Oral Glucose Tolerance Test
ITT	Insulin tolerance test
DMSO	Dimethyl sulfoxide
NP reagent	Natural product reagent
MIC	Minimum inhibitory concentration
FIC	Fraction inhibitory concentration
PBS	Phosphate buffer
PEHI	Petroleum Ether fraction of *Habenaria intermedia*
CHI	Chloroform fraction of *Habenaria intermedia*
EAHI	Ethyl acetate fraction of *Habenaria intermedia*
EtHI	Ethanol (95%) fraction of *Habenaria intermedia*

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Introduction to Tetrahydroquinolines

Chapter - 1

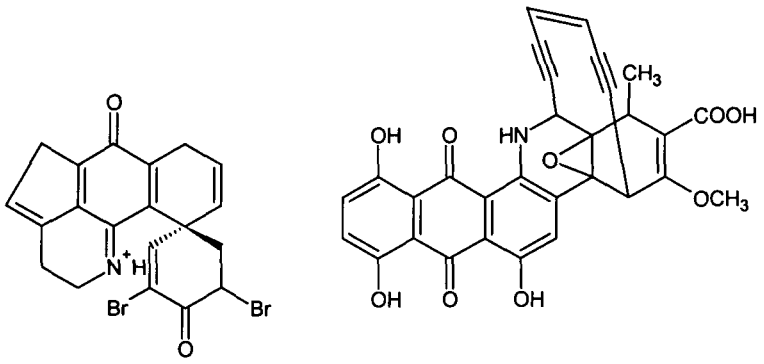
Introduction to Tetrahydroquinolines

1.1. Tetrahydroquinolines as Natural and Pharmaceutical Products

The greatest interest in 1,2,3,4-tetrahydroquinolines is due to their biological activities. Several of these compounds are naturally occurring. 2-Methyl-1,2,3,4-tetrahydroquinoline is present in human brain. **Discohabdin C (1)** , a polycyclic system based on tetrahdyroquinoline having antibiotic and cytotoxic activity and influencing sea star (*Perknaster fuscus*) feeding behavior were isolated from the Antarctic sponge *Latrunculia apicalis* Ridley and Dendy from McMurdo Sound[1-6]. **Dynemicin A (2)**, an enediyne class of antibiotic, is a potent antibacterial and antitumor agent recently isolated from *Micromonospora chersina* and from *Micromonospora globosa*[7]. It is a hybrid containing enediyne and anthraquinone. There are three species which comprise the tropical genus, *Martinella* (Bignoniaceae), *M. iquitosensis* and *M. obovata.* The use of *Martinella* as eye medication by Amazon Indian tribes has been reported[8]. According to folklore, juice obtained from the bark of M. *obovata,* when administered to the eye, has an immediate effect on inflammation and will eventually cure conjunctivitis. In 1995, Witherup and coworkers successfully isolated and characterized two biologically active compounds from the roots of *Martinella iquitosensis,* **Martinelline (3)** and **Martinellic acid (4)**[9].

Biological assays and in *vitro* antagonist studies[10] were performed to determine the properties of (**3**) and (**4**). Martinelline was found to inhibit the binding of radioligands to bradykinin B_1 and B_2, muscarinic and α_1-adrenergic receptors. As well, organ bath assays with (**3**) indicated histaminergic receptor antagonism. Inflammation properties of (**3**) would be expected *in vivo* due to the inhibition of bradykinin and histaminergic

receptor systems, which mediate inflammatory reactions. In addition, bradykinin has potent nociceptive activity which suggests that antagonism of this receptor system would result in (**3**), possessed antibiotic activity. The combined effects of analgesia, antibiotic activity and control of inflammation provides the potential for (**3**) to have therapeutic value in the treatment of ailments including conjunctivitis.



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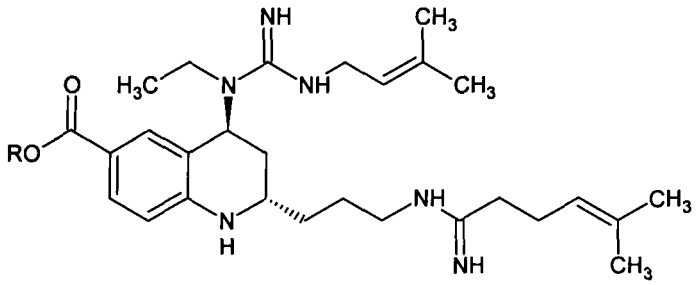
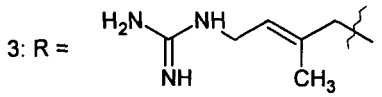


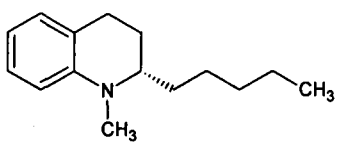


3: R =

4: R = H

Angustureine (5), galipeine (6), galipinine (7) and cuspareine (8) are the four 2-substituted 1-Methyltetrahydroquinoline alkaloids isolated from the bark of *Galipea officinalis* Hancock by Ingrid Jacquemond-Collet *et al.* in 1999[11]. *Galipea officinalis* Hancock is a shrub indigenous to the mountains of Venezuela that has marked influence on the spinal motor nerves, thus, serves a cure for paralytic affections.[61] Further biological testing indicated that these four compounds possessed promising *in vitro*

antimalarial activity for the chloroquine-resistant and chloroquine-resistant strains[12]. Therefore, these alkaloids can serve as new leads for development of new antimalarial drugs. *Plasmodium falciparum*, with galipinine being the most active compound. The same four alkaloids were cytotoxic towards human fibroblast cells, but in this case cuspareine was the most effective. Zhou and his co-workers have reported the first synthesis and stereochemical assignments of (-)-angustureine **(5)**, (-)-galipinine **(7)** and (-)-cuspareine **(8)** by using their newly developed enantioselective hydrogenation of substituted quinolines[13]. Hartmut Laatsch *et al.* has shown that the ethyl acetate extract of cultures of *Janibacter limosus* showed a high biological activity against bacteria, and fungi and delivered two new natural products, a tetrahydroquinoline derivative designated as **helquinoline (9)**, and the *N*-acetylkynuramine along with other known secondary metabolites[14]. The isolated helquinoline showed potent activity against *Bacillus subtilis*, *Streptomyces viridochromogenes* Tü 57 and *Staphylococcus aureus*.



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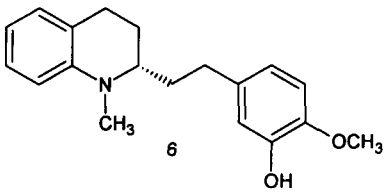

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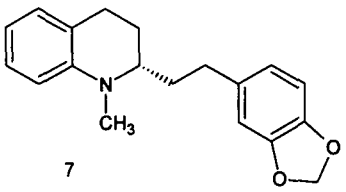

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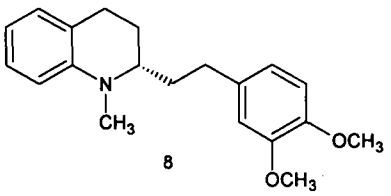

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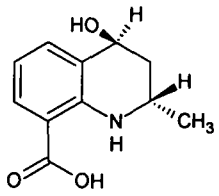

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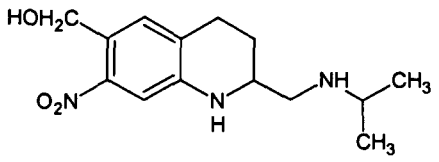
Many relatively simple synthetic of 1,2,3,4-tetrahydroquinolines are already used or have been tested as potential drugs. Among them, **oxamniquine (10)**, a schistosomicide[15-20], **nicainoprol (11)**, an antiarrhytmic drug[21], and **virantmycin (12)** a novel antibiotic [22-24] are the best known. **RG-120499 (13)** is most potent ecdysone receptor mutant. Tetrahydroquinoline **L-6X9.560 (14)** is one of the most potent NMDA antagonists yet found[25-32]. The compounds of the type **(15)** inhibits *Pf*-PFT enzymatic activity *in vitro* with an IC_{50} (concentration that causes 50% enzyme inhibition) of 0.5 nM and arrests the growth of *P. Falciparum* in human red cells with an ED_{50} (concentration that causes 50% inhibition of growth) of 15 nM[33,34].

Researchers at Pfizer Co. have identified a completely distinct but related series of 4-amino-substituted 1,2,3,4-tetrahydroquinoline carbamates where the partial saturation occurs in the heterocyclic ring rather than the carbocyclic ring found in the Bayer leads.

Optimization of this series led to an exciting new potent (IC_{50}) 0.05 *i*M, human plasma) chiral CETP inhibitor, **(16)** (torcetrapib, CP-529,414), for clinical development. The discovery and early optimization of torcetrapib have been reported [35] starting from the initial 6,7-dimethoxy-4-aminotetrahydroquinoline [36] lead **(17)** that had micromolar potency (IC_{50}) 10 *i*M) under buffered conditions and retained its activity in the presence of human plasma (IC_{50}) 25 *i*M)[35]. Additional biochemical studies indicate that torcetrapib is a potent, reversible inhibitor of CETP and that the binding of **(16)** to CETP enhances its interaction with HDL[35]. Patent applications describing processes to isolate anhydrous torcetrapib have also appeared, including the conditions required to isolate single crystalline, polymorphic forms[37].



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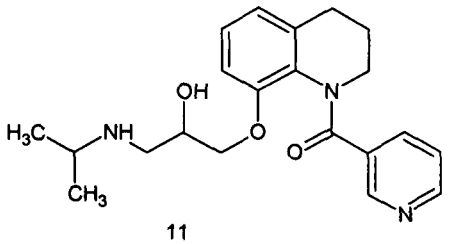

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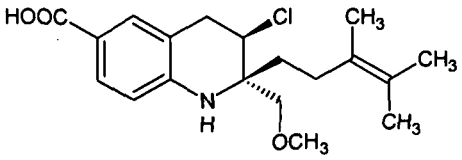

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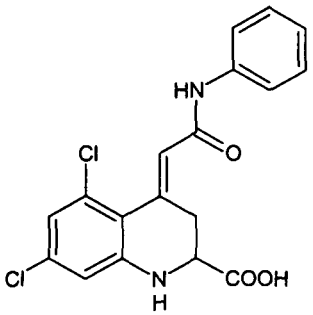

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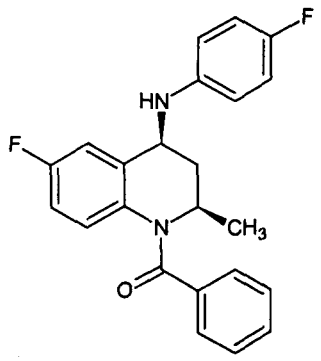

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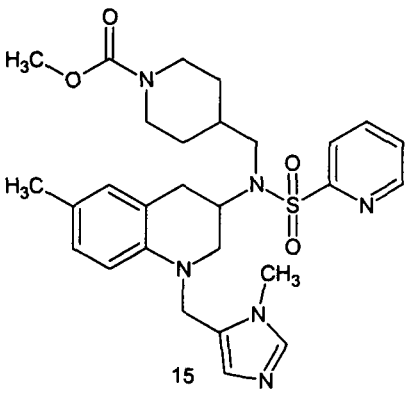

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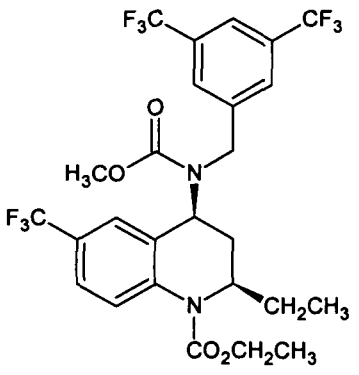
The positioning of the 2*R*-methyl substituent in **(17)** was preferred for activity over the corresponding 2*S*-methyl enantiomer **(18)**. In contrast to the geminal 2,2-dimethyl group as in **(19)** significantly reduced activity in the Pfizer series. Overall activity was relatively insensitive to changes in either of the carbamate ester groups. In contrast, the introduction of one or more trifluoromethyl substituents into the benzylic ring of **(17)** significantly increased potency. The monotrifluoromethyl analogue **(20)** was approximately 20-fold more potent than **(17)**, and the **3,5-bistrifluoromethyl** derivative **(21)** was about 100-fold more potent than **(17)**[35].



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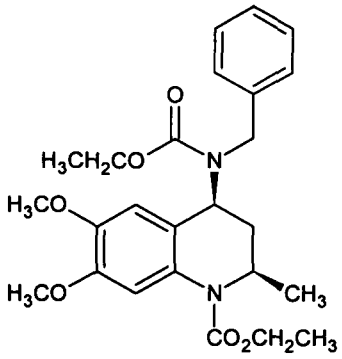

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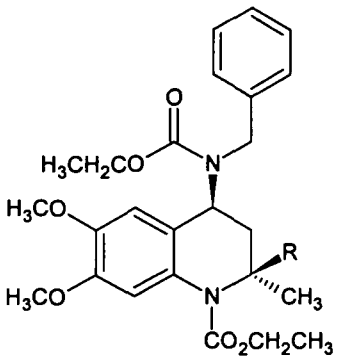

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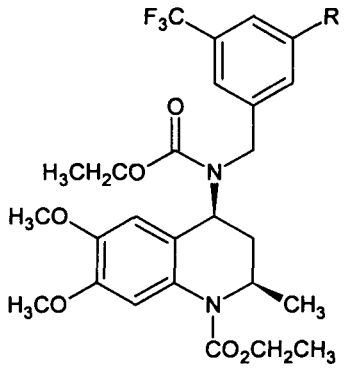
19 R = CH_3



20 R = H
21 R = CF_3

Hundreds of tetrahydroquinolines bearing various simple or complex substituents have interesting biochemical activity; some are potential pharmaceutical agents. Thus, a very simple derivative, 2-methyl-5-hydroxy-1,2,3,4-tetrahydroquinoline, exhibits analgestic activity one eighth as potent as morphine[38]. 1,2,3,4-Tetrahydroquinoline-4-carboxylic acid is used in tissue irrigating solutions[39]. Some tetrahydroquinolines are potent inhibitors of (H^++K^+) adenosine triphosphatase[40], blood serum monoamine oxidase[41], angiotensin-I converting enzyme[42], lipoxygenase[43], lipid peroxidation[44], bone resorption[45], leukotriene synthesis[46,47] and bacterial dihydrofolate reductase[48]. Other

tetrahydroquinolines are antagonists of vasopresin[49], adrenergic cl,-receptor[50], and calcium[51], or agonists of dopamine D_2[52,53]. Tetrahydroquinolines are potential antidepressants[54], nervous system depressants[55], potent antiulcer[56,57], cardiovascular[58], positive inotropic[59], antithrombotic[60,61], antiarrhytmic[62], antiallergenic[63], antitumor[64], antirheumatic[65], immunosuppressant[66,67], anticonvulsant, [68] or antifertility[69] agents. Some tetrahydroquinolines are recognized as high affinity ligands at the glycine site of the NMDA receptor[70], other as facilitators of noradrenergic transmissions[71,72], or myotilament sensitizers without affecting cell Ca^{++} loading[73]. In this group are also promising drugs for the treatment of cerebral ischemia[74] and osteoporosis[75]

1.2. Other Applications of Tetrahydroquinolines

Besides pharmaceutical applications, tetrahydroquinoline derivatives are useful as pesticides[80], antioxidants[81-84], and corrosion inhibitors[85]. 2,2,4-Trimethyl-5-hydroxy-1,2,3,4-tetrahydroquinoline is a specific reagent for photometric determination of iron(III) salts[86]. Tetrahydroquinolines are use as active components in various types of dyes: for hair[87], acrylic fibers[88,89], for polyesters[90,91], and for polyamides[92,93]. (2s)-2,6-Dimethyl-1,2,3,4-tetrahydroquinoline was used in synthesis of chinal polymethine dyes with interesting optical properties[94].

Tetrahydroquinoline derivatives are also widely used in modern recording technologies: as charge-transporting agents for electro photographic photoconductors[95-101], as leuco dyes for thermal and pressure sensitive materials[101-106], as antiirradiation filter dyes in photography[107,108], in the preparation of optical information recording media[109,110], as intermediates for photographic couplers[111], as dyes for colored electrostatographic toners[112], and as high sensitivity photosensitizers in photography[113].

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Chapter - 2

Synthesis, Characterisation of 2-phenyl-1,2,3,4-tetrahydroquinolines and 2-phenylquinolines and Screening for Hepatoprotective and Anticonvulsant Activities

Chapter - 2

Synthesis, Characterisation of 2-phenyl-1,2,3,4-tetrahydroquinolines and 2-phenylquinolines and Screening for Hepatoprotective and Anticonvulsant Activities

2.1. Introduction

The structural core of quinoline has generally been synthesized by various conventional named reactions such as Skraup[1], Doebner-von Miller[2], Friedländer[3], Pfitzinger[4], Conrad-Limpach[5] and Combes[6] syntheses. These classical syntheses are well-known and frequently used for the preparation of pharmaceutical agents, ligands and functional materials bearing a quinoline backbone. However, Imines current methods for quinoline synthesis often do not allow for adequate diversity and substitution on the quinoline ring system[7]. Recent developments in the chemistry of quinoline derivatives have demonstrated that new metal-catalyzed coupling cyclizations or acid catalyzed cycloaddition of appropriate precursors could compete with classical synthesis in the efficacy and rapidity of the quinoline construction[8-15]. These new developments have prompted us to explore novel methods and modifications of existing reactions in synthesising of substituted quinolines utilising $BiCl_3$ as an efficient catalyst.

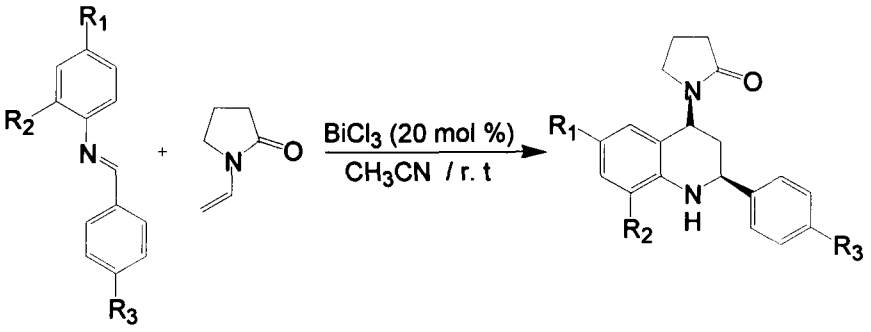
In this context, we are interested in the design of new methodologies to prepare 4-aminoquinolines containing various substituents. These substituents could regulate important biological functions and could increase the biological activity of these types of compounds, in a way similar to that reported for other pharmaceuticals[16].

Hence the present study has been focussed on the investigation of $BiCl_3$ catalysed method of synthesis of quinoline nucleus involving directly introduce an amino or a

substituted amino group at 4-position of tetrahydroquinolines, and a general method for a one pot synthesis of 2-phenylquinolines by suing sulphur in acetonitrile medium.

2.2. Present work

The imino-Diels-Alder reaction of *N*-vinylpyrrolidin-2-one in the synthesis of tetrahydroquinolines with *N*-benzylideneaniline shows stereoselective preference analogous to those of other dienophiles[17, 18]. Recently, X. Jia. *et al.* reported synthesis of 4-lactam-*N*-yl tetrahydroquinolines with *N*-Arylimines and *N*-vinyllactams *via* imino Diels-Alder reaction[19]. Initially, we found that, the reaction of *N*-benzylideneaniline **1a** (1 mmol) with *N*-vinylpyrroledin-2-one **2a** (1 mmol) in acetonitrile in presence of $BiCl_3$ (20 mole %) at room temperature produced compound **3a-h** in good to excellent yields, this reaction is found to be faster than the reaction of the other reported imino-Diels-Alder reaction [20] (Scheme-1) and the results of our study are summarized in Table 2.1.



1 **2a** **3a-h**
90 - 95 %

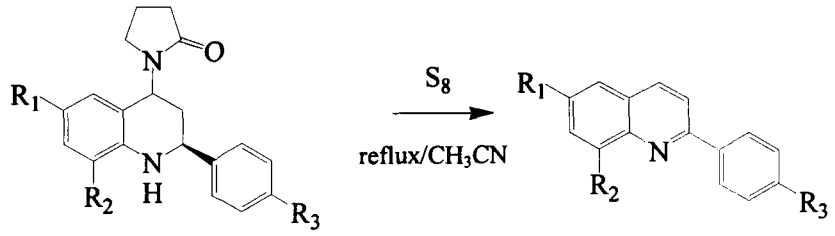
Scheme 1

Table 2.1. $BiCl_3$-catalysed cycloaddition of *N*-benzylideneaniline 1a with *N*-vinyl pyrrolidin-2-one 2a in acetonitrile at room temperature

Entry	R_1	R_2	R_3	Product	Time / min	Yield / (%)[a]
1	H	H	H	**3a**	30	93
2	Cl	H	H	**3b**	40	96
3	OCH_3	H	H	**3c**	25	91
4	H	H	NO_2	**3d**	25	91
5	CH_3	H	H	**3e**	25	90
6	H	CH_3	H	**3f**	25	87
7	H	H	Cl	**3g**	30	93
8	Br	H	H	**3h**	40	91

[a] Isolated Yield.

In order to obtain various 2-phenyl quinolines, the compounds **3a-h** were subjected to reflux with sulphur in acetonitrile as solvent. Thus various 2-phenylquinoline **4a-g** were prepared in high yield (Scheme-2).The results of this study are summarized in (Table 2.2).



3a-g **4a-g**

Scheme 2

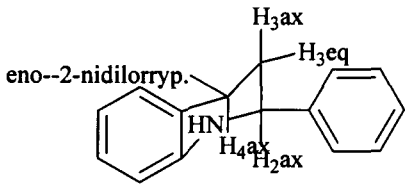
Table 2.2 Synthesis of 2-phenylquinoline from 4a-g on refluxing with sulphur in acetonitrile as solvent

Entry	R_1	R_2	R_3	Product	Time / min	Yield (%)[a]
1	H	H	H	**4a**	35	65
2	H	H	Cl	**4b**	40	72
3	F	H	H	**4c**	30	75
4	Br	H	NO_2	**4d**	45	75
5	CH_3	H	H	**4e**	75	80
6	OCH_3	H	H	**4f**	70	75
7	OCH_3	H	F	**4g**	80	72

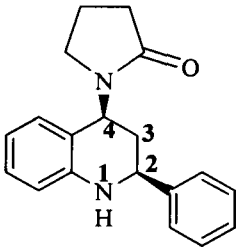
[a] Isolated yield

All the substituted 2-pheny-1,2,3,4-tetrahydroquinolines **(3a-h),** and 2-phenylquinolines **(4a-g)** obtained were purified by SiO_2 column chromatography (pet-ether / ethyl acetate 9:1 v/v). From the spectral analysis it is found that 2-pheny-1,2,3,4-tetrahydroquinolines exist as *cis*-isomers. Hence the reaction is highly stereoselective in nature.

Finally the structures of all the new products were established on the basis of spectroscopic evidence and all the products provided satisfactory elemental analysis. The exclusive *cis* diastereoselectivity of the products were established by the ^{1}H NMR spectral analysis. The ^{1}H NMR spectrum of compound **3a** showed large vicinal coupling constants between H-4 and H-3ax (11.1 Hz) and H-2 and H-3ax (10.2 Hz), which remain consistent with a trans-diaxial relationship between these protons and hence with a cis relationship between the caprolactam ring and phenyl groups[18]

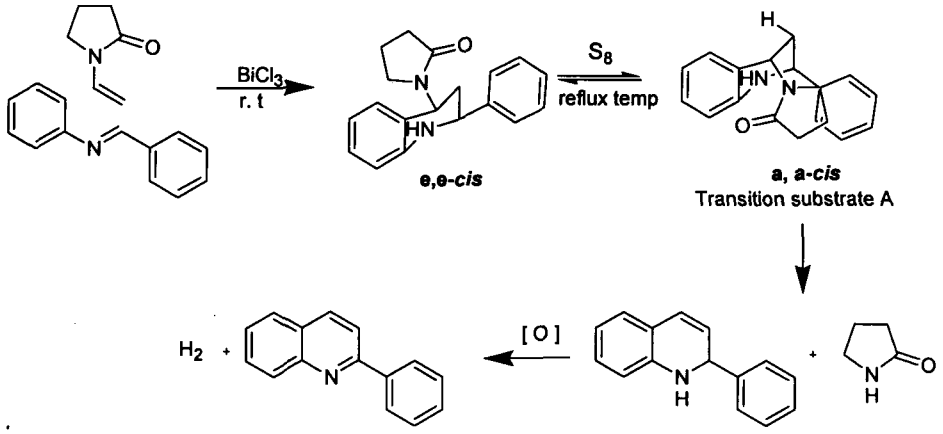


3a



3a

Thus with the forgone evidences, we have proposed a plausible mechanism for synthesis of 3a-h and subsequent oxidation of 3a-g with sulphur leading to 2-phenylquinolines 4a-g (scheme-3).



Scheme 3

Finally all the newly synthesised 2-phenyl-1,2,3,4-tetrahydroquinolines and 2-phenylquinolines have been screened for hepatoprotective and anticonvulsant activities due to their wide variety of biological activities as we have discussed in the introduction chapter.

2.3. Experimental

2.3.1 General procedure for the synthesis of tetrahydroquinolines

To a stirred solution of *N*-benzylideneaniline (1 mmol) and *N*-vinylpyrrolidin-2-one (1 mmol) in acetonitrile (10 ml) was added $BiCl_3$ (20 mol %) at room temperature. After completion of the reaction, (as indicated by TLC), the mixture was extracted with ethyl acetate (2x20 ml), dried over (anhydrous Na_2SO_4) and evaporated. The crude was purified by silica gel column chromatography using light petroleum ether-ethyl acetate (9/1, v/v).

2.3.2 General procedure for the synthesis of 2-phenylquinolines

To a solution of tetrahydroquinolines 3a-h (1 mmol) in acetonitrile (10 ml), sulphur 5g was added. The contents were refluxed. After completion of the reaction, (as indicated by TLC), the mixture was diluted with 100 ml water and extracted with ethyl acetate (2x20 ml), dried over (anhydrous Na_2SO_4) and evaporated. The crude was subjected to silica gel column chromatography with a petroleum ether–ethyl acetate (9/1, v/v) as eluent.

2.4. Pharmacological Evaluation

2.4.1. Anticonvulsant activity of 2-phenylquinolines and tetrahydroquinolines

Epilepsy, one of the most common neurologic diseases, is characterized by epileptic seizures, which are evoked by unexpected, high-level neuronal discharges in the brain[21]. Since the anticonvulsant agents currently used in the treatment of epilepsy have certain disadvantages such as notable side effects and inefficient therapy in some seizure types, a clear need for safer and more effective antiepileptic drugs is well known[21-23].

Therefore the development of new antiepileptic drugs with approved therapeutic properties is an important challenge for medicinal chemists.

Even though considerable research work has been done on the synthesis, pharmacological activities of 2-phenyl tetrahydroquinolines and 2-phenyl quinolines derivatives still remains thrust area of research. Hence it is thought to explore the synthesized 2-phenyl tetrahydroquinolines and 2-phenyl quinolines derivatives for anticonvulsant activity using animal models. The anticonvulsant activity was evaluated using the maximal electroshock (MES) test and the subcutaneous pentylenetetrezol (PTZ) test.

2.4.1.1 Materials and Methods

a) Animals

Male Wistar albino rats (150-200 gms) and Swiss albino mice weighing 18-25 gms were used. The animals were housed 6 per cage at 25°±1°C and 55±5% RH. A 12:12 dark:light cycle was followed during the experiments. The animals were fed with standard pellet diet (Lipton India Ltd. Mumbai) and water *ad libitum*. The institutional ethics committee approved the protocol for study.

b) Anticonvulsant activity[24,25]

i) Maximal Electric Shock (MES) induced seizures

Male Wistar albino rats were used in the study. Animals were divided in to control, standard and test groups. The tested compounds were dissolved in polyethylene glycol-400. In the MES test, seizures were elicited with a 60 Hz alternating current of 150 mA intensity in rats. The current was applied *via* corneal electrodes for 0.2 s.

Abolition of the hind-leg tonic-extensor component of the seizure indicated protection against the spread of MES-induced seizures.

ii) Pentylenetetrazole (PTZ) induced seizures

Swiss albino mice weighing 18-25 gms were used in the study. Animals were divided in to control, standard and test groups. Test compounds were dissolved in polyethylene glycol-400 before administration. The PTZ test involved subcutaneous injection of a convulsant dose of pentylenetetrazole (70 mg/kg s.c.) after 60 min of test drug administration[26]. Falling and jerking were considered as beginning of seizures[27]. Elevation of the pentylenetrazol-induced seizure threshold was indicated by the absence of clonic spasms for at least 5-s duration over a 30-min period following administration of the test compound.

c) Statistical analysis

The data were analyzed by using one way ANOVA followed by Tukey's multiple comparison test. The level of significance was set at $P<0.05$.

2.4.1.2 Results and Discussion

Different types of epilepsies, i.e., grandmal, petit mal or psychomotor type, can be studied using laboratory animal models. Based on previous reports we knew that quinolines have activity against both major and minor seizures[28]. The MES test is regarded as the pharmacologic model of grand mal epilepsy. Similarly chemo-convulsions due to pentelynetetrazol (PTZ) which produce clonic type of convulsions resemble petit mal in humans[29]. Therefore we carried out these two tests to evaluate the anticonvulsant activity of the synthesized quinoline compounds.

The compounds were tested for anticonvulsant activity using the procedures described previously. Results revealed that 2-phenyl quinolines derivatives such as **4c,**

4d, 4f and **4g** significantly reduced the tonic extensor phase and the incidence of convulsions in MES induced seizures (Table 2.3). Among them **4c** ($P< 0.001$), **4d** and **4g** ($P< 0.001$) were found to be more potent than other compounds. Synthesised 2-phenyl tetrahydroquinolines such as **3b** ($P< 0.001$), **3c** ($P<0.01$), **3d** ($P<0.01$) and **3g** ($P< 0.001$) were found to show better anticonvulsant activity than the other tested derivatives (Table 2.4). The antiepileptic property of these compounds may be due to the blockage of MES-induced tonic extension phase by blocking seizure spread[30].

In PTZ test, **4c** and **4f** ($P<0.01$) of 2-phenyl quinolines significantly protected mice against all phases of seizure activity defined by tonic, clonic, hind paw extension and lethality in PTZ induced convulsions. Table 2.5 and 2.6 depicts the time of onset of clonic phases and lethality induced by PTZ. Among the 2-phenyl tetrahydroquinolines **3b** ($P<0.01$), **3c** ($P< 0.001$), **3d** ($P<0.05$) and **3g** ($P<0.01$) significantly enhanced the time of onset of clonic phase. Some of these derivatives showed a high degree of protection against MES-induced seizures, although they were less effective against PTZ-induced seizures.

2.4.1.3 Structure activity relation ship

2-phenyl 1,2,3,4-tetrahydroquinoline moiety as such did not show any activity in MES and PTZ tests. Whereas, chlorine substitution on quinoline nucleus at 6 position in **(3b)** and on 2-phenyl ring at para position **(3g)** significantly enhanced the anticonvulsant activity. However substitution with OCH_3 **(3c)** and NO_2 to 2-phenyl ring **(3d)** shown moderate anticonvulsant activity. The substitution such as CH_3 at 6^{th} and 8^{th} position and Br at 6^{th} position of 2-phenyl tetrahydroquinoline did not show any activity in MES as well as PTZ tests.

Table 2.3. Effect of 2-phenyl quinolines on MES induced seizures in rats

Treatment	Dose (mg/kg)	Duration of tonic extensor phase (sec) ± SE	Incidence of convulsions
Control (MES)	-	12.67±0.80	6/6
Phenytoin	25 mg/kg i.p.	0.16±0.16[c]	1/6
4a	25 mg/kg p.o.	11.43±1.03	6/6
4b	25 mg/kg p.o.	9.50±0.62	6/6
4c	25 mg/kg p.o.	4.33±1.28[c]	3/6
4d	25 mg/kg p.o.	3.33±0.55[c]	2/6
4e	25 mg/kg p.o.	12.32±0.41	6/6
4f	25 mg/kg p.o.	6.00±0.36[b]	3/6
4g	25 mg/kg p.o.	4.83±0.60[c]	5/6

Values are mean ± SE from 6 animals in each group. [a] P< 0.05, [b] P< 0.01, [c] P< 0.001 compared to MES control group.

Table 2.4. Effect of 2-phenyl-1,2,3,4-tetrahydroquinolines on MES induced seizures in rats

Treatment	Dose (mg/kg)	Duration of tonic extensor phase (sec) ± SE	Incidence of convulsions
Control (MES)	-	12.67±0.80	6/6
Phenytoin	25 mg/kg i.p.	0.166±0.16[c]	1/6
3a	25 mg/kg p.o.	12.83±1.60	6/6
3b	25 mg/kg p.o.	3.40±1.07[c]	5/6
3c	25 mg/kg p.o.	6.00±0.93[b]	4/6
3d	25 mg/kg p.o.	6.00±1.06[b]	4/6
3e	25 mg/kg p.o.	11.83±2.08	6/6
3f	25 mg/kg p.o.	13.50±0.67	6/6
3g	25 mg/kg p.o.	3.66±0.42[c]	2/6
3h	25 mg/kg p.o.	11.33±1.47	6/6

Values are mean ± SE from 6 animals in each group. [a] P< 0.05, [b] P< 0.01, [c] P< 0.001 compared to MES control group.

Table 2.5. Effect of 2-phenyl quinolines on PTZ induced seizures in mice

Treatment	Dose (mg/kg)	Onset of clonic phase (min) ± SE	Death/Recovery n = 6
Control (PTZ)	70 mg/kg s.c.	10.49±0.40[c]	5/1
Phenytoin	25 mg.kg i.p.	27.74±1.0[c]	0/6
4a	25 mg/kg p.o.	15.34±0.52	5/1
4b	25 mg/kg p.o.	16.36±0.54	5/1
4c	25 mg/kg p.o.	18.37±1.14[b]	0/6
4d	25 mg/kg p.o.	11.33±1.91	6/0
4e	25 mg/kg p.o.	13.48±1.54	3/3
4f	25 mg/kg p.o.	18.68±0.90[b]	1/5
4g	25 mg/kg p.o.	16.04±1.43	3/3

Values are mean ± SE from 6 animals in each group. [b] P< 0.01, [c] P< 0.001 compared to PTZ treated group.

Table 2.6. Effect of 2-phenyl-1,2,3,4-tetrahydroquinolines on PTZ induced seizures in mice

Treatment	Dose (mg/kg)	Onset of clonic phase (min) ± SE	Death/Recovery n = 6
Control (PTZ)	70 mg/kg s.c.	10.49±0.40[c]	5/1
Phenytoin	25 mg/kgi.p.	27.74±1.0	0/6
3a	25 mg/kg p.o.	15.17±0.65	4/2
3b	25 mg/kg p.o.	18.23±1.08[b]	1/5
3c	25 mg/kg p.o.	20.16±1.49[c]	0/6
3d	25 mg/kg p.o.	16.83±1.36[a]	1/5
3e	25 mg/kg p.o.	15.15±0.78	5/1
3f	25 mg/kg p.o.	18.42±0.87[b]	2/4
3g	25 mg/kg p.o.	15.11±1.78	4/2
3h	25 mg/kg p.o.	13.26±2.12	4/2

Values are mean ± SE from 6 animals in each group. [a] P< 0.05, [b] P< 0.01, [c] P< 0.001 compared to PTZ treated group.

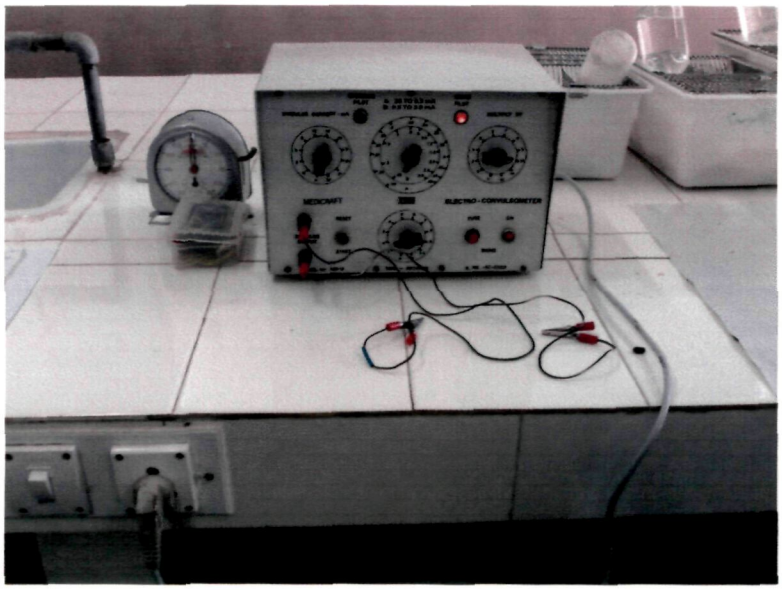


Fig. 2.1 Photograph of Electroconvulsiometer used in MES test.

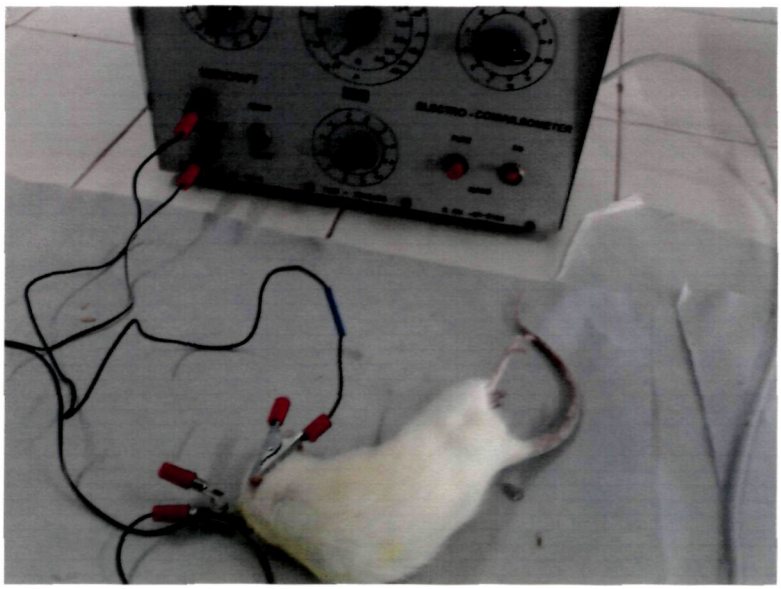


Fig. 2.2. Photograph showing the activity of rat (Hind paw extension) in MES test.

2.4.2 Hepatoprotective activity of 2-phenylquinolines and tetrahydroquinolines in CCl_4 induced hepatotoxicity in mice

Hundreds of tetrahydroquinolines bearing various simple or complex substituents have interesting biochemical activity, some are potential pharmaceutical agents. Recent studies demonstrated the biological importance of quinoline and tetrahydroquinoline derivatives[31-35]. Hence we thought to screen the synthesized 2-phenyl quinolines and 2-phenyl-1,2,3,4-tetrahydroquinolines for their ability to protect the liver in CCl_4 induced hepatotoxicity.

2.4.2.1 Materials and methods

a) Animals

Random-bred, male Swiss mice (6-8 weeks old) were obtained from Venkateshwara enterprises, Bangalore (India). The animals were provided with standard mice feed and tap water *ad libitum*. Once in a fortnight tetracycline water was given as a preventive measure against infection.

b) Experimental protocol (subacute study- 5 days)

Hepatoprotective activity was carried out using Swiss mice (6-8 weeks old). Animals were divided into various groups of six animals in each group. **Group I:** Served as control (normal saline 5 ml/kg, p.o.). **Group II:** Served as negative control (CCl_4/olive oil (1:1), 1 ml/kg, i.p.) on 3rd and 4th day). **Group III**: Treated with Silymarin 100 mg/kg, p.o for successive five days. All the animals except control group received CCl_4 (1 ml/kg, i.p.) on 3rd and 4th day. **Test groups:** Suspensions of 2-phenyl quinolines and 2-phenyl-1,2,3,4-tetrahydroquinolines in polyethylene glycol-400 at the dose of 25 mg/kg p.o. for five days. On the fifth day 2 hr after the administration of the last dose, blood samples were collected from the retro-orbital plexus, serum was separated by centrifugation and used for biochemical studies[36,37].

c) Biochemical studies

The SGPT and SGOT activity was measured by the method of Reitman & Frankel (1957) using DNPH as a colour reagent which can be measured colorimetrically. Serum alkaline phosphatase activity was measured by Kind & King's method (Varley, 1975), using commercially accessible kits (span Diagnostics Ltd., Surat, India). Optical density was measured at 510 nm.

2.4.2.2 Results and discussion

Hepatic cells appear to participate in a variety of enzymatic metabolic activities and carbon tetra chloride produced marked liver damage in the given dose as expected [40,41]. Administration of CCl_4 elevated the serum levels of SGPT, SGOT and ALP and bilirubin significantly, due to its enzymatic activation of CCl_3 free radical, which in turns alters the function of liver cells[42]. Serum activities of GOT and GPT are the most commonly used biochemical markers of liver injuries. ALP activities on the other hand are related to functioning of hepatocytes, increase in its activity is due to increased synthesis in presence of increased biliary pressure[43]. A highly significant ($P<0.001$) elevation was observed for all the parameters in CCl_4 intoxicated mice compared to normal control. Pretreatment with **4c, 4f** and **4g** derivatives of 2-phenyl quinolines a significant decline was observed in SGOT, SGPT, ALP and total and direct bilirubin activity with respect to CCl_4 intoxicated mice. Suppression of increased ALP activity with concurrent depletion of raised bilirubin level suggests the stability of biliary dysfunction in rat liver during chronic hepatic injury with CCl_4. The results are shown in Table 2.7. Among 2-phenyl-1,2,3,4-tetrahydroquinolines tested **3b, 3c, 3e** and **3f** also significantly attenuated the CCl_4 toxicity (Table 2.8). This protection by these compounds against CCl_4 may be due to interference with cytochrome P450, resulting in the hindrance of the formation of hepatotoxic free radicals[44,45].

2.4.2.3 Structure activity relationship

· Substitution with fluoro, methoxy at 6th position of 2-phenyl quinoline ring and fluro on phenyl ring at para position significantly protected the liver by decreasing the increased levels of serum parameters. However, substitution with methyl and bromo at 6th position of 2-phenyl quinoline did not protect the liver in CCl_4 induced hepatotoxicity.

Table 2.7. Effect of 2-phenyl quinolines on serum biochemical parameters in CCl_4 induced hepatotoxicity in mice (Acute study- 5 days)

Design of treatment	SGOT (U/ml)	SGPT (U/ml)	ALP (U/ml)	T. Bil (mg/dl)	D. Bil. (mg/dl)
Normal control	127.43±2.3	62.60±0.30	140.0±0.63	1.00±0.02	0.186±0.01
Carbon tetra-Chloride(1ml/kg on 3rd and 4th day	423.45±7.31^c	291.1± 1.44^c	437.6±2.19	3.66±0.15^c	1.585±0.072
Silymarin (100 mg/kg)	152.22 ± 3.10^c	75.34±2.00^c	163.81±4.28^c	0.96±0.05^c	0.24±0.02^c
4a (25 mg/kg p.o.)	395.4±3.21	285.3±4.28	421.1±2.31	3.161±0.020	1.352±0.052
4b (25 mg/kg p.o.)	373.4±8.896	284.6±2.82	431.3±1.60	3.043±0.09^a	1.380±0.08
4c (25 mg/kg p.o.)	366.0±1.147^b	279.0±0.86^a	419.7±1.39^a	2.972±0.05^b	1.228±0.03^b
4d (25 mg/kg p.o.)	390.5±3.432	291.2±2.45	428.6±2.63	3.108±0.059^a	1.357±0.05
4e (25 mg/kg p.o.)	400.9±2.318	291.3±1.84	429.4±3.61	3.178±0.016	1.322±0.045
4f (25 mg/kg p.o.)	352.2±6.962^c	274.4±4.58^b	331.1±2.62^c	2.487±0.11^c	1.285±0.09^a
4g (25 mg/kg p.o.)	345.3±1.191^c	266.6±2.27^c	416.0±3.93^b	3.115±0.17^a	1.182±0.07^b

Values are mean ± SE from 6 animals in each group. ^{a} P< 0.05, ^{b} P< 0.01, ^{c} P< 0.001 compared to CCl_4 treated group.

Table 2.8. Effect of 2-phenyl-1,2,3,4-tetrahydroquinolines on serum biochemical parameters in CCl_4 induced hepatotoxicity in mice (Acute study- 5 days)

Design of treatment	SGOT (U/ml)	SGPT (U/ml)	ALP (U/ml)	T. Bil (mg/dl)	D. Bil. (mg/dl)
Normal control	127.43±2.33	62.60±0.30	140.0±0.63	1.00±0.02	0.186±0.01
Carbon tetra-Chloride(1ml/kg on 3rd and 4th day	423.45±7.31^c	291.1± 1.44^c	437.6±2.19	3.66±0.15^c	1.585±0.072
Silymarin (100 mg/kg)	152.22± 3.10^c	75.34±2.00^c	163.81±4.28^c	0.96±0.05^c	0.24±0.02^c
3a (25 mg/kg p.o.)	412.51±5.21	277.87±5.32	427.22±7.68	1.74±0.42	0.87± 0.04
3b (25 mg/kg p.o.)	369.32± 4.43^a	275.22± 4.56^a	415 ± 6.65^a	2.74±0.34^a	1.40±0.25^c
3c (25 mg/kg p.o.)	367.13± 5.65^a	265 ± 6.56^a	422.36±3.63^a	2.80±0.54^a	1.25± 0.45^a
3d (25 mg/kg p.o.)	422.34± 3.54	285.21± 3.58	428.54± 2.56	2.56 ± 0.43	1.12 ± 0.54
3e (25 mg/kg p.o.)	325.55± 3.45^c	253.35± 5.64^c	414 ± 4.50^a	0.85 ±0.32^a	0.95 ± 0.05^a
3f (25 mg/kg p.o.)	391.58±5.63 ^{b}	278.40± 7.84^b	390.25±3.65^c	2.63±0.23^b	1.54±0.54^b
3g (25 mg/kg p.o.)	452.0±7.57^c	274.2±3.94^a	276.8±5.88^c	2.508±0.12	1.42±0.0
3h (25 mg/kg p.o.)	380.5±5.371^c	277.2±2.58^a	425.6±3.50^c	3.063±0.04	1.30±0.06

Values are mean ± SE from 6 animals in each group. a P< 0.05, b P< 0.01, c P< 0.001 compared to CCl4 treated group.

2.5 Spectral Data

cis-4-(2'-oxopyrrolinidyl-1')-2-phenyl-1,2,3,4-tetrahydroquinoline: 3a

Colorless needles, M. P.: 142-144 °C; IR (KBr): $\bar{\nu}$ = 3315 (NH) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 1.99-2.13 (m, 4H), 2.42-2.56 (m, 2H), 3.19-3.25 (m, 2H), 4.02 (brs, 1H, NH), 4.60 (dd, 1H, J = 10.2, 3.3 Hz, H-2), 5.73 (dd, J = 11.1, 6.6 Hz, 1H), 6.58 (d, J = 7.6 Hz, 1H), 6.72 (t, J = 7.8 Hz, 1H), 6.87 (d, J = 7.8 Hz, 1H), 7.06 (t, J = 7.8 Hz, 1H), 7.28-7.45 (m, 5H); ^{13}C NMR ($CDCl_3$): δ = 18.2, 31.4, 35.2, 42.3, 48.4, 56.3, 114.9, 118.1, 118.8, 126.4, 126.7, 127.9, 128.2, 128.7, 143.0, 145.9, 175.8; MS: *m/z* = 293 (M+1); Anal. Calcd for $C_{19}H_20N_2O$: C, 78.05; H, 6.89; N, 9.58. Found: C, 77.82; H, 6.88; N, 9.51% (Fig. 2.3, 2.4 and 2.5).

cis-6-Chloro-4-(2'-oxopyrrolinidyl-1')-2-phenyl-1,2,3,4-tetrahydroquinoline: 3b

Colorless needles, M. P.: 175-177 °C; IR (KBr): $\bar{\nu}$ = 3315 (NH) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 1.95–2.17 (m, 4H), 2.39-2.58 (m, 2H), 3.20-3.26 (m, 2H), 4.07 (br, NH, 1H), 4.57 (dd, J = 10.5, 3.8 Hz, 1H), 5.23 (dd, 1H, J = 11.4, 6.3 Hz, H-4), 6.51 (d, 1H, J = 8.6 Hz, H-8), 6.82 (d, 1H, J = 2.4 Hz, H-5), 7.01 (dd, 1H, J = 8.2, 2.4 Hz, H-7), 7.30-7.42 (m, 5H) ppm; ^{13}C NMR ($CDCl_3$): δ = 18.4, 31.5, 35.1, 42.5, 48.5, 56.6, 116.3, 120.7, 123.0, 126.5, 126.7, 128.3, 128.5, 129.0, 142.8, 144.7, 176.1 ppm; MS: *m/z* = 328 (M+1); Anal. Calcd for $C_{19}H_{19}N_2OCl$: C, 69.83; H, 5.86; N, 8.57. Found: C, 69.67; H, 5.95; N, 8.48.

cis-6-Methoxy-4-(2'-oxopyrrolinidyl-1')-2-phenyl-1,2,3,4-tetrahydroquinoline: 3c

Colorless needles, M. P.: 156-158 °C; IR (KBr): $\bar{\nu}$ = 3315 cm^{-1}(NH); ^1H NMR ($CDCl_3$): δ= 1.92-2.16 (m, 4H), 2.43-2.53 (m, 2H), 3.18-3.26 (m, 2H), 3.71 (s, 3H), 3.98 (brs, 1H), 4.51 (dd, , J = 9.6, 3.2 Hz, 1H), 5.70 (dd, J = 10.5, 6.8 Hz, 1H), 6.45 (d, J = 8.8

Hz, 1H), 6.57 (d, J = 2.8 Hz, 1H), 7.01 (dd, J = 8.8, 2.4 Hz, 1H), 7.44-7.27 (m, 5H); ^{13}C NMR ($CDCl_3$): δ = 18.1, 31.3, 35.2, 42.3, 48.6, 55.8, 112.1, 114.4, 116.1, 120.1, 126.4, 127.8, 128.6, 140.1, 143.1, 152.6, 175.7; MS: *m/z* = 322 (M+); Anal. Calcd for $C_{20}H_{22}N_2O_2$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.33; H, 7.01; N, 8.61.

cis-2-(p-Nitrophenyl)-4-(2'-oxopyrrolinidyl-1')-1,2,3,4-tetrahydroquinoline: 3d

Yellow needles, mp: 218-220 °C; IR (KBr): $\bar{\nu}$ = 3315 cm^{-1} (NH); ^1H NMR ($CDCl_3$): δ = 1.97–2.16 (m, 4H), 2.39-2.56 (m, 2H), 3.22 (dd, *J* = 7.2, 6.9 Hz, 2H), 4.12 (brs, NH, 1H), 4.71 (dd, *J* = 10.5, 3.3 Hz, 1H), 5.72 (dd, *J* = 11.7, 6.3 Hz, 1H), 6.65 (d, 1H, *J* = 7.6 Hz, H-8), 6.76 (dd, , *J* = 7.6, 7.2 Hz, 1H), 6.88 (d, *J* = 7.6 Hz, 1H), 7.09 (dd, J = 7.6, 7.2 Hz, 1H), 7.35 (d, J = 8.62 Hz, 2H), 8.22 (d, J = 8.6 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ = 18.4, 31.6, 35.4, 42.5, 48.4, 56.6, 115.6, 119.0, 119.1, 124.3, 127.0, 127.6, 128.7, 145.5, 147.8, 150.7, 176.2; MS: *m/z* = 337 (M+); Anal. Calcd for $C_{19}H_{19}N_3O_3$: C, 67.64; H, 5.68; N, 12.45. Found: C, 67.77; H, 5.76; N, 12.48.

cis-6-Methyl-2-phenyl-4-(2'-oxopyrrolinidyl-1')-1,2,3,4-tetrahydroquinoline: 3e

Colorless crystalline solid, M.p.: 164-166°C; IR (KBr): $\bar{\nu}$ = 3356 (NH) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 1.96-2.01 (m, 2H), 2.05-2.09 (m, 2H), 2.26 (s, 3H), 2.41-2.52 (m, 2H), 3.17-3.28 (m, 2H), 4.02 (brs, NH), 4.55 (dd, *J* = 9.0, 3.9 Hz, 1H), 5.22 (dd, *J* = 10.8, 6.4 Hz, 1H), 6.61 (d, *J* = 8.1 Hz, 1H), 6.99 (dd, *J* = 8.1, 2.8 Hz, 1H), 7.25 (d, *J* = 6.6 Hz, 1H), 7.33-7.51 (m, 5H) ppm; ^{13}C NMR ($CDCl_3$): δ = 175.6, 143.1, 142.5, 129.5, 129.4, 128.7, 127.9, 127.7, 126.6, 122.2, 121.8, 118.4, 55.3, 48.1, 42.8, 35.5, 31.3, 21.4, 18.2 ppm; MS: *m/z* = 306 (M+).

cis-8-Methyl-2-phenyl-4-(2'-oxopyrrolinidyl-1')-1,2,3,4-tetrahydroquinoline:3f

Colorless crystalline solid, M.p.: 172-174°C; IR (KBr): $\bar{\nu}$ = 3348 (NH) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 2.01-2.05 (m, 2H), 2.15-2.33 (m, 5H), 2.45-2.55 (m, 2H), 3.18-3.33 (m, 2H), 4.09 (brs, NH), 4.35 (dd, J = 8.6, 3.8 Hz, 1H), 5.24 (dd, J = 11.2, 2.8 Hz, 1H), 6.65 (t, J = 7.6 Hz, 1H), 7.12 (d, J = 7.8 Hz, 1H), 7.29-7.42 (m, 6H) ppm; ^{13}C NMR ($CDCl_3$): δ = 175.4, 141.9, 141.0, 129.3, 129.2, 128.8, 128.1, 127.8, 121.9, 118.1, 117.0, 55.1, 47.6, 42.1, 34.6, 31.1, 20.9, 17.22 ppm; MS: *m/z* = 306 (M+).

cis-2-(4-Chlorophenyl)-4-(2'-oxopyrrolinidyl-1')-1,2,3,4-tetrahydroquinoline:3g

Colorless crystalline solid, M.p.: 148-150°C; IR (KBr): $\bar{\nu}$ = 3328 (NH) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 2.04-2.09 (m, 2H), 2.11-2.15 (m, 2H), 2.45-2.52 (m, 2H), 3.19-3.34 (m, 2H), 4.10 (brs, NH), 4.26 (dd, J = 8.8, 3.8 Hz, 1H), 5.21 (dd, J = 11.8, 2.6 Hz, 1H), 6.77 (t, J = 7.4, 1.0 Hz, 1H), 7.13 (dt, J = 7.4, 1.7 Hz, 1H), 7.25-7.54 (m, 6H) ppm; ^{13}C NMR ($CDCl_3$): δ = 175.4, 144.7, 140.6, 132.2, 130.0, 129.5, 128.7, 128.3, 122.5, 119.7, 114.9, 55.5, 47.2, 42.3, 34.4, 32.1, 18.2 ppm; MS: *m/z* = 328 (M+1).

cis-6-Bromo-2-phenyl-4-(2'-oxopyrrolinidyl-1')-1,2,3,4-tetrahydroquinoline:3h

Colorless crystalline solid, M.p.: 185-187°C; IR (KBr): $\bar{\nu}$ = 3315 (NH) cm^{-1}; ^1H NMR ($CDCl_3$): δ = 2.08-2.22 (m, 4H), 2.29-2.48 (m, 2H), 3.15-3.21 (m, 2H), 4.12 (brs, 1H, NH), 4.38 (dd, J = 10.6, 2.8 Hz, 1H), 5.38 (dd, J = 11.6, 5.9 Hz, 1H), 6.50 (d, J = 7.8 Hz, 1H), 7.2 (dd, J = 7.6, 2.6 Hz, 1H), 7.32-7.44 (m, 5H), 7.48 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$): δ = 175.8, 144.1, 140.7, 128.3, 127.9, 127.6, 127.2, 126.9, 123.1, 121.6, 116.6, 56.6, 49.1, 42.3, 35.1, 31.6, 18.8 ppm; MS: *m/z* = 372 (M+1).

2-phenylquinoline:4a

mp:; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 7.49 -7.61 (m, 4H), 7.75-7.79 (m, 1H), 7.98-8.28 (m, 5H), 8.44-8.47 (m, 1H); MS m/z (%) = 207 (M+2); ^{13}C NMR (100 MHz, $CDCl_3$) 120.0, 126.0, 127.5, 127.7, 127.9, 127.9, 128.9, 129.8, 130.3, 130.3, 130.9, 137.0, 138.6, 148.0, 157.0.; Anal. Calcd. for ($C_{15}H_{11}N$); C, 87.77; H, 5.40; N, 6.82; Found; C, 87.75; H, 5.30; N, 6.52.

2-(4-chlorophenyl) quinolines:4b

mp: 107-108 ^{0}C; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.49-7.57 (m, 3H), 7.74-7.75 (m, 1H), 7.83-7.87 (m, 2H), 8.12-8.17 (m, 3H), 8.23-8.26 (m, 1H); MS m/z (%) = 240 (M+1). Anal. Calcd. for ($C_{15}H_{10}ClN$); C, 75.16; H, 4.21; N, 5.84. Found; C, 74.95; H, 3.94; N, 5.53.

6-fluoro-2-phenylquinoline:4c

mp: 85-87 ^{0}C; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7. 47-7. 54 (m, 5H), 7.92 (s, 1H), 8. 13-8. 20 (m, 4H); MS m/z (%) = 224.0 (M+1). ^{13}C NMR (100 MHz, $CDCl_3$) 110.7, 119.2, 120.4, 127.1, 127.6, 127.8, 127.8, 130.2, 130.2, 131.8, 137.0, 138.0, 145.1, 156.2, 161.2.; Anal. Calcd. for: $C_{15}H_{10}FN$: C, 80.70; H, 4.51; N 6.27. Found: C, 80.44; H, 4.25, N, 6.08 (Fig. 2.6 and 2.7).

6-bromo-2-(3-nitrophenyl) quinolines:4d

mp: 147–149 8^{0}C; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.72 (t, J = 8.0 Hz 1H), 7.84 (dd, J = 9.0, 2.2 Hz, 1H), 8.0 (d, J = 8.6 Hz, 1H), 8.04-8.08 (m, 2H), 8.22 (d, J = 8.6 Hz, 1H), 8.32-8.35 (m, 1H), 8.53-8.56 (m, 1H), 9.04-9.05 (m, 1H); MS m/z (%) = 329 (M+1); ^{13}C NMR (100 MHz, $CDCl_3$) 119.8, 120.0, 121.2, 122.5, 123.8, 129.9, 130.8, 131.3, 134.0, 134.0, 137.6, 138.0, 148.1, 149.5, 157.2.; Anal. Calcd. for:($C_{15}H_9BrN_2O_2$) : C, 54.74; H, 2.76; N, 8.51. Found; C, 54.4; H, 2.57; N, 8.22.

6-methyl-2-phenylquinoline:4e

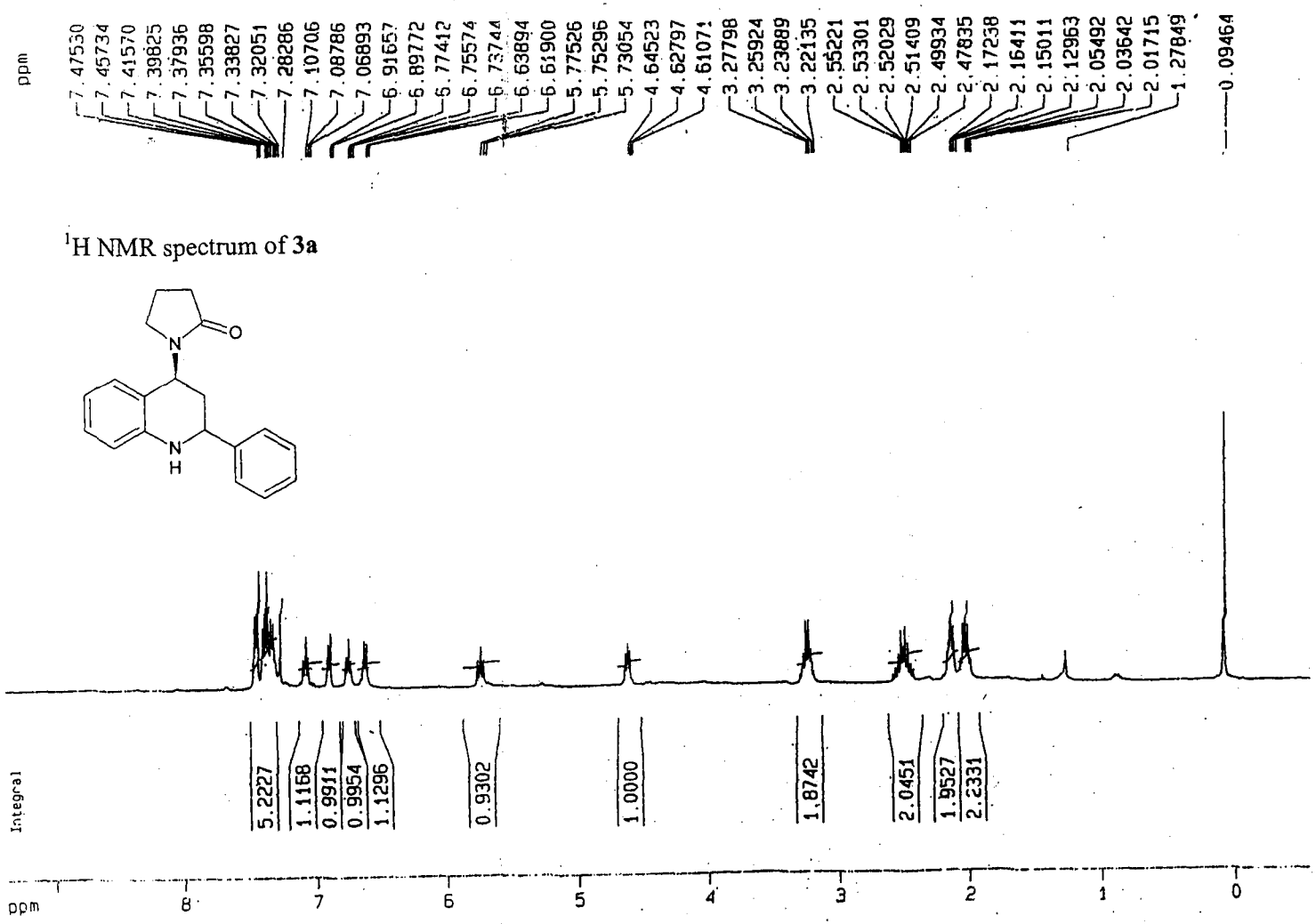
mp: 147–149 8^0C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 2.56 (s, 3H), 7.46-7.60 (m, 5H), 7.85 (d, J = 8.6 Hz, 1H), 8.08 (d, J = 8.5 Hz, 1H), 8.07-8.17 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 119.8, 120.0, 121.2, 122.5, 123.8, 129.9, 130.8, 131.3, 134.0, 134.0, 137.6, 138.0, 148.1, 149.5, 157.2; MS m/z (%) = 220.2 (M+1). Anal. Calcd. for: ($C_{16}H_{13}N$): C, 87.64; H, 5.98; N, 6.39. Found: C, 87.42; H, 5.68; N,6.2.

6-methoxy-2-phenylquinoline:4f

mp: 128 ^{0}C; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 3.96 (s, 3H), 7.11 (d, J = 2.8 Hz, 1H), 7.40-7.52 (m, 4H), 7.86 (d, J = 8.6 Hz, 1H), 8.06-8.15 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) 56, 106.2, 120.5, 123.0, 127.0, 127.6, 127.8, 127.8, 130.2, 130.2, 131.3, 136.8, 137.6, 143.0, 155.3, 158.1; MS m/z (%) = 236.2 (M+1). Anal. Calcd. for: ($C_{16}H_{13}NO$): C, 81.68; H, 5.57; N, 5.95. Found: C, 81.32; H, 5.38; N, 5.77 (Fig. 2.8 and 2.9).

2-(4-fluorophenyl)-6-methoxyquinoline:4g

mp: 147–149 8^0C; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 3.95 (s, 3H), 7.10 (d, J = 2.7 Hz, 1H), 7.17-7.26 (m, 2H), 7.40 (dd, J = 9.2, 3.0 Hz, 1H), 7.80 (d, J = 8.6 Hz, 1H) 8.03-8.15 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) 56, 106.3, 116.5, 116.5, 120.5, 123.0, 127.0, 129.3, 129.3, 130.8, 131.5, 137.4, 142.6, 155.2, 158.0, 162.0; MS m/z (%) = 254.0 (M+1). Anal. Calcd. for: ($C_{16}H_{12}FNO$): C, 75.88; H, 4.78; N, 5.53. Found; C, 75.51; H, 4.62; N, 5.38.



^{1}H NMR spectrum of **3a**

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NAME
EXPNO                  1
PROCNO                 1

F2 - Acquisition Parameters
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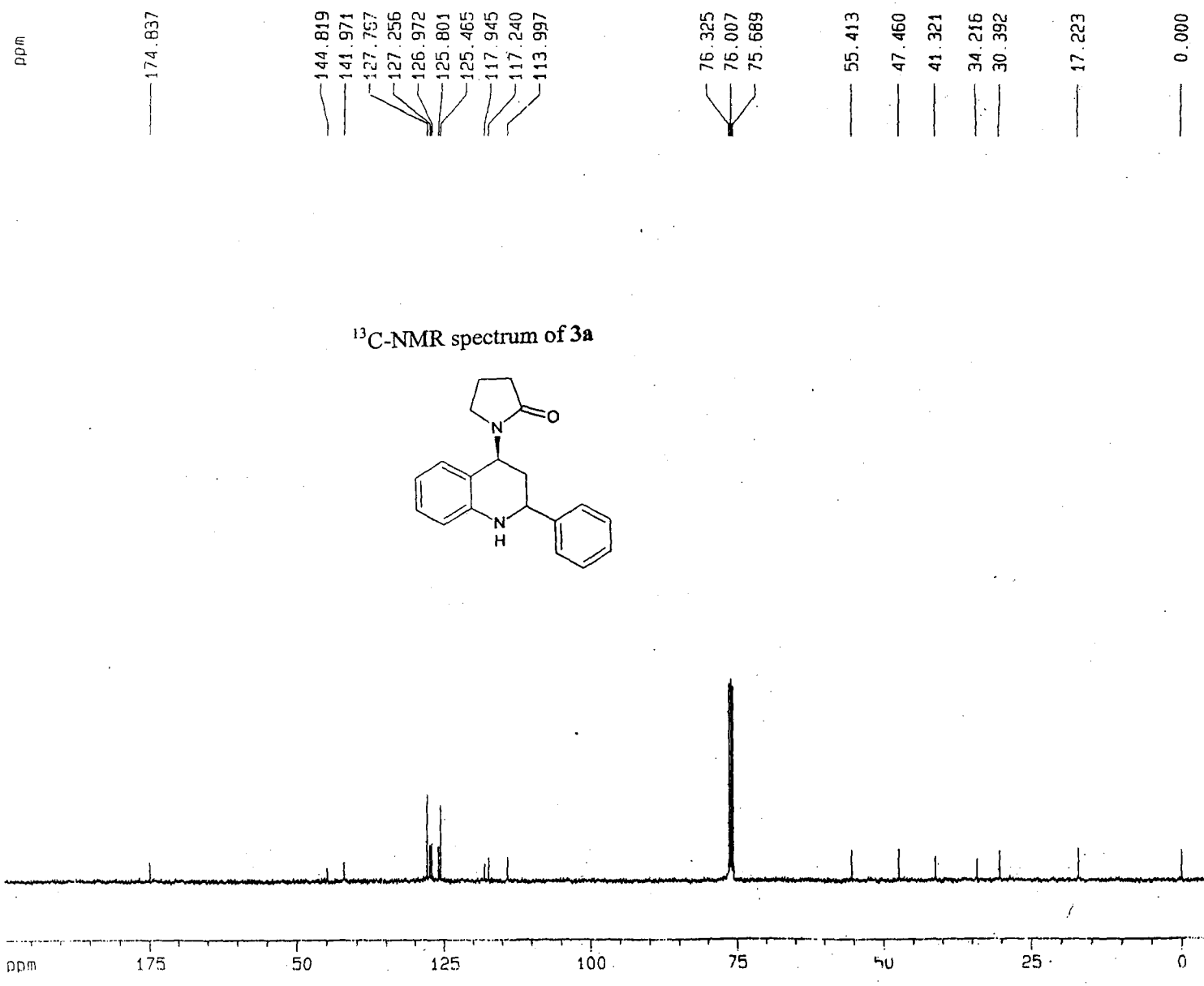
PULPROG             zg30
TD                 32768
SOLVENT            CDCl3
NS                     8
DS                     0
SWH           9980.040 Hz
FIDRES        0.304567 Hz
AQ            1.6417269 sec
RG                   128
DW             50.100 usec
DE              6.00 usec
TE                 0.0 K
D1            2.00000000 sec
MCREST        0.00000000 sec
MCWRK         0.01500000 sec

======== CHANNEL f1 ========
NUC1                  1H
P1             12.00 usec
PL1             0.00 dB
SFO1      400.1336012 MHz

F2 - Processing parameters
SI                 16384
SF        400.1300000 MHz
WDW                   EM
SSB                    0
LB              0.50 Hz
GB                     0
PC                  0.50

1D NMR plot parameters
CX                20.00 cm
CY                 0.00 cm
F1P               9.408 ppm
F1              3764.40 Hz
F2P              -0.557 ppm
F2             -222.96 Hz
PPMCM          0.49826 ppm/cm
HZCM          199.36763 Hz/cm
```

Fig 2.3. ^{1}H NMR Spectrum of *cis*-4-(2'-oxopyrrolinidyl-1')-2-phenyl-1,2,3,4-



^{13}C-NMR spectrum of **3a**


```
Current Data Parameters
NAME
EXPNO                   2
PROCNO                  1

F2 - Acquisition Parameters
Date_            20070330
Time                18.59
INSTRUM             spect
PROBHD   5 mm BBO BB-1H
PULPROG            zgpg30
TD                  65536
SOLVENT             CDCl3
NS                   1000
DS                      4
SWH           23980.814 Hz
FIDRES        0.365918 Hz
AQ            1.3664756 sec
RG                 1824.6
DW                20.850 usec
DE                 6.00 usec
TE                 _0.0 K
D1            2.00000000 sec
d11           0.03000000 sec
MCREST        0.00000000 sec
MCWRK         0.01500000 sec

======== CHANNEL f1 ========
NUC1                  13C
P1                 9.25 usec
PL1               -2.00 dB
SFO1        100.6228298 MHz

======== CHANNEL f2 ========
CPDPRG2          waltz16
NUC2                  1H
PCPD2            100.00 usec
PL2                0.00 dB
PL12              18.42 dB
PL13             120.00 dB
SFO2        400.1316005 MHz

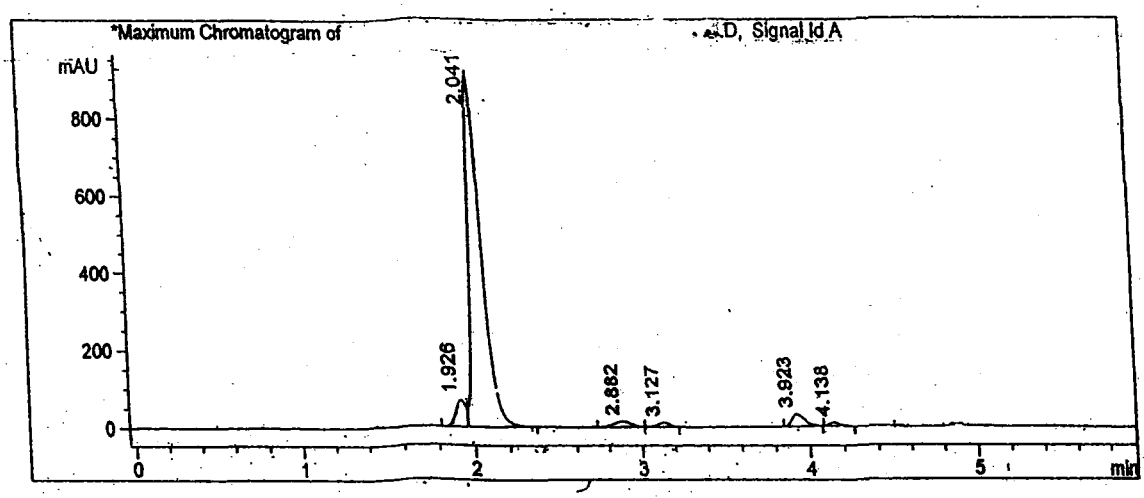
F2 - Processing parameters
SI                   32768
SF          100.6128715 MHz
WDW                    EM
SSB                     0
LB                 3.00 Hz
GB                      0
PC                   1.40

1D NMR plot parameters
CX                20.00 cm
CY                 0.00 cm
F1P              199.495 ppm
F1              20071.72 Hz
F2P               -8.224 ppm
F2              -827.44 Hz
PPMCM            10.38593 ppm/cm
HZCM          1044.95837 Hz/cm
```

Fig 2.4 . 13C NMR Spectrum of *cis*-4-(2'-oxopyrrolinidyl-1')-2-phenyl-1,2,3,4-tetrahydroquinoline

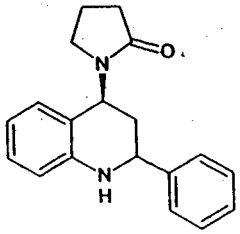
Method info :A-0.1%HCOOH;B-ACN Flow = 0.8ML/MIN ISD - 09
Column-Atlantis dC18 (75X4.6mm-5µm) Positive Mode

Time (min.):	0--1.0	1.0--1.5	1.5--2.5	2.5--3.0	3.0--5.0
% B :	70	70--95	95	95--70	70



Peak No	RT min	Area	Area %
1	1.926	2.724e+002	5.302
2	2.041	4.468e+003	86.969
3	2.882	1.132e+002	2.204
4	3.127	5.848e+001	1.138
5	3.923	1.783e+002	3.471
6	4.138	4.708e+001	0.916

Mass spectrum of 3a



Mass Calcd m/z=292

Found m/z=293(M+1)

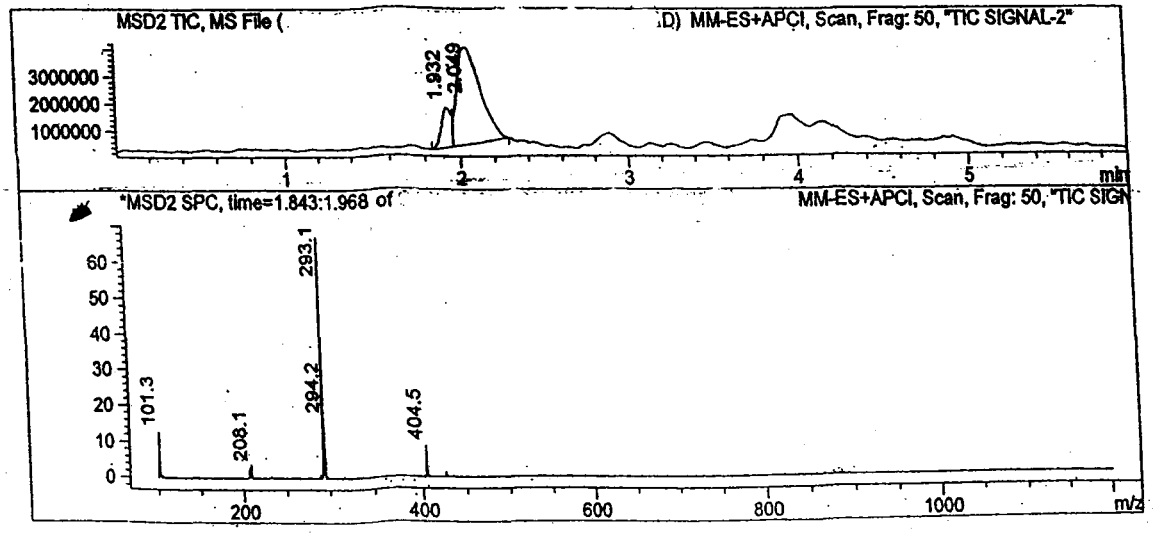
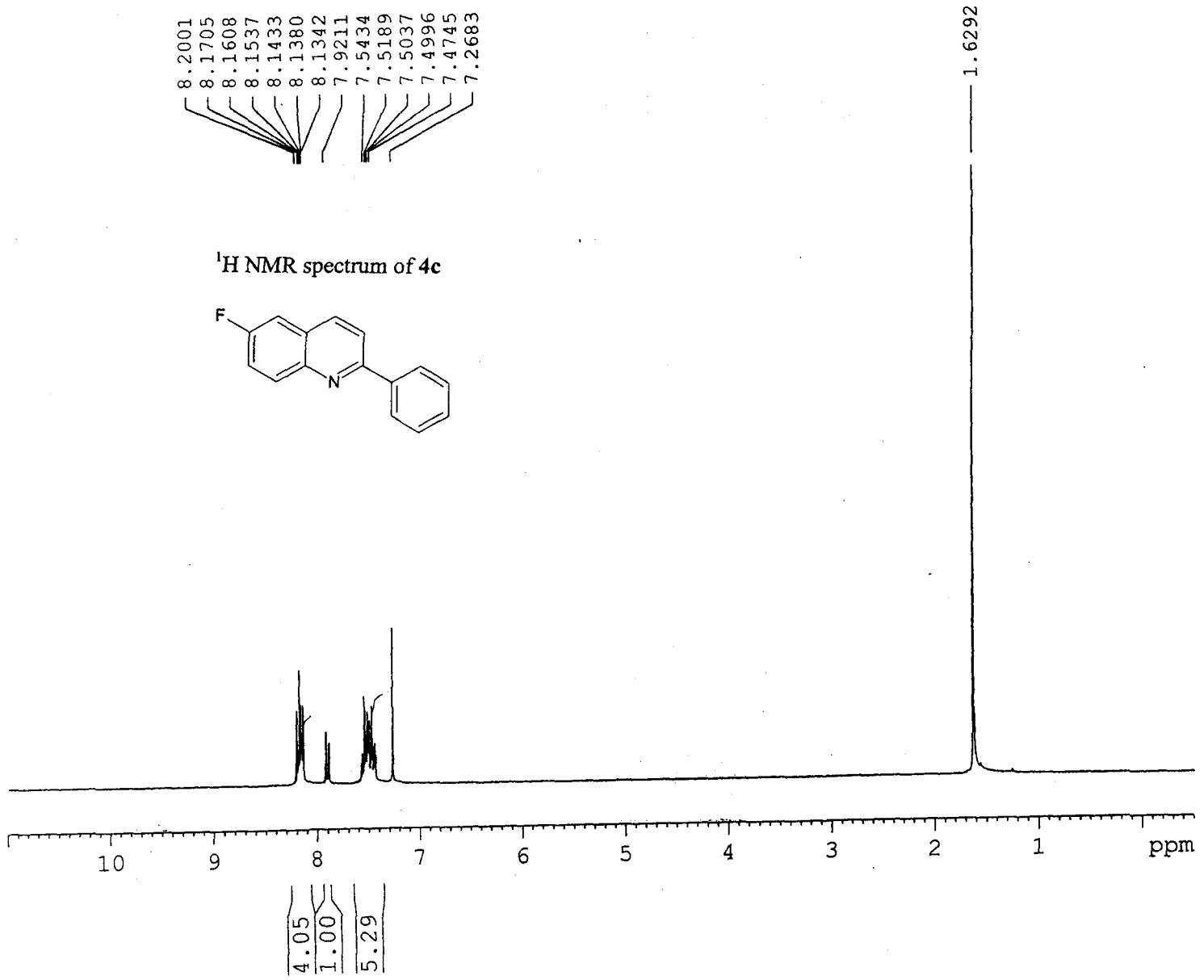


Fig 2.5. Mass Spectrum of *cis*-4-(2'-oxopyrrolinidyl-1')-2-phenyl-1,2,3,4-tetrahydroquinoline



Current Data Parameters

NAME	
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20081024
Time	19.28
INSTRUM	spect
PROBHD	5 mm DUL BB-1H
PULPROG	zg30
TD	32768
SOLVENT	CDCl3
NS	8
DS	2
SWH	7183.908 Hz
FIDRES	0.219235 Hz
AQ	2.2807028 sec
RG	574.7
DW	69.600 usec
DE	6.00 usec
TE	0.0 K
D1	2.00000000 sec
MCREST	0.00000000 sec
MCWRK	0.01500000 sec

======== CHANNEL f1 ========

NUC1	1H
P1	10.30 usec
PL1	0.00 dB
SFO1	300.1327012 MHz

F2 - Processing parameters

SI	32768
SF	300.1298168 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.40

Fig 2.6. ^{1}H NMR Spectrum of 6-fluoro-2-phenylquinoline

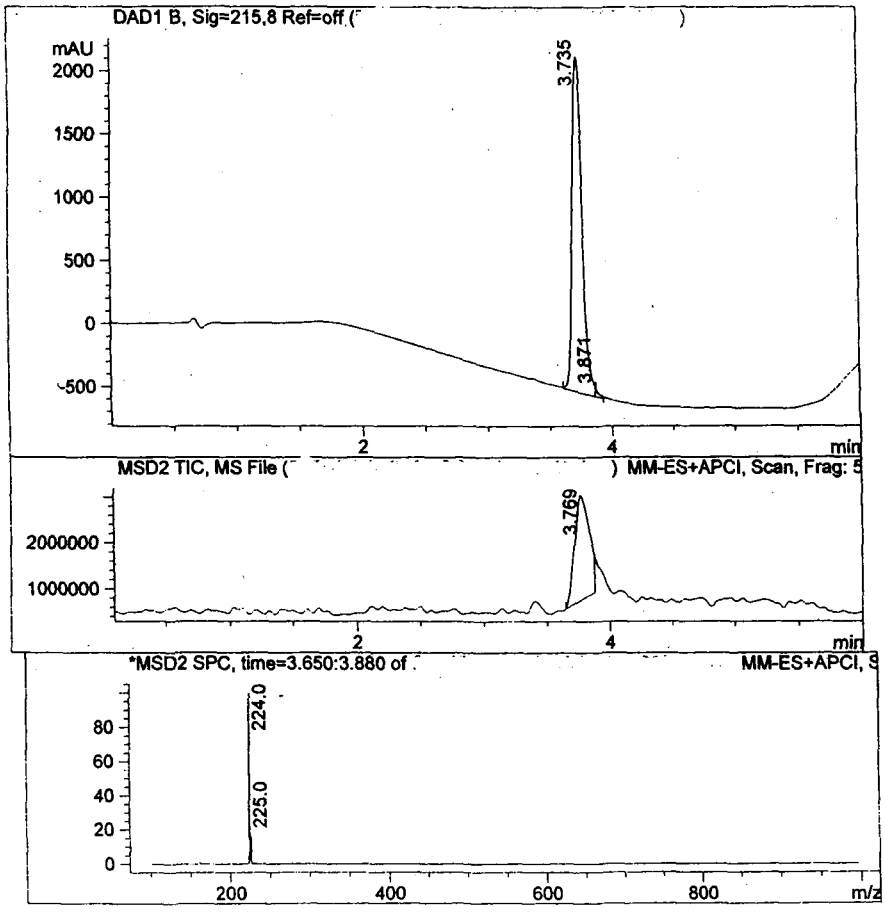
LC/MS REPORT

```
Data file        :
Injection Date   :  10/25/2008
Sample Name      :
Vial No.         :  P1-A-07
Acq Method       :  D:\METHODS\AT_3070FA.M
Injection vol    :  2.0ul
```

Method info : A-0.1%HCOOH;B-ACN Flow = 1.0ml/min, Column-Atlatis d C18 (50X4.6mm-5µm,) ,positive mode

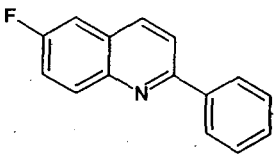
Time (min.): 0--2.5 2.5--4.0 4.0--4.5 4.5--6.0

% B : 30-95 95 95-30 30



Peak No	RT min	Area	Area %
1	3.73	14842.35	99.23
2	3.87	114.50	0.77

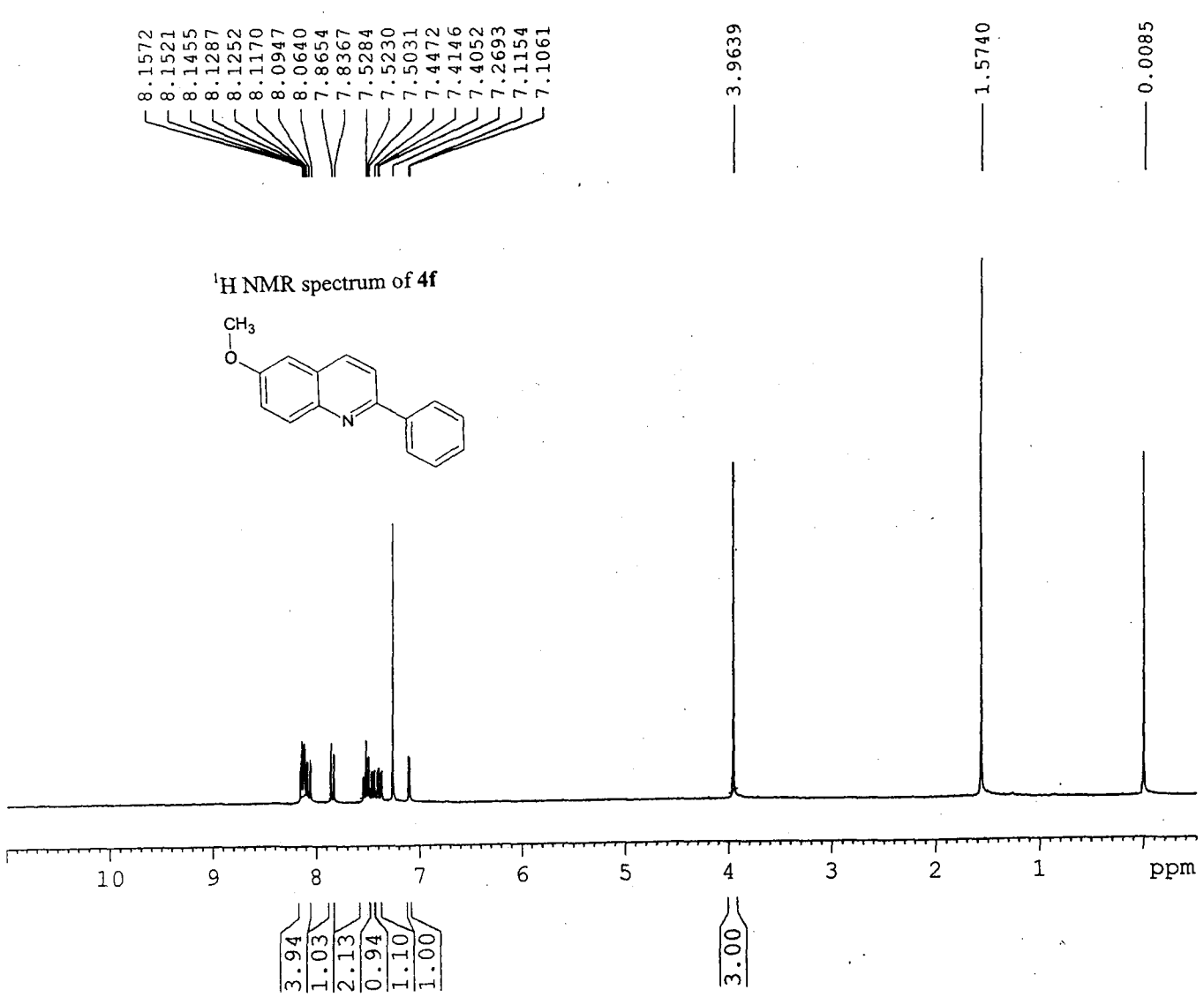
Mass spectrum of **4c**



Mass Calcd m/z=223.00

Found m/z=224.0(M+1)

Fig 2.7. Mass Spectrum of 6-fluoro-2-phenylquinoline

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Current Data Parameters
NAME
EXPNO                  1
PROCNO                 1

F2 - Acquisition Parameters
Date_           20081023
Time              14.39
INSTRUM           spect
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PULPROG            zg30
TD                32768
SOLVENT           CDCl3
NS                   16
DS                    2
SWH            7183.908 Hz
FIDRES        0.219235 Hz
AQ            2.2807028 sec
RG                645.1
DW               69.600 usec
DE                6.00 usec
TE                 0.0 K
D1            2.00000000 sec
MCREST        0.00000000 sec
MCWRK         0.01500000 sec

======== CHANNEL f1 ========
NUC1                  1H
P1                10.30 usec
PL1                0.00 dB
SFO1         300.1327012 MHz

F2 - Processing parameters
SI                32768
SF           300.1298168 MHz
WDW                  EM
SSB                   0
LB                 0.30 Hz
GB                    0
PC                 1.40
```

Fig 2.8. ^{1}H NMR Spectrum of 6-methoxy-2-phenylquinoline

```
Data file       :
Injection Date  :  10/23/2008
Sample Name     :
Vial No.        :  P1-E-02
Acq Method      :  C:\CHEM32\1\METHODS\AT_3070FA.M
Injection vol   :  2.0ul
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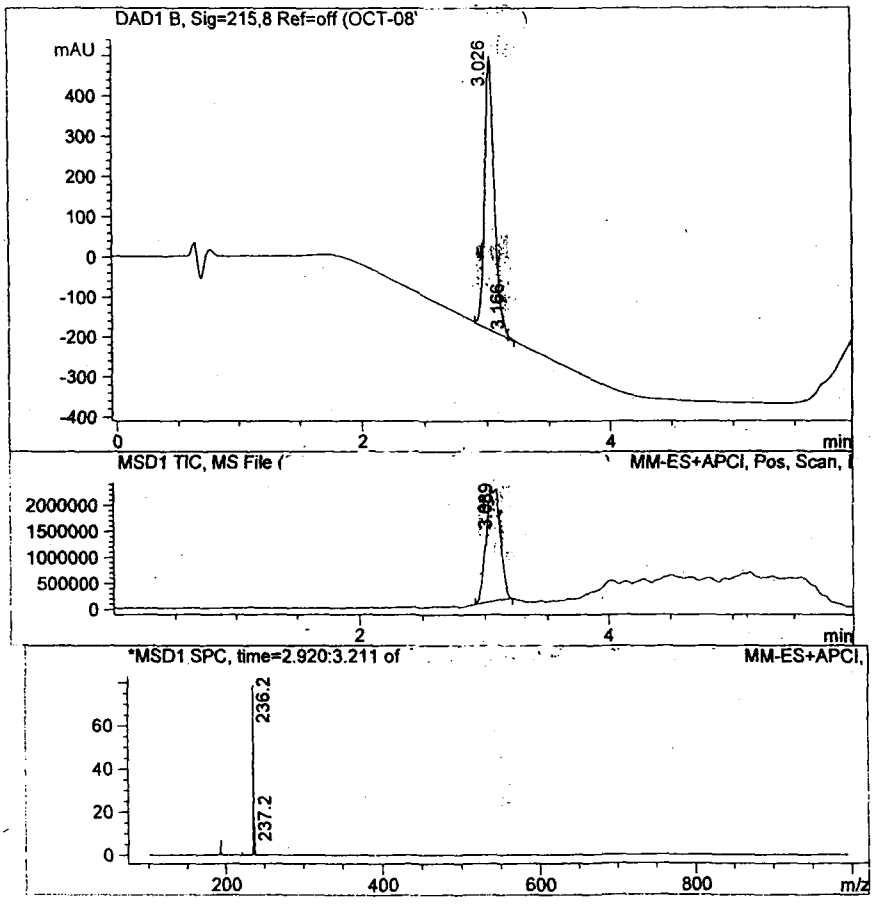
Method info : A-0.1%HCOOH;B-ACN Flow = 1.0ml/min, Column-Atlatis d C18 (50X4.6mm-5µm,) Positive Mode

Time (min.): 0--2.5 2.5--4.0 4.0--4.5 4.5--6.0

% B : 30-95 95 95-30 30



Peak No	RT min	Area	Area %
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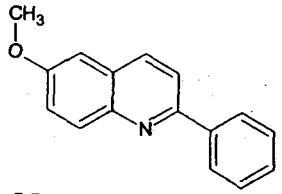
2	3.17	11.65	0.32

Mass spectrum of **4f**



Mass Calcd m/z=235.09

Found m/z=236.2(M+1)

Fig 2.9. Mass Spectrum of 6-methoxy-2-phenylquinoline

2.6 References

1. Manske. RHF, Kukla, M. *Org. React.* 1953;7:59.
2. Bergstrom FW. *Chem. Rev.* 1944;35:153.
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Chapter - 3

Synthesis, Characterization of 2-methyl-4-amino-tetrahydroquinoline Derivatives and Screening for Hepatoprotective and Anti-convulsant Activities

Chapter - 3

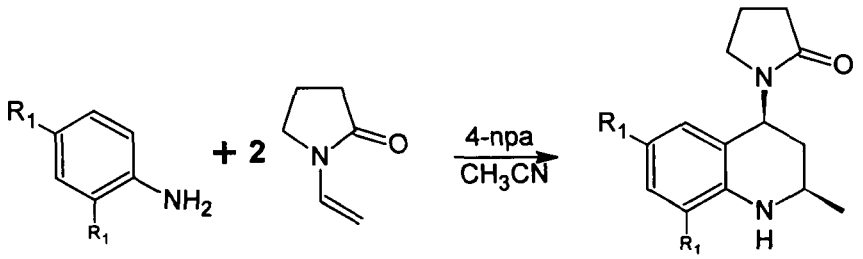
Synthesis, Characterization of 2-methyl-4-amino-tetrahydroquinoline Derivatives and Screening for Hepatoprotective and Anti-convulsant Activities

3.1 Introduction

Tetrahydroquinoline derivatives are an important class of natural products that exhibit biological activities[1]. Imino Diels–Alder reaction is a well-established route for the synthesis of nitrogen containing six membered heterocycles. Lewis acids[2] (BF_3 -OEt_3, $InCl_3$) and protic acids[3] (TFA, TsOH) has been found to catalyze imino Diels–Alder reaction with electron-rich dienophiles. Although the imino Diels–Alder reaction promoted by Lewis acids has been reported, more than a stoichiometric amount of Lewis acid is required coordinate the Lewis acids to imine nitrogen. To overcome this problem, the imino Diels–Alder reaction has been carried out successfully using urea nitrate[4], montmorillonite K-10 clay[5], oxalic acid[6], I_2[7], and rare-earth catalysts [8]. Recently, photochemically catalyzed Diels–Alder reaction of arylimines with *N*-vinylpyrrolidin-2-one has also been reported [9]. Recently, we have reported the utility of 4-nitro phthalic acidas catalyst in imino Diels–Alder reaction for the synthesis of pyrano and furanoquinolines[10]. In continuation of our work, we thought of using 4-nitro phthalic acid and other nitro derivatives of phthalic acid in synthesis of 2,4-substituted-1,2,3,4-tetrahydroquinolines.

3.2 Present Work

Since the 2-methyl tetrahydoquinolines are medicinally very important compounds, here we used our reported[11] protocol to obtain quantitative yield of 2-methyl-4-amino-tetrahydroquinolines **(scheme-1)** to evaluate for the hepatoprotective and anti-convulsant activities.



1 2 3

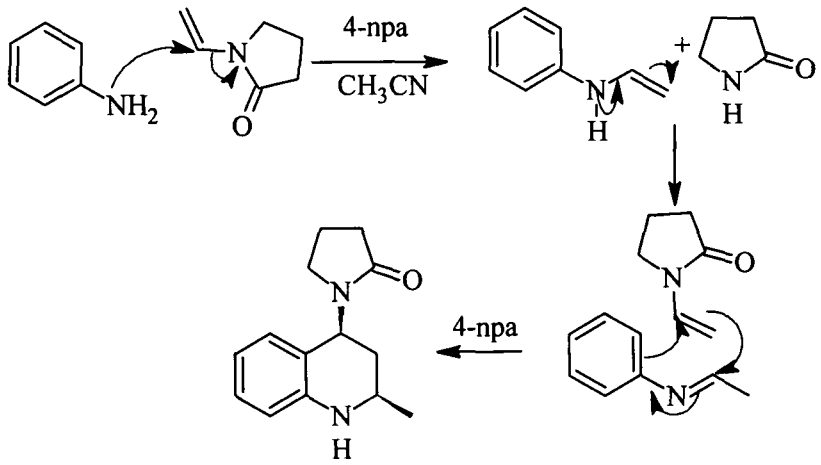
Scheme 1

Thus several aryl amines **(Table 3.1)** reacted smoothly with NVP to give corresponding tetrahydroquinolines in 88–95% yields in the presence of 50 mol% of 4-npa in acetonitrile at 50°C **(Scheme 1).** In all cases, the products **3a–3i** were obtained as cis-diastereoisomers. Their structural elucidations are based on ^{1}H NMR spectral data of the column-purified products. The relative trans orientations of H2, H3, and H4 were established from the large vicinal coupling constants between H-4 and H-3 (J = 11.9) and H-2 and H-3 (J = 12.3). From these results, we propose the possible following mechanism to account for the reaction. An aromatic amine first reacts with NVP to afford *N*-vinyl aniline, and the second step proceeds via the imino Diels–Alder reaction between *N*-acetylidene phenylamine and another molecule of NVP **(Scheme 2).** In conclusion, a very interesting and a facile synthesis of 1-(2-methyl-1,2,3,4-tetrahydroquinolin-4-yl) pyrrolidin-2-ones via 4-nitro phthalic acid–catalyzed hetero cyclization addition reaction between aryl amines and *N*-vinyl pyrrolidin-2-one has been developed.

Table 3.1. Synthesis of 2-methyl tetrahydroquinolines[a]

Entries	Product	R_1	R_2	Reaction time (h)	Yields (%)[a]
1	3a	Cl	H	3.5	92
2	3b	H	H	3.5	95
3	3c	OCH_3	H	2.5	88
4	3d	CH_3	H	2.0	90
5	3e	F	H	4.0	90
6	3f	Br	H	4.5	88
7	3g	H	CH_3	4.0	92
8	3h	H	OCH_3	3.0	90
9	3i	H	F	4.0	92

[a]Isolated yields.



Scheme 2

3.3 Experimental

All the melting points were recorded in open capillaries. The purity of the compounds was checked by thin-layer chromatography (TLC) on silica gel, and they were purified by column chromatography. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker 400-Hz spectrometer using TMS as an internal standard. IR spectra were

obtained using a FTS-135 spectrometer instrument. Mass spectra were recorded on a LC-MS instrument.

3.3.1 General Reaction Protocol

The mixture of aryl amines (5 mmol), N-vinyl pyrrolidin-2-one (12 mmol), and 4-nitro phthalic acid (2.5 mmol) in acetonitrile (5 ml) was stirred at 50^0 C for the appropriate time (Table 2). After completion of reaction as indicated by TLC, the reaction mixture was quenched with saturated aqueous $NaHCO_3$ solution (20 ml) and extracted with ethyl acetate (2-15 ml). The combined organic layer was dried over anhydrous sodium sulphate and concentrated, and the crude product was purified by column chromatography on silica gel (60–120 mesh, ethyl acetate–petroleum ether, 3:7) to afford 2-methyl-1,2,3,4-tetrahydroquinolines.

Finally all the newly synthesized 2-methyl-tetrahydroquinolines have been screened for hepatoprotective and anticonvulsant activities in animal models

3.4 Pharmacological Evaluation

3.4.1 Anticonvulsant activity of 2-methyl-tetrahydroquinolines

Anticonvulsant activity for the newly synthesized 2-methyl tetrahydroquinolines was carried out using MES and PTZ tests as described in chapter-2.

3.4.1.1 Materials and methods

a) Animals

As described in 2.4.1.1a

b) Anticonvulsant activity[12,13]

As described in 2.4.1.1b

c) Statistical analysis

The data were analyzed by using one way ANOVA followed by Tukey's multiple comparison test. The level of significance was set at $P<0.05$.

3.4.1.2 Results

Results revealed that 2-methyl tetrahydroquinolines such as **2a, 2c, 2e, 2h** and **2i** significantly reduced the tonic extensor phase and the incidence of convulsions in MES induced seizures (Table 3.2). Among them **2a** ($P< 0.001$), **2c** and **2h** ($P< 0.001$) were found to be more potent than other compounds. **2e** and **2i** exhibited moderate activity.

Among the 2-methyl tetrahydroquinolines 2a, ($P<0.01$), 2c ($P<0.01$), 2f ($P<0.05$) and 2i ($P<0.01$) significantly enhanced the time of onset of clonic phase and protected mice at all levels of seizure activity induced by PTZ (Table 3.3).

3.4.1.3 Structure activity relation ship

Substitution of Chlorine **(2a),** OCH_3 **(2c),** and fluorine **(2e)** groups at 6^{th} position of 2-methyl tetrahydroquinoline significantly decreased the tonic extensor phase, thus protecting the animal from MES induced seizures. OCH_3 **(2h)** and fluorine **(2i)** substitution at 8^{th} position of 2-methyl tetrahydroquinoline also potentiated the anticonvulsant activity in MES test. In PTZ test substitution at 6^{th} position with chlorine **(2a),** bromine **(2f)** and methoxy **(2c),** and fluorine **(2i)** on 8^{th} position of 2-methyl tetrahydroquinoline significantly increased the onset of all phases of seizures and clonic phase thus inhibiting the action of PTZ.

Table 3.2. Effect of 2-methyl-4-amino-tetrahydroquinolines on MES induced seizures in rats

Treatment	Dose (mg/kg)	Duration of tonic extensor phase (sec) ± SE	Incidence of convulsions
Control (MES)	-	12.67±0.80	6/6
Phenytoin	25 mg/kg i.p.	0.166±0.16[c]	1/6
2a	25 mg/kg p.o.	4.55±0.44[c]	2/6
2b	25 mg/kg p.o.	10.50±1.05	6/6
2c	25 mg/kg p.o.	3.66±0.33[c]	2/6
2d	25 mg/kg p.o.	10.45±2.12	6/6
2e	25 mg/kg p.o.	5.50.22±0.52[b]	4/6
2f	25 mg/kg p.o.	11.25±1.35	6/6
2g	25 mg/kg p.o.	9.75±1.58	6/6
2h	25 mg/kg p.o.	3.33±0.49[c]	2/6
2i	25 mg/kg p.o.	6.66±0.84[a]	5/6

Values are mean ± SE from 6 animals in each group. [a] P< 0.05, [b] P< 0.01, [c] P< 0.001 compared to MES control group.

Table 3.3. Effect of 2-methyl-4-amino-tetrahydroquinolines on PTZ induced seizures in mice

Treatment	Dose (mg/kg)	Onset of clonic phase (min) ± SE	Death/Recovery n = 6
Control (PTZ)	70 mg/kg s.c.	10.49±0.40[c]	5/1
Phenytoin	25 mg/kg i.p.	27.74±1.0	0/6
2a	25 mg/kg p.o.	18.45±1.76[b]	2/4
2b	25 mg/kg p.o.	17.87±0.72[b]	0/6
2c	25 mg/kg p.o.	18.61±1.65[b]	2/4
2d	25 mg/kg p.o.	14.22±0.54	4/2
2e	25 mg/kg p.o.	15.33±1.04	5/1
2f	25 mg/kg p.o.	17.32±0.83[a]	3/3
2g	25 mg/kg p.o.	17.89±1.31[b]	1/5
2h	25 mg/kg p.o.	15.26±0.74	4/2
2i	25 mg/kg p.o.	19.32±2.63[b]	2/4

Values are mean ± SE from 6 animals in each group. a P< 0.05, b P< 0.01, [c] P< 0.001 compared to PTZ treated group.

3.4.2 Hepatoprotective activity of 2-methyl-4-amino-tetrahydroquinolines

Antihepatotoxic activity of 2-methyl-4-amino-tetrahydroquinolines was studied in CCl_4 induced hepatotoxicity at subacute level using mice.

3.4.2.1 Materials and methods

a) Animals

As described in 2.5.1a

b) Experimental protocol (sub acute study- 5 days)

Hepatoprotective activity was carried out using Swiss mice (6-8 weeks old). Animals were divided into various groups of six animals in each group. **Group I:** Served as control (normal saline 5 ml/kg, p.o.). **Group II:** Served as negative control (CCl_4/olive oil (1:1), 1 ml/kg, i.p.) on 3rd and 4th day). **Group III**: Treated with Silymarin 100 mg/kg, p.o for successive five days. All the animals except control group received CCl_4 (1 ml/kg, i.p.) on 3rd and 4th day. **Test groups:** suspensions of 2-methyl-4-amino-tetrahydroquinolines in polyethylene glycol-400 at the dose of 25 mg/kg p.o. for five days. On the fifth day 2 hr after the administration of the last dose, blood samples were collected from the retro-orbital plexus, serum was separated by centrifugation and used for biochemical studies[14,15].

c) Biochemical studies

As described in 2.5.1c

3.4.2.2 Results

A highly significant ($P<0.001$) elevation was observed for all the parameters in CCl_4 intoxicated mice compared to normal control. Pretreatment with **2a, 2c** and **2f** derivatives of 2-methyl-4-amino-tetrahydroquinolines a significant decline was observed in SGOT, SGPT, ALP and total and direct bilirubin activity with respect to CCl_4 intoxicated mice. The results are shown in Table 3.4.

3.4.2.3 Structure activity relationship

Substitution with chlorine **(2a),** OCH_3 **(2c)** and bromine **(2f)** at 6th position of 2-methyl tetrahydroquinoline nucleus significantly protected the liver by decreasing the elevated levels of serum GOT, GPT, ALP, total and direct bilirubin thus protecting the liver by toxic effects induced by CCl_4. However, OCH_3 at 8th position **(2h),** fluorine at 6th and 8th position **(2e and 2i),** CH_3 at 6th and 8th position **(2d and 2g)** of 2-methyl tetrahydroquinoline did not show any protection against CCl_4 induced toxicity.

Table 3.4. Effect of 2-methyl-4-amino-tetrahydroquinolines on serum biochemical parameters in CCl_4 induced hepatotoxicity in mice (Acute study- 5 days)

Design of treatment	SGOT (U/ml)	SGPT (U/ml)	ALP (U/ml)	T. Bil (mg/dl)	D. Bil. (mg/dl)
Normal control	127.43±2.33	62.60±0.30	140.0±0.63	1.00±0.02	0.186±0.01
Carbon tetra-Chloride (1ml/kg on 3rd and 4th day	423.45±7.31^c	291.1± 1.44^c	437.6±2.19	3.66±0.15^c	1.585±0.072
Silymarin (100 mg/kg)	152.22 ± 3.10^c	75.34±2.00^c	163.81±4.28^c	0.96±0.05^c	0.24±0.02^c
2a	341.5±6.294^c	268.4±0.82^a	375.9±5.16^c	2.95±0.03^b	1.275±0.05^a
2b	381.3±9.250	285.9±2.26	426.0±2.89	3.09±0.07	1.447±0.02
2c	396.4±2.808	286.4±1.57	425.5±4.34	3.20±0.02	1.292±0.039^a
2d	367.2±4.05^a	266.32±4.30^a	378.1±5.62^c	2.88±0.04^a	1.19±0.05^b
2e	397.23±5.07	278.54±6.72	426.21±6.21	3.10±0.08	1.32±0.04
2f	355.3±2.33^c	257.1±6.22^c	285.5±3.22^c	2.54±0.32	1.25±0.06^b
2g	390.5±8.299	287.2±1.33	430.6±2.45	3.182±0.23	1.448±0.02
2h	394.5±8.32	277.2±3.33	423.6±4.21	3.04±0.30	1.55±0.04
2i	394.23±5.07	288.36±6.72	422.25±7.21	3.20±0.10	1.34±0.05

Values are mean ± SE from 6 animals in each group. ^{a} P< 0.05, ^{b} P< 0.01, ^{c} P< 0.001 compared to CCl_4 treated group.

3.5 Spectral Data

cis-1-(2-Methyl-6-chloro-1,2,3,4-tetrahydroquinolin-4-yl)pyrrolidin-2-one:3a

Colorless crystalline solid, mp 150–152 ^{0}C; IR (KBr): ν = 3395 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.24 (d, 3H, J = 6.2 Hz), 1.75 (ddd, 1H, J = 12.3, 5.5, 2.2 Hz), 1.95 (ddd, 1H, J = 11.6, 5.9, 2.4 Hz), 1.99–2.15 (m, 2H), 2.42–2.59 (m, 2H,), 3.11–3.30 (m, 2H), 3.47–3.61 (m, 1H), 4.5 (brs, 1H), 5.5 (dd, 1H, J = 11.9, 5.9 Hz), 6.48 (d, 1H, J = 8.5 Hz), 6.78 (s, 1H), 6.96 (dd, 1H, J = 8.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 18.2, 22.0, 31.3, 33.5, 42.2, 47.0, 47.8, 116.0, 120.8, 122.7, 126.3, 129.0, 144.0, 175.8; MS: m/z = 265 (M+1).

cis-1-(2-Methyl-1,2,3,4-tetrahydroquinolin-4-yl)pyrrolidin-2-one: 3b

Colorless crystalline solid, mp 70–72 ^{0}C; IR (KBr): ν = 3348 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.26 (d, 3H, J = 6.0 Hz), 1.65 (ddd, 1H, J = 12.1, 5.7, 1.9 Hz), 1.94 (ddd, 1H, J = 12.0, 5.79, 2.3 Hz), 1.99–2.16 (m, 2H), 2.44–2.68 (m, 2H), 2.98– 3.13 (m, 2H), 3.43–3.54 (m, 1H), 4.34 (brs, 1H), 5.65 (dd, 1H, J = 11.5, 5.8 Hz), 6.67 (td, 1H, J = 8.0), 6.8 (t, 1H, J = 7.5 Hz), 6.99 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$): δ = 18.2, 22.0, 31.3, 33.5, 42.2, 47.0, 47.8, 114.3, 117.9, 122.9, 127.7, 128.5, 145.0, 175.8 ppm; MS: m/z = 231 (M+1).

cis-1-(2-Methyl-6-methoxy-1,2,3,4-tetrahydroquinolin-4-yl)pyrrolidin-2-one:3c

Pale yellow crystalline solid, mp 90–92 ^{0}C; IR (KBr): ν= 3341 cm^{-1}; ^{1}H NMR (400MHz, $CDCl_3$): δ = 1.24 (d, 3H, J = 6.2 Hz), 1.75 (ddd, 1H, J = 12.1, 5.4, 2.0 Hz), 1.98 (ddd, 1H, J = 12.0, 5.8, 2.2 Hz), 2.01–2.19 (m, 2H), 2.41–2.57 (m, 2H), 3.04–3.16 (m, 2H), 3.41–3.61 (m, 1H), 3.8 (s, 3H), 4.22 (brs, 1H), 5.43 (dd, 1H, J = 11.4, 6.0 Hz), 6.47 (d, 1H, J = 8.7 Hz), 6.67 (dd, 1H, J = 8.7, 2.7 Hz), 6.98 (d, 1H, J = 2.9 Hz); ^{13}C

NMR (400 MHz, $CDCl_3$): δ = 17.8, 22.0, 31.8, 33.4, 42.2, 46.7, 48.8, 56.7, 112.6, 115.2, 115.7, 124.3, 139.1, 152.7, 175.6 ppm; MS: m/z = 261 (M+1).

cis-1-(2,6-Methyl-1,2,3,4-tetrahydroquinolin-4-yl)pyrrolidin-2-one:3d

Pale yellow crystalline solid, mp 95–96 _C; IR (KBr): v = 3422 cm^{-1}; ^1H NMR (400 MHz, DMSO): δ = 1.15 (d, 3H, J =5.1 Hz), 1.64 (q, 1H, J = 11.9 Hz), 1.76 (ddd, 1H, J = 12.3, 5.6, 2.3 Hz), 1.97–2.12 (m, 2H), 2.21 (s, 3H), 2.25–2.45 (m, 2H), 2.95–3.22 (m, 2H), 3.39–3.55 (m, 1H), 5.25 (dd, 1H, J = 11.6, 6.0 Hz), 5.47 (brs, 1H), 6.42 (m, 2H), 6.72 (d, 1H, J = 8.0 Hz; ^{13}C NMR (100MHz, DMSO): δ = 17.72, 20.2, 21.78, 30.74, 33.8, 41.6, 46.1, 47.4, 114.1, 124.2, 126.0, 128.1, 129.3, 144.1, 174.4 ppm; MS: m/z = 245 (M+1).

cis-1-(2-Methyl-6-fluoro-1,2,3,4-tetrahydroquinolin-4-yl)pyrrolidin-2-one: 3e

Colorless crystalline solid, mp 138–140 ^0C; IR (KBr): v = 3258 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 1.22 (d, 3H, J = 5.8 Hz), 1.74 (ddd, 1H, J = 11.8, 5.6, 2.2 Hz), 1.91 (ddd, 1H, J = 12.0, 5.6, 2.1 Hz), 2.09–2.21 (m, 2H), 2.45–2.61 (m, 2H), 3.04– 3.18 (m, 2H), 3.42–3.55 (m, 1H), 4.25 (brs, 1H), 5.48 (dd, 1H, J = 11.4, 5.8 Hz), 6.60 (m, 2H) 6.78 (td, 1H, J = 8.5, 2.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.6, 21.6, 30.6, 33.2, 41.6, 46.3, 47.4, 116.0, 120.8, 122.7, 126.3, 129.0, 142.7, 174.7 ppm; MS: m/z = 249 (M+1).

***cis*-1-(6-bromo-2-methyl-1,2,3,4-tetrahydroquinolin-4-yl)pyrrolidin-2-one (3f)**

White crystalline solid; mp.158-162 ^0C; ^1H NMR (300 MHz, $CDCl_3$): δ = 1.22 (d, J = 6.3 Hz, 3H), 1.67-1.75 (m, 1H), 2.0 (ddd, J = 8.3, 6.0, 2.3 Hz, 1H), 2.00-2.10 (m, 2H), 2.42-2.59 (m, 2H), 3.12-3.29 (m, 2H), 3.56 (dqd, J = 8.4, 6.3, 2.2 Hz, 1H), 3.76 (s, 1H), 5.52 (dd, J = 12.0, 6.0 Hz, 1H), 6.40 (d, J = 8.5 Hz, 1H) 6.89-6.90 (m, 1H), 7.07-

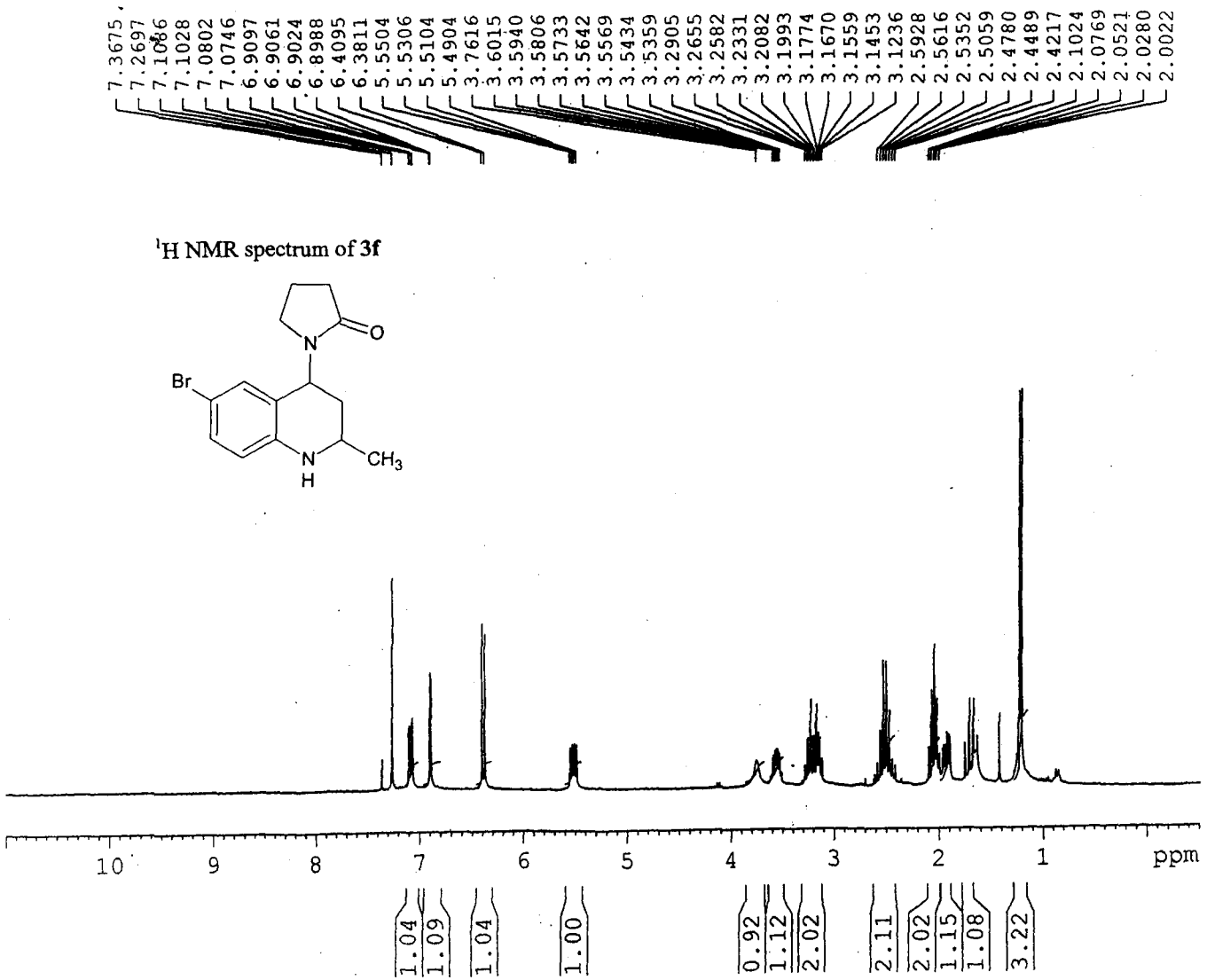
7.10 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.1, 21.7, 37.2, 38.3, 44.0, 47.6, 51.1, 111.8, 114.5, 125.9, 130.1, 133, 142.5, 173.3; MS: *m/z* = 309 (M+1). Anal. Calcd for $C_{14}H_{17}BrN_2O$: C, 54.38, H, 5.54, N, 9.06. Found: C, 54.21, H, 5.56, N, 9.1 (Fig. 3.1 and 3.2).

cis-1-(2,8-Methyl-1,2,3,4-tetrahydroquinolin-4-yl)pyrrolidin-2-one:3g

Colorless crystalline solid, mp 86–88 ^{0}C; IR (KBr): ν = 3415 cm^{-1}; ^{1}H NMR (400 MHz, CDCl3): δ = 1.28 (d, 3H, J = 5.8 Hz), 1.71 (q, 1H, J = 11.2 Hz), 1.78–1.89 (m, 1H), 1.94–2.08 (m, 2H), 2.16 (s, 3H), 2.24–2.42 (m, 2H), 2.78–2.97 (m, 2H), 3.32– 3.41 (m, 1H), 3.78 (brs, 1H), 4.99 (dd, 1H, J = 11.1, 5.9 Hz), 6.42 (m, 2H), 6.95 (dd, 1H, J = 7.9, 1.5 Hz), 7.28 (dd, 1H, J = 8.0, 1.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.81, 20.5, 23.6, 31.4, 33.5, 40.7, 46.1, 47.4, 116.9, 121.6, 122.5, 125.7, 130.1, 143.3, 174.5 ppm; MS: m/z = 245 (M+1).

cis-1-(2-Methyl-8-methoxy-1,2,3,4-tetrahydroquinolin-4-yl)pyrrolidin-2-one:3h

Colorless crystalline solid, mp 78–80 ^{0}C; IR (KBr): ν = 3341 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.24 (d, 3H, J = 6.2 Hz), 1.77 (ddd, 1H, J = 11.9, 5.3, 1.8 Hz), 1.94–2.17 (m, 3H), 2.36–2.49 (m, 2H), 3.08–3.19 (m, 2H), 3.39–3.58 (m, 1H), 3.76 (s, 3H), 4.09 (brs, 1H), 5.42 (dd, 1H, J = 11.7, 6.2 Hz), 6.62–6.83 (m, 2H), 6.99–7.11 (m, 1H); ^{13}C NMR (100 MHz, CDCl3): δ = 17.4, 22.5, 33.4, 30.8, 42.5, 46.5, 49.3, 56.1, 109.1, 116.4, 119.6,123.3, 135.5, 146.7, 175.6 ppm; MS: m/z = 261 (M+1).



^{1}H NMR spectrum of **3f**

```
Current Data Parameters
NAME
EXPNO                  1
PROCNO                 1

F2 - Acquisition Parameters
Date_           20081023
Time              14.16
INSTRUM           spect
PROBHD   5 mm DUL BB-1H
PULPROG           zg30
TD                32768
SOLVENT           CDCl3
NS                    8
DS                    2
SWH            7183.908 Hz
FIDRES        0.219235 Hz
AQ            2.2807028 sec
RG                  362
DW               69.600 usec
DE                6.00 usec
TE                 0.0 K
D1           2.00000000 sec
MCREST       0.00000000 sec
MCWRK        0.01500000 sec

======== CHANNEL f1 ========
NUC1                1H
P1              10.30 usec
PL1              0.00 dB
SFO1     300.1327012 MHz

F2 - Processing parameters
SI                32768
SF       300.1298168 MHz
WDW                EM
SSB                 0
LB               0.30 Hz
GB                  0
PC                1.40
```

Fig 3.1. ^{1}H NMR Spectrum of *cis*-1-(6-bromo-2-methyl-1,2,3,4-tetrahydroquinolin-4-yl)pyrrolidin-2-one

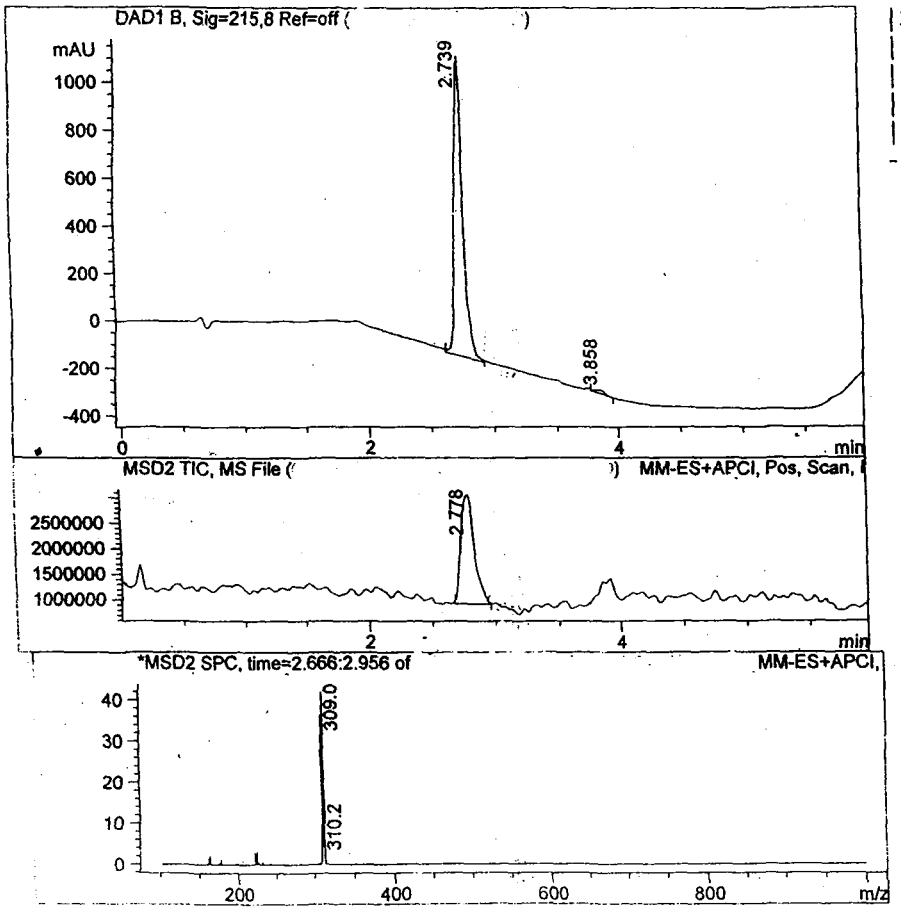
LC/MS REPORT

```
Data file       :
Injection Date  :  10/23/2008
Sample Name     :
Vial No.        :
Acq Method      :  C:\CHEM32\1\METHODS\AT_3070FA.M
Injection vol   :  2.0ul
```

Method info : A-0.1%HCOOH;B-ACN Flow = 1.0ml/min, Column-Atlatis d C18 (50X4.6mm-5µm,) Positive Mode

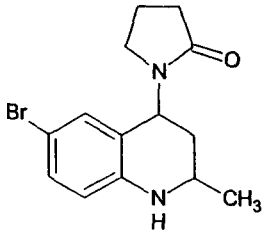
Time (min.): 0--2.5 2.5--4.0 4.0--4.5 4.5--6.0

% B : 30-95 95 95-30 30



Peak No	RT min	Area	Area %
1	2.74	5991.76	98.47
2	3.86	93.14	1.53

Mass spectrum of **3f**



Mass Calcd m/z=309

Found m/z=309

Fig 3.2. Mass Spectrum of *cis*-1-(6-bromo-2-methyl-1,2,3,4-tetrahydroquinolin-4-yl)pyrrolidin-2-one (3f)

3.6 References

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Chapter - 4

Introduction to Medicinal Plants

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Introduction to Medicinal Plants

4.1 Historical Prospective

The relationship between man and other elements of nature is as old as the history of mankind itself. The material culture of nearly all the civilizations throughout the world is based more on plants than on animals. The people have long dependent on plants for food, clothing, shelter, transportation, medicines, rituals and traditions[1]. From the dawn of human existence, countless generations of humanity patiently experimented with and discovered wide variety of plants that cure various diseases[2].

The use of plants as medicinal agents presumably predates the earliest recorded history as the earliest humans used various, but specific plants to treat illness. The earliest mention of medicinal uses of the plants was from the Indian subcontinent. *Rig veda* was written between 4500 and 1600 B.C. was considered as oldest text with number of references about the medicinal herbs[3]. *Susruta samhita* written around 1000 B.C contains a comprehensive chapter on natural therapeutics, where as *Charaka samhita* written in the same period provides a comprehensive description of the *Materia Medica* and it was practiced by ancient Indians. Later during the Buddhist period considerable progress was made in this field and medicinal plants were cultivated under the supervision of specialists. Further relationship of Indians with Greeks and Romans and later with Arabs and Persians further enriched Indian Materia medica and large number of plants and natural products for the curative purposes. The Egyptians described the use of medicinal plants for the treatment of various diseases as early as 1550 B.C. in the *Ethers papyrus.* Traditional Chinese system with its complex range of pharmaceutical preparations called

'fongs' also utilized a wide variety of plants. The written texts of traditional Chinese medicine can be traced back *Shen Nong Ben Cao Jing* period (22- 250 A.D). The book *Ben Cao Gang Mu,* written by a great physician and naturalist, *Li Shizhen,* has been regarded as comprehensive pharmacopoeia containing information on thousands of medicinal plants. *Ben Cao Gang Mu* is considered as a classic and still serves as a valuable reference for the teaching and practices of medicinal plants used in China. Many Arab-Muslim scientists contributed remarkably in the field of natural products. Ibn-Al-baitar, a great botanist and pharmacist listed medicinal plants and their traditional importance *"Kitab-al-jami fi al-Mufradat"*. This book was compiled by referring the work of 150 authors including 20 early Greek scientists. Another well known scientist Al-Idrisi (1099-1166 A.D) contributed the field by his work entitled *"Kitab al jami-li-sifat ashtatal-nabata"*, which includes several plant derived drugs in different languages. Al-Tabari (883-880 A.D.) wrote a book *"Firdous al Hakima"* comprising of seven parts and explaining about drugs and poisons. Ibne Sina (980-1037) described 769 herbal drugs in his famous book *Quanum fi al-Tibb* which become known as canon in the west. It was considered to be the most authentic materia medica of that era. Another great physician Muhammed Ibn Zakariya Al-Razi (864-930 A.D) wrote a very famous voluminous book called *"Kitab Al Mansoori"* which mainly explains about Greco-Arab medicine. Besides, he was the first physician to use Opium as an anesthetic. In Western tradition, many authors including well known personalities as Dioscorides and Galen in the first and second centuries to Culpepper in the seventeenth century described the chemistry and medicinal properties of medicinal plants and their products. In Europe, after the tenth century, much of the medicinal lore was based in the church, particularly the monastic

orders, but by the 1500's, after the invention of the printing press, herbals available to the general public were popular, particularly in England. By the late 1700's, studies like William Withering's *An Account of the Foxglove and its Medicinal Uses* (1785) began to appear. These were based on case histories and described specific doses and gave administration instructions for herbal remedies. In the United States, before the advent of specific pharmaceuticals, herbal medicine was relied upon to treat many illnesses. Development of drugs based on natural products had a long history in the United States, and in 1991, almost half of the best selling drugs were natural products or derivatives of natural products. There has recently been a resurgence of interest in herbal remedies, and a Reuters/Zogby poll in 2000 showed that 40% of people in the U.S. had tried herbal remedies. In 1998, the U.S. market for natural supplements was over $12 billion in sales and increasing by as much as 10% per year. Herbs such as St. John's Wort, Ginkgo, Echinacea, and ginseng are among the most popular herbs. In 1999, Echinacea was reported to make up 38% of the U.S. market, with ginkgo a close second at 34%. The efficacy of these herbs is being investigated in many laboratories, and efforts are also being made to isolate and identify any active constituents.

4.2 Medicinal Plants and the Development of Novel Clinical Drugs

Higher plant-derived products represent around 25% of the total number of clinically used drugs and include the classical drugs atropine, codeine, digoxin, morphine and quinine etc. Investigation of novel compounds from plants utilizes the advanced techniques of phytochemical analysis. The separation, identification and structural determination of biologically active compounds has been facilitated by continual development of chromatographic and spectroscopic methods of analysis and these

techniques are becoming more sophisticated[4]. The NMR techniques of COSY and HECTOR are available for establishing connectivities between neighboring protons and between linked ^{1}H and ^{13}C, INEPT being used for long range heteronuclear correlations over 2-3 bonds. The applications of TLC, HPLC and HPLC coupled with UV photodiode array detection (LC-UV), LC-MS, electrospray and LC-NMR techniques for the separation and structure determination of enormous plant compounds are unquestionable. Computer modeling greatly assists spectrum interpretation and the generation of chemical structures meeting the spectral properties obtained. The computer system utilizes ^{1}H, ^{13}C, 2D-NMR, IR and MS spectral properties. Hyphenated chromatographic and spectroscopic techniques are powerful analytical tools that are combined with throughput biological screening in order to avoid re-isolation of known compounds. Hyphenated chromatographic and spectroscopic techniques include LC-UV-MS, LC-UV-NMR, LC-UV-MS and GC-MS.

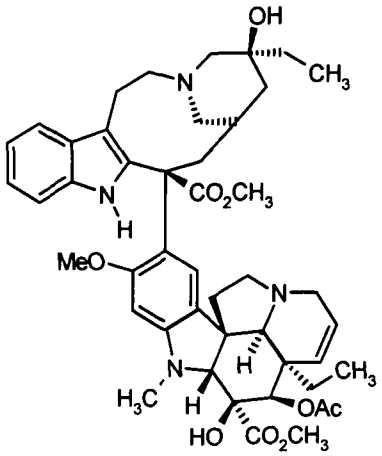
A successful strategy for investigating plants for biologically active compounds involves initial screening followed by bioassay-guided fractionation to aid isolation of active constituents. The ability to use automated throughput screening for biological activities helped the pharmaceutical industry to renew its interest in plants as potential sources of novel drugs. Bioassay guided fractionation resulted in the isolation of individual active compounds including indole alkaloids, proanthocyanidins, flavanoids and triterpenes [5, 6]. Biological activities were assessed by high throughput screening using receptors, enzymes and mechanism based cellular assays for cardiovascular, respiratory, gastrointestinal, autoimmune and analgesic activities. More than 200 biologically active compounds were identified and chemical structures of many compounds were established using NMR techniques[7]. In order to generate novel chemical entities with potential for new drug development, the technique of combinatorial synthesis has been developed.

Progress in isolation methods and structure elucidation has influenced an increase in the number of scientific publications dealing with pharmacological examinations of individual compounds of plant origin.

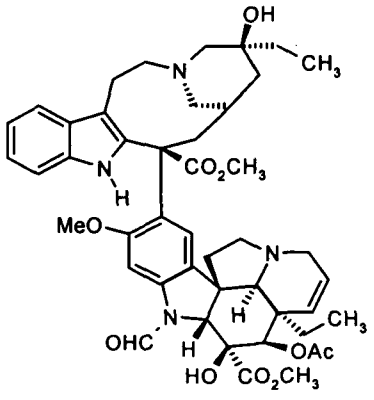
Higher plant extracts and their secondary metabolites like alkaloids, phenolics and terpenes etc. have been identified active compounds for anti-inflammatory, anticancer, immunostimulant, anti-infective, anti-stress, antidiabetic, hepatoprotective and cardioprotective activity [8].

4.2.1 Anticancer drugs

The *Catharanthus* alkaloids **Vinblastine (1)** and **Vincristine (2)** are currently used for the treatment of leukaemias, lymphomas and some solid tumors, were introduced through the Eli Lilly Company in the 1960s. Between 1960 and 1986 more than 35,000 species and thousands of extracts were screened against murine tumors and from 11 compounds approved for extensive tumour panel testing, 2 came in to clinical use. Semisynthetic analogues of vinblastine and vincristine in clinical use include vindesine, vinorelbine [9].

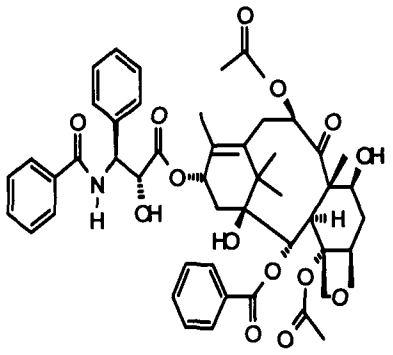


(1)



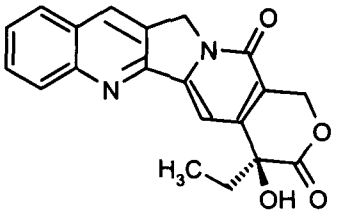
(2)

An extract of the bark of the *Taxus baccata* and *Taxus brevifolia* was shown to be highly active in the KB anti-tumour test [10]. It was not until 1971 that the chemical structure of the active compound **Taxol (3)** was determined. It is introduced in the clinical practice since it promoted the assembly of tubulin in to stable microtubules. Currently taxol is used for the treatment of ovarian cancer and in the secondary treatment of breast cancer. A semisynthetic analogue, taxotere , is used in adjuvant treatment of breast cancer and non-small cell lung cancer[10].



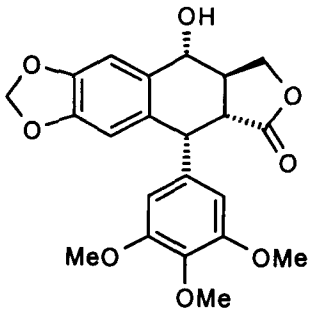
(3)

Camptothecin (4) was isolated from the fruits of *Camptotheca acuminate*. It was subsequently shown to be a topoisomerase I inhibitor[9]. It occurs along with 10-hydroxycamptothecin which proved to be more active in anti-cancer test systems. Further modifications to the molecule were made to improve water solubility and lower toxicity which resulted in two new clinical drugs, topotecan which is used in the treatment of metastasis ovarian cancer and irinotecan is used to treat colorectal cancer.



(4)

Podophylotoxin (5) isolated from *Podophyllum* species have reputations for treating cancers. Further attempts were made to improve the solubility and modify its toxicity. Its semisynthetic derivatives like Etoposide and teniposide are used in the treatment of small cell lung cancer, lymphomas, testicular cancer and brain tumors respectively. These derivatives are found to be topoisomerase II inhibitors and prevent the DNA synthesis.



(5)

4.2.2 Anti-infective drugs

Infectious diseases caused by bacteria, fungi, viruses and parasites are a major threat to public health despite tremendous growth in human chemotherapeutic medicine. Bacterial infections continue to pose a threat to health in many institutional and communal settings, including hospitals and other health care institutions, hotels, cruise liners, and damaged buildings, and epidemics are frequently reported.

Tuberculosis (TB), an infectious disease caused by different species of Mycobacterium, represents a worldwide public health problem and infects >30% of the global population[11]. Nearly 2 million people died of TB, with a global case fatality rate of 23% and reaching > 50% in some African countries due to high rates of coexisting HIV infection. Man infected with HIV is very susceptible to tuberculosis. Emergence of drug resistant strains of *Mycobacterium tuberculosis* has led to increased concern on current

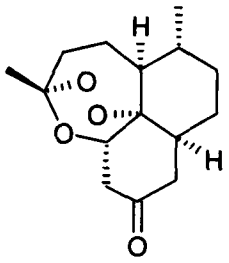
chemotherapy regimes[12]. Worldwide increase in the incidence of morbidity and mortality from tuberculosis prompted WHO to declare this disease a global emergency in the early 1990s[13]. The need for new antituberculosis agents is urgent due to increasing resistance of mycobacterium, together with increased incidence of severe disseminated infections produced by mycobacterium other than tuberculosis in immunocompromised patients, have prompted the search for new antimycobacterial agents, preferably those that can readily and simply be produced from some local natural plant sources.

An increase in the prevalence of antimicrobial resistance has made the selection of effective empiric antimicrobial therapy ever more challenging because the initiation of inadequate therapy (by in vitro susceptibilities) has been associated with much worse clinical outcomes, especially with bacteremia or pneumonia.[14,15].

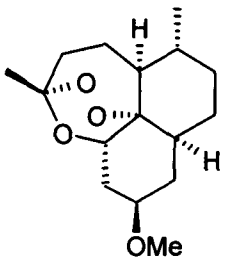
Plants with antimicrobial activity are also known to be numerous, minimal research had been conducted in the area of antimicrobial medicinal plants[16]. Considering the increased incidence of severe opportunistic fungal and bacterial infections in immunologically deficient patients together with the development of resistance among pathogenic gram positive, gram negative bacteria and *Candida albicans,* there is a great need in finding new classes of natural products that may be effective against antibiotic-resistant bacteria and fungi. Natural products or their semisynthetic derivatives provide novel examples of such anti-infective drugs, because of the resistance against antibiotics, there is a great interest in search of new antimicrobial agents from the nature[17,18,19].

Artemisinin (6) was isolated as the active principle of a traditional antimalarial herb *Artimisia annua.* Artemisinin is a sesqueterpene containing a highly unusual endoperoxide moiety and is currently in clinical use for the treatment of cerebral malaria.

Artemisinin is hydrophobic and some semisynthetic derivatives in ether form like **artemether (7), arteether (8)** and ester form like sodium artesunate has resulted in alternative antimalarial drugs[20].



(6)



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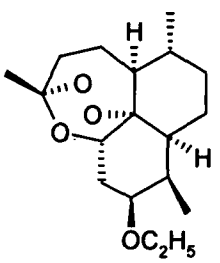
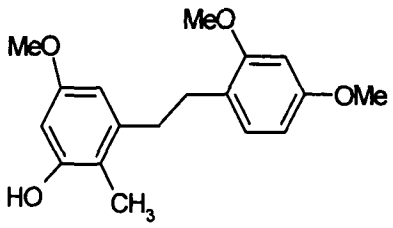


(8)

Further some synthetic tricyclic 1,2,4-trioxane based on the endoperoxide moiety of artemisinin have proved to be potently active against *Plasmodium berghei.* A readily synthesized trioxolone has been selected for clinical development as an antimalarial drug[21].

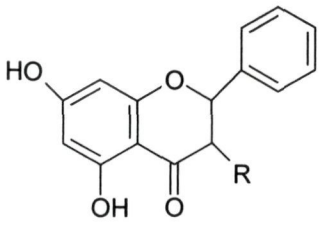
Antibacterial stilbenoids were isolated and identified from the roots of *Stemona tuberosa.* **Dihydrostilbene (9)** exhibited strong activity against *Bacillus pumilus* with MIT 12.5-25 μg/ml[22].



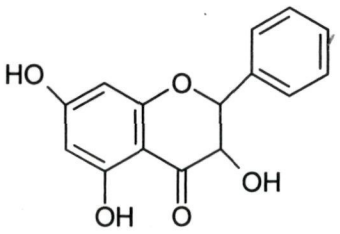
(9)

The antibacterial activity of Turkish propolis and its isolated chemical constituents was studied against *S. aureus* and *E. coli.* It was observed that flavanoids aglycones such as **pinocembrin (10), pinobanskin (11), galangin (12) ,** phenolic acids

such as *p*-coumaric acid , ferulic acid and cinnamyl cinnamate exhibited potent antibacterial activity[23].

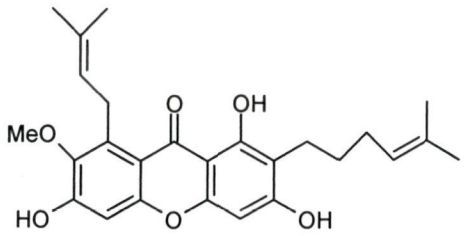


10. R= H
11. R=OH



(12)

α-Mangostin (13) and β- Mangostin (14) isolated from the stem bark of *Garcinia mangostana* were tested for their efficacy against vancomycin resistant *Enterococci* (VRE) and methicillin resistant *S. aureus* (MRSA). α-Mangostin was found to be active against these tested organisms with MIC values of 6.25 and 6.25 to 12.5 μg/ml respectively. Extended antibacterial activity indicated that α-Mangostin and β-Mangostin are inactive against gram negative bacteria[24].



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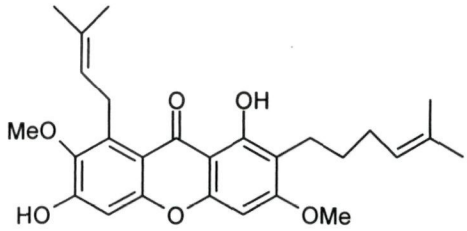

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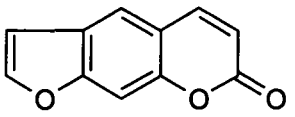
Crude extracts and eight isolated compounds from *Chresta scapigera* were evaluated for antibacterial and antifungal activities by the agar-well diffusion method. Twenty strains, including Gram-positive and Gram-negative bacteria and yeasts were used in the bioassay. Hexane extracts presented the best results while ethanol extracts did

not indicate inhibition of the microbial growth. Amongst the evaluated compounds β-amyrin acetate, tiliroside and luteolin showed the strongest antimicrobial effect.

Treculiaobovoidea N.E. Brown (Moraceae), one of the tree plants of the genus *Treculia* is traditionally used to treat skin diseases, dental allergy, amoebic dysentery and AIDS. Compounds were isolated from *Treculia obovoidea* and identified as **Psoralen (15), Bergapten (16), 7-methoxycoumarin (17), 4,2-,4- trihydroxy-3-prenylchalcone (18)**, and ***O*-[3-(2,2-dimethyl-3-oxo-2*H*-furan-5-yl) butyl] bergaptol (19).** These compounds together with the extract were tested for their antimicrobial activity against Gram-positive bacteria (six species), Gram-negative bacteria (12 species) and three *Candida* species using micro-dilution methods for the determination of the minimal inhibition concentration (MIC) and the minimal microbicidal concentration (MMC). The flavonoids isolated from this species also exhibited a very good antibacterial and anticandidal activities[25].



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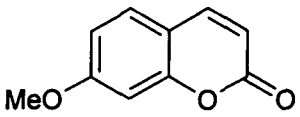

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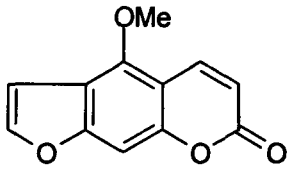

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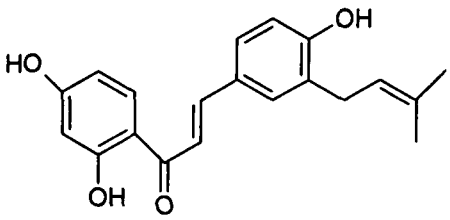

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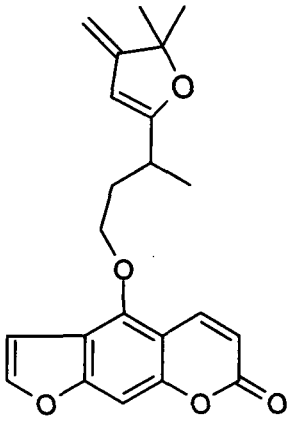

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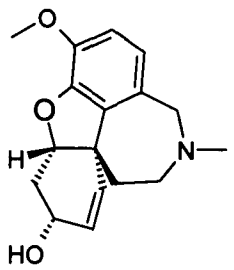
4.2.3 Anti-dementia drugs (Nootropic agents)

Learning generates an experience-dependent and long-lasting modification of the nervous system, which creates enduring memory representation. It involves the activation, by neurotransmitters such as acetylcholine, dopamine and serotonin, of receptor-linked enzymes responsible for the synthesis of inter-cellular messengers[26]. In humans and animals, the cholinergic neuronal system plays an important role in this process[27,28]. On the other hand, dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA), serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT), and cholinergic neurotoxin ethylcholine aziridinium administered intracisternally cause impairment of passive avoidance performance[29,30]. Many folk medicines, represented by Chinese and Ayurvedic medicines, are reputed to have cognitive-enhancing potential, and are referred to as nootropic or antiamnesic agents. Investigations of plant-based anti-dementia agents were relatively successful compared to other classes of herbal medicines.

Galantamine (20) is an alkaloid widely occurring in Amaryllidaceous plants, such as *Galanthus nivalis* L., *Lycoris radiata* herb, and species of the genus *Narcissus*[31,32,33]. Synthesized galantamine has been approved for the treatment of Alzheimer's disease in 29 countries[34]. Several clinical studies have shown the effectiveness of galantamine in the treatment of mild-to-moderate Alzheimer's disease[35]. A large body of evidence suggests that the cognition-enhancing effects of galantamine are largely derived from its specific inhibition of AChE and positive allosteric modulation of nicotinic cholinergic neurotransmission used in the treatment of Parkinson's disease. It has similar pharmacological action and clinical uses as galantamine.



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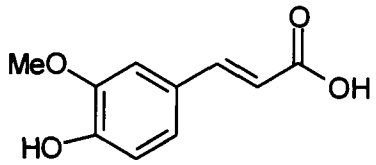
The n-hexane fraction of the extract from *A. sinensis* roots at a dose of 1 g/kg was administered orally to prolong the shortened step through latency of scopolamine (SCOP) and cycloheximide (CXM)-treated rats in the Passive avoidance test (PAT). The active constituent **ferulic acid (21)** reversed the step-through latency shortened by SCOP and CXM. Meanwhile, ferluic acid suppressed the Ah1–42 induced increase of the astrocyte marker glial fibrillary acidic protein (GAP) and interleukin-1a immunoreactivities in the hippocampus[36]. Ferulic acid has been known to be a potent antioxidant agent that protected against oxidative damages in synaptosomal membrane preparations and apoptosis in cultured neuronal cells[37,38].



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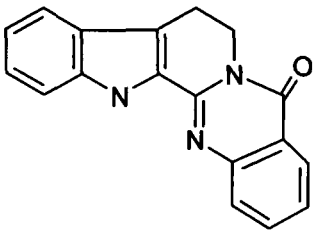
Dehydroevodiamine (22) is an active constituent identified from the fruit of *Evodia rutaecarpa.* It has been found to be a potent AChE inhibitor in a dose-dependent and non -competitive manner with an IC_{50} value of 37.8 micrograms. The antiamnesic effect of dehydroevodiamine was more potent than that of tacrine, an approved AChE inhibitor for patients with Alzheimer's disease[39]. *Gastrodia elata* has long been used in

traditional Chinese medicine for hypertension. The active ingredients isolated from *G. elata* roots, p-hydroxybenzyl alcohol and **gastrodin (23),** have been shown to possess antiamnesic activities in SCOP- and CXM-treated rats.



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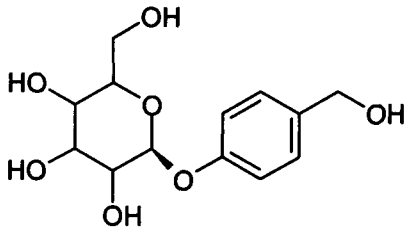

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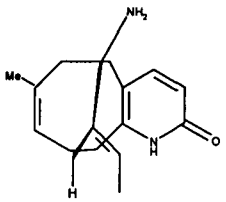
Huperzine-A (24) is a sesquiterpene alkaloid purified from the Chinese medicinal herb *Huperia serrata.* Huperzine-A had ameliorative effects on the impaired learning and memory induced by aging, SCOP, in the rat radial maze, PAT, and Morris water maze tests as well as in the chick PAT test[40,41] . Huperzine-A also improved spatial working memory in aged monkeys and young adult monkeys with experimental cognitive impairment in the delayed-response task test Huperzine-A, given daily at the dose of 0.05 or 0.1 mg/kg (i.p.) for 5 weeks, attenuated working memory impairments produced by hypoxic-ischemic brain injury in neonatal rats[42]. Huperzine-A is a potent and reversible AChE inhibitor and its potency has been reported to be superior to that of donepezil and tacrine[43,44]. Huperzine-A also possesses a broad range of neuroprotective actions. Since Huperzine-A has minimal adverse side effects, it has become a promising agent for use in patients with Alzheimer's disease.



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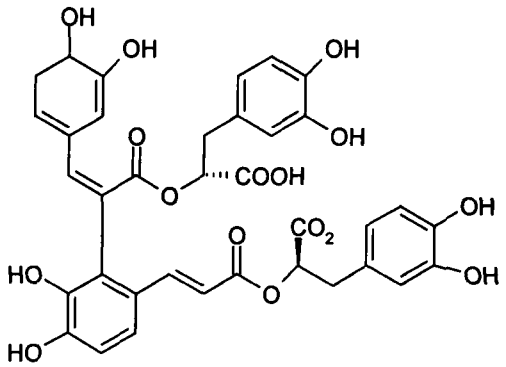
Ginseng has been used in traditional Chinese medicine for centuries, and is currently one of the most widely taken herbal products throughout the world. Among various ginseng species, *Panax ginseng* is the most extensively investigated member[45]. The nootropic effects of a variety of *P. ginseng* extracts and ginsenosides have been shown in aged rats, scopolamine and ethanol-treated rats[46], rats with medial prefrontal cortex lesions, scopolamine pretreated mice and ischemic gerbils, male chicks exhibited a significant reduction of the number of errors during retention trials in a visual discrimination task following intraperitoneal injections of the ginseng saponin Rb1[47].

The long-term treatment with *S. miltiorrhiza* methanol extract and its major ingredient **lithospermate-B (25)** has been found to be effective for the prevention of spatial learning deficits observed in senescence-accelerated mouse in the Morris's water maze task test[48]. Several constituents extracted from *S. lavandulaefolia* Vahl (Spanish sage), another species of the genus *Salvia*, also showed beneficial effects on dementia in both animal and human subjects[49].



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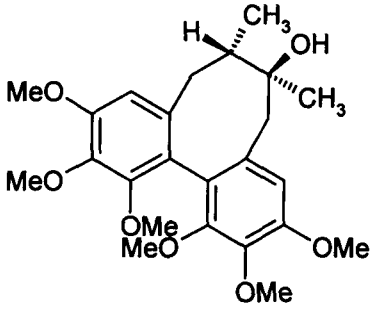
In traditional Chinese medicine, *Schisandra chinensis* was used to build up strength and remove fatigue. It has been found that *S. chinensis* extract at 0.25 and 0.75 g/kg and its water fraction at 25 mg/ kg administered for 1 week significantly

counteracted cycloheximide-induced cognitive impairment in the rat PAT test. In recent studies[50,51]. **Schizandrin (26)** (1 mg/kg) and **gomosin A (27)** (5 mg/kg, p.o) were shown to reverse significantly scopolamine-induced cognitive impairments in rodents. Gomosin A, C, D and Schizandrol B entirely inhibited acetylcholinesterase activity in a dose dependent manner.



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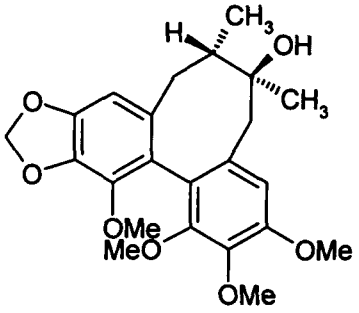

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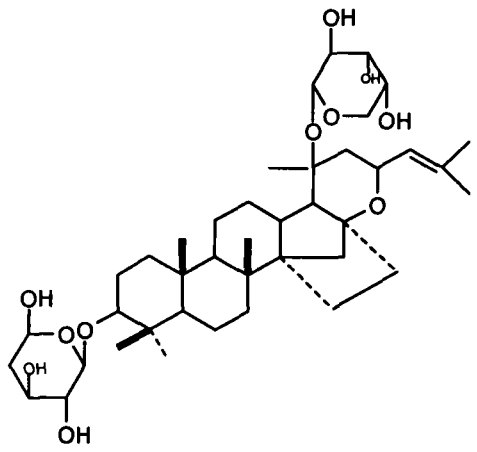
Bacopa monniera (BM), a traditional Ayurvedic medicine, used for centuries as a memory enhancing agent. It is used as *medyarasayana* in Indian system of medicine. The major chemical entity shown to be responsible for the memory-facilitating action of BM, **Bacoside-A (28),** was assigned as 3-(a-L-arabinopyranosyl)-O-b-D-glucopyranoside- 10, 20-dihydroxy-16-keto-dammar-24-ene[52]. In view of the increasing interest on this herbal drug, Chakravarty et al[53] undertook a thorough chemical reinvestigation of the glicosidic fraction of the methanol extract of the plant and were able to isolate two new pseudojujubogenin glycosides designated as **bacopaside I and II (29, 30)** . These active principles, apart from facilitating learning and memory in normal rats, inhibited the amnesic effects of scopolamine, electroshock and immobilization stress[54]. BM is known to lower norepinephrine and increase 5-hydroxytryptamine levels in the hippocampus, hypothalamus and cerebral cortex[55]. BM may thus, also indirectly, modify AchE concentrations, through its influence on other neurotransmitter systems.



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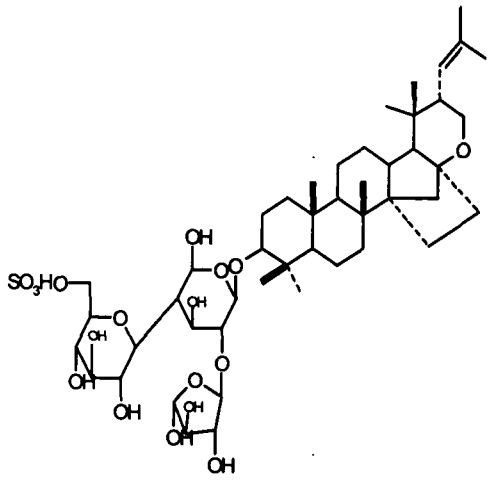

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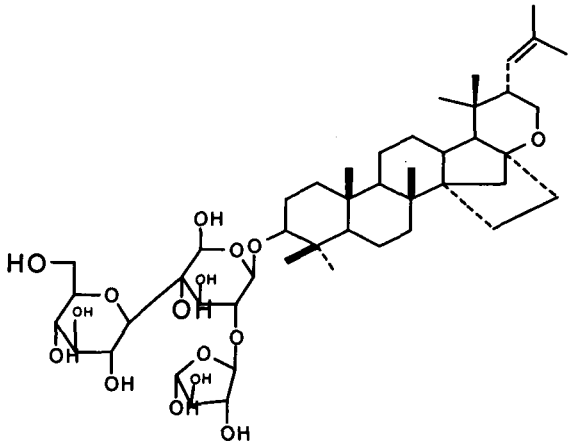

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4.2.4 Anti-diabetic drugs

Diabetes mellitus (DM) is defined as a state in which carbohydrate and lipid metabolism are improperly regulated by insulin[56]. There are two major categories of the disease, type 1 and type 2 according to insulin dependency. In type 2 diabetes mellitus (NIDDM), most common type of DM, it is generally accepted that resistance to insulin in peripheral tissues such as muscle and adipose develops initially, followed by decreased insulin secretion and other complications as a result of progress[57,58].

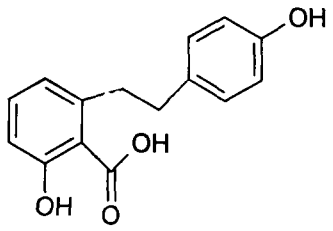
It is predicted that by 2030, India, China and the United States will have the largest number of people with diabetes[59]. In modern society, diabetes mellitus is a chronic and often undiagnosed or inadequately treated disease. Type 2 diabetes is closely associated with other metabolic disorders, such as hypertension, cardiovascular diseases, and atherosclerosis and its incidence is increasing worldwide. Insulin resistance is an important marker for developing type 2 diabetes.

Despite tremendous advances in medicine during the past century, there is still no cure, which means that effective prevention and treatment is of paramount importance to prevent future increases in disease burden[60,61]. Thiazolidinedione-type compounds such as pioglitazone are potent insulin sensitizers and currently used clinically to treat type 2 diabetes. Thiazolidinedione-type compounds were originally identified based on their antihyperglycemic activity, but they are also able to improve other abnormalities associated with type 2 diabetes, such as hyperlipidemia, atherosclerosis, hypertension, and chronic inflammation.

There is a rapidly increasing interest in finding new medicines, or even better finding prophylactic treatments. The latter is particularly in the food industry a major target, finding food that prevents or slows down the development of diabetes. As the symptoms of diabetes are quite characteristic, it is likely that the disease has been recognized in all medical systems. This is supported by the fact that worldwide numerous medicinal plants and their constituents have been reported for treatment of diabetes. Traditional medicine is thus an important source for novel leads for treating diabetes.

Ethyl acetate fraction of the processed leaves of *Hydrangea macrophylla* var. *thunbergi,i* which is listed in Japanese Pharmacopoeia XV and used as a sweetening agent for diabetic patients, promoted the accumulation of triglyceride in 3T3-L1 cells. Among the compounds isolated, the principal constituents, Hydrangenol and phyllodulcin

caused adipogenesis in 3T3-L1 cells similar to troglitazone. **Hydrangeic acid (31)**, a minor stilbene constituent of the plant also caused adipogenesis[62]. Hydrangeic acid significantly lowered blood glucose, triglyceride and free fatty acid levels 2 weeks after its administration at a dose of 200 mg/kg/d in mice. Hydrangeic acid, concentration-dependently increased the adipogenesis, the uptake of



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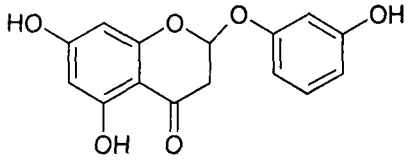
2- deoxyglucose into the cells, the translocation of GLUT4, and the amount of adiponectin released into the medium, and reduced the production of NO[63].

An ethanolic extract of *Artemisia dracunculus* L was shown to be hypoglycemic in animal models for Type 2 diabetes and contains at least 6 bioactive compounds responsible for its anti-diabetic properties. Extensive bioactivity guided fractionation the plant using *in vitro* assays, led to the isolation of 6 compounds, of which **6-demethoxy capillarisin (32)** and **2',4'dihydroxy-4-methoxydihydrochalcone (33)** may contribute to the anti-hyperglycemic activity and were found to decrease phosphoenolpyruvate carboxykinase mRNA expression in STZ-induced diabetic rats[64,65,66].



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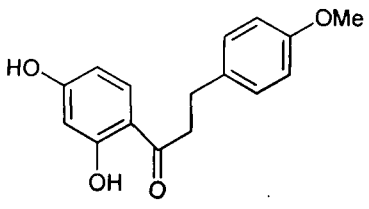

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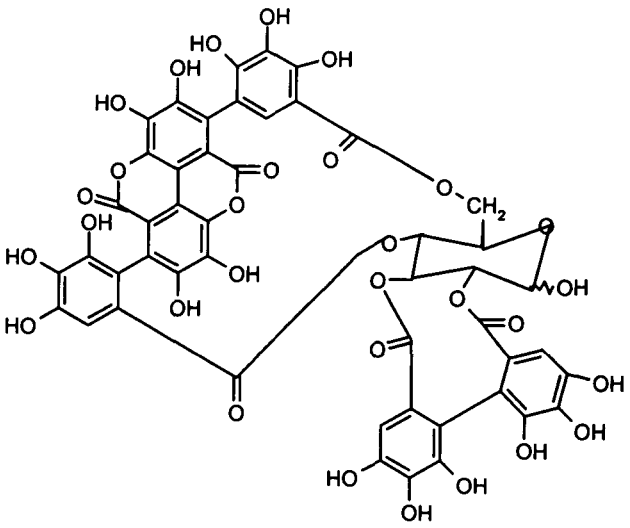
Pomegranate (*Punica granatum*) is a rich source of bioactive compounds and is used in folk medicine for the treatment of various diseases. Phenolic components, **punicalgins (34)**, ellagic acid and Urolithin-A are found to be useful metabolites of *P.ganatum* Oral administration of aqueous extract of Pomegranate at doses of 250 mg/kg and 500 mg/kg for 21 days resulted in a significant reduction in fasting blood glucose, TC, TG, LDL-C, VLDL-C and tissue LPO levels coupled with elevation of HDL-C, GSH content and antioxidant enzymes in comparison with diabetic control group[67].



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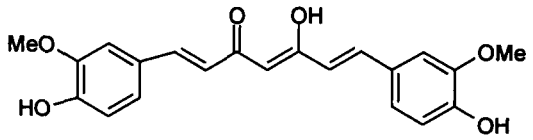
Panax ginseng has several pharmacologic and physiologic effects that are being disclosed gradually. Antihyperglycemic and antiobesity effects of ginsam, a component of *Panax ginseng* produced by vinegar extraction, which is enriched in the ginsenoside Rg_3 was evaluated using Otsuka Long-Evans Tokushima Fatty rats, an obese insulin-resistant rat model. Ginsam has distinct beneficial effects on glucose metabolism and body weight control in an obese animal model of insulin resistance by changing the expression of genes involved in glucose and fatty acid metabolism[68] .

Curcumin (35), a yellow pigment substance and component of turmeric (*Curcuma longa*), which was identified more than a century ago. For centuries it has been known that turmeric exhibits anti-inflammatory activity, but extensive research performed within the past two decades has shown that this activity of turmeric is due to curcumin (diferuloylmethane). In diabetes, curcumin can suppress blood glucose levels, increase the antioxidant status of pancreatic β-cells, and enhance the activation of PPAR-γ[69]. Curcumin treatment can induce hypoglycemia in rats with streptozotocin (STZ)-induced diabetes has been confirmed by many authors[70]. Both curcumin and its metabolite **tetrahydrocurcumin (36)** have been shown to decrease blood glucose levels, increase plasma insulin levels, and modulate hepatic key enzyme levels in STZ-induced diabetic rats[71] through modulation of oxidative stress[72] and reduction in lipids and lipid peroxidation. Studies have been conducted to determine whether curcumin's direct stimulatory effect on the pancreatic beta-cell can contribute to the hypoglycemic activity of this compound. Curcumin was also found be effective in the treatment of diabetic complications like nephropathy, retinopathy, cardiomyopathy etc.



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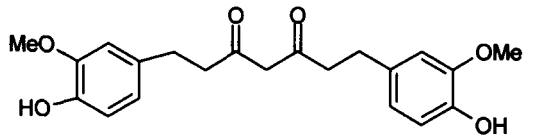

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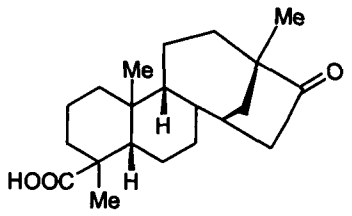
In a recent study, treatment with curcumin prevented diabetes-induced upregulation of these transcripts, suggesting the existence in curcumin of a novel glucose-induced epigenetic mechanism regulating gene expression and cardiomyocyte hypertrophy in diabetes.

4.2.5 Cardioprotective drugs

Isosteviol (37) (ent-16-ketobeyeran-19-oic acid) is a beyerane diterpene obtained by acid hydrolysis of **stevioside (38)** a traditional non-caloric sweetener extracted from *Stevia* rebaudiana[73]. Cardioprotective effect of Isosteviol was investigated in heart ischaemic-reperfusion injury in rats. Pretreatment with Isosteviol at the dose of 0.5, 1.0 and 2.0 mg/kg decreased the infract size, improved the haemodynamic parameters and the activities of serum enzymes[74].



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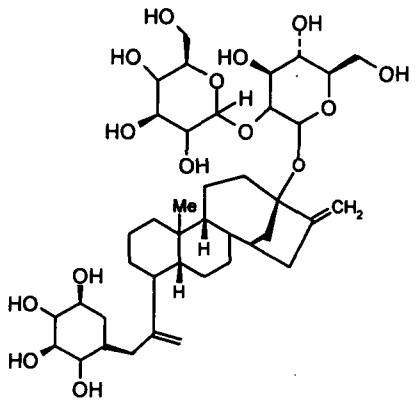

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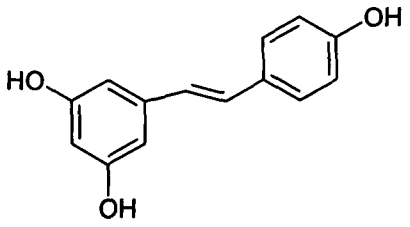
Resveratrol (39), a polyphenol found in red wine and grapes *(Vitis vinifera)*, is thought to be a major biologically active ingredient that contributes to the cardioprotective effects of red wine[75]. At the cellular and molecular level, resveratrol inhibits the oxidation of low-density lipoproteins, reduces platelet aggregation, promotes vasodilation and protects the heart. Daily intraperitoneal injection of 1 mg/kg/day resveratrol for four weeks had potent cardioprotective effects by suppressing the infract size and significantly improves the systolic function in echocardiography[76].



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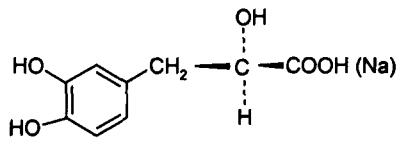
Salvia miltiorrhiza water soluble extract from *Salvia miltiorrhiza* contains 6 active bioactive ingredients such as **3, 4-dihydroxyphenyl lactate (40),** Salvianolic acid A , Salvianolic acid B, **Rosmarinic acid (41),** Protocatechualdehyde and Caffeic acid. Beside these 6 active water soluble ingredients, crude *Salvia miltiorrhiza* extract is mixed with other lipophilic ingredients like Tanshinone I, Tanshinone IIa and IIb, Cryptotanshinone, Isotanshinone I, Isotanshinone II, Isocryptotanshinone, Tanshinol I, Tanshinol II and Vitamin E[77,78,79]. After 2 weeks treatment with the extract, survival rates of rats with experimental myocardial infarction were marginally increased (68.2% and 71.4%) compared with saline (61.5%). The ratios of infarct size to left ventricular size in drug treated rats were significantly less than that in the saline-treated group. Activity of cardiac antioxidant enzyme superoxide dismutase (SOD) was significant higher while level of Thiobarbituric acid-reactive substances (TBARs) was lower in the standard extract treated group.



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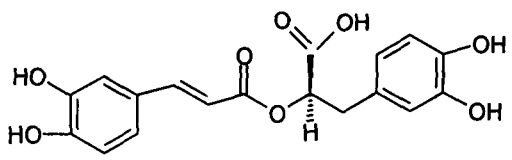

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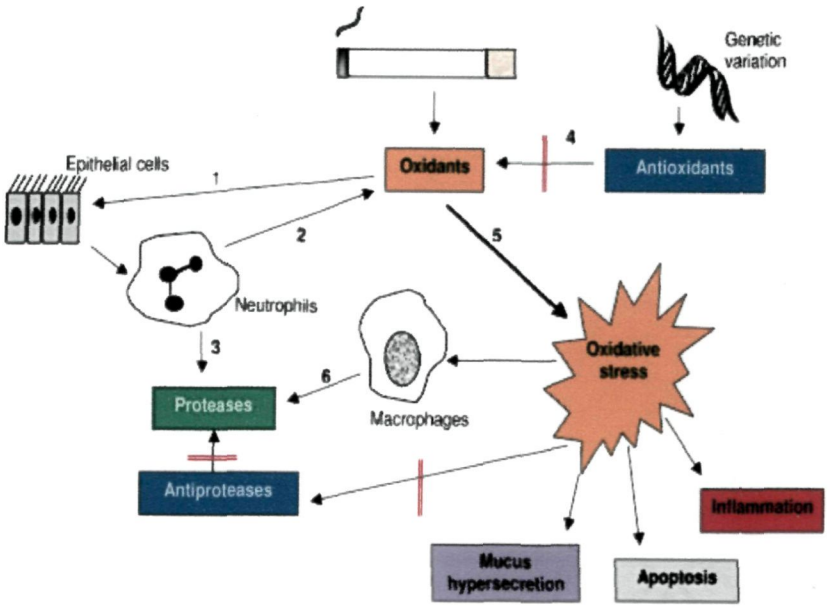
4.2.6 Anti-stress drugs (adaptogens or rejuvenators)

Stress is a broad, ambiguous, and often poorly understood concept. In its most simplified sense, stress is what one feels when life's demand exceeds one's ability to meet those demands. Stress has been postulated to be involved in the etiopathogenesis of a diverse variety of diseases, ranging from psychiatric disorders such as anxiety and depression, immunosuppression, endocrine disorders including diabetes mellitus, male sexual dysfunction, cognitive dysfunctions peptic ulcer, hypertension and ulcerative colitis[80].

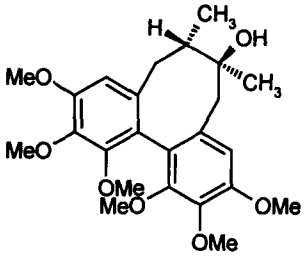


Benzodiazepines and anxiolytics, despite having significant anti-stress activity, have not proved effective against chronic stress induced adverse effects on immunity, behavior cognition, male sexual function, during pregnancy and lactation. Additionally, the problem of tolerance and physical dependence on their prolonged use, limits the clinical utility of these drugs. Therefore there is a need for an effective herbal antistress agent in the therapy of stress induced disorders[81].

The idea of using tonic remedies to restore balance and health in a person is an ancient idea. The word and concept of an **"adaptogen"** is a relatively new way of describing a type of remedy commonly found in traditional Chinese (Qi tonic), African (Manyasi), Tibetian, Ayurvedic (Rasayana), and Cherookee medicine. The term **"adaptogen"** was first coined by **Nikolai Lazarev** (1947), meaning a substance that can develop a state of raised resistance, enabling an organism to cope with different kinds of stressful situations[82].

Ayurveda is a science for living one's full potential and extreme longevity. Ayurvedic pharmacology classifies medicinal plants in to different groups according to their actions. Ayurvedic herbal or mineral preparations used for promoting general health, immunity, strength, vitality, virility and spiritual attainment are known as ***rasayanas***. Rasayanas are the agents which are reputed to promote health and longevity by augmenting defense against disease, arresting the aging process, revitalizing the body in debilitated conditions increasing the capability of the individual to resist the adverse environmental factors and by creating a sense of mental well being. Traditionally rasayana drugs are used against a plethora of seemingly diverse disorders with no pathophysiological connections according to modern medicine. Looking at these diverse applications adaptogenic agents from this group of Rasayana were identified[83]. It has been reported that the rasayanas are rejuvenators, nutritional supplements and possess antioxidant activity. Plants like *Panax ginseng, Elutherococcus senticosus, Schisandra chinensis, Withania somnifera, Bacopa monnieri, Asperagus racemosus, Evolvulus alsinoids* extracts and their constituents have been extensively studied for anti-stress/adaptogenic activity.

Schisandra chinensis was the first recognized plant as an adaptogen in the official medicine of USSR owing to its wide range of pharmacological activities. Its active principle **schizandrin (42)** was the first active component isolated from the seeds of *schisandra chinensis.* Schizandrin, **gomosin-A (43)** and other lignans, Nortriterpenoids like **Pre-schsanartanin (44),** schindilactones and **Wuwelizidilactones A (45) to F** have been isolated from the leaves and stems.



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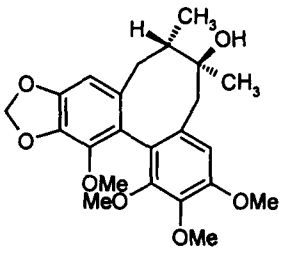

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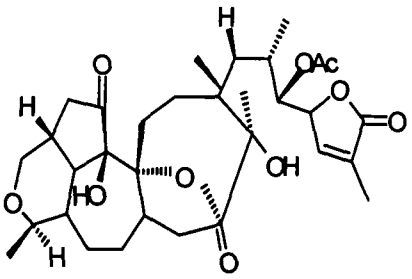
Anti-stress effects of the plant in both *in-vitro* and *in-vivo* have been proved to be due to the schizandrin lignans under various stress paradigms[84,85].



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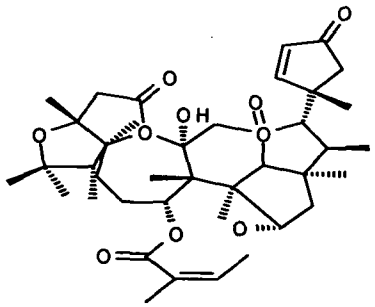
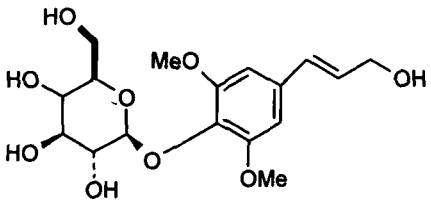


(45)

Elutherococcus senticosus (Siberian ginseng) is another potent adaptogen ever seen, because of its diversified pharmacological properties. **Syringin (46), Sesamin (47),** Isofraxidin, caffeic acid and coniferyl aldehyde are the important anti-stress components of the plant which act through various mechanisms[81].



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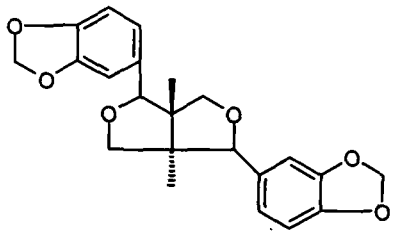

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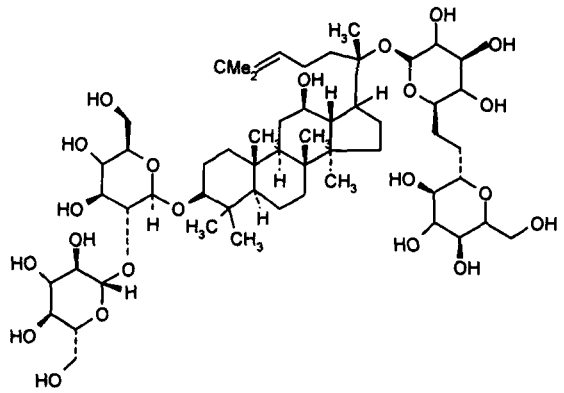
The use of *Panax ginseng* in herbal preparations as an adaptogen goes back to many centuries. Chemical nature of *panax ginseng* includes tetracyclic triterpenoid saponins (ginsenosides) which differ from each other by one or a few chemical groups. **Ginsenosides Rb_1 (48),** Rg_1, Rd, Rf, Rg_3 , Rh_2 have been extensively studied for various pharmacological activities using animal and human subjects. Ginseng total saponins and ginsenosides Rg3 and Rb1 found to attenuate the elevated polyamine levels after immobilization stress in gerbils[85].



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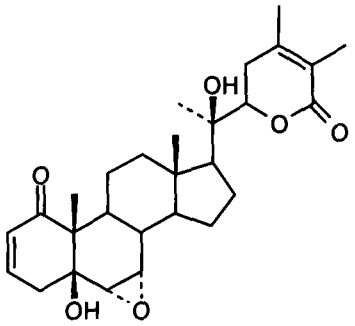
Withania somnifera (Indian ginseng) is extensively used for relieving stress in Indian system of medicine. Its efficacy as an adaptogen has been proved under various paradigms like chronic fatigue syndrome, chronic foot-shock induced stress, on neuronal degeneration, antioxidant action in animal models[86]. The major biochemical constituents

of *W. somnifera* are steroidal alkaloids and lactones, together known as Withanolides. The withanolides have the structural resemblance with the active constituents of *panax ginseng*. Withanolides have C-28 steroidal nucleus with C-9 side chain, having six membered lactone ring. **Withferin-A (49)** was reported to inhibit angiogenesis in certain types of cancers **Glycowithanolides (50) (sitoindosides IX and X)** possessed anti-stress activity[87].



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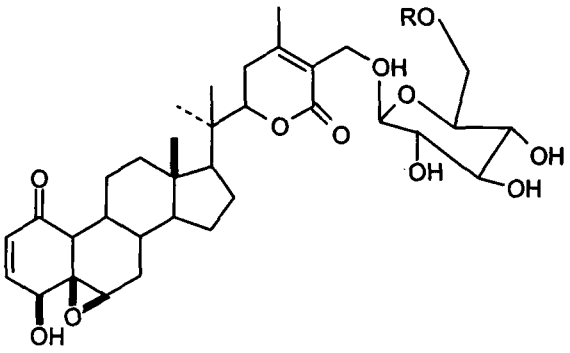

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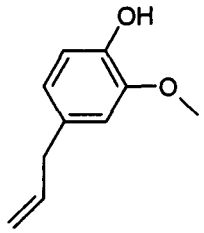
Bioactivity guided purification of n-BuOH soluble fraction from the ethanol extract of *Evolvulus alsinoids* resulted in the isolation of 2,3,4-trihydroxy-3-methylbutyl3-[3-hydroxy-4-(2,3,4-trihydroxy-2-methylbutoxy)-2-propeonate 1,3-di-O-caffeoyl quinic acid methyl ester , Caffeic acid and quercetin, kaempferol-glucosides and a Coumarin. Phenyl propanoids and caffeic acid showed potent anti-stress effects by normalizing hyperglycemia, plasma corticosterone, creatine kinase and adrenal hypertrophy[88].

Ocimum sanctum (Holy basil; Tulsi) have been recommended for the treatment of various diseases. It possesses antifertility, antidiabetic, hepatoprotective, cardioprotective, adaptogenic activity, and thus is used for stress disorders. **Eugenol (51)** (10-hydroxy-2-methoxy-4 allyl benzene) in Tulsi oil and **Ursolic acid (52)** present in leaves are the main

active constituents responsible for its pharmacological profile including anti-stress activity[89].



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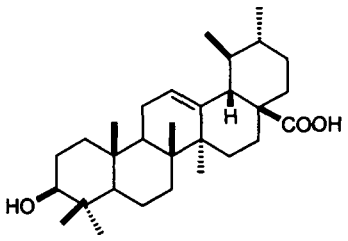

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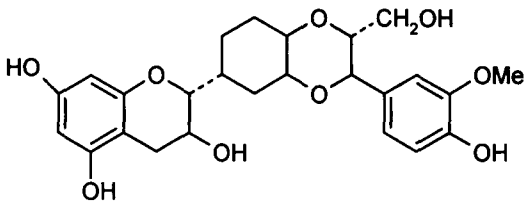
4.2.7 Hepatoprotective drugs

A wide range of flavonoid compounds have been isolated from plants which exhibit liver protective action. Flavonoids isolated from *silybum marianum* have been used for centuries in folk medicines for the treatment of liver disorders. The active constituents of this extract reside in a mixture **silymarin (53),** which in experimental models was demonstrated to exert not only a positive effect on intact liver cells, but also stimulated regenerative capacity after partial hepatectomy[90]. The hepatoprotective activity of **silybin (54),** the main flavolignan occurring in the mixture silymarin, was investigated in mice intoxicated with non-therapeutic doses of acetaminophen. However, it seems that silybin binds a subunit of the DNA-dependent RNA polymerase-I that activates this enzyme. As a consequence, protein synthesis is increased, leading to an accelerated regeneration and production of hepatocytes[91]. Combination of baicalein and silymarin eradicated tumor cells efficiently, has minimal deleterious effects to the surrounding normal cells, and offers mechanistic insight for the treatment of hepatocellular carcinoma treatment[92]. Silibinin is a 50:50 mixture of silybin A and silybin B. Silibinin has strong antioxidative and antifibrotic properties, which makes it a

potentially useful drug for treatment of chronic liver diseases. Oral Silibinin is widely used for treatment of hepatitis C, but its efficacy is unclear. In a clinical study silibinin was found to be a potent anti-viral drug in the treatment of hepatitis-C[93].



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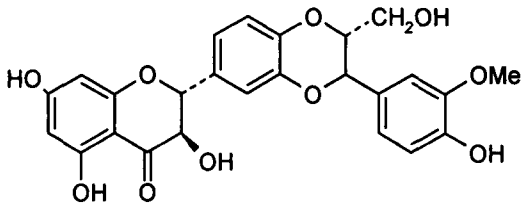

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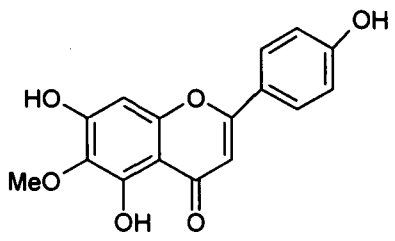
Flavonoids extracted from *Buccuris frimeru* were reported to protect mice from hepatic damage, **hispidulin (55)** appeared to be the most active flavonoid in this plant and protected mice intoxicated with phalloidin (80% of survival rate) while other flavonoids like quercetin, **luteolin (56),** nepetin and apigenin were less active or even inactive[94].



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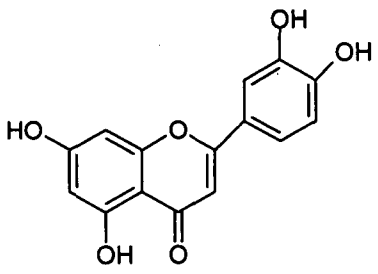
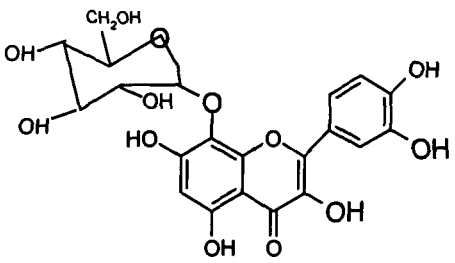


(56)

Administration of **gossypin (57)** and hydroxyethyl rutoside significantly increased the survival rate of mice thus treated, suggesting that flavonoids may be benefical in reducing the toxic effect of sulphur mustard[95]. Recently Gilani *et al.*[96] reported that **quercetin (58)** extracted from *Artemisia scoparia* possesses some protective activity on paracetamol-induced hepatotoxicity in mice and rats. The oral application quercetin in a nanocapsulated drug delivery system was found to be useful therapeutically to prevent arsenic-induced hepatic and cerebral oxidative damage[97]. Study indicated that a single dose of *Andrographis paniculata* extract and **andrographolide (59)** inhibited hepatic microsomal CYP2C/2E1-dependent aniline hydroxylase, CYP2B-dependent *N*-demethylation of *N,N*dimethyl aniline, and CYP2E1-dependent *O*-demethylation of *p*-nitroanisole in albino rats.



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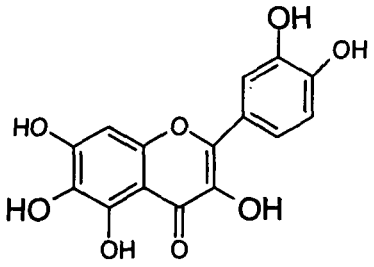
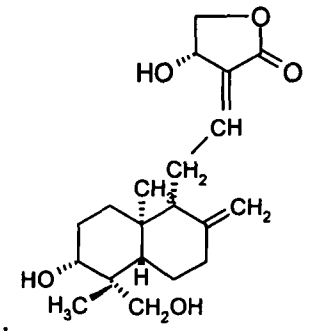


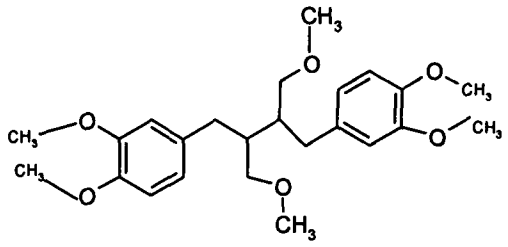
(58)

Andrographis paniculata extracts have recently been reported to increase CYP1A1-dependent ethoxyresorufin *O*-dealkylase and CYP2B10-pentoxyresorufin *O*-dealkylase activities *in vivo* in mice[98]. The inhibitory effect of *Andrographis paniculata* extract and andrographolide the most medicinally active phytochemical in the extract, on hepatic cytochrome P450s (CYPs) activities was examined using rat and human liver microsomes[99]. Andrographolide in combination with 5-fluorouracil potentiates the cytotoxic effect in hepatocellular carcinoma[100].



(59)

Phyllanthus amarus is a plant in the family Euphorbiaceae found in all regions. It contains many types of phenolic compounds, lignans such as **phyllanthin (60)** and hypophyllanthin, flavanoids such as quercetin and astragalin, ellagitannins such as amarinic acid, amarin and phyllanthisiin D[101,102]. *Phyllanthus amarus* is one of herbs with potent hepatoprotection against paracetamol[103], carbon tetrachloride[104] and galactosamine. It may enhance hepatic recovery after ethanol-induced liver injury. Phyllanthin and hypophyllanthin reported to reduce hepatotoxicity induced by carbon tetrachloride and galactosamine in rats and may be used as marker for hepatoprotection of *Phyllanthus amarus* [105,106].

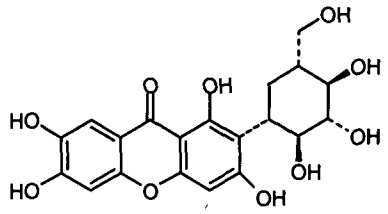


(60)

Glycyrrhizic acid (GA) is the main bioactive ingredient of licorice (*Glycyrrhiza glabra*). Protective effects of GA on tert-butyl hydroperoxide (t-BHP) induced oxidative injury leading to apoptosis in cultured primary rat hepatocytes was investigated. It was

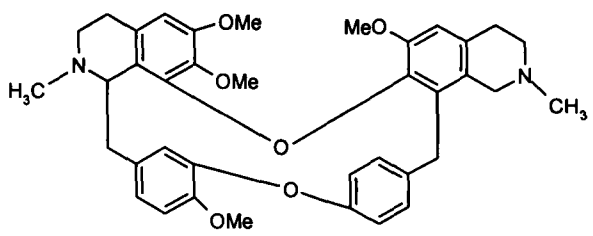
found to modulate critical end points of oxidative stress induced apoptosis and could be beneficial against liver diseases [107].

Mangiferin, (61) a glucosylxanthone present in large amounts in the leaves and edible mango fruits of *Mangifera indica.* It has potent cytoprotective and antigenotoxic effect against $CdCl_2$ induced toxicity in $HepG_2$ cell line. The cytoprotective and antigenotoxic property of Mangiferin may be attributed to decrease in $CdCl_2$ induced



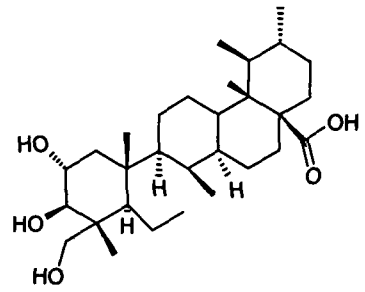
(61)

ROS levels and resultant oxidative stress[108]. **Tetrandrine (62)** is the major pharmacologically active compound of Chinese herb *Stephania tetrandra* which has been used traditionally for the treatment of rheumatic disorders, silicosis and hypertension. Tetrandrine is able to prevent T-cell-mediated liver injury *in vivo*. Pretreatment of mice with tetrandrine markedly reduced plasma transaminase release and the severity of liver damage. The beneficial effect may depend on suppressing the production of various inflammatory mediators in the liver through inhibiting of $NF\text{-}_kB$ activation[109].



(62)

Asiatic acid (63) is a triterpenoid component possessing antioxidative, anti-inflammatory and hepatoprotective activity. The protective effects of asiatic acid and the relative mechanism in the D-galactosamine/Lipopolysaccharide induced hepatotoxicity in hepatocytes and kupffer cells co-cultured system was explored. It was observed that asiatic acid protects against D-galactosamine/Lipopolysaccharide -induced hepatotoxicity in co-cultured system partly via cellular redox regulated LTC4S expression pathway[110].



(63)

Therefore, the roles of ethnobotany in drug development and of ethnopharmacology in drug discovery is very important since many people in developing countries continue to rely on traditional medicine practitioners and local medicinal plants for their primary health care. The expanding chemical and biological and analytical techniques has led to the focused research on providing scientific evidence for the presence of active principles in traditional medicines for their standardization and for assessment of toxicities. Identification of new biologically active compounds also provides leads for new drug development.

The last 50 years has seen the introduction of novel natural product drugs such as vincristine, vinblastine, taxol, artemisinin, galantamine etc. and their semisynthetic derivatives in to clinical use. This awakened the possibility that plants could be useful in drug discovery and development. The advent of high throughput screening methods for

assessment of large number plant extracts containing putative biologically active compounds further encouraged industrial interest in plant research. The unraveling of biosynthetic pathways has allowed greater understanding of the ways in which plants synthesize natural products and have enabled genetic technology to produce natural and related "unnatural" related compounds. There is now a parallel development to combinatorial synthesis, namely combinatorial biosynthesis[7]. Whatever new techniques come in to stream, it is evident that there will be a continued need for novel drugs in the treatment of various diseases. Medicinal plant research will continue to contribute to healthcare as well as producing novel clinically useful drugs. In view of this, part-B of our study was designed to explore some selected medicinal plants for phytochemcial and pharmacological investigation with following objectives:

Objectives

1) Identification of potential medicinal plants (rasayana herbs) used in Indian system of medicine through classical references, their collection and authentication.
2) Fractionation of the selected plants using polar and non-polar solvents.
3) Screening of the crude fractions of the plants for various pharmacological activities using *in vitro* and *in vivo* models, to establish a scientific data and to justify the traditional claim of these plants for various diseases.
4) Separation and identification of active secondary metabolites from potential fraction(s) of the plant showing promising results, through Chromatographic and instrumental analytical techniques (UV, IR, NMR and Mass spectra).
5) Screening of isolated compounds for possible biological activity.

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Chapter - 5

Preliminary Phytochemical and Pharmacological Studies of *Argyreia speciosa* (sweet) (Burm.) f. Boj.

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5.1 Ethnobotany of Convolvulaceae

The members of Convolvulaceae are mostly twining herbs or shrubs, sometimes with milky sap, comprising about 85 genera and 2,800 species that are further characterized by almost always having the flowers solitary or in terminal or axillary dichasia. The leaves are simple, though sometimes lobed to pinnatisect, and alternate; stipules are absent. The flowers are actinomorphic, often showy, and nearly always bisexual. The perianth and androecial whorls are 5-merous. The sepals of the calyx are usually distinct but the corolla is strongly sympetalous, plaited, and often rotate or trumpet shaped with inconspicuous lobes. The stamens are often unequal, and are adnate to the base of the corolla tube and alternate with the lobes. The gynoecium consists of a single compound pistil of 2 or rarely up to 5 carpels, usually an unbranched or 2-cleft style, and a superior ovary of 2 or sometimes up to 5 locules, each with 1 or 2 axile ovules. A prominent annular nectary disk is usually present around the base of the ovary. The fruit is usually a loculicidalapsule.

5.2 Chemical Nature of Convolvulaceae

The Convolvulaceae family is characterized by the wide spread occurrence of flavanols and their O-methylated derivatives and their glycosides[1,2]. Glycosides of the flavanols kaempferol and quercetin, especially 3-O-glyosides and 3-O-rutinosides are also characteristic constituents in the Convolvulaceae family. Predominantly quercetin also, kaempferol, though less frequent as well as their methyl ethers, and or their

glycosides were published with regard to 15 genera: *Argyreia, Bonamia, Catystegia, Convolvulus, Cressa, Cuccuta, Erycide, Ipomea* etc. Flavones predominantly apigenin and /or luteolin and /or their derivatives were described as constituents of only 8 genera like *Argyreia, Evolvulus, Ipomoea, Merreimia* etc. The known glycosides kaempferol-3-O (6-O-p-Coumaroyl, galactopyranoside and kaempferol as well as quercetin-3-O-β-glucopyranosyl β-galactopyranoside could be isolated for the first time from a convolvulacious species. *Argyreia capitata* (Vhal) choisy,[3] nine species of holopaeasitic genus C*uscuta* revealed characteristic pattern of soluble phenolic constituents. This is a special significance since many C*uscuta* species are difficult to identify or distinguish from related ones. Several new flavone glycosides could be discovered in the family eg: Acacetin-7-O-β-D-galactoside in the leaves of *Ipomea carnea* Jacq. species. Hispidulin 7-One-O-hyperoside (6-methoxy-5,7,4-trihydroxyflavone α-L-rhamnosyl- β-glucoside) in the flowers of *Ipomea purpurea,* 7-O-methyl luteolin 3-O-4-O-diglucoside in the vegetative part of *I. tamnifolia* L.Griseb. A novel flavone glycoside, eriodictoyol (5, 7-3'4'-tetrahydroxy flavonone) 7-O β-D-xylopyranosyl β-D-arabinopyranoside was characterized as a constituent of *Ipomea purpurea* seeds. The *Evolvulus* species found to contain delphinidin derivative as a marker component [4].

Flavanoid sulphates represent specific group of flavanoids which are linked co-valently via OH group to SO_3 thus forming ester moiety, were discovered and are documented in 160 species from 9 dicotyledonous and 7 monocotyledonous families including Convolvulaceae. *Ipomea regnellii* and *Ipomea reticulate* found to contain quercetin, kaempferol and their O- methylated sulfates[5] . Around 56 flavonol sulphates as well as 15 flavone sulphates have isolated usually in the form of their potassium salt

from various species of mono and dicotyledonous families. Recently a novel flavonol and two novel flavones have been discovered from the stem of *Erycibe expansa* which are identified as ericibenin-D and Ericibenin-F[6]. Anthocyanin found in *Ipomea* and *Evolvulus* in these two Convolvulaceae genera are family specific. *Ipomea* represent the most studied genus of this family as far as anthocyanins are connected. It is noticeable that the occurrence of anthocyanidin is confined to pelargonidin, cyanidin and its 3'-methyl ether peonidin[7].

5.3 Taxonomy of the genus *Argyreia*

Domain	:	Eukaryota
Kingdom	:	Plantae
Subkingdom	:	Viridaeplantae
Phylum	:	Tracheophyta
Subphylum	:	Euphyllophytina
Infraphylum	:	Radiatopses
Class	:	Magnoliopsida
Subclass	:	Lamiidae
Superorder	:	Solananae
Order	:	Convolvulales
Family	:	Convolvulaceae
Subfamily	:	Asteroideae
Tribe	:	Ipomoeeae
Genus	:	*Argyreia*

Members of *Argyreia* are woody climbers of tropical Asia to Australia. It is the genus of flowering plants having two cotyledons (embryonic leaves) in the seed which

usually appear at germination. These are twining shrubs having silvery leaves and showy purple flowers. About 190 species are available from this genus, majority of which are distributed in tropical Asia, with one species in Australia (Queensland) and 22 species in China. The genus *Argyreia* is further organized into finer groupings including 191 species, subspecies, varieties, forms and cultivars.

5.4 Earlier Studies on *Argyreia speciosa* (sweet), (Burm. f.) Bojer.

5.4.1 Pharmacological review

- The effect of aqueous and alcoholic extracts of *Argyria speciosa* on the spontaneous movements of both the whole worm and a nerve/muscle preparation of *Setaria cervi*, and on the survival of microfilariae *in vitro*, was studied. The concentration required to inhibit the movements of the whole worm preparation was 150 µg/ml for the aqueous, and 75 µg/ml for the alcoholic extract. The concentration of *A. speciosa* extract required to produce an equivalent effect on the nerve/muscle preparation was 25 µg/ml for aqueous and 50 µg/ml for the alcoholic extract[18].
- Immunomodulatory activity of ethanolic fraction (50, 100, 200 mg/kg) of *A. speciosa* was studied. *A. speciosa* significantly potentiated the delayed-type hypersensitivity reaction induced both by SRBC and Oxazolone. It significantly enhanced the production of circulating antibody titre in mice. Chronic administration of ethanolic fraction ameliorated the total WBC count and also restored the myelosuppressive effects[19].
- Ethanolic extract of *A. speciosa* at 50, 100, and 200 mg /kg p.o. was investigated for anti-inflammatory and antiarthritic activity in acute and chronic models in rats.

A. speciosa significantly inhibited the paw edema induced by carragenan and freund's complete adjuvant and also prevented the accumulation of inflammatory cells in carregenan induced peritonitis[20].

- Antifungal activity of hexadecanyl p-hydroxycinnamate and scopoletin isolated from n-hexane and ethyl acetate fractions of roots of *A. speciosa* was studied against three fingi namely, *Fusarium fusiformis, Fusarium semitectum and Alternaria alternata.* Both the compounds were found to be highly potent against *Alternaria alternate*[21].
- Effect of 50% ethanolic (100 to 200 mg/kg p.o.) extract of *A. speciosa* flowers was examined in different acute and chronic gastrointestinal ulcers in validated experimental models in rats. *A. speciosa* at a dose of 150 mg/kg showed healing effect against acetic acid induced ulcer index with decreased perforations[22].
- Wound healing and anti-inflammatory activity of 50% ethanolic extract was studied in Sprague–Dawley rats. The extract significantly showed anti-inflammatory activity at 3 hr in carragenan induced paw edema and enhanced the gain in breaking strength in dead space and incision wound, wound contraction area, epithelization time and hydroxyproline content of tissues[23].
- Antibacterial activity of *A. speciosa* seed oil against gram positive and gram negative organisms was investigated. Study revealed that oil is inactive against *Staphylococcus aureus* and *Staphylococcus albus.* The oil showed potent antifungal activity against *Geotrichum candidum, Alternaria solani* and *Colletotrichum dematum* [24].

5.4.2 Phytochemical review

- Two aryl esters, Sigmasteryl p-hydroxycinnamate and hexa decanyl p-hydroxy cinnamate and have been isolated from the n-hexane extract of *A. speciosa* root. Further ethyl acetate extract provided a coumarin called scopoletin [25].
- Disubstituted tetrahydofuran and a lipid ester were isolated from *A. speciosa* root fraction [26].
- Chromatographic (TLC and GLC) analysis of *A. speciosa* seed oil led to the identification of Methyl myristoleate, Myristate, 12-methyl myristate, Palmitate, Linolenate, Linoleate, Oleate, Stearate, 15-methyl stearate, nonadecanoate, epicosanoate, eicosanoate, heneicosanoate and behenoate. The oil is found to be rich in oleic acid and contains palmitic, stearic oleic and linoleic acid [24].
- The seeds are rich in protein (30.6%) and contain essential aminoacids. Besides the seeds contain n-triacontanol, β-sitosterol, p-hydroxycinnamo-L octadecanoate and β-hydroxycinnmic acid [27].

By considering the ethnobotanical, ethnopharmacolgical and phytochemical contributions of Convolvulaceae family, we have selected roots *Argyreia speciosa (sweet),* traditionally known as *Vrudhhadaru* and used as a rasayana in Ayurveda for its phytochemical and pharmacological investigations.

5.5 Procurement and authentication of plant

Roots of *Argyreia speciosa* were collected from hilly areas (900 meters) surrounding Dharwad and Kumata districts, Karnataka, and authentication of the plant was done by qualified taxonomist, Department of Botany, Karnatak University, Dharwad. A herbarium specimen of the plant was kept in Department of Pharmacognosy (SETCPD/Ph.cog/herb/33/2006), SET's College of Pharmacy, Dharwad, Karnataka,

India. The collected material was washed with running water. The roots were chopped in to small pieces and dried under shade. Dried roots were coarsely powdered and used for extraction.

5.6 Pharmacognostic evaluation of *Argyreia speciosa* (sweet), (Burm. f.) Bojer.

It is a very large climbing shrub with woody, white, tomentose stems. Leaves (7.5 cm to 30 cm long and 6.3 cm to 25 cm wide) are simple, large, ovate, acute, base cordate, glabrous above, white tomentose beneath. Flowers are large, purple, silky pubescent without in long-peduncled cymes (7.5 to 15 cm), corolla (1.7 cm long) tubular-infundibuliform. Fruits are dry, globose (2.0 cm diameter), apiculate.

5.6.1 Synonyms : *Argyreia nervosa* (Burm. f.) Bojer.

Convolvulus nervosus (Burm. f.) Bojer.

Convolvulus speciosus L. f.

Lettsomia nervosa (Burm. f.) Roxb.

5.6.2 Vernacular Names:

Sanskrit	:	*Vriddhadaruka, Vriddhadaru*
English	:	Elephant Creeper
Hindi	:	Samundara-ka-pat
Kannada	:	Chandarpada

5.6.3 Morphology or Macroscopical evaluation

External features, dimensions and organoleptic properties of root were studied.

5.6.4 Microscopical observations of Root

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10-12μm. Dewaxing of the sections was done[8] and sections were stained with toluidine blue[9] .

Since toluidine blue is a polychromatic stain, the staining results were remarkably good and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies. Whereever necessary sections were also stained with safranin and fast-green and Iodine.

Photomicrographs of the microscopic descriptions of the tissue were taken with Nikon labphoto-2 microscopic unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Magnifications of the figures are indicated by the scale bars. Descriptive terms of the anatomical are given as per the standard anatomy books[10,11].

Fig. 5.1 Shrub, aerial parts and roots of *Argyreia speciosa* (Burm.f.) Boj.

5.6.5 Determination of proximate values for *Argyreia speciosa* root [12]

a) Total Ash

Weigh accurately 2 gm of the air dried crude drug in a tared platinum or silica dish and incinerate at a temperature not exceeding 450°C until free from carbon, cool and weigh. If a carbon free ash cannot be obtained in this way, exhaust the charred mass with hot water, collect the residue on an ashless filter paper, incinerate the residue and the filter paper until the ash is white or nearly so, add the filtrate, evaporate to dryness and ignite at a temperature not exceeding 450°C. Calculate the percentage of total ash with reference to the air dried drug.

b) Acid Insoluble ash

Boil the total ash obtained with 25 ml of 2M hydrochloric acid for 5 min, collect the insoluble matter in a Gooch crucible or on an ashless filter paper, wash with hot water, and ignite, cool, in a descicator and weigh. The percentage of acid-insoluble ash with reference to the air dried drug was noted.

c) Sulphated ash

Weigh accurately 2 gm of the air dried crude drug in a tared platinum or silica dish and treat the powdered drug with dil. H_2SO_4 before incineration. Here all the oxides and carbonates are converted to sulphates. Then ignition is carried out at 600°C. The percentage of sulphated ash with reference to the air dried drug was calculated.

d) Water soluble ash

Boil the total ash obtained with 25 ml of distilled water for 5 min, filter and evaporate the filtrate cool, in a descicator and weigh. The percentage of water soluble ash with reference to the air dried drug was noted.

e) Ethanol soluble extractive

Macerate 5 gm of air dried drug, coarsely powdered, with 100 ml of ethanol of the specified strength in a closed flask for 24 hrs, shaking frequently during the first 6 hrs and allowed to stand for 18 hrs. Thereafter filter rapidly taking precautions against loss of ethanol, evaporate 25 ml of the filtrate to dryness in tared flat-bottomed shallow dish, dry at 105°C and weigh. Calculate the percentage of ethanol soluble extractive with reference to the air dried drug.

f) Water soluble extractive

Add 5 gm of air dried crude drug to 50 ml of water at 80°C in a stoppered flask. Shake well and allow to stand for 10 mins, cool, add 2 gm of keiselghur and filter. Transfer 5 ml of the filtrate to a tared evaporating dish, 7.5 cm in a diameter, evaporate the solvent on a waterbath, continue drying for 30 min, finally dry in a steam oven for 2 hrs and weigh the residue. Calculate the percentage of water-soluble extractive with reference to the air dried drug.

g) Loss on drying (Moisture content)

Weigh a glass stoppered, shallow weighing bottle that has been dried under the same conditions to be employed in the determination. Transfer to the bottle 5 gm of crude drug, cover it and accurately weigh the bottle and the contents. Distribute the drug as evenly as practicable by gentle sidewise shaking to a depth not exceeding 10 mm. Place the loaded bottle in the drying chamber (oven or descicator). Dry the drug to constant weight or for the specified time and at constant temperature. After drying is completed, open the drying chamber, close the bottle promptly and allow it to cool to room temperature in a descicator before weighing. Weigh the bottle and the contents.

h) Foreign organic matter

Foreign organic matter is the material consisting of any or all of the following

1. Parts of the organ or organs from which the drug is derived other than the parts named in the definition and description or for which the limit is prescribed.
2. Any organs other than those named in the definition and description.
3. Matter not containing from the source plant.
4. Moulds, Insects or other animal contamination.

Weigh 100 to 500 gm of the original crude drug and spread it out in a thin layer. Inspect the sample with the unaided eye or with the use of 6X lens and separate the foreign organic matter manually as completely as possible. Weigh and determine the percentage of foreign organic matter from the weight of the drug taken.

5.6.6 Traditional uses of the plant

The root is acrid, bitter, astringent digestive and appetizer. Traditional preparations include the roots for its rejuvenating, intellect promoting, brain tonic, anti-inflammatory, cardiotonic, hepatoprotective and antidiabetic properties. It is also used in the treatment of pulmonary tuberculosis, syphilis, bronchitis, pharyngitis and other infectious diseases because of its antimicrobial properties. It is also prescribed for leucorrhoea and general debility. Roots are also used for body pain and rheumatism and in the preparation of tonics in traditional systems of medicine. In western countries it is used as hypotensive, anabolic, aphrodisiac and hallucinogen. The leaves are emollient, vesicant antiphlogistic. The outer surface is irritant, hastens maturation and absorptive, hence externally applied for boils, carbuncles, foul ulcers, wounds as poultice. It is also used externally for ring worm infections and eczema. It is mixed with vinegar and the sap is rubbed to reduce obesity [13-17].

5.7 Extraction

The choice of extraction procedure depends on the type of crude drug (fresh or dried) and the type of chemical constituents present in the drug. Fresh plant (leaves) material if used should be homogenized in alcohol to stop the enzyme activity. Dried drug material should be powdered before extraction. Maceration, percolation, successive solvent extraction using Soxhlet apparatus and supercritical fluid extraction are the most widely used methods of extraction [28,29].

5.7.1 Choice of solvents for extraction

Various solvents either polar or non-polar are used for extraction of primary or secondary metabolites of plants. Choice of solvent also depends on the ability of solvents to extract the largest quantity of compounds with high biological activity. An important factor governing the choice of solvents used in an extraction is type of phytochemical groups that are to be extracted. Several researchers have used different solvents from non polar to polar while extracting compounds from plants.

A correct choice of solvent is fundamental for obtaining an optimal extraction process. When selecting a solvent consideration should be given to the interaction of the solvents with the matrix and the analyte of interest and exclude unwanted matrix components. Another important aspect is the compatibility of the extraction solvents with the analytical method used for the final analysis step. We need to know the type of compounds we are targeting in terms of polarity, if not we must use solvents of different polarities to have a wide range of compounds [30].

5.7.2 Temperature

Extraction temperature is one of the parameters, since an increase in temperature shortens of the time needed for the establishment partition equilibrium, for all extraction techniques. When extraction is conducted at high temperature it may reach well above the

boiling point of the solvent. This can only occur at high pressure in closed containers. These elevated temperatures results in improved extraction efficiencies, since desorption of analytes from active site in the matrix will increases. In addition solvents have higher capacity to solubilize analyte at high temperatures, while surface tension and solvent viscosity decreases with temperature, which will improve sample wetting and matrix penetration respectively. Room temperature was found to be optimum temperature to extract different compounds usually there is little effect when heat is used.

5.7.3 Time

There is an inverse relationship between the time required for efficient extraction and size of the sample particles. Extraction time differs and depends on the amount of sample used. Often 10 min are sufficient for extraction of organic pollutants but even 3 min have been demonstrated to give full recovery for pesticides from soils and sediments. When extracting amino acids from food no improvement in the extraction efficiency was observed even after applying longer radiation times. In addition there was no evidence of breakdown or alteration of the amino acids caused by longer extraction times. With thermolabile compounds long extraction time may results in degradation the optimum time for most plants extracts is 10 min for 1-2 gms sample.

5.7.4 Bioactivity guided fractionation of *Argyreia speciosa* roots

Authenticated roots of *A. speciosa* were shade dried and pulverized in to coarse material. Coarse plant material was cleaned by passing the powder material through 120 mesh sieve to remove any fine dust or powder, and coarse powder was used for extraction. Dried powder of root was exhaustively extracted successively using Petroleum ether (60-80°C) (PEAS), Chloroform (CAS), Ethyl acetate (EAAS) and Ethanol (95%) (EtAS) respectively in a Soxhlet apparatus. Aqueous fraction was

prepared by macerating the drug in chloroform water (AQAS). All the extracts were concentrated by rotary flash evaporator, under reduced pressure and controlled temperature, followed by freeze drying and stored in a descicator. Fig 5.2. Shows the scheme for successive fractionation of plant material with different solvents.

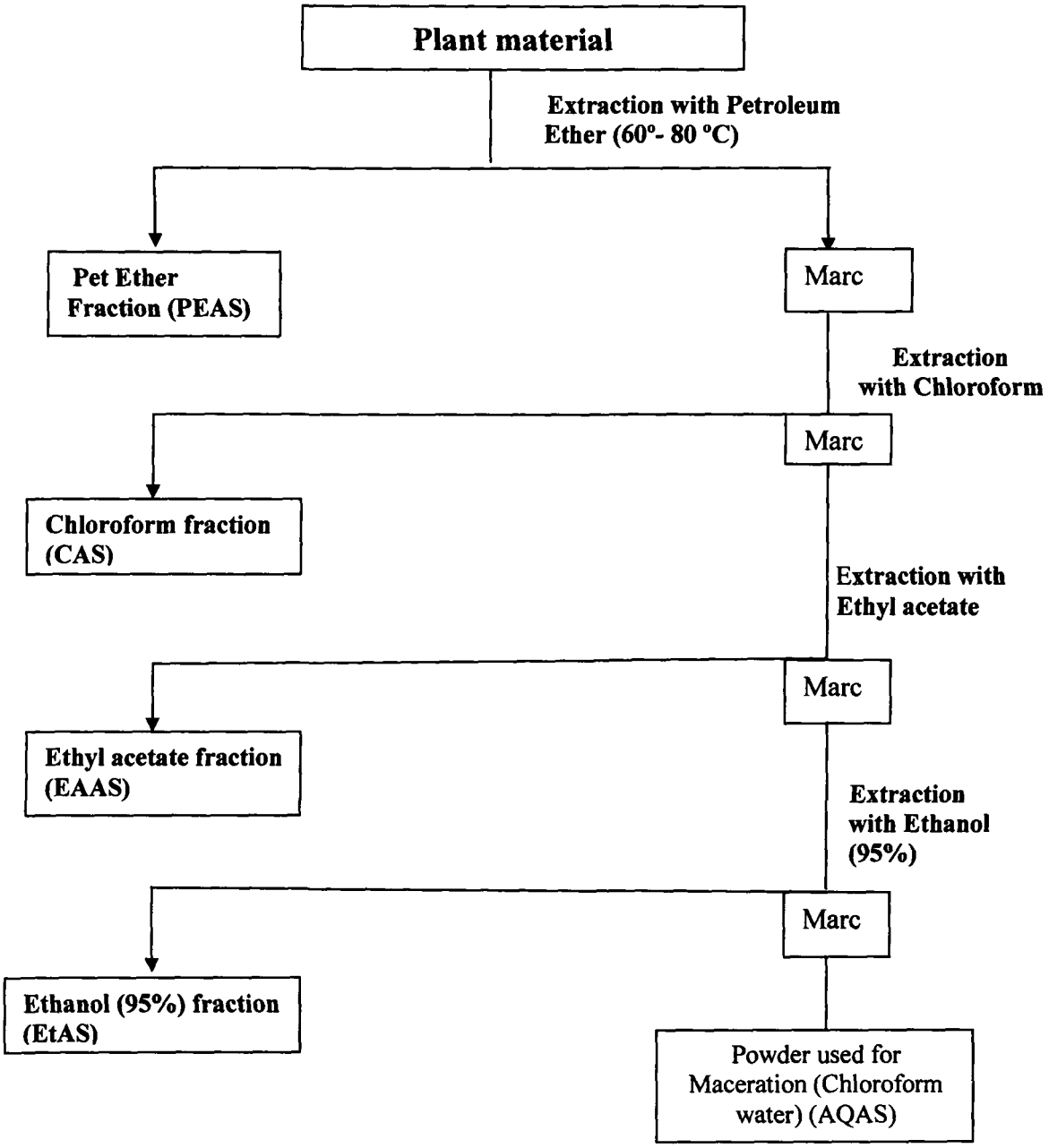

Fig. 5.2. Schematic diagram of Successive extraction of *Argyreia speciosa* root powder.

5.8 Preliminary Phytochemical investigation of crude fractions of *Argyreia Speciosa*

Roots of *A. speciosa* were collected according to their appropriate seasons and all the fractions were subjected to qualitative phytochemical investigation using following standard tests to identify the type(s) of phytoconstituents [31,32].

5.8.1 Test for carbohydrates

Molisch's test : Test solution + few drops of Molisch's reagent + 2ml of concentrated sulphuric acid along the sides of the test tube. A purple ring formed at the junction of two liquids indicates the presence of carbohydrates.

Fehling's test: Test solution + HCl heat + neutralize with NaOH + Fehling's solution A and B in equal propotions, Heat on waterbath. Reddish brown precipitate indicates the presence of carbohydrates.

Barfoed's test: Test solution + Barfoed's reagent, boiled on water bath. Brick red precipitate indicates the presence of carbohydrates.

Benedict's test: Test solution + Benedict's reagent, boiled on water bath. Reddish brown precipitate indicates the presence of carbohydrates.

5.8.2 Test for sterols

Salkowaski test: Test solution + concentrated sulphuric acid, shaken and allowed to stand. The lower layer turns red indicating the presence of sterols.

Liebermann-Burchard test: Test solution + few drops of acetic anhydride. + Concentrated sulphuric acid along the sides of the test tube. Brown ring forms at the junction of the two liquids and the upper layer turns green.

Sulphur test: Sulphur when added to the test solution, it sinks to the bottom indicating the presence of sterols.

5.8.3 Test for alkaloids (general)

Mayer's test: Test solution + Mayer's reagent (Potassium mercuric iodide) gives cream coloured precipitate.

Wagner's test: The acidic test solution with Wagner's reagent (Iodine in potassium iodide) gives brown precipitate.

Hager's test: The acidic test solution with Hager's reagent (Saturated picric acid solution) gives yellow precipitate.

Dragendorff's test: The acidic test solution with Dragendorff's reagent (Potassium bismuth iodide) shows reddish brown precipitate.

5.8.4 Test for proteins and amino acids

Millon's test: Test solution + Millon's reagent, heated on a water bath. Yellow colouration indicates the presence of protein.

Xanthoproteic test: Test solution + concentrated nitric acid, on boiling gives yellow precipitate.

Biuret test: Test solution + 40% sodium hydroxide + dilute copper sulphate solution. Blue colour indicates the presence of protein.

Ninhydrin test: Test solution + Ninhydrin reagent gives blue colour.

5.8.5 Test for tannins

Ferric chloride test: Test solution + few drops of ferric chloride solution gives dark red colour.

Gelatin test: Test solution + gelatin solution gives white precipitate.

5.8.6 Test for Saponin glycosides

Foam test: Saponins when mixed with water and shaken shows the formation of froth, which is stable at least for 15 min.

Haemolysis test: 2 ml each of 18% sodium chloride solution was taken in two test tubes. To one test tube 2 ml of distilled water and to another test tube 2 ml of test sample was added. A few drops of blood was added to both the test tubes, mixed and observed for haemolysis under microscope. Haemolysis of blood cells indicates the presence of saponin glycosides.

5.8.7 Test for triterpenoids

Salkowaski test: Test solution + few drops of concentrated sulphuric acid, shaken and allowed to stand, lower layer turns yellow indicating the presence of triterpenoids.

Liebermann-Burchard test: Test solution + few drops of acetic anhydride. + Concentrated sulphuric acid along the sides of the test tube. Development of deep red colour indicates the presence of triterpenoids.

5.8.8 Test for flavanoids

Ferric chloride test: Test solution + few drops of ferric chloride solution give intense green colour.

Shinoda test: Test solution + few fragments of magnesium ribbon + concentrated hydrochloric acid, shows pink to magenta red colour.

Zinc-Hydrochloric acid reduction test: Test solution + zinc dust + few drops of hydrochloric acid shows magenta red colour.

Alkaline reagent test: Test solution + sodium hydroxide solution shows increase in the intensity of yellow colour which becomes colourless on addition of few drops of dilute acid.

Lead acetate solution test: Test solution + few drops of lead acetate (10%) solution gives yellow precipitate.

5.9 Results

A) Pharmacognostic evaluation of roots of *Argyreia speciosa*

i) Morphological evaluation

Table 5.1. Details of morphological and organoleptic characters

Plant name and part used	Parameter studied	Feature
Argyreia speciosa root	Size	10-50 cm length 1-2 cm width
	Shape	Cylindrical pieces
	Colour	Brown
	Odour	none
	Taste	acrid, bitter
	External features	It is tuberous in nature

ii) Microscopical evaluation

The root is slightly features flattened or roughly circular in cross sectional view. It consists of fairly wide periderm and deeply cleaved vascular cylinder (Fig. 5.3.1). The periderm seems to have originated from deeper part and has pushed the cortex to a thin superficial dark rough and fissured layer (Fig. 5.3.3). It is heterogenous comprising of lignified thin cylinder of phelloids alternating with thin walled, suberized tabular phloem cells. In the root studied, there are two cylindrical zones of phloem and a thick cylinder of phelloid; a second layer of phelloids is found just appearing.

Secondary phloem follows immediately beneath the periderm. The secondary phloem occurs in the form of wide semicircular zone outside the xylem segments. The semicircular phloem zone has outer portion of collapsed tissue and minor zone of intact

noncollapsed tissue (Fig. 5.3.3 and 5.3.4). The collapsed phloem consists of thin, dark arcs of crushed phloem elements. The tissue immediately outside the xylem segments has intact, well preserved phloem cells (Fig. 5.3.2 and 5.3.4).

Secondary xylem is deeply lobed in to five or six, fan shaped radial segments. Each segment is narrow in the cube and widened towards the periphery (Fig. 5.3.1, 5.3.5 and 5.3.6). The xylem segments consist of wide, thin walled, circular or angular vessels and sclerenchymatous xylem vessels. The vessels in the centre are about 50μm wide, those towards the periphery are more than 150 μm wide. The xylem fibres are thick walled with wide lumen. The tissue beneath the vascular segments is the dilated ray. Due to longitudinal expansion of the rays, the vascular cylinder is cleaved in to fan shaped segments.

Cell inclusions

Calcium oxalate crystals of druses are seen all along the minor layers of the periderm as well as along the phloem sclerenchyma cylinder (Fig. 5.3.7). Crystals are also seen in the xylem fibres. Starch grains are sparsely seen in the dilated rays and phloem parenchyma (Fig. 5.3.8).

Fig 5.3 Microphotographs of histological characters of *Argyreia speciosa* (Burm.f.) Boj. root.

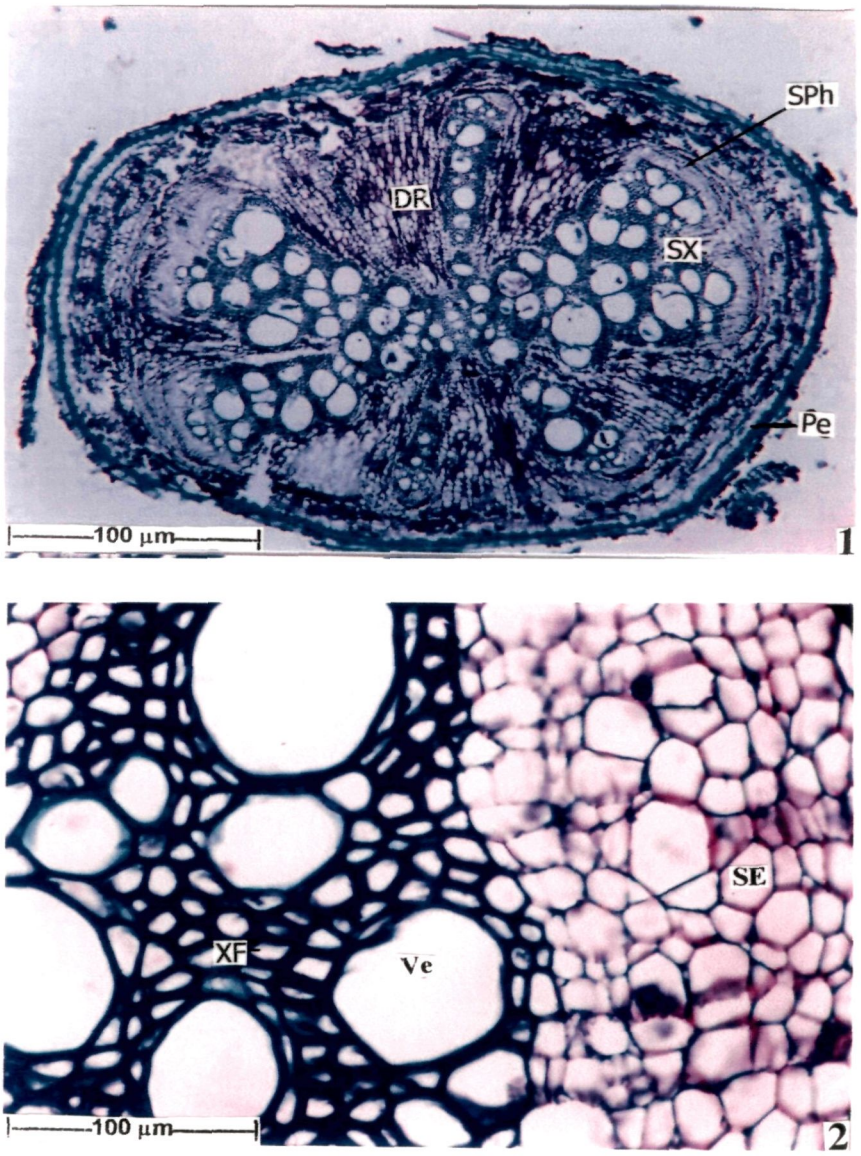

Fig. 5.3.1 and 5.3.2. Transverse section of root with entire view showing deeply divided fan shaped xylem segments and enlarged image of secondary phloem and xylem.
DR-Dilated ray; **Pe**-Periderm; **SE**-Sieve elements; **SPh**-Secondary Phloem; **Sx**-Secondary Xylem; **Ve**-Vessel; **XF**-Xylem Fibre.

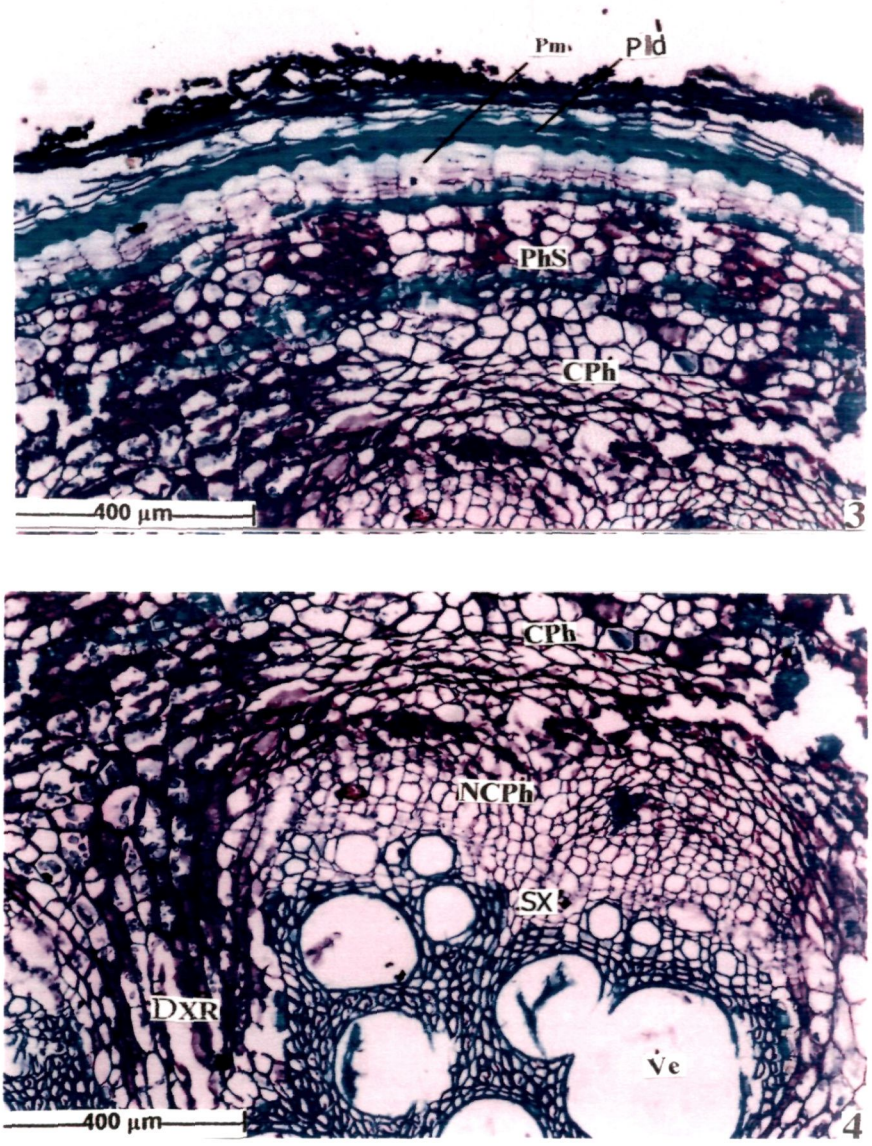

Fig. 5.3.3 and 5.3.4. Transverse section of root showing heterogeneous periderm and vascular segments enlarged with dilated ray.

Cph-Collapsed phloem; **DxR**-Dilated xylem ray; **NCph**-Non-Collapsed phloem; Phs-Phloem sclereides; PlD-Phelloderm; **Pm**-Phellem; **Sx**-Secondary xylem; **Ve**-Vessel

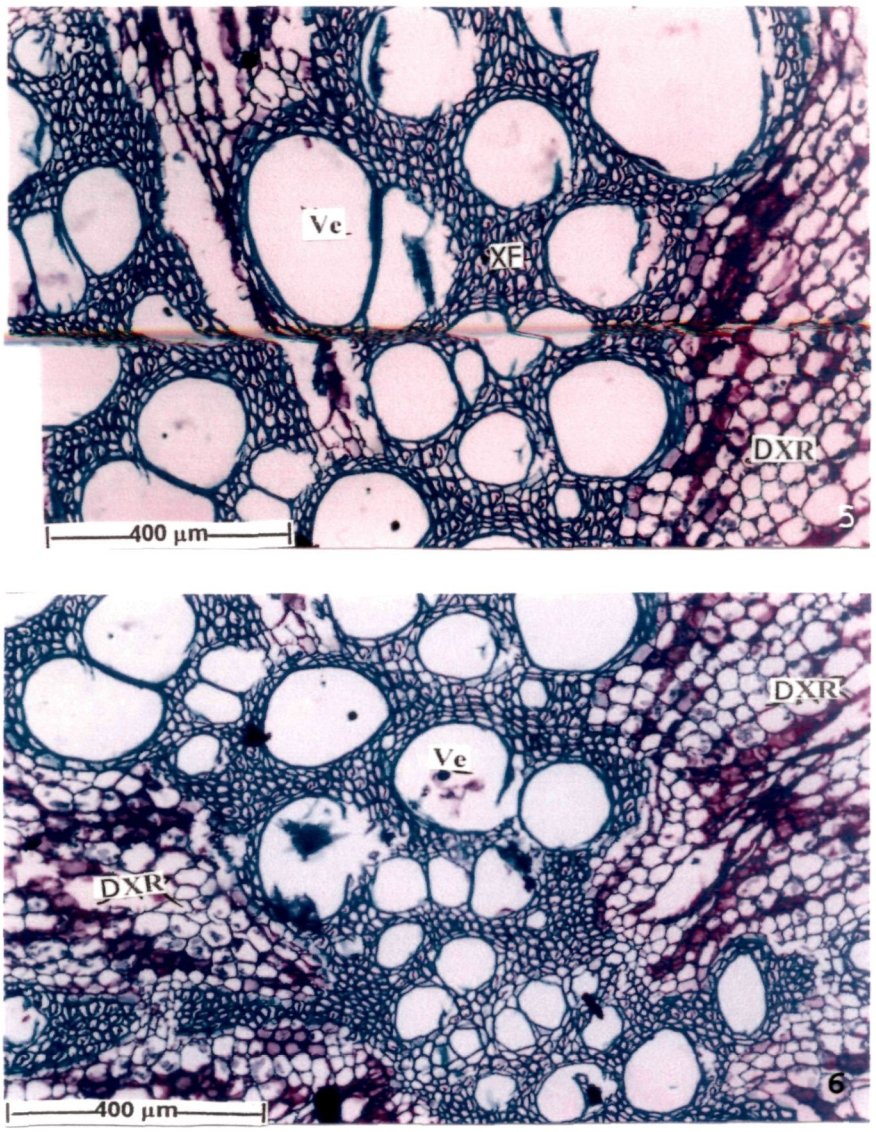

Fig. 5.3.5 and 5.3.6 Outer and central portion of secondary xylem

DxR-Dilated xylem ray; **Ve**-Vessel; **XF;** Xylem Fibres.

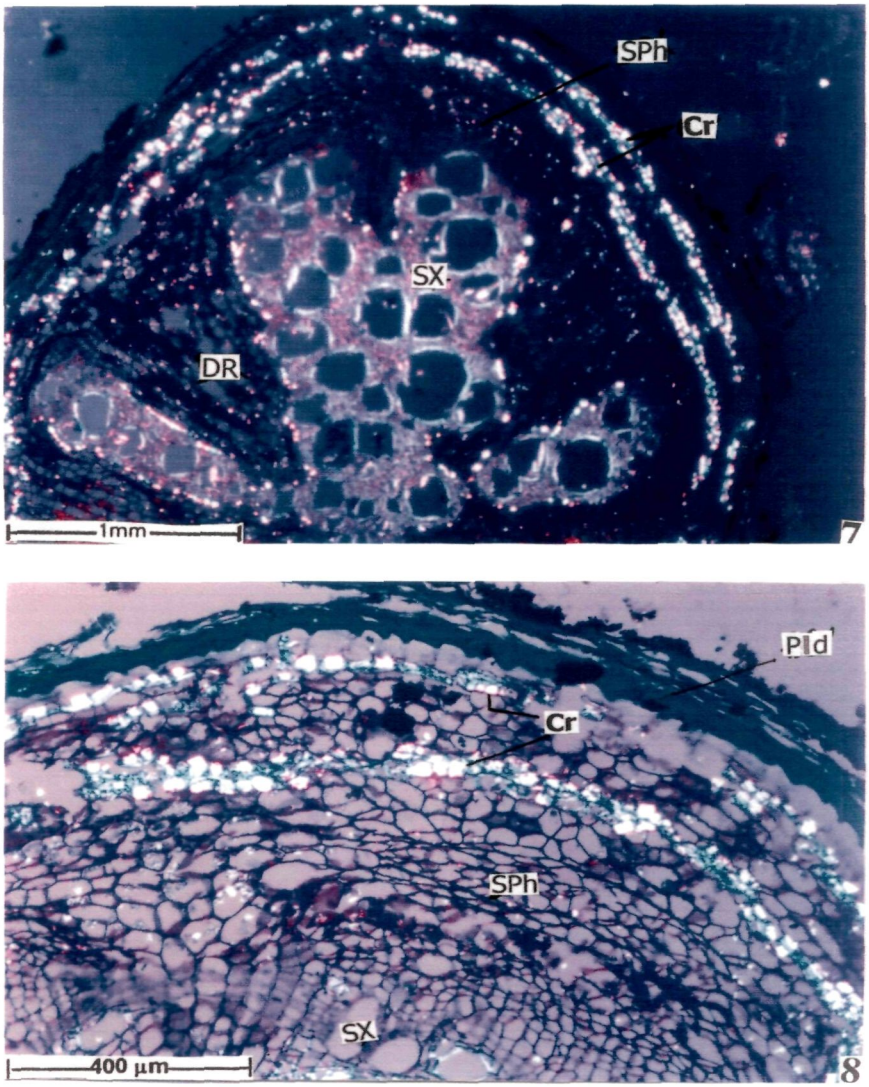

Fig 5.3.7 and 5.3.8 Transverse section of root showing crystal distribution in the cortical zone viewed under polarized light microscope.

Cr-Crystals; **DR**-Dilated ray; **PlD**-Phelloderm; **Sph**-Secondary phloem; **Sx**-Secondary xylem.

Table 5.2. Proximate values of *Argyreia speciosa* root. (Values are average of two replicates)

S.No	Evaluation parameter	Value (%w/w)
01	Total ash value	18-20
02	Acid insoluble ash value	2-2.5
03	Water soluble ash value	1.5-2
04	Sulphated ash value	0.8-1
05	Alcohol soluble extractive value	1.0-2.4
06	Water soluble extractive value	9.6
07	Moisture content	16
08	Foreign organic matter	0.5

Table 5.3. Percentage yield of different fractions of *Argyreia speciosa* root

S.No	Name of the fraction	Colour	Consistency	Yield (%w/w)
01	Petroleum Ether fraction (PEAS)	Reddish brown	Semisolid (waxy)	0.12
02	Chloroform fraction (CAS)	Dark green	Solid	0.14
03	Ethyl acetate fraction(EAAS)	Yellowish black	Semisolid	0.85
04	Ethanol fraction (EtAS)	Brown	Sticky solid	1.75
05	Aqueous fraction (AQAS)	Light brown	Semisolid	2.58

B) Preliminary Phytochemical analysis

Results of qualitative identification tests are summarized as follows

Table 5.4. Qualitative phytochemical analysis of different fractions of *Argyreia speciosa* root

S.No.	Name of the phytoconstituent	PEAS	CAS	EAAS	EtAS	AQAS
01	Carbohydrates	–	–	–	+	+
02	Steroids	+	+	–	–	–
03	Alkaloids	+	+	–	–	–
04	Proteins and Amino acids	–	–	–	+	+
05	Tannins	–	–	–	+	+
06	Saponins	–	–	–	+	+
07	Triterpenoids	–	+	–	–	–
08	Flavanoids	–	–	+	+	–
09	Coumarin glycosides	–	–	+	+	–

'+' = Present '-' = Absent

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5.11 Pharmacological evaluations of crude fractions of *Argyreia speciosa* (Burm.f.) Boj.

Crude fractions of *Argyreia speciosa* root were subjected to its traditionally claimed hepatoprotective (tonic), Nootropic (intellectual promoting), anti-stress (adaptogenic), antidiabetic, cardioprotective, antimicrobial and *in-vitro* and *in-vivo* antioxidant activities using animal models.

5.11.1 Hepatoprotective and *in-vivo* antioxidant activity of *Argyreia speciosa* fractions in CCl_4 induced hepatotoxicity in rats

5.11.1.1 Introduction

The liver, the body's largest internal organ, is responsible for many vital functions. It processes food and plays an important role in metabolism, including the breakdown, storage and release of sugar, fats, cholesterol and fat-soluble vitamins. It filters and processes numerous toxins, including alcohol and many drugs. In addition, it produces several crucial proteins such as enzymes, hormones, and factors that enable blood clotting. Since liver plays pivotal roles in a large number of metabolic and immune processes, the physiological and pathophysiological functions of reactive oxygen species (ROS) have attracted numerous investigations [1]

Hepatotoxicity implies chemical-driven liver damage. The liver plays a central role in transforming and clearing chemicals and is susceptible to the toxicity from these agents. Certain medicinal agents when taken in overdoses and sometimes even when introduced within therapeutic ranges may injure the organ. Other chemical agents such as those used in laboratories and industries, natural chemicals (e.g. microcystins) and herbal remedies can also induce hepatotoxicity. Chemicals that cause liver injury are called hepatotoxins [2]

5.11.1.2 Mechanism of liver damage

Drugs continue to be taken off the market due to late discovery of hepatotoxicity. Due to its unique metabolism and close relationship with the gastrointestinal tract, the liver is susceptible to injury from drugs and other substances. 75% of blood coming to the liver arrives directly from gastrointestinal organs and then spleen via portal veins which bring drugs and xenobiotics in concentrated form. Several mechanisms are responsible for either inducing hepatic injury or worsening the damage process. Many chemicals damage mitochondria, intracellular organelles that produce energy. Its dysfunction releases excessive amount of oxidants which in turn injures hepatic cells. Activation of some enzymes in the cytochrome P-450 system such as CYP2E1 also leads to oxidative stress [3] Injury to hepatocyte and bile duct cells lead to accumulation of bile acid inside liver. This promotes further liver damage [4] Non-parenchymal cells such as Kupffer cells, fat storing stellate cells and leukocytes (i.e. neutrophil and monocyte) also have role in the mechanism.

5.11.1.3 Role of Natural products in hepatic injury

Free radical induced lipid peroxidation is believed to be one of the major causes cell membrane damage leading to a number of pathological situations[5,6]. A number of recent reports clearly demonstrated that in addition to hepatic problems, CCl_4 also causes disorders in kidney, lungs, testis and brain as well as in blood generating free radicals [7,8]. Lipid peroxides produced from unsaturated fatty acids via radicals, cause histotoxicity and promote the formation of additional free radicals in a chain reaction type manner. It is thought that, if the *in vivo* activity of enzymes or scavengers is not high enough to inhibit the radicals, various diseases such as liver disease, diabetes and accelerated aging may result [9].

In spite of tremendous advances in modern medicine no effective drugs are available, which stimulate liver functions and offers protection to the liver from the damage or help to regenerate the hepatic cells[10]. In the modern medicine, plants occupy significant birth as raw materials for some important drug preparations [11,12]. The traditional Indian medicinal plants act as antiradicals and DNA cleavage protectors [13]. In the absence of a reliable modern hepatoprotective drugs, there are a number of traditional medicines recommended for the treatment of liver diseases [14]. Many herbs reportedly possess hepatoprotective activity such as *Silybum marianum*[15], *Tridax procumbens*[16], *Strychnous potatorum*[17], *Andrographis panic*ulata[18] *Picrorhiza kurroa*[19], *Aquilegia vulgaris*[20], *Moringa oleifera*[21], *Eclipt alba*[22], *Phyllanthus niruri* [23,24,25] possess hepatoprotective property against toxins and drugs induced hepatotoxicity. Herbal medicines are often used with modern drugs with the aim to decrease side effects or toxicity or to obtain a synergetic or additive effect in terms of pharmacological effects. For this reason herbal medicine has received increasing popularity [26].

In view of the importance of plants and their secondary metabolites in hepatic disorders, we investigated the antihepatotoxic activity *Argyreia speciosa* root fractions in CCl_4 induced hepatotoxicity and *in-vivo* antioxidant activity was also carried out to obtain the probable mode of action of the active fractions.

5.11.1.4 Materials and methods

a) Chemicals

All the solvents and chemicals used were of analytical grade/HPLC grade and obtained from Qualligens, Mumbai. Standard kits for SGOT, SGPT and Bilirubin were obtained from Teco Diagnostics, USA, and Cholesterol from Span Diagnostics, India. Standard drug Silymarin was obtained from Micro Laboratory, Bangalore India.

b) Experimental animals

All the experiments were carried out using male, Swiss albino mice (25-30 g) and wistar rats of either sex (180-200 g) procured from animal house of BLDEA Medical College, Bijapur, Karnataka. The animals had free access to food and water, and they were housed in a natural (12 h each) light/dark cycle. Food given to animals consisted of wheat flour kneaded with water and mixed with a small amount of refined vegetable oil. The animals were acclimatized for at least 5 days to the laboratory conditions before conducting experiments. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and the care of the laboratory animals was taken as per the CPCSEA regulations.

c) Preparation of drugs

Suspensions of Petroleum ether fraction (PEAS), Chloroform fraction (CAS), Ethyl acetate fraction (EAAS) and Ethanol (EtAS) were prepared in Tween-80 and distilled water (2:8) to assess hepatoprotective and *in-vivo* antioxidant activity.

d) Acute toxicity studies

Acute toxicity study was carried out using Swiss albino mice (25-30 g) by up and down / staircase method as per CPCSEA guidelines. Both the extracts were orally administered to different groups of mice at doses of 50 mg, 300 mg, 1000 mg and 2000 mg / kg body weight respectively. Animals were observed for 48 h to study the general behavior of animals, signs of discomfort and nervous manifestations.

e) Hepatoprotective and antioxidant activity

Hepatoprotective and antioxidant activity was carried out using Wister strain albino rats of either sex (180-200 g). Animals were divided into 7 groups of six animals in each group. Group I:served as control (normal saline 5 ml/kg, p.o.). Group II: Served

as negative control (CCl_4/olive oil (1:1), 0.7 ml/kg, i.p.) on alternate days for period of seven days). Group III: Treated with Silymarin 100 mg/kg, p.o for successive seven days. All the animals except control group received CCl_4 (0.7 ml/kg, i.p.) on every alternate days. Group IV and VI: EtAS (200 and 400 mg/kg) was orally administered for successive seven days. Group V and VII: EAAS (200 and 400 mg/kg) orally for seven days. On the seventh day 2 hr after the administration of the last dose, the animals were sacrificed by cervical dislocation, blood was withdrawn by intracardiac puncture. It was allowed to coagulate for 30 min, serum was separated by centrifugation and used to estimate serum glutamate pyruvate transaminsase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), serum cholesterol, total bilirubin and direct bilirubin. Livers were isolated to measure the levels of antioxidant enzymes and for histopathological studies [27,28,29].

f) Measurement of antioxidant enzymes

Five percent liver homogenate was prepared with 0.15 M KCl and centrifuged at 1000 rpm for 10 min. The cell free supernatant was used for the estimation of Super oxide dismutase (SOD)[30], Catalase [31] and Peroxidase [32].

SOD assay

Liver homogenate (0.5 ml) was taken, and 1 ml of 50 mM sodium carbonate, 0.4 ml of 24 µm NBT, and 0.2 ml of 0.1 mM EDTA were added. The reaction was initiated by adding 0.4 ml of 1mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560 nm followed by recording the absorbance after 5 min at 25°C. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of units per milligram of proteins.

Catalase assay

1 ml of liver homogenate was taken with 1.9 ml of phosphate buffer in test tubes (50 mM, pH 7.4). The reaction was initiated by the addition of 1ml of H_2O_2 (30 mM). Blank without liver homogenate was prepared with 2.9 ml of phosphate buffer and 1 ml of H_2O_2. The decrease in optical density due to decomposition of H_2O_2 was measured at the end of 1 min against the blank at 240 nm. Units of catalase were expressed as the amount of enzyme that decomposes 1μM H_2O_2 per min at 25°C. The specific activity expressed in terms of units per milligram of proteins.

Peroxidase assay

Liver homogenate (0.5ml) was taken, and to this were added 1ml of 10mM KI solution and 1ml of 40mM sodium acetate. The absorbance of Potassium per iodide was read at 353 nm, which indicates the amount of peroxidase. Then 20μl of H_2O_2 (15 mM) was added, and the change in the absorbance in 5 min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change the optical density by 1 unit per min. The specific activity expressed in terms of units per milligram of proteins.

g) Histopathological studies

For histopathopathological observation, sections were taken from each lobe of liver immediately. The tissue were fixed in 10% neutral formalin, dehydrated in graded alcohol and embedded in paraffin, cut into 4-5 μm thick sections and stained with Haematoxylin-Eosin for photomicroscopic assessment [33].

h) Statistical analysis

The data were expressed as the mean ± SEM, (n = 6). Data were analyzed using One way ANOVA followed by Dunnet's multiple comparison test. Values of $P< 0.05$ were considered statistically significant.

5.11.1.5 Results

The percentage yield of extracts was found to be 1.76% and 0.82% for ethanolic extract (EtAS) and ethyl acetate extract (EAAS) respectively. Preliminary phytochemical analysis revealed the presence of flavonoids, tannins, alkaloids coumarin glycosides in the crude fractions. EtAS and EAAS did not show any toxicity and behavioral changes in mice and hence doses of 200 and 400 mg/kg were selected for hepatoprotective and *in vivo* antioxidant activity. The activities of SGOT, SGPT, ALP and other biochemical parameters after administration of CCl_4 and test extracts is summarized in Table 5.5 and Fig. 5.5.1 to 5.5.6. There was a significant rise in the levels of all biochemical parameters after administration of CCl_4 injection compared to normal control animals. In contrast, pretreatment with suspensions of EtAS (200 mg/kg and 400 mg/kg) and EAAS (200 mg/kg and 400 mg/kg) of *A. speciosa* roots exhibited an ability to counteract the CCl_4 induced hepatotoxicity by decreasing serum enzyme levels compared CCl_4 group ($P<0.001$). Pretreatment of rats with suspensions of EtAS and EAAS at a dose of 200 mg/kg and 400 mg/kg preserved Catalase, SOD, and Peroxidase activity compared to control and CCl_4 group ($P<0.001$), thus providing protection against CCl_4 toxicity (Table 5.6). Histopathological examination demonstrated that CCl_4 treated group induces ballooning degeneration, centrilobular necrosis and apoptosis in hepatocytes (Fig 5.4.2). Groups treated with EtAS and EAAS showed recovery on ballooning degeneration and centrilobular bridging necrosis was occasionally present (Fig 5.4.3 & 5.4.4).

5.11.1.6 Discussion

The hepatic damage induced by CCl_4 is well known to be mediated by its free radical metabolites such as $\dot{C}Cl_3$ and CCl_3COO^-, which interact with unsaturated lipid membrane to produce lipid peroxidation and other cellular macromolecules leading to cell damage[34,35]. The free radicals in the presence of oxygen, leads to auto oxidation of the fatty acids present in the cytoplasmic membrane phospholipids[36] and causes functional and morphological changes in the cell membrane. Further more, influx of extracellular Ca^+ ions into cell is claimed to be an important step leading to cell death. In the present investigation CCl_4 intoxication significantly elevated the SGOT and SGPT activity in mice throughout the whole experimental period as compared to control animals. The increase in SGOT and SGPT in serum may be due to hepatocellular necrosis, which causes increase in the permeability of the cell membrane resulting in the release of transaminases in the blood stream[37,38,39]. Brzoska *et al.*[40] observed a significant elevation in SGOT and SGPT activity in rats following cadmium administration. Chanta *et al.*[41] in his investigation on rat liver by toxicant, reported that the greater the values of these serum enzymes, greater the damage on the liver cells. Elevated levels of SGOT and SGPT are indications of hepatocellular injury[42]. Among the two, GPT is a better index of liver injury, as liver GPT represents 90% of total enzyme present in the body[43]. Serum ALP activities on the other hand are related to functioning of hepatocytes, increase in its activity is due to increased synthesis in presence of increased biliary pressure[44]. Reduction in the levels of SGOT, SGPT towards the respective normal value by EtAS

($P<0.001$) and EAAS ($P<0.001$) is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damages caused by CCl_4. The suppression of increased ALP activity with concurrent depletion of raised bilirubin level suggests the stability of biliary dysfunction in rat liver during chronic hepatic injury with CCl_4[45].

The effect of free radical metabolites on the mean liver detoxificant enzymes like catalase, Super oxide dismutase (SOD) and peroxidase, reduced enzyme activity, due to enzyme inactivation during catalytic cycle. The results of the present work indicate that ethanolic extract (EtAS) and ethyl acetate extract (EAAS) (200 mg/kg, 400 mg/kg, p.o) of *A. speciosa* roots decreased the elevated enzyme levels induced by CCl_4, thus protecting the structural integrity of hepatocyte cell membrane or regeneration of damaged liver cells. These two extracts are found to be capable of enhancing or maintaining the activity of hepatic enzymes which are involved in combating ROS. The mode of action of EtAS and EAAS in affording the hepatoprotective activity against CCl_4 may be due to cell membrane stabilization, hepatic cell regeneration and activation of antioxidant enzymes such as SOD, catalase and peroxidase. The hepatoprotective effect of *A. speciosa* roots was evidenced by the amelioration of biochemical indicators of liver damage and pathological disturbances caused by CCl_4. From the study we can conclude that root extracts of *A. speciosa* protects liver from oxidative damage and could be used as an effective protector in chemical induced hepatic damage.

Table 5.5. Effect of *A. speciosa* root extracts on serum biochemical parameters in CCl_4 induced hepatotoxicity in rats

Group	Dose mg/kg	SGOT (U/ml)	SGPT (U/ml)	ALP (U/L)	Cholesterol (mg/dl)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
Control	5	170.3±3.66	74.45±4.40	201.0±7.99	62.96±0.69	1.20±0.63	0.47±0.21
CCl_4	0.7	696.0±11.87[a]	320.5±10.18[a]	382.9±7.18[a]	110.2±2.51[a]	2.23±0.12[a]	0.94±0.07[a]
Silymarin +CCl_4	100 0.7	292± 2.12[b]	77.64±0.92[b]	234.4±3.07[b]	74.14±1.37[b]	0.88±0.04[b]	0.60±0.01[b]
EtAS +CCl_4	200 0.7	355.0±11.29[b]	174.0±8.36[b]	267.0±3.18[b]	67.74±0.72[b]	1.51±0.02[b]	0.65±0.01[b]
EAAS +CCl_4	200 0.7	384.4± 3.96[b]	167.2±2.27[b]	261.4±3.42[b]	62.35±0.74[b]	1.65±0.13[b]	0.58±0.03[b]
EtAS +CCl_4	400 0.7	325±9.26[b]	163±6.36[b]	246±2.58[b]	65.25±0.62[b]	1.42±0.04[b]	0.56±0.02[b]
EAAS +CCl_4	400 0.7	365.0±2.96[b]	152.5±3.27[b]	238.4±4.42[b]	64.35±0.71[b]	1.52±0.12[b]	0.56±0.02[b]

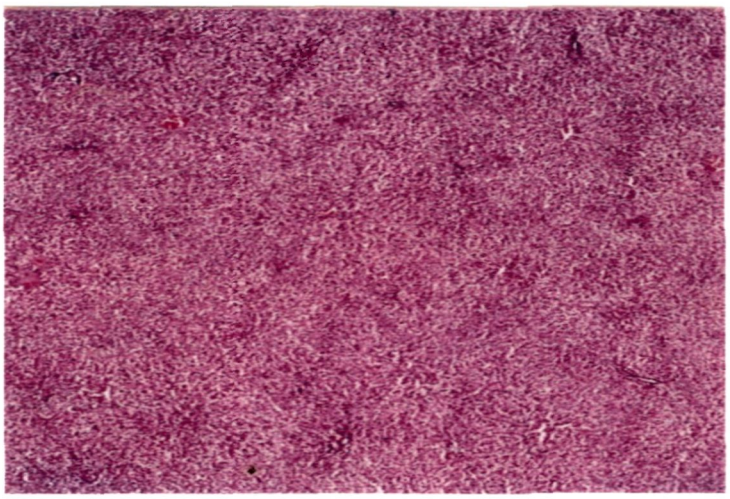
Values are mean ± SEM, n = 6, one way ANOVA followed by Dunnet's multiple comparison test. [a] $P<0.001$when compared with control, [b]$P<0.001$ when compared with control and CCl_4 group.

Table 5.6. Effect of *A. speciosa* root fractions on antioxidant enzymes in CCl_4 induced hepatotoxicity in rats

Group	Dose Mg/kg	Catalase (units/mg of protein)	Super oxide dismutase (units/mg of protein)	Peroxidase (units/mg of protein)
Control	5	461.7±6.36	21.0±0.89	165.8±3.36
CCl_4	0.7	109±1.73[a]	7.66±0.42[a]	35.73±2.54[a]
Silymarin +CCl_4	100 0.7	435.4±5.63[b]	18.6±0.53[b]	158.2±2.42[b]
EtAS +CCl_4	200 0.7	396±5.06[b]	15.5±0.76[b]	152.2±4.38[b]
EAAS +CCl_4	200 0.7	421.4±5.50[b]	16.17±1.07[b]	154.0±3.49[b]
EtAS +CCl_4	400 0.7	386.5±6.61[b]	16.25±0.62[b]	155.2±3.48[b]
EAAS +CCl_4	400 0.7	416.5±5.60[b]	17.12±2.02[b]	145.2±3.62[b]

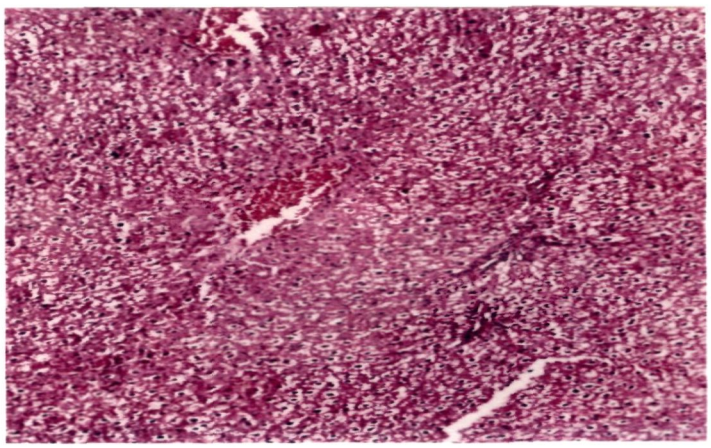
Values are mean ± SEM, n = 6, one way ANOVA followed by Dunnet's multiple comparison test. [a] $P<0.001$ when compared with control, [b]$P<0.001$ when compared with control and CCl_4 treated group.

Fig. 5.4.1. Histopathology of normal rat liver



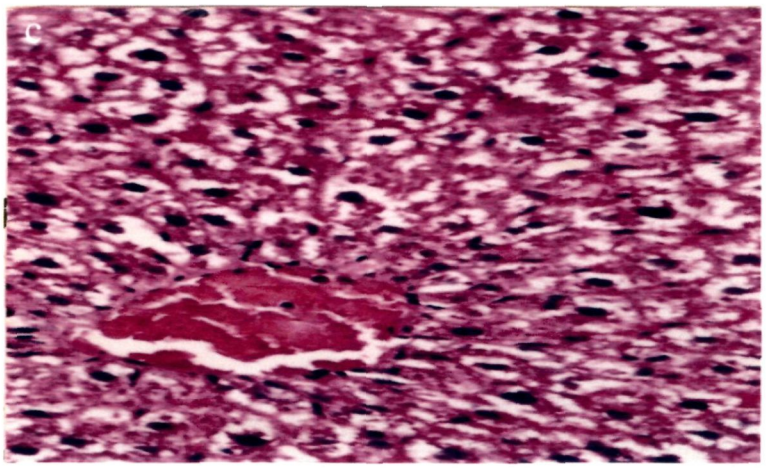
Central vein prominent, hepatocytes, sinusoids with normal texture stained with Haematoxyline- eosin with 25 X (original magnification).

Fig. 5.4.2. Histopathology of rat liver in CCl_4 treated group



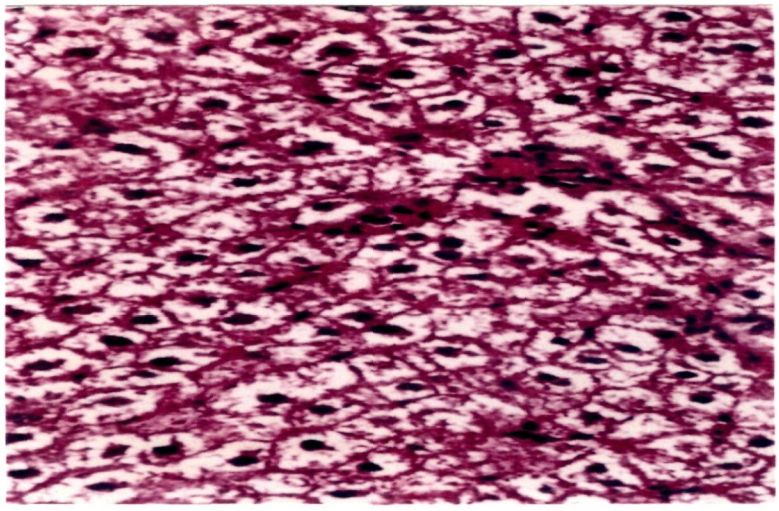
Centrilobular necrosis, fatty changes, ballooning degeneration, broad infiltration of kupffer cells around the central vein, stained with Haematoxyline- eosin with 50X (Original magnification).

Fig. 5.4.3. Histopathology of rat liver in EtAS treated group



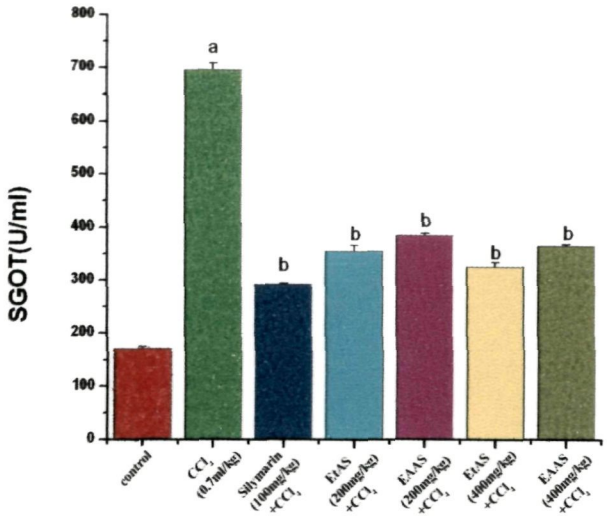
No centrilobular necrosis, no fatty change, sinusoids with mild recovery, Hepatocytes showing regenerative activity stained with Haematoxyline-eosin with 50X (original magnification).

Fig. 5.4.4. Histopathology of rat liver in EAAS treated group



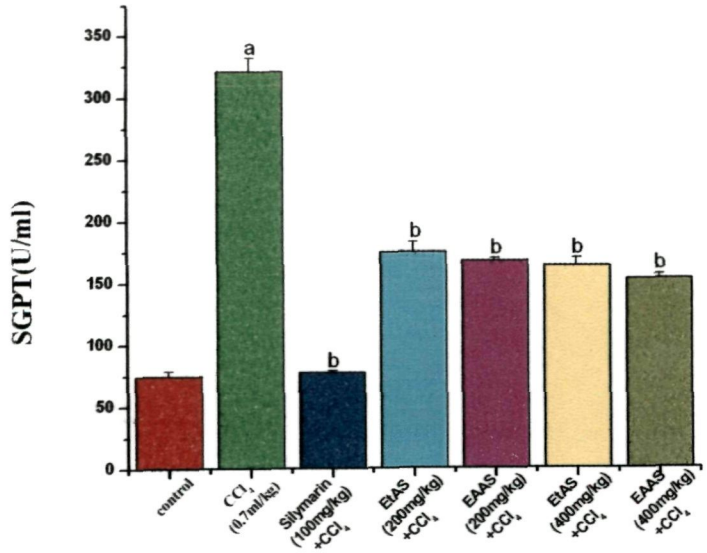
No centrilobular necrosis, no fatty change, sinusoids with recovered texture. Hepatocytes showing regenerative activity stained with Haematoxyline and Eosin with 50X (original magnification).

Fig. 5.5.1. Effect of *A. speciosa* root fractions on SGOT in CCl_4 induced hepatotoxicity in rats



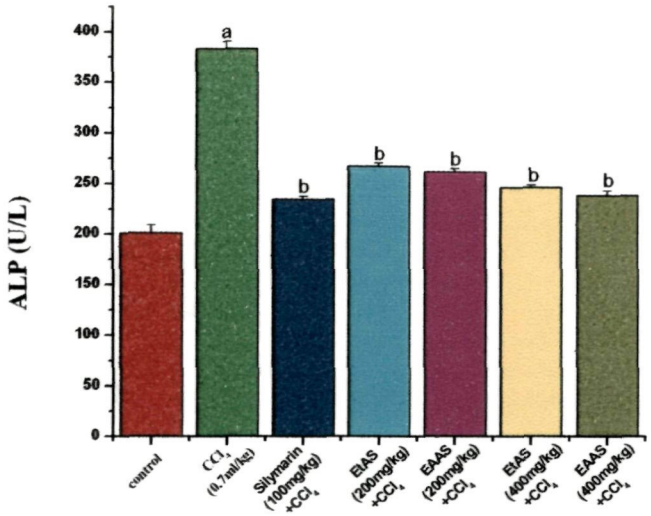
Values are mean ± SEM, n = 6, one way ANOVA followed by Dunnet's multiple comparison test. [a]P<0.001when compared with control, [b]P<0.001 when compared with control and CCl_4 group.

Fig. 5.5.2 Effect of *A. speciosa* root fractions on SGPT in CCl_4 induced hepatotoxicity in rats



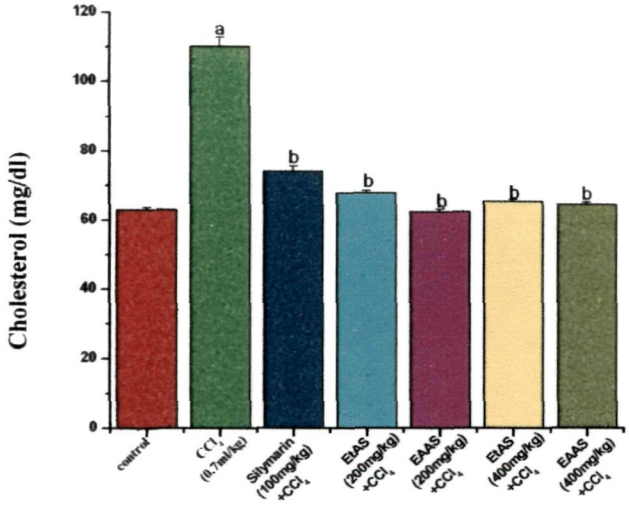
Values are mean ± SEM, n = 6, one way ANOVA followed by Dunnet's multiple comparison test. [a]P<0.001when compared with control, [b]P<0.001 when compared with control and CCl_4 group.

Fig. 5.5.3. Effect of *A. speciosa* root fractions on ALP in CCl_4 induced hepatotoxicity in rats



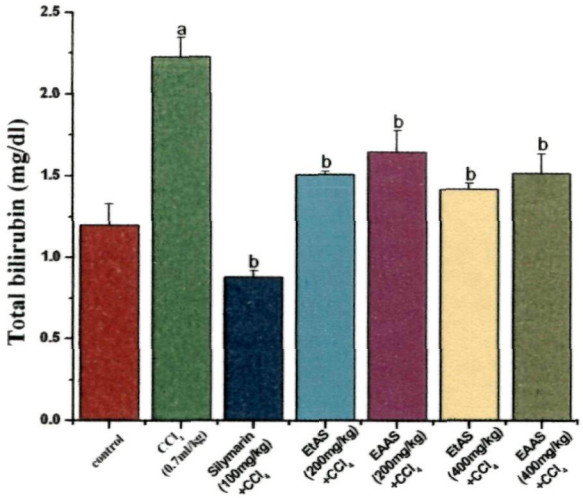
Values are mean ± SEM, n = 6, one way ANOVA followed by Dunnet's multiple comparison test. [a]P<0.001when compared with control, [b]P<0.001 when compared with control and CCl_4 group.

Fig. 5.5.4. Effect of *A. speciosa* root fractions on Total cholesterol in CCl_4 induced hepatotoxicity in rats



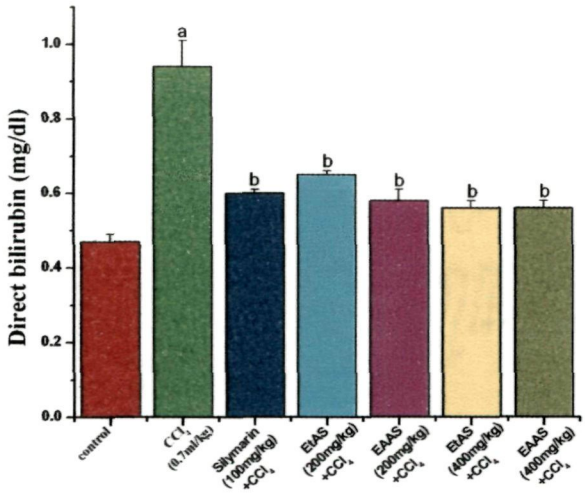
Values are mean ± SEM, n = 6, one way ANOVA followed by Dunnet's multiple comparison test. [a]P<0.001 when compared with control, [b]P<0.001 when compared with control and CCl_4 group.

Fig. 5.5.5. Effect of *A. speciosa* root fractions on Total bilirubin in CCl_4 induced hepatotoxicity in rats



Values are mean ± SEM, n = 6, one way ANOVA followed by Dunnet's multiple comparison test. $^{a}P<0.001$when compared with control, $^{b}P<0.001$ when compared with control and CCl_4 group.

Fig. 5.5.6. Effect of *A. speciosa* root fractions on direct bilirubin in CCl_4 induced hepatotoxicity in rats



Values are mean ± SEM, n = 6, one way ANOVA followed by Dunnet's multiple comparison test. $^{a}P<0.001$when compared with control, $^{b}P<0.001$ when compared with control and CCl_4 group.

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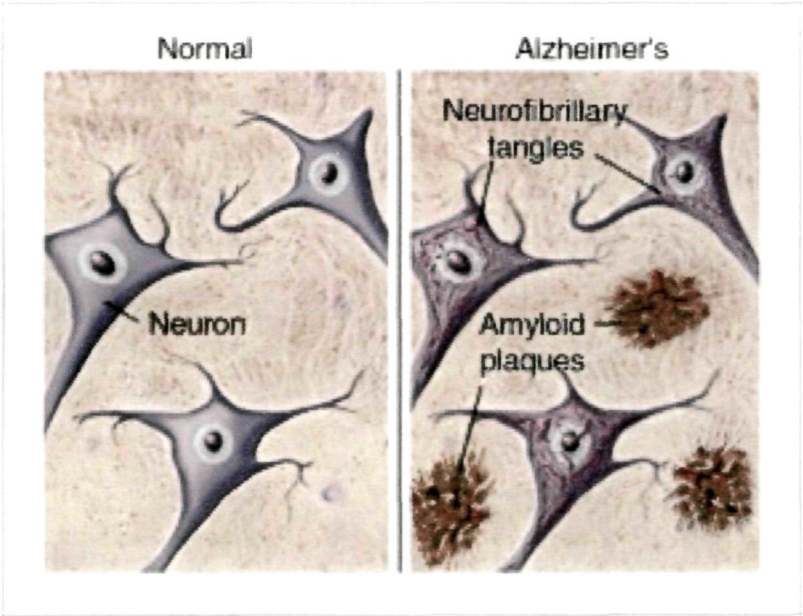
5.11.2 Antiamnesic (Nootropic) potentiality of *Argyreia speciosa* (Burm.f.) Boj. in mice

5.11.2.1 Introduction

Stress and free radicals have been implicated in the loss of memory, concentration and also Alzheimer's disease[1,2]. Alzheimer's disease, the most common form of dementia in the elderly population, is characterized by an insidious onset with memory impairment and an inexorable progression of cognitive decline. It is also known that Alzheimer's disease is associated with down-regulation of the cholinergic system in the brain. In addition to the cognitive impairment of functions such as memory and language, Alzheimer's disease patients frequently show abnormal behavior such as delusion agitation and wandering[3,4,5].



The personality distortions interfere with the patient's professional life, social activities and relationships[6]. Nootropic agents such as Piracetam, aniracetum and cholinesterase inhibitors like donepezil are being used for improving memory, mood and

behavior[7], but the resulting side effects (nausea, vomiting and diarrhea) associated with these agents have made their applicability limited. While pharmaceutical companies continue to invest enormous resources in identifying agents that could be used to alleviate debilitating disorders and retard mental deterioration afflicting numerous people around the world, a source of potentially beneficial agents, namely phytochemicals, would appear to have significant benefits that have yet to be fully elucidated. Galantamine, Huperzine-A and several flavanoids have been extensively studied for their beneficial effects in dementia models. Therefore several plants have been selected based on their use in traditional systems of medicine, and research has identified number of natural compounds that could act as nootropic or antiamnesic agents. Several *'rasayana'* herbs which enlisted in Indian system of medicine have been in use for the treatment of age related neurodegenerative disorders.

5.11.2.2 Herbal mixtures with nootropic activity

The mixed herbal preparations that have antidementia effects on animal models are listed: BR-16A (Mentat)[8,9], Bu-Zhong-Yi-Qi-Tang[10], Choto-san[11,12], DX-9386[13,14,15], Liuwei-Dihuang-Wan[16,17], Ninjin-yoei-to (Ren-Shen-Yang-Rong-Tang)[18], Oren-gedoku-to (Huang-Lian-Jie-Du-Tang)[19], S-113M[20], Shosaikoto[21], Yi-Zhi-Fang[22] and Zingicomb[23,24].

In the present study, the antiamnesic activity of Ethyl acetate (EAAS) and Ethanol (EtAS) fractions *A. speciosa* was investigated by employing both exteroceptive and interoceptive models. Elevated plus maze and Morris water maze are used to assess the short-term memory. Interoceptive behavioral models such as scopolamine and natural aging induced amnesia are widely used models to stimulate dementia in Alzheimer's disease.

5.11.2.3 Material and Methods

a) Preparation of Drugs

Suspensions of EAAS and EtAS fractions were prepared as described in **5.11.1.5c**

b) Drugs

Scopolamine hydrobromide (Sigma Aldrich, U.S.A.), Piracetam (Nootropil, UCB India Pvt. Ltd., Vapi, Gujarat), diazepam (Calmpose, Ranbaxy, India) Phenytoin (Dialantin suspension, Parke Davis, India) were diluted in normal saline. Volume of oral and i.p. administration was 1ml/100 g. of mouse.

c) Experimental animals

Swiss mice of either sex weighing around 18 gms (young ones, aged 8 weeks) and 25 gms (older ones, aged 28 weeks) were used in the present study. Animals were procured from disease free animal house of BLDEA's medical college and Research centre, Bijapur, Karnataka, India. They were acclimatized to the laboratory conditions for 5 days before behavioral studies. The animals had free access to food and water and were maintained under 12:12 hr light and dark cycles. All the readings were taken during the same time of the day i.e. between 6-8 pm. Institutional Animals Ethics Committee (IAEC) had approved the experimental protocol and care of animals was taken as per the CPCSEA guidelines, Animal welfare division, Ministry of Environment and forests, Govt. of India.

d) Elevated plus maze test

The elevated plus maze served as the exteroceptive behavioral model to evaluate learning and memory in mice. The apparatus consisted of two open arms (16x16 cm) and two covered arms (16x5x12 cm). The arms extended from a central platform (5x5 cm),

and maze was developed to a height of 25 cm from the floor. On the first day, each mouse was place at the end of open arm, facing away from the central platform. Transfer latency (TL) was taken as the time taken by mouse to move in to one of the covered arm with all its four legs. TL was recorded on the first day. If the animal did not enter one of the covered arms within the 90 sec, it was gently pushed in to one of the two covered arms and the TL was assigned as 90 sec. The mouse was allowed to explore the maze for 10 and then returned to its home cage. Memory retention was examined 24 hr after the first day trial on the second day[25,26].

Group I and Group XV: Represented Control groups for young and aged mice (n=6). 10ml/kg Distilled water, p.o, was administered for 8 days. TL was noted after 45 min of administration on 8th day and after 24 hr (9th day).

Group II and III: Piracetam, 200 mg/kg, i.p., was injected to both young and aged mice respectively. TL was noted after 45 min of injection and on the 9th day.

Group IV: Scopolamine hydrobromide (0.4 mg/kg, i.p) was administered to young mice and TL was noted after 45 min of injection on 8th day and after 24 h (9th day).

Group V and VI: EtAS extract, 100 mg and 200 mg/ kg, was administered orally to young mice for 8 days. The last dose was given 45 min before subjecting the animals to elevated plus maze test. TL was noted on 8th day and 9th day.

Group VII and VIII: EAAS extract, 100 mg and 200 mg/ kg, was administered orally to young mice for 8 days. The last dose was given 45 min before subjecting the animals to elevated plus maze test. TL was noted on 8th day and 9th day.

Group IX and X: EtAS extract, 100 mg and 200 mg/ kg, was administered orally to aged mice for 8 days. The last dose was given 45 min before subjecting the animals to elevated plus maze test. TL was noted on 8th day and 9th day.

Group XI and XII: EAAS extract, 100 mg and 200 mg/ kg, was administered orally to aged mice for 8 days. The last dose was given 45 min before subjecting the animals to elevated plus maze test. TL was noted on 8th day and 9th day.

Group XIII: EtAS 200 mg/kg, p.o. was administered to young mice for 8 days. After 45 min of administration of the last dose on 8th day, scopolamine hydrobromide (0.4 mg/ kg, i.p) was administered. TL was noted after 45 min of administration of scopolamine and on the 9th day.

Group XIV: EAAS 200 mg/kg, p.o. was administered to young mice for 8 days. After 45 min of administration of the last dose on 8th day, scopolamine hydrobromide (0.4 mg/ kg, i.p) was administered. TL was noted after 45 min of administration of scopolamine and on the 9th day.

e) Morris Water maze test

It consisted of a circular water tank (150 cm diameter, 45 cm height), filled with water maintained at 25° C. The water was made opaque with a white colored non toxic dye. The tank is divided in to four quadrants with the help of two threads, fixed at right angle to each other on the rim of the pool. A platform (10 cm^2) of 29 cm height was located in the center of one of these four quadrants. The position of platform was kept unaltered throughout the training sessions. In the present study the target quadrant was Q4. Each animal was subjected to four consecutive trails on each day with a gap of 5 min for four consecutive days, during which they are allowed to escape on to the hidden platform and to remain there for 20 sec. In case the animal was unable to locate the hidden platform within 120 sec, it was gently guided to the platform and allowed to remain on the platform for 20 sec. Escape latency time to locate the hidden platform in

water maze was taken as an index of acquisition or learning. Starting position on each day to conduct four acquisition trials was changed as described below and Q4 was maintained as target quadrant in all the acquisition trials. The starting point for dropping the mice in to water maze on day one for four consecutive acquisition trials was sequence Q1, Q2, Q3 Q4 and so on. Sequence change of starting point was as follows.

Day 1: Q1, Q2, Q3, Q4

Day2: Q2, Q3, Q4, Q1

Day 3: Q3, Q4, Q1, Q2

Day 4: Q4, Q1, Q2, Q3

Mean escape latency time (ELT) was calculated for each day of the trial. On fifth day the platform was removed, each mouse was placed in water for 120 sec. The animal was subjected to four such trials and each trial had a different starting point covering all the four quadrants. The mean time spent by animal in all four quadrants was recorded. The time spent in the target quadrants Q4 as compared to time spent in other Quadrants in search of missing platform was taken as an index of retrieval. Care was taken that relative location of water maze with respect to other objects in laboratory serving as visual clues was not disturbed during the total duration of the study[27].

f) Estimation of brain acetyl cholinesterase activity

The whole brain acetyl cholinesterase (AChE) activity was measured using the Ellman method[28]. Animals were euthanized on the 9th day by cervical dislocation and brain tissue was removed carefully to avoid any injuries. Tissue was homozenised in normal saline and centrifuged. The supernatant was used to estimate the AChE activity. The end point was the formation of yellow color due to the reaction of thiocholine from

acetylcholine iodide in the presence of dithiobisnitrobenzoate ions. The rate of formation of thiocholine from acetylcholine iodide in the presence of tissue cholinesterase was measured using spectrophotometer. The sample was first treated with 5, 5'-dinitrobenzoic acid (DTNB) and the optical density (OD) of the yellow color compound formed during the reaction at 412 nm every minute was measured. Protein estimation was done using Folin's method. AChE activity was calculated using the following formula.

$$R = \frac{\S O \times \text{volume of assay (5ml)}}{E \times \text{mg of protein}}$$

where, R = rate of enzyme activity in 'n' mole of acetyl choline iodide hydrolysed/min/mg protein. § O.D. = Change in absorbance /min, E = Extinction coefficient – 13,600/M/cm

Group I: Control group treated with normal saline

GroupII: Served as Phenytoin (12 mg/kg, p.o.) treated group

GroupIII and IV: EtAS 100 mg and 200 mg treated group

Group V and VI: EAAS, 100 and 200 mg treated group.

Group VII: treated with Piracetam (200 mg/ kg, p.o.)

g) Statistical analysis

The data were expressed as mean ± SEM. The data were analysed using one way ANOVA followed by Tukey-kramer test. $P < 0.01$ was considered significant.

5.11.2.4 Results

a) Effect on Transfer Latency (TL) using Elevated Plus maze

Aged mice showed higher TL values on first day and second day as compared to young mice, indicating impairment in learning (ageing induced amnesia). Scopolamine (0.4 mg/kg, i.p.) increased the transfer latency significantly ($P<0.01$) in young mice on

first and second day as compared to control indicating impairment of memory. Treatment with Piracetam (200 mg/kg, i.p.) for 8 days decreased TL as compared to control group, indicating improvement in both learning and memory. Pretreatment with EAAS (100 and 200 mg/kg, p.o.) decreased the TL on 8th and 9th day in young and aged mice ($P<0.01$) when compared to control groups. Higher dose of EAAS (200mg/kg, p.o.) significantly enhanced antiamnesic property in aged animals rather than young mice as reflected by marked decrease in TL on 8th and 9th day when subjected to EPM tests. EAAS (200 mg/kg, p.o.) exerted profound enhancement of memory in young mice and protected them from against scopolamine ($P<0.01$) and ageing induced amnesia. The results are summarized in Table 5.7 and 5.8 and fig 5.6.1

b) Effect of *A. speciosa* on Scopolamine induced enhancement on Escape latency Time (ELT) in mice using water maze

A significant decrease ($P<0.01$) in the ELT was observed in control group mice in their 4 day trial. Scopolamine produced impairment of acquisition and increased the ELT during successive training trials. The action of scopolamine was reversed by pretreatment with EAAS (100 and 200 mg/kg, p.o.) as reflected by significant decrease ($P<0.01$) in ELT of mice. The results are summarized in Table 5.10 and 5.11 and fig 5.6.3.

c) Effect of *A. speciosa* on scopolamine and ageing induced alterations in the Time Spent Target Quadrant (TSQT) during retrieval trials on water maze

The time spent by young control mice in target quadrant was more as compared to time spent on other quadrants during retrieval trial on 5th day. Further scopolamine (0.4 mg/kg, i.p) administered before retrieval trial produced significant decrease ($P<0.01$) in mean time spent in target quadrant in search of missing platform as compared to control

(young). Aged mice also decreased TSQT significantly compared to control (young). The results are shown in Table 5.12. These observations indicate that scopolamine and natural ageing produced anterograde and retrograde amnesia. Mice treated with Piracetam (200 mg/kg, i.p.) produced better effects only in aged mice by decreasing TSQT as compared to control (aged) mice. EAAS (100 and 200 mg/kg, p.o.) administered before training trial (from day 1 to day 4), significantly ($P<0.01$) attenuated scopolamine and ageing induced decrease in TSQT during retrieval test on 5th day. The results are shown in Table 5.13.

d) Effect on brain acetyl cholinesterase activity

EAAS (100 and 200 mg/kg, p.o.) significantly produced reduction in whole brain AChE activity of both young and aged mice as compared to respective control group. The brain AChE activity with phenytion (12 mg/kg, i.p.) exhibited significant elevation which was considered as negative control. Piracetam (200 mg/kg, i.p.) profoundly reduced AChE activity as compared to control groups (Table 5.9 and Fig. 5.6.2).

5.11.2.5 Discussion

The acquisition of the learned responses involves the modification of neurotransmission in the peripheral and central nervous systems[29,30]. It has long been suggested that the cholinergic neuronal system plays a major role in learning and memory in both humans and animals, especially in learning acquisition[31]. Cholinergic receptors are mainly grouped into two subtypes: muscarinic and nicotinic receptors. The muscarinic antagonist scopolamine impairs learning and memory in rodents and humans, especially learning acquisition[32], while the nicotinic antagonist mecamylamine also impairs cognitive performance, such as passive avoidance[33]. The serotonergic system has

likewise been implicated in learning and the decrease in serotonergic activity facilitates acquisition[34]. Similarly, the dopaminergic system is also involved in learning, where a decrease in dopaminergic activity facilitates acquisition performance[35]. Furthermore, peripheral or central cholinergic and dopaminergic systems also participate in learning and memory. The pre-training administration of scopolamine, a muscarinic receptor antagonist, is known to decrease cholinergic activity and impair learning acquisition in rodents[36].

Alzheimer's disease (AD) has been identified as a protein misfolding disease due to the accumulation of abnormally folded amyloid beta protein in the brains of AD patients[37]. This neuropathological disorder is increasingly diagnosed in all countries where the number of patients rises exponentially with life expectancy. It has been estimated that about 5% of the population aged more than 65 are affected by AD. Acetylcholinesterse inhibitors such as Donepezil, Rivastigmine and Galantamine are the only FDA approved drugs currently used for the treatment of mild or moderate cases of dementia. Recent reports established an important role for soluble Aβ in the development of AD. Earlier studies have demonstrated that exposure of cells to soluble Aβ could lead to neuronal apoptosis following oxidative stress, pro-inflammatory signals and cycloskeleton perturbations[38-41]. Due to its fusogenic properties, the amphiphilic non-aggregated Aβ oligomers could be the proximate effectors of the neuronal degeneration and death occurring in early stages of AD[42,43]. Therefore there is a need for development of novel therapeutic strategies that target or even better prevent the molecular mechanisms leading to dementia. Subgroups of Ayurvedic rasayanas, known as medhyarasayanas, are used to promote intellect and memory. The cognitive promoting

effect of *medhyarasayanas* is best seen in children with memory deficits, or when memory is compromised following head injury, prolonged illness or in old age[44].The present study indicates that Ethyl acetate fraction of *A. speciosa* is a potential Antiamnesic agent. It also possesses nootropic activity in view of its facilitatory effect on retention and acquired learning. EAAS (100 mg and 200 mg/kg, p.o.) decreased transfer latencies in both young but more profoundly in aged mice in dose dependent manner as compared to respective controls. Central cholinergic system plays an important role in learning and memory[45,46].

The acetylcholinesterase (AChE) enzyme exists in different molecular isoforms that are localized differentially in neuronal cell[47]. The two major isoforms are globular monomer (G1) protein and globular tetramer (G4) of the same monomer subunit. The G1 isoform is reported to be present as soluble form in the cytoplasm of the neuronal cells where as the G4 isoform that is predominantly a membrane bound enzyme[48]. Phenytoin is known to reduce the hippocampal AChE concentration[49,50], and causes cognitive impairment. In our study, phenytoin per se (12 mg.kg i.p.) significantly elevated brain AChE activity. Piracetam (200 mg/kg, i.p.) and EAAS (100 and 200 mg/kg, p.o.) on the other hand significantly ($P<0.01$) lowered this activity indicating the counteracting action of the drugs on cholinergic system. EAAS elicitated profound neuroprotective effect in scopolamine treated and older mice compared to control groups and Piracetam treated mice. It significantly inhibited AChE activity in the whole brain homogenate in mice indicating its potential in the attenuation of learning and memory deficits especially in aged mice.

In water maze model, a marked decrease in escape latency time (ELT), during subsequent trials as compared to the first exposure, denotes normal learning ability. The enhancement in the time spent by the animal in the target quadrant reflects successful retention of learned task (or memory).

Amnesia is inability to remember past experiences or loss of memory. Anterograde amnesia is impairment of memory for events occurring after accident/drug treatment. In such case, new memories are not formed. Retrograde amnesia is impairment of memory of events which have occurred before the accident or drug treatment. In such case new memories can be formed, but old memories are lost. In the present study, scopolamine (0.4 mg/kg, i.p.) showed anterograde amnesia as indicated by significant decrease in more time spent in target quadrant on 5^{th} day in Morris water maze model. Our observation suggested that EAAS (100 and 200 mg/kg, p.o) reversed the scopolamine and ageing induced amnesia.

The study justify the traditionally claimed intellect promoting and brain tonic potentiality of roots of *A. speciosa* and further investigations are warranted to explore the possible phytoconstituents from the potent fraction(s) responsible for the management of AD and other cognitive disorders.

Table 5.7. Effect of EtAS and EAAS on transfer latencies (TL) of young mice by elevated plus maze

Group	Treatment	Dose(mg/kg)	TLT(8^{th} day)	TLT(9^{th} day)
I	Control	10	29.4±13.45	22.5±3.57
II	Piracetam	200	25.2±4.57*	18.4±6.24*
IV	Scopolamine	0.4	48.4±4.56*	40.2±7.38*
V	EtAS	100	45.6±4.56	33.4±3.67
VI	EtAS	200	42.2±5.78	37.6±5.34
VII	EAAS	100	36.4±5.67†	20.8±4.89†
VIII	EAAS	200	31.2±7.64†	25.0±6.45†
XIII	EtAS+Scopolamine	200+0.4	39.8±4.79	38.0±6.25
XIV	EAAS+Scopolamine	200+0.4	29.6±3.65‡	25.1±4.93‡

Each group consists of 5 animals except group I (n=6). Values are mean ±SEM.*P< 0.001compared to control, †P< 0.01 compared to control, ‡P< 0.01 compared to control (scopolamine treated)

Table 5.8. Effect of EtAS and EAAS on transfer latencies (TL) of aged mice by elevated plus maze

Group	Treatment	Dose (mg/kg)	TLT(8^{th} day)	TLT(9^{th} day)
I	Control(Y)	10	29.4±13.45	22.5±3.57
XV	Control(A)	10	35.6±4.67*	31.7±5.65*
III	Piracetam	200	23.7±4.68*	18.6±2.13*
IX	EtAS	100	55.6±3.60	39.6±1.56
X	EtAS	200	54.2±3.54	41.2±1.21
XI	EAAS	100	35.2±1.02†	29.4±1.08†
XII	EAAS	200	30.4±1.32†	23.5±2.21†

Each group consists of 5 animals except group I and XV (n=6). Values are mean ±SEM.*P< 0.01compared to control, †P< 0.01 compared to control (young), †P< 0.01 compared to control (aged)

Table 5.9. Effect of EtAS, EAAS and piracetam on AChE activity in aged mice

Group	Treatment	Dose (mg/ kg, p.o.)	AChE (mM)
I	Control	10	128.4±7.40
II	Phenytoin	12	216.6±10.55*
III	EtAS	100	194.6±6.25
IV	EtAS	200	187.7±6.46
V	EAAS	100	138.7±6.26*
VI	EAAS	200	115.8±8.25*
VII	Piracetam	200	105.4±8.25*

Values are mean ± SEM., AChE- whole brain AChE activity, *P< 0.01compared to control.

Table 5.10. Effect of *A.speciosa* on Escape Latency Time (ELT) of young mice using Morris water maze

Group	Treatment	Dose (mg/kg, i.p./p.o.)	Escape Latency Time in secs	
			Day 1	Day4
I	Control	10	67.4±2.53	25.12±2.44
III	Piracetam	200	61.4±6.24*	23.4±4.55*
V	EtAS	100	68.7±1.54	50.5±4.46
VI	EtAS	200	66.4±2.47	53.8±6.67
VII	EAAS	100	65.7±2.21*	28.4±2.58*
VIII	EAAS	200	63.4±4.46*	22.3±5.58*

Each value represents ± SEM, * denotes P< 0.01as compared to control mice.

Table 5.11. Effect of *A.speciosa* on Escape Latency Time (ELT) of aged mice using Morris water maze

Group	Treatment	Dose (mg/kg, i.p./p.o.)	Escape Latency Time in secs	
			Day 1	Day4
I	Control	10	81.2±1.42	67.2±4.33
III	Piracetam	200	78.2±5.44*	66.5±7.23*
V	EtAS	100	64.3±1.27	54.23±1.23
VI	EtAS	200	66.2±1.37	52.23±4.23
VII	EAAS	100	55.4±3.64*	22.6±6.24*
VIII	EAAS	200	59.6±6.44*	25.4±5.54*

Each value represents ± SEM, * denotes P< 0.01as compared to control mice.

Table 5.12. Effect of *A. speciosa* on the mean Time spent in the Target Quadrants (TSTQ) Q4 in young mice using Morris Water maze

Group	Treatment	Dose (mg/kg, i.p./p.o.)	TSTQ (secs)
I	Control	10	68.7 ± 1.23
III	Piracetam	200	54.2±2.13*
V	Scopolamine	0.4	30.2±1.55
VI	EtAS	100	33.2±2.54
VII	EtAS	200	37.4±3.53
VIII	EAAS	100	58.4±2.45†
IX	EAAS	200	62.6±7.23†
X	EtAS + Scopolamine	200	36.3±1.24
XI	EAAS + Scopolamine	200	56.6±2.74†

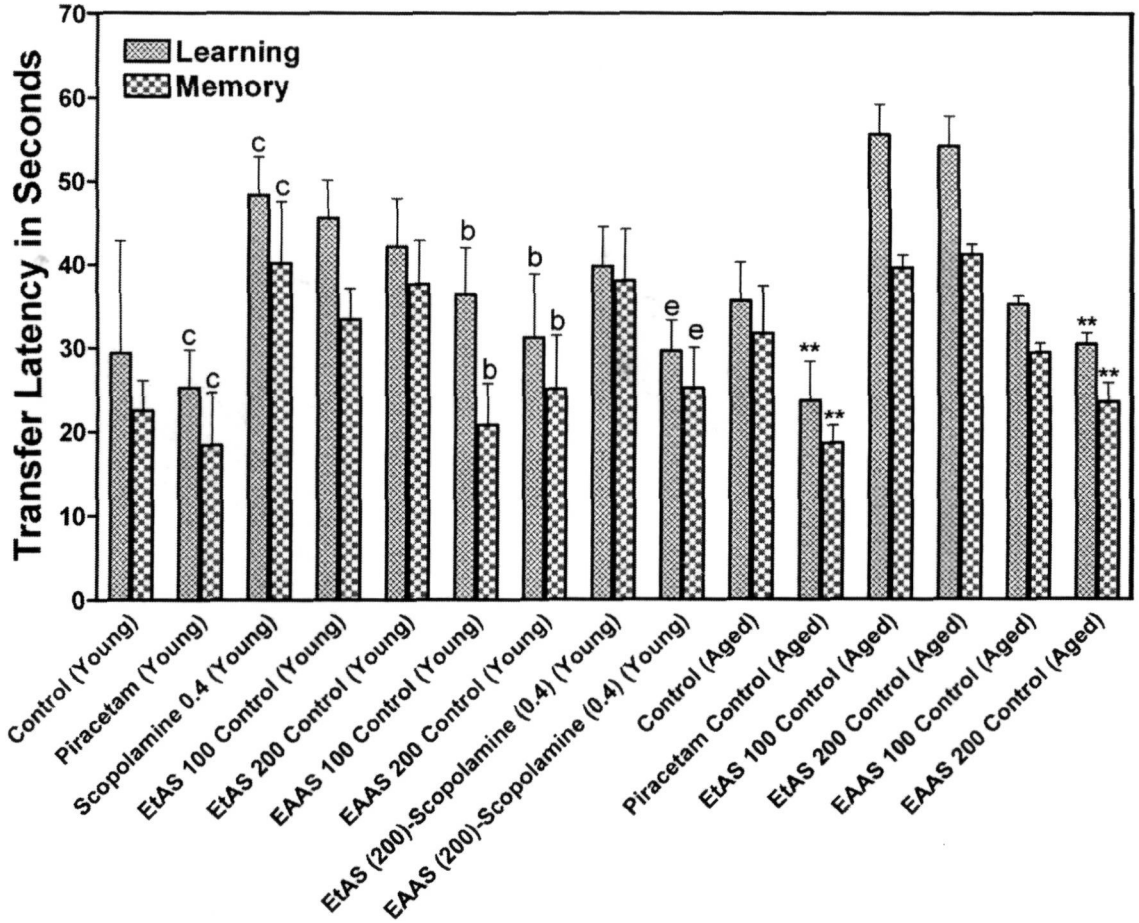
Each value represents mean ± SEM. * denotes P<0.01 as compared to control, † denotes P<0.01 as compared to scopolamine treated mice.

Table 5.13. Effect of *A.speciosa* on the mean Time spent in the Target Quadrants (TSTQ) Q4 in aged mice using Morris Water maze

Group	Treatment	Dose (mg/kg, i.p./p.o.)	TSTQ (secs)
II	Control	10	29.4±2.44
IV	Piracetam	200	52.4±1.75*
XII	EtAS	100	36.4±3.44
XIII	EtAS	200	38.2±4.34
XIV	EAAS	100	51.4±4.43*
XV	EAAS	200	58.4±2.33*

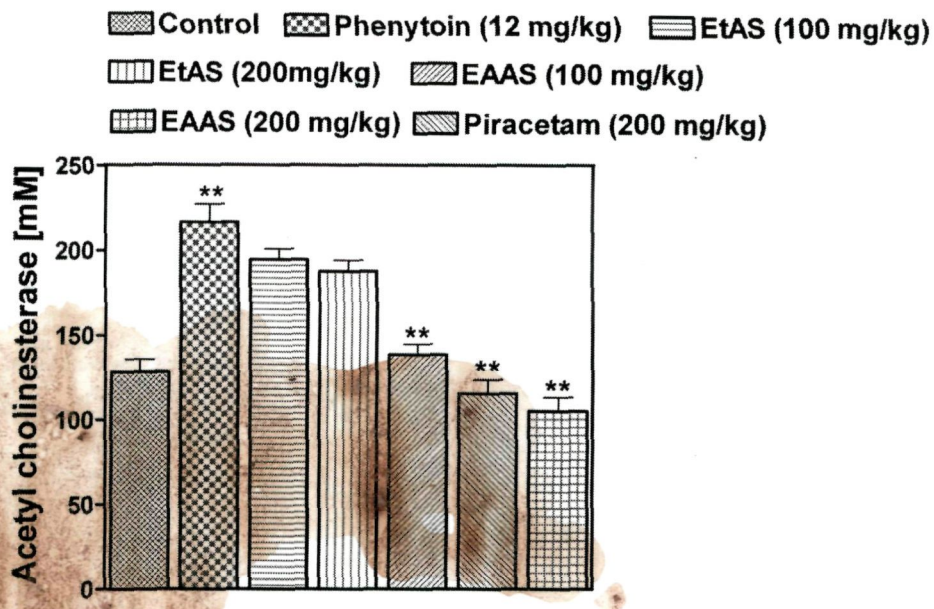
Each value represents mean ± SEM. * denotes p< 0.001 as compared to control.

Fig. 5.6.1. Effect of ethanol (EtAS) and ethyl acetate (EAAS) extracts of *Argyreia speciosa* on transfer latencies (TL) of young and aged mice in elevated plus maze model.



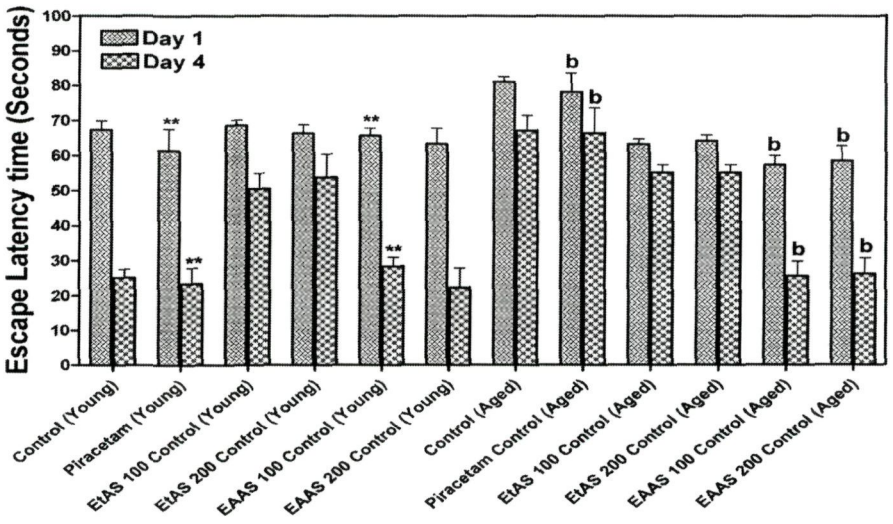
Values are mean ±SEM (n=6). $^{b}P<0.01$; $^{c}P< 0.001$ compared to control (Young); $^{e}P< 0.01$ compared to scopolamine treated group; $^{**}P<0.01$compared to control (Aged).

Fig. 5.6.2. Effect of ethanol (EtAS) and ethyl acetate (EAAS) extracts of *Argyreia speciosa* on Acetylcholinesterase (AChE) activity in aged mice.



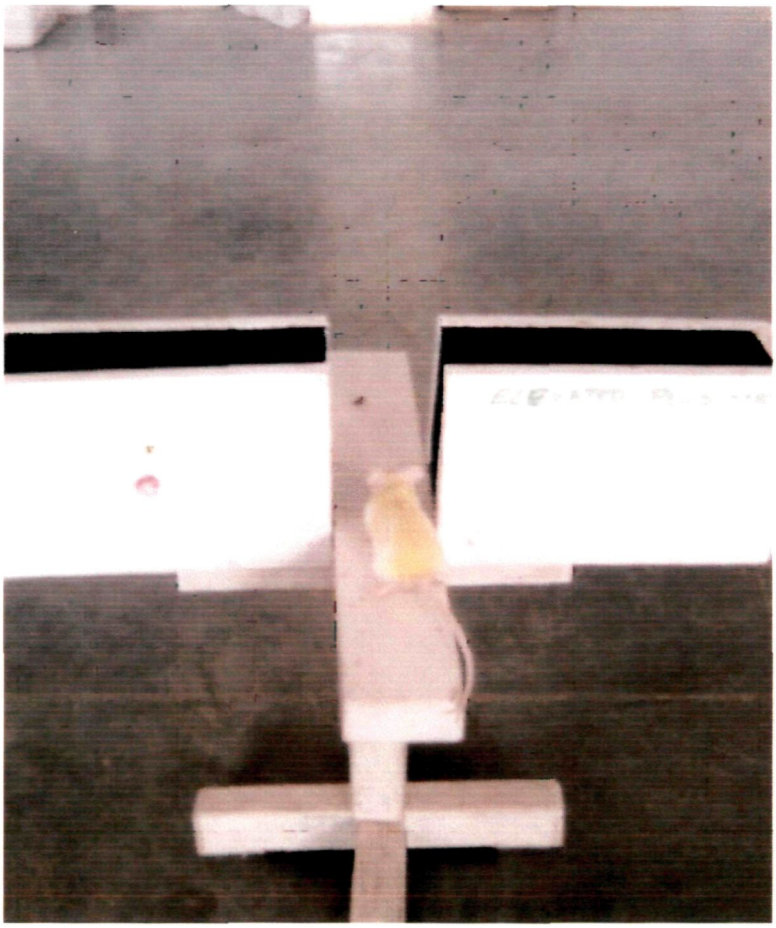
Values are mean ± SEM., **P<0.01compared to control.

Fig. 5.6.3. Effect of ethanol (EtAS) and ethyl acetate (EAAS) extracts of *Argyreia speciosa* on Escape Latency Time (ELT) of young and aged mice using Morris water maze



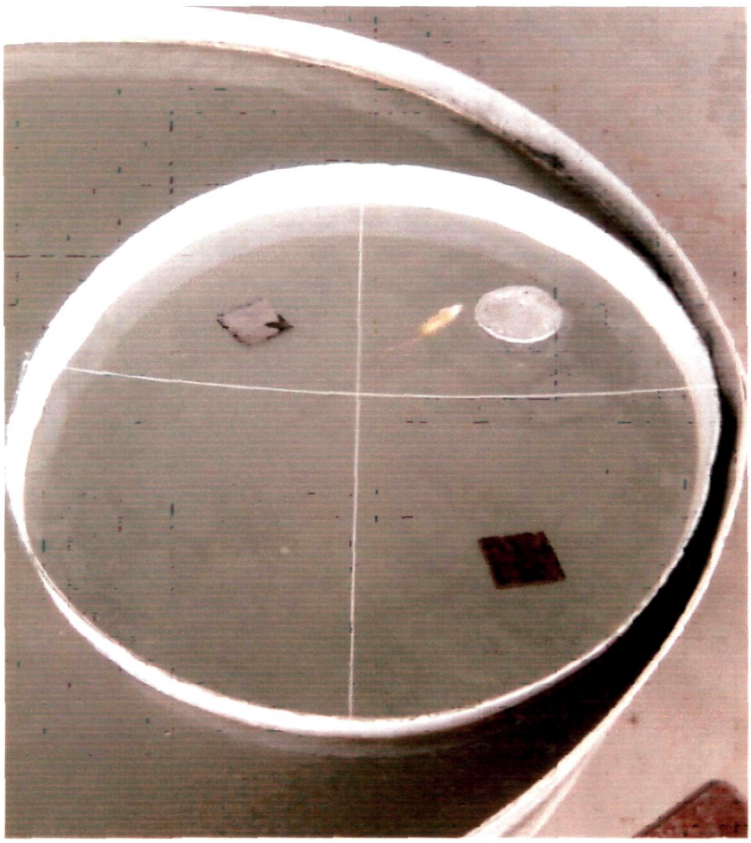
Each value represents ± SEM (n=6), **P<0.01as compared to control mice (Young). bP<0.01as compared to control mice (Aged).

Fig. 5.6.4. Photograph showing the activity of mice on Elevated plus maze



Open arms (16x16 cm) and two covered arms (16x5x12 cm). The arms extended from a central platform (5x5 cm), and maze was developed to a height of 25 cm from the floor.

Fig. 5.6.5. Photograph showing the activity of mice on Morris Water maze



Morris Water maze (150 cm diameter, 45 cm height). A platform (10 cm^2) of 29 cm height was located in the center of one of the four quadrants.

5.11.2.6. References

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5.11.3 Anti-stress activity of *Argyreia speciosa* (Burm.f) Boj. in acute and chronic stress paradigm in rodents

5.11.3.1 Introduction

Stress can be described as the sum total of all the reactions of the body, which disturb the normal physiological condition and result in a state of threatened homeostasis. There has long been an interest in the role of stress in production of human disease states, at least some of them being linked to suppression of the immune response[1]. Acute stress in any form will cause, at the very least, a temporary decrease in functioning of the immune system, while chronic stress will result in continued decline in immune system function[2]. Both humoral and cell mediated immune response are affected, indicating that may have an adverse effect on normal immune surveillance[1]. Overwhelming evidence has demonstrated that virtually any type of stress has a detrimental effect on the ability to maintain optimal levels of natural killer cell cytotoxic activity[3,4,5]. Secretary IgA (sIgA) is the first line defense and probably the single most important aspect of humoral immunity in the mucus secretions of digestive system, mouth, lungs, urinary tract and other body cavities. Any decline in the levels of sIgA decreases one's resistance to microbial pathogens[6].

It has been hypothesized that, a large number of human illness reflect the consequences of the generalized stress response brought about by failure of Selye's postulated resistance phase and failure of adaptation to stress, leading to the stage of exhaustion, resulting in a variety of diseases. Rasayanas or adaptogens may be effective anti-stress agents, because they appear to prolong, Selye's propounded second phase of the "General adaptation syndrome", the stage of resistance to stress, and prevent the final and third phase of exhaustion.

Argyreia speciosa is an important member of rasayana herbs in Ayurveda. Traditionally it is used in many herbal preparations for its rejuvenating properties. Hence, anti-stress potentiality of different fractions of *A.speciosa* was investigated using acute and chronic stress models in rodents.

5.11.3.2 Material and Methods

a) Drugs and chemicals

Diagnostic kits for the estimation of glucose, triglycerides, AST, ALT were purchased from ERBA diagnostic Mannheim Ltd.(Germany), Cholesterol (Span Diagnostics Ltd, India) and Creatinine kinase (Agappe Diagnostics Ltd.) A gift sample of standardized *Withania somnifera* (WS) extract was obtained from Natural remedies, Bangalore, India. DPPH was obtained from Sigma Chemical Co.(St Louis, MO USA), DNPH (SD-fine chemicals, India), Mannitol, Thio urea, Ascorbic acid, Deoxy ribose (Himedia, India), Oestradiol Valerate injection (German Remedies, India)

b) Preparation of drugs

EAAS and EtAS were suspended in 0.5% gum acacia, and a fine emulsion was made having uniform particle distribution. The emulsion of both the extracts was administered for orally daily for three days in case of acute stress (AS) and for 7 days in case of chronic stress (CS). Both the drugs were prepared fresh daily before administration.

c) Animals

Albino rats of either sex (150-200 gms) and Swiss albino mice (20-25 gms) were used in the study. They were housed three to four per cage at temperature 22± 2°C at 12/12 hr light/dark under controlled environment. Rats were fed standard laboratory food and water was given *ad libitum*. Rats were kept for 7 days in laboratory for habituation.

d) Stress Protocol [7]

The rats were divided in to non-stress, AS, CS, groups and drug treated groups for both AS and CS groups. Each group consists of 6 rats. The AS groups were fed with emulsions of EtAS (100 mg and 200 mg/kg p.o) and EAAS ((100 mg and 200 mg/kg p.o) or *W. somnifera* (100 mg/kg p.o) daily for 3 days. In AS model on the second day after feeding drug or vehicle, animals were fasted overnight with free access to water. On the third day, 45 mins after administering drug, rats were stressed. A parallel group of vehicle treated rats without exposure to any kind of stress and maintained under normal conditions served as control non-stress group. In CS drugs were fed daily 45 mins prior to stress regime for seven days except that the rats were fasted overnight on the sixth day after the completion of the experimental regimes of drug feeding and stress exposure. A parallel group of non-stress control group was also taken as described above and sacrificed on the seventh day along with CS group of rats.

Among the methods employed, immobilization has been used extensively and accepted widely for studying the stress induced physical and psychological alterations and consequences of the stress. In our experiments, the stress was produced by restraining the individual inside an acrylic hemicylindrical plastic tube (4.5 cm diameter, 12 cm long) for a period of 150 min once daily in AS and once daily for seven consecutive days in CS. The rats were sacrificed immediately after stress under ether anaesthesia, the abdomen and thorax were cut open, and blood was collected through cardiac puncture. The blood was centrifuged at 2000 rpm for 20 mins at 4° C and serum was separated. The serum was used to estimate glucose, triglycerides, cholesterol, alanine aminotransferase (ALT), asphartate aminotransferase (AST), and creatinine kinase (CK)

using semiautoanalyser (Maysun, MS-500E) with their respective kits. The adrenals, spleen and thymus were dissected and they were weighed after removing the adhering tissues.

e) Chronic stress induced sexual behavior[8]

Male rats were used in this paradigm. A male rat was placed in a cage for 10 min with six oestronized (sequentially treated with oestradiol valerate 5 mg/rat, followed 48 h later by hydroxyprogesterone 1.5 mg/rat sc) female rats (120–150 g), in a dimly lit room. The parameters observed included latency (in minutes) to lick female genitals, mounts and intromissions and the number of mounts and intromissions.

Group I - Normal control (unstressed)

Group II - Stress control

Group III - Stress + Ethyl acetate fraction 100 mg/kg, p.o. (EAAS 100 mg/kg)

Group IV - Stress + Ethyl acetate fraction 200 mg/kg, p.o. (EAAS 200 mg/kg)

Group V - Stress + Ethanolic fraction 100 mg/kg, p.o. (EtAS 100 mg/kg)

Group VI - Stress + Ethanolic fraction 200 mg/kg, p.o. (EtAS 100 mg/kg)

Group VII - Stress + *Withania somnifera* 100 mg/kg, p.o. (WS 100 mg/kg)

f) Swimming endurance test[9]

Swiss albino mice of 15 – 20 gm were selected and divided into seven groups of six animals each. Animals were divided in to Normal control (unstressed), Stress control, WS (100 mg/kg), and test groups (EAAS, EtAS-100 and 200 mg/kg p.o respectively).

Treatment was given to mice for seven days. On 7th day, 1 hr after drug administration the animals were forced to swim in glass chambers (30×30×15 cm) containing water at room temperature. The mice were allowed to swim till they got exhausted and the moment they drowned was considered as the endpoint. The mean swimming time for each group was noted. Adrenal glands are weighed and ascorbic acid and cortisol from the adrenal gland were estimated.

g) *In vitro* free radical scavenging activity[10]

A. Reaction with DPPH radical

Equal volume of 100 μM DPPH in methanol was added to different concentration of extracts/fractions (1-100 μg/ml) in methanol/water, mixed well and kept in dark for 20 min. The absorbance at 517 nm was measured with or without the extracts. Ascorbic acid (ASC) was used as standard for comparison. Different concentrations (1-5 μg/ml) of ASC were mixed with equal volumes of DPPH. Plotting the percentage DPPH scavenging against ASC concentration gave the standard cure.

B. Reaction with hydroxyl radical

Steady state $^{\bullet}OH$ scavenging activity of extracts/fraction (6-500 μg/ml) were measured by degradation of deoxy-D-ribose method, to the reaction mixture containing ascorbic acid (0.1 mM), deoxy-D-ribose (3 mM), ferric chloride (0.1 mM), EDTA (0.1 mM), hydrogen peroxide (2 mM) in phosphate buffer (20 mM, pH=7.4), various concentrations of the extract/fractions in a volume of 0.3 ml were added, to give a final volume of 3.0 ml. After incubation for 30 min at ambient temperature, 1.0 ml of TCA-TBA reagent (Equal volumes of TCA-2.8% and TBA-0.5% in 4mM NaOH) was added, followed by boiling the tubes in a water bath for 30 min. The tubes were then cooled and

the absorbance was measured at 532 nm. Mannitol was used as standard for comparison. Different concentrations (0.5-4.5 mg/ml) of mannitol were mixed as explained above. Plotting the percentage inhibition of $^{\bullet}OH$ scavenging against mannitol concentration gave the standard cure.

C. Lipid peroxidation (LPO) assay

Egg phospatidylcholine (20 mg) in chloroform (2 ml) was dried and further dispersed in normal saline (5 ml). The mixture was sonicated to get a homogeneous suspension of liposomes. Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid to a mixture containing liposome (0.1 ml), 150 mM potassium chloride, 0.2 mM ferric chloride, extracts/fractions (0.10-300 μg/ml) in a total volume of 0.4 ml. The reaction mixture was incubated for 40 min at 37°C. After incubation, the reaction was terminated by adding 1 ml of ice cold 0.25 M hydrochloric acid containing 20% w/v of trichloroacetic acid, 0.4% w/v of thiobarbituric acid and 0.05% w/v of butylated hydroxytoluene. After heating at 80°C for 20 min, the samples were cooled. The pink chromogen was extracted with a constant amount of n-butanol, and the absorbance of the upper organic layer was measured at 532 nm.

Trolox was used as standard for comparison. Different concentrations (4-15 μg/ml) of trolox were mixed as explained above. Plotting the percentage inhibition of LPO scavenging against trolox concentration gave the standard cure.

h) Statistical analysis

The results were expressed as mean ± S.E.M. The statistical significance was determined by two-way ANOVA followed by a post hoc Tukey's test. A probability 'P' value of less than 0.05 was taken to indicate statistical significance.

5.11.3.3 Results

A) Immobilization Induced Stress

i) Effect of drug treatment on acute stress (AS) and chronic stress (CS) induced alterations in biochemical parameters:

Table 5.14 and Fig. 5.7.1 showed that exposure to AS ($P<0.001$) resulted in a significant increase in the serum glucose level which is higher when compared to CS ($P<0.001$). Pretreatment with EAAS 100 and 200 mg/kg p.o. ($P<0.001$), EtAS 100 mg/kg p.o. ($P<0.001$), EtAS 200 mg/kg po ($P<0.001$) and WS 100 mg/kg p.o. ($P<0.001$) significantly decreased the circulating glucose level in AS. Where as increased level of glucose was also decreased by pretreatment with EtAS 100 mg/kg p.o. ($P<0.05$), EtAS 200 mg/kg p.o. ($P<0.01$) and WS 100 mg/kg p.o. ($P<0.001$)

Exposure to AS and CS resulted in significant increase in the total cholesterol ($P<0.001$) and triglyceride ($P<0.001$) levels compared to normal control. Pretreatment with EtAS 100 and 200 mg/kg po ($P<0.001$), EAAS 100 and 200 mg/kg ($P<0.01$) and WS 100 mg/kg p.o. ($P<0.05$) significantly decreased the circulating total cholesterol level in AS and CS. (Table 5.15 and Fig. 5.7.2). Moreover pretreatment with EtAS 200 mg/kg p.o. ($P<0.05$) only shown the significant reduction in triglyceride level in AS, Where as in CS, EtAS 100 mg/kg p.o. ($P<0.01$), and EtAS 200 mg/kg p.o. ($P<0.001$) significantly reduced elevated triglyceride levels in dose dependant manner (Table 5.16 and Fig. 5.7.3).

Exposure to AS ($P<0.001$) and CS ($P<0.01$) resulted in the significant increase in serum AST level as compare to respective control. Pretreatment with EtAS 100 mg/kg p.o. ($P<0.001$), EtAS 200 mg/kg p.o. ($P<0.001$) and WS 100 mg/kg p.o. ($P<0.001$) significantly decreased the AST level in AS. In CS EtAS mg/kg po 100 ($P<0.01$) and

EtAS 200 mg/kg p.o. (P<0.001) significantly reduced AST level in dose dependant manner (Table 5.17 and Fig. 5.7.4).

Exposure to AS (P<0.001) resulted in the significant increase in serum ALT level as compare to normal control. Pretreatment with EtAS 100 mg/kg p.o. (P<0.05), EtAS 200 mg/kg p.o. (P<0.001) and WS 100 mg/kg p.o. (P<0.01) significantly decreased the ALT level. In CS elevated levels 0f ALT was significantly lowered by pretreatment of EtAS 100 mg/kg p.o. (P<0.05), EtAS 200 mg/kg p.o. (P<0.01) and WS 100 mg/kg p.o. (P<0.05) (Table 5.18 and Fig. 5.7.5).

Exposure to AS (P<0.001) and CS (P<*0.*01) resulted in the significant increase in serum CK level as compare to respective control. In AS Pretreatment with EtAS 100 mg/kg p.o. (P<0.001), EtAS 200 mg/kg p.o. (P<0.001) and WS 100 mg/kg p.o. (P<0.01) significantly decreased the ALT level. Pretreatment with EtAS 100 mg/kg p.o. (P<0.05), EtAS 200 mg/kg p.o. (P<0.001) and WS 100 mg/kg p.o. (*P*<0.01) significantly decreased the ALT level in CS (Table 5.19 and Fig. 5.7.6).

ii) Effect of drug treatment on acute stress (AS) and chronic stress (CS) induced alterations in ulcer index and organ weight

Table 5.20 and Fig. 5.7.7 shows that AS and CS exposure resulted in a significant increase in score of ulcer index. Pretreatment with EtAS 100 mg/kg p.o. (P<0.001), EtAS 200 mg/kg p.o. (P<0.001) and WS 100 mg/kg p.o. (P<0.001) significantly decreased in ulcer index in comparison to AS and CS.

Exposure to AS (P<0.001) and CS (P<*0.*01) resulted in the significant increase the adrenal gland weight. Pretreatment with EtAS 100 mg/kg p.o. (P<0.01), EtAS 200 mg/kg p.o. (P<0.001) and WS 100 mg/kg p.o.(P<0.001) significantly restored the adrenal

weight in CS only, where as in AS none of the test drug shown any effect (Table 5.21 and Fig. 5.7.8).

A significant decreased was found on exposure to AS ($P<0.01$) and CS ($P<0.001$) in spleen weight. The weight was increased by EtAS 200 mg/kg p.o. ($P<0.05$) and WS 100 mg/kg p.o. ($P<0.05$) in AS and EtAS 100 mg/kg p.o. ($P<0.01$), EtAS 200 mg/kg p.o. ($P<0.001$) and WS 100 mg/kg p.o. ($P<0.001$) in CS (Table 5.22 and Fig. 5.7.9).

CS resulted in significant decrease in the weight of thymus weight ($P<0.001$). No significant change was observed after AS. The weight was only restored by the EtAS 200 mg/kg p.o. ($P<0.001$) and WS 100 mg/kg p.o. ($P<0.001$) in CS (Table 5.23 and Fig. 5.7.10).

iii) Chronic Stress Induced Sexual Behaviour

CS significantly inhibited the male sexual response indices, inducing decrease in latencies in licking female genitalia, mounting and intromission, number of mounts and intromissions. Pretreatment with WS 100 mg/kg po only reversed these change, where as none of the other test extract shown significant results (Table 5.28).

B) Swimming endurance test

The survival time of swimming mice increased significantly in dose dependent manner by pretreatment with EtAS 100 mg/kg p.o. ($P<0.01$) and EtAS 200 mg/kg p.o. ($P<0.001$) compared to normal (non-drug treated). The results of standard drug WS 100 was also found to be significant. Table 5.24 and Fig. 5.7.11 shows exposure to swimming stress causes hypertrophy of adrenal gland ($P<0.05$) which is associated with significant depletion of adrenal content viz. ascorbic acid ($P<0.001$) (Table 5.26 and Fig. 5.7.13) and cortisol ($P<0.001$) (Table 27 and Fig. 7.14). When compared to non swimmer group.

Pre-treatment with EtAS 100 mg/kg p.o. ($P<0.05$), EtAS 200 mg/kg p.o. ($P<0.05$) and WS 100 mg/kg p.o. ($P<0.05$) prevented the increase in adrenal weight significantly (Table 5.25 and Fig. 5.7.12). Depletion of adrenal ascorbic acid was attenuated significantly by pretreatment with EtAS 100 ($P<0.001$), EtAS 200 mg/kg p.o. ($P<0.001$) and WS 100 mg/kg p.o. ($P<0.001$). Pretreatment with EtAS 100 mg/kg p.o. ($P<0.01$), EtAS 200 mg/kg p.o. ($P<0.001$) and WS 100 mg/kg p.o. ($P<0.001$) also significantly increased cortisol contents of adrenals (Table 5.27 and Fig. 5.7.14).

C) *In-vitro* free radical scavenging activity

i) Reaction with DPPH radical

Ethanolic extract of *Argyreia speciosa* (EtAS) showed DPPH scavenging activity with IC_{50} levels of 36.54 µg/ml, whereas the IC_{50} value of the standard ascorbic acid was 2.94 µg/ml (Fig. 5.8.1), where as EAAS showed DPPH scavenging activity with higher IC_{50} levels of 200.93 µg/ml .

ii) Reaction with hydroxyl radical

The ability of the EtAS and EAAS to scavenge the hydroxyl radicals were found to be 121.18 µg/ml (Fig. 5.8.2), and 328.11 µg/ml respectively and IC_{50} value of standard Mannitol was found to be 4.99 mg/ml.

iii) Lipid peroxidation (LPO) assay

The inhibition of LPO by EtAS (IC_{50} = 41.83 µg/ml) was found to have comparable value with that of standard trolox (IC_{50}= 8.39 µg/ml) (Fig. 5.8.3) and ability of the EAAS to inhibit LPO was found to be 270 µg/ml.

5.11.3.4 Discussion

Abundant evidence accumulated over several decades has convincingly established a causally relevant linkage between recent stressful events and mental health.

During most of this history, this association was consistently found to be of modest magnitude, supporting the conclusion that variations in stress exposure are of only modest practical significance. However, more recent research that has considered additional dimensions of stress exposure appears to require a revised perspective. It indicates that the variance in mental health accounted for by level of stress exposure may be many times that suggested by prior work. Indeed, this evidence supports the hypotheses that differences in stress exposure represent a major factor driving population patterns of health and well-being and that it may represent the single most powerful social risk factor for mental health problems in the general population [11].

Stress is elicited by environmental, social, or pathological conditions occurring during the life of living beings and determines changes in the nervous, endocrine, and immune systems. Considerable evidence published in the last decade has focused on alterations of neurochemical, biochemical, and molecular effect caused by stress in these systems.The failure of successful adaptation during stressful situations has resulted in stress-related illnesses that result from, or are associated with, dysregulation of the stress response[12]. Various attempts have been made to counter the aversive effects of stress, ranging from yoga and meditation to antistress drugs, particularly the anxiolytic benzodiazepines. However, despite claims to the contrary, these nonpharmacological and pharmacological methods appear to have limited utility[13].

Due to the nonspecific nature of the stress pathogenesis, a separate class of therapeutic agents was evolved known as **"adaptogens"**. The term adaptogen was described by **Lazrev (1947)** as "the substance which can develop a state of raised resistance", enabling an organism to cope with stressful situations. Therapeutic approach for stress from ancient times has involved utilization of substances from natural origin,

rather than synthesis of new chemical compounds[14]. The general aims of adaptogen therapy appear to lie in their ability to reduce stress reactions during the alarm phase of the stress response, prevent or at least delay the state of exhaustion and, hence, provide a certain level of protection against long-term stress[15]. Ayurveda, the Indian system of medicine documents several herbs, which are categorized as rasayanas. The properties ascribed to rasayanas in Ayurveda are remarkably similar to those of adaptogens[16].

Several theories have been suggested to explain the effects of adaptogenic substances. One theory argues that adaptogens function primarily due to their antioxidant and free radical scavenging effects which is found to be partially accurate[17]. More recent research postulates that adaptogens work primarily by affecting the Hypothalamic/Pituitary/Adrenal (HPA) axis and the Sympathoadrenal System[18]. It has been reported that the rasayanas are rejuvenators, nutritional supplements and possess antioxidant activity. They also have antagonistic actions on the oxidative stressors which giving rise to the formation of different free radicals. The anti-stress/adaptogenic activity of plants made them therapeutically more important. The strong antioxidant activity of any rasayana was found to be 1000 times more potent than ascorbic acid, α-tocopherol and probucol [19].

A variety of stress situations have been employed to investigate the consequences of stress and to evaluate anti-stress agents and the lack of consistency of stress protocols and their biological consequences is astounding. Acute or short-duration stress appears to have limited aversive effects on the individual since the body sets in motion an array of physiological, biochemical and endocrine responses to counter stress effects. However, chronicity and excessiveness of the stressor, and the inability of the organism to cope with the stress, appear to induce the syndromal state described by Selye in 1936 [12,13].

Immobilization model used in our study found to cause long term desensitization of HPA response which affected both peripheral and central (paraventricular nucleus of the\hypothalamus, PVN) components of the HPA axis[20]. Immobilization has been the ideal choice for the induction of stress responses in animals and more specifically, for the investigation of drug effects, on typical stress-related gastrointestinal, neuroendocrine, and immunological pathology. The distinct advantage of using immobilization as a stressor lies in the fact that it produces both physical as well as inescapable psychological stress[21].

In our study we have selected acute and chronic immobilization stress to evaluate the immediate and chronic effects of *Argyreia speciosa* root extracts. Blood sugar level in response to stress is highly contradictory and studies related to stress in rats shows fluctuations in blood sugar level ranging from slight decrease, relative increase or no change[22]. However AS exposure in our study has elevated level of glucose when compare to CS. The hyperglycemic response in AS was due to release of glucocorticoids, as a result of HPA axis stimulation to compensate initial demand of energy[23]. The acute demand of glucose was fulfilled by the increase in glucogenolysis from liver during AS. During CS, this available source depletes. Thus, it utilizes fat as a secondary substrate and gluconeogenesis starts in response to corticosterone and, or due to the compensation of the energy demand during chronic conditions is from non-carbohydrate origin which are slow and rate limiting. Pretreatment with the EAAS 200 mg/kg, EtAS 100 mg/kg, EtAS 200 mg/kg and WS 100 mg/kg in AS, while during CS, EtAS 100 mg/kg po, EtAS 200 mg/kg and WS 100 mg/kg significantly decreased the circulating glucose level thus it seems to have a direct action on peripheral metabolism.

The AS and CS raised the serum cholesterol level through the enhanced activity of hypothalamo-hypophyseal axis resulting in increased liberation of catecholamine and corticosteroids. The effect of AS and CS stress on serum triglyceride has been shown to be variable probably due to mobilization of fats from adipose tissue by catecholamine. Treatment with EtAS 100 mg/kg, EtAS 200 mg/kg and WS 100 mg/kg ameliorated the elevated levels of cholesterol as well as triglyceride levels in both AS and CS. The suppression of stress induced triglyceride level may be due to the suppression of stress induced lipolysis.

The AS-induced significant increase in ALT, AST, and CK might be the outcome of AS induced secretion of corticosterone from cortex, epinephrine from medulla, and epinephrine from sympathetic nerve terminals to provide substrate for energy metabolism and the assurance of availability of ATP demand in the muscles, CNS, and organ of demand. ALT and AST enzymes catalyze the transfer of the γ-amino groups of alanine and aspartate, respectively, to the γ -keto group of ketogluterate, leading to the formation of oxaloacetic acid and pyruvic acid. In contrast to ALT, which is found primarily in liver, AST is present in many tissues, including the heart, kidney, brain, and skeletal muscles. As a result of transamination, amino acid can enter the citric acid cycle and then function in the intermediary metabolism of carbohydrate and lipids[24]. Stress hormones also increase CK activity during stress. The CK system is important in stabilizing the ATP levels and energy metabolism of the myocardium and other skeletal muscles of rats during stress. CK is known to act as an energy buffer and shuttle between sites of energy production, i.e. mitochondria and ion pumps. Perturbations of CK activity during extensive stress may result in ischemia due to the non-availability of ATP. A

maximum increase in CK activity was observed after AS exposure when compared to CS. A reduced CK activity in CS as compared to AS is due to partial habituation. Pretreatment with EtAS 100 mg/kg, EtAS 200 mg/kg and WS 100 mg/kg revert the AS and CS induced levels of AST, ALT and CK in blood. Reduction in ALT and AST may be due to direct action on the peripheral metabolism and of CK may be decrease in energy demand.

Stressful events activate autonomic and endocrine responses[25] responsible for gastric ulceration. In our study AS and CS induced ulceration in stomach with comparable intensity in both the models. This can be attributed to the stimulation of paraventricular nucleus of hypothalamus, increased intestinal motility, acid secretion and group of other factors[26]. Gastric damage induced by CS and AS has been reduced by EtAS 100, EtAS 200 and WS 100 as reflected by decreased mean ulcer severity score indicating their protective effects on gastric mucosa during stressful conditions.

Stress-induced adrenal hypertrophy found both in AS and CS was the result of activation of the HPA axis, which is highly responsive to stress and is one of the principal mechanism by which an organism mobilizes its defense against stress events[28]. The prolonged activation of HPA axis resulted in an increase in the adrenal hypertrophy in CS as compared to AS. The sympathetic nervous system in response to stress results in the secretion of corticosterone from adrenal cortex and epinephrine from adrenal medulla. These hormones are deemed as necessary manipulators in the body against stress response and help in combating stress. During stress, nerve terminals accelerate recruitment of lymphocytes to blood from spleen, which is a major storage pool of lymphocytes. This result in the squeezing of the spleen causing reduction in weight

observed in AS as well as in CS exposures. However, the atrophy of thymus was found only during CS exposure and not during AS. The transient activation of HPA axis and release of neurochemicals do not have profound impact on thymocytes as in AS, but persistent high level of corticosterone during CS causes apoptosis and necrosis in immature T and B cells resulting in the decline of thymus weight. Our study showed that during AS, there was no effect of any test drug on restoring the weight of adrenal and thymus gland, whereas pretreatment with EtAS 100 mg/kg, EtAS 200 mg/kg and WS 100 mg/kg significantly reverse the stress induced atrophy of spleen. CS leads to a prolonged activation of HPA axis; thus, more pronounced effect on ulcer index and adrenal gland weight was found. However during CS adrenal hypertrophy and spleen atrophy was reversed by the treatment with the EtAS 100 mg/kg, EtAS 200 mg/kg and WS 100 mg/kg, while the higher dose of EtAS and WS 100 mg.kg able to restore the weight of thymus gland. CS induced significant suppression of male sexual function in rats, which was only reversed by standard drug (WS 100 mg/kg).

Stress alters the normal functioning of the body. In a special contrivance, when an animal forced to swim becomes immobile after an initial period of vigorous activity. This resembles a state of mental depression. The adrenal glands contain relatively large amount of ascorbic acid and cortisol which are markedly reduced by stress and causes hypertrophy[27,28,29]. when they are stimulated by stress. Our results showed that pretreatment with EtAS 100, EtAS 200 and WS 100mg increased labor efficiency and increase of swimming performance, moreover, prevented the depletion of ascorbic acid, cortisol and hypertrophy of adrenal glands indicating that, drug having steroid sparing effects.

It has been shown that exposure to stress situations can stimulate numerous pathways leading to increased production of free radicals It is well known that free radicals generate a cascade, producing lipid peroxidation, protein oxidation, DNA damage and cell death, and contribute to the occurrence of pathological conditions. Stress may also impair antioxidant defenses, leading to oxidative damage, by changing the balance between oxidant and antioxidant factors. Both immobilization and variable stress are followed by an increase in lipid peroxidation, measured in plasma and in brain structures. In addition, decreased activities of the antioxidant enzymes have been observed in the brain of rats treated with glucocorticoids (steroid hormones released by the adrenals in response to physical and psychological stressors), and exposure to physiological levels of these hormones exacerbates reactive oxygen species generation[30]. Ethanolic extract of *Argyreia speciosa* have been shown to exert significant antioxidant activity induced by augmented activity of oxygen free radicals scavenging enzymes, superoxide dismutase, catalase and peroxidases[31]. Results of *in vitro* free radical scavenging activity carried, using DPPH , hydroxyl radical and LPO for ethanolic extract shown to have potent free radical scavenging effect when compared with there respective standard. Thus at least part of observed adaptogenic/ anti-stress effect of EtAS may be due to the antioxidant activity.

Table 5.14. Effect EtAS, EAAS on the serum glucose level on immobilization [Acute and chronic] induced stress in rats

Sr. No	Groups	Glucose (mg/dl)	
		Acute stress(AS)	Chronic stress(CS)
1	Normal control	82.92±1.22[c]	88.72±5.26[f]
2	Stress control	183.3±7.98	137.1± 4.23
3	EAAS 100	149.6±8.55	134.4±10.50
4	EAAS 200	102.6±5.46[c]	107.4± 5.15
5	EtAS 100	102.0±3.01[c]	98.73± 13.24[d]
6	EtAS 200	90.75±2.03[c]	90.56±12.98[e]
7	WS100	91.01±1.57[c]	87.41±6.01[f]

Results are represented as mean ± SEM (n=6). [c]P<0.001 as compared with stress control group for AS. [d]P<0.05, [e]P<0.01 and [f]P<0.001 as compared with stress control group for CS.

Fig. 5.7.1. Effect of EAAS and EtAS on the serum glucose level on immobilization [acute and chronic] induced stress in rats

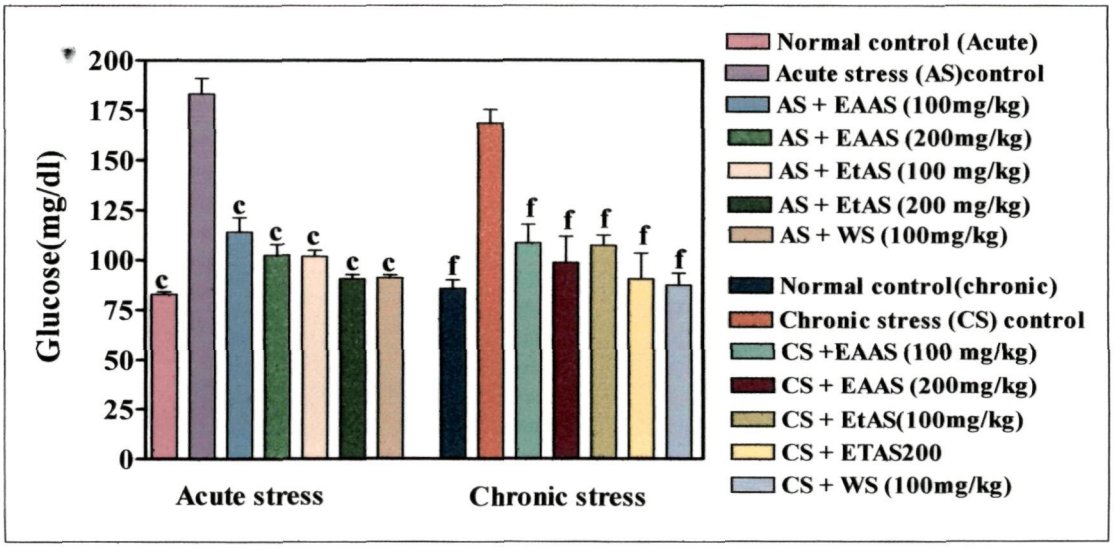


Table 5.15. Effect EtAS and EAAS on the serum total cholesterol level immobilization [Acute and chronic] induced stress in rats

Sr. No	Groups	Total cholesterol (mg/dl)	
		Acute stress(AS)	Chronic stress(CS)
1	Normal control	43.88±3.91[c]	33.61±3.92[f]
2	Stress control	75.55±5.71	67.49±3.22
3	EAAS 100	66.97±2.43	54.06±2.47
4	EAAS 200	60.42±5.27	44.13±3.41
5	EtAS 100	51.31±2.23[b]	48.49±1.95[d]
6	EtAS 200	52.69±6.26[b]	46.56±3.64[d]
7	WS100	55.15±3.03[a]	45.17±2.46[e]

Results are represented as mean ± SEM (n=6). [a]P<0.05, [b]P<0.01 and [c]P<0.001as compared with stress control group for AS. [d]P<0.05,[e]P<0.01and [f]P<0.001as compared with stress control group for CS.

Fig 5.7.2. Effect of EtAS and EAAS on the serum total cholesterol level on immobilization [acute and chronic] induced stress in rats

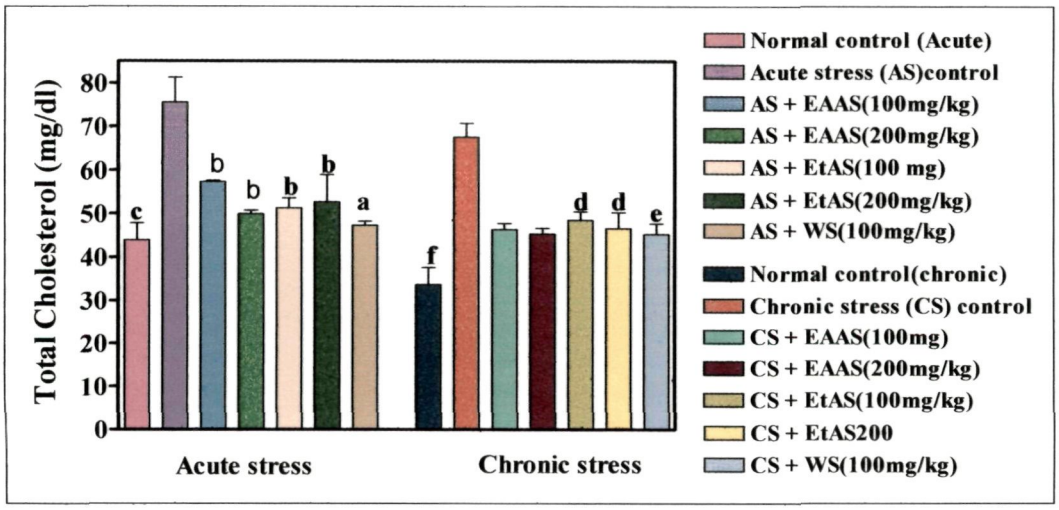


Table 5.16. Effect of EtAS and EAAS on the serum triglyceride level on immobilization [Acute and chronic] induced stress in rats

Sr. No	Groups	Trigyceride (mg/dl)	
		Acute stress(AS)	Chronic stress(CS)
1	Normal control	31.38±2.52^{c}	36.18±5.03^{f}
2	Stress control	77.50±5.04	88.09±15.98
3	EAAS 100	56.59±5.92	65.63±10.66
4	EAAS 200	44.74±3.02	56.73±8.54
5	EtAS 100	43.86±3.78	43.23±3.27^{e}
6	EtAS 200	42.82±3.78^{a}	38.13±7.56^{f}
7	WS100	42.23±1.99^{a}	36.82±0.10^{f}

Results are represented as mean ± SEM (n=6). $^{a}P<0.05$ and $^{c}P<0.001$as compared with stress control group for AS. $^{e}P<0.01$and $^{f}P<0.001$as compared with stress control group for CS.

Fig. 5.7.3. Effect of EtAS and EAAS on the serum triglyceride level on immobilization [acute and chronic] induced stress in rats

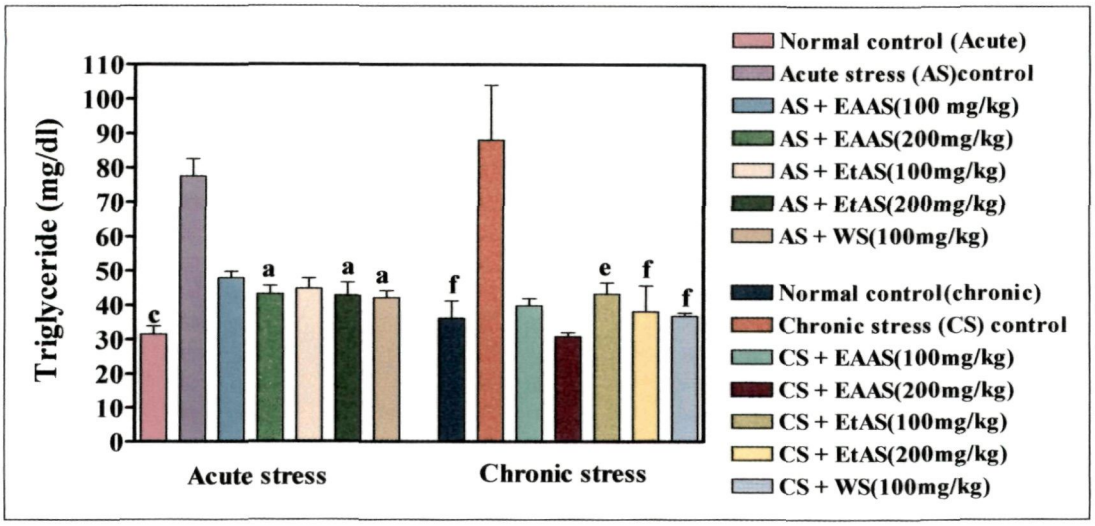


Table 5.17. Effect EtAS and EAAS on the serum AST level on immobilization [Acute and chronic] induced stress in rats

Sr. No	Groups	AST (IU/L)	
		Acute stress(AS)	Chronic stress(CS)
1	Normal control	66.83±6.66[c]	34.68±1.55[e]
2	Stress control	162.7±5.92	86.78±20.76
3	EAAS 100	135.01±12.45	76.69±4.19
4	EAAS 200	125.4±12.71	72.79± 7.23
5	EtAS 100	77.72±3.14[c]	36.67±3.89[e]
6	EtAS 200	57.94±3.39[c]	34.53±4.02[f]
7	WS100	66.01±3.28[c]	42.98±5.52[d]

Results are represented as mean ± SEM (n=6). [c]P<0.001as compared with stress control group for AS. [d]P<0.05, [e]P<0.01and [f]P<0.001as compared with stress control group for CS.

Fig. 5.7.4. Effect of EtAS and EAAS on the serum AST level on immobilization [acute and chronic] induced stress in rats

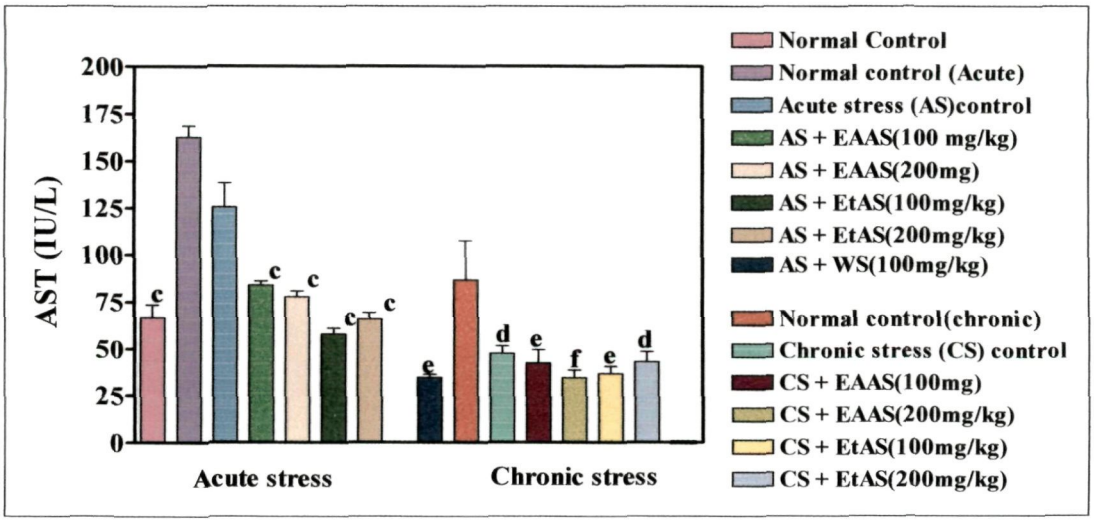


Table 5.18. Effect EtAS and EAAS on the serum ALT level on immobilization [Acute and chronic] induced stress in rats

Sr. No	Groups	ALT (IU/L)	
		Acute stress(AS)	Chronic stress(CS)
1	Normal control	20.06± 0.49[c]	25.84±2.96 [e]
2	Stress control	44.20±0.88	41.89±2.59
3	WAS 100	37.83±3.28	39.33±2.66
4	WAS 200	37.36±2.06	30.12±2.87
5	EtAS 100	30.03±1.50[a]	27.98±2.08[d]
6	EtAS 200	26.60±2.05[c]	27.50±2.89[e]
7	WS100	27.60±2.78[b]	28.66±1.59[d]

Results are represented as mean ± SEM (n=6). [a]P<0.05, [b]P<0.01and [c]P<0.001 as compared with stress control group for AS. [d]P<0.05 and [e]P<0.01 as compared with stress control group for CS.

Fig. 5.7.5. Effect of EtAS and EAAS on serum ALT level on immobilization [acute and chronic] induced stress in rats

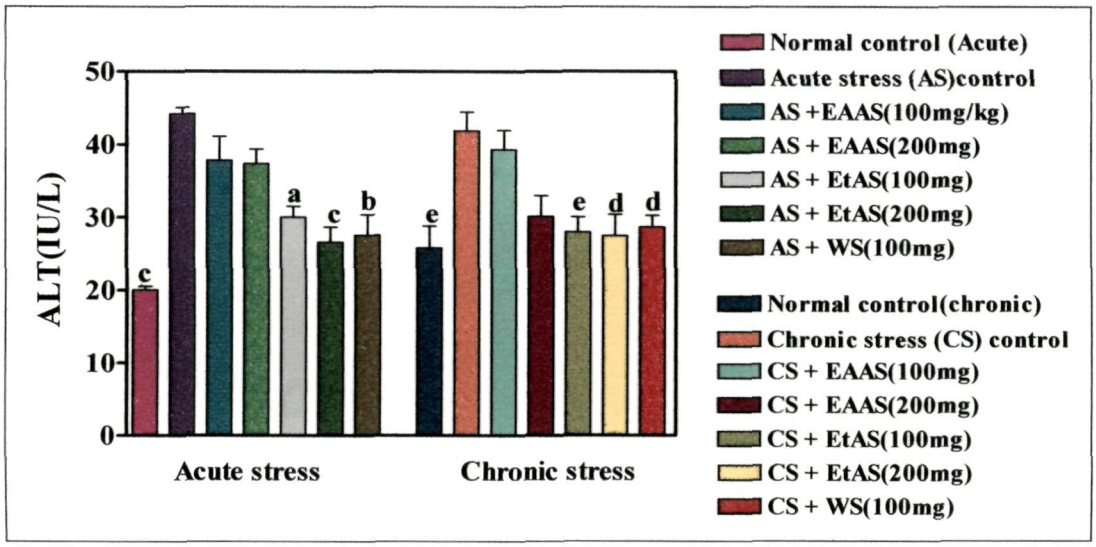


Table 5.19. Effect of EtAS and EAAS on the serum creatine kinase (CK) level on immobilization [Acute and chronic] induced stress in rats

Sr. No	Groups	CK (IU/L)	
		Acute stress(AS)	Chronic stress(CS)
1	Normal control	136.0±4.62^{c}	147.0±5.69^{e}
2	Stress control	250.5±10.63	202.7±8.79
3	EAAS 100	227.3±10.81	197.3±16.16
4	EAAS 200	222.0±6.51	170.3±11.12
5	EtAS 100	179.5±5.38^{c}	156.7±5.98^{d}
6	EtAS 200	162.2±6.77^{c}	158.2±4.28^{d}
7	WS100	160.8±6.39^{c}	147.7±4.25^{e}

Results are represented as mean ± SEM (n=6). $^{a}P<0.05$ and $^{c}P<0.001$as compared with stress control group for AS. $^{d}P<0.05$ and $^{e}P<0.01$as compared with stress control group for CS.

Fig. 5.7.6. Effect of EtAS and EAAS on serum CK level on immobilization [acute and chronic] induced stress in rats

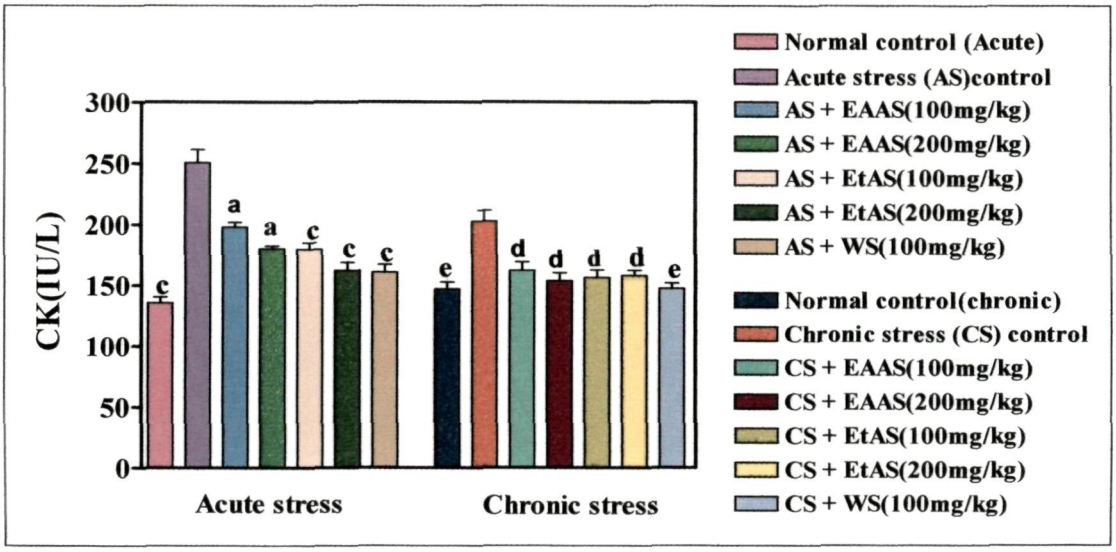


Table 5.20. Effect EtAS and EAAS on ulcer index on immobilization [Acute and chronic] induced stress in rats

Sr. No	Groups	Ulcer index	
		Acute stress(AS)	Chronic stress(CS)
1	Normal control	0.0±0.0	0.0±0.0
2	Stress control	10.17±0.70	13.00±0.81
3	EAAS 100	8.333±0.33	12.17±0.79
4	EAAS 200	7.55±0.42	10.50 ±0.76
5	EtAS 100	5.333±0.61^{c}	5.50±0.76^{f}
6	EtAS 200	3.667± 0.42^{c}	3.167±0.47^{f}
7	WS100	3.333±0.55^{c}	3.500±3.50^{f}

Results are represented as mean ± SEM (n=6). $^{b}P<0.01$and $^{c}P<0.001$as compared with stress control group for AS. $^{e}P<0.01$and $^{f}P<0.001$as compared with stress control group for CS.

Fig. 5.7.7. Effect of EtAS and EAAS on ulcer index on immobilization [acute and chronic] induced stress in rats

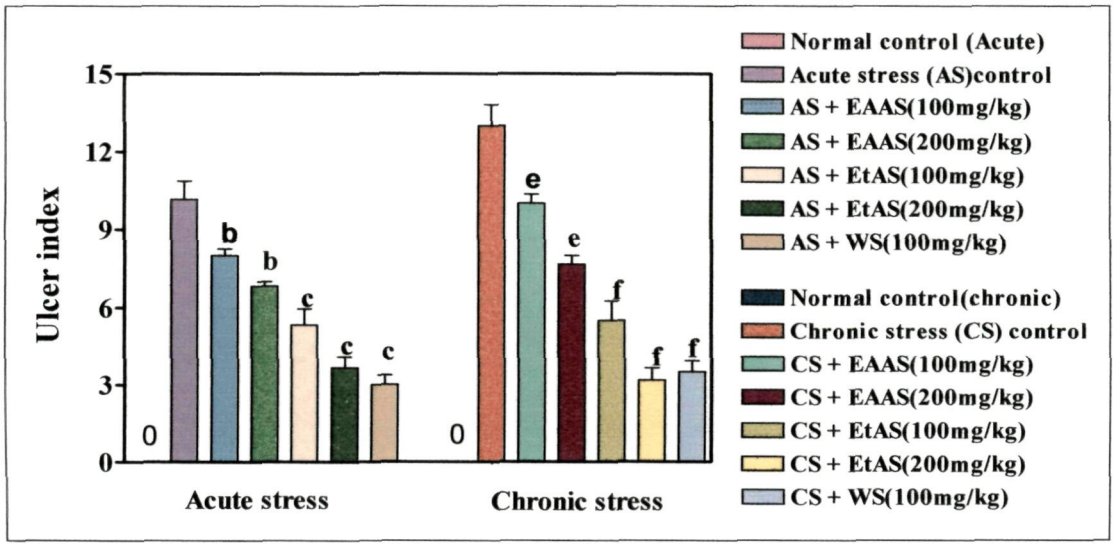


Table 5.21. Effect EtAS and EAAS on the weight of adrenal gland on immobilization [Acute and chronic] induced stress in rats

Sr. No	Groups	weight of adrenal gland(mg)	
		Acute stress(AS)	Chronic stress(CS)
1	Normal control	14.00±0.36[c]	17.00±0.93[e]
2	Stress control	23.17±1.25	21.33±0.91
3	EAAS 100	20.67±0.95	18.17±1.11
4	EAAS 200	19.83±0.79	20.17±0.94
5	EtAS 100	20.50±0.92	17.67±0.76[e]
6	EtAS 200	19.00±1.31	15.17±0.47[f]
7	WS100	19.50 ±1.176	15.67± 0.76[f]

Results are represented as mean ± SEM (n=6). [c]P<0.001as compared with stress control group for AS. [d]P<0.05, [e]P<0.01and [f]P<0.001as compared with stress control group for CS.

Fig. 5.7.8. Effect of EtAS and EAAS on the weight of adrenal gland on immobilization [acute and chronic] induced stress in rats

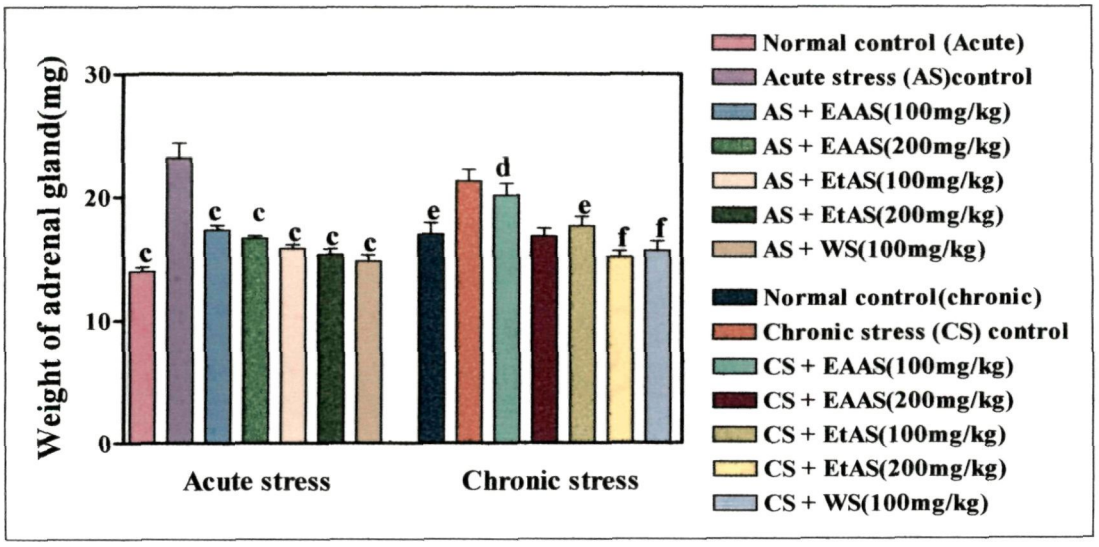


Table 5.22. Effect of EtAS and EAAS on the weight of spleen on immobilization [Acute and chronic] induced stress in rats

Sr. No	Groups	weight of spleen (mg)	
		Acute stress(AS)	Chronic stress(CS)
1	Normal control	644.5±15.05[b]	664.0±16.08[f]
2	Stress control	473.7±33.59	444.0±39.21
3	EAAS 100	488.5±5.23	459.2±18.47
4	EAAS 200	554.8±20.97	533.0±22.50
5	EtAS 100	605.0±6.90	630.3±57.76[e]
6	EtAS 200	612.7±7.67[a]	603.8±48.79[d]
7	WS100	620.0±14.17[a]	599.7±10.92[d]

Results are represented as mean ± SEM (n=6). [a]P<0.05 and [b]P<0.01 as compared with stress control group for AS. [d]P<0.05, [e]P<0.01and [f]P<0.001as compared with stress control group for CS.

Fig. 5.7.9. Effect of EtAS and EAAS on the weight of spleen on immobilization [acute and chronic] induced stress in rats

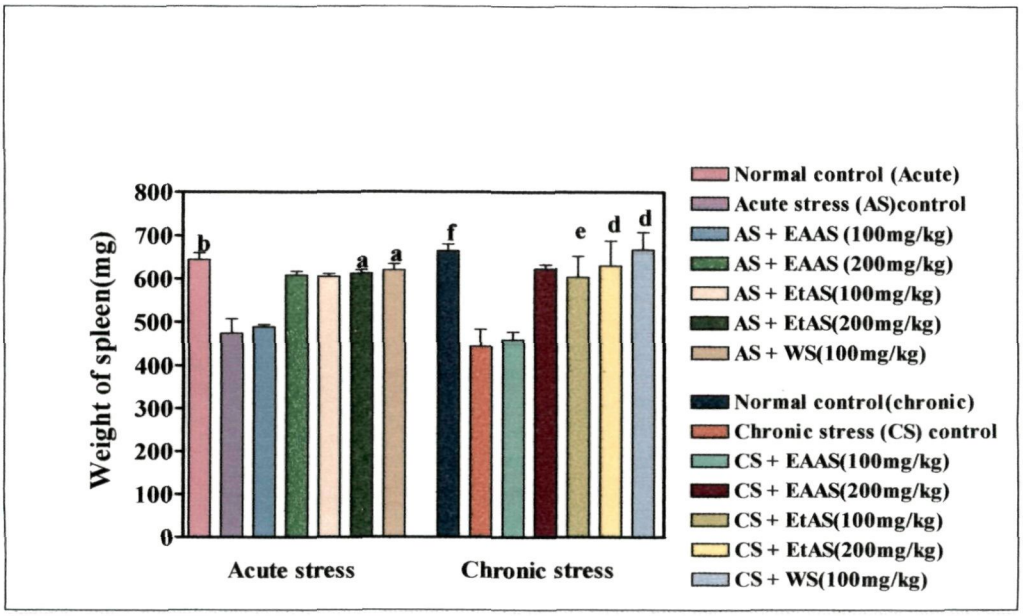


Table 5.23. Effect of EtAS and EAAS on the weight of thymus gland on immobilization [Acute and chronic] induced stress in rats

Sr. No	Groups	weight of thymus gland (mg)	
		Acute stress(AS)	Chronic stress(CS)
1	Normal control	667.7±9.28	651.5±19.33[f]
2	Stress control	619.3±27.93	386.3±10.48
3	EAAS 100	578.2±22.97	446.3±8.81
4	EAAS 200	570.0±7.64	437.7 ±8.82
5	EtAS 100	563.0±12.68	445±6.33
6	EtAS 200	583.5±15.40	615.0±17.23[f]
7	WS100	608.3±8.11	623.8±19.37[f]

Results are represented as mean ± SEM (n=6). [f]P<0.001as compared with stress control group for CS.

Fig. 5.7.10. Effect of EtAS and EAAS on the weight of thymus gland on immobilization [acute and chronic] induced stress in rats

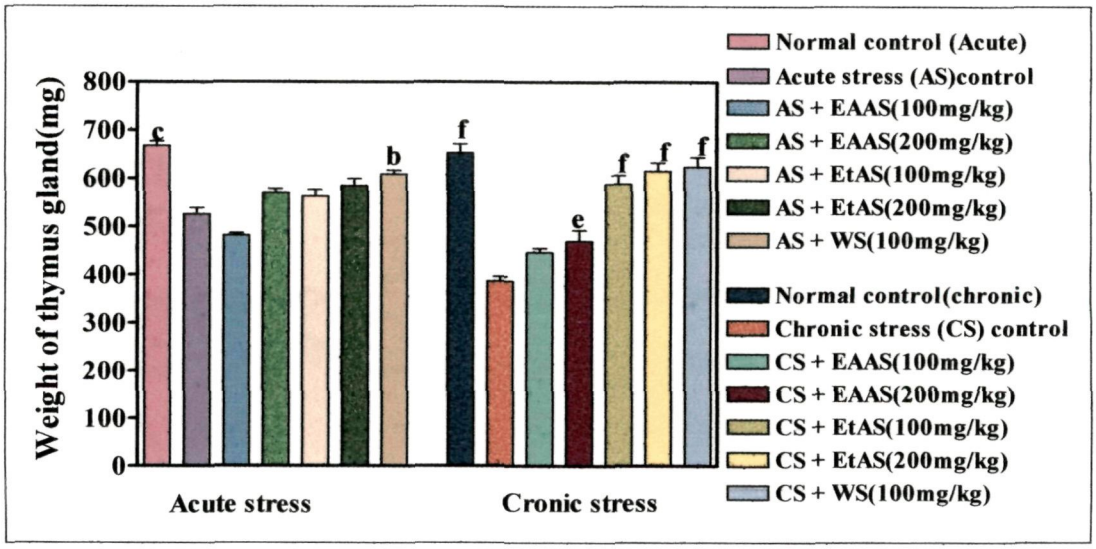


Table 5.24. Effect EtAS and EAAS on mean swimming time (min) on swimming induced stress in mice

Sr. No	Groups	Mean Swimming Time (min)
1	Normal	206.5±8.30
2	EAAS 100	167.7±5.98
3	EAAS 200	174.7±6.68
4	EtAS 100	238.5±4.11[b]
5	EtAS 200	252.2±4.16[c]
6	WS100	267.3±3.77[c]

Results are represented as mean ± SEM (n=6). [b]P<0.01and [c]P<0.001as compared with normal group.

Fig. 5.7.11. Effect of EtAS and EAAS on mean swimming time (min) on swimming induced stress in mice

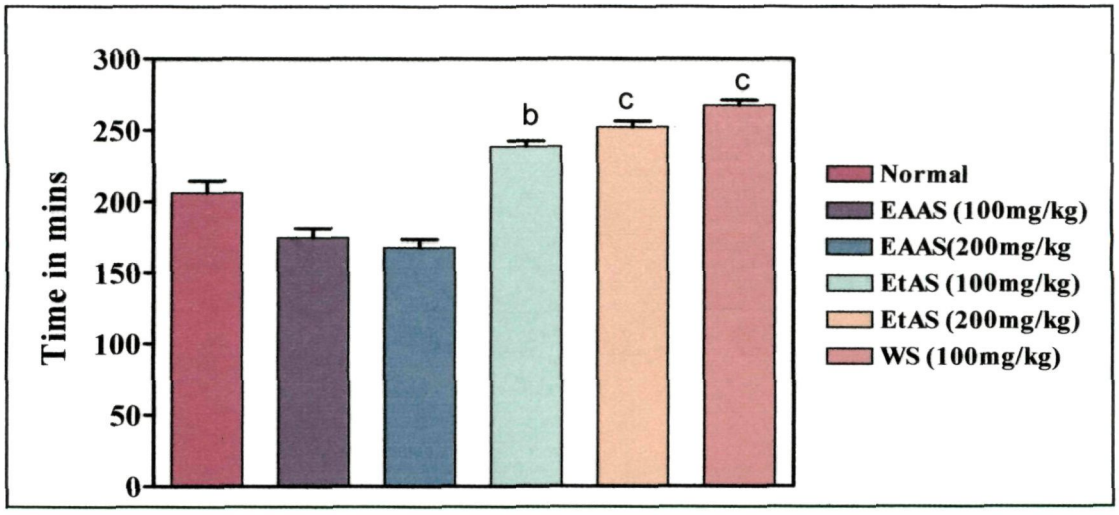


Table 5.25. Effect of EtAS and EAAS on weight of adrenal gland (mg/100 body weight) on swimming induced stress in mice

Sr. No	Groups	Weight of adrenal gland(mg/100 gm BW)
1	Normal control	13.66±1.47[a]
2	Stress control	20.13±1.79
3	EAAS 100	18.00±1.27
4	EAAS 200	17.89±1.41
5	EtAS 100	14.22±0.83[a]
6	EtAS 200	13.25±1.10[a]
7	WS100	12.51±1.07[b]

Results are represented as mean ± SEM (n=6). [a]P<0.05and [b]P<0.01as compared with stress control group.

Fig. 5.7.12. Effect of EtAS and EAAS on weight of adrenal gland mg/100g bodyweight on swimming induced stress in mice

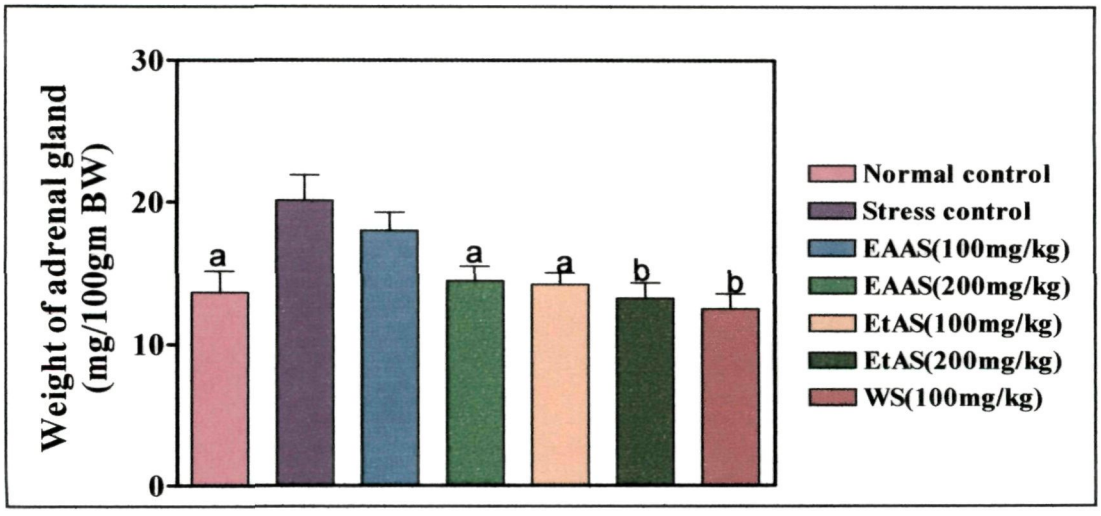


Table 5.26. Effect EtAS and EAAS on level of ascorbic acid (mg/100g of tissue) in adrenal gland on swimming induced stress in mice

Sr. No	Groups	Ascorbic acid (mg/100gm of tissue)
1	Normal control	196.8 ±16.76^{c}
2	Stress control	22.03±4.04
3	EAAS 100	40.28±3.10
4	EAAS 200	80.09±12.58
5	EtAS 100	156.2±19.78^{c}
6	EtAS 200	201.1±21.74^{c}
7	WS100	224.4±19.42^{c}

Results are represented as mean ± SEM (n=6). $^{c}P<0.001$as compared with stress control group.

Fig. 5.7.13. Effect of EtAS and WAS on level of in adrenal ascorbic acid (mg/100 g of tissue) on swimming induced stress in mice

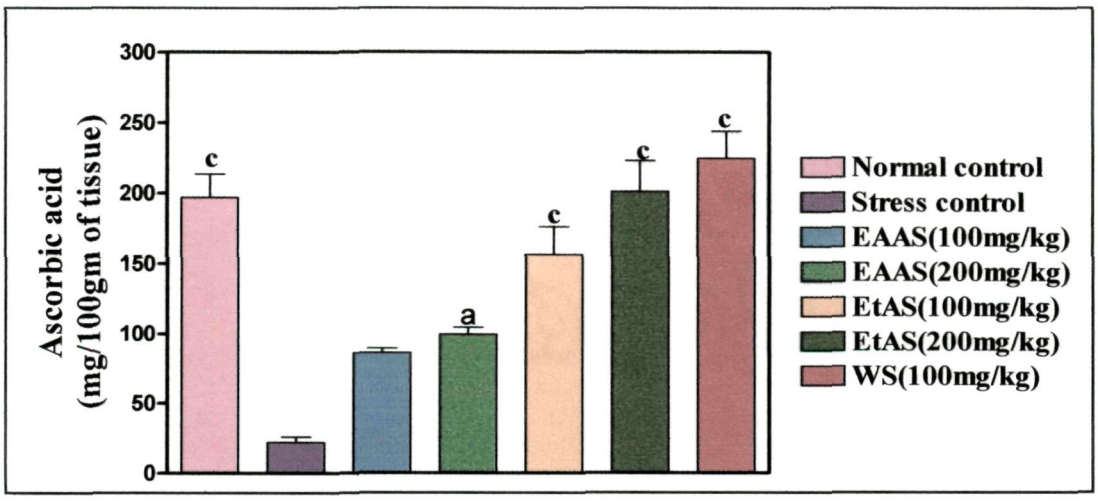


Table 5.27. Effect of EtAS and EAAS on level of cortisol (mg/100g of tissue) in adrenal gland on swimming induced stress in mice

Sr. No	Groups	Cortisol (mg/100g of tissue)
1	Normal control	2.65±0.40^{c}
2	Stress control	0.61±0.09
3	EAAS 100	0.82±0.07
4	EAAS 200	1.03±0.15
5	EtAS 100	2.18±0.24^{b}
6	EtAS 200	2.61±0.23^{c}
7	WS100	2.45±0.27^{c}

Results are represented as mean ± SEM (n=6). $^{b}P<0.01$ and $^{c}P<0.001$as compared with normal group.

Fig. 5.7.14. Effect of EtAS and EAAS on level of adrenal cortisol (mg/100 g of tissue) on swimming induced stress in mice

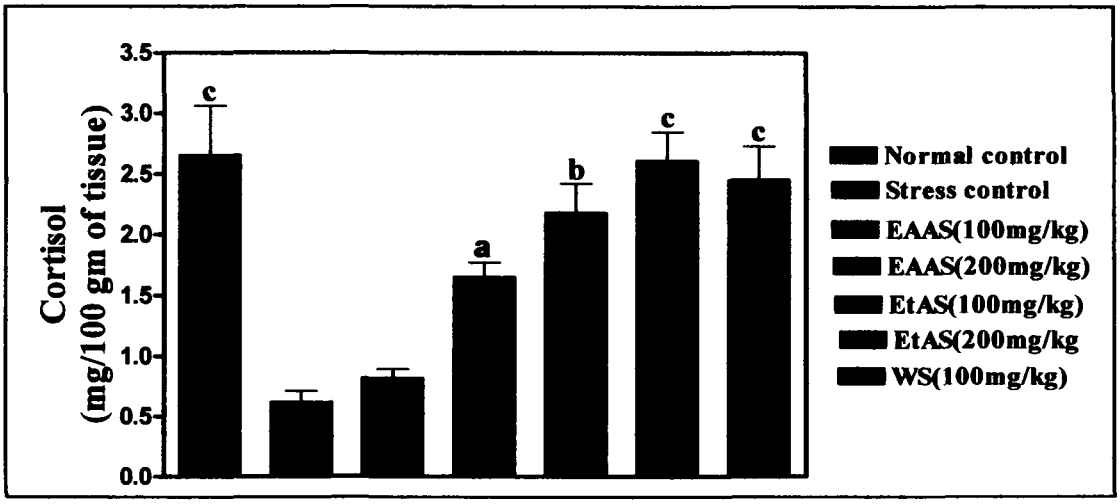


Table 5.28. Effect of EtAS and EAAS fractions on sexual behavior on immobilization [chronic] induced stress in rats

Groups	Latency in min			Number of action	
	Licking	Mounting	Intromission	Mounting	Intromission
Normal control	3.45±0.38^{c}	5.13±0.17^{c}	4.63±0.24^{c}	4.33±0.33^{c}	3.66±0.42^{b}
Chronic Stress	1.64±0.08	2.32±0.19	1.76±0.20	1.83±0.40	2.0± 0.25
EAAS 100	1.60±0.14	2.07±0.21	1.5±0.057	1.16±±0.30	1.0±0.36
EAAS 200	1.73±0.12	2.57±0.15	1.58±0.09	1.50±0.22	1.83± 0.30
EtAS 100	1.60±0.06	2.78±0.19	1.83±0.08	1.16±0.16	1.66±0.21
EtAS 100	1.51±0.12	2.78±0.19	1.58±0.11	1.33±0.21	1.66±0.21
WS 100	2.66±0.30^{a}	4.78±0.31^{c}	4.36±0.38^{c}	3.50±0.34^{b}	3.5±0.34^{c}

Results are represented as mean ± SEM (n=6). $^{a}P<0.05$, $^{b}P<0.01$and $^{c}P<0.001$as compared with stress control group.

IN-VITRO FREE RADICAL SCAVENGING ACTIVITY

Fig 5.8.1. Scavenging effect of ethanolic fraction (EtAS) of *Argyreia speciosa* on DPPH radical

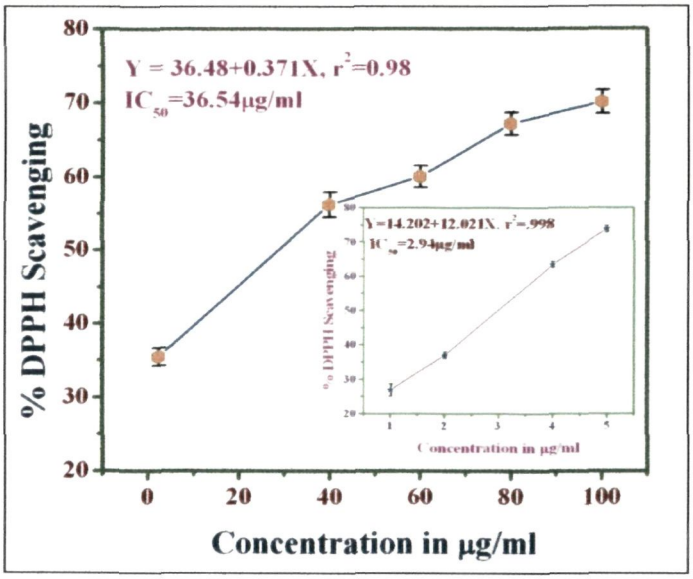


Fig. 5.8.2. Scavenging effect of ethanolic fraction (EtAS) of *Argyreia speciosa* on Hydroxyl radical

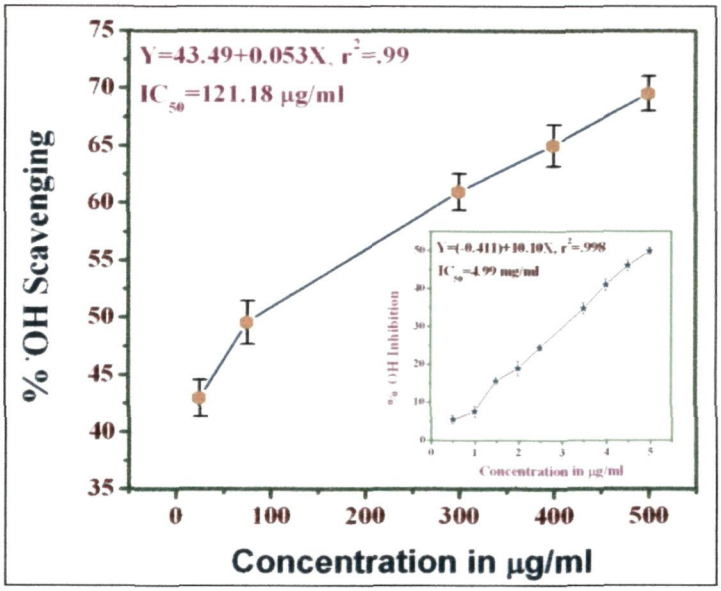
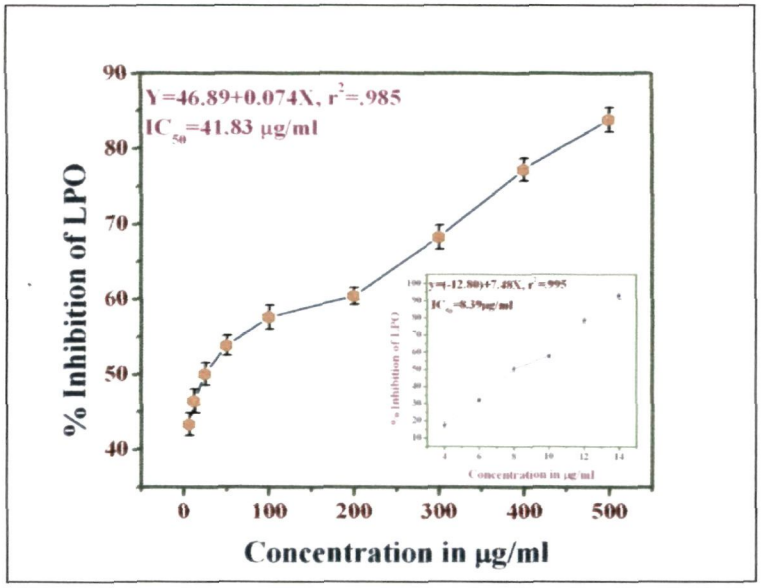


Fig. 5.8.3. Scavenging effect of ethanolic fraction (EtAS) of *Argyreia speciosa* on LPO



5.11.3.6. References

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5.11.4 Cardioprotective effect of *Argyreia speciosa* (Burm.f.) Boj. in Isoproterenol-induced myocardial infraction in rats

5.11.4.1 Introduction

Ischaemic heart diseases, especially acute myocardial infarction (MI), remain the leading cause of death in both developed and developing countries as seen over the past quarter century[1,2]. Ischemic heart disease (IHD) is the leading cause of morbidity and mortality in world wide, and according to the world health organization it will be the major cause of death in the world by the year 2020[3]. Myocardial infarction (MI) results from the prolonged myocardial ischemia with necrosis of myocytes due to interruption of blood supply to an area of heart[4].

Isoproterenol (ISO) induced myocardial necrosis is a well known standard model to study the beneficial effect of many drugs on cardiac dysfunction[5]. ISO is a β-adrenergic agonist that causes severe stress in myocardium and necrotic lesions in the heart muscles. ISO induced myocardial injury involves membrane permeability alterations, which brings about the loss of functions and integrity of myocardial membranes [6]. MI induced by ISO in rats has been shown to be accompanied by hyperglycemia, hyperlipidemia, and increase in serum creatine phosphokinase, alanine aminotransferase, aspertate aminotransferase and lactate dehydrogenase activities[7,8]. The mechanism proposed to explain isoproterenol induced cardiac damage involves generation of highly cytotoxic free radicals through auto-oxidation of catecholamine and has been implicated as one of the causative factor[7].

Reduction of mortality rate and prevention of myocardial infarction are of utmost importance. Western drugs such as angiotensin-converting enzyme (ACE) inhibitors,

calcium channel blockers, angiotensin II receptor antagonists, etc. have been proven to have cardioprotective effects in both preclinical and clinical studies. ACE inhibitors have been gradually introduced into the treatment of hypertension, congestive heart failure and myocardial infarction since the 1970s[8,9,10,11]. Experimental studies also showed that ACE inhibitors administered chronically before acute MI might limit myocardial infarct size, improve cardiac function and prevent cardiac hypertrophy [8,12].

Cardiovascular diseases (CVD) are the secondary cause of deaths in many parts of the world, although modern drugs are effective in preventing the disorders, their use is often limited because of their side effects, and adverse reactions. However, there is a growing interest in the use of alternative medicine for long-term prevention of heart attack in high risk patients. A wide array of plants and its active principles, with minimal side effects, provide an alternate therapy for Ischemic heart diseases. Moreover, the plant kingdom represents a largely unexplored reservoir of biologically active compounds in cardiovascular diseases. The present study was designed to investigate the modulation of different root fractions of *Argyreia speciosa* in ISO-induced electrocardiographic, biochemical and histopathological changes.

5.11.4.2 Materials and Methods

a) Drugs and chemicals

Isoproterenol hydrochloride (ISO) purchased from Sigma Chemical Company, St louis MO USA, ECG Electrodes procured from Biopac Santa barbara California. Sodium carboxy methyl cellulose (Na-CMC) from Loba Chemie, Mumbai, India, Pentobarbitone, Anaesthetic ether were used. All chemicals were of analytical grade.

b) Experimental animals

Male Wistar rats of 150-200 gms were used for the study. The inbred colonies of rats were purchased from Venkateshwara Enterprises, Bangalore. They were acclimatized to controlled conditions of temperature (23±2°C), humidity (50±5%) and 12-h light-dark cycles. The animals were randomized into experimental and control groups and housed two each in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellets as basal diet and water *ad libitum.* We selected male rats for our studies, since females are shown to be protected from cardiovascular complication[13].

c) Preparation of Drugs

EAAS, EtAS and AQAS suspensions prepared in Sodium carboxy methyl cellulose (Na CMC) and distilled water, were used to assess the cardioprotective effect of the plant.

d) Induction of myocardial injury

Rats were treated with different doses EtAS, EAAS and AQAS orally using an intra-gastric tube daily for 14 days. On 14^{th} day, myocardial injury was induced in experimental rats by injection of Isoproterenol (200 mg/kg, s.c) twice at an interval of 24 hrs (i.e., on 14 and 15^{th} day of drug treatment) while normal control and Isoproterenol treated rats were given an equivalent volume of the vehicle.

e) Treatment protocol

The experimental rats were divided into eleven groups of six animal each and treated as follows :

Group 1: Normal Control Rats treated with 1% Na CMC [~2 ml/kg/day, p.o.]

Group 2: Rats treated with EAAS (200 mg/kg/day, p.o.)

Group 3: Rats treated with AQAS (200 mg/kg/day, p.o.)

Group 4: Rats treated with EtAS (200 mg/kg/day, p.o.)

Group 5: Rats treated with 1% Na CMC [~2 ml/kg/day, p.o.] and then ISO (200mg/kg/day; for two days, s.c.)

Group 6: Rats pretreated with EAAS (100 mg/kg/day, p.o.) and then ISO (200mg/kg/day; for two days, s.c.)

Group 7: Rats pretreated with AQAS (100 mg/kg/day, p.o.) and then ISO (200mg/kg/day; for two days, s.c.)

Group 8: Rats pretreated with EtAS (100 mg/kg/day, p.o.) and then ISO (200mg/kg/day; for two days, s.c.)

Group 9: Rats pretreated with EAAS (200 mg/kg/day, p.o.) and then ISO (200mg/kg/day; for two days, s.c.).

Group 10: Rats pretreated with AQAS (200 mg/kg/day, p.o.) and then ISO (200mg/kg/day; for two days, s.c.).

Group 11: Rats pretreated with EtAS (200 mg/kg/day, p.o.) and then ISO (200mg/kg/day; for two days, s.c.)

f) Measurement of ECG

At the end of experimental period (after 24 hr of second ISO injection or 16th day of extract/vehicle treatment) the rats were anaesthetized with light anaesthetic ether and ECGs were recorded using computerized data acquisition system (Biopac MP 35, Santa Barbara, California). Recordings were made on the bi-polar standard lead-I, lead-II and lead- III. In all cases of myocardial infarction, Lead II show the clear, distinct individual waves than Lead I & III. Therefore, ECG was monitored on Lead II only.

g) Biochemical analysis

After recording the ECG, blood was collected from retro-orbital plexus, serum was separated for estimation of marker enzymes. The activities of SGOT, SGPT Lactate dehydrogenase (LDH), Creatine kinase (CK), glucose, triglycerides (TG) and total cholesterol were measured by using standard kits (Erba diagnostics,Germany).

h) Histopathological Studies

The hearts were removed, washed immediately with saline and then fixed in 10% buffered formalin. The hearts, stored in 10% buffered formalin, were embedded in paraffin, sections cut at 5 μm and stained with hematoxylin and eosin. These sections were then examined under a light microscope for histological changes.

i) Statistical analysis

The data were expressed as Mean ± S.E.M for six rats in each group. Statistical comparisons were performed by one-way ANOVA followed by Tukey's post-test using Graph Pad Prism version 4.0, USA.

5.11.4.3 Results

a) Effect of *Argyreia speciosa* on different ECG parameters

Fig. 5.10 represents the electrocardiographic pattern of control & experimental animals. Normal control and different doses of EtAS, EAAS and AQAS (100 and 200 mg/kg) alone treated rats showed a normal ECG pattern, where as animals treated with ISO alone showed significant elevation in ST segment, reduction in P wave, QRS complex and R-R interval. In addition there was an increase in heart rate, prolongation of QT interval and cardiac cycles compared to normal control animals. Pretreatment of EtAS, EAAS and AQAS (200 mg/kg) for 14 days and two doses of ISO (200 mg/kg) administered rats exhibited normal ECG pattern with a slight elevation in ST segment. Furthermore, treatment also resulted in significant ($P<0.001$) increase in P wave, QRS complex and R-R interval, whereas heart rate, QT interval and cardiac cycle were maintained near to normal values. The data of the experimental animals such as P wave, QRS complex, QT interval, R-R interval, heart rate & cardiac cycle are represented in Table 5.30.

b) Effect of *Argyreia speciosa* on serum marker enzymes

ISO treated rats exhibited significantly ($P<0.001$) higher levels of serum myocardial injury marker enzymes such as AST (195.2±7.04), ALT (120.6±8.24U/L), LDH (1924±191.2) CK (713.8±21.10), glucose (137.0±2.34), triglycerides(119.0±3.24) and total cholesterol (77.00±3.80) compared to normal control rats (Table 5.29). Pretreatment with EtAS, EAAS (200 mg/kg) for 14 days and ISO (200 mg/kg, for two days) administration showed significant ($P<0.05$; $P<0.001$) reduction in all the tested diagnostic markers. However there was no change in any of these marker enzyme levels

in EtAS (200 mg), EAAS (200mg) and AQAS (200 mg) alone treated group as compared to normal control group.

c) Histopathological findings

Histopathological examination of myocardial tissue obtained from normal control animals and animals treated with *A. speciosa* fractions depicted clear integrity of myocardial membrane and an infiltration of inflammatory cells were not seen in these experimental groups (Fig. 5.9.1.). The histological sections obtained from the hearts of animals receiving ISO alone (Fig. 5.9.2) shows various degrees of focal lesions in many sections consisting of molten staining, fragmentation of muscle fibers with confluent retrogressive lesions were observed. In addition marked sequestering mucoid oedema and vacuolar changes along with hyaline necrosis were clearly visible in ISO treated rats. Pretreatment with EtAS, EAAS and AQAS (200 mg/kg p.o.) respectively demonstrated marked improvement in ISO-induced alterations such as vacuolar changes, edema, capillary dilatation and leukocyte infiltration compared to ISO administered group (Fig. 5.9.3 to 5.9.5).

5.11.4.4 Discussion

Oxidative stress is one of the major concerns in the treatment of ischemic heart diseases. There is ample evidence for a detrimental role of ROS in cardiovascular disease[14,15]. Isoproterenol is well known cardiotoxic agent due to its ability it will destruct myocardial cells. As a result of this, cytosolic enzymes such as lactate dehydrogenase (LDH), transaminases (ALT, AST) and creatine kinase (CK) were released into blood stream and serve as the diagnostic markers of myocardial tissue damage[16,17]. The amount of these cellular enzymes present in blood reflects the alterations in plasma membrane

integrity and/or permeability. Drug treatments such as naringin, silibinin, and squalene evidenced by a decline in lactate dehydrogenase, glutamic oxalacetic transaminase and creatine kinase levels indicated their membrane stabilizing action [16, 18,19].

In the present study, ISO treated rats showed significant elevation in the levels of these diagnostic marker enzymes (AST, ALT, LDH and CK). Moreover, elevated levels of these enzymes are an indicator of the severity of ISO-induced myocardial membrane necrosis, which is in line with an earlier report[16,17]. The prior administration of EAAS and EtAS (200 mg/kg p.o) showed significant reduction in ISO-induced elevated serum marker enzymes. This reduction in enzyme levels could be due to its action on maintaining membrane integrity thereby restricting the leakage of these enzymes. It is well known that isoproterenol-induced myocardial injury is mediated primarily via the β1-adrenergic receptor. Acute β-adrenergic receptor stimulation not only rapidly generates reactive oxygen species, but also depresses total cellular antioxidant capacity, down regulates copper–zinc superoxide dismutase enzyme activity, protein and mRNA, and reduces glutathione level, leading to the loss of membrane integrity and inducing heart contractile dysfunction and myocyte toxicity finally producing myocardial necrosis[20,21]. In the present study, we found that *A. speciosa* fractions protected myocardium from isoproterenol-induced myocardial functional and structural injury via normalization levels of diagnostic marker enzymes.

Current epidemiological evidence suggest that inadequate intake of certain nutrients predispose humans to chronic degenerative diseases[22]. In particular it was demonstrated that intake of an adequate diet rich in vegetable and fruit reduces the likelihood of cardiovascular diseases, but the exact mechanisms for this protective effect are inadequately understood. However, increased circulating antioxidants are believed to be important. This is supported by recent trials reporting that the intake of antioxidant

flavonols predicts a reduced rate of coronary-heart disease mortality in elderly male: in particular, those epidemiological studies show that dietary intake of flavanoids (quercetin, catechin and epicatechin), notably present in red wine but also in fruits and vegetables) is inversely associated with subsequent coronary heart disease[23,24]. This effect seems to be in part related to their antioxidant activity. Therefore, the observed myocardial protective effect of EAAS and EtAS could be due to the flavanoids such as quercetin, kaempferol and coumarin like scopoletin[25 26,] which are known free radical scavengers.

Electrocardiograph-abnormalities are the main criteria generally used for the definite diagnosis of myocardial infarction. ST-segment elevation was observed either in patient with acute myocardial ischemia[27] or in isoproterenol-induced myocardial infarction in rat[28]. The study shows significant alterations of ECG patterns in ISO administered rats as compared to normal control rats. The characteristic findings were reductions in the P wave intensity, QRS complex, R-R intervals, QT interval and prolongation of cardiac cycle. We also observed a significant elevation in the ST segment and increase in heart rate. These alterations could be due to the consecutive loss of cell membrane in injured myocardium[29]. In the present study, we observed an elevation of ST-segments in isoproterenol-induced rat, and pretreatment with *A.speciosa* fractions markedly inhibited isoproterenol-induced ST-segment elevation suggestive of its cell membrane protecting effects. The appearance of Q wave & ST segment elevation are some of the indicative signs of ischemia. In the present study we did not observe pathological Q wave due to conditions of ischemia. The prominent Q wave were seen only on severe ischemia, infarction and in patients with severe heart diseases. The consecutive loss of cellular membrane damage due to oxidative stress might be

characterized by ST elevation[30,31]. EAAS, and EtAS (200mg/kg p.o) administration showed a protective effect against ISO-induced altered ECG pattern and eliminated the acute fatal complications by protecting the cell membrane damage.

Electrocardiograph and biochemical findings were further confirmed by histopathological studies. Histopathological examination of myocardial tissue in control depicted clear integrity of the myocardial cell membrane. No inflammatory cells infiltration was seen in the rat heart of normal control. In ISO administered group, focal lesions in many sections consisting of molted staining and fragmentation of muscle fibres with confluent retrogressive lesions, hyaline necrosis, sequestering mucoid edema were observed. Pretreatment with *A. speciosa* EtAS and EAAS fractions at 200mg/kg demonstrated reversal of focal lesions, fragmentation of muscle fibres and retrogressive lesions with hyaline necrosis seen with ISO treated group. Inflammatory cells were seen with reduced density in EAAS, and EtAS treated group confirming further the cardioprotective activity exerted by *A. speciosa*. However EAAS and EtAS (200mg/kg p.o.) treated normal rats had no toxic effects on cardiac architecture. Higher dose of isoproterenol induce subendocardial ischemia, hypoxia, necrosis, and finally fibroblastic hyperplasia with decreased myocardial compliance and inhibition of diastolic and systolic function, which closely resembles local myocardial infarction-like pathological changes seen in human myocardial infarction[32]. In the present study, we found that *A. speciosa* root fractions protected myocardium from isoproterenol-induced myocardial functional and structural injury. The data of the present study clearly showed that these fractions modulated most of the electrophysiological, biochemical and histopathological parameters were maintained to normal status in isoproterenol rats, suggesting the beneficial action of *A. speciosa* as a cardioprotective agent.

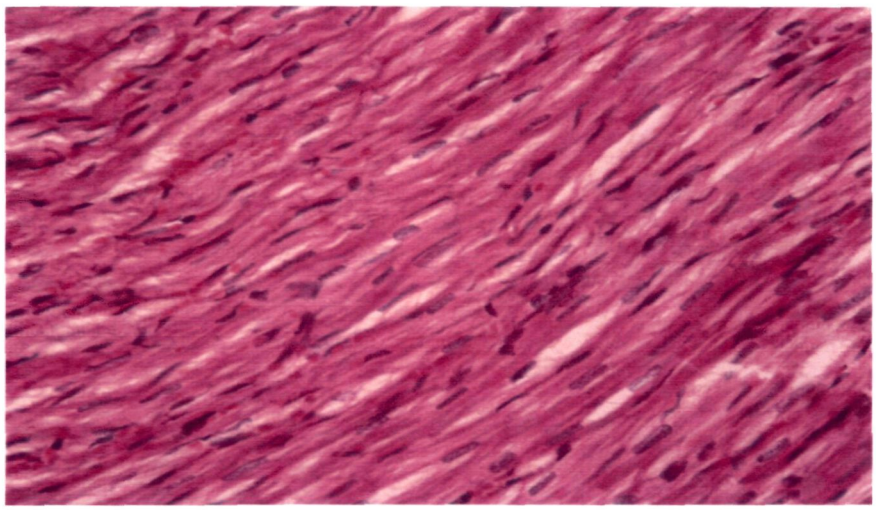
Table 5.29. Effect of *Argyreia speciosa* root fractions on serum biochemical parameters in Isoproterenol induced myocardial infracted rats

Group	Dose mg/kg (p.o., s.c.)	SGPT IU/L	SGOT IU/L	LDH IU/L	CK IU/L	Glucose mg/dl	TG mg/dl	TC mg/dl
Normal control	2 ml/kg	66.40±10.04	97.31±9.437	1021±27.34	389.8±11.74	88.75±2.955	42.25±4.76	37.25±4.51
EAAS	200	63.75±10.52	106.8±5.67	1039±55.66	386.5±5.107	92.00±6.48	96.25±3.25	31.75±3.32
AQAS	200	52.20±6.55	105.3±5.36	995.3±51.69	390.8±5.313	187.3±25.78	322.5±12.61	56.50±6.19
EtAS	200	52.20±6.55	121.5±10.05	836.3±28.10	363.5±17.04	91.50±4.73	176.8±39.94	41.25±2.78
ISO	200	120.6±8.24	195.2±7.04	1924±191.2	713.8±21.10	137.0±2.34	119.0±3.24	77.00±3.80
ISO+ EAAS	200 100	93.20±2.95	128.3±7.07^{c}	1786±34.82^{a}	674.0±43.21^{b}	127.5±4.62^{a}	22.25±4.55^{c}	40.75±5.77^{b}
ISO+ AQAS	200 100	93.50±5.26	93.75±7.29	1883±137.4	545.5±28.03	120.3±3.09	18.20±4.62	48.00±4.50
ISO+ EtAS	200 100	79.75±5.51^{a}	104.8±8.35^{c}	1535±84.30^{a}	591.3±54.80^{b}	99.00±3.67^{a}	20.75±3.902^{c}	42.50±5.23^{a}
ISO+ EAAS	200 200	70.20±9.10^{b}	130.3±10.1^{c}	1139±204.8^{b}	658.3±15.93^{c}	105.5±7.75^{b}	31.14±3.96^{c}	57.60±3.26^{c}
ISO+ AQAS	200 200	77.60±4.70	145.8±7.66	2441±87.26^{b}	517.0±42.59	108.0±8.38	52.96±15.21	67.60±3.20
ISO+ EtAS	200 200	76.80±9.383^{b}	130.3±10.19^{c}	1229±204.7^{b}	545.3±26.05^{c}	100.0±4.56^{b}	89.80±3.90^{b}	65.40±2.42^{b}

Values are mean ± S.E (n=6), ^{a} P<0.05, ^{b} P< 0.01, ^{c} P<0.001 compared to Isoproterenol treated group.

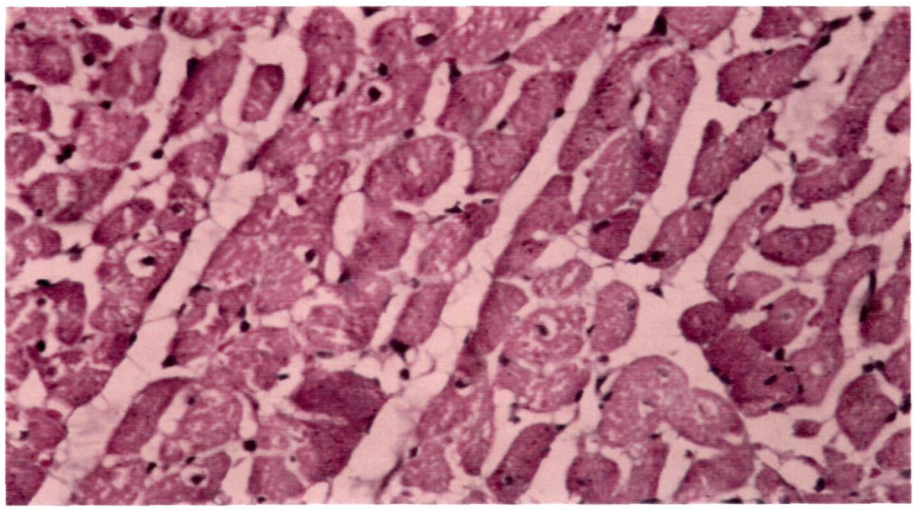
Fig. 5.9. Effect of different fractions of *Argyreia speciosa* on histopathological changes in rat myocardial tissue [H & E stain, X100]

Fig. 5.9.1. Normal control



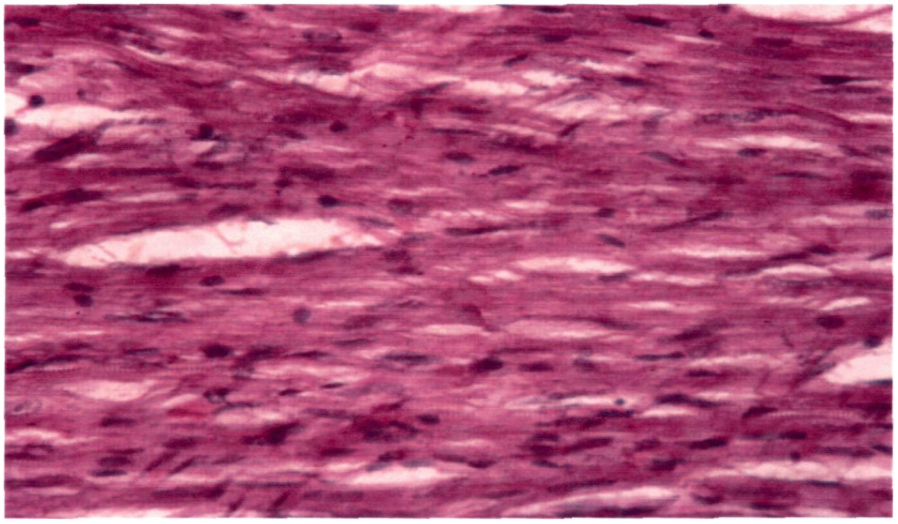
Myocardial tissue with normal features

Fig. 5.9.2. Isoproterenol induced heart



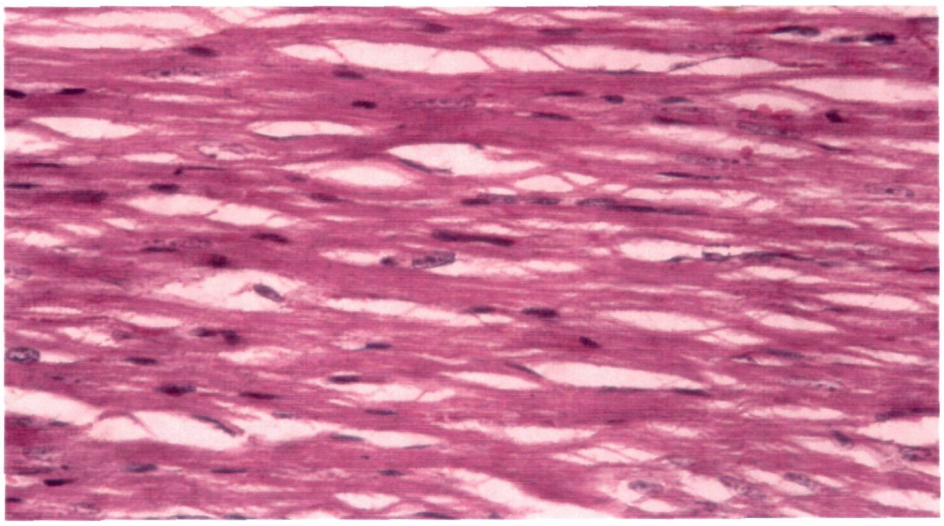
Myocardial tissue showing focal necrosis, oedema, sweeling and deformation of fibres

Fig. 5.9.3. EtAS (200 mg/kg) treated group



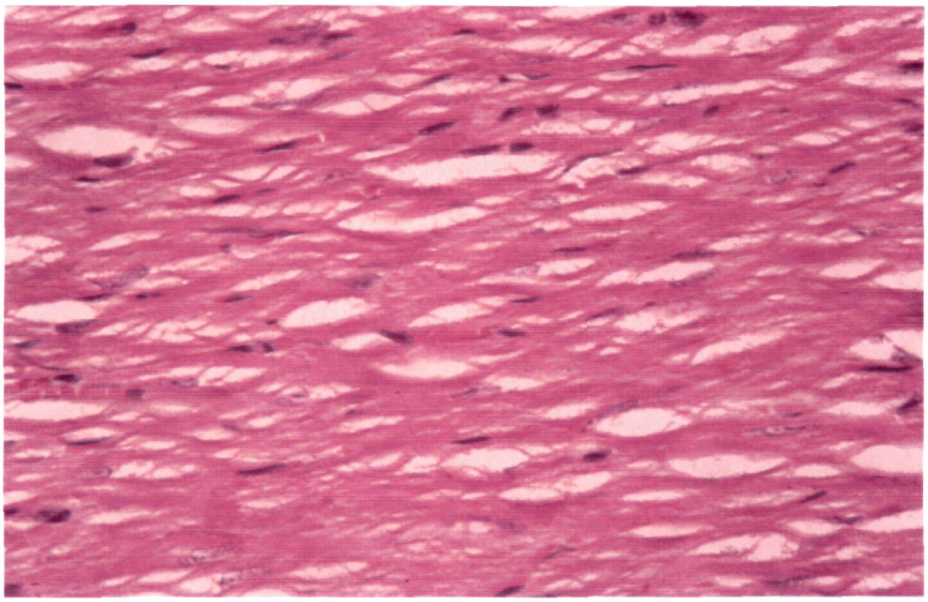
Pretreatment with EtAS (200 mg/kg) showing marked improvement in ISO-induced alterations on myocardial tissue

Fig. 5.9.4 EAAS (200 mg/kg) treated group



Pretreatment with EAAS (200 mg/kg) showing marked improvement in ISO-Induced alterations on myocardial tissue

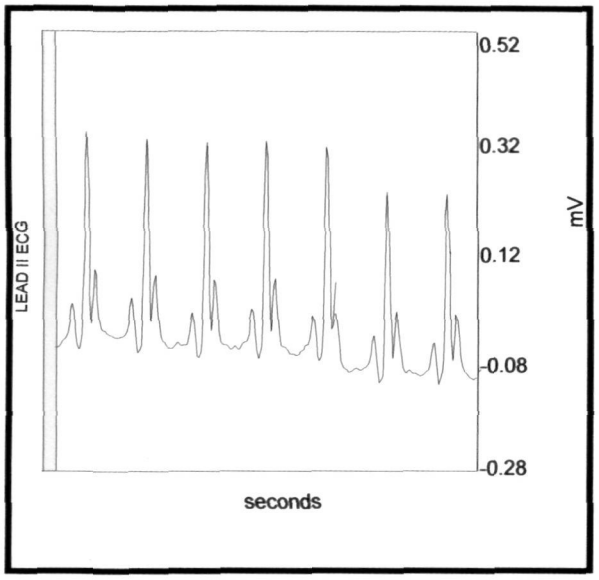
Fig. 5.9.5. AQAS (200 mg/kg) treated group



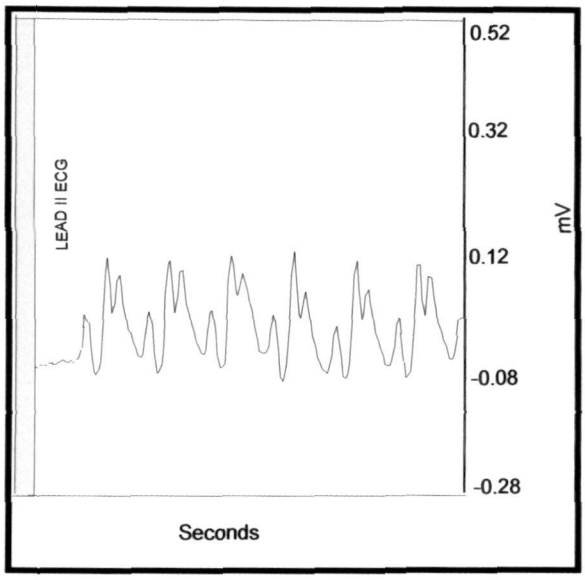
Pretreatment with AQAS (200 mg/kg) showing marked improvement in ISO-Induced alterations on myocardial tissue.

Fig. 5.10. Effect of *Argyreia speciosa* root fractions on different ECG parameters in ISO-induced myocardial infarction in rats

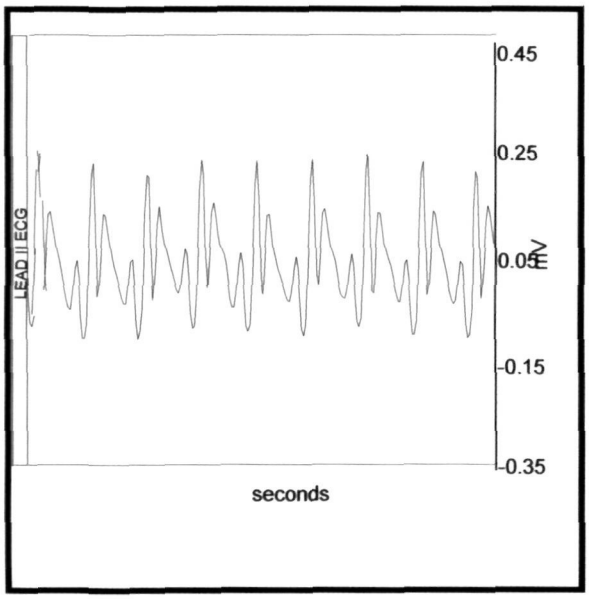
Normal control



ISO treated



EtAS (200 mg/kg)



EAAS (200 mg/kg)

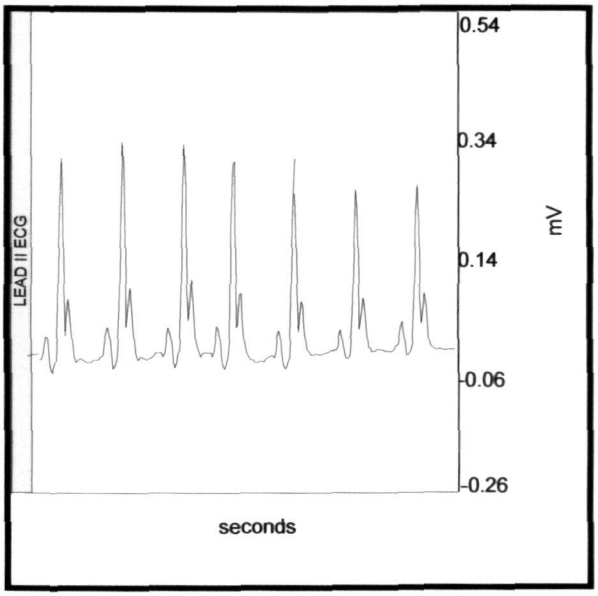


Table 5.30. Effect of *Argyreia speciosa* root fractions on different ECG parameters in ISO-induced myocardial infarction in rats

Treatment	P Wave	QRS Complex	QT Interval	R-R Interval	Heart Rate	Cardiac Cycle
Normal control	0.03750 ±0.0014[a]	0.04292±0.0009[a]	0.08500±0.0033[b]	0.1879±0.0033[b]	321.2±5.87[b]	0.1213±0.0037[b]
EtAS(200 mg/kg,p.o)	0.03756 ±0.0012	0.04132±0.0007	0.0834±0.0032	0.1800±0.0079	325.3±19.25	0.1203±0.0019
EAAS (200mg/kg,p.o)	0.03889 ±0.0011	0.04278±0.0008	0.08278±0.0031	0.1837±0.0086	328.0±17.61	0.1200±0.0029
ISO (200 mg/kg, s.c)	0.03278 ±0.0009	0.03833±0.0014	0.09944±0.0019	0.1367±0.0030	417.9±6.90	0.1361±0.0033
ISO + EtAS (200 mg/kg,p.o)	0.03467 ±0.0089	0.03963±0.0011	0.08713±0.0012[a]	0.1215±0.0021	378.0±8.41	0.1284±0.0038
ISO + EAAS (200 g/kg,p.o)	0.03833 ±0.0014[a]	0.04250±0.0012[a]	0.08233±0.0016[a]	0.1551±0.0052[a]	365.0±9.35[b]	0.1221±0.0040[a]

Values are expressed as mean ± S.E.M for 6 animals in each group. The ECG parameters are expressed in seconds (sec) and the Heart rate as Beats Per Minute (B.P.M). [a] $P<0.05$, [b]$P<0.01$ when compared with ISO treated group.

5.11.4.5. References

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5.11.5 Antidiabetic activity of *Argyreia speciosa* (sweet) (Burm.f.).Boj. in normoglycemic and streptozotocin induced diabetes in rats

5.11.5.1 Introduction

Diabetes is defined as a state in which homeostasis of carbohydrate and lipid metabolism is improperly regulated by insulin. This results primarily in elevated fasting and postprandial blood glucose levels. If this imbalanced homeostasis does not return to normalcy and continues for a protracted period of time, it leads to hyperglycemia that in due course turns into a syndrome called Diabetes Mellitus[1].

The three main types of diabetes are:

- Type 1 diabetes
- Type 2 diabetes
- Gestational diabetes

a) Type 1 diabetes

Type 1 diabetes commonly called insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes. Type 1 diabetes is an autoimmune disease. An autoimmune disease results when the body's system for fighting infection (the immune system) turns against a part of the body. In diabetes, the immune system attacks and destroys the insulin-producing beta cells in the pancreas. The pancreas then produces little or no insulin. A person who has typel diabetes must take insulin daily to live. At present, it is not know exactly what causes the body's immune system to attack the beta cells, but it is believed that autoimmune, genetic, and environmental factors, possibly viruses, are involved. It develops most often in children and young adults but can appear at any age. Symptoms of type 1 diabetes usually develop over a short period, although beta cell

destruction can begin years earlier. Symptoms may include increased thirst and urination, constant hunger, weight loss, blurred vision, and extreme fatigue. If not diagnosed and treated with insulin, a person with type 1 diabetes can lapse into a life-threatening diabetic coma, also known as diabetic ketoacidosis.

b) Type 2 Diabetes

Type 2 diabetes is commonly called non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes. The most common form of diabetes is type 2 diabetes. About 90 to 95 percent of people with diabetes have type 2. This form of diabetes is most often associated with older age, obesity, family history of diabetes, previous history of gestational diabetes, physical inactivity, and certain ethnicities. About 80 percent of people with type 2 diabetes are overweight. Type 2 diabetes is increasingly being diagnosed in children and adolescents. When type 2 diabetes is diagnosed, the pancreas is usually producing enough insulin, but for unknown reasons the body cannot use the insulin effectively, a condition called insulin resistance. After several years, insulin production decreases. The result is the same as for type 1 diabetes: glucose builds up in the blood and the body cannot make efficient use of its main source of fuel. The symptoms of type 2 diabetes develop gradually. Their onset is not as sudden as in type 1 diabetes. Symptoms may include fatigue, frequent urination, increased thirst and hunger, weight loss, blurred vision, and slow healing of wounds or sores. Some people have no symptoms.

c) Gestational Diabetes

Some women develop gestational diabetes late in pregnancy. Although this form of diabetes usually disappears after the birth of the baby, women who have had gestational diabetes have a 20 to 50 percent chance of developing type 2 diabetes within

5 to 10 years. Maintaining a reasonable body weight and being physically active may help prevent development of type 2 diabetes. About 3 to 8 percent of pregnant women develop gestational diabetes. As with type 2 diabetes, gestational diabetes occurs more often in some ethnic groups and among women with a family history of diabetes. Gestational diabetes is caused by the hormones of pregnancy or a shortage of insulin. Women with gestational diabetes may not experience any symptoms[2].

In contrast to substitution therapy with insulin in IDDM, therapy for type-2 diabetes relies mainly on several approaches intended to reduce the hyperglycemia itself. Although diet and exercise are the first steps toward achieving treatment goals of diabetics, 90% of patients with diabetes cannot maintain long term glycemic control with diet and exercise alone. Thus, antihyperglycemic drugs are necessary for the treatment of diabetes[3].

It is well known that treatment with insulin preparation has many disadvantages viz., needed constant refrigeration, insulin shock, revert hypoglycemia, allergic reactions, lipodystrophy, and gain in weight. Moreover, in the treatment of type 2 diabetes, drugs such as sulfonylureas (insulin secretagogues), metformin (acts to reduce hepatic glucose production) and acarbose (interferes with glucose absorption and metabolism) TZD (improve sensitivity to insulin) were used. These oral hypoglycemics are also not free from adverse effects such as gastrointestinal disturbances, blood dyscrasia, hepatic dysfunction, cardiotoxicity, hypoglycemia, etc. In view of one or the other adverse effects of insulin and oral hypoglycemics, search continues to develop newer agents for the treatment of diabetes mellitus. Plant derivatives with hypoglycemic properties have been used in folk medicine and traditional healing systems around the world from very ancient time.

5.11.5.2. The impact of Diabetes

The global prevalence of diabetes is estimated to be 2.8% in 2000 and 4.4% in 2030. The total number of Diabetes is projected to rise from 171 million in 2000 to 366 million in 2030. Diabetes is a major threat to global public health that is rapidly getting worse and also it is a life threatening condition, at least one in 20 deaths is attributed to diabetes and 8,700 deaths every day, six deaths every minute. Moreover, it is a common condition and its frequency is dramatically rising burden all over the world. Medication often has an important role to play particularly for the control of blood glucose and lipids profiles[5]. According to recent estimates, the human population worldwide appears to be in the midst of an epidemic of diabetes. Despite the great strides that have been made in the understanding and management of diabetes, the disease and disease-related complications are increasing. Parallel to this, recent developments in understanding the pathophysiology of the disease process have opened up several new avenues to identify and develop novel therapies to combat the diabetic plague. Concurrently, phytochemicals identified from traditional medicinal plants are presenting an exciting opportunity for the development of new types of therapeutics. This has accelerated the global effort to harness and harvest those medicinal plants that bear substantial amount of potential phytochemicals showing multiple beneficial effects in combating diabetes and diabetes-related complications. Therefore, as the disease is progressing unabated, there is an urgent need of identifying indigenous natural resources in order to procure them, and study in detail, their potential on different newly identified targets in order to develop them as new therapeutics[6].

External insulin preparations can be used to treat type 1 diabetes mellitus as well as in some of the type 2 diabetes mellitus patients. The oral hypoglycemic agents like

Sulfonylureas (Tolbutamide Glibenclamide, Glipizide), *Biguanides* (Phenformin, Metformin), *Thiazolidinediones* (Troglitazone, Rosiglitazone), *α-Glucosidsase inhibiotrs* (Acarbose, Miglitol) have been extensively used in type-2 Diabetes mellitus. But drawback of insulin and oral hypoglycemic agents is, none of them are ideal in correcting blood glucose levels round the clock, with single dose administration. In large doses, these agents cause hypoglycemia, while, in smaller doses, unchecked hyperglycemia and both these extremes are equally life threatening. Moreover, it has been clearly established that insulin and sulfonylureas, even with excellent glycemic control do not normalise diabetes dyslipidemia or improve hypertension[7].

In the Indigenous system of medicine like Ayurveda, many herbal medicines have been recommended for the treatment of diabetes or madhumeha and some of them experimentally evaluated [8]. In recent years, there has been renewed interest in the treatment against different diseases using herbal drugs as they are generally non-toxic and WHO has also recommended the evaluation of the effectiveness of plants in condition where we lack safe modern drugs. Researches conducted in last few decades on plants mentioned in ancient literature or used traditionally for diabetes have shown anti-diabetic property. Many pants like *Allium cepa, Allium sativum, Aloe vera, Cajanus cajan, Coccinia indica, Caesalpinia bonducella, Ficus bengalenesis, Gymnema sylvestre, Momordica charantia, Ocimum sanctum, Pterocarpus marsupium, Swertia chirayita, Syzigium cumini, Tinospora cordifolia,Trigonella foenum graecum, M. charantia, Eugenia jambolana, Mucuna pruriens, T. cordifolia, T. foenum graecum, Murraya koeingii Tapinanthus butungii and Brassica juncea.* All these plants have shown varying degree of hypoglycemic and anti-hyperglycemic activity.

Due to paucity of scientific information regarding the effect of *Argyreia speciosa* on blood glucose and lipid levels, the present study is planned to investigate the hypoglycemic and hypolipidemic activity in normoglycemic and diabetic animals.

5.11.5.3 Materials and Methods

a) Drugs and Chemicals

Streptozotocin (STZ) chemical name 2 deosyl-2-(3-methyl-3-nitroso urea) -1-d-glucopyranose (commonly known (N-Methylnitroso carboxyl)-D glucose C_8 $H_{15}N_3O_7$ Mol.wt 265.2 CAS No- 18883-66-4 Min Assay 98% PurchEtASd from HI-Media Mumbai. **Insulin** injection biphasic isophane, Ph. Eur. Human mixtard manufactured by Torrent Pharmaceuticals, Ltd Indrad. Standard drug **Glidenclamide 5mg** (Daonil) obtained from Avents Pharma, Ltd Goa. **Acarbose** (glucobay) Bayer Pharma, Pvt Ltd. Tragacanth, Glucose, Sucrose, Sodium Chloride, Potassium Chloride, Calcium Chloride, Magnesium Chloride, Sodium Bicarbonate, Sodium Hypo Phosphate, Citric acid, Trisodium Citrate, Hi-Media Laboratories, Ltd Mumbai India. Standard kits for Glucose, Triglycerides, Cholesterol, and HDL-Cholesterol were obtained from Erba Mannheim, Manufactured by Transasia Biomedical Ltd Baddi, India.

b) Preparation of drugs

Weighed quantity of ethanol (EtAS) and water (AQAS) extracts of *Argyeria Speciosa* were suspended in water using 0.5% tragacanth and administered orally to experimental animals. Suspension of extract was prepared freshly. The extracts were administered at a constant volume of ~10 ml/kg for each animal.

c) Animals

Young adult male Wistar rats 7-8 weeks old, weighing 150-200g were obtained from inbred animal house Venkateshwara enterprises, Bangalore, Karnataka. The animals were housed in polypropylene cages in standard environmental conditions, 12 hr light

and 12 hr dark cycle at 25 ± 2 °C. Before and during the experiments, the rats were fed with standard laboratory pellet diet and water *ad libitum* obtained from Venkateshwara enterprises, Bangalore. Animals were acclimatized to the laboratory condition for at least 15 days prior to the experiment and were maintained in a well ventilated animal house. The experimental protocol was approved by the Institutional animal Ethical Committee (IAEC) animals and the care of the laboratory was taken as per the CPCSEA regulation.

d) Effect of crude fractions in normoglycemic rats

i) Oral Glucose Tolerance Test (OGTT) in normal rats

The experimental rats were divided into six groups of five rats each and treated as follows.

Group 1: Normal control (NC) received 0.5% tragacanth (10 ml/kg, p.o.)

Group 2: NC rats treated with EtAS (100 mg/kg, p.o.)

Group 3: NC rats treated with EtAS (200 mg/kg, p.o.)

Group 4: NC rats treated with AQAS (100 mg/kg, p.o.)

Group 5: NC rats treated with AQAS (200 mg/kg, p.o.)

Group 6: NC rats treated with glibenclamide (10 mg/kg, p.o.)

Different doses of EtAS/AQAS/glibenclamide (GLB) and vehicle were given orally to normoglycemic rats fasted for 18 hr. Thirty minutes later, glucose (2 g/kg in distilled water) was administered orally. Blood samples were collected from the retro-orbital plexus at 0 min (i.e. immediately after glucose load), 30, 60 and 120 mins after glucose administration. Serum glucose (SG) was estimated by the enzymatic glucose oxidEtAS method using diagnostic reagent kit. The results are expressed as integrated area under curve for glucose ($AUC_{glucose}$) calculated by trapezoid rule.

$$AUC_{glucose} = \frac{(C_1 + C_2)}{2} X (t_2 - t_1)$$

ii) Experimental design for Multiple-dose fifteen-day study in Normoglycemic rats

The animals treated with respective doses of EtAS, AQAS and GLB were further treated for fifteen consecutive days **[Multiple-dose fifteen-day study]**. The following groups of animals were further treated with single daily doses for another 15 days in order to evaluate the chronic effect of extracts/GLB treatment

Group 1: Normal control (NC) received 0.5% tragacanth (10 ml/kg, p.o./day)

Group 2: NC rats treated with EtAS (100 mg/kg, p.o./day)

Group 3: NC rats treated with EtAS (200 mg/kg, p.o./day)

Group 4: NC rats treated with AQAS (100 mg/kg, p.o./day)

Group 5: NC rats treated with AQAS (200 mg/kg, p.o./day)

Group 6: NC rats treated with glibenclamide (0.5 mg/kg, p.o./day)

iii) Oral Sucrose Tolerance Test (OSTT) in normal rats

The experimental rats were divided into six groups of five rats each and treated as follows.

Group 1: Normal control (NC) received 0.5% tragacanth (10 ml/kg, p.o.)

Group 2: NC rats treated with EtAS (100 mg/kg, p.o.)

Group 3: NC rats treated with EtAS (200 mg/kg, p.o.)

Group 4: NC rats treated with AQAS (100 mg/kg, p.o.)

Group 5: NC rats treated with AQAS (200 mg/kg, p.o.)

Group 6: NC rats treated with Acarbose (7 mg/kg, p.o.)

Different doses of EtAS/AQAS/Acarbose and vehicle were given orally to normal rats fasted for 18 hr. Thirty minutes later, sucrose (2 g/kg in distilled water) was administered orally. Blood samples were collected from the retro-orbital plexus at 0 min

(i.e. immediately after sucrose load), 30, 60 and 120 mins post sucrose administration. SG was estimated by the enzymatic glucose oxidase method using diagnostic reagent kit. The results are expressed as integrated area under curve for glucose ($AUC_{glucose}$) calculated by trapezoid rule using formula as mentioned above [9].

e) Evaluation of anti-diabetic effect of EtAS and AQAS in standardized STZ-induced diabetic rats

i) Induction of Diabetes mellitus

Diabetic condition was induced in male Wistar rats by single intravenous injection of STZ (50 mg/kg) [Chosen optimum dose: In house data] after overnight fasting for 12 hr [10]. Rats showing SG level > 200 mg/dl seven days after STZ administration were considered diabetic and included in the study. Diabetic rats were randomized into different groups based on their SG levels.

ii) Experimental design for Single-dose one-day study

The experimental rats were divided into seven groups of five rats each and treated as follows.

Group 1: Normal control (NC) received 0.5% tragacanth (10 ml/kg, p.o.)

Group 2: Diabetic control (DC) received 0.5% tragacanth (10 ml/kg, p.o.)

Group 3: DC rats treated with EtAS (100 mg/kg, p.o.)

Group 4: DC rats treated with EtAS (200 mg/kg, p.o.)

Group 5: DC rats treated with AQAS (100 mg/kg, p.o.)

Group 6: DC rats treated with AQAS (200 mg/kg, p.o.)

Group 7: DC rats treated with Glibenclamide (10 mg/kg, p.o.)

Blood samples were collected at 0, 2 and 4 h after extracts/GLB administration **[single-dose one-day study]**. SG was estimated by the enzymatic glucose oxidase

method. Percent reduction in glycemia was calculated with respect to the initial (0 hr) level by above mentioned formula.

iii) Experimental design for Multiple-dose fifteen-day study

The animals treated with respective doses of EtAS AQAS and GLB were further treated for fifteen consecutive days **[Multiple-dose fifteen-day study]**. The following groups of animals were further treated with single daily doses for another 15 days in order to evaluate the chronic effect of extracts/GLB treatment on hyperglycemia.

Group 1: Normal control (NC) received 0.5% tragacanth (10 ml/kg, p.o./day)

Group 2: Diabetic control (DC) received 0.5% tragacanth (10 ml/kg, p.o./day)

Group 3: DC rats treated with EtAS (100 mg/kg, p.o./day)

Group 4: DC rats treated with EtAS (200 mg/kg, p.o./day)

Group 5: DC rats treated with AQAS (100 mg/kg, p.o./day)

Group 6: DC rats treated with AQAS (200 mg/kg, p.o./day)

Group 7: DC rats treated with glibenclamide (0.5 mg/kg, p.o./day)

iv) Oral glucose tolerance test (OGTT)

On 10^{th} day, glucose tolerance of various groups was estimated by a simple OGTT. Glucose (2 gms/kg) was administered to 12 hr fasted rats and blood samples were collected from the retro-orbital plexus at 0 (before glucose load), 30, 60 and 120 mins after glucose administration. SG was estimated by the enzymatic glucose oxidase method. The results were expressed as integrated area under curve for glucose ($AUC_{glucose}$), which was calculated by trapezoid rule,

$$AUC_{glucose} = \frac{(C_1 + C_2)}{2} X (t_2 - t_1)$$

Also serum insulin was estimated 0 (before glucose load), 30 and 60 mins after glucose administration. Serum insulin (SI) was estimated by radioimmunoassay method using the kit from Bhabha Atomic Research Centre, Mumbai, India. The results were expressed as integrated area under curve for insulin ($AUC_{insulin}$), which was calculated by trapezoid rule.

$$AUC_{insulin} = \frac{(C_1 + C_2)}{2} X (t_2 - t_1)$$

v) Insulin tolerance test (ITT)

Insulin tolerance test is a measure of the extent of peripheral utilization of glucose. On 13th day, insulin (2 U/kg, i.v) was administered to six h-fasted rats. Blood samples were collected from the retro-orbital plexus at 0 (just before insulin load), 10, 20 and 30 mins after insulin injection. SG was estimated by the enzymatic glucose oxidase method. The results were expressed as integrated area under curve for glucose ($AUC_{glucose}$), which was calculated by trapezoid rule using formula as give above.

f) Estimation of biochemical parameters

At the end of the treatment schedule, blood samples were collected from retro-orbital plexus. Serum was separated and analysed spectrophotometrically for triglyceride (STG), total cholesterol (STC), HDL-cholesterol (HDL-c), using diagnostic reagent kit ERBA diagnostics Mannheim GMBH, Germany. Serum insulin (SI) was estimated by radioimmunoassay method using the kit from Bhabha Atomic Research Centre, Mumbai, India. Homeostatic Model Assessment (HOMA) as a measure of insulin resistance was calculated by the following formula.

$$HOMA = \frac{\text{insulin } \mu U/ml \text{ X glucose mmol/L}}{22.5}$$

VLDL-cholesterol (VLDL-c) and LDL-cholesterol (LDL-c) in serum were calculated as per Friedewald's equation.[87]

$$VLDL - c = \frac{Triglyceride}{5}$$

$$LDL - c = Total\ cholestero\ l - \frac{Triglyceride}{5} - HDL - c$$

The markers of dyslipidemia such as TC/HDL-c and LDL-c/HDL-c ratios were also calculated.

g) Glucose uptake by isolated hemi-diaphragm of diabetic rats

i) Isolation of diaphragm

At the end of the study, the overnight fasted experimental rats were killed by cervical dislocation. The diaphragms were dissected out quickly with minimal trauma and divided into two equal halves. The hemi-diaphragms were then rinsed in cold Tyrode solution (without glucose) to remove any blood clots[11]. Two diaphragms of five animals (10 diaphragms) in each group were used for the study.

ii) Experimental design

Seven sets of ten graduated test tubes each, were grouped as follows,

Group 1: 2 ml of Tyrode solution with 2 gm % glucose [Normal control]

Group 2: 2 ml of Tyrode solution with 2 gm % glucose [Diabetic control]

Group 3: 2 ml of Tyrode solution with 2 gm % glucose + insulin (Nova Nordisk) 0.62ml of 0.4 U/ml solution [Insulin treated group]

Group 4: 2 ml of Tyrode solution with 2 gm % glucose + EtAS (50 µg/ml)

Group 5: 2 ml of Tyrode solution with 2 gm % glucose + EtAS (100 µg/ml)

Group 6: 2 ml of Tyrode solution with 2 gm % glucose + AQAS (50 µg/ml)

Group 7: 2 ml of Tyrode solution with 2 gm % glucose + AQAS (100 µg/ml)

The volumes of all graduated test tubes were made upto 4 ml with distilled water and estimated the initial glucose concentration by commercially available glucose kit based on glucose-oxidase method (ERBA diagnostics Mannheim GMBH, Germany). The hemi-diaphragms were placed in test tubes and incubated for 30 min at 37°C in bubbled with oxygen with continuous shaking. Glucose uptake per gram of tissue was calculated as the difference between the initial and final glucose content in the incubated medium [12,13].

h) Statistical analysis

The data were expressed as Mean ± S.E.M for six rats in each group. Statistical comparisons were performed by one-way ANOVA followed by Tukey's post-test using Graph Pad Prism version 4.0, USA.

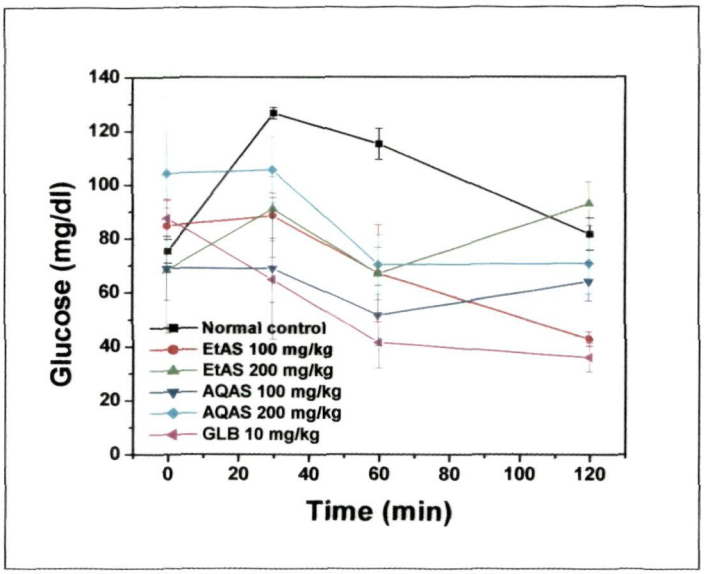
5.11.5.4 Results

A) Effect of extracts in normoglycemic rats

i) Oral Glucose Tolerance Test (OGTT) in normal rats

Administration of glucose (2 gm/kg) produces significant change in SG level of normal rats. Treatment with lower dose of EtAS (100 mg/kg) and AQAS (100 mg/kg) and GLB (10 mg/kg) significantly ($P<0.01$; $P<0.001$) improve the glucose tolerance (Fig. 5.11.1 A & B), whereas, treatment with higher dose of EtAS and AQAS (200 mg/kg) did not significantly reduced the $AUC_{glucose}$ compared to normal control group.

[A]



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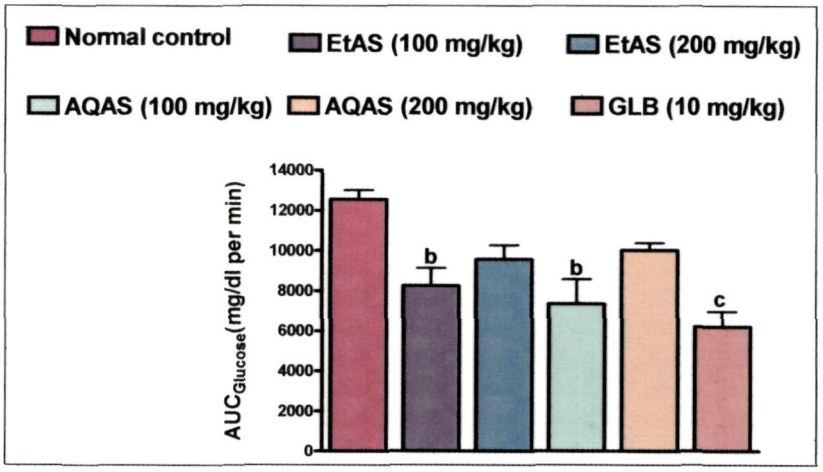


Fig. 5.11.1. Effect of ethanol (EtAS) and water extract (AQAS) of *Argyreia speciosa* on glucose tolerance in fasted normal rats. [A] SG levels were measured prior to, and after p.o. administration of glucose alone (2 gm/kg body weight), or in combination with EtAS, AQAS or Glibenclamide [GLB]. **[B]** Area under curve for glucose ($AUC_{glucose}$) values for 0-120 min post glucose load. Data represent the mean ± S.E.M., for 5 rats. ^{a} $P < 0.05$; ^{b} $P < 0.01$; ^{c} $P < 0.001$ as compared with normal rats (one way ANOVA followed by Tukey's post-test).

ii) Multiple-dose fifteen-day study in Normoglycemic rats

Treatment with EtAS (100 and 200 mg/kg) and AQAS (100 and 200 mg/kg) once daily for fifteen days showed hypoglycemic activity (P<0.05; P<0.01; P<0.001) EtAS (100 mg/kg) showed significant (P<0.05; P<0.01) percentage reduction (15.14% and 30.39%) in SG levels at 7th and 15th days, compared to basal values (0 day) and activity is better than ETAS (200 mg/kg). Whereas, higher dose of AQAS (200 mg/kg) exhibited higher activity than AQAS (100 mg/kg) at all tested days (Fig 5.11.2) (Table 5.31).

Table 5.31. Effect of EtAS and AQAS on Serum glucose levels in normoglycemic rats [Multiple-dose fifteen-day study]

Treatment [dose/kg b.w]	SGL levels [mg/dl]		
	0 day	7th day	15th day
Normal Control	95.32±6.50	92.47±5.15 [1.88]	88.77±5.89 [5.50]
EtAS [100 mg]	90.05±5.31	76.09±3.90 [15.14]	62.04±4.17** [30.39]
EtAS [200 mg]	70.23±2.43	61.54±1.60 [11.98]	57.57±2.99* [17.35]
AQAS [100mg]	85.00±7.20	77.72±5.18 [8.09]	64.31±5.99* [23.54]
AQAS [200 mg]	101.88±2.42	74.55±1.60 [23.60]	66.02±2.99** [33.71]
GLB [0.5 mg]	90.15±3.96	68.40±5.40** [23.57]	56.56±2.25*** [36.70]

Each value represents Mean ± S.E.M., n=5. Values in parentheses indicate Percent reduction in glycaemia and *P<0.05; **P<0.01; ***P<0.001 compared to basal values [0 day] of the same group. One-way ANOVA followed by tukey post-test.

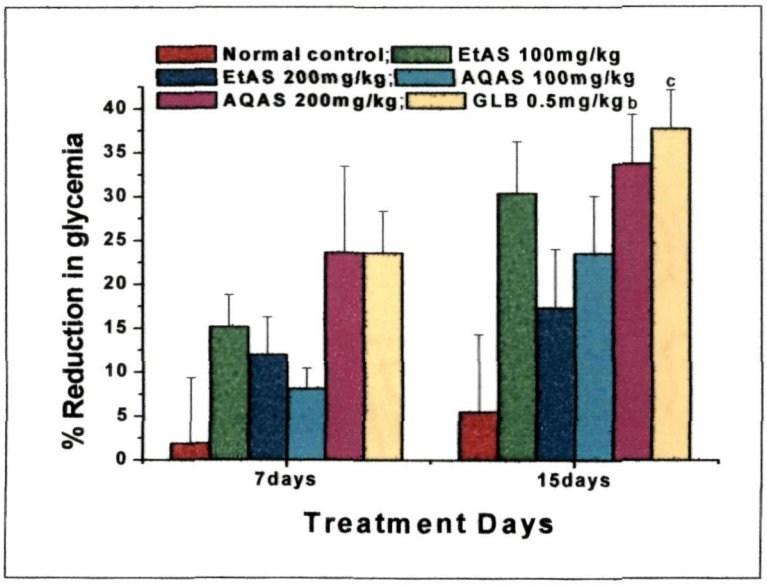


Fig. 5.11.2. Effect of ethanol (EtAS) and water extract (AQAS) of *Argyreia speciosa* in Normoglycemic rats. Bar graph represents the percentage reduction in glycemia with respect to the initial (0 day) level. Each value represents Mean ± S.E.M., n=5. ^{b} P<0.01,compared to normal control of the same time interval. One-way ANOVA followed by Tukey's post-test.

iii) Estimation of Lipid parameter

Treatment with EtAS (100 and 200 mg/kg) and AQAS (100 and 200 mg/kg) once daily for fifteen days showed reduction in tested lipid parameters but activity was not significant (Table 5.32).

Table 5.32. Effect of ethanol (EtAS) and water extract (AQAS) of *Argyreia speciosa* on lipid profile in Normoglycemic rats model (multiple dose-fifteen-day study)

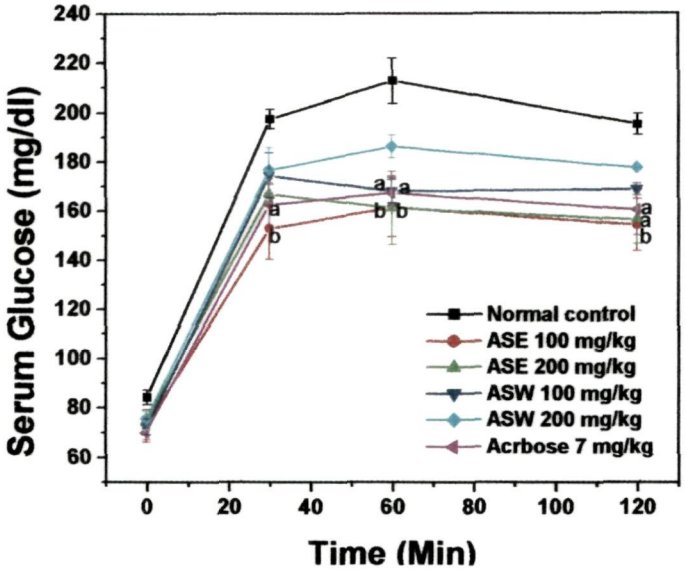
Serum parameter	Normal control	EtAS 100mg/kg	EtAS 200mg/kg	AQAS 200mg/kg	AQAS 100mg/kg	GLB 10mg/kg
STG (mg/dl)	77.91±14.22	52.56±2.95	79.33±8.77	83.63±10.02	54.42±8.41	63.20±15.90
STC (mg/dl)	57.14±6.16	71.28±12.46	60.50±15.13	72.42±5.10	74.69±9.84	53.07±4.70
HDL-c (mg/dl)	16.68±3.67	13.07±1.50	17.80±3.19	16.55±2.06	17.96±1.28	17.15±2.88
VLDL-c (mg/dl)	15.58±2.84	10.51±0.59	15.87±1.75	16.73±2.00	10.88±1.68	12.64±3.18
LDL-c (mg/dl)	24.88±8.18	47.70±11.67	26.83±14.31	39.14±7.01	45.84±9.02	23.28±5.66
TC/HDL -c ratio	3.91±0.92	5.38±0.46	3.86±1.31	1.14±0.57	4.18±0.50	3.39±0.61
LDL-c/HDL- c ratio	1.90±0.75	3.52±0.56	1.89±1.15	2.50±0.65	2.58±0.52	1.58±0.45

Each value represent Mean± S.E.M n=5 $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ compared to normal control. One way ANOVA followed by Tukey's test

iv) Oral Sucrose Tolerance Test (OSTT) in normal rats

Animals subjected to Sucrose (2 gm/kg) load produces significant change in SG level of normal rats. Treatment with different dose of EtAS (100 and 200 mg/kg), AQAS (100 and 200 mg/kg) and Acarbose (7 mg/kg) exhibited significant ($P<0.05$; $P<0.01$) reduction in SG level over the period of 120 min compared to normal control group (Fig. 5.11. 3 A). Further, treatment of EtAS /AQAS/Acarbose significantly ($P<0.05$; $P<0.001$) improve the Sucrose tolerance (Fig 5.11.3 B) suggested that the components of extracts may inhibit α-glucosidase. Moreover, treatment of lower dose of EtAS and AQAS were better than higher dose to improve sucrose tolerance in normal rats.

[A]



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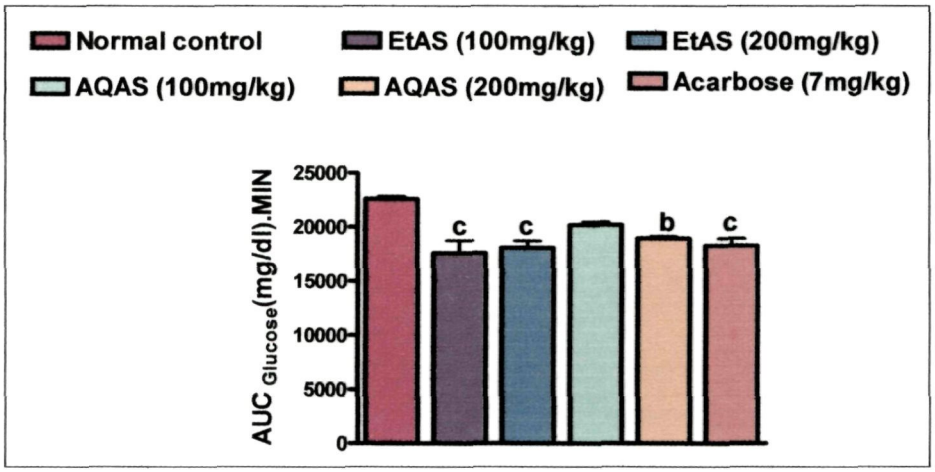


Fig. 5.11.3. Effect of ethanol (EtAS) and water extract (AQAS) of *Argyreia speciosa* on sucrose tolerance in fasted normal rats. [A] SG levels were measured prior to, and after p.o. administration of sucrose alone (2g/kg body weight), or in combination with EtAS, AQAS or Acarbose. [B] Area under curve for glucose ($AUC_{glucose}$) values for 0-120 min post sucrose load. Data represent the mean ± S.E.M., for 5 rats. [a] $P<0.05$; [b] $P < 0.01$; [c] $P < 0.001$ as compared with normal rats (one way ANOVA followed by Tukey's post-test).

B) Evaluation of anti-diabetic effect of EtAS and AQAS in standardized STZ-induced diabetic rats

i) Single-dose one-day study

A single dose of EtAS (100 and 200 mg/kg) and AQAS (100 and 200 mg/kg) treatment exhibited reduction in SG levels at different time intervals compared to basal levels (0 hr). However, administration of GLB showed significant ($P<0.05$; $P<0.001$) reduction is SG levels with maximum reduction (50.94%) at 4 hr post GLB treatment compared to their basal levels, whereas, EtAS treated animals showed dose dependent percentage reduction in SG levels compared to their basal levels (Table 5.33 and Fig. 5.11.4).

Table 5.33. Effect of EtAS AQAS on SGL levels in STZ-induced MD rats [Single-dose one-day study]

Treatment [dose/kg b.w]	SGL levels [mg/dl]		
	0 h	2h	4h
Normal Control	86.38±3.15	87.18±3.31 [-1.0]	86.4±2.8 [-0.1]
Diabetic control (DC)	344.01±29.26	348.5±29.3 [-1.37]	350.65±29.2 [-1.98]
DC+ EtAS [100 mg]	337.5±27.7	311.2±24.2 [7.6]	298.0±20.9 [11.3]
DC+ EtAS [200 mg]	331.7±24.3	277.7±22.8 [17.8]	260.3±26.8 [22.1]
DC+AQAS [100 mg]	282.3±24.0	255.8±14.3 [8.9]	237.0±23.6 [16.1]
DC+AQAS [200 mg]	286.31±19.4	257.0±18.1 [10.8]	253.4±24.2 [13.0]
DC+GLB [10 mg]	342.3±28.3	240.7±18.25* [29.52]	160.04±8.29*** [50.94]

Each value represents Mean ± S.E.M., n=5. Values in parentheses indicate Percent reduction in glycaemia and ***P<0.001, **P<0.01 AND *P<0.05 compared to basal values [0 hr] of the same group. One-way ANOVA followed by Tukey's post test.

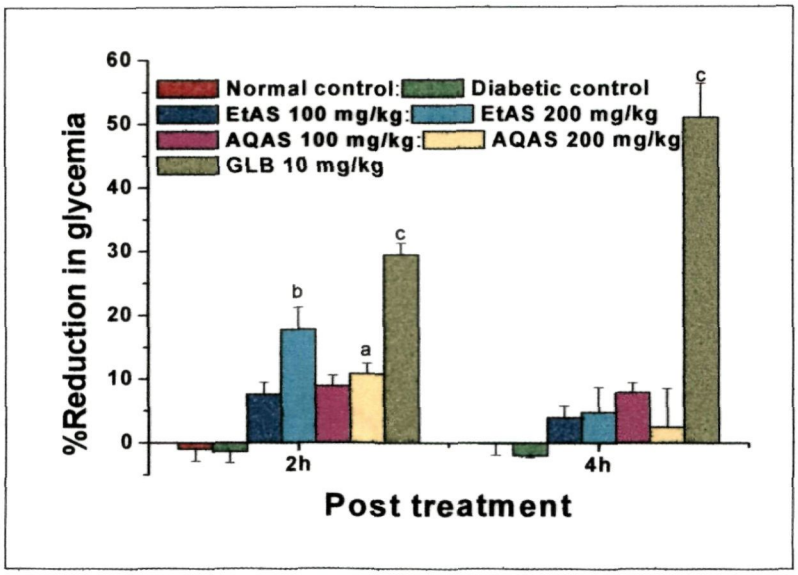


Fig. 5.11.4. Effect of ethanol (EtAS) and water extract (AQAS) of *Argyreia speciosa* on SG levels in STZ-induced rats [Single-dose one-day study]. Bar graph represents the percentage reduction in glycemia with respect to the initial (0 hr) level. Each value represents Mean ± S.E.M., n=5. $^{a}P<0.05$; $^{b}P<0.01$;$^{c}P<0.001$ compared to diabetic control of the same time interval. One-way ANOVA followed by Tukey's post-test.

ii) Multiple-dose fifteen-day study

Repeated administration of EtAS (100 and 200 mg/kg) and AQAS (100 and 200 mg/kg) for 15 days, showed significantly ($P<0.05$; $P<0.01$) reduced levels of SG compared to respective basal values (0 day) (Table 5.34). On 15th day, tested doses of EtAS and AQAS showed significantly ($P<0.001$) greater percentage reduction in glycemia (24.6%: 24.7% and 23.9%:21.9% respectively) compared to diabetic control (Fig. 5.11.5).

Table 5.34. Effect of EtAS AQAS on SG levels in STZ-induced MD rats [Multiple-dose fifteen-day study]

Treatment [dose/kg b.w]	SGL levels [mg/dl]			
	0 day	7th day	10th day	15th day
Normal Control	86.38±3.15	87.95±4.2 [-1.77]	91.45±3.7 [-6.75]	98.98±4.9 [-15.64]
Diabetic control (DC)	344.01±29.6	323.7±18.64 [5.89]	345s.42±15.95 [-0.78]	388.97±12.98 [-14.76]
DC + EtAS [100 mg]	337.5±20.4	265.0±23.6 [20.8]	256.7±19.4 [23.4]	250±14.9* [24.6]
DC+EtAS [200 mg]	331.7±18.5	269.9±18.8 [20.2]	237.4±11.5** [28.5]	243.3±10.5** [24.7]
DC+AQAS [100mg]	282.3±21.5	237.2±9.6 [15.5]	211.1±14.0* [24.8]	210.3±9.5* [23.9]
DC+AQAS [200 mg]	286.31±11.3	240.73±12.1 [17.4]	235.62±10.2* [18.6]	225.6±8.4* [21.9]
DC+GLB [0.5 mg]	342.3±28.3	275.5±8.22 [19.2]	247.9±8.9* [27.57]	195.0±10.4 *** [43.0]

Each value represents Mean ± S.E.M., n=5. Values in parentheses indicate Percent reduction in glycaemia and *P<0.05;**P<0.01; ***P<0.001 compared to basal values [0 day] of the same group. One-way ANOVA followed by Tukey's post test.

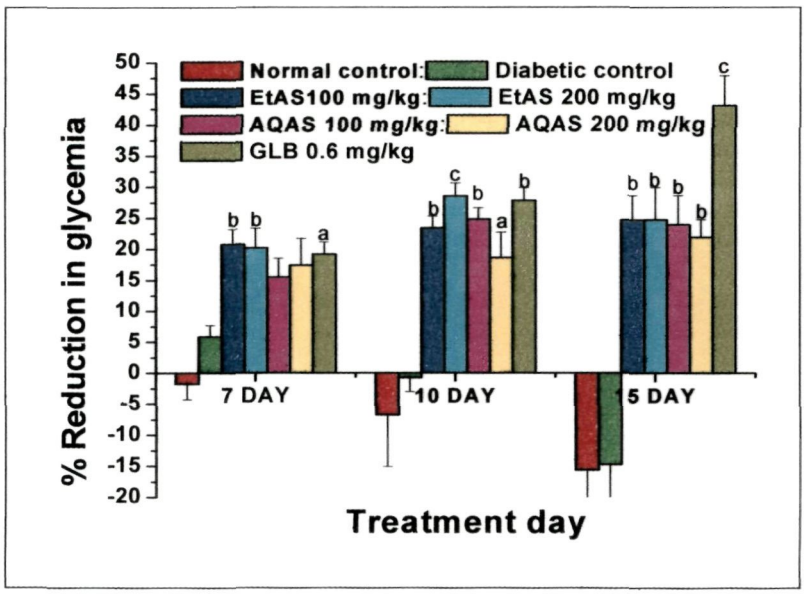


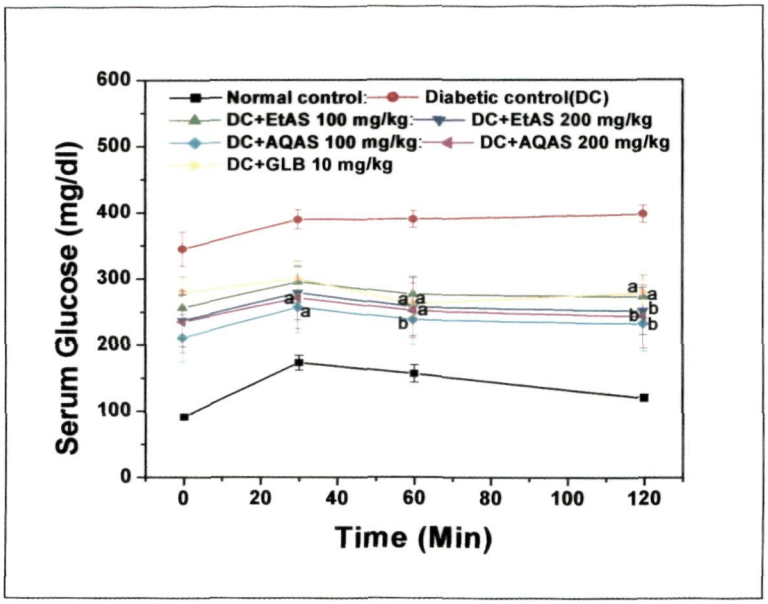
Fig. 5.11.5. Effect of ethanol (EtAS) and water extract (AQAS) of *Argyreia speciosa* on SG levels in STZ-induced diabetic rats [Multiple-dose fifteen-day study]. Bar graph represents the percentage reduction in glycaemia with respect to the initial (0 day) level. Each value represents Mean ± S.E.M., n=5. [a] P<0.05; [b] P<0.01, [c] P<0.001 compared to diabetic control of the same time interval. One-way ANOVA followed by Tukey's post-test.

iii) Oral glucose tolerance test (OGTT)

On 10th day, oral administration of glucose (2 gm/kg) did not produced significant change in SG level of normal control rats and AUC for the 120 min interval was not altered. The diabetic rats exhibited significant elevation in fasting SG (at time zero) and showed significant impairment in glucose tolerance to exogenously administered glucose compared to Normal rats (Fig. 5.11.6 A). Treatment with different dose of EtAS AQAS (100 and 200 mg/kg), and GLB (10 mg/kg) significantly (P<0.05; P<0.01) improve the glucose tolerance (Fig. 5.11.6 B). Further, treatment of ETAS and AQAS exhibited significant (P<0.05; P<0.01) reduction in SG level over the period of 120 min compared to diabetic control group (Fig. 5.11.6 A).

Administration of glucose (2 gm/kg) stimulated the release of higher levels of insulin in normal control rats, whereas glucose load was ineffective in stimulating the release of insulin in diabetic rats, suggesting that these diabetic rats resembled severe diabetic (type I) condition in which a maximum pancreatic damage occurred, whereas, treatment of EtAS and AQAS to diabetic rats enhanced the glucose stimulated insulin release frcm pancreatic β-cells but response was not significant (Fig. 5.11.6 A). Integrated areas under the insulin curve over 60 min ($AUC_{insulin}$) of diabetic group was significantly lower ($P<0.001$) compared to normal control. Treatment with GLB produced a significantly ($P<0.05$) increased $AUC_{insulin}$ compared to diabetic control, whereas, administration of different doses of EtAS and AQAS fail to increase significantly (Fig. 5.11.7 B).

[A]



[B]

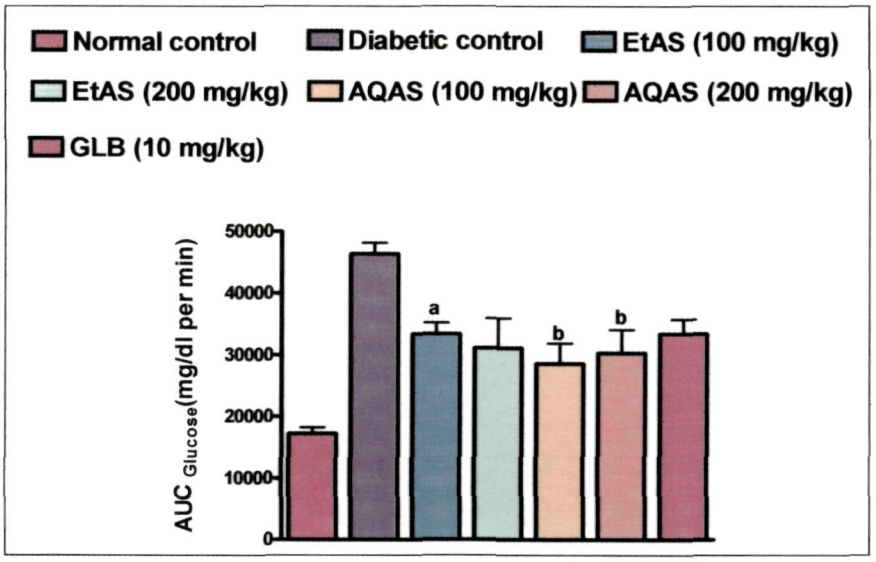
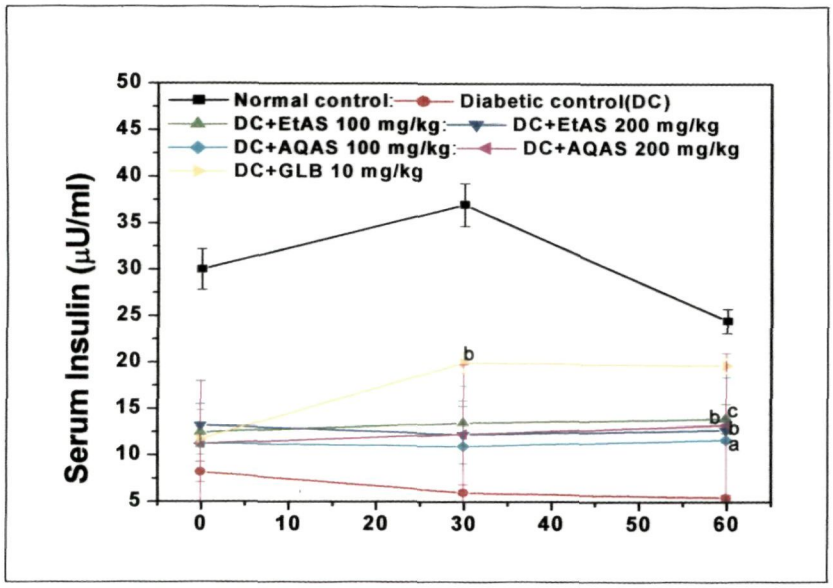


Fig. 5.11.6. Effect of ethanol (EtAS) and water extract (AQAS) of *Argyreia speciosa* on glucose tolerance in fasted diabetic rats. [A] SG levels were measured prior to, and after p.o. administration of glucose alone (2g/kg body weight), or in combination with EtAS, AQAS or Glibenclamide [GLB]. [B] Area under curve for glucose ($AUC_{glucose}$) values for 0-120 min post glucose load. Data represent the mean ± S.E.M., for 5 rats. ^{a} $P < 0.05$; $^{b}P < 0.01$; ^{c} $P < 0.001$ as compared with normal rats (one way ANOVA followed by Tukey's post-test).

[A]



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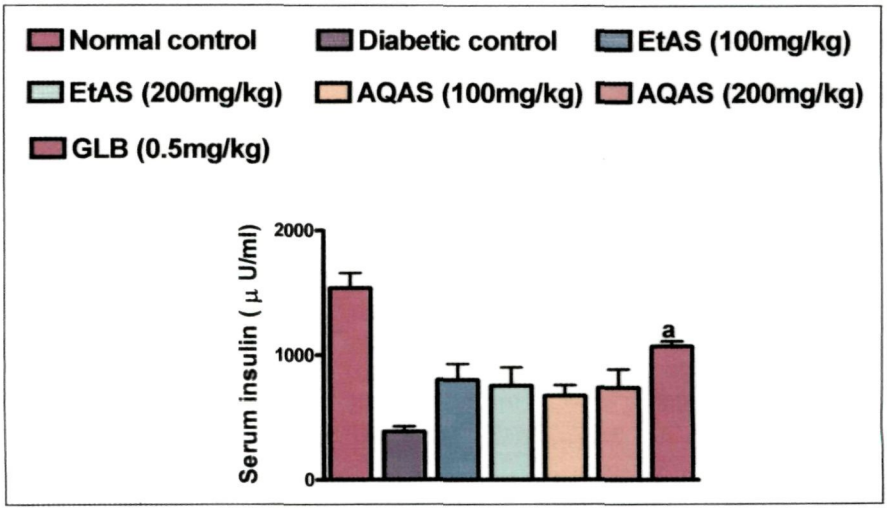
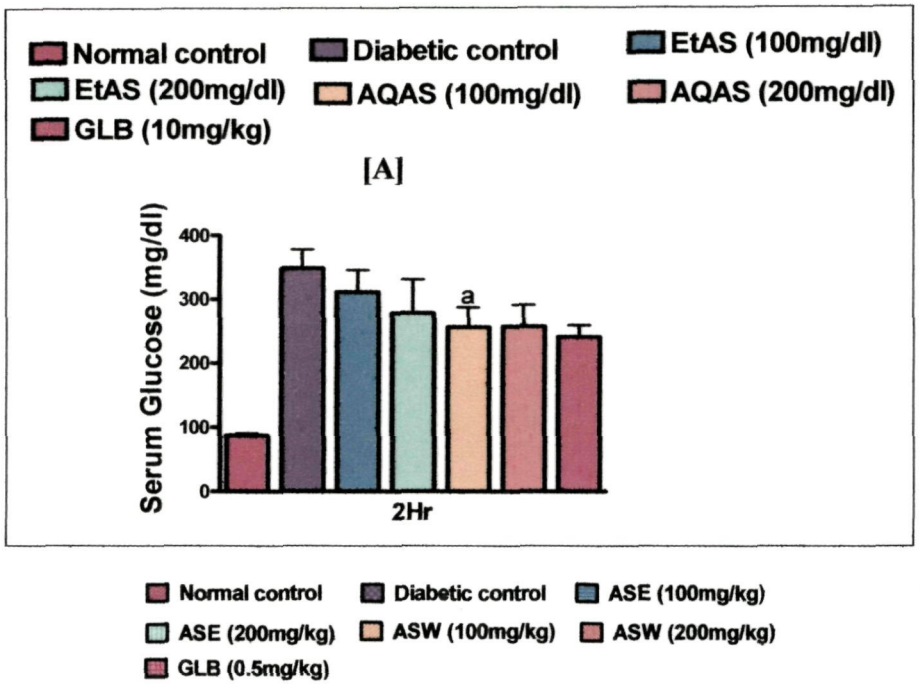


Fig. 5.11.7. Serum insulin (SI) levels [A] Incremental AUC $_{insulin}$ values for 0-60 min. [B] post glucose (2g/kg body weight) challenge performed on tenth day of treatment with ethanol (EtAS) and water extract (AQAS) of *Argyreia speciosa.* Data represent the mean ± S.E.M., for 5 rats. [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ as compared with normal rats (one way ANOVA followed by Tukey's post-test).

iv) Blood glucose, serum insulin and HOMA

On 10^{th} day, diabetic rats exhibited significant ($P<0.001$) hyperglycemia (354.42±15.95; SG levels rose to between 296 to 406 mg/dl) and hypoinsulinemia (8.25±1.1) as compared to normal control rats. The degree of insulin resistance as calculated by HOMA values were similar in both diabetic and normal control rats suggested that, the diabetic rats were not under insulin resistance condition (i.e. peripheral utilization of glucose was not compromised) (Fig. 5.11.8).

Oral administration of AQAS (100 mg/kg) to diabetic rats, significantly ($P<0.05$) decreased SG levels, whereas all other tested doses of EtAS and AQAS fail to produce significant effect. Moreover, tested doses of EtAS and AQAS did not significantly increase SI levels. However, HOMA values were near to normal levels (Fig. 5.11.8).



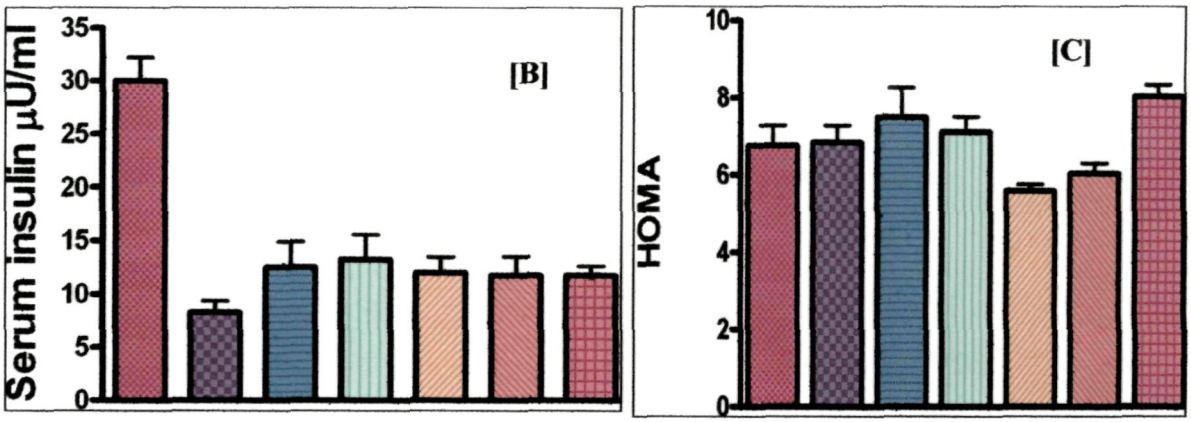


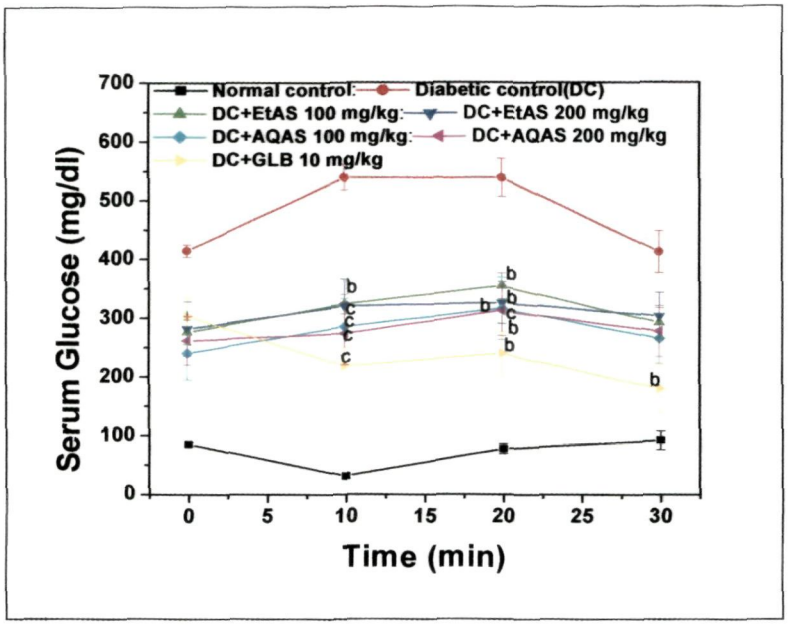
Fig. 5.11.8. Effect of ethanol (EtAS) and water extract (AQAS) of *Argyreia speciosa* on [A] Serum glucose (SGL) [B] Serum insulin (SI) [C] Homeostatic model assessment (HOMA) levels in diabetic rats.

v) Insulin tolerance test (ITT)

On 13th day, SG levels were measured following insulin challenge (2 U/kg, i.v). Surprisingly, in contrast to established reports, severe (type I) diabetic rats subjected to insulin challenge did not exhibit a marked fall in SG levels suggested that, these diabetic rats were not able utilize the exogenously administered insulin to reduce the SG levels. This contrary observation may be due to the marginal loss of insulin sensitivity in diabetic rats, even though these diabetic rats were in type I diabetic condition (as evident by lower insulin levels after glucose challenge and HOMA values).

Moreover, the blood glucose levels and AUC $_{glucose}$ in diabetic rats treated with EtAS and AQAS were significantly ($P<0.01$; $P<0.001$) lower at 10, 20 min compared to the glucose levels at the corresponding time points in the diabetic rats receiving the vehicle (Fig. 5.11.9 A and B).

[A]



[B]

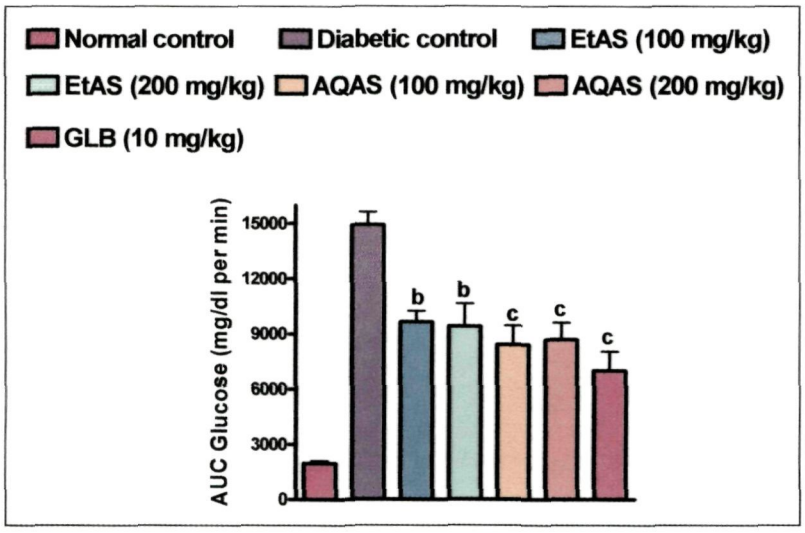


Fig. 5.11.9. Effect of ethanol (EtAS) and water extract (AQAS) of *Argyreia speciosa* on insulin tolerance 6hr in fasted diabetic rats. [A] SG levels were measured prior to, and after p.o. administration of insulin alone (2U/kg body weight), or in combination with EtAS, AQAS or Glibenclamide [GLB]. [B] Area under curve for glucose ($AUC_{glucose}$) values for 0-30 min post insulin injection. Data represent the mean ± S.E.M., for 5 rats. [a] $P < 0.05$; [b] $P < 0.001$; [c] $P < 0.001$ as compared with normal rats (one way ANOVA followed by Tukey's post-test).

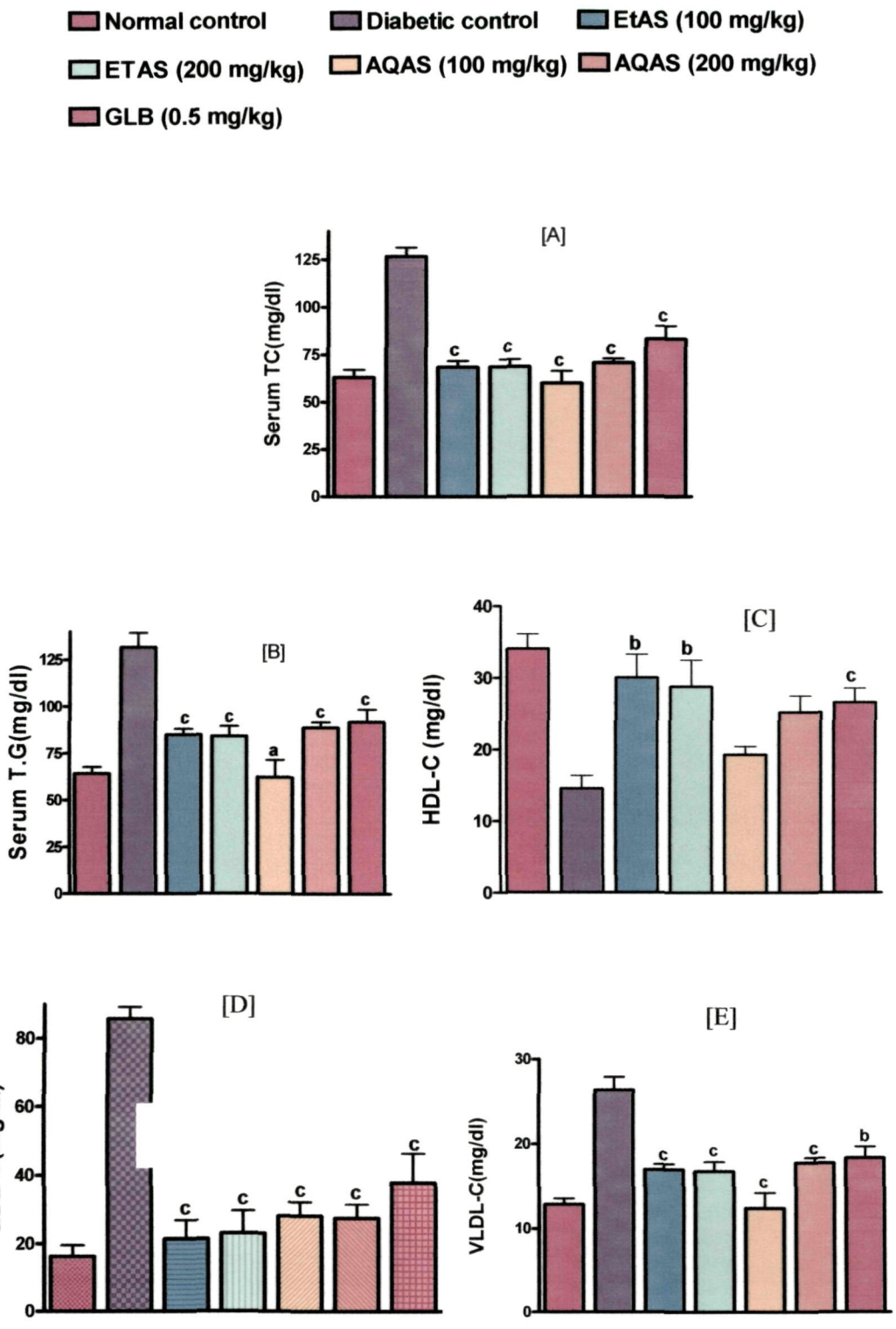
vi) Estimation of Lipid parameter

Diabetic rats showed significantly ($P<0.001$) increased levels of STG, STC, VLDL-c and LDL-c levels, whereas HDL-c was decreased in diabetic rats compared to normal rats (Table 5.35 and Fig. 5.11.10). The markers of dyslipidemia such as TC/HDL-c and LDL-c/HDL-c ratios were significantly elevated in the diabetic group. Oral administration of different doses of EtAS and AQAS for fifteen-days exhibited significant reduction ($P<0.001$) in all tested lipid parameters and restoring them to near-normal values (Table 5.35 and Fig. 5.11.10).

Table 5.35. Effect of ethanol (EtAS) and water fraction(AQAS) of *Argyreia speciosa* on Lipid Profile in STZ-induced model (Multiple Dose fifteen-days Study)

Serum parameter	Normal control	Diabetic control	EtAS 100 mg/kg	EtAS 200 mg/kg	AQAS 100 mg/kg	AQAS 200 mg/kg	GLB 10 mg/kg
STG (mg/dl)	84.9±3.13	131.77±7.5	84.9±3.13***	84.04±5.5***	62.2±13.0***	88.9±4.3***	91.9±6.7***
STC (mg/dl)	68.41±3.9	126.62±4.7	68.41±3.13***	68.68±3.8***	59.9±8.9***	70.5±3.2***	82.9±6.9***
HDL-c (mg/dl)	34.1±2.0	14.52±1.8	30.03±3.24	28.75±3.8	19.3±1.6	25.2±3.2	26.6±1.9
VLDL-c (mg/dl)	12.8±0.7	26.35±1.5	16.98±0.63***	16.81±1.1***	12.4±2.6***	17.8±0.9***	18.4±1.3***
LDL-c (mg/dl)	16.3±3.3	85.75±3.4	21.4±5.46***	23.12±6.5***	28.1±5.8***	27.6±5.6***	37.9±8.5***
TC/HDL-c ratio	1.9±0.1	9.17±1.2	2.35±0.24***	2.57±0.5***	3.1±0.4***	2.9±0.5***	3.2±0.4***
LDL-c/HDL-c ratio	0.5±0.1	6.26±0.9	0.77±0.21***	0.96±0.4***	1.5±0.3***	1.2±0.4***	1.5±0.4***

Each value represent Mean± S.E.M n=5 ***P<0.001 compared to Diabetic control. One way ANOVA followed by Tukey's post test


Normal control
Diabetic control
EtAS (100 mg/kg)
ETAS (200 mg/kg)
AQAS (100 mg/kg)
AQAS (200 mg/kg)
GLB (0.5 mg/kg)
[A]
Serum TC(mg/dl)
[B]
Serum T.G(mg/dl)
[C]
HDL-C (mg/dl)
[D]
LDL-C(mg/dl)
[E]
VLDL-C(mg/dl)

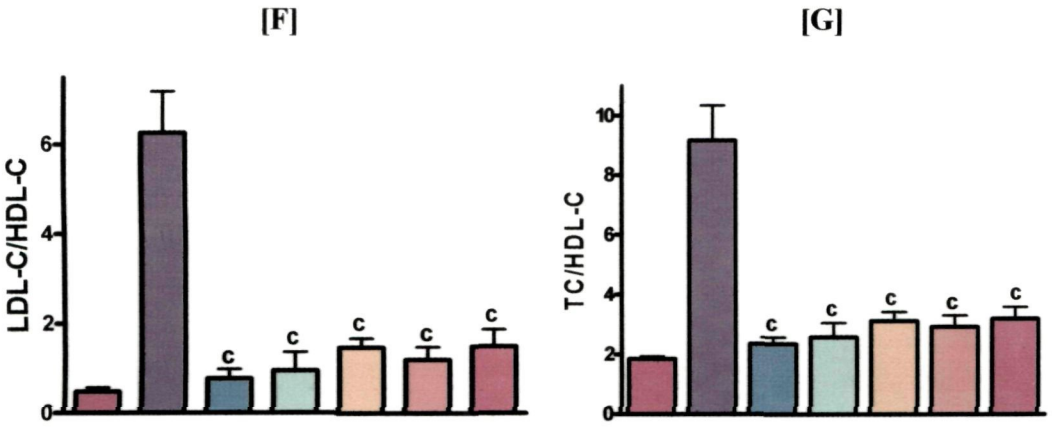


Fig. 5.11.10. Effect of Fifteen-day treatment with ethanol EtAS and water (AQAS) extract of *Argyreia speciosa* on [A] Serum TG [B] Serum TC [C] Serum HDL-c [D] VLDL-c [E] LDL-c [F] TC/HDL-c [G] LDL-c/HDL-c levels in STZ-induced diabetic rats. Each bar represent the Mean ± S.E.M. (n = 5). ^{a} P < 0.05; ^{c} P < 0.001 compared with diabetic control.

C) Glucose uptake by isolated hemi-diaphragm of diabetic rats

Normal rats showed 2.154 mg glucose uptake per gram of diaphragm tissue, whereas fifteen-day post STZ (50 mg/kg, i.v) treated rats showed significant (P<0.01) reduction in glucose uptake (0.328 mg/g of tissue) (Fig. 5.11). This observation suggested that these STZ administered rats were under insulin resistance condition (even though these STZ rats exhibited type I diabetic condition as evident by lower insulin levels post glucose challenge and HOMA values). Furthermore, this observation (reduction in glucose uptake by diaphragm of diabetic rats) was in line with data of insulin tolerance test.

Treatment of insulin (62 μU) caused significant (P<0.001) stimulation of glucose uptake leading to ~5 fold increase compared to diabetic control values Whereas, treatment of different doses of EtAS (50 and 100 μg/ml) and AQAS (50 and 100 μg/ml) failed to stimulate glucose uptake.

5.11.5.4 Discussion

Diabetes mellitus is a metabolic disease as old as mankind and is characterized by hyperglycemia associated with impairment in insulin secretion/action along with altered carbohydrate, protein and lipid metabolism. The function of insulin is to maintain normal blood glucose levels either by suppression of glucose output from liver or by the stimulation of glucose uptake and its metabolism. Insufficient release of insulin or loss of insulin action at target tissues causes abnormal glucose and lipid metabolism. This results in elevated glucose levels in blood, the hallmark of diabetes. Type-1 diabetes results from autoimmune destruction of pancreatic β-cells resulting in insulin deficiency. Before the introduction of insulin in 1922 the treatment of diabetes mellitus relied heavily on dietary measures which included the use of traditional plant therapies. Many traditional plant treatments for diabetes exist[14,15,16]. However, few have received scientific or medical scrutiny and the World Health Organization has recommended that traditional plant treatments for diabetes warrant further evaluation[17]. Insulin therapy affords effective glycemic control, yet its drawbacks such as ineffectiveness on oral administration, short shelf life, need for constant refrigeration and hypoglycemia on excess dosage limits its usage[18]. Therefore efforts continue to find insulin substitutes from synthetic or plant sources.

Numerous animal models have been developed to mimic human disease states. The underlying assumption in using these animal models in medical research is that they will provide additional knowledge about and insight into disease processes and, hopefully, better methods for treatment or prevention of diseases in humans[19]. Wessler defines an animal model as "a living organism with an inherited, naturally acquired, or

induced pathological process that in one or more respects closely resembles the same phenomenon occurring in man"[20]. He emphasizes that animal models offer only approximations of human disease and can never actually duplicate the same process. In attempting to approximate type 1 diabetes in animals, both spontaneous and induced models have been developed. The means by which the disease is induced or how it develops in an animal is important in determining how the model is to be studied [20].

STZ-induced experimental diabetes is a valuable model for induction of type 1 diabetes. Further it is generally accepted that severe diabetes (SD) is similar to type 1 and mild diabetes (MD) is similar to type 2 diabetes [21,22].

To our knowledge, this is the first detailed study to investigate the effect of ethanol (EtAS) and water (AQAS) extracts of *Argyreia speciosa* on the glucose levels, lipid profile in STZ-induced diabetic rats.

The severity and insulin resistance (reduced peripheral utilization of glucose) condition in diabetic rats induced by STZ (50 mg/kg, i.v) was confirmed by lower insulin levels post glucose-challenge, HOMA (measure of insulin resistance) values and lack of glucose uptake by isolated hemi-diaphragm of diabetic rats.

Both EtAS and AQAS produced hypoglycemia and improved glucose tolerance in normal rats in spite of counter regulatory factors avoiding reduction in blood glucose levels. Therefore hypoglycemic activity of EtAS and AQAS could be mediated by stimulation of surviving β-cells to release more insulin and may be through extra-pancreatic mechanisms. Furthermore, treatment of EtAS and AQAS significantly improve the sucrose tolerance in normal rats, suggested that the components of extracts may inhibit α-glucosidase, a membrane bound enzyme located on the epithelium of the

small intestine, catalyzing the cleavage of disaccharides to form glucose. Therefore, inhibitors can retard the uptake of dietary carbohydrates and suppress post-prandial hyperglycemia. STZ is well known for its selective pancreatic islet β-cell cytotoxicity and has been extensively used to induce DM in animals. It interferes with cellular metabolic oxidative mechanisms.

The present data suggested that EtAS and AQAS significantly reduced hyperglycemia in both single-dose one-day and multiple-dose fifteen-day diabetic studies. The efficacy of the EtAS is better than AQAS. This could be mediated by improving the glycemic control mechanisms (extra-pancreatic) and increasing insulin secretion from remnant pancreatic β-cells in diabetic rats.

DM is often linked with abnormal lipid metabolism. The impairment of insulin secretion results in enhanced metabolism of lipids from the adipose tissue to the plasma [23]. It has been demonstrated that insulin deficiency in diabetes leads to a variety of disruptive changes in metabolic and regulatory processes, which in turn lead to accumulation of lipids[24]. It has also been shown that insulin significantly normalizes lipid levels in diabetic rats[25]. The extract supplementation also results in significant attenuation in STG, STC, VLDL-c, and LDL-c. These effects may be due to low activity of cholesterol biosynthesis enzymes or low levels of lipolysis. Increased TC/HDL-c and LDL-c/HDL-c ratios are well known markers of dyslipidemia in STZ-induced diabetic rats[26]. EtAS and AQAS administration reinstated dyslipidemic markers to near-normal values.

The phytochemical examination of ethanol (EtAS) and water (AQAS) extract of *Argyreia speciosa* revealed the presence of alkaloids, carbohydrate, flavanoids, tannins

and phenolics. Several authors have reported flavanoids, phenolics and steroidal glycoside as bioactive antidiabetic principles[27,28]. The observed antidiabetic and hypolipidaemic activity of title plant may be attributed to the presence of these bioactive principles and their synergistic properties. Therefore we conclude that the ethanol (EtAS) and water (AQAS) extract of *Argyreia speciosa* root has endowed with anti-diabetic (single-dose one-day study and multi-dose fifteen-day study), anti-hyperlipidaemic activity in standardized STZ-induced diabetic rats, justifying its use in the traditional system of medicine.

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Chapter - 6

Isolation and Pharmacological Activity of Secondary Metabolites from A*rgyreia speciosa* (Burm.f.) Boj.

Chapter - 6

Isolation and Pharmacological Activity of Secondary Metabolites from *Argyreia speciosa* (Burm.f.) Boj.

6.1 Introduction to chromatography

Over the past 100 years, chromatography has developed from a rudimentary tool for the separation of pigments into an array of techniques capable of dealing with the most complex analytical and purification problems in phytochemistry. **Tswett** introduced column adsorption chromatography at the beginning of the twentieth century, initially for the separation of plant pigments. By the end of the 1930s, column adsorption chromatography (nowadays known as normal phase chromatography) had become a widely used separation technique for plant extracts and natural products.

Martin and co-workers introduced paper chromatography using filter paper sheets impregnated with water or other liquids, this became the first chromatographic microanalytical technique. However, the slow migration rates of paper chromatography have resulted in this technique being overtaken by thin-layer chromatography (TLC), in which the application of thin layers of adsorbent on glass plates originated in Russia.

GLC was finally introduced in 1952 and became widely available in the 1960s. It is suitable for small, volatile compounds. Sensitive universal detection was provided by flame ionization (FID). GLC (or GC) is ideal for the analysis of complex mixtures such as those found in essential oils. In one run, it is possible to separate hundreds of constituents and identify them by comparison with a data base.

Since Ethanolic (EtAS) and ethyl acetate (EAAS) fractions of *Argyreia speciosa* were found to be potent in various pharmacological activities, we further undertook the isolation and characterization of important secondary metabolites using column chromatography and spectral data.

6.2 Materials and Methods

6.2.1 Preparation of crude fraction

Root powder of *A. speciosa* was extracted with 80% methanol at room temperature. After evaporation the residue was dissolved in water and extracted successively with Petroleum ether, CH_2Cl_2 and n-butanol. The n-butanol fraction was used for separation of flavanoids by column chromatography.

6.2.2 Column chromatography

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids when carrying out small-scale experiments. Column chromatography is the most widely used to isolate the component of complex mixture (preparative chromatography). Column chromatography can also be used to determine the quantity of different compounds in the mixture (analytical chromatography). Column chromatography is another solid-liquid technique in which the two phases are a solid (stationary phase) and a liquid (mobile phase). The theory of column chromatography is analogous to that of thin-layer chromatography. The most common adsorbents silica gel and alumina are the same ones used in TLC. Various stationary phases are used to separate compounds either according to polarity (normal and reverse phase silica gel, Sephadex) or size of compounds (Toyopearl- size- exclusion chromatography).

6.2.3 Column packing

Glass column with length 45 cm and diameter 2.5 cm was used for separation of constituents from active extract. With a long stirring rod, small plug of cotton was placed at the bottom of the column, and bottom of the column was closed with a small piece of rubber tubing and a screw clamp. The ¾ of column was filled with eluting solvent..

Approximately 40 grams of Silica (Merck, 60-120 mesh size) gel activated for 24 h at 80°C in an oven was transferred into the column. Conical flask was placed under column while adding mixture to the column. Tubing and clamp were removed from the tip of the column and allowed the solvent to flow freely for the rest of the experiment.

6.2.4 Gradient elution

Dried n-butanol fraction of *A. speciosa* was chromatographed over silica gel column (45 cm x 2.5 cm) using EtOAc-MeOH- H_2O (80:10:10) as eluent. The eluting solvent was drained to approximately 1 cm above the top of the silica bed. Using a long pipette extract was transferred to the top of the silica bed making sure not to squirt mixture onto the inner sides of the column. Later, mixture was allowed to adsorb on the top of the silica gel before adding more eluting solvent. Whattmann filter paper was cut to size of column diameter and inserted at top end of the stationary phase to prevent disruption during addition of solvent. Once addition of solvent to the column, was started the solvent level was maintained not to go below the top of the silica bed. Gradient elution was carried out by using EtOAc-MeOH- H_2O (80:10:10). The elution rate was adjusted to 50 ml/min till 200 ml of several 10 ml fractions were collected, and then 15-18 drops per minute up to 450 ml. These sub fractions were further chromatographed on Sephadex LH-20 by stepwise gradient elution with MeOH-H_2O.

6.2.5 Monitoring the column with TLC

Several 10 ml fractions were collected and TLC was checked for every fraction and grouped according to their homogeneity. The isolation was subjected to TLC using silica gel GF_{254} EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).TLC was carried out on glass slides coated with 0.25 mm silica (Merck, silica gel for TLC). Using capillary tube place approximately 5 µL column eluted fraction was loaded on silica gel 3 mm above from

bottom. Flavanoids were identified in fractions between 270-420 ml. The TLC plates were checked under UV light (254 nm) and then sprayed with 2-aminoethyl diphenyl borinate (Natural product reagent, Sigma Aldrich, USA) and observed for yellow fluorescent spots at 366 nm. Fractions with similar TLC pattern were pooled together and concentrated at reduced pressure and temperature. The concentrated components were further dried in vacuum descicator. Completely dried components were weighed to calculate the total mass isolated. Isolated flavanoids were dissolved in 2 ml MeOH and heated with 5 ml 3% HCl under reflux for 15 min. After evaporation of MeOH the resultant aglycones were extracted with aliquots of ethyl acetate (10 ml, twice) and analyzed by TLC on silica gel with CH_2Cl_2:Me_2CO:HCO_2H (76:16:8).

6.2.6 Characterization of isolated compounds

The structures of flavanoids were confirmed by spectral data

Infrared spectroscopy

The infrared spectrum of the isolated compounds was recorded using KBr in the range of 4000-400 cm^{-1} on FTIR (Nicolet 5700, Thermo electron corporation).

Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (^1H and ^{13}C) of the isolated compounds was recorded using Bruker AV 300 MHz spectrophotometer. All spectra were recorded at 25°C and the chemical shifts were recorded in δ ppm with the solvent shift. Deuterated chloroform ($CDCl_3$) and DMSO (HPLC grade) were used as solvents.

Mass spectrometry

Electron ionization mass spectrometry of the compounds was performed by direct inlet at 70 eV on the LCMS-QP2010.

6.2.7 Results

Table 6.1. Elution of flavanoids from *Argyreia speciosa* (Burm.f.). Boj.

Fraction no	Composition & proportion of the solvent system	Color of the elute	TLC Studies Solvent system	No of spots & Rf values	Amount
1-10	Methanol (100%)	No color	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	–
11-20	Methanol:H_2O (99-1%)	No color	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	–
21-50	Methanol:H_2O (95-5%)	No color	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	–
50-75	Methanol:H_2O (90-10%)	No color	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	–
76-100	Methanol:H_2O (85-15%)	No color	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	–
100-150	Methanol:H_2O (90-10%)	No color	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	–
150-200	Methanol:H_2O (80-20%)	No color	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	–
201-250	Methanol:H_2O (80-20%)	Light yellow	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	--
251-275	Methanol:H_2O (75-25%)	Light yellow	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	–
276-300	Methanol:H_2O (75-25%)	**fluoroscent Yellow**	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	**Single spot (0.7)**	**100 mg**
301-355	Methanol:H_2O (70-30%)	Light yellow	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	--
356-375	Methanol:H_2O (70-30%)	Light yellow	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	---
376-400	Methanol:H_2O (70-30%)	No colour	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	--
400-425	Methanol:H_2O (65-35%)	**Yellowish brown**	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	**Single spot (0.39)**	**85mg**
426-450	Methanol:H_2O (60-40%)	Light brown	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	--
451-475	Methanol:H_2O (50-50%)	No colour	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	---
476-500	Methanol:H_2O (25-75%)	No colour	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	--
500-525	H_2O (100%)	No colour	EtOAc-Me-CO-Et-HCOOH-H_2O (5:3:1:1).	No spot	--

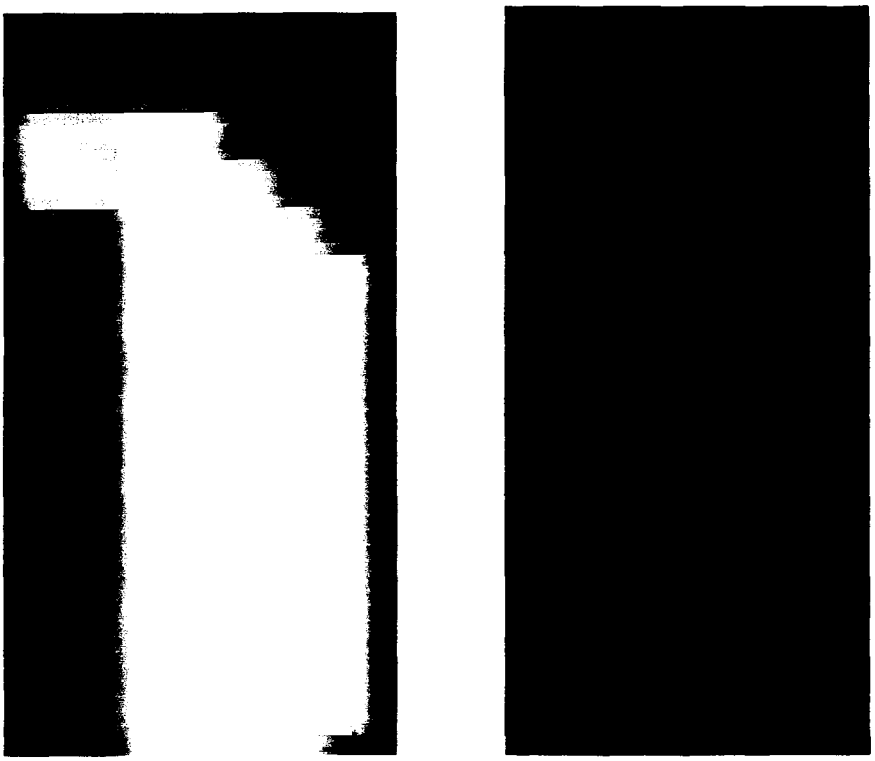
6.2.7.1 TLC Profile of ASB1 (Quercetin) and ASB2 (Kaempferol)

Adsorbent : Silica Gel GF_{254}

Mobile phase : CH_2Cl_2:Me_2CO:HCO_2H (76:16:8).

Spray reagent : 2-aminoethyl diphenyl borinate (NP reagent)

R_f Value : ASB1- 0.7, ASB2- 0.3



ASB1 **ASB2**

Fig. 6.1.Thin layer chromatogram of ASB1 and ASB2

6.2.8 Spectral data

Structures of isolated compounds were established based on IR, ^{1}HNMR and mass spectral studies.

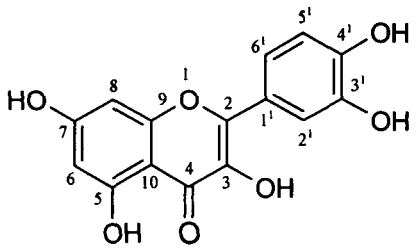
Spectral data of Quercetin (ASB1)

Yellow crystals, 100 mg. R_f 0.71, mp. found 314°C, (literature data 315 °C)[1,2] IR spectrum **(Fig.6.2)** of ASB1 showed characteristic absorption band at 3406 cm^{-1} due to hydroxyl group. Another band at 1609 cm^{-1} attributed to stretching frequency of carbonyl group. The CH=CH stretching peak appeared at 2912 cm^{-1}

The ^{1}H NMR spectrum **(Fig. 6.3)** of revealed the presence of a hydrogen bonded hydroxyl signal with C=O (C_5- OH) at δ 12.48. A singlet at δ 10.77 was assigned to C_3-OH proton. A singlet due to C_7-OH proton resonated at δ 9.58. A broad singlet at δ 9.36 which integrated for two protons was assigned to C_3' and C_4' hydroxyl groups. Two singlets at δ 6.18 and δ 6.40 which integrated for a proton each were due to protons present on phenyl ring of flavanoid nucleus (H-6 and H-8). Two doublets at δ 6.88 and δ 7.67 were attributed to H_5' and H_6' protons. A peak due to C_2' proton appeared as singlet at δ 7.67.

The Mass spectrum **(Fig. 6.4)** of ASB1 showed molecular ion peak at m/z at 303.0 which corresponds to its molecular formula (C_{15} H_{10} O_7) and molecular weight.

All the above spectral data were consistent with the earlier reported data[3-6] and the structure assigned to the molecule as **Quercetin (1)**



(1)

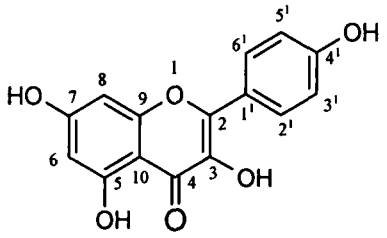
Spectral data Kaempferol (ASB2)

Yellow needles, 85 mg. R_f 0.30, mp. 277°C, (literature data 275-278 °C)[1,2] IR spectrum **(Fig. 6.5)** of ASB2 exhibited a characteristic absorption band at 3247 cm^{-1} due to hydroxyl groups. Another band at 1612 cm^{-1} attributed to stretching frequency of carbonyl group. The CH=CH stretching appeared at 2925 cm^{-1}.

The ^1H NMR spectrum **(Fig. 6.6)** of revealed the presence of a hydrogen bonded hydroxyl signal with C=O (C_5- OH) at δ 12.47. A singlet at δ 10.78 was assigned to C_3-OH proton. A singlet due to C_7-OH proton resonated at δ 10.10. A singlet at δ 9.39 was assigned to C_4' hydroxyl group. Two singlets at δ 6.18 and δ 6.43 which integrated for a proton each, were due to protons present on phenyl ring of flavanoid nucleus (C_6-H and C_8-H). Doublet at δ 6.90 was attributed to C_3' and C5' protons. Doublet due to C_5' proton and C6' protons appeared at δ 8.05(2H).

The Mass spectrum **(Fig. 6.7)** of ASB1 showed molecular ion peak at m/z at 287.0 which corresponds to its molecular formula (C_{15} H_{10} O6) and molecular weight.

Hence, by comparing the spectral data obtained and earlier reported data[3-6] the structure assigned was in good agreement with **Kaempferol(2).**



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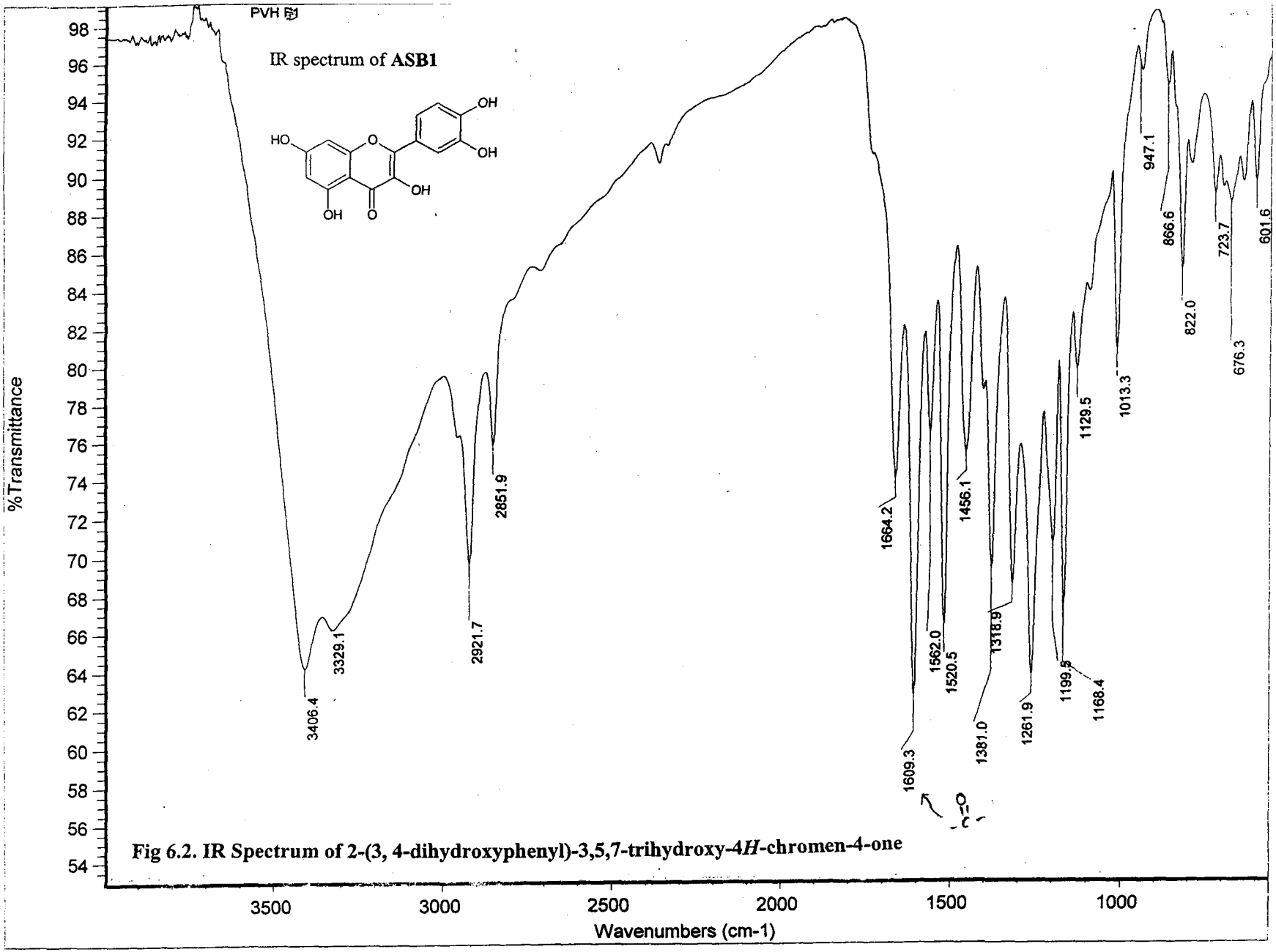
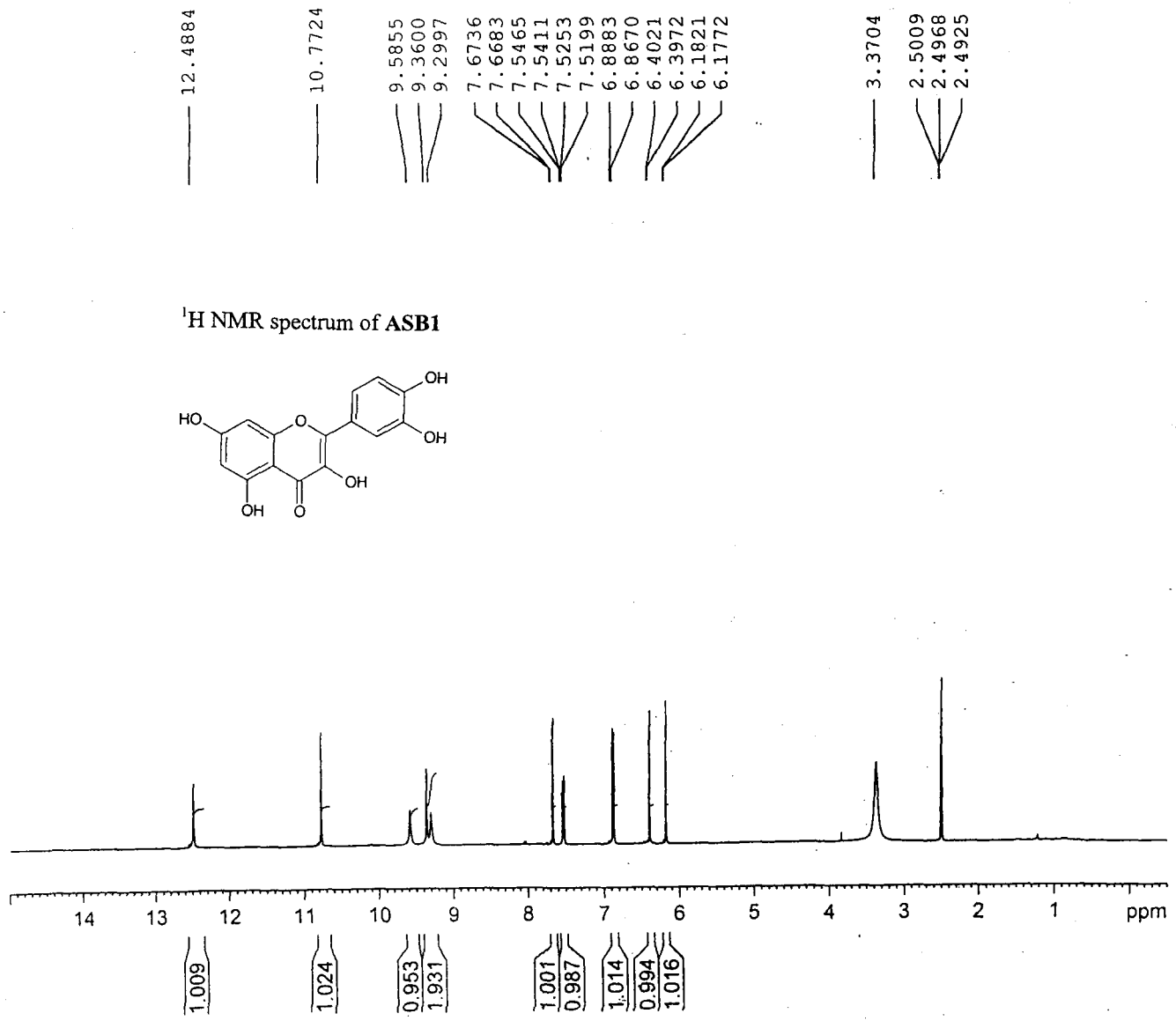


Fig 6.2. IR Spectrum of 2-(3, 4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one



^{1}H NMR spectrum of **ASB1**

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DS                     2
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RG                645.1
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DE                 6.00 usec
TE                296.0 K
D1             2.00000000 sec
TD0                    1

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PL1                0.00 dB
SFO1          400.2340023 MHz

F2 - Processing parameters
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SF            400.2300000 MHz
WDW                   EM
SSB                    0
LB                0.30 Hz
GB                     0
PC                1.00
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Fig 6.3. ^{1}H NMR Spectrum of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one

LC/MS REPORT

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Injection vol   :  2.0ul
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               % B :         5-90         90           90              90--5
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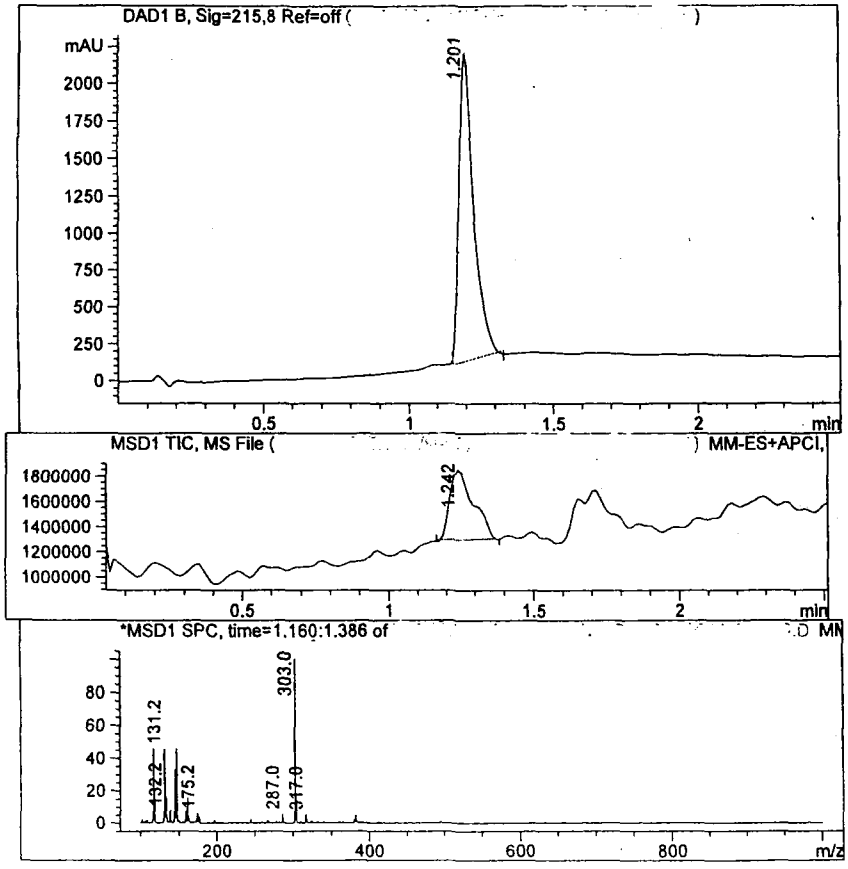

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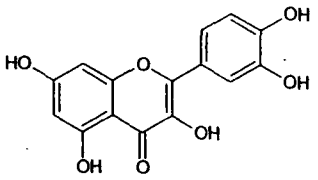
Mass spectrum of **ASB1**



Mass Calcd m/z=302

Found m/z=303(M+1)

Fig 6.4. Mass Spectrum of 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one

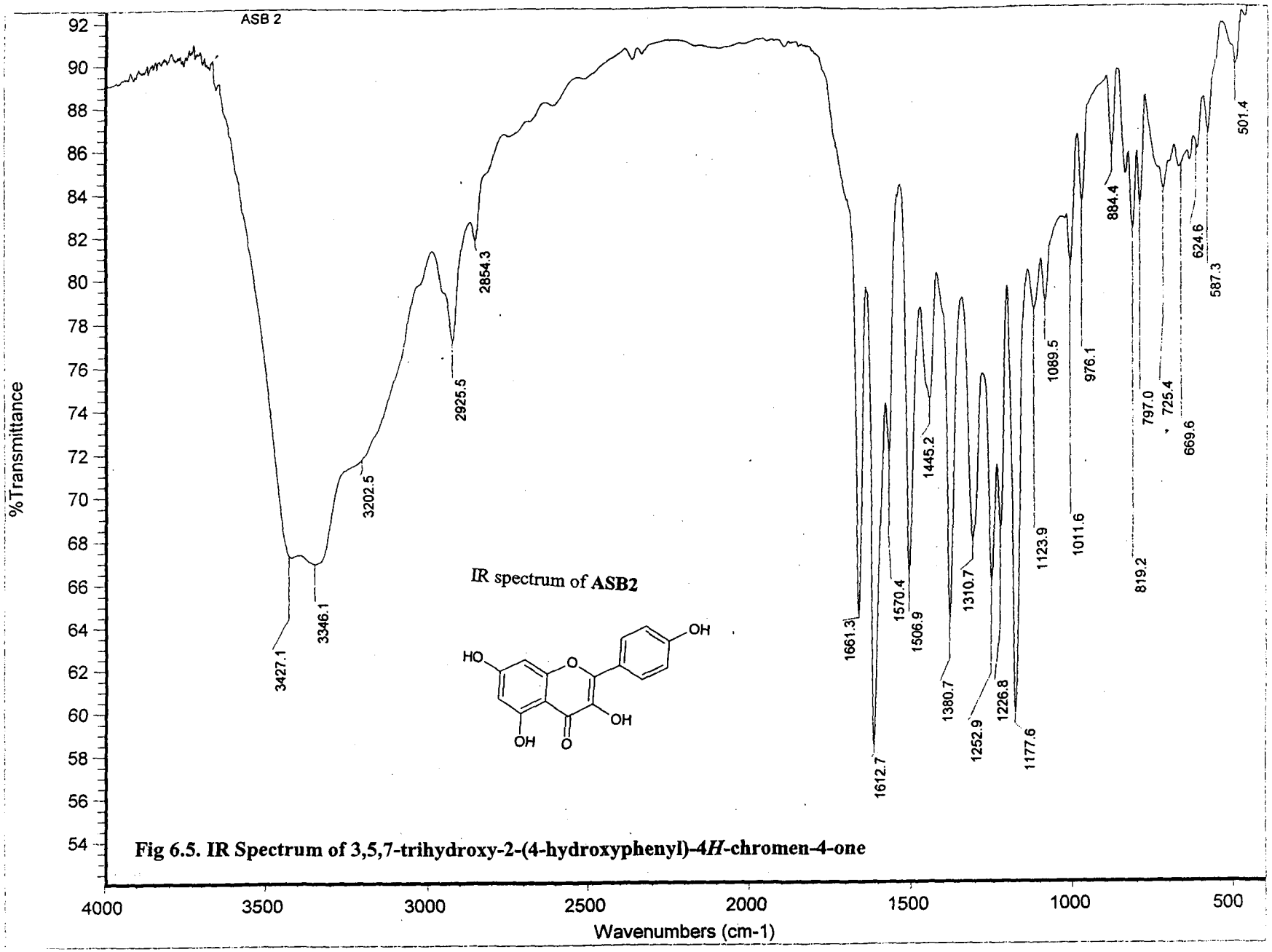
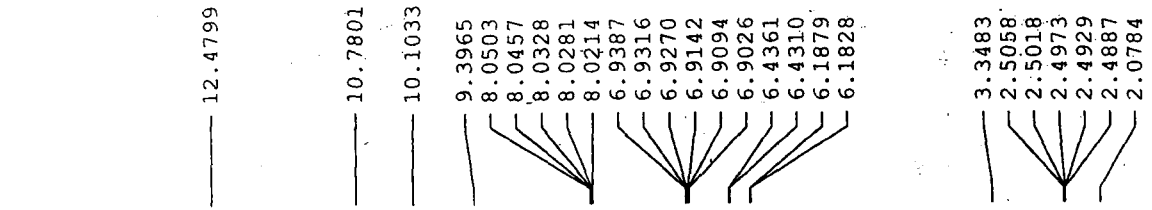
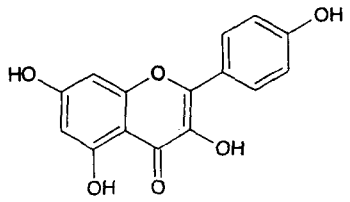
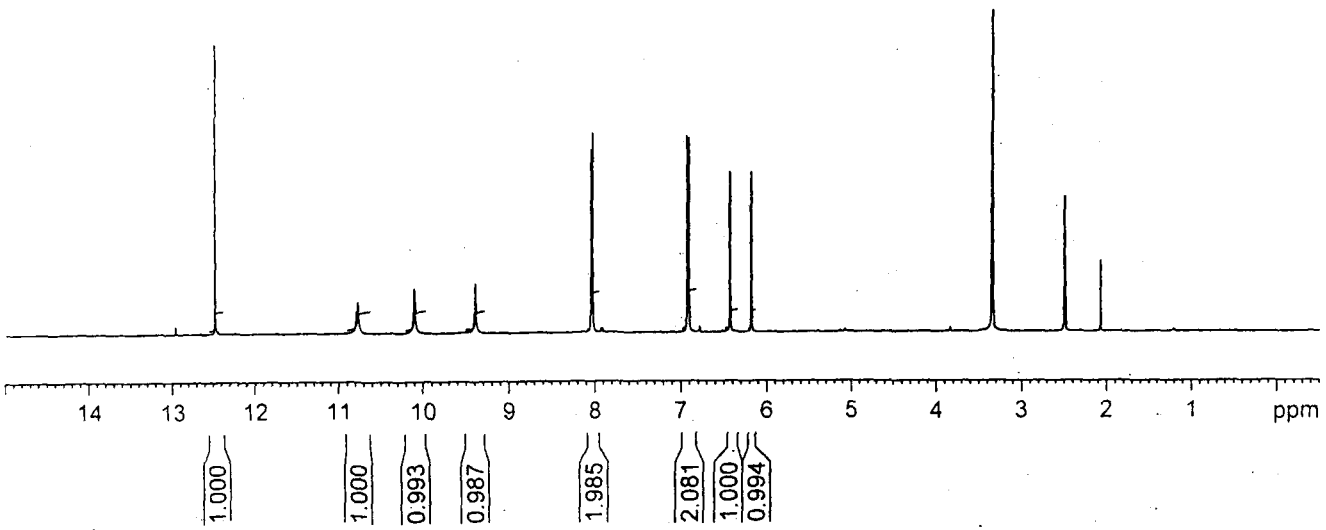


Fig 6.5. IR Spectrum of 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one



^{1}H NMR spectrum of **ASB2**



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DS                      2
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FIDRES           0.294814 Hz
AQ              1.6960351 sec
RG                  574.7
DW                 50.100 usec
DE                  6.00 usec
TE                 296.0 K
D1              2.00000000 sec
TD0                     1

======== CHANNEL f1 ========
NUC1                   1H
P1                  13.70 usec
PL1                  0.00 dB
SFO1          400.2340023 MHz

F2 - Processing parameters
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SF            400.2300000 MHz
WDW                    EM
SSB                     0
LB                   0.30 Hz
GB                      0
PC                   1.00
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Fig 6.6. ^{1}H NMR Spectrum of 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one

LC/MS REPORT

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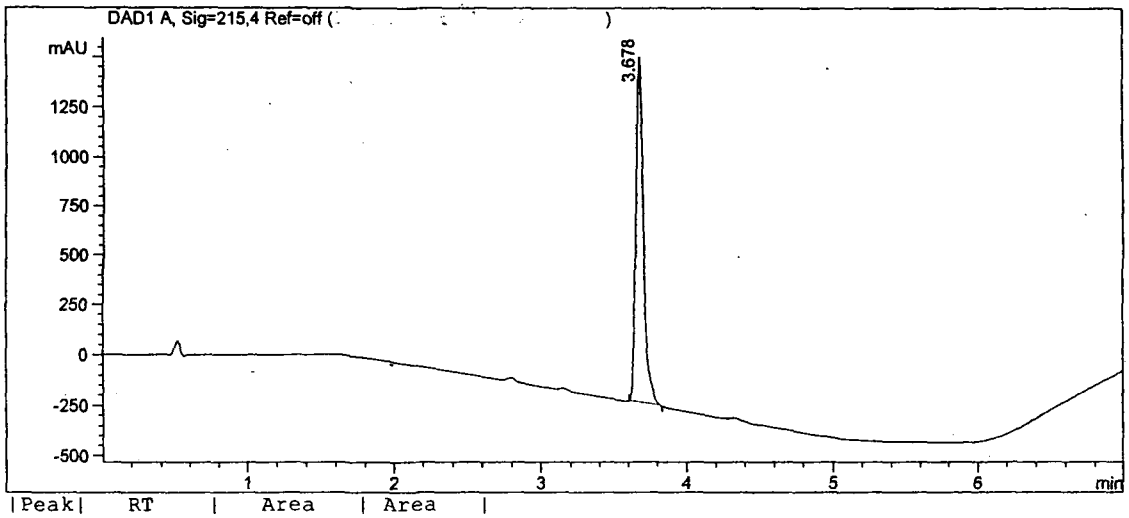

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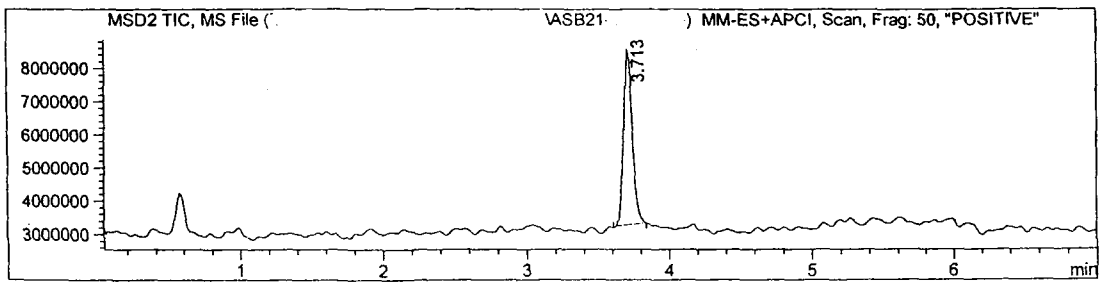
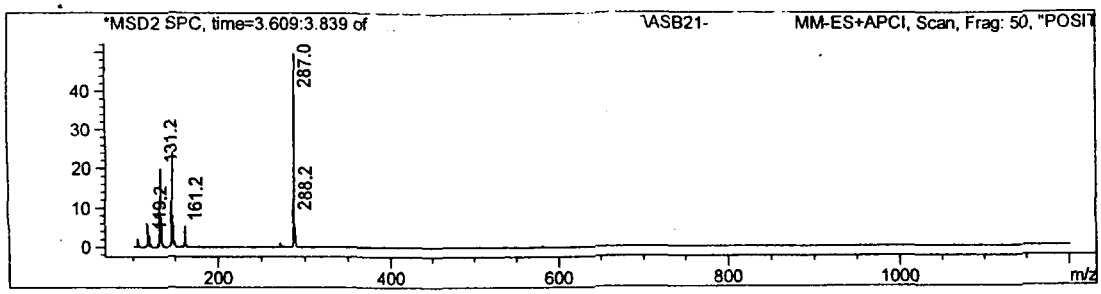
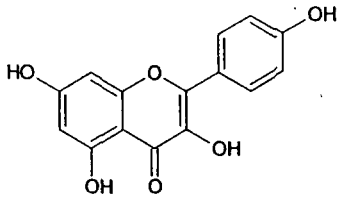


LC/MS REPORT



Mass spectrum of **ASB2**



Mass Calcd m/z=286

Found m/z=287(M+1)

Fig 6.7. Mass Spectrum of 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one

6.2.9 References

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6.3 Pharmacological activities of flavanoids of *Argyreia speciosa* (Burm.f.) Boj.

Flavanols isolated from *Argyreia speciosa* (Burm).f. Boj. were subjected to pharmacological screening for antimicrobial and adaptogenic/anti-stress activity using *in-vitro* and *in-vivo* methods.

6.3.1 Adaptogenic activity

Since Alcoholic fraction (EtAS) of *Argyreia speciosa* exhibited significant anti-stress activity, we further extended our study to prove the efficacy of the isolated flavanols as adaptogen constituents.

6.3.1.1 Materials and Methods

a) Drugs and chemicals

Diagnostic kits for the estimation of glucose, triglycerides, AST, ALT were purchased from ERBA diagnostic Mannheim Ltd.(Germany), Cholesterol (Span Diagnostics Ltd, India) and Creatinine kinase (Agappe Diagnostics Ltd.) A gift sample of standardized *Withania somnifera* (WS) extract was obtained from Natural remedies, Bangalore, India.

b) Preparation of drugs

Isolated flavanols were suspended in 0.5% gum acacia, and a fine emulsion was made having uniform particle distribution. The emulsion of both the compounds was administered for orally daily for three days in case of acute stress (AS) and for 7 days in case of chronic stress (CS). Both the drugs were prepared fresh, daily before administration.

c) Animals

Albino rats of either sex weighing 150-200 gms and Swiss albino mice (15-20 gms) were used in the study. They were housed three to four per cage at temperature 22± 2°C at 12/12 hr light/dark under controlled environment. Animals were fed standard

laboratory food and water was given *ad libitum*. Rats and mice were kept for 7 days in laboratory for habituation.

d) Stress Protocol [1]

As described in 5.11.3.2d

f) Swimming endurance test [2]

As described in 5.11.3.2f

6.3.1.2 Results

i) Effect of drug treatment on acute stress (AS) and chronic stress (CS) induced alterations in biochemical parameters:

Table 6.2 and 6.3 showed that exposure to AS ($P<0.001$) resulted in a significant increase in the serum glucose level which is higher when compared to CS ($P<0.001$). Pretreatment with quercetin 25 mg/kg p.o. ($P<0.001$), kaempferol 25 mg/kg p.o. ($P<0.001$), and WS 100 mg/kg p.o. ($P<0.001$) significantly decreased the circulating glucose level in Acute and chronic stress.

Exposure to AS and CS resulted in significant increase in the total cholesterol ($P<0.001$) level compared to normal control. Pretreatment with quercetin 25 mg/kg p.o. ($P<0.001$), kaempferol 25 mg/kg p.o.($P<0.001$) and WS 100 mg/kg po ($P<0.001$) significantly decreased the circulating total cholesterol level in AS.(Table 6.2).In CS, quercetin 25 mg/kg p.o ($P<0.001$), and kaempferol 25 mg/kg p.o. ($P<0.05$) and WS 100 mg/kg p.o. ($P<0.05$) significantly reduced elevated total cholesterol level (Table 6.3).

Exposure to AS and CS resulted in significant increase in the serum triglyceride ($P<0.001$) level compared to normal control. Pretreatment with quercetin 25 mg/kg p.o. ($P<0.01$), kaempferol 25 mg/kg p.o. ($P<0.01$) and WS 100 mg/kg p.o. ($P<0.05$)

significantly decreased the serum triglyceride level in AS.(Table 6.2). In CS, quercetin 25 mg/kg p.o. (P<0.01), and kaempferol 25 mg/kg p.o. (P<0.01) and WS 100 mg/kg p.o. (P<0.05) significantly reduced elevated triglyceride level (Table 6.3).

Exposure to AS (P<0.001) and CS (P<0.001) resulted in the significant increase in serum AST level as compare to respective control. Pretreatment with quercetin 25 mg/kg p.o. (P<0.001), kaempferol 25 mg/kg p.o. (P<0.001) and WS 100 mg/kg p.o. (P<0.001) significantly decreased the AST level in AS. Quercetin 25 mg/kg p.o. (P<0.001), kaempferol 25 mg/kg p.o. (P<0.001) and WS 100 mg/kg p.o. (P<0.001) significantly decreased the elevated level of AST in chronic stress (Table 6.2& 6.3).

Exposure to AS (P<0.001) resulted in the significant increase in serum ALT level as compare to normal control. Pretreatment with quercetin 25 mg/kg p.o. (P<0.01), kaempferol 25 mg/kg p.o. (P<0.01) and WS 100 mg/kg p.o. (P<0.001) significantly decreased the ALT level. In CS elevated levels of ALT was significantly lowered by pretreatment of quercetin 25 mg/kg p.o. (P<0.001), kaempferol 25 mg/kg p.o. (P<0.001) and WS 100 mg/kg p.o. (P<0.001) (Table 6.2 & 6.3).

Exposure to AS (P<0.001) and CS (P<0.01) resulted in the significant increase in serum CK level as compare to respective control. In AS Pretreatment with quercetin 25 mg/kg p.o. (P<0.001), kaempferol 25 mg/kg p.o. (P<0.001) and WS 100 mg/kg p.o. (P<0.01) significantly decreased the CK level. Pretreatment with quercetin 25 mg/kg p.o. (P<0.05), kaempferol 25 mg/kg p.o. (P<0.01) and WS 100 mg/kg p.o. (P<0.01) significantly decreased the CK level in CS (Table 6.2 and 6.3).

ii) Effect of drug treatment on acute stress (AS) and chronic stress (CS) induced alterations in ulcer index and organ weight

AS and CS exposure resulted in a significant increase in score of ulcer index. Pretreatment with quercetin 25 mg/kg p.o. ($P<0.001$), kaempferol 25 mg/kg p.o. ($P<0.001$) and WS 100 mg/kg p.o. ($P<0.01$) significantly decreased in ulcer index in comparison to AS and CS.

Exposure to AS ($P<0.001$) and CS ($P<0.01$) resulted in the significant increase the adrenal gland weight. In acute stress, pretreatment with quercetin 25 mg/kg p.o. ($P<0.05$), kaempferol 25 mg/kg p.o. ($P<0.001$) and WS 100 mg/kg p.o. ($P<0.01$) significantly restored the adrenal weight, where as in chronic stress pretreatment with kaempferol 25 mg/kg p.o. ($P<0.001$) and WS 100 mg/kg p.o. ($P<0.05$) significantly increased the reduced weight of adrenal gland (Table 6.4).

A significant decrease was found on exposure to AS ($P<0.01$) and CS ($P<0.001$) in spleen weight. The weight was increased by quercetin 25 mg/kg p.o. ($P<0.05$), only in AS and quercetin 25 mg/kg p.o. ($P<0.05$), kaempferol 25 mg/kg p.o. ($P<0.01$) and WS 100 mg/kg p.o. ($P<0.01$) in CS (Table 6.4).

A significant decrease was found on exposure to AS ($P<0.01$) and CS ($P<0.001$) in thymus weight. Pretreatment with quercetin 25 mg/kg p.o. ($P<0.05$), kaempferol 25 mg/kg p.o. ($P<0.01$) and WS 100 mg/kg p.o. ($P<0.001$) resulted a significant increase in acute stress. Chronic stress resulted in significant decrease in the weight of thymus weight ($P<0.001$). The weight was restored by the quercetin 25 mg/kg p.o. ($P<0.001$), kaempferol 25 mg/kg p.o. ($P<0.001$) and WS 100 mg/kg p.o. ($P<0.01$) CS (Table 6.4).

iii) Swimming endurance model

The survival time of swimming mice increased significantly by pretreatment with quercetin 25 mg/kg ($P<0.001$), kaempferol 25 mg/kg ($P<0.01$) and WS 100 mg/kg ($P<0.001$) compared to normal (non-drug treated)(Table 6.5). Table 6.6 shows exposure to swimming stress causes hypertrophy of adrenal gland ($P<0.01$) which is associated with significant depletion of adrenal content viz. ascorbic acid ($P<0.001$) and cortisol ($P<0.001$) compared to non swimmer group. Pre-treatment with quercetin 25 mg/kg ($P<0.001$), kaempferol 25 mg/kg ($P<0.001$) and WS 100 mg/kg ($P<0.001$) prevented the increase in adrenal weight significantly. Depletion of adrenal ascorbic acid was attenuated significantly by pretreatment with quercetin 25 mg/kg ($P<0.001$), kaempferol 25 mg/kg ($P<0.001$) and WS 100 mg/kg ($P<0.001$) Pretreatment with quercetin 25 g/kg p.o. ($P<0.001$), kaempferol 25 mg/kg ($P<0.001$) and WS 100 mg/kg ($P<0.001$) also significantly increased cortisol contents of adrenals (Table 6.7).

6.3.1.3 Discussion

Stress is nonspecific response of the body known to alter the physiological homeostasis of the organism resulting in various neuronal, endocrine and visceral dysfunctions[3]. The ability to develop and maintain resistance against a variety of stressors encountered in human life is crucial for survival. Since the characterization of the generalized adaptation syndrome by Hans Syele, the issue of stress has come to be viewed as a risk factor in both etiology and progression of a wide variety of diseases[4].

A potentially beneficial use of herbal medicine involves the use of herbs or their secondary metabolites as adaptogens in order to prevent stress induced morbidity. Herbs are not the only potential adaptogens as other agents like vitamins and even amino acids

have also exihibited this type of activity[5]. Phytochemicals play an important role in attenuating various disorders. Glycowithanolides[6,7,8], oligosaccharides[9], sitoindosides[10], ginsenosides, syringin, sesamine, caffeic acid[11] and eugenol[12] were already proved for their efficacy in combating the stress response in animal models.

Simple phenolics such as hydroxycinnamic acid conjugates and flavanoids are important constituents of fruits, vegetable and beverages. These compounds show a wide range of biological activities which is attributed to their antioxidant activity. Fruits and vegetables rich in anthocyanins demonstrated the highest antioxidant activities followed by those rich in flavanones and flavanols[13]. Flavonoids are becoming very popular because they have many health promoting effects. Some of the activities attributed to flavonoids include, anti-allergic, anti-cancer, antioxidant, anti-inflammatory and anti-viral and as adaptogen. The flavonols like quercetin and kaempferol are known for their ability to relieve hay fever, eszema, sinusitis and asthma. Epidemiological studies have illustrated that heart diseases are inversely related to flavonoid intake. Studies have shown that flavonoids prevent the oxidation of low-density lipoprotein thereby reducing the risk for the development of atherosclerosis.

Several theories have been suggested to explain the effects of adaptogenic substances. One theory argues that adaptogens function primarily due to their antioxidant and free radical scavenging effects which is found to be partially accurate[14]. More recent research postulates that adaptogens work primarily by affecting the Hypothalamic/Pituitary/Adrenal (HPA) axis and the Sympathoadrenal System. It has

been reported that the rasayanas are rejuvenators, nutritional supplements and possess antioxidant activity[15]. They also have antagonistic actions on the oxidative stressors which giving rise to the formation of different free radicals. The anti-stress/adaptogenic activity of plants made them therapeutically more important. The strong antioxidant activity of any rasayana was found to be 1000 times more potent than ascorbic acid, α-tocopherol and probucol[16].

Immobilization has been the ideal choice for the induction of stress responses in animals and more specifically, for the investigation of drug effects, on typical stress-related gastrointestinal, neuroendocrine, and immunological pathology. The distinct advantage of using immobilization as a stressor lies in the fact that it produces both physical as well as inescapable psychological stress[17].

Previous study in our laboratory proved the anti-stress activity of ethanolic fraction of *Argyreia speciosa* roots in acute and chronic stress models. Further we extended our investigation to study the anti-stress potential of two flavanols, quercetin and kaempferol isolated from *Argyreia speciosa.*

AS exposure in our study has elevated level of glucose when compare to CS. The hyperglycemic response in AS was due to release of glucocorticoids, as a result of HPA axis stimulation to compensate initial demand of energy[18]. Pretreatment with quercetin (25 mg/kg), kaempferol (25 mg/kg) and WS (100 mg/kg) significantly decreased the elevated glucose levels in blood. Stress induced hyperglycemia was inhibited by flavanols. A significant increase in serum cholesterol and triglyceride was found when

animals are exposed to acute and chronic stress. Pretreatment with quercetin (25 mg/kg), kaempferol (25 mg/kg) and WS (100 mg/kg) significantly ameliorated the elevated cholesterol and triglycerides. AS and CS significantly increased the levels of AST, ALT and CK which might be due the direct action on sympathetic nerve terminals to release the adrenaline. Pretreatment with quercetin (25 mg/kg), kaempferol (25 mg/kg) and WS (100 mg/kg) significantly reduced the elevated levels of these biochemical markers.

Acute and chronic stress increased the weight of adrenal gland and thymus, whereas the weight of spleen in decreased. Pretreatment with quercetin (25 mg/kg), kaempferol (25 mg/kg) and WS (100 mg/kg) maintained the altered weights of adrenal, thymus and spleen. The adrenal glands contain a large amounts of cholesterol, ascorbic acid and cortical steroids which are markedly decreased and cause hypertrophy when they are stimulated by stress[19,20]. Pretreatment with quercetin (25 mg/kg), kaempferol (25 mg/kg) and WS (100 mg/kg) significantly prevented the depletion of ascorbic acid and cortisol and hypertrophy of adrenal glands indicating that these flavanols have corticosteroid sparing effects.

Independently, several reports suggest the involvement of flavanoids in the prevention of various diseases. This study clearly demonstrates the value of adaptogen flvanoids for increasing the nonspecific resistance and accelerating the recovery process after physical and psychological exertion.

Table 6.2. Effect of flavanoids of *Argyreia speciosa* on the serum biochemical parameters in acute immobilization induced stress in rats

S.No	Groups/dose (mg/kg)	AST (IU/L)	ALT (IU/L)	TG (mg/dl)	TC (mg/dl)	CK (IU/L)	Glucose (mg/dl)
1	Normal control	67.45±4.74	30.25±2.13	36.02±5.12	55.03±3.12	139.0±5.29	79.55±9.08
2	Acute stress control	160.8±4.80	57.18±3.49	77.40±2.68	81.97±3.30	249.2±11.01	177.2±3.59
3	Quercetin 25	76.70±4.23^{c}	34.31±3.24^{c}	57.87±1.07^{c}	57.02±3.80^{c}	156.7±5.98^{c}	101.5±2.79^{c}
4	Kaempferol 25	72.79±8.21^{c}	34.45±1.80^{c}	53.17±1.54^{c}	56.14±2.67^{c}	158.2±4.28^{c}	90.75±2.02^{c}
5	WS100	64.13±1.89^{c}	36.46±3.24^{c}	60.64±2.08^{b}	43.88±3.91^{c}	208.3±6.54^{b}	109.1±4.40^{c}

Results are represented as mean ± SEM (n=6). $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$as compared with acute stress control group.

Table 6.3. Effect of flavanoids of *Argyreia speciosa* on the serum biochemical parameters in chronic immobilization induced stress in rats

S.No	Groups/dose (mg/kg)	AST (IU/L)	ALT (IU/L)	TG (mg/dl)	TC (mg/dl)	CK (IU/L)	Glucose (mg/dl)
1	Normal control	58.83±4.44	28.99±1.68	32.47±2.36	47.14±2.39	147.0±5.69	91.36±1.95
2	Chronic stress control	126.1±10.94	68.96±4.28	54.17±3.67	65.82±1.95	201.8±8.71	145.3±3.00
3	Quercetin 25	76.27±2.02^{f}	36.29±3.57^{f}	32.93±1.23^{f}	45.35±2.88^{f}	168.0±4.64^{d}	98.71±3.21^{f}
4	Kaempferol 25	66.41±6.44^{f}	39.99±2.63^{f}	31.66±1.47^{f}	48.76±1.00^{e}	162.2±6.77^{e}	96.30±3.02^{f}
5	WS100	72.22±4.25^{f}	39.42±0.83^{f}	35.68±2.62^{f}	49.39±1.01^{e}	160.8±6.39^{e}	91.35±1.32^{f}

Results are represented as mean ± SEM (n=6). $^{d}P < 0.05$ and $^{e}P < 0.01$ $^{f}P<0.001$ as compared with chronic stress control group.

Table 6.4. Effect of flavanoids on the weight of adrenal gland, spleen and thymus on immobilization [Acute and chronic] induced stress in rats

S. No	Groups/dose (mg/kg)	Acute immobilization stress (AS)			Chronic immobilization stress (CS)		
		Weight of adrenal gland (mg)	Weight of spleen (mg)	Weight of thymus gland (mg)	Weight of adrenal gland (mg)	Weight of spleen (mg)	Weight of thymus gland (mg)
01	Normal control	14.00±0.365	644.5±15.05	667.7±9.280	17.00±0.93^{e}	664.0±16.08^{f}	651.5±19.33
02	Acute stress control	25.17±0.477	473.7±33.59	457.2±49.81	----	-------	------
03	Chronic stress control	---	--	----	21.33±0.91	444.0±39.21	386.3±10.48
04	Quercetin 25	20.50±0.922^{a}	630.3±57.76^{c}	563.0±12.68^{a}	17.67±0.76	605.0±6.90^{d}	469.3±25.91
05	Kaempferol 25	19.00±1.31^{c}	603.8±48.79^{c}	583.5±15.40^{b}	15.17±0.47^{f}	612.7±7.67^{e}	615.0±17.23^{c}
06	WS100	19.50±1.17^{b}	599.7±10.92^{b}	608.3±8.11^{c}	15.67±0.76^{e}	620.0±14.17^{e}	623.8±19.37^{c}

Results are represented as mean ± SEM (n=6). $^{a}P<0.05$, $^{b}P<0.01$ $^{c}P<0.001$ as compared with stress control group for AS. $^{d}P<0.05$, $^{e}P<0.01$and $^{f}P<0.001$as compared with stress control group for CS.

Table 6.5. Effect flavanoids on mean swimming time (min) on swimming induced stress in mice

Sr. No	Groups	Mean Swimming Time (min)
1	Normal	198.8±10.32
2	Quercetin 25 mg/kg	248.5±6.41^{c}
3	Kaempferol 25 mg/kg	241.4±3.76^{c}
4	WS 100 mg/kg	256.3±5.43^{c}

Results are represented as mean ± SEM (n=6). $^{c}P<0.001$as compared with normal group.

Table 6.6. Effect of flavanoids on level of ascorbic acid (mg/100g of tissue) in adrenal gland on swimming induced stress in mice

Sr. No	Groups	Ascorbic acid (mg/100gm of tissue)
1	Normal control	208.14 ±10.32^c
2	Stress control	45.12±6.44
3	Quercetin 25mg/kg	168.4±8.54^c
4	Kaempferol 25 mg/kg	198.21±10.31^c
5	WS 100 mg/kg	215.5±11.30^c

Results are represented as mean ± SEM (n=6). ^{c}P<0.001as compared with stress control group.

Table 6.7. Effect of flavanoids on level of cortisol (mg/100g of tissue) in adrenal gland on swimming induced stress in mice

Sr. No	Groups	Cortisol (mg/100g of tissue)
1	Normal control	2.88±0.54^c
2	Stress control	1.05±0.05
3	Quercetin 25mg/kg	2.48±0.34^c
4	Kaempferol 25 mg/kg	2.51±0.29^c
5	WS100 mg/kg	2.58±0.40^c

Results are represented as mean ± SEM (n=6). ^{c}P<0.001 as compared with normal group.

6.3.1.4 References

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6.3.2 Antimicrobial activity of flavanoids and other fractions of *Argyreia speciosa* (Burm.f) Boj.

6.3.2.1 Introduction

Infectious diseases caused by bacteria, fungi, viruses and parasites are a major threat to public health despite tremendous growth in human chemotherapeutic medicine. Bacterial infections continue to pose a threat to health in many institutional and communal settings, including hospitals and other health care institutions, hotels, cruise liners, and damaged buildings, and epidemics are frequently reported. In addition to the increasingly frequent episodes of antibiotic-resistant Clostridium difficile–associated diarrhea (CDAD)[1-3] and methicillin-resistant *Staphylococcus aureus* (MRSA),[4] there have been recent epidemics associated with various other genera, including *Acinetobacter* and *Klebsiella*.[5-8] These and many other recent incidents demonstrate the often severe impact of persistent contamination by potentially pathogenic bacteria. There is a desperate need for a simple, effective, and safe way to remove infectious organisms within these settings. Modeling studies have shown that early intervention by appropriate decontamination also could have a substantial economic impact[9].

Tuberculosis (TB), an infectious disease caused by different species of Mycobacterium, represents a worldwide public health problem and infects >30% of the global population[10]. Nearly 2 million people died of TB, with a global case fatality rate of 23% and reaching > 50% in some African countries due to high rates of coexisting HIV infection. Man infected with HIV is very susceptible to tuberculosis. Emergence of drug resistant strains of *Mycobacterium tuberculosis* has led to increased concern on current chemotherapy regimes[11]. Worldwide increase in the incidence of morbidity and mortality

from tuberculosis prompted WHO to declare this disease a global emergency in the early 1990s[12]. The need for new antituberculosis agents is urgent due to increasing resistance of mycobacterium, together with increased incidence of severe disseminated infections produced by mycobacterium other than tuberculosis in immunocompromised patients, have prompted the search for new antimycobacterial agents, preferably those that can readily and simply be produced from some local natural plant sources. In addition to mycobacterial infectious diseases, other bacterial diseases and systemic mycoses are also difficult to medicate. An increase in the prevalence of antimicrobial resistance has made the selection of effective empiric antimicrobial therapy ever more challenging because the initiation of inadequate therapy (by in vitro susceptibilities) has been associated with much worse clinical outcomes, especially with bacteremia or pneumonia[13,14]. In most cases, the reason why an initial empiric antimicrobial regimen is inadequate and associated with therapeutic failure is resistance of the infecting isolate or isolates to the agent or agents in the regimen chosen[13,14]. Giving 2 drugs at the outset increases the likelihood that the potentially infecting microorganism will susceptible to the regimen.

Throughout history and across the globe, the plant kingdom has provided a variety of medicines. In modern times, plants have been a source of analgesics, anti-inflammatories, antineoplastic drugs, medicines for asthma, antiarrhythmic agents, and antihypertensives. Plants with antimicrobial activity are also known to be numerous, minimal research had been conducted in the area of antimicrobial medicinal plants[15]. Considering the increased incidence of severe opportunistic fungal and bacterial infections in immunologically deficient patients together with the development of

resistance among pathogenic gram positive, gram negative bacteria and *Candida albicans,* there is a great need in finding new classes of natural products that may be effective against antibiotic-resistant bacteria and fungi. Natural products or their semisynthetic derivatives provide novel examples of such anti-infective drugs[16]. Because of the resistance against antibiotics, there is a great interest in search of new antimicrobial agents from the nature[17].

6.3.2.2 Materials and Methods

a) Test microorganisms

In the present study strains used were, *Mycobacterium tuberculosis* H_{37} Rv sensitive strain ATCC 27294, Gram-positive bacteria - *Staphylococcus aureus* ATCC-11632, *Enterococcus feculis* ATCC-35550. Gram-negative bacteria - *Klebsiella pnuemoniae* ATCC-10031, *Escherichia coli* ATCC-10536, and fungi *Candida albicans* ATCC-2091, *Aspergelless fumigatus* ATCC-13073.

b) Culture medium

For *Mycobacterium tuberculosis* bioassay, Middle brook7H9 broth supplemented with 10% of albumin-dextrose-catalase and 0.2% of glycerol was used as culture medium[18]. Whereas, for the bioassay of other microorganisms, Mueller-Hinton agar for bacteria[11] and Sabraudus-dextrose agar medium for fungi was used[19]. All the test samples were sterilized by filtration using 13mm nylon acrodiscs (0.22 μm pore size).

c) Culture and growth conditions

Stock strains of mycobacterium were maintained in 7H9 broth with 0.2% glycerol at -20^{0}C. Subcultures of the microorganisms were made in Middlebrook 7H9 broth (Difco, Becton Dickinson and Co. USA) containing 10% ADC (albumin-dextrose-catalase) enrichment (Difco, BD, USA), 0.05% Tween 80, and 20 mg/ml of kanamycin

(Sigma, St. Louis,USA). Cultures of *Mycobacterium tuberculosis* were incubated for 24 hr at 37^{0}C. Following incubation, the culture suspension was sonicated for 10 sec with a Sonicator (Cole-Parmer India). To prepare the inoculum, the sonicated culture was diluted in Middlebrook 7H9 broth without kanamycin to an absorbance at 540 nm of 0.05 absorbance. This procedure yielded a suspension containing approximately 10^5 CFU/ml. This diluted suspension was used to inoculate test trays as described below.

d) Preparation of extracts

Different fractions of *Argyreia speciosa* were prepared as described earlier. Stock solution of each fraction was prepared by dissolving preweighed samples of the fraction in dimethyl sulfoxide (DMSO) to attain final concentrations of 1 mg/ml. These stock solutions were stored at -20^{0}C until further study.

e) Isolation of flavanoids

As described in 6.2

f) Antimycobacterial activity

Antitubercular screening of plant fractions and isolated compounds was obtained for *Mycobacterium tuberculosis* H_{37} Rv ATCC 27294 sensitive strain by broth dilution assay[21,22]. A frozen culture in Middle brook 7H9 broth supplemented with 10% albumin-dextrose-catalase and 0.2% glycerol was thawed and diluted in broth to 10^5 CFU/ml for *M. tuberculosis* and used as the inoculum. For assay, U-tubes (1ml) were used to accommodate test fractions and isolated compounds in the concentrations of 10, 25, 50 and 100 µg/ml. Each U-tube was then inoculated with 0.05 ml of standardized culture and then incubated at 37°C for 21 days. The Bacterial growth in U-tubes was compared with visibility against positive control (without drug), negative control (without drug and inoculum) and with standard isoniazid (INH) and rifampacin. INH, rifampacin, were solubilized in distilled water and stored at -20^{0}C.

g) Antibacterial activity

Antibacterial activity of different fractions and isolated compounds of *A. speciosa* was carried out by broth microdilution method[23]. Serial dilutions of the test fractions, isolated compounds and reference drugs were prepared in DMSO to attain a final concentration of 1mg/ml. Further progressive dilutions with Mueller-Hinton agar were performed to obtain the required concentrations of 1, 2, 4, 16, 31.25, 62.5, 125, 250 and 500 μg/ml. The tubes were inoculated with 10^5 CFU/ml (colony forming unit/ml) of each microorganism and incubated at 37°C for 18 hr. To ensure that whether solvent had any effect on the bacterial growth, a respective parallel control was performed. Minimum inhibitory concentration (MIC) of the fractions was determined. Ciprofloxacin and norfloxacin were used as standards to compare the antibacterial activity of the fractions of the plant.

h) Antifungal activity

Antifungal activity of different fractions and isolated compounds of *A. speciosa* was carried out by broth microdilution method[24]. Serial dilutions of the test fractions, isolated compounds and reference drugs were prepared in DMSO to attain a concentration of 1mg/ml. Fungal growth inhibition was determined at 25, 50, 100, 250 and 500 μg/ml concentrations The tubes were inoculated with 10^5cfu/ml (colony forming unit/ml) of each microorganism and incubated at 37°C for 18 hr. To ensure that solvent had no effect on fungal growth, a respective control was performed. Minimum inhibitory concentration (MIC) of the fractions was determined. Flucanazole was used as standard to compare the antifungal activity.

i) Synergism between flavanoids and antitubercular drugs

Solutions of flavanoids alone (50% dimethyl sulfoxide in water), and flavanoid in combination with respective antitubercular drugs were prepared by the doubling dilution method with sterilized water and were poured in to petridishes separately. Sterilized Mueller-Hinton agar (8 ml) was poured in to the above petridishes and mixed. MIC of flavanoids alone, antitubercular compounds alone and flavanoids in combination with each drug were determined. Fraction inhibitory concentration (FIC) was calculated and the interactive effects between the flavanoids and antitubercular drugs were examined[25].

j) Synergism between flavanoids and other fractions with antibiotics

Solutions of flavanoids alone (50% dimethyl sulfoxide in water), and flavanoids in combination with respective antibiotics (ciprofloxacin and norfloxacin), other fractions alone and other fractions with antibiotics were prepared by the doubling dilution method with sterilized water and were poured in to petridishes separately. Sterilized Mueller-Hinton agar (8 ml) was poured in to the above petridishes and mixed. MIC of flavanoids alone, antibiotics alone, other fractions of the plant alone and other fractions in combination with antibiotics were examined and fraction inhibitory concentration (FIC) were determined.

k) Determination of cellular toxicity to human erythrocytes

Since EAAS and isolated flavanoids showed good antibacterial activity, we investigated the cellular toxicity of RBCs. Blood was obtained from blood bank of Karanataka Medical College, Hospital and Research Centre, Hubli, India. Human erythrocytes were isolated from the blood by removing buffy coat and suspended in PBS (10mM phosphate, 150mM sodium chloride, pH 7.4) which were dispensed in sugar

tubes (10^{10} cells/500 µl/tube). The serial dilutions of EAAS and flavanoids were made and mixed with erythrocytes keeping final volume of 1 ml. The cells were incubated for 1 hr at 37°C and finally centrifuged at 1500*g* for 10 min. Lysis of the cells was observed by determining absorbance at 600 nm using colorimeter. The respective dilutions of test compounds (without erythrocytes) were used as blank for determination of absorbance. The erythrocytes were completely lysed by treatment with 1% Triton-X100 and absorbance of the released hemoglobin was taken as 100% lysis[26].

l) Assessment of *in-vivo* antimicrobial activity

Since PEAS, EAAS, EtAS and isolated flavanoids showed good activity during *in vitro* studies against *Klebseilla pnuemoniae*, we planned to assess the activity of these compounds using animals. Swiss Mice of either sex (20-22 gms) were used in the study. All the animals were given a standard pellet diet and water *ad libitum*. Animals were checked daily for their mortality and morbidity prior to commencement of the study and only healthy animals were included in the experiment. Techniques used for the bleeding, injection as well as sacrifice of the animals were approved by the Animals Ethics Committee as per CPCSEA guidelines. Each animal was challenged by 5 X 10^5 viable *Klebseilla pnuemoniae* bacteria in 200 µl of normal saline (0.9%) through intravenous route. The drug treatment was started 24 hr post infection. Suspension of PEAS, EAAS and EtAS was prepared in Tween 80 and administered orally at a dose of 100 mg/kg body weight, whereas the isolated flavanoids were dissolved in DMSO and administered at a dose of 2 µg/ml. Control group animals were given normal saline. All the test extracts were administered for 7 days and necessary precautions were taken to administer specified dose of the drug to the experimental animals.

m) Statistical analysis

Effect of treatment on the survival rate of the animals was tested by Mantel Haenzel test[27]. $P<0.05$ was considered statistically significant.

6.3.2.3 Results

EAAS fraction and flavanoids inhibited the growth of *Mycobacterium tuberculosis* H_{37} Rv ATCC 27294 sensitive strain at MIC values 50 and 25μg/ml, respectively (Table 6.9). The tested fractions inhibited the growth of bacteria at different MIC values. Among the four fractions EAAS and EtAS fractions and flavanoids of the plant showed significant activity. MIC values of fractions and flavanoids for antibacterial and antifungal activity have been represented in Table 6.8. and figure 6.9 and 6.10. All the tested fractions and flavanoids showed better activity against *Kelbsiella pneumoniae* than other organisms tested. Among the fractions tested against fungi, chloroform fraction showed significant inhibition with a lesser MIC (100 μg/ml) compared to other fractions against both the tested fungi, whereas flavanoids of the plant did not show any inhibition against fungi tested. Since flavanoids and EAAS fraction showed active against *Mycobacterium tuberculosis*, we extended our investigation to study the synergetic effect between these active compounds and commercially available antitubercular drugs. FIC index calculations, which are widely accepted method to evaluate *in vitro* synergistic studies between antitubercular compounds used in our experiments and the results, have been given in Table 6.10. A synergistic effect between flavanoids and commercially available antitubercular drugs was observed having FIC index of 0.443±0.245, 0.487±0.247 for isoniazid and 0.468±0.333, 0.417±0.345 for rifampicin whereas EAAS fraction showed partial synergistic effect having FIC index of 0.612±0.204 and 0.735± 0.247 for isoniazid and rifampicin, respectively.

Synergism study for different fractions and isolated compounds was also studied with antibiotics (ciprofloxacin and norfloxacin). A synergistic effect was observed for EAAS fraction and flavanoids having FIC index < 0.5 and partial synergism with antibiotics for other fractions having FIC index between 0.5 and 1.0. The results have been summarized in Table 6.11.

Results of hemolysis assay suggested that EAAS and flavanoids caused least hemolysis of erythrocytes as compared to chloramphenicol (Fig 6.8). Survival data showed in Table 6.12 clearly demonstrated that on day 10^{th} of post treatment of different fractions and isolated compounds of *A. speciosa*, about 60% of the animals treated with EAAS, 40% of animals treated with EtAS and 70% of animals treated with flavanoids were survived. All the control animals were died within 6 days.

6.3.2.4 Discussion

Antimicrobial activities of various plants have been reported earlier[25,26,28]. Plant derived compounds have been attracting much attention as potent alternatives for infectious diseases. Phytoconstituents present in plant extracts namely polyphenols, flavanoids, flavones, quinones, alkaloids, tannins, triterpenoids, lectins, latex, lignan, lactones, resins, monosaccharide, organic acid, coumarin, polypeptides and essential oils are providing excellent opportunity for the expansion of modern chemotherapies against wide range of resistant microorganisms[29-31]. Quercetin and kaempferol are widely distributed polyphenolic flavanoid compounds in nature. These flavanols possess anti-inflammatory[32] analgesic[33], cytotoxic[34], antioxidant and antimicrobial[35,36] activity. These compounds possessed significant action against variety of gram positive and gram negative microbes. Further, antimicrobial combinations of quercetin with antibiotics resulted in synergism without any antagonism. In the present study, Gram positive and

Gram negative bacteria, *Mycobacterium tuberculosis* and fungal strains were selected for the screening of antimicrobial effect of *A. speciosa* extracts and flavanoids to perceive the antimicrobial spectrum as well to validate ethnomedicinal assertion. Fractions were considered as active if they gave MIC≤ 500 μg/ml against bacteria and a MIC ≤ 200 μg/ml against fungal strains and a MIC ≤ 100 μg/ml against the strain of *Mycobacterium tuberculosis*. Although, some consensus on these values can be found in the literature concerning *Mycobacterium tuberculosis,* in the case of other microorganisms tested, there does not appear to be a clear criterion for determining the lower concentrations of plant extracts that can be considered as having an adequate antibacterial activity. In a recent review Rios[37] has suggested to avoid experiments with quantities higher than 1000 μg/ml. *Mycobacterium tuberculosis* H_{37} Rv strain is a standard strain used around the world to study the preliminary antituberculosis activity of different chemical entities and plant extracts. In our study, EAAS and flavanoids isolated from the n-butanol fraction of *A. speciosa* showed better activity at MIC value of 50 and 25 μg/ml, respectively. *Staphylococcus aureus,* and *Klebesiella pneumonia* were included in the study, because these species are some of the most common bacterial agents for pneumonia. Treatment of patients infected with these strains is difficult because bacteria are resistant to variety of penicillins[38-39]. *Enterococcus feculis* and *Escherichia coli* are the common bacterial strains causing GIT infections and other diseases. EAAS and EtAS fractions of *A. speciosa* showed best activity against these organisms at acceptable MIC values (4-62.5 μg/ml).

The opportunistic fungi that cause the more common pulmonary infections are *Candida albicans* and *Aspergillus fumigatus*. Fungal resistance to antibiotics in clinical use is rising, demanding the development of new antifungal agents. The activity of crude

extracts from plants against diverse microorganisms has been reported. Cimanga and Bruyne evaluated the activity and *Candida albicans*[40]. Fenner *et al.* also studied the activity of extracts from different species belonging to the genus *Hypericum* against *Candida albicans* and *Aspergillus fumigatus* [41]. Several reports regarding the antifungal activity of plant extracts against *Candida albicans* and *Aspergillus fumigatus* have been made[42]. In our study, only the chloroform fraction has shown antifungal activity against the fungi tested, at MIC value of 100 μg/ml which warrants further investigation and opportunities for the treatment of refractory infections such as those caused by *Candida albicans* and *Aspergillus fumigatus* . Isolated flavanoids were found inactive against these fungi.

Combination of antimicrobial agents with different modes of action is useful in the treatment of infectious diseases. One benefit is decrease of the administration dose of each individual agent due to synergetic effect, reducing the appearance of side effects and resistant mutants[43-44]. Fraction inhibitory concentration (FIC) was determined to study synergistic effects of active fractions and flavanoids with commercially available antitubercular drugs and antibiotics against *Mycobacterium tuberculosis* and other organisms. The EAAS fraction and flavanoids showed better synergism with drugs and organism studied. Keeping in to consideration the fact that antibiotics exert serious untoward effects to the host tissues leading to the systemic toxicity, we performed hemolysis assay which revealed that administration of *A. speciosa* fraction and isolated compounds did not lead to the unfavorable biochemical changes against human erythrocytes[26]. We extended this study in animal system as well and established the potential of *A. speciosa* fractions and isolated compounds to cure experimentally induced pneumonia infection in mice. The results clearly demonstrated that EAAS and flavanoids were significantly active against experimental pneumonia.

Hence, the results of the present investigation revealed that *A. speciosa* had antibacterial, antifungal, and antituberculosis activity. Although phytotoxic hexadecanyl p-hydroxy cinnamate and scopoletin have been isolated from the plant, this is the first report on antimicrobial, antitubercolsis activity of flavanoids from the plant. Results of the study show a good correlation between the reported uses of *Argyreia speciosa* roots for respiratory and other infections in Indian system of medicine. Finally it can be concluded that the active chemical compounds present in *A. speciosa* are useful for the treatment of bacterial infections, particularly pulmonary tuberculosis and *pneumococcal* infections.

Table 6.8. Efficacy of *A. speciosa* fractions and flavanoids against bacteria and fungi (Values are mean of two replications)

Plant fraction/ isolated compounds	MIC in µg /ml					
	Bacteria				Fungi	
	Staphylococcus aureus	*Enterococcus feculis*	*Klebsiella pnuemoniae*	*Escherichia coli*	*Candida albicans*	*Aspergelles s fumigatus*
PEAS	250	>500	4	250	>500	>500
CAS	125	62.5	31.25	250	100	100
EAAS	62.5	31.25	4	62.5	500	500
EtAS	31.25	31.25	4	62.5	250	250
Quercetin	62.5	125	2	62.5	nil	nil
Kaempferol	62.5	125	2	62.5	nil	nil
Ciprofloxacin	<5	<5	≤1	≤1	-	-
Norfloxacin	<5	<5	≤1	≤1	-	-
Flucanazole	-	-	-	-	8	8

PEAS: Petroleum Ether fraction, **CAS:** Chloroform fraction, **EAAS:** Ethyl acetate fraction, **EtAS:** Ethanol fraction.

Table 6.9. Antitubercular activity of flavanoids and different fractions of *Argyreia speciosa* against *M. tuberculosis* H_{37} Rv strain

Plant fraction/isolated compounds	MIC in µg /ml.
PEAS	>100
CAS	>100
EAAS	50
EtAS	>100
Isoniazid	0.25
Rifampacin	0.1
Quercetin	25
Kaempferol	25

PEAS: Petroleum Ether fraction, **CAS:** Chloroform fraction, **EAAS:** Ethyl acetate fraction, **EtAS:** Ethanol fraction.

Table 6.10. Synergism between potential fractions of *A. speciosa* and commercially available antitubercular drugs against *Mycobacterium tuberculosis* strain [Values are mean ± SE of two replications]

Plant fraction/isolated compounds	FIC index	
	Isoniazid	Rifampicin
EAAS	0.612±0.204	0.735±0.242
Quercetin	0.443±0.245	0.487±0.333
Kaempferol	0.487±0.247	0.467±0.345

Synergistic effect: FIC index ≤ 0.5, Partially synergistic effect: $0.5 <$ FIC index $<$ 1.0, no synergistic effect: FIC index >1.0, Antagonistic effect: FIC index ≥ 2.0.

Table 6.11. Synergism between potential fractions and flavanoids of *speciosa* with commercially available antibiotics against Bacteria [Values are mean ± SE of two replications]

Plant fraction/isolated compounds	FIC index	
	Ciprofloxacin	**Norfloxacin**
PEAS	0.842±0.322	0.752±0.208
CAS	0.654±0.291	0.545±0.372
EAAS	0.423±0.191	0.542±0.195
EtAS	0.673±0.303	0.726±0.274
Quercetin	0.324±0.246	0.425±0.328
Kaempferol	0.343±.354	0.453±0.292

PEAS: Petroleum Ether fraction, **CAS:** Chloroform fraction, **EAAS:** Ethyl acetate fraction, **EtAS:** Ethanol fraction, Synergistic effect: FIC index $\leq$ 0.5, Partially synergistic effect: 0.5 < FIC index < 1.0, no synergistic effect: FIC index >1.0, Antagonistic effect: FIC index $\geq$ 2.0.

Table 6.12. Efficacy of *A. speciosa* root fractions and isolated compounds on *K. pneumoniae* infection in mice

Plant fraction/isolated compounds	No of days/ Percentage of protection										
	0	1	2	3	4	5	6	7	8	9	10
Control	100	100	100	80	60	20	NS	NS	NS	NS	NS
PEAS	100	100	100	80	60	60	40	20	NS	NS	NS
EAAS	100	100	100	100	100	100	80	60	60	60	60
EtAS	100	100	100	100	100	80	60	60	60	40	40
Quercetin (QAS)	100	100	100	100	100	100	80	80	80	70	70
Kaempferol (KAS)	100	100	100	100	100	100	90	80	80	70	70

Swiss albino mice (n=60) were challenged with 5 x 10^5 cfu of *K. pneumoniae*. The animals were treated orally with different fractions of *A. speciosa* (100 mg/kg body weight) and flavanoids sulphates (2 mg /kg) daily for 7 days. 'P' value: Control vs. EAAS, P<0.001; Control vs. QAS, P<0.001; Control vs. KAS, P<0.001. NS: Not survived. **PEAS:** Petroleum Ether fraction, **CAS:** Chloroform fraction, **EAAS:** Ethyl acetate fraction, **EtAS:** Ethanol fraction.

Fig. 6.8. Cellular toxicity of Ethyl acetate fraction and flavanoids of *A. speciosa*

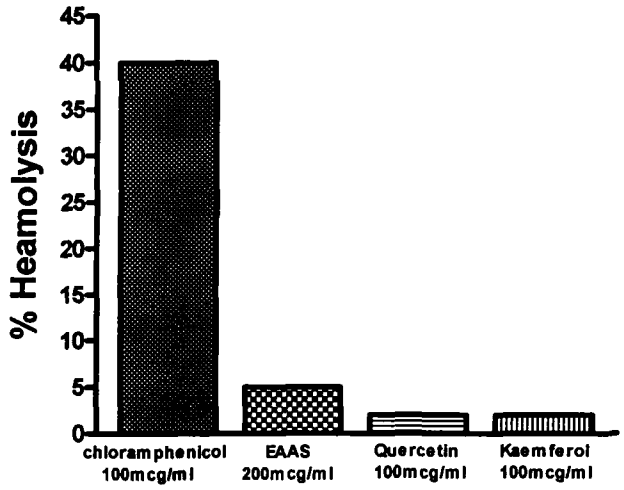
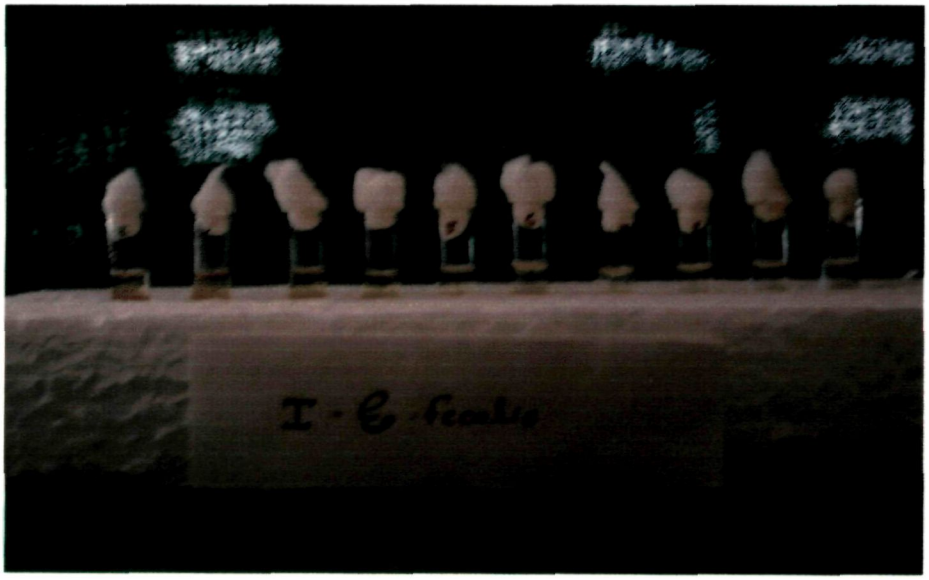


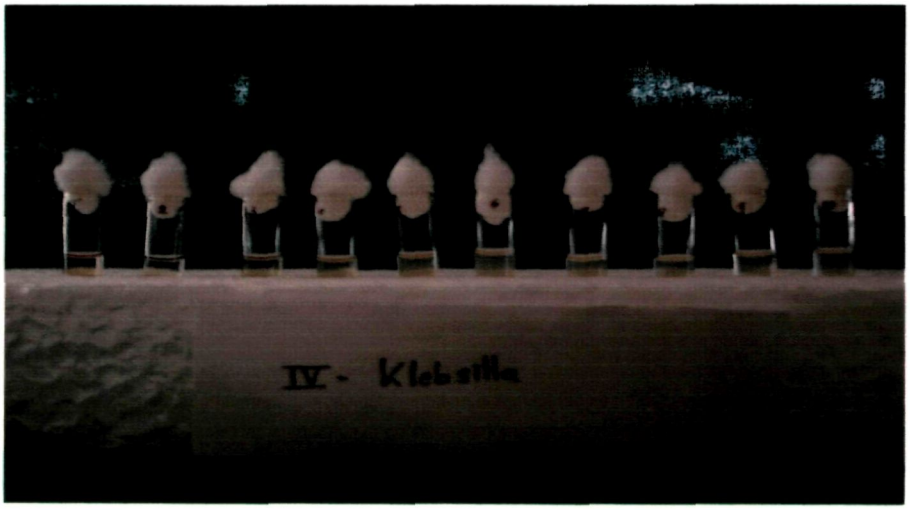
Fig. 6.9. Antibacterial activity of *Argyreia speciosa* root fractions against gram positive and Gram negative bacteria

a) ***Enterococcus feculis***



b) ***Escherichia coli***

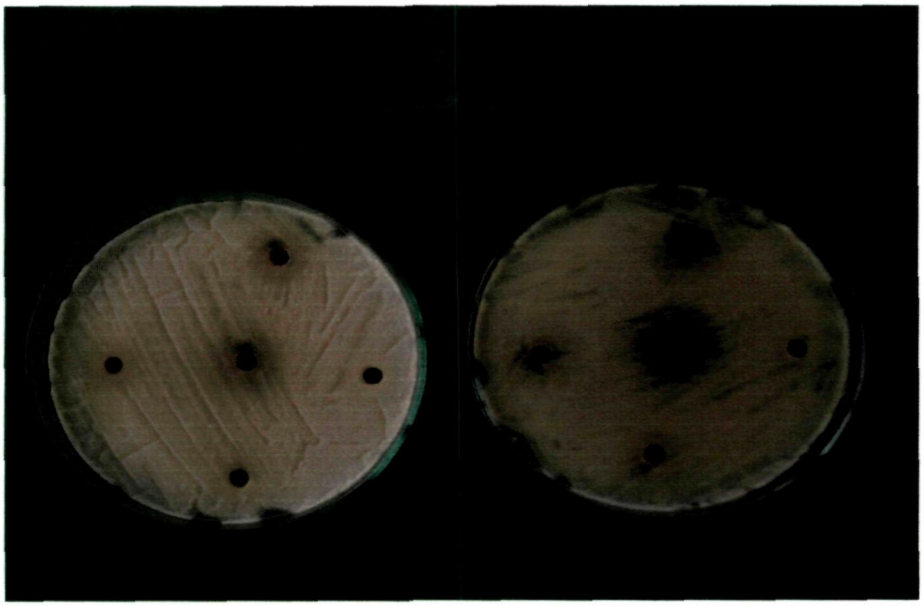
c) ***Klebsiella pnuemoniae***



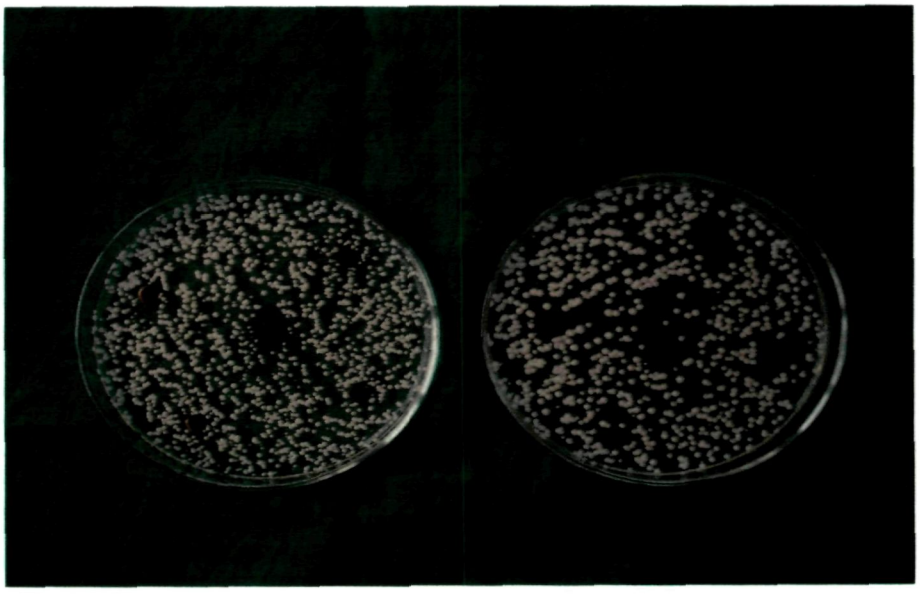
d) ***Staphylococcus aureus***

Fig. 6.10. Antifungal activity of *Argyreia speciosa* root fractions against fungi

a) ***Aspergelless fumigatus***



b) ***Candida albicans***



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Chapter - 7

Phytochemical Investigations and Pharmacological Studies of Crude Fractions and Isolated Compounds of *Habenaria intermedia* D. Don.

Chapter - 7

Phytochemical Investigations and Pharmacological Studies of Crude Fractions and Isolated Compounds of *Habenaria intermedia* D. Don.

7.1 Ethnobotany of Orchidaceae

The term orchid was coined by **Theophrastus** as anatomy of the plants resemble with testicles. Greek word orchid literally means testicles[1].This may account for use of orchids as aphrodisiacs in ancient civilizations. When we study the history of ancient alternative systems of medicine Ayurveda and Traditional Chinese Medicine (TCM) are on the forefront[3].

Orchidaeae is one of the largest families among angiosperms. According to one estimate the family includes 800 genera and 25,000 species[1]. Orchids are well known for there economic importance and widely cultivated for ornamental purposes. Orchids are cosmopolitan in distribution. *Vanilla planifolia* is commercially important orchid as it is source of vanillin used as a foodstuff flavoring [2]. *Ashtavarga* (group of eight medicinal plants) is vital part of Ayurvedic formulations like *Chyvanprasha* and four plants viz, *Riddhi (Habenaria edgeworthii* H.f.*), Vriddhi (Habenaria intermedia* D.Don), *Jivaka* (*Malaxis acuminta* D.Don) and *Rishbhaka* (*Microstylis muscifera* Ridley) have been discussed as possible members of family Orchidaceae.

7.2 Chemical nature of Orchidaceae

Traditional views in freshwater ecology have held that rates of grazing on freshwater plants are low, and therefore, chemical defenses against herbivores among these plants would be uncommon[4]. Because of this view, secondary metabolites of freshwater plants have been investigated little. Genobiotic medicinal plants of orchidaceae family are found to be more important therapeutically because of their

diversified chemical nature. These members contain phenanthrene, bibenzene, flavone, sterol, terpenes, alkoloids [5]. The characteristic feature of the large number of species from Orchidaceae family is, that their nectar contains a group of alkaloids of various chemical origins. So far over 1500 different compounds with overpowering properties, coming from the orchids, have been described [1]. The majority of them belong to two classes of compounds: pyrrolizidine alkaloids and dendrobine alkaloids[6,7,8,9]. Pyrrolizidine alkaloids are typical compounds of plant secondary metabolism and are believed to be part of the plant's chemical defense. Within the monocotyledonous plants, these alkaloids have been described in only a few genera, mainly orchids, including *Phalaenopsis species*[10]. The freshwater orchid, *Habenaria repens*, produces the structurally unusual compound, habenariol, which protects this plant against grazing by crayfish and provides one of the few examples of chemical defenses in freshwater plants[11]. Only the terrestrial climbing orchid, *Galeola faberi*, produces a structurally related compound, viz., 2-[1-methylpropyl] malate ester[12]. Five compounds were isolated from the $CHCl_3$ soluble portion of *Anoectochilus roxburghii* Wall. Lindl. and were identified as p-hydroxybenzaldehyde , ferulic acid , quercetin , daucosterol and cirsilineol. All these compounds were isolated from the plant for the first time, cirsilineol was isolated from the Orchid Family for the first time and the other compounds were isolated from this genus for the first time[13]. A novel flavanoid glucoside quercetin-7-O-β-D-[6"-O-(trans-feruloyl)]-glucopyranoside was isolated from n-butanol fraction of Anoectochilus *roxburghii*[14] .

Phenanthrene constituents are one of the marker compounds of Orchidaceae family. Viz: 7-hydroxy-2, 4-dimethoxy-9, 10-dihydrophenanthrene, 4, 7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene,2,7-dihydroxy-4-methoxy-9,10-dihydro phenanthrene, 7-hydroxy-2-methoxyphenanthrene-1,4-dione, and 7-hydroxy-2-methoxy-

9, 10-dihydrophenanthrene-1,4-dione and a novel benzyldihydrophenanthrene named arundinaol, triacontanol, 3-hydroxy-5-methoxybibenzyl were isolated from the rhizomes of *Arundina graminifolia*[15,16,17] and Cypritibetquinones A and B were isolated from the ethyl acetate residue of *Cypripedium tibeticum*[18]. Phenolic constituents like 3',4'7-trimethoxy-3,5-dihydroxyflavone, isorhamnetin-3-O-beta-D-rutinoside and rutin, steroidal compounds like lanosterol, daucosterol, beta-sitosterol were isolated from the whole herb of *Anoectochilus roxburghii* (Wall) Lindl.[19]. *Dendrobium candidum* Wall. ex Lindl. is one of the most popular and valuable *Dendrobium* species and has been recorded in the Chinese Pharmacopoeia. Two bibenzyls namely Dendrocandin-A, Dendrocandin-B have been isolated from the stems of the plant[20]. Thus, members Orchidaceae are more important chemotaxonomically for their varied chemical nature and therapeutic utility to the mankind.

7.3 Taxonomy of genus *Habenaria*

Domain	:	Eukaryota
Kingdom	:	Plantae
Subkingdom	:	Viridaeplantae
Phylum	:	Tracheophyta
Subphylum	:	Euphyllophytina
Infraphylum	:	Radiatopses
Class	:	Magnoliopsida
Subclass	:	Lamiidae
Order	:	Asparagales
Family	:	Orchidaceae
Subfamily	:	Orchidoideae
Genus	:	*Habenaria*

A large genus of orchids found in tropical and temperate parts of the world. About species are reported in India, of which two are of minor economic importance.

Fig. 7.1 Photographs showing aerial parts and tubers of *Habenaria intermedia* D.Don.

7.4 Traditional uses of the tubers

Habenaria Intermedia D.Don. (Orchidaceae), is commonly known as *Vrddhi* in Indian system of medicine. The edible tubers are sweet, emollient, and used as intellect promoting, aphrodisiac, depurative, anthelmintic, rejuvenating and tonic. Tubers are also useful in asthma, leprosy and skin diseases. This plant is an important ingredient of *Chyavanprasha,* a well known polyherbal rejuvenator[22,23].

7.5 Earlier studies on *Habenaria intermedia* D. Don

- Antioxidant activity of polyherbal formulation containing tubers of *Habenaria intermedia* was investigated in nitric oxide scavenging activity[24].

7.6 Procurement and authentication of the plant material

Tubers of *H. Intermedia* D. Don. were obtained from Forest research institute, Dehra Dun, India and authenticated by qualified taxonomist, Department of Botany, Karnataka University, Dharwad. A herbarium specimen was kept in Dept. of Pharmacognosy (SETCPD/Ph.cog/herb/36/2007). The collected tubers were washed with running water. The tubers were chopped in to small pieces and dried under shade. Dried tubers were coarsely powdered and used for extraction.

7.7 Extraction

The tubers were dried under shade and powdered. Dried powder was exhaustively extracted successively using petroleum ether (PEHI) (60-80), Chloroform (CHI) Ethyl acetate (EAHI) and Ethanol (95%) (EtHI) respectively. All the extracts were concentrated by rotary flash evaporator, under reduced pressure and controlled temperature, followed by freeze drying and stored in a descicator.

7.8 Preliminary Phytochemical investigation of crude fractions of *Habenaria intermedia* D. Don

Tubers were collected according to their appropriate seasons and both the fractions were subjected to qualitative phytochemical investigation using following standard tests to identify the type(s) of phytoconstituents as described in **5.8** [25,26].

7.9 Results

Table 7.1. Percentage yield of crude fractions of *Habenaria intermedia* tuber

S.No	Name of the fraction	Colour	Consistency	Percentage yield (w/w)
01	Petroleum Ether fraction (PEHI)	Reddish brown	Semisolid	1.5
02	Chloroform fraction (CHI)	Dark green	Solid (light green)	2.0
03	Ethyl acetate fraction (EAHI)	Yellowish black	Semisolid	0.9
04	Ethanol fraction (EtHI)	Brown	Sticky solid	2.5

Table 7.2. Qualitative phytochemical analysis of crude fractions of *Habenaria intermedia* tuber

S.No.	Name of the phytoconstituent	PEHI	CHI	EAHI	EtHI
01	Carbohydrates	-	-	-	+
02	Steroids	-	+	-	-
03	Alkaloids	+	+	-	-
04	Proteins and Amino acids	-	-	-	+
05	Tannins	-	-	-	+
06	Saponins	-	-	-	-
07	Triterpenoids	-	-	-	-
08	Flavanoids	-	-	+	+
09	Coumarin glycosides	-	-	+	+

7.10 References

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7.11 Pharmacological evaluation of *Habenaria intermedia* D. Don.

Crude fractions of *Habenaria intermedia* tuber were subjected to its traditionally claimed Nootropic (intellectual promoting), anti-stress (rejuvenator), activities using animal models.

7.11.1 Anti-stress activity of *Habenaria intermedia* D. Don.in acute and chronic stress paradigm in rats

7.11.1.1 Introduction

Habenaria intermedia D.Don (Orchidaceae) is an important member of rasayana herbs in Ayurveda. Traditionally it is used in many herbal preparations for its rejuvenating properties. Hence, anti-stress potentiality of crude fractions of *H. intermedia* was investigated using acute and chronic stress models in rodents.

7.11.1.2 Material and Methods

a) Drugs and chemicals

As described in 5.11.3.2a

b) Acute toxicity studies

Acute toxicity study was carried out using Swiss albino mice (25-30 gms) by up and down / staircase method as per CPCSEA guidelines. EtHI and EAHI fractions were orally administered to different groups of young and aged mice at doses of 50 mg, 300 mg, 1000 mg and 2000 mg / kg body weight respectively. Animals were observed for 48 hrs to study the general behavior of animals, signs of discomfort and nervous manifestations.

c) Preparation of drugs

EAHI and EtHI were suspended in 0.5% gum acacia, and a fine emulsion was made having uniform particle distribution. The emulsion of both the extracts was administered for orally daily for three days in case of acute stress (AS) and for 7 days in

case of chronic stress (CS). Both the drugs were prepared fresh daily before administration.

d) Animals

Albino rats of either sex (150-200 gms) and Swiss albino mice (20-25 gms) were used in the study. They were housed three to four per cage at temperature 22±2°C at 12:12 hr, light: dark under controlled environment. Rats were fed standard laboratory food and water was given *ad libitum*. Rats were kept for 7 days in laboratory for habituation.

e) Stress Protocol [1]

As described in 5.11.3.2d

f) Swimming endurance test [2]

As described in 5.11.3.2f

g) *In-vitro* anti-oxidant activity[3]

As described in 5.11.3.2g

h) Statistical analysis

The results were expressed as mean ± S.E.M. The statistical significance was determined by two-way ANOVA followed by a post hoc Tukey's test. A probability P value of less than 0.05 was taken to indicate statistical significance.

7.11.1.3 Results

A) Immobilization Induced Stress

i) Effect of drug treatment on acute stress (AS) and chronic stress (CS) induced alterations in biochemical parameters:

Table 7.3 and 7.4 showed that exposure to AS ($P<0.001$) resulted in a significant increase in the serum glucose level which is higher when compared to CS ($P<0.001$). Pretreatment with EAHI 200 mg/kg ($P<0.001$), EtHI 100 mg/kg ($P<0.001$), EtHI 200 mg/kg ($P<0.001$) and WS 100 mg/kg ($P<0.001$) significantly decreased the circulating glucose level in AS. Where as increased level of glucose was also decreased by pretreatment with EAHI 200 mg/kg, EtHI 100 mg/kg ($P<0.001$), EtHI 200 mg/kg ($P<0.001$) and WS 100 mg/kg ($P<0.001$) in CS.

Exposure to AS and CS resulted in significant increase in the total cholesterol ($P<0.001$) and triglyceride ($P<0.001$) levels compared to normal control. Pretreatment with EtHI 100 and 200 mg/kg ($P<0.001$), EAHI 100 and 200 mg/kg ($P<0.01$) and WS 100 mg/kg ($P<0.001$) significantly decreased the circulating total cholesterol level in AS and CS. Moreover pretreatment with EAHI 200 mg/kg ($P<0.05$) only shown the significant reduction in triglyceride level in AS, Where as in CS, EtHI 100 mg/kg ($P<0.01$), and EtHI 200 mg/kg ($P<0.001$) significantly reduced elevated triglyceride levels in dose dependant manner. (Table 7.3 and 7.4).

Exposure to AS ($P<0.001$) and CS ($P<0.01$) resulted in the significant increase in serum AST level as compare to respective control. Pretreatment with EAHI 200 mg/kg, ($P<0.05$), EtHI 100 mg/kg ($P<0.001$), EtHI 200 mg/kg ($P<0.001$) and WS 100 mg/kg ($P<0.001$) significantly decreased the AST level in AS. In CS, EtHI mg/kg 100 ($P<0.01$)

and EtHI 200 mg/kg (P<0.001) significantly reduced AST level in dose dependant manner (Table 7.3 and 7.4).

Exposure to AS (P<0.001) resulted in the significant increase in serum ALT level as compare to normal control. Pretreatment with EAHI 200 mg/kg (P<0.05) EtHI 100 mg/kg (P<0.01), EtHI 200 mg/kg (P<0.001) and WS 100 mg/kg (P<0.001) significantly decreased the ALT level. In CS elevated levels of ALT was significantly lowered by pretreatment of EtHI 100 mg/kg (P<0.01), EtHI 200 mg/kg (P<0.01) and WS 100 mg/kg po (P<0.01) (Table 7.3 and 7.4).

Exposure to AS (P<0.001) and CS (P<*0.*01) resulted in the significant increase in serum CK level as compare to respective control. In AS Pretreatment with EAHI 200 mg/kg (P<0.01), EtHI 100 mg/kg (P<0.001), EtHI 200 mg/kg (P<0.001) and WS 100 mg/kg (P<0.001) significantly decreased the CK level. Pretreatment with EtAS 100 mg/kg (P<0.01), EtHI 200 mg/kg (P<0.001) and WS 100 mg/kg (P<0.01) significantly decreased the CK level in CS (Table 7.3 and 7.4).

ii) Effect of drug treatment on acute stress (AS) and chronic stress (CS) induced alterations in ulcer index and organ weight

Table 7.6 and shows that AS and CS exposure resulted in a significant increase in score of ulcer index. Pretreatment with EAHI 200 mg/kg (P<0.001), EtHI 100 mg/kg (P<0.001), EtHI 200 mg/kg (P<0.001) and WS 100 mg/kg (P<0.001) significantly decreased in ulcer index in comparison to AS and CS.

Exposure to AS (P<0.001) and CS (P<*0.*01) resulted in the significant increase the adrenal gland weight. Pretreatment with EAHI 200 mg/kg (P<0.001), EtHI 100 mg/kg (P<0.001), EtHI 200 mg/kg (P<0.001) and WS 100 mg/kg (P<0.001) significantly reduced the increased adrenal weight in AS where as in CS, EtHI 100 mg/kg (P<0.001),

EtHI 200 mg/kg (P<0.001) and WS 100 mg/kg (P<0.001) significantly restored the adrenal weight (Table 7.5).

. A significant decreased was found on exposure to AS (P<0.01) and CS (P<0.001) in spleen weight. The weight was increased by EAHI 100 and 200 mg/kg (P<0.05), EtHI 100 and 200 mg/kg (P<0.05) and WS 100 mg/kg (P<0.05) in AS and EAHI 200 mg/kg (P<0.05), EtAS 100 mg/kg (P<0.01), EtHI 200 mg/kg (P<0.01) and WS 100 mg/kg (P<0.001) in CS, (Table 7.5).

. CS resulted in significant decrease in the weight of thymus weight (P<0.001). In AS EAHI 200 mg/kg (P<0.01), EtHI 200 mg/kg and WS 100 mg/kg increased the thymus weight. The weight was restored by the EtHI 100 and 200mg/kg (P<0.001) and WS 100 mg/kg (P<0.001) in CS, (Table 7.5).

B) Swimming endurance model

The survival time of swimming mice increased significantly in dose dependent manner by pretreatment with EAHI 200 mg/kg (P<0.05), EtHI 100 mg/kg (P<0.05) and EtHI 200 mg/kg (P<0.001) compared to normal (non-drug treated) (Table 7.7, figure 7.2). The results of standard drug WS 100 was also found to be significant. Table 7.8 and figure 7.3 shows exposure to swimming stress causes hypertrophy of adrenal gland (P<0.05) which is associated with significant depletion of adrenal content viz. ascorbic acid (P<0.001) (Table 7.9 and Fig. 7.4) and cortisol (P<0.001) (Table 7.10 and Fig. 7.5). When compared to non swimmer group. Pre-treatment with EAHI 200 mg/kg (P<0.001), EtHI 100 mg/kg (P<0.05), EtHI 200 mg/kg (P<0.001) and WS 100 mg/kg (P<0.01) prevented the increase in adrenal weight significantly (Table 7.9, figure 7.4) Depletion of adrenal ascorbic acid was attenuated significantly by pretreatment with EAHI 200 mg/kg (P<0.05), EtHI 100 (P<0.001), EtHI 200 mg/kg (P<0.001) and WS 100 mg/kg (P<0.01).

Pretreatment with EAHI 200 mg/kg ($P<0.05$), EtHI 100 mg/kg ($P<0.001$), EtAS 200 mg/kg ($P<0.001$) and WS 100 mg/kg ($P<0.001$) also significantly increased cortisol contents of adrenals.

C) *In-vitro* free radical scavenging activity

i) Reaction with DPPH radical

Ethanolic extract of *Habenaria intermedia* (EtHI) showed DPPH scavenging activity with IC_{50} levels of 35.46 µg/ml, as compared with the IC_{50} value of the standard ascorbic acid (2.94 µg/ml) , where as EAHI showed DPPH scavenging activity with IC_{50} levels of 32.88 µg/ml (figure 7.6a and 7.7a) .

ii) Reaction with hydroxyl radical

The ability of the EtHI and EAHI to scavenge the hydroxyl radical was found to be 52.38 µg/ml and 11.28 µg/ml respectively and IC_{50} value of standard Mannitol was found to be 4.99 µg/ml (figure 7.6c and 7.7c).

iii) Lipid peroxidation (LPO) assay

The inhibition of LPO by EtHI was found to be 122.62 µg/ml where as EAHI inhibit LPO with IC_{50} 42.75 µg/ml (figure 7.6b and 7.7b).

7.11.1.4 Discussion

Biological stress is a response to physical, chemical, biological and emotional changes, consisting of a pattern of metabolic and behavioral reactions that helps to strengthen the organism. The management of unusual stress therefore has acquired enormous significance in day-to-day life[4]. Various attempts have been made to counter the aversive effects of stress, ranging from yoga and meditation to anti-stress drugs, particularly the anxiolytic benzodiazepines.

Due to the nonspecific nature of the stress pathogenesis, a separate class of therapeutic agents was evolved known as **"adaptogens"**. Ayurveda, the Indian system of medicine documents several herbs, which are categorized as rasayanas. The properties ascribed to rasayanas in Ayurveda are remarkably similar to those of adaptogens[5]. However, supplementation with various macro and micronutrients and herbal preparations has been evaluated for their adaptogenic activity during exposure to a stressful environment [6,7,8,9].

A variety of stress situations have been employed to investigate the consequences of stress and to evaluate anti-stress agents and the lack of consistency of stress protocols and their biological consequences is astounding. Immobilization has been the ideal choice for the induction of stress responses in animals and more specifically, for the investigation of drug effects, on typical stress-related gastrointestinal, neuroendocrine, and immunological pathology. Immobilization model used in our study found to cause long term desensitization of HPA response which affected both peripheral and central components of the HPA axis[10]. The distinct advantage of using immobilization as a stressor lies in the fact that it produces both physical as well as inescapable psychological stress [11].

In this investigation, the effect of Ethyl acetate (EAHI) and ethanol (EtHI) fractions of *Habenaria intermedia* was evaluated using acute and chronic immobilization stress immobilization stress. AS exposure in our study has elevated level of glucose compared to CS. The hyperglycemic response in AS was due to release of glucocorticoids, as a result of HPA axis stimulation to compensate initial demand of energy[23]. The acute demand of glucose was fulfilled by the increase in glucogenolysis from liver during AS. During CS, this available source depletes. Thus, it utilizes fat as a secondary substrate and gluconeogenesis starts in response to corticosterone and, or due

to the compensation of the energy demand during chronic conditions is from non-carbohydrate origin which are slow and rate limiting. Pretreatment with the EAHI 100 and 200 mg/kg , EtHI 100 mg/kg, EtHI 200 mg/kg and WS 100 mg/kg in AS, while during CS, EAHI 200 mg/kg, EtHI 100 mg/kg , EtHI 200 mg/kg and WS 100 mg/kg significantly decreased the circulating glucose level thus it seems to have a direct action on peripheral metabolism.

The AS and CS raised the serum cholesterol level through the enhanced activity of hypothalamo-hypophyseal axis resulting in increased liberation of catecholamine and corticosteroids. The effect of AS and CS stress on serum triglyceride has been shown to be variable probably due to mobilization of fats from adipose tissue by catecholamine. Treatment with EAHI 200 mg/kg, EtHI 100 mg/kg, EtHI 200 mg/kg and WS 100 mg/kg ameliorated the elevated levels of cholesterol as well as triglyceride levels in both AS and CS. The suppression of stress induced triglyceride level may be due to the suppression of stress induced lipolysis.

The AS-induced significant increase in ALT, AST, and CK might be the outcome of AS induced secretion of corticosterone from cortex, epinephrine from medulla, and epinephrine from sympathetic nerve terminals to provide substrate for energy metabolism and the assurance of availability of ATP demand in the muscles, CNS, and organ of demand. In contrast to ALT, which is found primarily in liver, AST is present in many tissues, including the heart, kidney, brain, and skeletal muscles. Stress hormones also increase CK activity during stress. The CK system is important in stabilizing the ATP levels and energy metabolism of the myocardium and other skeletal muscles of rats during stress. Perturbations of CK activity during extensive stress may result in ischemia due to the non-availability of ATP. A maximum increase in CK activity was observed after AS exposure when compared to CS. A reduced CK activity in

CS as compared to AS is due to partial habituation. Pretreatment with EAHI 200 mg/kg, EtHI 100 mg/kg and 200 mg/kg, WS 100 mg/kg revert the AS induced levels of AST, ALT and CK in blood. In CS, EtHI 100 mg/kg and 200 mg/kg, WS 100 mg/kg were found to be effective. Reduction in ALT and AST by the test fractions may be due to direct action on the peripheral metabolism and of CK may be due to decrease in energy demand.

Stressful events activate autonomic and endocrine responses[12] responsible for gastric ulceration. In our study AS and CS induced ulceration in stomach with comparable intensity in both the models. Gastric damage induced by CS and AS has been reduced by EAHI 200 mg/kg, EtHI 100&200 mg/kg and WS 100 mg/kg by decreasing mean ulcer index indicating their protective effects on gastric mucosa during stressful conditions.

Stress-induced adrenal hypertrophy found both in AS and CS was the result of activation of the HPA axis, which is highly responsive to stress and is one of the principal mechanism by which an organism mobilizes its defense against stress events[14]. The prolonged activation of HPA axis resulted in an increase in the adrenal hypertrophy in CS as compared to AS. During stress, nerve terminals accelerate recruitment of lymphocytes to blood from spleen, which is a major storage pool of lymphocytes. This result in the squeezing of the spleen causing reduction in weight observed in AS and CS exposures. The atrophy of thymus was found only during AS & CS exposure may be due to apoptosis and necrosis in immature T and B cells resulting in the decline of thymus weight. Our study showed that Pretreatment with EAHI 200 mg/kg EtHI 100 mg/kg, EtHI 200 mg/kg and WS 100 mg/kg significantly reduced the increased adrenal weight and increased the decreased weight of thymus in AS and CS, where as pretreatment with

EAHI 100 and 200 mg/kg, EtHI 100 and 200 mg/kg , and WS 100 mg/kg significantly reverse the stress induced atrophy of spleen.

Forced swimming stress makes the individual immobile after an initial period of vigorous activity. This resembles a state of mental depression. The adrenal glands contain relatively large amount of ascorbic acid and cortisol which are markedly reduced by stress and causes hypertrophy[13,14]. Our results showed that pretreatment with EAHI 200 mg/kg, EtHI 100 and 200 mg/kg, WS 100mg increased labor efficiency and increase of swimming performance. Moreover, prevented the depletion of ascorbic acid, cortisol and hypertrophy of adrenal glands indicating that, drug having steroid sparing effects.

It has been shown that exposure to stress situations can stimulate numerous pathways leading to increased production of free radicals. Both immobilization and variable stress are followed by an increase in lipid peroxidation, measured in plasma and in brain structures. Previous studies on *Habenaria intermedia* containing formulation have shown the NO scavenging activity [16]. Results of *in vitro* free radical scavenging activity carried, using DPPH , hydroxyl radical and LPO for ethanolic extract (EtHI) and ethyl acetate extract (EAHI) shown to have potent free radical scavenging effect when compared with there respective standard.

The result of the present study suggestd that Ethyl acetate fraction (EAHI) at 200 mg/kg significantly balanced the altered biochemical parameters in acute stress, where as the Ethanol fraction (EtHI) at 100 and 200 mg/kg was effective in both acute and chronic stress models. The observed anti-stress activity of *Habenaria intermedia* may be due to its potent anti-oxidative effects.

Table 7.3. Effect of EtHI, EAHI on the serum biochemical parameters in acute immobilization induced stress in rats

S.No	Groups/dose (mg/kg)	AST (IU/L)	ALT (IU/L)	TG (mg/dl)	TC (mg/dl)	CK (IU/L)	Glucose (mg/dl)
1	Normal control	66.83±6.66^{c}	20.06± 0.49^{c}	33.68±2.38^{c}	43.88±3.91^{c}	138.7± 3.57^{c}	78.92±1.18^{c}
2	Acute stress control	162.7±5.92	44.20±0.88	77.50±5.04	75.55±5.71	261.5± 8.87	163.5±4.38
3	EAHI 100	125.4±12.71	38.09±1.88	55.85±1.49	65.99±2.45	268.2±11.15	149.6±8.55
4	EAHI 200	116.0±8.04^{a}	32.83±2.30^{a}	48.64±1.66^{a}	64.03±2.16	218.7± 4.27^{b}	112.6±6.46^{c}
5	EtHI 100	94.83±4.188^{c}	30.37±1.31^{c}	44.74±3.02^{b}	51.31±2.23^{c}	165.8±2.81^{c}	107.0±6.01^{c}
6	EtHI 200	81.61±37.63^{c}	26.93±1.76^{c}	42.82±3.79^{b}	52.69±6.25^{c}	155.7± 3.84^{c}	92.31±1.03^{c}
7	WS100	66.01±3.28^{c}	27.24±2.38^{c}	42.23±1.987^{b}	47.33±0.87^{c}	160.8±6.39^{c}	89.12±1.37^{c}

Results are represented as mean ± SEM (n=6). $^{d}P<0.05$ and $^{e}P<0.01$ $^{f}P<0.001$as compared with chronic stress control group.

Table 7.4. Effect of EtHI, EAHI on the serum biochemical parameters in chronic immobilization induced stress in rats

S.No	Groups/dose (mg/kg)	AST (IU/L)	ALT (IU/L)	TG (mg/dl)	TC (mg/dl)	CK (IU/L)	Glucose (mg/dl)
1	Normal control	34.68±1.55^{f}	27.03±2.77^{f}	36.18±5.03^{f}	33.61±3.92^{f}	147.0±5.69^{e}	85.75±4.26^{f}
2	Chronic stress control	86.78±20.76	42.35±2.23	88.09±15.98	67.49±3.22	202.7±8.79	137.1± 4.23
3	EAHI 100	76.69±4.19	40.62±2.59	39.77±2.229	48.64±1.30^{d}	197.3±16.16	134.4±10.50
4	EAHI 200	72.79± 7.23	29.62±0.526^{f}	30.83±1.19^{f}	45.31±31.32^{e}	170.3±11.12	107.4± 5.15
5	EtHI 100	22.48±0.653^{e}	27.84±1.76^{f}	43.27±3.26^{e}	48.49±1.94^{d}	156.7±5.98^{f}	95.40± 12.12^{f}
6	EtHI 200	21.20±0.77^{f}	26.91±2.31^{f}	38.13±7.56^{f}	46.56±3.64^{e}	158.2±4.28^{f}	91.36±10.41^{f}
7	WS100	20.88±0.83^{d}	26.63±0.97^{f}	36.82±0.10^{f}	45.17±2.46^{f}	147.7±4.25^{f}	89.12±5.07^{f}

Results are represented as mean ± SEM (n=6). $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$as compared with acute stress control group.

Table 7.5. Effect of EtHI, EAHI on the weight of adrenal gland, spleen and thymus on immobilization [Acute and chronic] induced stress in rats

S. No	Groups/dose (mg/kg)	Acute immobilization stress (AS)			Chronic immobilization stress (CS)		
		Weight of adrenal gland (mg)	Weight of spleen (mg)	Weight of thymus gland (mg)	Weight of adrenal gland (mg)	Weight of spleen (mg)	Weight of thymus gland (mg)
01	Normal control	14.00±0.36^{c}	644.5±15.05^{b}	667.7±9.28^{c}	17.00±0.93^{e}	664.0±16.08^{f}	651.5±19.33^{f}
02	Acute stress control	23.17±1.25	473.7±33.59	619.3±27.93	----	-------	------
03	Chronic stress control	---	--	----	21.33±0.91	444.0±39.21	386.3±10.48
04	EAHI 100	20.67±0.95	559.5±13.61	578.2±22.97	21.50±0.84	459.2±18.47	446.3±8.81
05	EAHI 200	17.33±0.55^{c}	617.5±5.00^{a}	599.2 ±3.19^{b}	19.33±1.25	623.3±9.73^{e}	437.7 ±8.82
06	EtHI 100	16.33±0.33^{c}	605.0±6.90	563.0±12.68	16.33±0.55^{d}	603.8±48.79^{d}	579.8 ±7.44^{f}
07	EtHI 200	15.33±0.49^{c}	622.5±7.11^{a}	604.5 ±7.22^{b}	15.17±0.47^{f}	630.3±57.76^{e}	607.0 ±14.39^{f}
08	WS100	14.83 ±0.47^{c}	620.0±14.17^{b}	608.3±8.11^{b}	15.67± 0.76^{f}	667.3±40.36^{f}	617.3 ±10.86^{f}

Results are represented as mean ± SEM (n=6). $^{a}P<0.05$, $^{b}P<0.01$ $^{c}P<0.001$ as compared with stress control group for AS. $^{d}P<0.05$, $^{e}P<0.01$and $^{f}P<0.001$ as compared with stress control group for CS.

Table 7.6. Effect EtHI and EAHI on ulcer index on immobilization [Acute and chronic] induced stress in rats

Sr. No	Groups	Ulcer index	
		Acute stress(AS)	Chronic stress(CS)
1	Normal control	0.0±0.0	0.0±0.0
2	Stress control	11.00± 0.89	14.33± 1.1
3	EAHI 100	9.500± 0.61	14.17± 0.47
4	EAHI 200	7.00± 0.68^{c}	8.50± 0.42^{f}
5	EtHI 100	5.50± 0.42^{c}	5.83± 0.60^{f}
6	EtHI 200	3.50± 0.42^{c}	3.167±0.47^{f}
7	WS100	2.83± 0.47^{c}	2.83± 0.47^{f}

Results are represented as mean ± SEM (n=6). $^{c}P<0.001$ as compared with stress control group for AS. $^{f}P<0.001$ as compared with stress control group for CS.

Table 7.7 Effect EtHI and EAHI on mean swimming time (min) on swimming induced stress in mice

Sr. No	Groups	Mean Swimming Time (min)
1	Normal	209.0±7.63
2	EAHI 100	190.3±10.79
3	EAHI 200	236.3±3.18^{a}
4	EtHI 100	235.7±1.54^{a}
5	EtHI 200	250.8±5.25^{c}
6	WS100	267.3±3.77^{c}

Results are represented as mean ± SEM (n=6) $^{a}P<0.05$, and $^{c}P<0.001$ as compared with normal group.

Fig. 7.2. Effect of EtHI and EAHI on mean swimming time (min) on swimming induced stress in mice

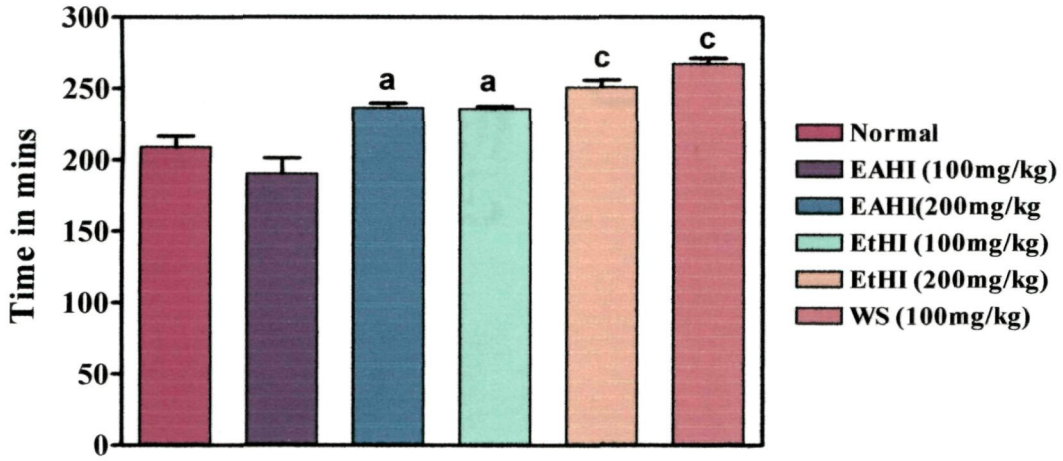


Table 7.8. Effect of EtHI and EAHI on weight of adrenal gland (mg/100 body weight) on swimming induced stress in mice

Sr. No	Groups	Weight of adrenal gland (mg/100 gm BW)
1	Normal control	1.312 ± 1.31^{c}
2	Stress control	21.84 ± 1.79
3	EAHI 100	16.69 ± 0.473
4	EAHI 200	14.25 ± 0.89^{c}
5	EtHI 100	14.22 ± 0.83^{c}
6	EtHI 200	13.25 ± 1.10^{c}
7	WS100	12.51 ± 1.07^{b}

Results are represented as mean ± SEM (n=6). $^{b}P<0.01$ and $^{c}P<0.001$as compared with stress control group.

Fig. 7.3 Effect of EtHI and EAHI on weight of adrenal gland mg/100gm bodyweight on swimming induced stress in mice

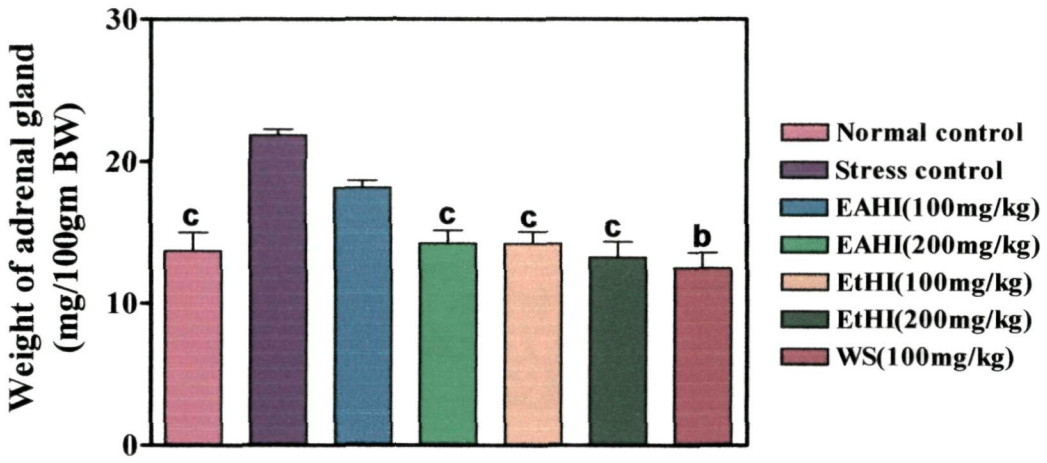


Table 7.9. Effect EtHI and EAHI on level of ascorbic acid (mg/100g of tissue) in adrenal gland on swimming induced stress in mice

Sr. No	Groups	Ascorbic acid (mg/100gm of tissue)
1	Normal control	196.8 ±16.76^{c}
2	Stress control	22.03±4.04
3	EAHI 100	65.62±8.51
4	EAHI 200	99.06±4.73^{a}
5	EtHI 100	156.2±19.79^{c}
6	EtHI 200	204.1±19.88^{c}
7	WS100	229.4±16.94^{c}

Results are represented as mean ± SEM (n=6).^{a}P<0.05, ^{c}P<0.001 as compared with stress control group.

Fig. 7.4. Effect of EtHI and EAHI on level of in adrenal ascorbic acid (mg/100 gm of tissue) on swimming induced stress in mice

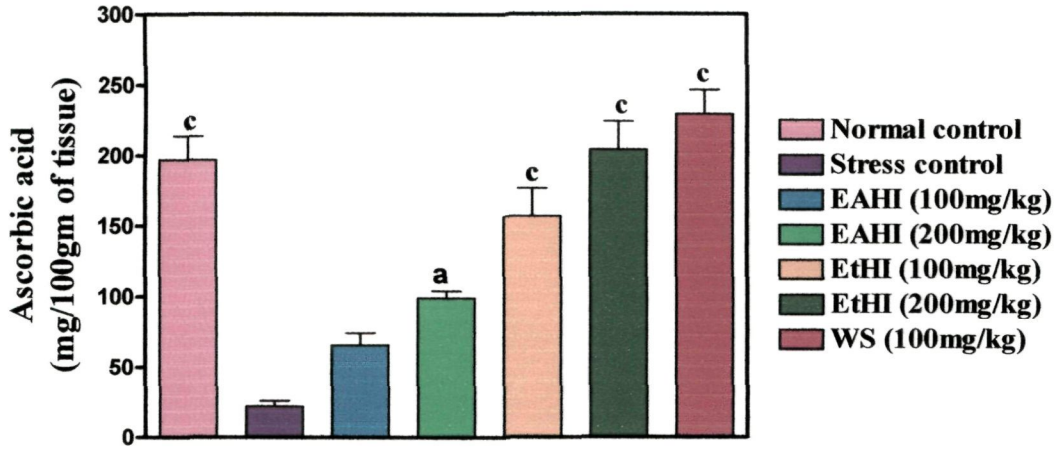
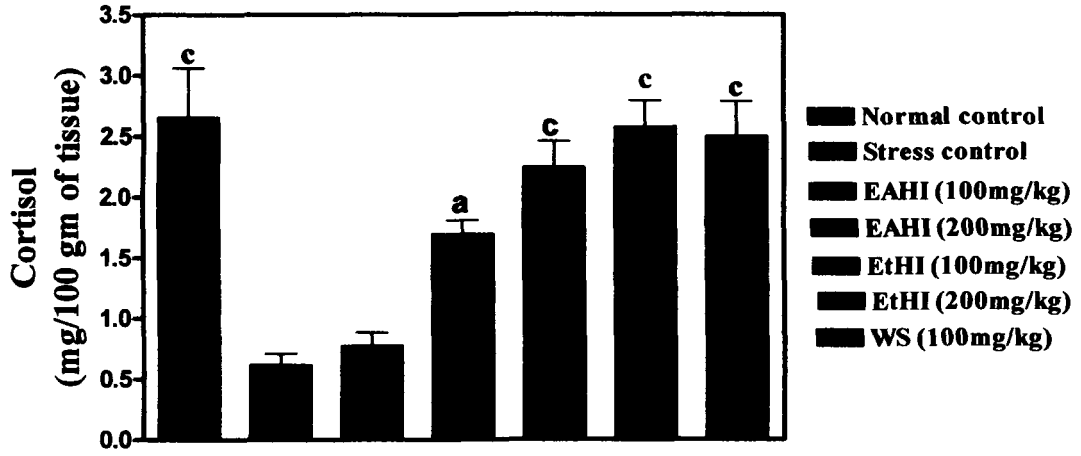


Table 7.10. Effect of EtHI and EAHI on level of cortisol (mg/100g of tissue) in adrenal gland on swimming induced stress in mice

Sr. No	Groups	Cortisol (mg/100g of tissue)
1	Normal control	2.65±0.40^{c}
2	Stress control	0.61±0.09
3	EAHI 100	0.77±0.10
4	EAHI 200	1.69±0.11^{a}
5	EtHI 100	2.24±0.21^{c}
6	EtHI 200	2.57±0.21^{c}
7	WS100	2.49±0.28^{c}

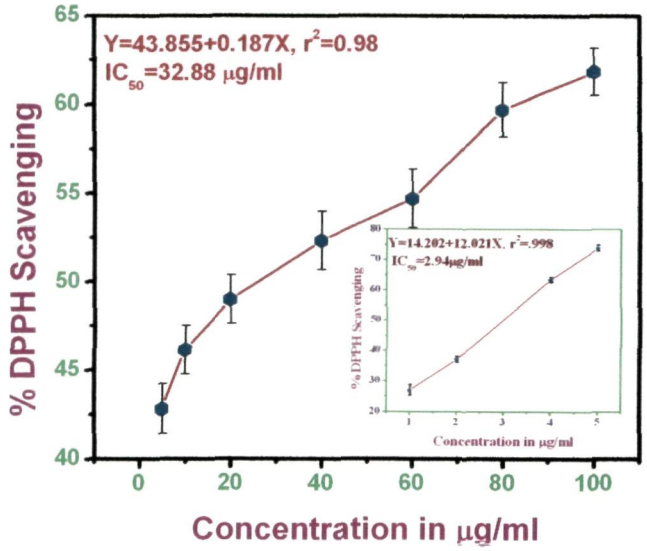
Results are represented as mean ± SEM (n=6). $^{a}P<0.05$ and $^{c}P<0.001$as compared with normal group.

Fig. 7.5. Effect of EtHI and EAHI on level of adrenal cortisol (mg/100 g of tissue) on swimming induced stress in mice

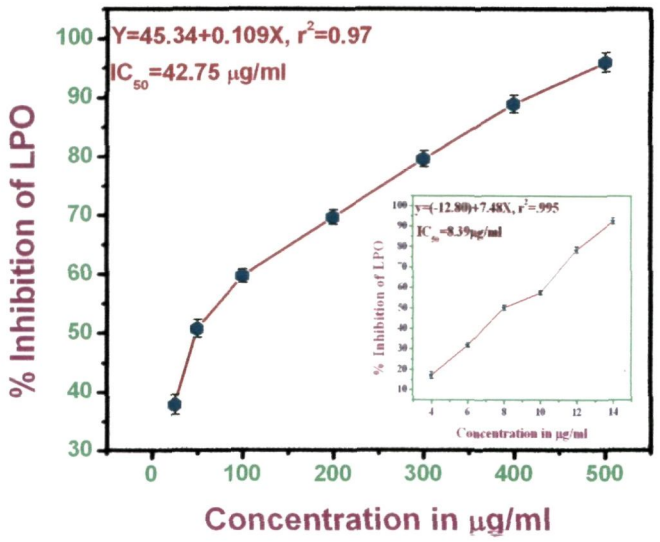


IN-VITRO FREE RADICAL SCAVENGING ACTIVITY

Fig. 7.6. a) Scavenging effect of ethyl acetate fraction (EAHI) of *Habenaria intermedia* on DPPH radical



b) Scavenging effect of ethyl acetate fraction (EAHI) of *Habenaria intermedia* on LPO.



c) **Scavenging effect of ethyl acetate fraction (EAHI) of *Habenaria intermedia* on Hydroxy radical**

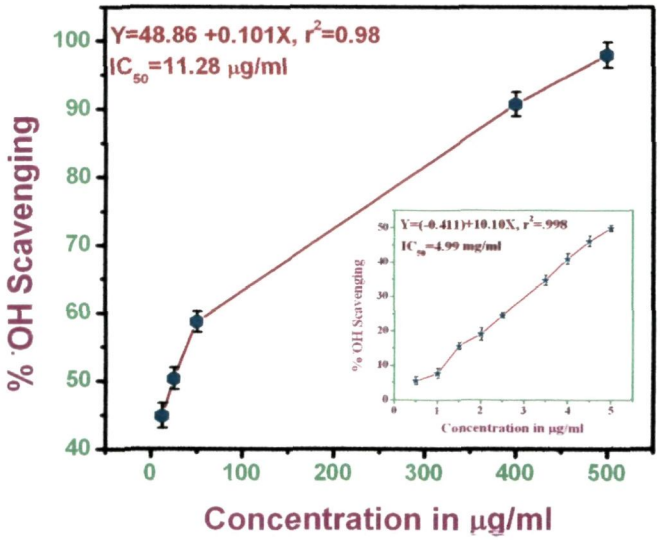
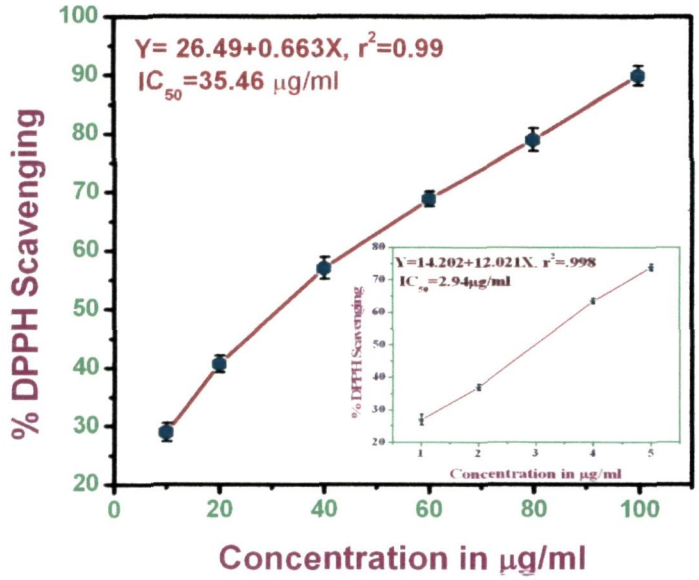
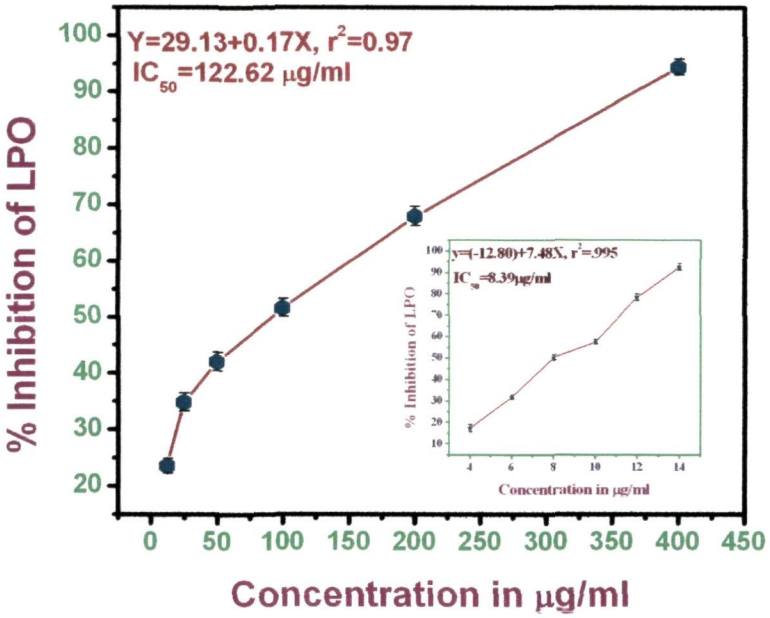


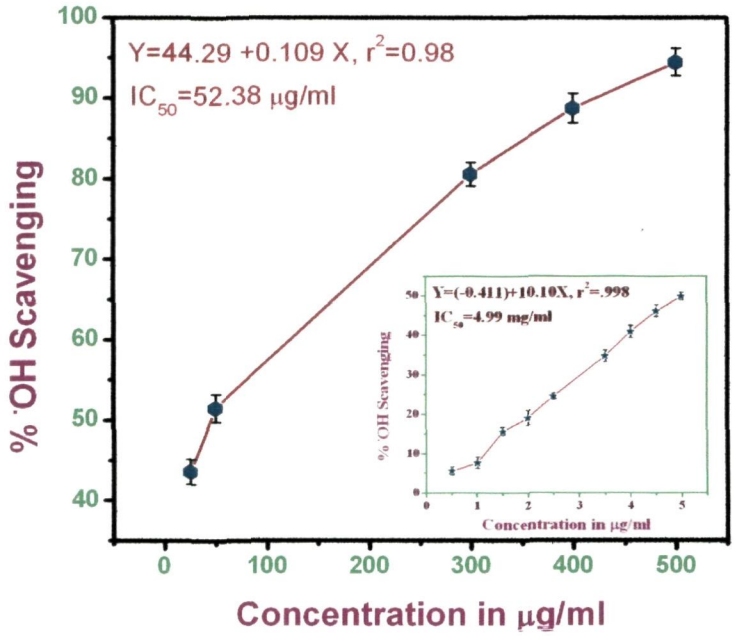
Fig. 7.7. a) Scavenging effect of ethanol fraction (EtHI) of *Habenaria intermedia* on DPPH radical



b) Scavenging effect of ethanol fraction (EtHI) of *Habenaria intermedia* on LPO assay



c) Scavenging effect of ethanol fraction (EtHI) of *Habenaria intermedia* on Hydroxyl radical



7.11.1.5 References

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7.11.2 Nootropic activity of *Habenaria Intermedia* D.Don. in mice

7.11.2.1 Introduction

Since *Habenaria intermedia* tuber is an important ingredient of *Chyavanprasha,* a well known polyherbal rejuvenator and memory enhancer, nootropic activity of the crude fractions was investigated by employing both exteroceptive and interoceptive models. Elevated plus maze and Morris water maze are used to assess the short-term memory. Interoceptive behavioral models such as scopolamine and natural aging induced amnesia are widely used models to stimulate dementia in Alzheimer's disease.

7.11.2.2 Methods and Material

a) Preparation of drugs

Suspensions of the extracts were prepared in Tween-80 and distilled water (2:8) and used to assess nootropic activity.

b) Drugs

Scopolamine hydrobromide (Sigma Aldrich, U.S.A.), Piracetam (Nootropil, UCB India Pvt. Ltd., Vapi, Gujarat), diazepam (Calmpose, Ranbaxy, India) Phenytoin (Dialantin suspension, Parke Davis) wee diluted in normal saline. Volume of oral and i.p. administration was 1ml/100 g. of mouse.

c) Experimental animals

Swiss mice of either sex weighing around 18 gms (young ones, aged 8 weeks) and 25 gms (older ones, aged 28 weeks) were used in the present study. They were acclimatized to the laboratory conditions for 5 days before behavioral studies. The animals had free access to food and water and were maintained under 12:12 h light and dark cycles. All the readings were taken during the same time of the day i.e. between 6- 8

PM. Institutional Animals Ethics Committee (IAEC) had approved the experimental protocol and care of animals was taken as per the CPCSEA guidelines, Animal welfare division, Ministry of Environment and forests, Govt. of India.

d) Elevated plus maze [1,2]

As described in 5.11.2.3d

e) Morris Water maze [3]

As described in 5.11.2.3e

f) Estimation of brain acetyl cholinesterase activity

The whole brain acetyl cholinesterase (AChE) activity was measured using the Ellman method [4] as described in 5.11.2.3f.

g) Statistical analysis

The data were expressed as mean ± SEM. The data were analysed using one way ANOVA followed by Tukey's test. $P<0.01$ was considered significant.

7.11.2.3 Results

i) Effect on Transfer Latency (TL) using Elevated Plus maze

Aged mice showed higher TL values on first day and second day as compared to young mice, indicating impairment in learning (ageing induced amnesia). Scopolamine (0.4 mg/kg, i.p.) increased the transfer latency significantly ($P<0.01$) in young mice on first and second day as compared to control indicating impairment of memory. Treatment with Piracetam (200 mg/kg, i.p.) for 8 days decreased TL as compared to control group, indicating improvement in both learning and memory. Pretreatment with EAHI (100 and 200 mg/kg, p.o.) decreased the TL on 8^{th} and 9^{th} day in young and aged mice ($P<0.01$) when compared to control groups. Higher dose of EAHI (200mg/kg, p.o.) significantly enhanced antiamnesic property in aged animals rather than young mice as reflected by

marked decrease in TL on 8th and 9th day when subjected to EPM tests. EAHI (200 mg/kg, p.o.) exerted profound enhancement of memory in young mice and protected them from against scopolamine ($P < 0.01$) and ageing induced amnesia. The results are summarized in Table 7.10 and 7.11.

ii) Effect on brain acetyl cholinesterase activity

EAHI(100 and 200 mg/kg, p.o.) significantly produced reduction in whole brain AChE activity of both young and aged mice as compared to respective control group. The brain AChE activity with phenytion (12 mg/kg, i.p.) exhibited significant elevation which was considered as negative control. Piracetam (200 mg/kg, i.p.) profoundly reduced AChE activity as compared to control groups (Table.7.12).

iii) Effect of *H. intermdeia* on Scopolamine induced enhancement on Escape latency Time (ELT) in mice using water maze

A significant decrease ($P< 0.01$) in the ELT was observed in control group mice in their 4 day trial. Scopolamine produced impairment of acquisition and increased the ELT during successive training trials. The action of scopolamine was reversed by pretreatment with EAHI (100 and 200 mg/kg, p.o.) as reflected by significant decrease ($P< 0.01$) in ELT of mice. The results are summarized in Table 7.13 and 7.14.

iv) Effect of *H. intermdeia* on scopolamine and ageing induced alterations in the Time Spent Target Quadrant (TSQT) during retrieval trials on water maze

The time spent by young control mice in target quadrant was more as compared to time spent on other quadrants during retrieval trial on 5th day. Further scopolamine (0.4 mg/kg, i.p) administered before retrieval trial produced significant decrease ($P< 0.01$) in mean time spent in target quadrant in search of missing platform as compared to control (young). Aged mice also decreased TSQT significantly compared to control (young). The

results are shown in Table 7.15. These observations indicate that scopolamine and natural ageing produced anterograde and retrograde amnesia. Mice treated with Piracetam (200 mg/kg, i.p.) produced better effects only in aged mice by decreasing TSQT as compared to control (aged) mice. EAHI (100 and 200 mg/kg, p.o.) administered before training trial (from day 1 to day 4), significantly ($P<0.01$) attenuated scopolamine and ageing induced decrease in TSQT during retrieval test on 5th day. The results are shown in Table 7.16.

7.11.2.4 Discussion

Alzheimer's disease (AD) neuropathological disorder which identified by accumulation of beta amyloid protein in the brain and is increasingly diagnosed in all countries. It has been estimated that about 5% of the population aged more than 65 are affected by AD[4]. Acetylcholinesterse inhibitors such as Donepezil, Rivastigmine and Galantamine are the only FDA approved drugs currently used for the treatment of mild or moderate cases of dementia. Due to its fusogenic properties, the amphiphilic non-aggregated Aβ oligomers could be the proximate effectors of the neuronal degeneration and death occurring in early stages of AD[5,6].

Therefore there is a need for development of novel therapeutic strategies that target or even better prevent the molecular mechanisms leading to dementia. There has been a plenty of research on the plants used as Rasayana drugs in order to reason them in the modern context[7]. Subgroups of Ayurvedic rasayanas, known as *medhyarasayanas*, are used to promote intellect and memory. The cognitive promoting effect of *medhyarasayanas* is best seen in children with memory deficits, or when memory is compromised following head injury, prolonged illness or in old age[8] .The present study indicates that Ethyl acetate fraction (EAHI) of *H. intermedia* is a potential anti-amnesic agent. It also possesses nootropic activity in view of its facilitatory effect on retention and acquired learning. EAHI (100 mg and 200 mg/kg, p.o.) decreased transfer latencies in

both young but more profoundly in aged mice in dose dependent manner as compared to respective controls. Central cholinergic system plays an important role in learning and memory[9,10]. Phenytoin is known to reduce the hippocampal AChE concentration[11,12] , and causes cognitive impairment. In our study, phenytoin per se (12 mg.kg i.p.) significantly elevated brain AChE activity. Piracetam (200 mg/kg, i.p.) and EAHI (100 and 200 mg/kg, p.o.) on the other hand significantly ($P<0.01$) lowered this activity indicating the counteracting action of the drugs on cholinergic system. EAHI elicitated profound neuroprotective effect in scopolamine treated and older mice compared to control groups and Piracetam treated mice. It significantly inhibited AChE activity in the whole brain homogenate in mice indicating its potential in the attenuation of learning and memory deficits especially in aged mice.

In water maze model, a marked decrease in escape latency time (ELT), during subsequent trials as compared to the first exposure, denotes normal learning ability. The enhancement in the time spent by the animal in the target quadrant reflects successful retention of learned task (or memory).

Amnesia is inability to remember past experiences or loss of memory. Anterograde amnesia is impairment of memory for events occurring after accident/drug treatment. In such case, new memories are not formed. Retrograde amnesia is impairment of memory of events which have occurred before the accident or drug treatment. In such case new memories can be formed, but old memories are lost. In the present study, scopolamine (0.4 mg/kg, i.p.) showed anterograde amnesia as indicated by significant decrease in more time spent in target quadrant on 5^{th} day in Morris water maze model. Our observation suggested that EAHI (100 and 200 mg/kg, p.o) reversed the scopolamine and ageing induced amnesia.

Since ROS play a key in the development of around 100 diseases including Alzeimer's disease, Parkinson's disease and many CNS disorders, the observed nootropic activity of the EAHI may be due to its anti-oxidative properties. The study justify the traditionally claimed intellect promoting potentiality of tubers of *H intermedia* and further investigations are warranted to explore the possible phytoconstituents from the potent fraction(s) responsible for the management of AD and other cognitive disorders.

Table 7.11 Effect of EtHI and EAHI on transfer latencies (TL) of young mice by elevated plus maze

Group	Treatment	Dose(mg/kg)	TLT(8^{th} day)	TLT(9^{th} day)
I	Control (Young)	10	29.4±1.45	22.5±2.57
II	Piracetam	200	23.2±2.37*	17.4±1.24*
IV	Scopolamine	0.4	51.4±1.56*	41.2±3.38*
V	EtHI	100	41.6±2.34	38.4±3.67
VI	EtHI	200	40.2±3.48	38.6±5.34
VII	EAHI	100	27.4±3.43[a]	21.8±1.89[a]
VIII	EAHI	200	26.2±2.44[a]	22.0±1.45[a]
XIII	EtHI+Scopolamine	200+0.4	39.8±3.79	39.0±6.25
XIV	EAHI+Scopolamine	200+0.4	29.6±3.65[b]	22.1±2.93[b]

Each group consists of 6 animals (n=6). Values are mean ±SEM. *P<0.001compared to control, [a]P<0.01 compared to control, [b]P<0.01 compared to control (scopolamine treated)

Table 7.12. Effect of EtHI and EAHI on transfer latencies of aged mice by elevated plus maze

Group	Treatment	Dose(mg/kg)	TLT(8^{th} day)	TLT(9^{th} day)
I	Control(Y)	10	29.4±2.45	22.5±2.57
XV	Control(A)	10	33.6±1.67*	31.7±2.65*
III	Piracetam	200	26.7±3.68*	19.6±2.13*
IX	EtHI	100	52.6±3.60	41.6±1.56
X	EtHI	200	50.2±3.54	43.2±1.21
XI	EAHI	100	33.2±1.02[a]	28.4±1.08[a]
XII	EAHI	200	31.4±1.32[b]	25.5±2.21[b]

Each group consists of 6 animals (n=6). Values are mean ±SEM. *P<0.01compared to control, [a]P<0.01 compared to control (young), [b]P<0.01 compared to control (aged).

Table 7.13. Effect of *H. intermedia* fractions and Piracetam on AChE activity in aged mice

Group	Treatment	Dose (mg/ kg, p.o.)	AChE (mM)
I	Control	10	128.4±7.40
II	Phenytoin	12	216.6±10.55*
III	EtHI	100	196.3±7.53
IV	EtHI	200	197.7±5.44
V	EAHI	100	141.5±2.12*
VI	EAHI	200	118.34±6.25*
VII	Piracetam	200	107.21±5.14*

Values are mean ± SEM., AChE- whole brain AChE activity, *P<0.01compared to control.

Table 7.14. Effect of *H.intermedia* on Escape Latency Time (ELT) of young mice using Morris water maze

Group	Treatment	Dose (mg/kg, i.p./p.o.)	Escape Latency Time in secs	
			Day 1	Day4
I	Control	10	67.4±2.53	25.12±2.44
III	Piracetam	200	61.4±6.24*	23.4±4.55*
V	EtHI	100	70.7±1.54	51.5±4.32
VI	EtHI	200	68.4±2.47	55.8±6.19
VII	EAHI	100	55.7±4.56*	24.4±1.54*
VIII	EAHI	200	61.4±3.54*	21.6±3.54*

Each value represents ± SEM, *P<0.01as compared to control mice.

Table 7.15. Effect of *H.intermedia* on Escape Latency Time (ELT) of aged mice using Morris water maze

Group	Treatment	Dose (mg/kg, i.p./p.o.)	Escape Latency Time in secs	
			Day 1	Day 4
I	Control	10	81.2±1.42	67.2±4.33
III	Piracetam	200	78.2±5.44*	66.5±7.23*
V	EtHI	100	63.3±1.47	55.23±2.23
VI	EtHI	200	64.2±1.67	55.23±2.23
VII	EAHI	100	57.4±2.64*	25.6±4.24*
VIII	EAHI	200	58.6±4.24*	26.2±4.54*

Each value represents ± SEM, * P< 0.01 as compared to control mice.

Table 7.16. Effect of *H.intermedia* on the mean Time spent in the Target Quadrants (TSTQ) Q4 in young mice using Morris Water maze

Group	Treatment	Dose (mg/kg, i.p./p.o.)	TSTQ (secs)
I	Control	10	68.7 ± 1.23
III	Piracetam	200	54.2±2.13*
V	Scopolamine	0.4	30.2±1.55
VI	EtHI	100	36.6±3.54
VII	EtHI	200	34.6±3.53
VIII	EAHI	100	55.4±2.45[a]
IX	EAHI	200	61.54±4.23[a]
X	EtHI + Scopolamine	200	33.3±1.43
XI	EAHI + Scopolamine	200	58.2±4.76[a]

Each value represents mean ± SEM. *P<0.01 as compared to control, [a]P<0.01 as compared to scopolamine treated mice.

Table 7.17. Effect of *H. intermedia* on the mean Time spent in the Target Quadrants (TSTQ) Q4 in aged mice using Morris Water maze

Group	Treatment	Dose (mg/kg, i.p./p.o.)	TSTQ (secs)
II	Control	10	29.4±2.44
IV	Piracetam	200	52.4±1.75*
XII	EtHI	100	36.4±3.44
XIII	EtHI	200	38.2±4.34
XIV	EAHI	100	50.2±2.44*
XV	EAHI	200	52.7±5.63*

Each value represents mean ± SEM. *P< 0.001 as compared to control.

7.11.2.5 References

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7.12 Isolation of secondary metabolites from *Habenaria intermedia* D. Don.

Preliminary phytochemical investigation of crude fractions of *Habenaria intermedia* revealed the presence of phenolic components and coumarin glycosides. Since Ethyl acetate (EAHI) and Ethanol (EtHI) fractions were identified as potent fractions in adaptogenic and nootropic activities, we further extended our study to isolate secondary metabolites by column chromatography.

7.12.1 Isolation of Scopoletin (ASB3)

Adsorbent	:	Silica gel for column chromatography
Activation	:	110°C for 1 hr.
Length of the column	:	45 cm
Diameter	:	Outer- 4.2 cm, Inner- 3.8 cm
Length of the adsorbent	:	30 cm
Rate of elution	:	15-18 drops/min
Volume of elute collected	:	10 ml each
Type of elution	:	Gradient elution

a) Gradient elution

5gm of ethyl acetate fraction was dissolved in 10ml of methanol and mixed with 5gm of silica gel (60-120mesh size) and dried in vacuum oven at 45°C. The adsorbed material obtained was transferred to the column and elution was carried out with Chloroform, chloroform-methanol mixture in different proportions. The elution rate was adjusted to 15-18 drops/min, several 10ml fractions were collected up to 200 ml. A yellow colored compound was eluted from $CHCl_3$-MeOH (90:10) fractions 60-69. Pale yellow colored needles were obtained after recrystalisation with methanol. The identity of the compound was established by TLC and spectral data.

b) Thin Layer Chromatography

Identification of isolated component was carried out on TLC plates (0.25 mm) precoated with silica gel GF_{254} (Merck, silica gel for TLC). Chromatogram was run using Toluene: Ether (1:1) saturated with 10% acetic acid as mobile phase. The TLC plates were checked under UV light (365 nm) and then sprayed with 10% ethanolic KOH reagent. Fractions with similar TLC pattern were pooled together and concentrated at reduced pressure and temperature. The concentrated components were further dried in vacuum descicator. Completely dried components were weighed to calculate the total mass isolated.

7.12.2 Isolation of Gallic acid (ASPVH4)

a) Gradient elution

Adsorbent	:	Silica gel for column chromatography
Activation	:	110°C for 1 hr.
Length of the column	:	45 cm
Diameter	:	Outer- 4.2 cm, Inner - 3.8 cm
Length of the adsorbent	:	30 cm
Rate of elution	:	50 ml/min upto 100 ml
		15-18 drops/min upto 350 ml
Volume of elute collected	:	10 ml each.
Type of elution	:	Gradient elution.

5 gm of ethanolic fraction was subjected to acid hydrolysis with 2M HCl for 0.5 hrs. Extraction was carried with ether (10 ml aliquots, three times), evaporated to get light yellow colored residue. 2gm of residue was dissolved in 10ml of methanol and

mixed with 2gm of silica gel (60-120mesh size) and dried in vacuum oven at 45°C. The adsorbed material obtained was transferred to the column and elution was carried out by gradient method with ethyl acetate: benzene in different proportions. The elution rate was adjusted to 50 ml/min, for several 10ml fractions up to 100 ml and then 18-20 drops/min up to 350 ml. A pale yellow colored compound was eluted from ethyl acetate: benzene (80:20) fractions 210-224. The identity of the compound was established by TLC and spectral data.

b) Thin Layer Chromatography

Identification of isolated component was carried out on TLC plates (0.25 mm) precoated with silica gel GF_{254} (Merck, silica gel for TLC). Chromatogram was run using Ethyl acetate:Benzene (9:11) as mobile phase. The TLC plates were then sprayed with Folin-Ciocalteu reagent. Fractions with similar TLC pattern were pooled together and concentrated at reduced pressure and temperature. The concentrated components were further dried in vacuum descicator. Completely dried components were weighed to calculate the total mass isolated.

7.12.3 Characterization of isolated compounds

The structures isolated compounds were confirmed by spectral data

Infrared spectroscopy

The infrared spectrum of the isolated compounds was recorded using KBr in the range of 4000-400 cm^{-1} on FTIR (Nicolet 5700, Thermo Electron Corporation).

Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C) of the isolated compounds was recorded using Bruker AV 300 MHz spectrophotometer. All spectra were recorded at 25°C and the chemical shifts were recorded in δ ppm with the solvent shift. Deuterated chloroform ($CDCl_3$) and DMSO (HPLC grade) were used as solvents.

Mass spectrometry

Electron ionization mass spectrometry of the compounds was performed by direct inlet at 70 eV on the LCMS-QP2010.

7.12.4 Results

1) Table 7.18. Elution of coumarin from *Habenaria intermedia* D. Don.

Fraction no	Composition & proportion of the solvent system	Color of the elute	TLC Studies Solvent system	No of spots & R_f values	Amount
1-10	Chloroform (100%)	No color	Toluene:Ether (1:1) saturated with 10% acetic acid	No spot	–
11-15	Chloroform: Methanol (99-1%)	No color	Toluene:Ether (1:1) saturated with 10% acetic acid	No spot	–
15-20	Chloroform; Methanol (98-2%)	No color	Toluene:Ether (1:1) saturated with 10% acetic acid	No spot	–
21-40	Chloroform; Methanol (95-5%)	No color	Toluene:Ether (1:1) saturated with 10% acetic acid	No spot	–
41-60	Chloroform; Methanol (90-10%)	Pale yellow	Toluene:Ether (1:1) saturated with 10% acetic acid	No spot	–
61-70	**Chloroform; Methanol (90-10%)**	**Dark yellow**	Toluene:Ether (1:1) saturated with 10% acetic acid	**Single spot (0.30) Blue fluroscence under UV**	**85 mg**
71-80	Chloroform; Methanol (85-15%)	No color	Toluene:Ether (1:1) saturated with 10% acetic acid	No spot	–
81-100	Chloroform; Methanol (80-20%)	Light yellow	Toluene:Ether (1:1) saturated with 10% acetic acid	No spot	--

2) **Table 7.19. Elution of phenolic acid from *Habenaria intermedia* D. Don.**

Fraction no	Composition & proportion of the solvent system	Color of the elute	TLC Studies Solvent system	No of spots & R_f values	Amount
1-10	Ethyl acetate (100%)	No color	Ethyl acetate: Benzene (9:11)	No spot	–
11-20	Ethyl acetate: benzene (99-1%)	No color	Ethyl acetate: Benzene (9:11)	No spot	–
21-50	Ethyl acetate: benzene (98-2%)	No color	Ethyl acetate: Benzene (9:11)	No spot	–
50-75	Ethyl acetate: benzene (96-4%)	No color	Ethyl acetate: Benzene (9:11)	No spot	–
76-100	Ethyl acetate: benzene (96-4%)	No color	Ethyl acetate: Benzene (9:11)	No spot	–
100-150	Ethyl acetate: benzene (90-10%)	Light brown	Ethyl acetate: Benzene (9:11)	No spot	–
150-200	Ethyl acetate: benzene (85-15%)	No color	Ethyl acetate: Benzene (9:11)	No spot	–
201-225	**Ethyl acetate: benzene (80-20%)**	**Pale yellow**	Ethyl acetate: Benzene (9:11)	**Single spot (0.4) blue spot with Folin reagent and ammonia**	**65 mg**
225-250	Ethyl acetate: benzene (75-25%)	Light yellow	Ethyl acetate: Benzene (9:11)	No spot	–
276-300	Ethyl acetate: benzene (50-50%)	No color	Ethyl acetate: Benzene (9:11)	No spot	-
301-325	Ethyl acetate: benzene (25-75%)	Light green	Ethyl acetate: Benzene (9:11)	No spot	

7.12.5 Spectral data

Structures of isolated compounds were established based on IR, ^{1}HNMR and mass spectral studies.

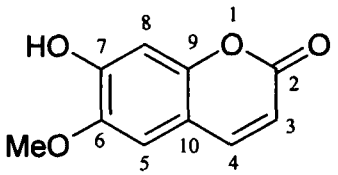
Spectral data of Scopoletin (ASB3)

Light yellow powder (fluorescent under UV), 85 mg. R_f 0.30, mp. 203°C (literature data 203-205°C) IR spectrum **(Fig 7.9)** of ASB3 showed characteristic absorption band at 3338 cm^{-1} due to hydroxyl group. Another band at 1703 cm^{-1} attributed to stretching frequency of carbonyl group. The CH=CH stretching peak appeared at 2944 cm^{-1}.

Proton NMR spectrum **(Fig. 7.10)** showed a doublet at δ 7.8-7.9 (1-H) which integrated for one proton and was assigned to C_3 proton. One more doublet at δ 6.20-6.22 (1-H) which corresponds to one proton was attributed to C_4 proton. Two singlets which appeared at δ 7.21 and δ 6.77 were assigned to C_8 and C_5 protons. Hydroxyl proton at C_7 resonated as singlet at 10.30. A singlet at δ 3.80 which integrated for three protons was due to $-OCH_3$ group.

The Mass spectrum **(Fig. 7.11)** of ASB3 showed a molecular ion peak at m/z 193, which was due to its molecular formula ($C_{10}H_8O_4$) and molecular weight.

Hence, by comparing the spectral data obtained and earlier reported[1] data the structure assigned was in good agreement with a coumarin called **scopoletin (3)**



(3)

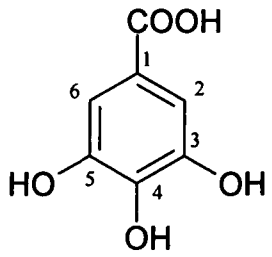
Spectral data of Gallic acid (ASPVH4)

Buff white colored powder (fluroscent under UV), 65 mg, R_f 0.40, MP 249°C, IR spectrum **(Fig. 7.12)** exhibited a characteristic peak at 1612 cm^{-1} which was due to carbonyl peak of COOH group. A broad peak at 3387 cm-1 was due to hydroxyl groups.

^1H NMR spectrum **(Fig. 7.13)** displayed a singlet at δ 6.9 (2-H) was assigned to two aromatic protons at C_2 and C_6. Three hydroxyl groups comes to resonate at δ 9.5, broad singlet corresponds to three –OH groups at C_3, C_4 and C_5. The carboxylic acid – OH group also appeared as broad singlet at δ 12.3 respectively.

The Mass spectrum **(Fig. 7.14)** of ASPVH4 showed a molecular ion peak at m/z 170, which was due to its molecular formula (C_7 H_6 O_5) and molecular weight.

Hence, by comparing the spectral data obtained and earlier reported[2,3] data the structure assigned was in good agreement with **gallic acid (4)**



(4)

Hence the structures of the new compounds found in *Habenaria intermedia* D. Don. were found to be scopoletin and gallic acid. These compounds were also reported in various other medicinal plants like *Sanguisorba officinalis*[3] *Rosa rugosa*[2] *Rhus chinensis, Terminalia chebula, Artemisia capillaris* [4] with wide range of biological activities. This is the first report of these compounds in tuber of *Habenaria intermedia.* All the spectral interpretations are in comparison with the earlier reported data.

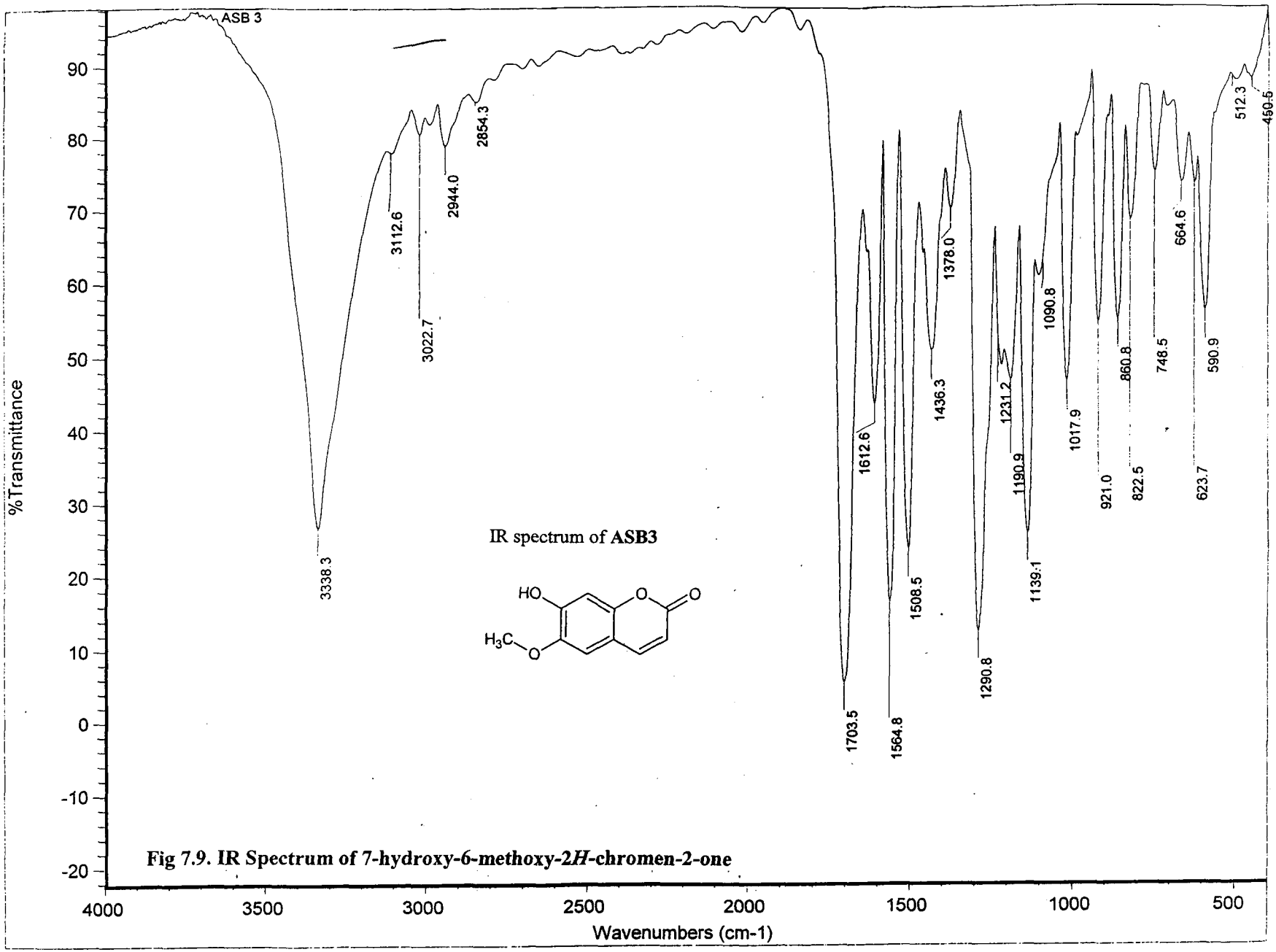
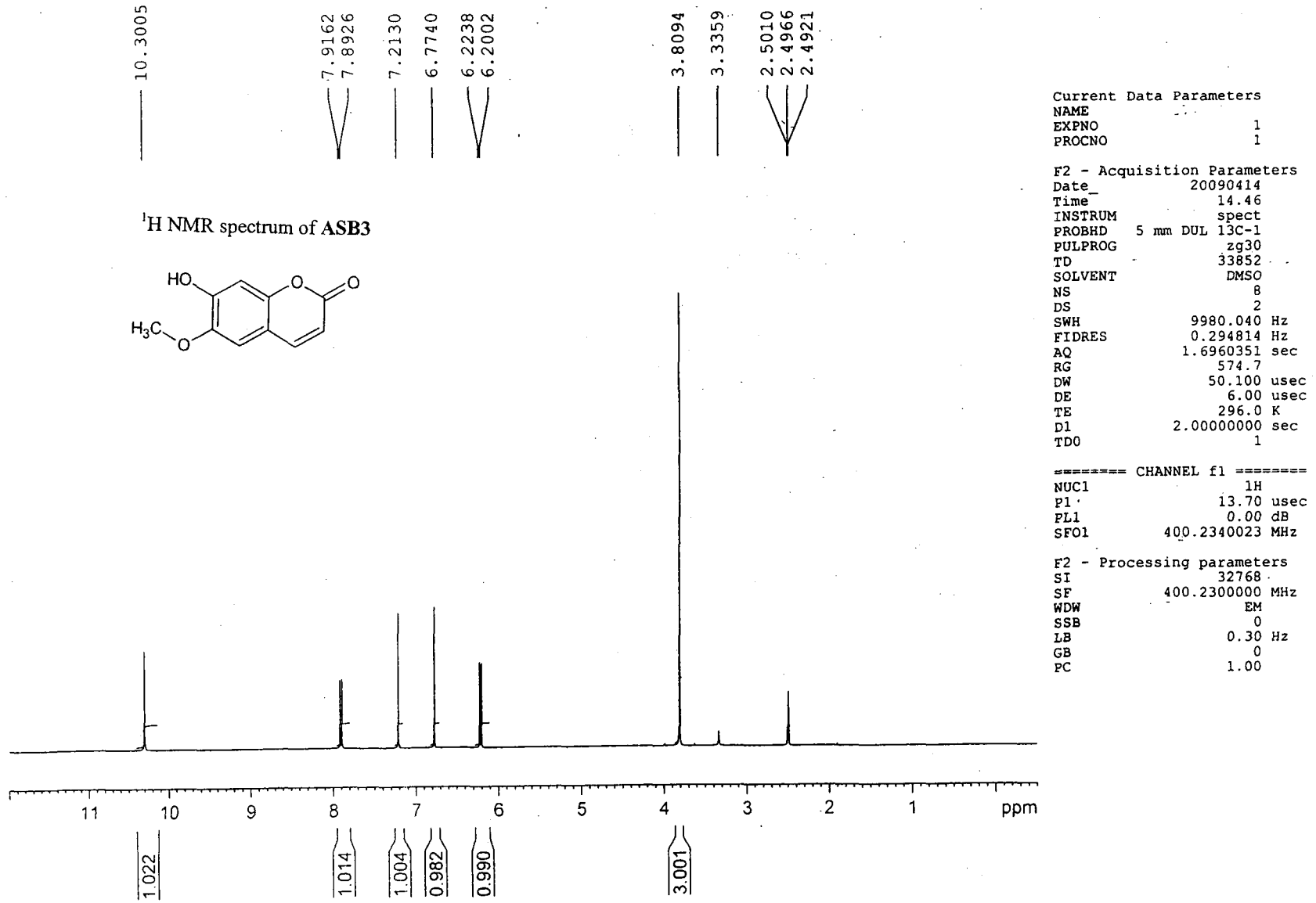


Fig 7.9. IR Spectrum of 7-hydroxy-6-methoxy-2*H*-chromen-2-one



^{1}H NMR spectrum of **ASB3**

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PULPROG           zg30
TD               33852
SOLVENT           DMSO
NS                   8
DS                   2
SWH            9980.040 Hz
FIDRES         0.294814 Hz
AQ             1.6960351 sec
RG               574.7
DW              50.100 usec
DE               6.00 usec
TE              296.0 K
D1             2.00000000 sec
TD0                  1

======== CHANNEL f1 ========
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P1                13.70 usec
PL1                0.00 dB
SFO1         400.2340023 MHz

F2 - Processing parameters
SI                32768
SF           400.2300000 MHz
WDW                  EM
SSB                  0
LB                0.30 Hz
GB                   0
PC                1.00
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Fig 7.10. ^{1}H NMR Spectrum of 7-hydroxy-6-methoxy-2*H*-chromen-2-one

LC/MS REPORT

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Injection vol   :  2.0ul
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               % B :         5-90          90           90              90--5
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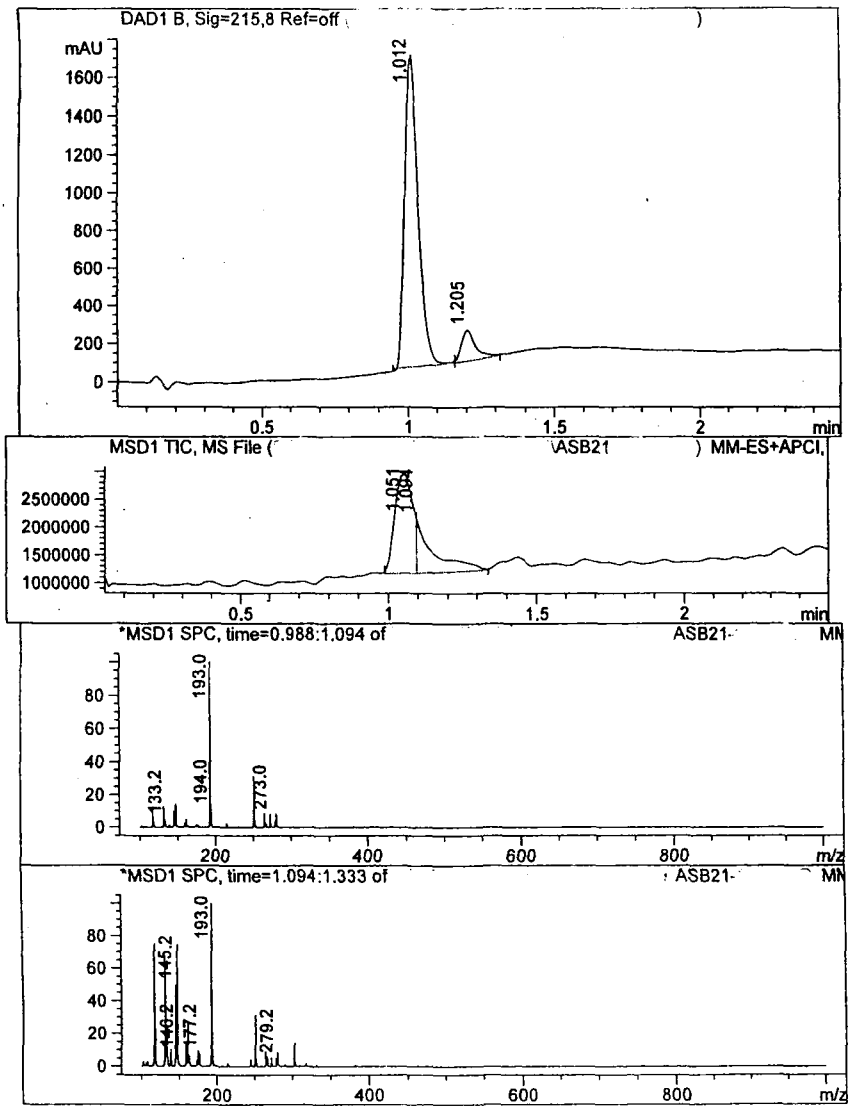

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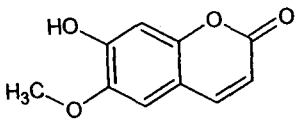
Mass spectrum of **ASB3**



Mass Calcd m/z=192

Found m/z=193(M+1)

Fig 7.11. Mass Spectrum of 7-hydroxy-6-methoxy-2*H*-chromen-2-one

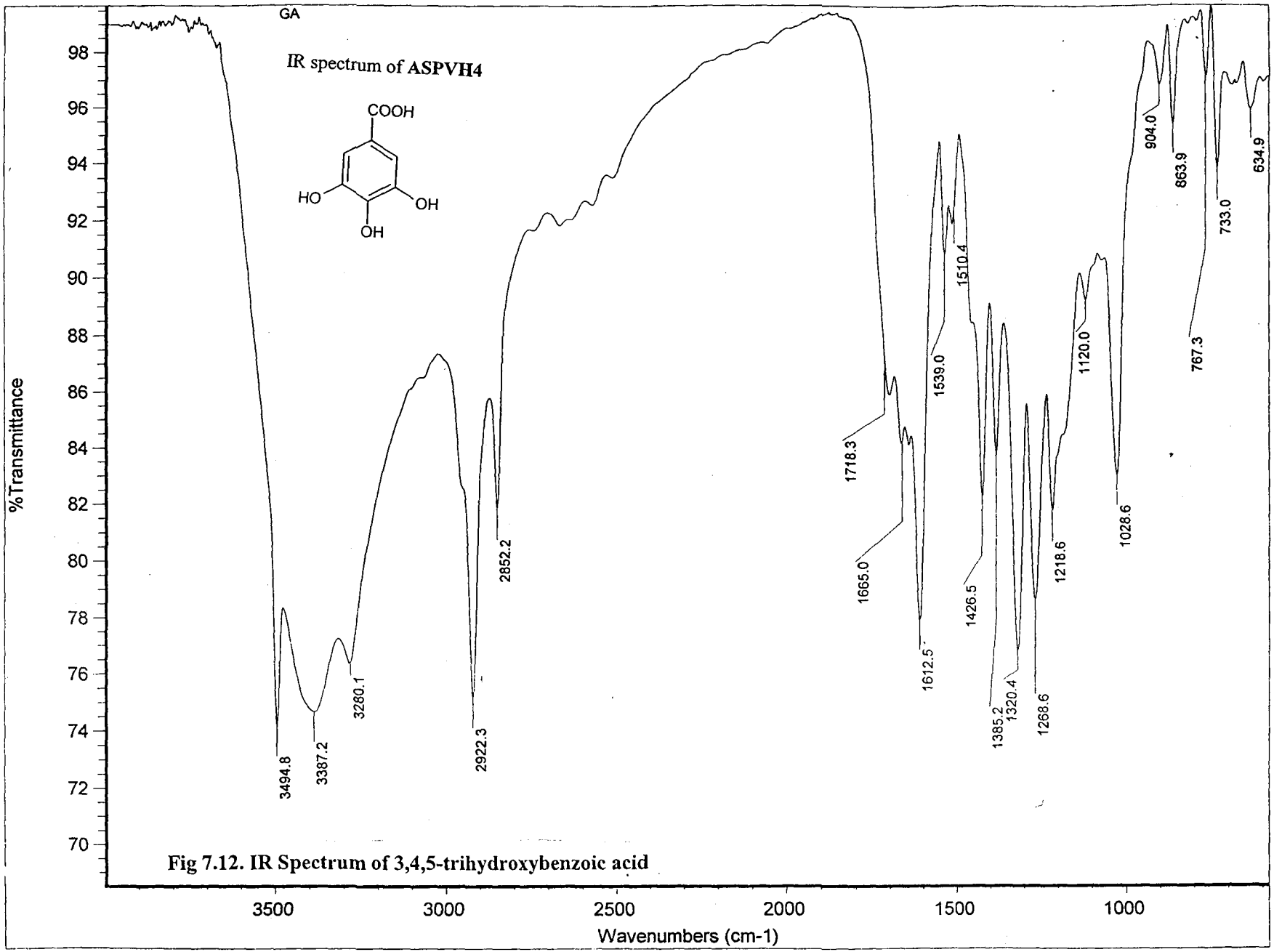
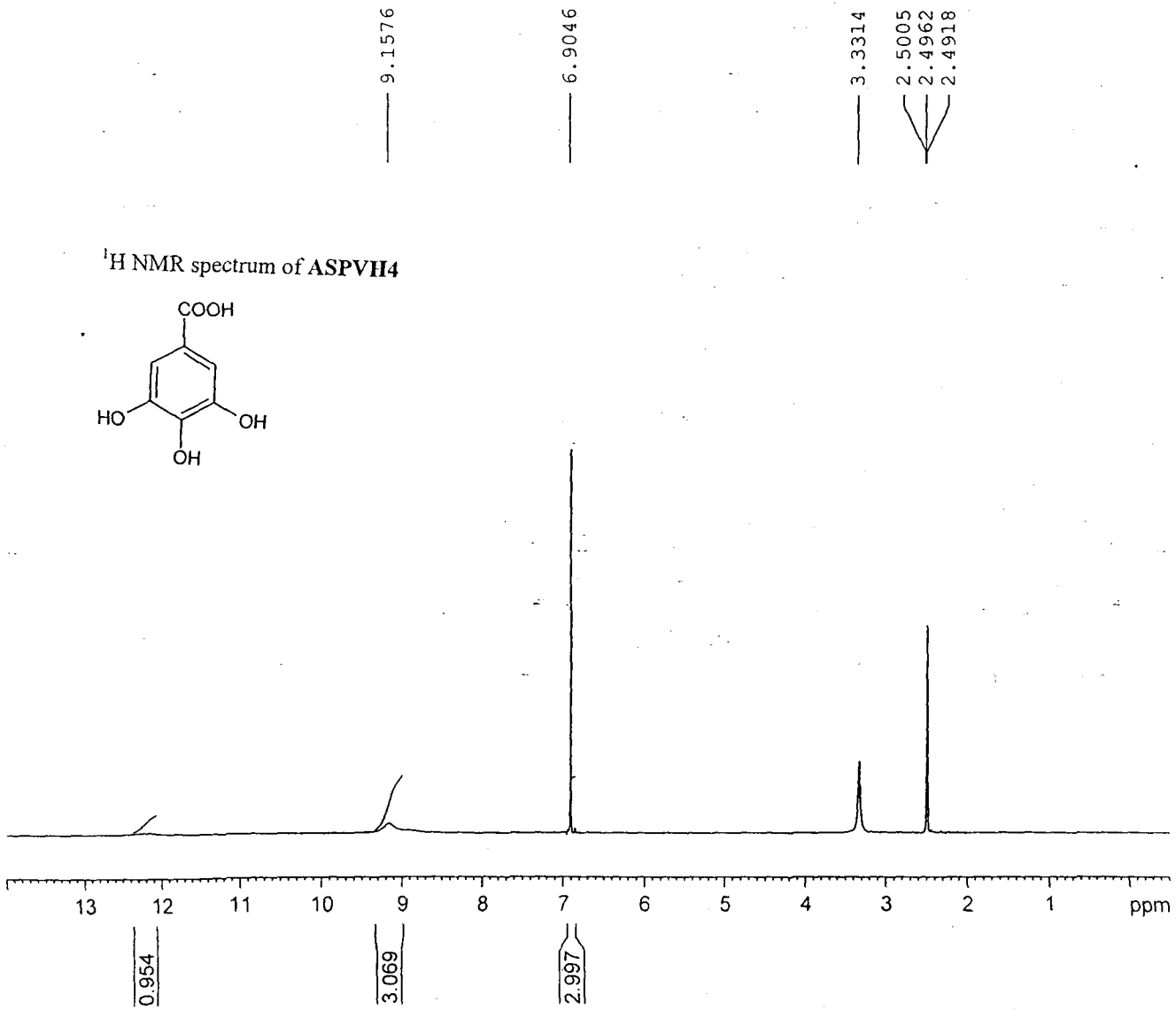


Fig 7.12. IR Spectrum of 3,4,5-trihydroxybenzoic acid

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PROCNO                   1

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TD                    33852
SOLVENT                DMSO
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DS                         2
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FIDRES              0.294814 Hz
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RG                      1024
DW                    50.100 usec
DE                     6.00 usec
TE                     296.1 K
D1                2.00000000 sec
TD0                        1

======== CHANNEL f1 ========
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PL1                     0.00 dB
SFO1           400.2340023 MHz

F2 - Processing parameters
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SSB                        0
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GB                         0
PC                      1.00
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Fig 7.13. ^{1}H NMR Spectrum of 3,4,5-trihydroxybenzoic acid

LC/MS REPORT

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Injection vol   :  2ul
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Acq Method      :  D:\METHODS\NONPOLAR.M
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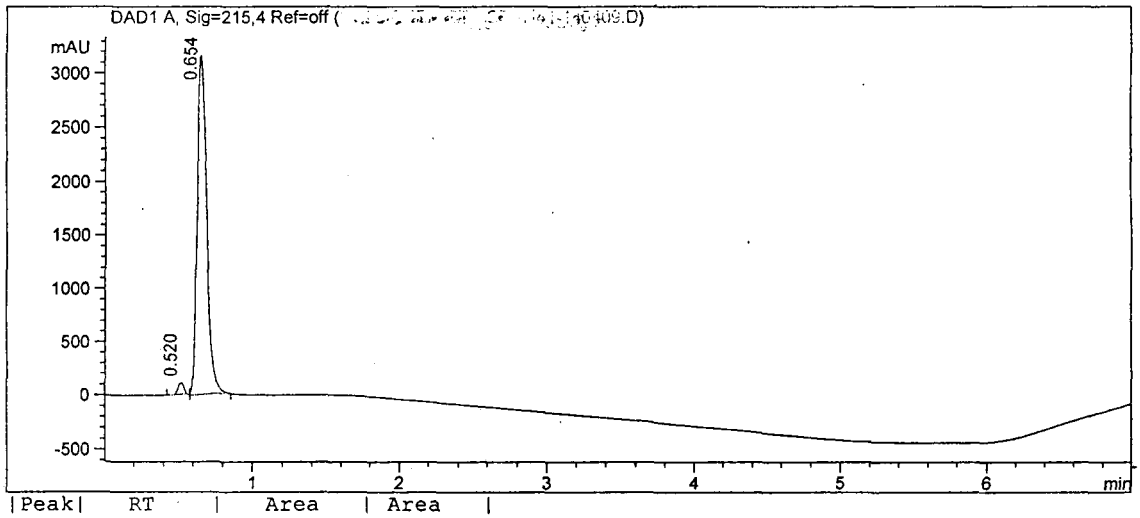

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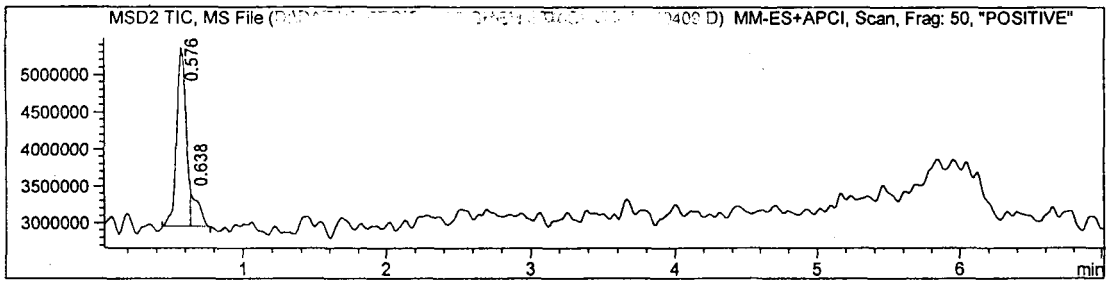
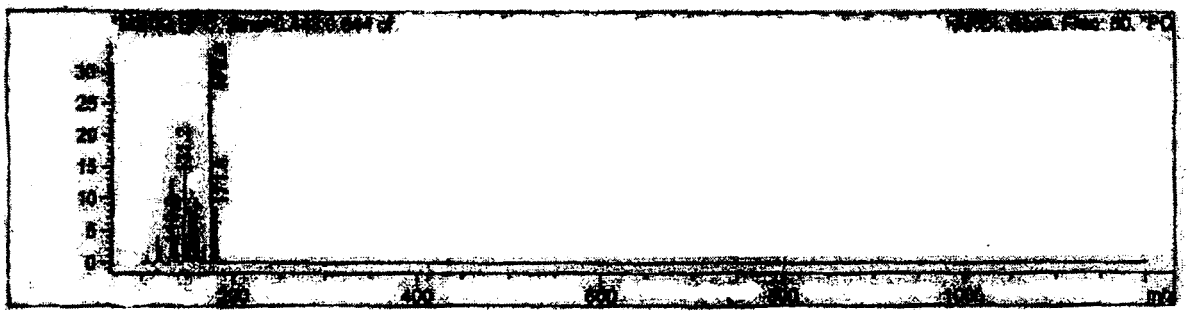
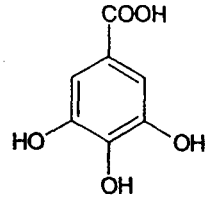




Mass spectrum of **ASPVH4**



Mass Calcd m/z=170

Found m/z=170

Fig 7.14. Mass Spectrum of 3,4,5-trihydroxybenzoic acid

7.12.6 References

1. Deniz Tasdemir, Marcel Kaiser, Reto Brun, Vanessa Yardley, Thomas J. Schmidt, Fatma Tosun, and Peter Ru¨edi. *Antimicr. Agents Chemother.* 2006;1352.

2. Lia L. Ng TB, Gao W, Lia W, Fu M, Niu SM, Zhao L, Chen RR,, Liu. F. *Life Sciences.* 2005;77:230.

3. Yi-Zhong Cai, Mei Sun, Jie Xing , Qiong Luo, Harold Corke. *Life Sciences.* 2006;78:2872.

4. Yizhong Cai, Qiong Luo, Mei Sun, Harold Corke. *Life Sciences.* 2004;742157.

LIST OF PUBLICATIONS

1. **P.V. Habbu**, K.M. Mahadevan R.A. Shastry and H Manjunath. Antimicrobial activity of flavanoids and other fractions of *Argyreia speciosa* (Burm. f) Boj. *Ind. J. Expt. Biol.* 2009;47: 121-127.

2. **P.V. Habbu,** K.M. Mahadevanl R.A. Shastry, Hanumanthachar Joshi, S.K. Das. Hepatoprotective and antioxidant effects of A*rgyreia speciosa* in rats. *Afr. J. Trad. CAM* 2008;5 (2): 158-64.

3. **P.V. Habbu,** K.M. Mahadevanl R.A. Shastry and S. R. Chilakwad. Anti-amnesic Potentiality of *Argyreia speciosa* (Burm.f) Boj. in mice. *Int. J. Green Pharm.* **(Accepted, In press)**

4. **P. V. Habbu,** H K Joshi, Navneet Kaur, J B. Chauhan, K M Mahadevan, M Krupa, S K Das, V H Kulkarni. Memory improving effect of Argyreia speciosa in mice. *Natural products*- An Indian journal. 2007;3 (1): 1-5.

5. **P V Habbu** , K M Mahadevan , P V Kulkarni , Daulatsingh C , V P Veerapur and R A Shastry. Adaptogenic and *in-vitro* antioxidant activity of flavanoids and other fractions of *Argyreia speciosa* (Burm.f) Boj. in acute and chronic stress paradigms in rodents. (Communicated).

CONFERENCE/SEMINAR PRESENTATIONS

1. Presented paper entitled "Antibacterial, antifungal, and antitubercular activity of *Argyreia speciosa*" in 59th Indian Pharmaceutical Congress, at Banaras Hindu University, Varanasi, India from 20th to 23rd December, C-224, 2007.

2. Presented paper entitled "Hepatoprotective and antioxidant activity of *Argyreia speciosa* in rats" during National conference in Emerging Areas in Chemical and Biological Sciences, Kuvempu University, Shankaraghatta, Shimoga. India from 23rd and 24th March 2007.

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SETCP/IAEC/2006 - 2007 / 035 Date: 12.09.2006

CERTIFICATE

This is to certify that ***Mr. PRASANNA. V. HABBU*** M.Pharm, is permitted to carry out animal experiments for the Ph.D., thesis work entitled ***"INVESTIGATION OF NOVEL QUINOLINE HETEROCYCLES AND SOME SELECTED MEDICINAL PLANTS FOR VARIOUS PHARMACOLOGICAL ACTIVITIES"*** as per the details mentioned and after observing the usual formalities laid down by IAEC as per the provisions made by CPCSEA.

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