PHYTOCHEMICAL, ANTIMICROBIAL AND PHARMACOLOGICAL INVESTIGATIONS IN *Phyllanthus wightianus* Muell. Arg. (EUPHORBIACEAE)

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Dedicated
To
Ever Memorable
Affectionate Father

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CERTIFICATE

This Thesis entitled "**PHYTOCHEMICAL, ANTIMICROBIAL AND PHARMACOLOGICAL INVESTIGATIONS IN *Phyllanthus wightianus* Muell. Arg. (EUPHORBIACEAE)**", "submitted by Mrs. O. Siva Priya for the award of Degree of Doctor of Philosophy in Microbiology-Environmental Sciences of Manonmaniam Sundaranar University is a record of bona fide research work done by her and it has not been submitted for the award of any Degree, Diploma, Associateship, Fellowship of any University/Institution.

Place: Tiruchirappalli

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DECLARARTION

I hereby declare that the Thesis entitled, **"PHYTOCHEMICAL, ANTIMICROBIAL AND PHARMACOLOGICAL INVESTIGATIONS IN *Phyllanthus wightianus* Muell. Arg. (EUPHORBIACEAE)"**, submitted by me for the Degree of Doctor of Philosophy in Microbiology-Environmental Sciences is the result of my original and independent research work carried out under the guidance of **Dr. M.B. Viswanathan**, Professor, Coordinator, Centre for Herbal Drug Discovery and Development (CHDDD), Department of Plant Science, Bharathidasan University, Tiruchirappalli 620 024 formerly Reader, Division of Biodiversity, Drug Discovery and Development, Sri Paramakalyani Centre for Environmental Sciences, Manonmaniam Sundaranar University, Alwarkurichi – 627 412, and it has not been submitted for the award of any Degree, Diploma, Associateship, Fellowship of any University or Institution.

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CONTENTS

CHAPTER 4

CHAPTER 1

INTRODUCTION

Plants form the basis of several systems of traditional medicines throughout the world for thousands of years and continue to provide new remedies to mankind. Their entry on an apprenticeship system of information has been passed on to the next generation through a shaman, curandero, traditional healer, or herbalist. Uses of the plants are often kept secret by the practitioner, so little information about them is recorded. An estimate of the World Health Organization reports that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care needs and traditional medicines also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developing countries. Ayurveda, Unani, Kampo and Chinese medicine which have flourished as systems of medicine in use for thousands of years emphasize education based on an established, frequently revised body of written knowledge and theory. These systems are still in place today because of their organizational strengths and focus primarily on multi-component mixtures (Bannerman *et al.*, 1983).

Natural Products and Drug Discovery

The history of drug development has its foundation firmly set in the study of natural remedies used to treat human disease over centuries. Analysis of medicinal plants, bioactive cultures, and increased understanding of micronutrients in the food chain have opened the door to the development of purified and defined chemical compounds as dose-controlled medicines. Thus, with the early discovery of cardiotonics from Foxglove, salicylic acid from Willow bark, morphine from Poppies, and penicillin from Mould has lead to launching of the pharmaceutical industry.

These small molecules not only served to treat diseases but also ultimately acted as pharmacologic tools for better understanding of the biochemical pathways and disease mechanisms. In contrast, modern drug discovery technologies coupled with the powerful tools of biotechnology have prompted drug discovery organizations to focus on target-driven drug discovery at the molecular level by launching high-throughput screening programs using artificial biochemical assays. At a time when the pharmaceutical industry has come under scrutiny for high rates of drug development failures it is interesting to see that natural products drug discovery has been marginalized in favor of this high-throughput biochemical screening paradigm. If modern drug development is once again to benefit from natural products as a source, then the limitations of artificial biochemical assays as applied to the screening of natural extracts must be realized in order to capitalize on the vast natural molecular diversity and rich ethnobotanic data that have emerged worldwide. Natural compounds can again become central players in the treatment of disease and in the understanding of disease mechanisms including the most dramatic impact in the area of cancer.

A more recent study, of the top 150 proprietary drugs used in the USA in 1993, found that 57% of all prescriptions contained at least one major active compound currently or once derived from (or patterned after) compounds derived from biological diversity (Table 1). A recent analysis of natural products as a source of new drugs over the period 1981–2002 shows that 67% or the 877 small molecules, new chemical entities are formally synthetic but 16.4% correspond to synthetic molecules containing a pharmacophore derived directly from natural products. Furthermore, 12% are actually modeled on a natural product inhibitor of the molecule target of interest, or mimic the endogenous substrate or the active site, such as ATP.

Table 1. Drugs Developed from Traditional Medicinal Plants

Active Principles	Clinical use	Plant source
Acetyldigoxin, Deslanoside	Cardiotonic	*Digitalis lanata* Ehrh.
Adoniside	Cardiotonic	*Adonis vernalis* L.
Aescin	Anti-inflammatory	*Aesculus hippocastanum* L.
Aesculetin	Antidysentery	*Fraxinus rhynchophylla* Hance
Agrimophol	Anthelmintic	*Agrimonia eupatoria* L.
Ajmalicine	Treatment for circulatory disorders	*Rauvolfia serpentina* (L.) Benth. ex Kurz
Allyl isothiocyanate	Rubefacient	*Brassica nigra* (L.) Koch
Anabesine	Skeletal muscle relaxant	*Anabasis aphylla* L.
Andrographolide	Treatment for bacillary dysentery	*Andrographis paniculata* Nees
Anisodamine, Anisodine	Anticholinergic	*Anisodus tanguticus* (Maxim.) Pascher
Arecoline	Anthelmintic	*Areca catechu* L.
Artemisinin	Angina pectoris and bronchial asthma	*Artemisia annua* Pall.
Asiaticoside	Vulnerary	*Centella asiatica* (L.) Urban
Atropine	Antibacterial; Anticholinergic	*Atropa belladonna* L.
Berberine	Antibacterial and treatment for bacillary dysentery	*Berberis vulgaris* L.
Bergenin	Antitussive	*Ardisia japonica* Bl.
Betulinic acid	Anticancerous	*Betula alba* L.
Bromelain	Anti-inflammatory, proteolytic	*Ananas comosus* (L.) Merrill
Caffeine	CNS stimulant	*Camellia sinensis* (L.) Kuntze
Camptothecin	Anticancerous	*Camptotheca acuminata* Decne.
Chymopapain, papain	Proteolytic and mycolytic	*Carica papaya* L.
Cissampeline	Skeletal muscle relaxant	*Cissampelos pareira* L.
Cocaine	Local anesthetic	*Erythroxylum coca* Lam.
Codeine, Morphine, Noscapine	Analgesic, antitussive, cough, pain and sedative	*Papaver somniferum* L.
Colchicine	Antitumor, antigout	*Colchicum autumnale* L.
Convallatoxin	Cardiotonic	*Convallaria majalis* L.
Curcumin	Choleretic	*Curcuma longa* L.
Cynarin	Choleretic	*Cynara scolymus* L.
Demecolcine	Antitumor agent	*Colchicum autumnale* L.
Deserpidine	Antihypertensive, tranquilizer	*Rauvolfia canescens* L.
Digitalin, Digitoxin, Digoxin	Cardiotonic	*Digitalis purpurea* L.
Emetine	Amoebicide, emetic	*Cephaelis ipecacuanha* (Brotero) A. Richard

Ephedrine	Sympathomimetic, antihistamine	*Ephedra sinica* Stapf
Ephedrine, Psedudoephedrine	Sympathomimetic, Bronchodilator	*Ephedra sinica* Stapf
Etoposide	Antitumor agent	*Podophyllum peltatum* L.
Galanthamine	Cholinesterase inhibitor	*Lycoris squamigera* Maxim.
Ginkgolides	Dementia, cerebral	*Ginkgo biloba* L.
Glasiovine	Antidepressant	*Octea glaziovii* Mez
Glaucarubin	Amoebicide	*Simarouba glauca* DC.
Glaucine	Antitussive	*Glaucium flavum* Crantz
Glycyrrhizin	Sweetener	*Glycyrrhiza glabra* L.
Gossypol	Male contraceptive	*Gossypium* species
Harpagoside, Caffeic acid	Pain, rheumatism	*Harpagophytum procumbens* DC.
Hemsleyadin	Treatment for bacillary dysentery	*Helmsleya amabilis* Diels
Hesperidin	Treatment for capillary fragility	*Citrus* species
Hydrastine	Hemostatic, astringent	*Hydrastis canadensis* L.
Hyoscyamine	Anticholinergic	*Hyoscamus niger* L.
Irinotecan	Anticancer, antitumor agent	*Camptotheca acuminata* Decne.
Kaibic acud	Ascaricide	*Digenea simplex* (Wulf.) Agardh
Kava pyrones	Anxiolytic, mild stimulant	*Piper methysticum* G. Forst.
Kawain	Tranquilizer	*Piper methysticum* G. Forst.
Khellin	Anticholinergic; Bronchodilator	*Ammi visnaga* (L.) Lam.
Lanatosides A, B, C	Cardiotonic	*Digitalis lanata* Ehrh.
Lapachol	Anticancer, antitumor	*Tabebuia* species
L-Dopa	Anti-parkinsonism	*Mucuna* species
Menthol	Rubefacient	*Mentha* species
Methyl salicylate	Rubefacient	*Gaultheria procumbens* L.
Monocrotaline	Topical antitumor agent	*Crotalaria sessiliflora* L.
Morphine	Analgesic	*Papaver somniferum* L.
Neoandrographolide	Treatment of dysentery	*Andrographis paniculata* Nees
Nicotine	Insecticide	*Nicotiana tabacum* L.
Nicotinic acid	Neuromuscular blocking Malaria	*Alangium chinense* Rehder
Nordihydroguaiaretic acid	Antioxidant	*Larrea divaricata* Cav.
Nor-pseudoephedrine	Sympathomimetic	*Ephedra sinica* Stapf
Noscapine	Antitussive	*Papaver somniferum* L.
Ouabain	Cardiotonic	*Strophanthus gratus* Franch.
Pachycarpine	Oxytoxic	*Sophora pachycarpa* Schrenk ex C.A. Mey.

Palmatine	Antipyretic, detoxicant	*Coptis japonica* Makino
Papain	Proteolytic, mucolytic	*Carica papaya* L.
Papavarine	Smooth muscle relaxant	*Papaver somniferum* L.
Phyllodulcin	Sweetener	*Hydrangea macrophylla* Ser.
Physostigmine	Cholinesterase inhibitor	*Physostigma venenosum* Balf.
Picrotoxin	Analeptic	*Anamirta cocculus* Wight & Arn.
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi* Holmes
Pinitol	Expectorant	Several plants
Podophyllotoxin	Antitumor, anticancer agent	*Podophyllum peltatum* L.
Prostratin	HIV infection	*Homalanthus nutans* Benth. & Hook. f. ex Drake
Protoveratrines A, B	Antihypertensives	*Veratrum album* S. Watson
Pseudoephredrine	Sympathomimetic	*Ephedra sinica* Stapf
Quinidine, Quinine	Antiarrhythmic, Antimalarial, antipyretic	*Cinchona ledgeriana* Moens ex Trimen
Quisqualic acid	Anthelmintic	*Quisqualis indica* Blanco
Rescinnamine, Reserpine	Antihypertensive, tranquilizer	*Rauvolfia serpentina* (L.) Benth. ex Kurz
Rhomitoxin	Antihypertensive, tranquilizer	*Rhododendron molle* Siebold & Zucc.
Rorifone	Antitussive	*Rorippa indica* (L.) Stehle
Rotenone	Piscicide, Insecticide	*Lonchocarpus nicou* (Aubl.) DC. & A. Chev.
Rotundine	Analagesic, sedative. traquilizer	*Stephania sinica* Diels
Rutin	Treatment for capillary fragility	*Citrus* species
Salicin	Analgesic	*Salix alba* L.
Sanguinarine	Dental plaque inhibitor	*Sanguinaria canadensis* L.
Santonin	Ascaricide	*Artemisia maritima* L.
Scillarin A	Cardiotonic	*Urginea maritima* (L.) Baker
Scopolamine	Motion sickness, Sedative	*Datura stramonium* L., *D. metel* L.
Sennosides A, B	Laxative	*Cassia* species
Silymarin	Antihepatotoxic	*Silybum marianum* Gaertn.
Sitosterol	Prostate hyperplasia	*Prunus africana* (Hook.f.) Kalkman
Sparteine	Oxytocic	*Cytisus scoparius* (L.) Link
Spegatrine, verticillatine	Hypotensive	*Rauvolfia verticillata* A. Chev., *R. yunnanensis* Tsiang
Stevioside	Sweetener	*Stevia rebaudiana* Bertoni
Strychnine	CNS stimulant	*Strychnos nux-vomica* L.
Taxol	Ovarian cancer	*Taxus brevifolia* L.
Teniposide	Antitumor agent	*Podophyllum peltatum* L.

Tetrahydropalmatine	Analgesic, sedative, tranquilizer	*Corydalis ambigua* Cham. & Schltdl.
Tetrandrine	Antihypertensive	*Stephania tetrandra* S. Moore
Theobromine	Diuretic, vasodilator	*Theobroma cacao* L.
Theophylline	Open bronchial passage	*Camellia sinensis* (L.) Kuntze
Theophylline	Diuretic, bronchodilator	*Theobroma cacao* L.
Thymol	Topical antifungal	*Thymus vulgaris* L.
Topotecan	Antitumor, anticancer agent	*Camptotheca acuminata* Decne.
Trichosanthin	Abortifacient	*Trichosanthes kirilowii* Maxim.
Tubocurarine	Skeletal muscle relaxant	*Chondodendron tomentosum* Ruiz & Pav.
Valepotriates	Sedative	*Valeriana officinalis* L. & Maillefer
Vasicin	Antispasmodic, cough suppressant	*Adhatoda vasica* Nees
Vasicine	Cerebral stimulant	*Vinca minor* Sm.
Vinblastine,Vincristine	Antitumor, Antileukemic agent	*Catharanthus roseus* (L.) G. Don
Yuanhuacine	Abortifacient	*Daphne genkwa* Siebold & Zucc.

Thus only 39% or the 877 molecules can be classified as truly synthetic in origin. In the area of the antiinfectives (antibacterial, antifungal, parasitic, and viral), close to 70% are naturally derived or inspired, while in the cancer treatment area 67% are in this category. In recent years, there has been a steady decline in the output of the Research & Development programs of the pharmaceutical industry and the number of new active substances, also known as the new chemical entities hit a 20-year low or 37 in 2001. This downturn has been attributed in part to disruption of laboratory activities by the surge in company merges and acquisitions, the mounting costs of drug development, and the FDA over-caution in the drug approval process. Recently, there has been rekindling of interest in rediscovering natural products. As stated by one authority "we would not have the top-selling drug class today, the statins; the whole field of angiotensin antagonists and angiotensin-converting enzyme inhibitors; the whole area or immunosuppressives, nor most or the anticancer and antibacterial drugs". It is clear that nature has played and will continue to play a vital role in the drug discovery process (Cragg and Newman, 2005). From 155 anticancer drugs developed since the 1940s, only 27% could not be traced to natural products, with 47% being either a natural product or a direct derivation thereof. Only one drug, the anticancer compound sorafenib, could be traced to completely de novo combinatorial chemistry (Newman and Cragg, 2006).

Challenges in Drug Discovery from Medicinal Plants

The process of drug discovery has been estimated to take an average of 10 years upwards (Reichert, 2003) and costs more than 800 million dollars (Dickson and Gagnon, 2004). Much of this time and money is spent on the numerous leads that are discarded during the drug discovery process. In fact, it has been estimated that only one in 5000 lead compounds will successfully advance through clinical trials and be

approved for use. Lead identification is the first step in a lengthy drug development process. Lead optimization (involving medicinal and combinatorial chemistry), lead development (including pharmacology, toxicology, pharmacokinetics, ADME [absorption, distribution, metabolism, and excretion], and drug delivery), and clinical trials all take a considerable length of time. Drug discovery from medicinal plants has traditionally been lengthier and more complicated than other drug discovery methods. As such, many pharmaceutical companies have eliminated or scaled down their natural product research (Butler, 2004; Koehn and Carter, 2005). At National Cancer Institute, contracts for the collection of plants that have been operating for nearly 20 years in the Americas, Africa, Madagascar, and Southeast Asia were recently suspended due to reallocation of National Cancer Institute funds for new initiatives aimed at improving diagnosis and prevention, as well as expediting the translation of drugs from the development phase to clinical use. In addition, as academic pharmacy departments redirect their focus towards the production of clinical and community pharmacists, the emphasis on pharmaceutical research and development related to medicinal plant and natural product drug discovery in academic pharmacy departments is declining. Although the trend towards a loss of teaching and research positions in pharmacognosy has been in evidence at U.S. institutions of pharmacy education, several positive steps can be seen recently towards reversing this trend, such as the development of the National Center for Natural Products Research at the University of Mississippi, the establishment of National Institutes of Health (NIH). Challenges in bioassay screening remain an important issue in the future of drug discovery from medicinal plants. Improving the speed of active compound isolation will necessitate the incorporation of new technologies. Although nuclear magnetic resonance (NMR) and mass spectrometry (MS) are currently in wide use for

compound identification, new methods of using NMR and MS could be applied to medicinal plant drug discovery to facilitate compound isolation (Eldridge *et al.*, 2002; Pellecchia *et al.*, 2002; Glish and Vachet, 2003). Also, the use of high-throughput X-ray crystallography could be applied to medicinal plant lead discovery (Blundell *et al.*, 2002). Compound development of drugs discovered from medicinal plants also faces unique challenges. Natural products are typically isolated in small quantities that are insufficient for lead optimization, lead development, and clinical trials. Collaborating with synthetic and medicinal chemists is necessary to determine if synthesis or semi-synthesis might be possible (Ley and Baxendale, 2002; Federsel, 2003; Lombardino and Lowe, 2004). Another technique to improve natural product compound development may involve the creation of natural product and natural-product-like libraries that combine the features of natural products with combinatorial chemistry (Hall *et al.*, 2001; Lee and Schneider, 2001; Eldridge *et al.*, 2002; Feher and Schmidt, 2003; Burke *et al.*, 2004; Ganesan, 2004; Piggott and Karuso, 2004; Tan, 2004; Koehn and Carter, 2005). In conclusion, natural products discovered from medicinal plants (and derivatives thereof) have provided numerous clinically used medicines. Even with all the challenges facing drug discovery from medicinal plants, natural products isolated from medicinal plants can be predicted to remain an essential component in the search for new medicines. Therefore, the world has been witnessing the growth of scientific and commercial interests in plant-based medicines mainly due to their immense economic potential and the widespread cultural acceptability.

Traditional Uses of *Phyllanthus* species

The genus *Phyllanthus* (Euphorbiacae) is widely distributed in most tropical and subtropical countries and is estimated to be about 750 species in the world and

about 175 species in India. It has long been extensively used in folk medicine in most of the countries of the world and in India for thousands of years to treat broad spectrum of diseases such as disturbances of the kidney and urinary bladder, intestinal infections, diabetes and the hepatitis B virus (Unander *et al.,* 1995; Calixto *et al.,* 1998). Various therapeutic activities have been reported in ethnobotany such as leaves as expectorant, diaphoretic and useful in strangury and sweats, seeds as carminative, laxative, astringent to the bowels, tonic to the liver, diuretic, diaphoretic, bronchitis, earache, griping, ophthalmia and ascites (Kirtikar and Basu, 2001). Modern research focuses on its potential for fighting viruses, specifically the hepatitis B virus. In recent years, the interest has increased considerably, especially regarding their therapeutic potential for the management of many diseases and its greater diversity of secondary metabolites.

Therefore, one of the *Phyllanthus* species, *P. wightianus* used by the Malayali tribe in the Javadi hills of the Vellore District in Tamil Nadu for bone setting and to treat diarrhoea was chosen with the following objectives for the present study:

- To find out the bioactive extracts/fractions/principles or active compounds from *P. wightianus* using phytochemical methods;
- To test the bioactive extracts/fractions/principles or active compounds for antimicrobial and pharmacologic studies at preclinical level to substantiate the claims mentioned in ethnobotanical information and to generate scientific evidence for curing diseases such as hepatitis, diabetes, arthritis, intestinal and skin infections and painful disorders; and
- To identify new activities that can be used as leads for developing new drugs not recorded in the reported literature.

CHAPTER 2

REVIEW OF LITERATURE

2.1 PHYTOCHEMISTRY

According to the published and available literatures, 299 compounds have been reported under various classes from *Phyllanthus* species (Table-2).

2.2 ANTIMICROBIAL STUDIES

Antibacterial properties have been reported in 4 species such as *P. acidus* (Melendez and Capriles, 2006), *P. amarus* (Vinayagamoorthy, 1982; Kannan and Venkatakrishnan, 2002; Chukwujekwu *et al.,* 2005; Kloucek *et al.,* 2005; Mazumder *et al.,* 2006), *P. discoideus* (Mensah *et al.,* 1990; Olukoya *et al.,* 1993) and *P. emblica* (Thakare, 1980). Equal number of species have been reported to contain antifungal properties such as *P. amarus* (Agrawal *et al.,* 2004), *P. emblica* (Mehmood *et al.,* 1999), *P. fraternus* (Bhowmick and Vardhan, 1981, 1982) and *P. niruri* (Rodriguez and Sanabria, 2005). However, antibacterial and antifungal properties have been reported increasingly in 7 species such as *P. acuminatus* (Goun *et al.,* 2003), *P. amarus* (Verpoorte and Dihal, 1987; Houghton *et al.,* 1996), *P. emblica* (Sankaranarayanan and Jolly, 1993; Jasril *et al.,* 1999), *P. flexuosus* (Ito, 2005), *P. fraternus* (Ramchandani and Chungath, 1988), *P. piscatorum* (Gertsch *et al.,* 2004) and *P. urinaria* (Verpoorte and Dihal, 1987; Cruz *et al.,* 1994).

2.3 PHARAMACOLOGY

Several biological activities have been reported for the species of *Phyllanthus.* They are given below:

Table 2. Chemical Constituents Isolated from Plants of the Genus *Phyllanthus*

S. No.	Compound Name	Species	References
		Alkaloids	
1	Allosecurinine	*P. niruri*	Hassarajani and Mulchandani, 1990
2	Dihydro securinine	*P. discoideus*	Mensah *et al.*, 1988
		P.niruri	Hassarajani and Mulchandani, 1990
3	Epibubbialine	*P. amarus*	Houghton *et al.*, 1996
4	Isobubbialine		
5	Nirurine		Thuy *et al.*, 2005
6	Niruroidine	*P. niruroides*	Bila *et al.*, 1996
7	Norsecurinine	*P. amarus*	Houghton *et al.*, 1996
		P. discoideus	Bevan *et al.*, 1964
			Mensah *et al.*, 1988
		P. niruri	Hassarajani and Mulchandani, 1990
8	Phyllanthimide	*P. sellowianus*	Tempesta *et al.*, 1988
9	Phyllanthine	*P. amarus*	Houghton *et al.*, 1996
		P. niruri	Hassarajani and Mulchandani, 1990
		P. simplex	Negi and Fakhir, 1988
10	Phyllantidine	*P. discoideus*	Parello and Munavalli, 1965
		P. emblica	Khanna and Bansal, 1975
11	Phyllantine	*P. discoideus*	Parello and Munavalli, 1965
		P. emblica	Khanna and Bansal, 1975
12	Phyllochrysine	*P. discoideus*	Parello *et al.*, 1963
13	Phyllochrysine – HCl		Foussard Blanpin *et al.*, 1967
14	Phyrrolizidine alkaloids	*P. amarus*	Arun *et al.*, 2001
15	Securinine	*P. amarus*	Houghton *et al.*, 1996
		P. discoideus	Parello *et al.*, 1963
			Mensah *et al.*, 1988
			Foussard Blanpin *et al.*, 1967
			Quevauviller *et al.*, 1967
			Mensah *et al.*, 1990
		P. niruri	Hassarajani and Mulchandani, 1990
16	Securinol – A	*P. niruri*	Hassarajani and Mulchandani, 1990
17	Securinol – B		
18	Simplexine	*P. simplex*	Negi and Fakhir, 1988

19	Tetra hydro securinine	*P. niruri*	Hassarajani and Mulchandani, 1990
20	Tricontanol	*P. urinaria*	Satyan *et al.*, 1995
21	Viroallosecurinine	*P. discoideus*	Mensah *et al.*, 1988, 1990
22	Zeatin	*P .emblica*	Ram and Rao, 1976
23	Zeatin nucleotide		
24	Zeatin riboside		
25	14 – hydroxy – 4 methoxy – 13, 24 dihydro nor securinine	*P. simplex*	Negi and Fakhir, 1988
26	14, 15 – dihydroallo securinine – 15 - β- 01	*P. discoideus*	Mensah *et al.*, 1988
27	4 – hdroxyl securinine	*P. niruri*	Hassarajani and Mulchandani, 1990
28	4 – methoxy dihydro nor securinine		
29	4 – methoxy tetra hydro securinine		
Benzenoids			
30	Amlaic acid	*P. emblica*	Desai *et al.*, 1977
31	β-glucogallin		Theresa *et al.*, 1967
			Srivastava and Ranjan, 1967
32	Chebulic acid		Theresa *et al.*, 1965, 1967
33	Corilagin	*P. amarus*	Foo, 1993 b
		P. debilis	Kumaran and Karunakaran, 2006 a
		P. emblica	Srivastava and Ranjan, 1967
		P. myrtifolius	Chen Liu *et al.*, 1999
		P. niruri	Ishimaru *et al.*, 1992
			Wei *et al.*, 2004
			Than *et al.*, 2006
		P. urinaria	Chen Liu *et al.*, 1999
34	Digallic acid	*P. emblica*	El – Mekkawy *et al.*,1995
35	Ethyl gallate		Srivastava and Ranjan, 1967
36	Gallic acid	*P. debilis*	Kumaran and Karunakaran, 2006 a
		P. emblica	Theresa *et al.*, 1965, 1967
			Basa and Srinivasulu, 1987
		P. niruri	Ueno *et al.*, 1988
			Ishimaru *et al.*, 1992; Than *et al.*,2006
		P. ussuriensis	Whang *et al.*, 1994

37	Isocorilagin	*P. niruri*	Than *et al.*, 2006
38	Putranjivain A	*P. emblica*	El – Mekkawy *et al.*, 1995
39	1 – di – O – galloyl - β - D – glucose		
40	1,6 – di – O – galloyl - β - D – glucose		
41	3-6 – di – O – galloyl – glucose		Srivastava and Ranjan, 1967
Carbohydrates			
42	Acidic and nuetral polysaccharides	*P. emblica*	Nizzamuddin *et al.*, 1982
			Theresa *et al.*, 1967
Coumarins			
43	Bergenin	*P. flexuosus*	Tanaka and Matsunaga, 1988
44	Ellagic acid	*P. emblica*	Theresa *et al.*, 1965
			Hui and Sung, 1968
			Subramanian *et al.*, 1971.
		P. niruri	Ueno *et al* ., 1988; Wei and Pan, 2002
Diterpenes			
45	Gibberellin A-1	*P. emblica*	Ram and Raja, 1978
46	Gibberellin A-3		
47	Gibberellin A-4		
48	Gibberellin A-7		
49	Gibberellin A-9		
Flavonoids			
50	Aglycone	*P. niruri*	Gupta and Ahmed, 1984 b
51	Epicatechin – (2β- 8) gallocatechin	*P. emblica*	Zhang *et al.*, 2000 a
52	Epigallocatechin–(2β–8) gallogatatechin (Prodephinidin A-1)		
53	Fisetin – 4' – 0 – glycoside	*p. niruri*	Gupta and Ahmed, 1984 a
54	Flavanoids	*P.chamaecristoides*	Alonsa *et al.*, 1995
		P.simplex	Bharadwaj, 1994
55	Flavanoids FG 1	*P. fraternus*	Hukeri *et al.*, 1988
56	Flavanoids FG 2	*P. niruri*	Than *et al.*, 2006
57	Galangin – 3 – 0-β–D – glucoside – 8 - sulfonate	*P. virgatus*	Huang *et al.*, 1998 b
58	Galangin – 8 – sulfonate		

59	Iso quercetin	*P. niruri*	Than *et al.*, 2006
60	Kaempherol	*P. emblica*	Subramanian *et al.*, 1971
61	Kaempherol – 3 – O - β - D – glucoside	*P. emblica*	El – Mekkawy *et al.*, 1995
62	Kamferol – 8 – Sulfonate	*P. virgatus*	Huang *et al.*, 1998 b
63	Leucodelphinidin	*P. emblica*	Laumas and Seshadri, 1958
64	Niruriflavone	*P. niruri*	Than *et al.*, 2006
65	Nirurin		Gupta and Ahmed, 1984 b
66	Nirurinetin		
67	Pholoroglucinol	*P. ussuriensis*	Whang *et al.*, 1994
68	Protocatechunic acid	*P. klotzschianus*	Kuster *et al.*, 1996
69	Quercetin		
70	Quercetin – 3 – 0 glucopyranoside	*P. amarus*	Foo, 1993 b
71	Quercetin -3– 0 – β – D – glucopyranosyl – rhamno pyrananoside	*P. niruri*	Than *et al.*, 2006
72	Quercetin -3– O - β - D – glucoside	*P. emblica*	El – Mekkawy *et al.*, 1995
73	Rutin	*P. amarus*	Foo, 1993 b
		P. debilis	Kumaran and Karunakaran, 2006 a
		P. klotzschianus	Kuster *et al.*, 1996
		P. sellowianus	Miguel *et al.*, 1995 a
		P. ussuriensis	Whang *et al.*, 1994
74	(-) – epicatechin	*P. klotzschianus*	Kuster *et al.*, 1996
75	(-) – epigallocatechin 3–0-gallate	*P. niruri*	Ishimaru *et al.*, 1992
76	(+) – Catechin	*P. klotzschianus*	Kuster *et al.*, 1996
77	(+) – gallocatechin	*p. niruri*	Ishimaru *et al.*, 1992
78	1 – 0 - β - (6' acetyl) – glucosylorcinol	*P. klotzschianus*	Kuster *et al.*, 1996
79	1 – 0 - β - Glucosylorcinol		
80	4', 4" – di – 0 – methyl cupressulflavone	*P. sellowianus*	Hnatyszyn *et al.*, 1987, 1995
81	7 – hydroxy flavanone		
Lignans			
82	Cleistanthin A methyl ether	*P. taxodiifolius*	Tuchinda *et al.*, 2006
83	Cleistanthin B		
84	Cleistanthoside A		

85	Cubebin dimethyl ether	*P. niruri*	Elfahmi *et al.*, 2006
86	Demethyl enedioxynirganthin		Satyanarayana and Venkateshwarlu, 1991
87	Dextrobursehernin	*P. urinaria*	Chang *et al.*, 2003
88	Heliobuphthalmin lactone		
89	Hydroxy niranthin	*P. niruri*	Satyanarayana *et al.*, 1988
90	Hypophyllanthin	*P. amarus*	Deb and Mandal, 1996
			Murali *et al.*, 2001
			Sharma *et al.*, 1993
		P. niruri	Row and Srinivasulu, 1964; Row *et al.*, 1966
			Anjaneyulu *et al.*, 1973
			Bhadbhade *et al.*, 1980
			Singh *et al.*, 1986
			Srinivasulu, 1992
			Huang *et al.*, 1992
			Hussain *et al.*, 1995
			Than *et al.*, 2006
		P. urinaria	Chang *et al.*, 2003
91	Iso lintetralin	*P. niruri*	Huang *et al.*, 1992
		P. urinaria	Chang *et al.*, 2003
92	Justicidin A	*P. acuminatus*	Pettit and Schaufelberger, 1988
		P. myrtifolius	Lin *et al.*, 1995
93	Justicidin B	*P. anisoloubs*	Bachmann *et al.*, 1993
		P. myrtifolius	Lin *et al.*, 1995
94	Linnanthin	*P. niruri*	Satyanarayana and Venkateshwarlu, 1991
95	Linoleinin	*P. niruri*	Huang *et al.*, 1992
96	Lintetralin	*P. niruri*	Singh *et al.*, 1986
			Satyanarayana *et al.*, 1988
			Huang *et al.*, 1992
		P. urinaria	Chang *et al.*, 2003
97	Niranthin	*P. niruri*	Anjaneyulu *et al.*, 1973
			Singh *et al.*, 1986
			Huang *et al.*, 1992
		P. urinaria	Chang *et al.*, 2003
98	Nirphyllin	*P. niruri*	Singh *et al.*, 1989 a
99	Nirtetralin	*P. niruri*	Anjaneyulu *et al.*, 1973
			Singh *et al.*, 1986
			Huang *et al.*, 1992

			Hussain *et al.*, 1995
		P. urinaria	Chang *et al.*, 2003
100	Phyllamyricin A	*P. myrtifolius*	Lin *et al.*, 1995
101	Phyllamyricin B		
102	Phyllamyricin C		
103	Phyllamyricin D		
104	Phyllamyricin E		
105	Phyllamyricin F		
106	Phyllamyricoside A		
107	Phyllamyricoside B		
108	Phyllamyricoside C		
109	Phyllanthin	*P. amarus*	Sharma *et al.*, 1993
			Deb and Mandal, 1996
			Bagchi *et al.*, 1999
			Patel *et al.*, 2000
			Murali *et al.*, 2001
		P. niruri	Row and Srinivasulu, 1964; Row *et al.*, 1966
			Anjaneyulu *et al.*, 1973
			Singh *et al.*, 1986
			Srinivasulu, 1992
			Huang *et al.*, 1992
			Hussain *et al.*, 1995
		P. urinaria	Chang *et al.*, 2003
110	Phyllanthoside	*P. acuminatus*	Pettit *et al.*, 1983, 1984, 1990
		P. verminiatus	George, 1983
111	Phyllanthostatin 1	*P. acuminatus*	Pettit *et al.*, 1983
112	Phyllanthostatin 2	*P. verminiatus*	George, 1983
113	Phyllanthostatin 3	*P. acuminatus*	Pettit *et al.*, 1984, 1990
114	Phyllanthostatin 6		Pettit *et al.*, 1990
115	Phyllanthostatin A		Pettit and Schaufelberger, 1988
		P. anisolobus	Bachmann *et al.*, 1993
116	Phyllanthusmin A	*P. oligospermus*	Wu and Wu, 2006
117	Phyllanthusmin B		
118	Phyllanthusmin C		
119	Phyllnirurin	*P. niruri*	Singh *et al.*, 1989 a
120	Phylteralin	*P. niruri*	Anjaneyulu *et al.*, 1973
			Singh *et al.*, 1986
		P. urinaria	Chang *et al.*, 2003
121	Retrojusticidin B	*P. myrtifolius*	Lin *et al.*, 1995

122	Seco – 4 – hydroxyl – interalin	*P. niruri*	Satyanarayana *et al.*, 1988
123	Seco – isolari – ciresinol trimethyl ether		
124	Taxodiifoloside	*P. taxodiifolius*	Tuchinda *et al.*, 2006
125	Urinaligran	*P. urinaria*	Chang *et al.*, 2003
126	Urinatetralin	*P.niruri*	Elfahmi *et al.*, 2006
		P.urinaria	Chang *et al.*, 2003
127	Virgatusin	*P. virgatus*	Huang *et al.*, 1996
128	Virgatyne		Huang *et al.*, 1998 b
129	(+) – 3, 3' 4, 4' 9, 9' – hexamethoxy – 8 : 8' – butyrolignan	*P. niruri*	Row *et al.*, 1966
130	2, 3 – de methoxy seco-isolintetralin	*P. niruri*	Satyanarayana and Venkateshwarlu, 1991
131	2, 3 – demethoxy seco – isolintetralin diacetate		
132	5 – demethoxy niranthin	*P. urinaria*	Chang *et al.*, 2003
Sterols			
133	Campesterol	*P. flexuosus*	Tanaka and Matsunaga, 1988
134	Ester of β - Sitosterol	*P. maderaspatensis*	Jain *et al.*, 2005
135	Estradiol	*P. niruri*	Mannan and Ahmed, 1978
			Singh *et al.*, 1986
136	Fraternu sterol	*P. fraternus*	Gupta and Ali, 1999 b
137	Phyllanthoesterol		
138	Phyllanthosecosteryl ester		
139	Phyllanthostigma sterol		
140	Sito sterol - β - (D) – glucoside	*P. watsonii*	Matsunaga *et al.*, 1993
141	Sitosterol	*P. reticulatus*	Hui *et al.*, 1976
		P. watsonii	Matsunaga *et al.*, 1993
142	Stigma sterol	*P. flexuosus*	Tanaka and Matsunaga, 1988
143	Stigma sterol acetate	*P. corcovadensis*	Santos *et al.*, 1995 a
144	Tara xerol	*P. maderaspatensis*	Jain *et al.*, 2005
145	24 – isopropyl cholesterol	*P. niruri*	Singh *et al.*, 1986
146	7 – dehydro - β - sitosterol	*P. amarus*	Liengjayetz and Teerasukaporn, 2000
147	α - sitosterol	*P. corcovadensis*	Santos *et al.*, 1995a
148	β – sitosterol	*P. emblica*	Hui and Sung, 1968

		P. flexuosus	Tanaka and Matsunaga, 1988
		P. maderaspatensis	Jain *et al.*, 2005
		P. muellerianus	Adesida *et al.*, 1972
		P. niruri	Singh *et al.*, 1986
		P. reticulatus	Joshi *et al.*, 1981
		P. urinaria	Satyan *et al.*, 1995
Tannins			
149	Amariinic acid	*P. amarus*	Foo, 1995
150	Amarulone		Foo, 1993 a
151	Catechuic tannin	*P. emblica*	Heimde Balsac *et al.*, 1930
152	Chebulagic acid	*P. emblica*	Theresa *et al.*, 1965, 1967
		P. myrtifolius	Liu *et al.*, 1999
		P. urinaria	
153	Elecocarpusin	*P. amarus*	Foo, 1995
154	Ellagitannins	*P. urinaria*	Liu *et al.*, 1999
155	Furosin	*P. amarus*	Foo, 1995
		P. debilis	Kumaran and Karunakaran, 2006 a
156	Geraniin	*P. amarus*	Foo, 1993 b
		P. debilis	Kumaran and Karunakaran, 2006 a
		P. niruri	Ueno *et al.*, 1988
		P. myrtifolius	Liu *et al.*, 1999
		P.urinaria	
157	Geraniinic acid B	*P. amarus*	Foo, 1995
158	Mallotusinin	*P. myrtifolius*	Liu *et al.*, 1999
		P. urinaria	
159	Phyllanemblinin A	*P. emblica*	Zhang *et al.*, 2001 a, b
160	Phyllanemblinin B		
161	Phyllanemblinin C		
162	Phyllanemblinin D		
163	Phyllanemblinin E		
164	Phyllanemblinin F		
165	Phyllanthusiin	*P. urinaria*	Chen *et al.*, 1999
166	Phyllanthusiin A	*P. flexuosus*	Yoshida *et al.*, 1992
167	Phyllanthusiin B		
168	Phyllanthusiin C	*P. flexuosus*	Yoshida *et al.*, 1992
		P. myrtifolius	Liu *et al.*, 1999
		P. urinaria	

169	Phyllanthusiin D	*P. amarus*	Foo and Wong, 1992
170	Phyllanthusiin E	*P. flexuosus*	Yoshida *et al.*, 1992
171	Pinocembrin – 7 – O – [3" – O – galloyl – 4", 6" – (S) – hexa hydroxy diphenoyl] - β-D-glucose	*P. tenellus*	Huang *et al.*, 1998 a
172	Pinocembrin – 7 – O – [4", 6" – (S) – hexa hydroxy diphenoyl] β - D – glucose		
173	Pyrogallic tannin	*P. emblica*	Heimde Balsac *et al.*, 1930
174	Pyrogalloyl	*P. urinaria*	Chen *et al.*, 1999
175	Repanudusiinic acid A	*P. amarus*	Foo, 1995
176	Virganin	*P. viragatus*	Huang *et al.*, 1998 b
177	1 – galloyl – 2, 4 – (acetonyl – de hydro hexa hydroxyl diphenyl) – 3, 6 hexa hydroxy diphenoyl glucopyranoside	*P. amarus*	Foo and Wong, 1992
178	1 – galloyl – 2, 4 : 3, 6 – bis – DHHDP – glucopyranoside (Amariin)		Foo, 1993 b
179	1 – galloyl – 3, 6 hexa hydroxy diphenoyl – 4 – O – brevifolin Carboxyl - β - D – glucopyranose	*P. myrtifolius* *P.urinaria*	Liu *et al.*, 1999
180	1, 6 – di galloyl glucopyranoside	*P. amarus*	Foo, 1993 b
181	1-O- galloyl – 2, 4 – de hydrohexahydroxy di phenoyl gluco pyranose		Foo, 1995
182	2 – galloyl – 3, 6 hexa hydroxy diphenoyl – 4 – O – brevifolin Carboxyl - β - D – glucopyranose	*P. urinaria*	Liu *et al.*, 1999
183	4 – O – galloylduinic acid	*P. amarus*	Foo, 1995
Triterpenes			
184	Betulin	*P. flexuosus*	Tanaka *et al.*, 1988 a; Wada *et al.*, 2001

		P.reticulatus	Joshi *et al.*, 1981
185	Betulinic acid	*P. reticulatus*	Hui *et al.*, 1976
186	ent - 3β - hydroxykaur – 16 – ene	*P. flexuosus*	Tanaka and Matsunaga, 1988
187	Epifriedelinol	*P. reticulatus*	Joshi *et al.*, 1981
		P. watsonii	Matsunaga *et al.*, 1993
188	Friedelan – 3 β - ol	*P. reticulatus*	Hui *et al.*, 1976
189	Friedelin	*P. amarus*	Liengjayetz and Teerasukaporn, 2000
		P. flexuosus	Tanaka and Matsunaga, 1988
		P. reticulatus	Hui *et al.*, 1976
			Joshi *et al.*, 1981
		P. watsonii	Matsunaga *et al.*, 1993
190	Glochidonol	*P. reticulatus*	Hui *et al.*, 1976; Joshi *et al.*, 1981
		P. watsonii	Matsunaga *et al.*, 1993
191	Hexacosane	*P.maderas patensis*	Jain *et al.*, 2005
192	lup – 20 (29) – en - 1β, 3β - diol	*p. watsonii*	Matsunaga *et al.*, 1993
193	Lup – 20 (29) – en - 3β, 24 – diol	*P. flexuosus*	Tanaka *et al.*, 1988 a
194	lup – 20 (29) – ene - 1β, 3α - diol	*P. sellowianus*	Filho *et al.*, 1998
195	Lupa – 1, 20 (29) - dien 3 – one (Glochidone)	*P. flexuosus*	Tanaka and Matsunaga, 1988
		P. sellowianus	Miguel *et al.*, 1995 a
		P. taxodiifolius	Tuchinda *et al.*, 2006
		P. watsonii	Matsunaga *et al.*, 1993
196	Lupa – 20 (29) – ene – 3 - β - 01	*P. niruri*	Chauhan *et al.*, 1979
197	Lupenone	*P. emblica*	Hui and Sung, 1968
198	Lupenyl palmitate	*p. watsonii*	Matsunaga *et al.*, 1993
199	Lupeol	*P. emblica*	Desai *et al.*, 1977
			Hui and Sung, 1968
		P. flexuosus	Tanaka and Matsunaga, 1988
			Wada *et al.*, 2001
		P. niruri	Chauhan *et al.*, 1979
		P. watsonii	Matsunaga *et al.*, 1993
200	Lupeol acetate	*P. urinaria*	Satyan *et al.*, 1995
201	n-tetracosane	*P.maderas patensis*	Jain *et al.*, 2005

202	Octacosanol	*P. reticulatus*	Joshi *et al.*, 1981
203	Olean – 12 – en - 3β, 15α - 24 triol	*P. flexuosus*	Tanaka *et al.*, 1988 a
			Wada *et al.*, 2001
204	Olean – 12 – en - 3β, 15α - diol	*P. flexuosus*	Tanaka *et al.*, 1988 a
			Wada *et al.*, 2001
205	Olean – 12 – en - 3β, 24 – diol	*P. flexuosus*	Tanaka *et al.*, 1988 a
206	Oleana – 11 – 13(18) dienc – 3p, 24 – diol	*P. maderaspatensis*	Jain *et al.*, 2005
207	Oleana – 11 : 13 (18) – dien - 3β - ol	*P. flexuosus*	Tanaka and Matsunaga, 1988
208	Oleana – 11:13 (18) – dien - 3β1, 24 – driol	*P. flexuosus*	Tanaka *et al.*, 1988 a
209	Oleana – 9 (11) : 12 – dien - 3β - ol	*P. flexuosus*	Tanaka and Matsunaga, 1988
210	Phyllanthenol	*P. amarus*	Liengjayetz and Teerasukaporn, 2000
		P. niruri	Singh *et al.*, 1989 b
211	Phyllanthenone	*P. niruri*	Singh *et al.*, 1989 b
212	Phyllantheol		
213	Phyllanthol	*P. engleri*	Cole, 1954
		P. sellowianus	Hnatyszyn and Ferraro, 1985
214	Tara xenone	*P. reticulatus*	Joshi *et al.*, 1981
215	Tara xeryl acetate	*P.maderas patensis*	Jain *et al.*, 2005
		P. reticulatus	Joshi *et al.*, 1981
216	Tetracos – 20 (cn) – 1, 18 – diol	*P. maderaspatensis*	Jain *et al.*, 2005
217	Triterpene arabinosides 1	*P. polyphyllus var siamensis*	Youkwan *et al.*, 2005
218	Triterpene arabinosides 2		
219	β–amyrin	*P. flexuosus*	Tanaka and Matsunaga, 1988
		P. urinaria	Satyan *et al.*, 1995
220	11 – hydroxy hexacosan – 3 – one	*P. maderaspatensis*	Jain *et al.*, 2005
221	11 β - hydroxy D : A friedo olean – 1 – en – 3 – one	*P. flexuosus*	Tanaka *et al.*, 1994
222	1β - hydroxy lupa 20 (29) – en – 3 one (glochidonol)	*P. sellowianus*	Filho *et al.*, 1998
223	21 α - hydroxy friedel – 4 (23) – en – 3 – one	*P. reticulatus*	Hui *et al.*, 1976

224	21 α - hydroxy friedelan – 3 – one		
225	21 α hydroxy friedelen – 3 – one		Joshi *et al.*, 1981
226	26 – nor – D : A – friedo olean 14 – en – 3 – one	*P. watsonii*	Matsunaga *et al.*, 1993
227	26 – nor – D : A – friedo olean 14 – en - 3β - ol		
228	26 – nor – D : A – friedo oleanane	*P. watsonii*	Matsunaga *et al.*, 1993
229	29 – nor – 3, 4 – Seco – friedelan – 4 (23), 20 (30) – dien – 3 – oic acid	*P. oxyphyllus*	Sutthivaiyakit *et al.*, 2003
230	3, 7, 11, 15, 19, 23 – hexamethyl – 2z, 6z, 10z, 14E, 18E, 22E tetra-coshexan – 1 – ol	*P. niruri*	Singh *et al.*, 1989 c
231	3β - hydroxy – D:A – friedo oleanan – 27 – oic acid (Trichadenic acid B)	*P. flexuosus*	Tanaka *et al.*, 1988 b
Sesquiterpenes			
232	Methyl ester of Phyllaemblic acid	*p. emblica*	Zhang *et al.*, 2000 b
233	Phyllaemblic acid	*P. emblica*	Pillay and Iyer, 1958
			Zhang *et al.*, 2000 a, b
234	Phyllaemblic acids B	*P. emblica*	Zhang *et al.*, 2001 a, b
235	Phyllaemblic acids C		
236	Phyllaemblicin A	*P. emblica*	Zhang *et al.*, 2000 a
237	Phyllaemblicin B		
238	Phyllaemblicin C		
239	Phyllaemblicin D	*P. emblica*	Zhang *et al.*, 2001 a, b
240	Phyllanthocin (derivative of phyllanthoside)	*P. brasiliensis*	Kupchan *et al.*, 1977
Glycosides			
241	Arabinogalactan	*P. niruri*	Mellinger *et al.*, 2005
242	Phyllurine	*P. urinaria*	Ueda *et al.*, 1998
243	Phyllanthurinolactone	*P. urinaria*	Ueda *et al.*, 1995, 1998; Mori *et al.*, 1997
244	2 – Carboxymethyl Phenol 1 – O - β - D – glucopyranoside	*P. emblica*	Zhang *et al.*, 2001 a, b

245	2, 6 – dimethyloxy – 4 – (2-hydroxy ethyl) phenol 1-O-β-D-gluco pyranoside		
246	(S) – eriodictyol 7 – O – (6" – O – trans – P – coumaroyl) - β - D – gluco pyranoside		
247	(S) – eriodictyol 7 – O – (6" – O – galloyl) – β – D – gluco pyranoside	*P. emblica*	Zhang *et al.*, 2002
248	2 – (2 – methyl butyryl) – phloroglucinol 1 – O - (6" – O – β - D – apifuranosyl) - β - D – gluco pyranoside		
249	5 – Hydroxy–6, 9–epoxyguaiane	*P. oxyphyllus*	Sutthivaiyakit *et al.*, 2003
250	5 – O – acetyl – 6, 9 – epoxy guaiane		
Lactones			
251	Aquilegiolide	*P. anisolobus*	Bachmann *et al.*, 1993
		P. klotzschianus	Kuster *et al.*, 1997
252	Dibenzylbutyro lactone	*P. niruri*	Satyanarayana *et al.*, 1988
253	Menisdaurilide	*P. anisolobus*	Bachmann *et al.*, 1993
		P. klotzschianus	Kuster *et al.*, 1997
254	Simaronbolides	*P. braziliensis*	Kinghorn, 1983
Phytallates			
255	Daphnane ester	*P. brazilinesis*	Kinghorn, 1983
256	Heptacosanoic acid	*P. fraternus*	Gupta and Ali, 1999 a
257	Montanoic acid methyl ester	*P. urinaria*	Satyan *et al.*, 1995
258	Pentacosanyl ester	*P. fraternus*	Gupta and Ali, 1999 a
259	Phyllanterpenyle ester		
260	Phyllanthusone		
261	Phyllester	*P. niruri*	Singh *et al.*, 1986
262	Pthalic acid bis (2, 5 – di methyl hexyl) ester	*P. urinaria*	Satyan *et al.*, 1995
Alkanols			
263	Heptacosanol	*P. maderaspatensis*	Jain *et al.*, 2005
264	n – alkanols	*P. flexuosus*	Tanaka and Matsunaga, 1988
265	n – alkane		
267	32–methyl tritria contanol -1	*P. maderaspatensis*	Jain *et al.*, 2005

Alkamides			
268	E, E-2, 4 – Octadien amide	*P. fraternus*	Sittie *et al.*, 1998
269	E, Z – 2, 4 – decadien amide		
Acids			
270	Ascorbic acid	*P. emblica*	Damoradan and Shrinivasan, 1935
			Quadry *et al.*, 1962
			Shah and Hamid, 1968
			Basa and Srinivasulu, 1987
			Reddy and Laxminarayana, 1984
			Roy *et al.*, 1991
			Prasad and Kumar, 1980 – 1981
			Suresh and Vasudevan, 1994
			Singh *et al.*, 1984
			Roy *et al.*, 1987
			Mishra *et al.*, 1996
			Shishoo *et al.*, 1997
		P. niruri	Sinha and Dogra, 1981
			Bharadwaj, 1994
		P. urinaria	Shishoo *et al.*, 1997
			Dogra and Sinha, 1979, 1983
		P. simplex	Bharadwaj, 1994
271	Aspartic acid	*P. niruri*	Bharadwaj, 1994
272	Brerifolin carboxylic acid	*P. urinaria*	Chen *et al.*, 1999
273	Caffeic acid	*P. sellowianus*	Hnatyszyn *et al.*, 1995
		P. urinaria	Chen *et al.*, 1999
274	Chlorinergic acid	*P. sellowiancus*	Hnatyszyn *et al.*, 1995
275	Dotriacontanoic acid	*P. niruri*	Singh *et al.*, 1986
276	Hexacosanic acid	*P. urinaria*	Satyan *et al.*, 1995
277	Indole – 3 – carboxylic acid	*p. virgatus*	Huang *et al.*, 1996
278	Inuciic acid	*P. emblica*	Basa and Srinivasulu, 1987
279	Keto acid	*P. urinaria*	Dogra and Sinha, 1979
280	Oxalic acid	*P. niruri*	Bharadwaj, 1994
281	Tartaric acid	*P. simplex*	
Sugars			

282	Galloyl glucose	*P. niruri*	Ishimaru *et al.*, 1992
283	Galactose	*P. sellowianus*	Hnatyszyn *et al.*, 1995
284	Glucose	*P. emblica*	Theresa *et al.*, 1967
		P. sellowianus	Hnatyszyn *et al.*, 1995
285	Levulose		
286	Saccharose		
Other Miscellaneous Compounds			
287	Brevifolin Carboxylic acid	*P. niruri*	Than *et al.*, 2006
288	Camphor Sulfonate	*P. discoideus*	Oletta, 1962
289	Di-hexyl benzene – 1, 2 – dicarboxylate	*P.niruri*	Wei *et al.*, 2004
290	Ethylene diamine Camphor Sulphanate	*P. discoideus*	Oletta, 1962
291	Gallic acid ethyl ester	*P. sellowianus*	Miguel *et al.*, 1995 b
292	Isofraxidin		Hnatyszyn *et al.*, 1993
293	Methyl brevifolin carboxylate	*P. niruri*	Iizuka *et al.*, 2006
294	Scopoletin	*P. sellowianus*	Hnatyszyn *et al.*, 1993
295	1, 12 – Diaza – Cyclodocosane – 2, 11 – dione	*P.niruri*	Wei *et al.*, 2004
296	2, 3, 5, 6 – tetra hydroxy benzyl acetate		
297	2, 4, 5 – trihydroxy – 3 – (4, 6, 7 – tri hydroxy – 3 – OXO – 1, 3 – dihydro benzofuran – 5 – yl) – benzonic acid methyl ester (Phyllangin)		
298	3, 4, 5 – trihydroxy benzonic acid		
299	6, 10, 14 – trimethyl 2 – penta decanone		Than *et al.*, 2006

2.3.1 Hepatoprotective Activity

Hepatoprotective activity has been reported such as *P. acidus* (Lee *et al.*, 2006), *P. amarus* (Raja Reddy, 1988; De and Datta, 1990; Saraf *et al.*, 1991; Bansiddhi, 1992; Tripathi *et al.*, 1992; Sane and Kuber, 1993; Sharma *et al.*, 1993; Unander *et al.*, 1993; Doreswamy and Sharma, 1995; Sane *et al.*, 1995 a, b; Farooqi *et al.*, 2000; Rajeshkumar and Kuttan 2000 a, b; Trimbark, 2000; Yeh *et al.*, 2002; Lee *et al.*, 2006; Wongnawa *et al.*, 2006), *P. asperlatus* (Hemadri and Rao, 1984), *P. debilis* (Bansiddhi, 1992; Sane *et al.*, 1995 b; Kuber *et al.*, 1997; Trimbark, 2000; Lee *et al.*, 2006), *P. emblica* (Patra *et al.*, 1991; Roy *et al.*, 1991; Gulati *et al.*, 1995; Jose *et al.*, 1997, 1999; Dimri *et al.*, 1999; Sultana *et al.*, 2005; Tasduq *et al.*, 2005 a, b; Lee *et al.*, 2006; Pramyothin *et al.*, 2006). *P. embergeri* (Lee *et al.*, 2006), *P. fraternus* (Sane and Kuber, 1993; Kuber *et al.*, 1997; Padma and Setty, 1999; Sen *et al.*, 2000; Trimbark, 2000), *P. hookeri* (Lee *et al.*, 2006), *P. maderaspatensis* (Trimbark, 2000; Asha *et al.*, 2004, 2007), *P. multiflorus* (Lee *et al.*, 2006), *P. myrtifolius* (Lee *et al.*, 2006), *P. niruri* (Sreenivasa Rao, 1985; Syamsundar *et al.*, 1985; Umarani *et al.*, 1985; Shyam *et al.*, 1986; Chauhan *et al.*, 1992; Kohale *et al.*, 1993; Reddy *et al.*, 1993; Kapur *et al.*, 1994; Doreswamy and Sharma, 1995; Prakash *et al.*, 1995; Majgaonkar and Phadake, 1998; Adithan *et al.*, 1999; Agarwal, 1999; Latha *et al.*, 1999; Shukla and Srivastava, 1999; Singh and Dubey, 1999; Kale *et al.*, 2001; Pradhan, 2001; Upadhyay *et al.*, 2001; Tabassum *et al.*, 2005; Harish and Shivanandappa, 2006; Bhattacharjee and Sil, 2006 a, b; 2007), *P. simplex* (Prakash *et al.*, 1995), *P. tenellus* (Lee *et al.*, 2006), *P. urinaria* (Bansiddhi, 1992; Prakash *et al.*, 1995; Kuber *et al.*, 1997; Trimbark, 2000), *P. urinaria* subsp. *nudicarpus* (Lee *et al.*, 2006), and *P. urinaria* subsp. *urinaria* (Lee *et al.*, 2006).

Anti-hepatitis B virus activity has been reported to *P. amarus* (Thyagarajan *et al.*, 1988, 1990; Blumberg *et al.*,1989; Jayaram *et al.*, 1990, 1997; Jayaram and Thyagarajan, 1996; Leelarasamee *et al.*, 1990; Niu *et al.*, 1990; Mehrotra *et al.*, 1991; Munshi *et al.*,1993; Yeh *et al.*, 1993; Doshi *et al.*, 1994; Milne *et al.*, 1994; Meixia *et al.*, 1995; Jayaram and Thyagarajan, 1996; Lee *et al.*, 1996; Thabrew and Hughes, 1996; Ott *et al.*, 1997; Jeena *et al.*, 1999; Xin-Hua *et al.*, 2001), *P. chamaecristoides* (Alonsa *et al.*, 1995), *P. emblica* (Jeena *et al.*, 1999), *P. formosus* (Alonsa *et al.*, 1995), *P. fraternus* (Yelne *et al.*, 1993), *P. maderaspatensis* (Munshi *et al.*, 1993), *P. microdictyus* (Alonsa *et al.*, 1995), *P. nanus* (Lam *et al.*, 2006), *P. niruri* (Thyagarajan *et al.*, 1982; Jayaram *et al.*, 1987; Venkateswaran *et al.*, 1987; Mehrotra *et al.*, 1990; Jhou and Krishnamoorthy, 1993; Meixia *et al.*, 1995; Thabrew *et al.*, 1991), and, *P. urinaria* (Ji *et al.*, 1993; Chen *et al.*, 1995; Meixia *et al.*, 1995; Thabrew *et al.*, 1991; Wang *et al.*, 1997; Kim *et al.*, 1999; Wang, 2000; Shin *et al.*, 2005).

2.3.2 Hypoglycaemic Activity

Hypoglycaemic activity has been reported to *P. amarus* (Srividya and Periwal, 1995; Sivaprakasam *et al.*, 1995; Moshi *et al.*, 1997, 2001; Adeneye *et al.*, 2006; Ali *et al.*, 2006), *P. emblica* (Saley and Nalgirkar, 1982; Sivaprakasam *et al.*, 1984; Janjua, 1991, 1998; Anila and Vijayalakshmi, 2000; Raj Kapoor and Kavimani, 2001), *P. fraternus* (Hukeri *et al.*, 1988), *P. niruri* (Kumar *et al.*, 1989, 2001; Raphael *et al.*, 2000), and, *P. sellowianus* (Gonalons and Fontana, 1927; Hnatyszyn *et al.*, 1997, 2002).

2.3.3 Hypocholesterolemic Activity

Hypocholesterolemic activity has been reported to *P. amarus* (Adneneye *et al.*, 2006), *P. emblica* (Shanmugasundaram *et al.*, 1983 a, b; Anila and Vijayalakshmi,

2000) and *P. niruri* (Umarani *et al.,* 1985; Khanna *et al.,* 2002). Specific activities include α-amylase inhibition in *P. maderaspatensis* (Prashanth *et al.,* 2001), and hypolipidemic activity in *P. emblica* (Thakur, 1985; Jacob *et al.,* 1988; Mand *et al.,* 1991; Mathur *et al.*, 1996).

2.3.4 Antimalarial Activity

Antimalarial activity has been reported to *P. acuminatus* (Munoz *et al.,* 2000), *P. amarus* (Adjobimey *et al.,* 2004; Chukwujekwu *et al.,* 2005), *P. fraternus* (Sittie *et al.,* 1998), *P. muellerianus* (Zirihi *et al.,* 2005), *P. niruri* (Tona *et al.,* 1999, 2004; Totte *et al.,* 2001; Usha Devi *et al.,* 2001; Cimanga *et al.,* 2004; Subeki *et al.,* 2005), *P. piscatorum* (Gertsch *et al.,* 2004), *P. reticulatus* (Omulokoli *et al.,* 1997), and, *P. urinaria* (Hout *et al.,* 2006).

2.3.5 Anti-oxidant/Free Radical Scavenging Activity

Anti-oxidant/free radical scavenging activity has been reported to *P. acidus* (Lee *et al.,* 2006), *P. amarus* (Kumaran and Karunakaran, 2007; Lim and Murtijaya, 2007), *P. emblica* (Bhattacharya *et al.*, 2000; Jose and Kuttan, 1995; Ghosal *et al.*, 1996; Kumar and Muller, 1999; Bandyopadhyay *et al.,* 2000; Khopde *et al.,* 2001; Bafna and Balaraman, 2005; Bajpai *et al.,* 2005; Kumaran and Karunakaran, 2006 b, 2007), *P. debilis* (Kumaran and Karunakaran, 2006 a), *P. fraternus* (Sailaja and Setty, 2006), *P. maderaspatensis* (Kumaran and Karunakaran, 2007), *P. niruri* (Johu and Krishnamoorthy, 1993; Joy and Kuttan, 1995; Sarkar *et al.,* 2005; Bhattacharjee and Sil, 2006 b, 2007; Chatterjee *et al.,* 2006; Harish and Shivanandappa, 2006; Than *et al.,* 2006), *P. orbicularis* (Ferrer *et al.,* 2002), *P. oxyphyllus* (Sutthivaiyakit *et al.,* 2003), *P. sellowianus* (Paya *et al.,* 1996), *P. stipulatus* (Ignacio *et al.,* 2001), *P. urinaria* (Chularojmontri *et al.,* 2005; Kumaran and Karunakaran, 2007), *P. urinaria*

subsp. *urinaria* (Lee *et al.*, 2006), and, *P. virgatus* (Kumaran and Karunakaran, 2007).

2.3.6 Anticancer and Antitumour Activity

Anticancer and antitumour activity has been reported to *P. acuminatus* (Pettit *et al.*, 1984, 1990), *P. amarus* (Jeena *et al.*, 1999; Rajeshkumar and Kuttan, 2000 a,b; Kumar and Kuttan, 2000; Rajeshkumar *et al.*, 2002; Sripanidkulchai *et al.*, 2002), *P. brasiliensis* (Kinghorn, 1983), *P. emblica* (Suresh and Vasudevan, 1994; Jeena *et al.*, 1999; Jose *et al.*, 2001; Sancheti *et al.*, 2005), *P. flexuosus* (Wada *et al.*, 2001), *P. niruri* (Satyanarayana *et al.*, 1988), *P. oligospermus* (Wu and Wu, 2006), *P. polyphyllus* var. *siamensis* (Youkwan *et al.*, 2005), *P. sellowianus* (Fernandez *et al.*, 2002), *P. urinaria* (Huang *et al.*, 2003, 2004, 2006), and, *P. verminiatus* (George, 1983).

2.3.7 Antimutagenic Activity

Antimutagenic activity has been reported to *P. amarus* (Sripanidkulchai *et al.*, 2002) and *P. orbicularis* (Sanchez-Lamar *et al.*, 1999; Ferrer *et al.*, 2001, 2002, 2004).

2.3.8 Chemoprotective/Anticytotoxic Properties

Chemoprotective/anticytotoxic properties have been reported to *P. amarus* (Kumar and Kuttan, 2005), *P. emblica* (Giri and Banerjee, 1986; Yadav, 1987; Dhir *et al.*, 1990, 1991; Agarwal *et al.*, 1992; Roy *et al.*, 1992; Ghosh *et al.*, 1993; Nandi *et al.*, 1997; Jasril *et al.*, 1999; Sai Ram *et al.*, 2003), *P. maderaspatensis* (Chandrasekar *et al.*, 2006), *P. niruri* (Dhir *et al.*, 1990; Hussain *et al.*, 1995), *P. orbicularis* (Sanchez-Lamar *et al.*, 1999, 2002), and, *P. piscatorum* (Gertsch *et al.*, 2004).

2.3.9 Antinociceptive Activity

Antinociceptive activity has been reported to *P. amarus* (Filho *et al.*, 1996; Santos *et al.*, 2000), *P. corcovadensis* (Gorski *et al.*, 1993; Santos *et al.*, 1994, 1995 c), *P. debilis* (Chandrashekar *et al.*, 2005), *P emblica* (Perianayagam *et al.*, 2004), *P. fraternus* (Santos *et al.*, 2000), *P. niruri* (Santos *et al.*, 1994, 1995 b), *P. orbiculatus* (Santos *et al.*, 2000), *P. sellowianus* (Santos *et al.*, 1995 a), *P. stipulatus* (Santos *et al.*, 2000), *P. tenellus* (Santos *et al.*, 1994, 1995 a), and *P. urinaria* (Santos *et al.*, 1999 a, b; 1999).

2.3.10 Antipyretic Activity

Antipyretic activity has been reported to *P. emblica* (Ihantola – Vormisto *et al.*, 1997; Perianayagam *et al.*, 2004).

2.3.11 Anti-inflammatory Activity

Anti-inflammatory activity has been reported to *P. amarus* (Kassuya *et al.*, 2003; Kiemer *et al.*, 2003; Raphael and Kuttan, 2003; Chukwujekwu *et al.*, 2005; Kassuya *et al.*, 2005, 2006; Mahat and Patil, 2007), *P. debilis* (Chandrashekar *et al.*, 2005), *P. emblica* (Purushottam Dev, 1979; Asmawi *et al.*, 1993; Jantan *et al.*, 1996; Ihantola-Vormisto *et al.*, 1997), *P. polyphyllus* (Rao *et al.*, 2006), and *P. singampattiyana* (Maridass *et al.*, 2005).

2.3.12 Immunomodulatary Properties

Immuomodulatory properties have been reported to *P. debilis* (Thabrew *et al.*, 1991), *P. emblica* (Suresh and Vasudevan, 1994), *P. sellowianus* (Fernandez *et al.*, 2002), and, *P. tenellus* (Ignacio *et al.*, 2001).

2.3.13 Antiviral Activities

Possessing antiviral property is a unique feature of the genus *Phyllanthus*. Some of the species such as *P. amarus* (Unander, 1991; Yeh *et al.*, 2002;

Bhattacharyya *et al.,* 2003), *P. emblica* (Ramfi *et al.,* 1992; Anonymous, 2000), *P. fraternus* (Saigopal *et al.,* 1986; Louis and Balakrishnan, 1996), *P. oribicularis* (Del Barrio and Parra, 2000; Ferrer *et al.,* 2001), *P. myrtifolius* (Liu *et al.,* 1999; Jassim and Naji, 2003), *P. niruri* (Thabrew and Hughes, 1996*), P. urinaria* (Zhibao *et al.,* 1995; Qian – Cutrone *et al.,* 1996; Chen *et al.,* 1999; Liu *et al.,* 1999; Jassim and Naji, 2003; Wang *et al.,* 2005; Yang *et al.,* 2005) have been reported to possess this property. Anti–HIV activity has been reported in *P. amarus* (Notka *et al.,* 2003, 2004), *P. emblica* (El–Mekkaway *et al.,* 1995; Kusumoto *et al.,* 1995; Rama Rao, 1998), *P. myrtifolius* (Chang *et al.,* 1995), and, *P. niruri* (Nataraj, 2000).

2.3.14 Anti-diarrheal Activity

Anti-diarrheal activity has been reported to *P. amarus* (Obasi *et al.,* 1993) and *P. emblica* (Perianayagam *et al.,* 2005).

2.3.15 Antiulcer/Gastrointestinal Disturbances

Antiulcer activity and activity against gastrointestinal disturbances have been reported to *P. amarus* (Obasi *et al.,* 1993; Raphael and Kuttan, 2003), *P. emblica* (Maroli and Javale, 1982; Saley and Nalgirkar, 1982; Date and Kulkarni, 1994, 1995 b; Kulkarni *et al.,* 1995 a, b; Pakrashi and Bandyopadhyaya, 1996 – 1997; Rao *et al.,* 2000; Bandyopadhyay, 2001; Al-Rehaily *et al.,* 2002).

2.3.16 Activity against Renal Disorders

Activities that have been reported include *P. amarus* for kidney problems (De and Datta, 1990), *P. amarus* (Srividya and Periwal, 1995), *P. discoideus* (Oletta, 1962), *P. niruri* (Jhou and Krishnamoorthy, 1993) and *P. sellowianus* (Hnatyszyn *et al.,* 1999) for diuretic activity, *P. emblica* (Roy *et al.,* 1991) for modifying renotoxicity, *P. niruri* (Freitas *et al.,* 2002) for treating urolithiasis and *P.*

urinaria (Cruz *et al.,* 1994) for renal infections and contractile response in guinea pig urinary bladder (Dias *et al.,* 1995).

2.3.17 Activity against Reproductive Disorders

P. amarus in antifertility (Shah *et al.,* 1995; Rao *et al.,* 1997; Rao and Alice, 2001), and genitourinary (De and Datta, 1990), *P. discoideus* in uterotonic (Corallo *et al.,* 1988) and *P. emblica* in pregnancy anemia (Gupta and Nandyala, 1984), reproductive system (Maroli and Javale, 1982), and to treat leukorrhea (Singh and Londhe, 1993).

2.3.18 Activity against Dental Infections

P. emblica (Date and Kulkarni, 1995 b) and *P. muellerianus* (Lorougnon and Akeasni, 1989).

2.3.19 Activity against other Inflammatory Disorders

P. emblica for allergic rhinitis (Amit *et al.,* 2005), prevention of acute pancreatitis (Thorat *et al.,* 1995), chronic otorrhea and pharyngitis (Roy, 1989), otitis media (Suryawanshi and Suryawanshi, 1989) and rheumatoid arthritis (Chandrasekera, 1982).

2.3.20 Activity against Skin Infections

P. amarus for anti-healing (Nadig and Rao, 1999), *P. emblica* for wound healing (Suguna *et al.,* 2000) and to treat wounds and dermatitis (Roy, 1989) and to treat vitiligo (Nair *et al.,* 1987) and *P. niruri* for wound healing (Devi *et al.,* 2005).

2.3.21 Activity against Ophthalmic Complaints

P. emblica (Tambvekar, 1985; Nair *et al.,* 1987).

2.3.22 Activity against Cardiovascular Defects

P. discoideus in cardiotonic (Oletta, 1962) and *P. emblica* in antiatherogenic (Shanmugasundaram *et al.,* 1983 a, b; Duan *et al.,* 2005), myocardial necrosis (Tandon *et al.,* 1996) and cardioprotective (Maroli and Javale, 1982).

2.3.23 Activity against Disorders in Central Nervous System

P. amarus in hypotensive (Srividya and Periwal, 1995), *P. discoideus* in excitant CNS activity (Foussard-Blanpin *et al.,* 1967; Quevauviller *et al.,* 1967) and *P. emblica* in neuroprotective (Maroli and Javale, 1982).

2.3.24 Activity against Defects of Respiratory System

P. emblica in respiroprotective activity (Roy, 1989; Bhasin, 1991), *P. emblica* for anti-respiratory tract infections (Ingle *et al.,* 1980; Suryawanshi and Suryawanshi, 1989; Abhang, 1993, 1994), *P. discoideus* in respiroprotective action (Oletta, 1962; Foussard – Blanpin *et al.,* 1967; Quevauviller *et al.,* 1967), *P. urinaria* in contractile response in guinea–pig trachea (Paulino *et al.,* 1996 a), relaxant effect in guinea–pig trachea (Paulino *et al.,* 1996 b).

2.3.25 Activity against Blood-related Disorders

P. emblica for haemopoietic (Maroli and Javale, 1982), *P. niruri* as vasorelaxant (Iizuka *et al.,* 2006) and resist to osmotic haemolysis (Jhou and Krishnamoorthy, 1993), and, *P. reticulatus* for blood purification (Joseph *et al.,* 1994).

2.3.26 Radioprotective Activity

P. emblica (Jagetia *et al.,* 2002) and *P. niruri* (Uma Devi *et al.,* 2000).

2.3.27 Anti-protozoal Activity

P. amarus (Kolodziej *et al.,* 2005) and *P. niruri* (Rajendran *et al.,* 2001) for anti- Leishmanial, *P. maderaspatensis* for antiameobic (Siddique and Hokim, 1991), *P. niruri* for antibabesiol (Subeki *et al.,* 2005) and *P. piscatorum* for antitrypanosomidal (Gertsch *et al.,* 2004).

2.3.28 Enzyme Inhibition and Induction

P. amarus for inducing nitric oxide synthase and cytokines gene expressions (Kolodzie *et al.,* 2005), inhibition of signal regulated protein kinase (Polya *et al.,* 1995) and *P. emblica* for enhancing cholinergic enzymes, acetyl choline esterase and choline acetyl transferase (Vohra *et al.,* 2001).

2.3.29 As Biopesticide

P. debilis (Bansiddhi, 1992)

2.3.30 Protective Effect on Anorexia, Debility and Emaciation

P. niruri (Anilkumar *et al.,* 1997).

2.3.31 Molluscicidal and Larvicidal Activity

Molluscicidal activity was reported in *P.nummulariifolius* (Chifundera *et al.,* 1993). Molluscicidal (El Hadi *et al.,* 1984) and larvicidal (Goswami and Mohan, 1998) has been reported in *P. niruri.*

2.4 Reported Biological Activities of Bergenin

Bergenin has been reported so far from 12 species. Various biological activities of the compound are given in detail (Table 3).

Table 3. Reported Biological Activities of Bergenin

S. No.	Plant Name	Activity	Range	Model	References
1	*Ardisia japonica*	Anti-HIV	Weak	*In vitro*	Piacente *et al.*, 1996
		PTP1 B inhibitors	Moderate	-	Li *et al.*, 2005
2	*Astilbe thunbergii*	Wound healing	Significant	Burn wounds in mice	Kimura *et al.*, 2006
		Lipolytic	Significant	-	Han *et al.*, 1998
3	*Ficus racemosa*	Anti-inflammatory	Significant	*In vitro* inhibition of COX – 1 and 5 – LOX	Li *et al.*, 2004
4	*Flueggea microcarpa*	Anti-fungal (against plant pathogenic Fungi)	Significant	Inhibition of spore germination.	Prithiviraj *et al.*, 1997
		Antiulcer	Significant	*In vivo*, aspirin induced gastric ulcers in rats & cold restraint stress induced gastric ulcers in rats and guinea-pig.	Goel and Maiti, 1997
		Hypolipidaemic	Significant	*In vivo*	Jahromi *et al.*, 1992
5	*Fluggea virosa*	Antiarrhythmic	Significant	$BaCl_2$ – induced arrhythmias in rats	Pu *et al.*, 2002
		Antiprotozoal (*Trypanosoma brucei*)	Significant	-	Nyasse *et al.*, 2004
6	*Humiria balsamifera*	Antimalarial	Significant	*In vivo / In vitro*	Da Silva *et al.*, 2004
7	*Mallotus japonicus*	Hepato protective	Significant	D-galactosamine intoxicated rat hepatocytes-*In vitro*	Lim *et al.*, 2000 a
		Hepato protective	Significant	CCl_4 induced cyto toxicity in rat hepatocytes – *In vitro*	Kim *et al.*, 2000
		Hepato protective	Significant	D-galactosamine intoxicated rats-*In vivo* comparative activity of Bergenin and acetylbergenin.	Lim *et al.*,2000 c
		Hepato	Significant	D – galactosamine	Lim *et al.*, 2001

		protective		induced rats *in vivo*	
		Hepato protective	Significant	CCl_4 induced toxicity in rats – *In vivo*	Lim *et al.*, 2000 b
		Inhibition of bovine adrenal tyrosine hydroxylase (Peptic ulcers)	Significant	*In vitro*	Zhang *et al.*, 2003
8	*Mallotus roxburghianus*	Antioxidant	Significant	-	Rana *et al.*, 2005
9	*Peltophorum africanum*	Anti-HIV1	Inactive on both enzymes	Inhibition of HIV – I Reverse transcriptase and (RT), HIV – 1 Integrase (IN)	Bessong *et al.*, 2005
10	*Peltophorum petrocarpum*	Anti-inflammatory	Significant	Carrageenan induced rat paw oedema	Swarnalakshmi *et al.*, 1984
11	*Sacoglottis gabonensis*	Antioxidant.	Significant	*In vivo*	Maduka *et al.*, 2002
12	*Saxifraga melanocentra*	Anti HCV	Significant	*In vitro* ELISA	Zuo *et al.*, 2005

CHAPTER 3

MATERIALS AND METHODS

3.1 PHYTOCHEMISTRY

3.1.1 Selection of Plant Material

Phyllanthus wightianus Muell. Arg., an ethnomedicine which is used by Malayali tribals in the Javadi Hills of Vellore District, Tamil Nadu, India, was selected for the present study.

3.1.2 Ethnobotanical Uses

P. wightianus belongs to the family of Euphorbiaceae and is locally known as 'Elumbotti' by the Malayali tribals from the Vellore District of Tamil Nadu in India. They use plant paste for bone setting and to treat diarrhoea.

3.1.3 Collection of Plant Material

Whole plant material in required quantity has been collected during the months of December and January from the Javadi Hills (± 866 MSL) in the Vellore District. A voucher specimen (MBV & OSP 18632) was identified and authenticated by Dr. M.B. Viswanathan and has been deposited in the Herbarium of the Sri Paramakalyani Centre for Environmental Sciences, Manonmaniam Sundaranar University, Alwarkurichi, Tamil Nadu, India.

3.1.4 Description

Phyllanthus wightianus Muell. Arg. in Linnaea 32: 47. 1863 *et* in DC., Prodr. 15(2): 425.1866 (non *P. wightianus* Muell. Arg in Linnaea 32: 6. 1863 et in DC., Prodr. 15(2): 334.1866); Hook.f., Fl. Brit. India 5: 303. 1887. *Reidia floribunda* Wight, Ic. t. 1903. 1852 (non *Phyllanthus floribundus* H.B.K. 1817, nec Muell. Arg.

1863); Gamble, Fl. Pres. Madras 1293. 1925 (2:905. 1957 repr. ed.). *Phyllanthus pinnatus* sensu Chandrabose in Henry, Kumari and Chithra, Fl. Tamil Nadu, Analysis 2: 238 .1987, quoad syn. *Phyllanthus wightianus* et *Reidia floribunda*, non (Wight) Webster.

Subshrubs, villous, up to 1.5 m high; branches spirally arranged towards stem tips. Leaves alternate, membranous, green above, pale green beneath, obliquely elliptic to oblong, obliquely truncate at base, entire, ciliate, obtuse, apiculate at apex, subsessile, puberulous above, pubescent beneath, 1.5 – 3 x 0.7 – 1.7 cm; stipules lanceolate, acuminate, persistent, *c.* 1.5 mm long. Flowers red, solitary, axillary. Male: pedicels densely puberulous, *c.* 2.6 cm long. Tepals 4 – 7, oblong–ovate or lanceolate, bluntly acuminate, glabrous within, villous without, ciliate along margins, *c.* 3 x 1.5 mm. Disc segments 4 – 7, truncate, dotted, *c.* 1.5 mm long. Stamens usually 2, rarely 3; filaments connate, 1 or 0.5 mm long; anthers 2 or 3, subglobose, dehiscing transversely, 0.5 or 1 x 1.5 mm. Female: pedicels densely puberulous, *c.* 2.7 cm long. Tepals 5 or 6, ovate, acuminate, glabrous within, villous without, ciliate along margins, *c.* 1 x 2 mm. Ovary globose, 3-lobed, hairy, *c.*1 mm long; styles 3, each 2-fid, divided up to base, glabrous, *c.* 1.2 mm long. Capsules globose, 3–valved, hairy, *c.* 6 x 6 mm; hairs soft, short, dense, branched, multicellular. Seeds 6, 3-gonous, appressed hairy, *c.* 3 x 1.5 mm.

Distribution: Tamil Nadu and Karnataka in India.

3.1.5 Chemicals

Chemicals (E. Merck, Shiv Sagar Estate A, Worli, Mumbai, India, and S.D. Fine Chemicals, Mumbai, India) used were of AR Grade. Silica gel (ACME's and S.D. Fine Chemicals, Mumbai) 60 – 120 mesh and 100 – 200 mesh for Column Chromatography (CC) and Silica gel^{G} were used for thin layer Chromatography

(TLC). Pre-coated aluminium plates coated with silica gel 60 F254 (E. Merck, Shiv Sagar Estate A, Worli, Mumbai, India) for 0.2 mm layer thickness were used for HPTLC analysis.

3.1.6 Extraction

Shade-dried and coarsely powdered whole plant material was successively extracted (hexane, chloroform and methanol) using Soxhlet apparatus. The solvent extracts were recovered, concentrated under reduced pressure and the last traces of the solvents were removed *in vacuo*.

3.1.7 Preliminary Phytochemical Screening

All the extracts such as hexane, chloroform and methanol of *P. wightianus* (whole plant) were subjected to routine qualitative chemical analysis to identify the nature of phytochemical constituents present in it (Brindha *et al.*, 1982.).

3.1.8 Qualitative Analysis

3.1.8.1 Determination of Extractive Value

This method was used to determine the amount of active constituents present in each plant solvent extract such as hexane, chloroform and methanol. Plant material weighing 100 g was successively extracted with organic solvents in the order of increasing polarity using a Soxhlet apparatus, following the British Pharmacopoeia (Anonymous, 1993). The percentage solubility for the extracts was calculated. The extractive values of the crude drugs thus calculated are very much useful in their evaluation where the constituents of a drug can not be readily estimated by any other means.

3.1.8.2 Fluorescent Analysis

Organic solvents such as hexane, benzene, chloroform, ethyl acetate, alcohol and acetone, water, 1 N HCl and 1:1% H_2SO_4, HNO_3 and alkaline solutions of aqueous and alcoholic 1 N NaOH were treated individually with desired quantity preferably one g each of the drug powder in separate test tubes. After 24 hours, fluorescence of each extraction observed under day light and UV light (254 nm) was recorded.

3.1.8.3 Determination of Ash Value

The presence of ash in the plant materials can be determined by three different ways such as determination of total ash, acid insoluble ash and water soluble ash (Anonymous, 1996).

Total Ash

Total ash was determined by the method of the Association of Official Analytical Chemists (Horwitz, 1980). This method was designed to measure the amount of material remaining after ignition. Physiological ash is derived from the plant tissue itself and non- physiological ash is the residue after ignition of the extraneous matter (e.g. sand and soil) adhering to the surface.

About 2 g of the plant powder was accurately weighed in a tarred silica crucible previously ignited and weighed. The powder was scattered in a fine even layer on the bottom of the crucible. It was ignited by gradually increasing the heat to 500°C. The ignition was repeated until constant weight was obtained, cooled in a desiccator and weighed. Percentage of ash with reference to the air-dried drug was calculated.

Acid Insoluble Ash

The total ash obtained above was boiled for 5 min with 25 ml of dilute hydrochloric acid. The insoluble matter was collected on an ash less filter paper and washed with hot water. Then, it was ignited and weighed after cooling in a desiccator. The percentage of acid insoluble ash was calculated with reference to the air-dried drug and tabulated.

Water Soluble Ash

The total ash obtained as above was boiled with 25 ml of water for 5 min. The insoluble matter was collected in an ash less filter paper and washed with hot water. This was then ignited to constant weight at temperature not exceeding 450°C. The weight of insoluble matter was subtracted from the weight of total ash. The difference in weight represents the water soluble ash. The percentage of it was calculated with reference to air-dried drug. The above tests were repeated thrice and the average value was calculated.

3.1.8.4 Determination of Loss of Weight on Drying

An excess of water in medicinal plant materials will lead to microbial growth, insect attack and deterioration by hydrolysis. Hence, loss of weight on drying was determined. This test was used to determine both water and volatile matter.

A known quantity of 500g of fresh plant material was weighed and dried under shade until a constant weight was obtained. The loss of weight on drying was calculated.

3.1.8.5 Tests for Inorganic Elements

The total ash value was determined for the plant material and the total ash content represents the inorganic salts, which usually consist of carbonates, phosphates and silicates of sodium, potassium, magnesium, calcium, etc. These salts may be

occurring naturally or may be present as impurity or adulterant. Hence, the following tests were performed using the total ash. Tests for arsenic, borate, copper, calcium, magnesium, lead, iron, potassium, mercury, sodium, sulphate, phosphate, chloride, carbonate and nitrates were analyzed and the results were tabulated.

3.1.9 Quantitative Analysis of Inorganic Elements (Salts and Minerals)

Apart from that, column chromatography of the methanol extract when eluted continuously with ethyl acetate: methanol 3:1 yielded fractions 1, 2, 3, and 4 with a high content of salts. Therefore, the plant material was subjected to inorganic mineral analysis to know the quantity of minerals present in 1 g of plant material.

Preparation of Sample Solution for Inorganic Mineral Analysis

The plant material (1 g) was digested with 10 ml of nitric acid and left over night. It was then heated on a hot plate until the reddish brown fumes ceased and cooled. A small volume of perchloric acid was added and transferred to a 50 ml volumetric flask and made up to volume with double distilled water (McKenzie, 1982).

Determination of Sodium and Potassium by Flame Photometry (Anonymous, 1996)

The instrument used was Systronics Flame Photometer. A series of standard solutions containing the element to be determined in increasing concentrations within the concentration range recommended for the instrument was prepared. Nitric acid and perchloric acid used for the preparation of the sample solution of the plant material were also added in the same concentrations to the standard solutions. The appropriate filter was chosen, water was sprayed into the flame and the galvanometer reading was adjusted to zero. The most concentrated solution was then sprayed into the flame and the galvanometer reading was recorded. Again, water was sprayed till

the galvanometer reading was zero. Then the standard solution was sprayed into the flame and the procedure was repeated thrice for each concentration. A calibration curve was prepared by plotting the mean of three readings of each standard against the concentration. The sample solution prepared above was then aspirated into the flame thrice followed by recording the galvanometer reading. The apparatus was washed thoroughly with water after each aspiration. Using the mean of three readings, the concentration of the element being examined was determined from the calibration curve. To confirm the concentration thus obtained, the operation was repeated with the standard solution of the same concentration as that of the solution being examined.

Determination of Calcium, Cobalt, Copper, Iron, Magnesium and Manganese by Atomic Absorption Spectroscopy (Anonymous, 1996)

The instrument used was Perkin Elmer Atomic Absorption Spectrophotometer. Three standard solutions of the element to be determined covering the concentration range recommended for the instrument for the element were prepared. Nitric acid and perchloric acid used in the preparation of the solution of the substance being examined were also added to the standard solutions in the same concentration. After calibration of the instrument, each standard solution was introduced into the flame for three times and the steady readings were recorded. The apparatus was thoroughly washed after each introduction. A calibration curve was prepared by plotting the mean of each group of three readings against the concentration. The plant extract prepared above was then introduced into the flame. The reading recorded and the apparatus washed with water. The sequence was then repeated twice. Using the mean of the three readings, the concentration of the element was determined from the calibration curve. The process was repeated for determination of other elements using different lamps.

3.1.10 Separation Methods

3.1.10.1 Paper Chromatography

Amino acids were identified following Jayaraman (1981) and Sadasivam and Manickam (1996). The mobile phase used for identification of amino acids was a mixture of n-butanol, glacial acetic acid and water in the ratio of 4:1:5 v/v. SD's Amino acid reference collection (Kit of 24 items) was used. The collection contains 24 chromatographically homogenous amino acids. Each vial contains 1 g except 3 (3, 4–dihydroxy phenyl)–DL–alanine and L-hydroxy proline which contains 0.1 g each.

The twenty four standard amino acids were dissolved with distilled water at a concentration of 1 mg/ml in different test tubes. Very dilute hydrochloric acid was used for dissolving tyrosine and phenylalanine and dilute sodium hydroxide was used to dissolve tryptophan.

A known quantity of plant material was ground using a mortar and pestle with 10 fold volume of 70% ethanol. The contents were shaken at 55°C for 30 min. The contents were centrifuged and the supernatant was collected. The extraction was repeated again twice. The supernatants were pooled and evaporated to dryness under vacuum. The residue was dissolved in a known volume of absolute ethanol or water for analysis. ninhydrin solution (1 mg/ml) in acetone was used as spraying agent. The chromatography sheet was cut into a convenient size, a line was drawn about 5 cm away from one end and points were marked at regular intervals of 3 cm.

A small volume of the each standard amino acid and sample were applied using a microsyringe. The spots were allowed to dry fast using a stream of hot air from a dryer. The sheet was then placed inside of a chamber containing the mobile phase and the chromatogram was developed. The paper was then removed and the solvent front was recorded. The chromatogram was dried and sprayed with ninhydrin

reagent using an atomizer. The paper was dried at room temperature and then in an Oven for 2-3 min. Amino acids appeared as purple spots whereas hydroxy proline and proline give yellow spots. The spots were marked and their Rf values were calculated. The amino acids present in the sample were then identified by comparing the Rf obtained with that of the authentic amino acids and also by co-chromatography.

3.1.10.2 Thin Layer Chromatography

A slurry of the adsorbent (Silica gel^{G}) was prepared with water (1:2). Dried and clean glass plates (20 cm x 5 cm) were laid in a row as a template, the suspension was poured into Stahl TLC spreader, which was adjusted to 0.25 mm thickness and coated in a single passage on the spreader over them. The prepared plates were allowed to air-dry and placed in an Oven at 110°C for 30 min after drying towards activation. Then, they were transferred into a dust free chamber. Aluminium plates coated with silica gel^{G} F254 (E. Merck, Shiv Sagar Estate A, Worli, Mumbai, India) were also used. The application of spots was done using capillary tubes about 2 cm above the bottom of the plate.

Chromatograms were detected with vanillin-H_2SO_4 reagent (1 g vanillin dissolved in 100 ml H_2SO_4, heated at 110°C after spraying). The fractions of similar TLC patterns were combined, concentrated and re-chromatographed repeatedly over silica gel to isolate pure compounds.

3.1.10.3 Column Chromatography

The adsorbent used was Silica gel 60 – 120 mesh and finer than 200 mesh. The columns of different sizes were used for the isolation of constituents in the present investigation. The column elutes were collected in fractions with components of small elution volumes from 25 to 50 ml. The fractions were analyzed

using Thin Layer Chromatography. Fractions containing the individual pure components were combined and the eluting solvents removed using rotary evaporator.

3.1.10.4 High Performance Thin Layer Chromatography (HPTLC)

HPTLC fingerprinting was performed on CAMAG TLC scanner3 instrument, equipped with Linomat IV applicator and CATS 3.1 Software. The various extracts used for taking HPTLC fingerprints were hexane, chloroform and methanol extracts of the test plant.

Stationary Phase

Aluminium sheets pre-coated with silica gel 60 F254 (E. Merck, Shiv Sagar Estate A, Worli, Mumbai, India) of 0.2 mm layer thickness were used as the stationary phase.

Mobile Phase

The HPTLC chromatograms for the different solvent extracts were developed with the following solvent systems:

Hexane Extract

Developing System	:	Hexane: Toluene 3:2
Scanning wave length	:	200 nm
Marker compound	:	Friedelin

Chloroform Extract

Developing System	:	Chloroform
Scanning Wave Length	:	200 nm
Marker compound	:	Lupeol

Methanol Extract

Developing System	:	Ethyl acetate

Scanning Wave Length : 254 nm

Marker compounds : Bergenin and gallic acid

Fingerprints were obtained by development in CAMAG twin trough glass chamber. The developed TLC plates were dipped in vanillin-H_2SO_4 reagent and heated at 110°C for 5 min. The development of spots on the TLC plates was given (Fig. 8).

Preparation of Extracts

The dried whole plant material was powdered and passed through 60 mesh. A quantity of 5 g powder was successively extracted with hexane, chloroform and methanol in Soxhlet apparatus. Each extract was concentrated and made up to 50 ml in a standard flask with respective solvents. All the marker compounds such as friedelin, lupeol, bergenin and gallic acid, each 10 mg was dissolved in 10 ml of suitable solvent (friedelin and lupeol were dissolved with chloroform, while bergenin and gallic acid were dissolved with methanol). A 10 µl of the extract solution and marker compound solutions were spotted for the chromatogram. The scanning wavelength was 200 nm for hexane and chloroform extracts while for methanol extract it was 254 nm.

3.1.10.5 High Performance Liquid Chromatography (HPLC) of Tannins and Lignans

HPLC analysis of total tannins and lignans was done using Shimadzu HPLC–LC 2010 CHT with class VP version 6.2–6.3 with auto injector.

Operational Conditions

Instrument : Shimadzu LC-2010 (Quaternary gradient)

Stationery phase : Lichrocart RP 18E (02/04)

Mobile phase	: Binary Gradient
Pump A	: 0.05% Orthophosphoric acid in acetonitrile
Pump B	: 0.05% Orthophosphoric acid in water gradient elution
Flow rate	: 1.0 ml/minute
Detection wavelength	: 220 & 254 nm

Reagents and Chemicals

Tanins	: Gallic acid (GA), corillagin (C), geranin (G) and ellagic acid (EA) were procured from SPIC Pharma, Chennai.
Acetonitrile	: HPLC grade
Orthophosphoric acid	: AR grade
Water	: HPLC grade

Estimation of Lignans

Lignans	: Hypophyllanthin and Phyllanthin
Instrument	: Shimadzu 10 AD
Stationary Phase	: Spherisorb ODS-2
Mobile phase	: 65 CH_3OH:35 H_2O
Flow rate	: 1.3 ml/minute
Detection wavelength	: 230 nm
Standard dilution	: Hypophyllanthin – 0.25 mg/ml and Phyllanthin – 0.50 mg/ml in CH_3CN
Volume injected	: 20 µl
Sample dilution	: 200 mg in 20 ml

Standard Stock Solution An accurately weighed quantity of gallic acid (GA), corillagin (C), geraniin (G) and ellagic acid (EA) standards were dissolved in methanol to prepare a known concentration of 0.125, 0.25, 0.30 and 0.05 mg/ml respectively.

Sample Preparation

About 200 mg of sample was transferred to a 25 ml volumetric flask, dissolved with diluents by sonication and made up to volume with diluents and filtered the above solution through syringe filter.

Estimation method

Standard solutions such as gallic acid (GA), corillagin (C), geraniin (G) at 220 nm and ellagic acid (EA) at 254 nm were injected.

Formula

$$= \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Standard concentration}}{\text{Sample concentration}} \times \text{standard purity}$$

3.1.10.6 Gas Chromatography–Mass Spectrometry (GC-MS) Analysis of Lipids

GC-MS was recorded on Agilent instrument by the direct inlet method.

Operating Parameters

Name of the instrument	:	GCMS D 5973 Agilent
Detector	:	Mass selective detector
Column specification	:	DB_5 – MS
Column thickness	:	0.25 μ (film thickness)
Column length	:	30 m
Internal diameter	:	0.25 mm
Column thickness	:	0.25 μm
Carrier gas	:	Helium

Temperature programme	:	70°C for 2 min, 10°C/min up to 280°C for 10 min
Injection temperature	:	250°C
Flow Rate	:	1 ml/minute
Library software	:	NIST

3.1.11 Identification of the Compounds

3.1.11.1 Melting Point

Melting points were recorded on an INLAB Melting point apparatus and paraffin was used as a solution.

3.1.11.2 Structural Elucidation by Spectral Analysis

The structure of the isolated compounds was elucidated by analyzing the spectral data and in comparison with compounds previously reported in literature.

3.1.11.3 Ultraviolet–Visible Spectroscopy

The absorption spectra were measured in very dilute solution against a solvent blank using an automatic recording spectrophotometer. For colorless compounds, measurements were made in the range of 200 to 400 nm (nanometers) and for coloured compounds, the range was 200 to 700 nm. The wavelengths of the maxima and minima of the absorption spectrum so obtained were recorded (in nm). The instrument used for taking UV–Visible spectra was Shimadzu UV-Vis Spectrophotometer Model UV–1601, with Spectroscopic grade methanol in the range of 200–600 nm.

3.1.11.4 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra were taken on Shimadzu FTIR grating spectrophotometer, model FTIR 8101 A, with potassium bromide (KBr disc).

3.1.11.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^{1}H and ^{13}C NMR Spectra were taken on Bruker (400 Mz) or JOEL (400 Mz) instruments. The solvents used were ($CDCL_3$ or DMSO – d6 with tetra methyl silane (TMS) as the internal standard chemical shifts were recorded δ in scale. Proton shifts in organic compounds ranged from 0 ppm to 14 ppm, i.e. from a δ value of 0 – 14. The spread of resonance for ^{13}C was from 0 to 180 ppm.

3.1.11.6 X-ray Crystallography

Suitable single crystals of compound (6) were obtained from ETOAC – EtOH 9:1 mixture was subjected to X-ray diffraction analysis carried out on an Enrafnonius (CAD) – 4 diffractometer with the $\theta/2\theta$ scan mode λ (μoKα) = 0.71069 A°.

3.2 ANTIMICROBIAL STUDIES

The various solvent extracts of the plant such as hexane, chloroform and methanol were tested for antibacterial, antifungal and antidermatophytic activities.

3.2.1 Test Microorganisms

Bacterial Strains

The bacterial strains employed in the biological assays were gram-positive bacteria such as *Bacillus cereus* (MTCC 430), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (*Staph. aureus,* MTCC 96), *Staphylococcus epidermidis* (MTCC 435) and gram-negative bacteria such as *Aeromonas hydrophila* (MTCC 646), *Enterobacter aerogenes* (MTCC 111), *Escherichia coli* (MTCC 724), *Klebsiella pneumoniae* (MTCC 432), *Proteus mirabilis* (MTCC 425), *Proteus vulgaris* (MTCC 426), *Pseudomonas aureoginosa* (MTCC 741), *Salmonella paratyphi* (MTCC 735), *Salmonella typhi* (MTCC 733), *Vibrio cholerae*, *Vibrio parahaemolyticus* (MTCC 451) and *Vibrio vulnificus* (MTCC 1146).

Fungal Strains

Aspergillus flavus (MTCC 277), *Aspergillus fumigatus* (MTCC 343), *Aspergillus niger* (MTCC 1344) and *Candida albicans* (MTCC 227).

Dermatophytes

Epidermophyton floccosum. Microsporum canis (MTCC 2820), *Microsporum gypseum* (MTCC 2819), *Trichophyton mentagrophytes* and *Trichophyton rubram* (MTCC F 296).

Vibrio cholerae was procured from the Department of Environmental Sciences, Bharathiar University, Coimbatore 641 046, Tamil Nadu, *Epidermophyton floccosum* and *Trichophyton mentagrophytes* were obtained from the Department of Dermatology, Sri Ramachandra Medical College and Research Institute, Porur, Tamil Nadu, and the remaining strains were procured from Microbial Type culture collection (MTCC) at the Institute of Microbial Technology (IMTECH), Chandigarh.

3.2.2. Media Used

Mueller–Hinton Agar (MHA), Mueller–Hinton Broth (MHB), Nutrient Broth (NB), Sabourad Dextrose Agar (SDA), and Sabourad Dextrose Broth (SDB) were procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India, and used for testing the antibacterial and antifungal activities.

3.2.3 Determination of Antibacterial Activity

Concentration of the Test Extracts

Different concentrations of the test extracts were prepared by two-fold dilution method for agar-well diffusion (100 mg/ml – 12.5 mg/ml) and minimal inhibitory concentration (MIC) (20 mg/ml – 1.25 mg/ml) assays.

Preparation of Inoculum

The inoculum size of the test strains were standardized according to the National Committee for Clinical Laboratory Standards (NCCLS, 1993) guidelines.

Each test bacterial strain was inoculated into Mueller-Hinton Broth medium and incubated for 3-6 hours at 35°C in a shaker water bath until the culture attained a turbidity of 0.5 McFarland unit. The final inoculum size was adjusted to 10^8 CFU/ml.

Agar-well Diffusion Assay

Susceptibility tests were performed following Perez *et al.* (1990) with modifications according to the present experimental conditions (Okunji *et al.*, 1990; Okeke *et al.*, 2001).

A one ml volume of the standard suspension of test bacterial strain was spread evenly on Mueller-Hinton Agar plate using a sterile glass rod spreader and the plates were allowed to dry at room temperature. Subsequently, 6 mm diameter wells were bored in the agar of each plate. Different concentrations of the solvent extracts were (100 mg/ml – 12.5 mg/ml) added into the wells using micropipettes and allowed for diffusion at room temperature for 2 h. The plates were incubated at 37°C for 24 h. The solvent without extracts served as negative control. Standard antibiotics such as ampicillin – 10 µ/disc, erythromycin 10 µ/disc, kanamycin – 30 µ/disc, methicillin – 5µ/disc, nalidixic acid – 30 µ/disc, rifampicin – 30 µ/disc, tetracycline 10 µ/disc, gentamicin – 10 µ/disc and trimethoprim – 10 µ/disc were used as positive controls. After 24 h of incubation, diameter of the inhibition zone was recorded in mm. The experiment was repeated thrice and the average values were calculated.

Determination of Minimal Inhibitory Concentration (MIC)

MICs of the plant test extracts were determined by macro broth dilution assay method (NCCLS, 1993). Two fold serial dilutions of the test extracts (20 mg/ml – 1.25 mg/ml) were prepared in tubes with Mueller-Hinton Broth (MHB) as diluent. Each dilution was seeded with test organism to the standard concentration (10^8 CFU/ml). The tubes were incubated at 37°C for 24h. MIC was taken as the lowest concentration of extract that completely inhibited the bacterial growth, indicated by the lack of visual turbidity.

Determination of Minimal Bactericidal Concentration (MBC)

MBC determination was done by aspirating 0.1 ml of the culture medium from each tube (in the Macrobroth MIC assay) showing no apparent growth and sub-culturing it on fresh MHA. The latter was incubated at 37°C for 24 h. The MBC was read as the least concentration showing no visible growth on MHA subculture.

3.2.4 Determination of Antifungal Activity

Agar-well Diffusion Assay

The antifungal activity was determined by the Agar-well diffusion method following Perez *et al.* (1990) with modifications according to the present experimental conditions (Okunji *et al.*, 1990; Okeke *et al.*, 2001). All the stock cultures were maintained in sabourad dextrose agar. Inoculums for *Candida albicans* were prepared by spread plating of 24 h old culture grown in sabourad broth. For *Aspergillus flavus, Aspergillus fumigatus,* and *Aspergillus niger*, 10^4 spore/ml of fungi was uniformly distributed on the surface of SDA plates with the help of sterile cotton swab. For the dermatophytes, inoculation was done by taking a piece of fungal colony on a sterile cotton swab and gently swabbing on the surface uniformly.

The plates were allowed to dry at room temperature. Subsequently, 6 mm diameter wells were bored in the agar of each plate. Different concentrations (100 mg/ml – 12.5 mg/ml) of the solvent extracts were added into the wells using micropipettes and allowed for diffusion, as was done for the antibacterial assay. The plates were incubated at similar temperature of 28°C but different time periods such as 24–48 h for *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger* and *Candida albicans,* and 72–96 h for dermatophytes (and more depending on the incubation time required for a visible growth). The solvent without extracts served as negative control. Standard antibiotics of ketoconzole (20 µg/disc) and chloromphenicol (30 µg/disc) were used as positive controls. After the required incubation period, diameter of the inhibition zone was recorded in mm. The experiment was repeated thrice and the average values were calculated.

Determination of Minimal Inhibitory Concentration

The assay was performed by NCCLS (1997) protocol as per Rajarajan *et al.* (2002). Inoculums of each test fungus were standardized adopting spectrophotometric method. All the test strains, which were freshly sub-cultured on sabourad dextrose agar plates, were used for the study. A 100 µl each of the standardized inoculum was added to the respective tubes, including the control which was devoid of extract. The inoculated test tubes with various concentrations of 20 mg/ml–1.25 mg/ml of the test extracts and respective test strains were incubated at 25°C till 48 h in the case of *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger* and *Candida albicans*) and 25°C for 96 h in the case of dermatophytes. Then, the test tubes were observed for the presence or absence of turbidity in comparison to that of the control. The lowest concentration of the extract that inhibited the growth

of each fungus, detected by the lack of visual turbidity, was taken as MIC value of the extract for the test fungus.

Determination of Minimum Fungicidal Concentration (MFC)

The MFC was determined using the method of Rotimi *et al.* (1988). The test tubes which showed no visible growth (in the MIC assay) after 96 h of incubation were sub-cultured on extract free SDA plates and incubated at room temperature for 5–7 days. The MFC was regarded as the lowest concentration that prevented the growth of any fungal colony on the solid medium.

3.3 PHARMACOLOGY

3.3.1 Plant Material

The various solvent extracts of the plant (hexane, chloroform and methanol) and an isolated compound, bergenin, were used for pharmacological studies.

3.3.2 Preparation of the Drugs

The various solvent extracts of the plant such as hexane, chloroform, methanol and bergenin and other standard drugs such as silymarin, indomethacin and glibenclamide were administered orally in the form of suspension in water with 1% w/v sodium carboxy methyl cellulose (SCMC) as the suspending agent.

3.3.3 Animals

Male Wistar albino rats (150–250 g) and male and female Swiss albino mice (25–35 g) were used depending upon the study. The animals were maintained in the Animal House of Arulmigu Kalasalingam College of Pharmacy (Registration No. 509/01/C/CPCSEA/2002), Department of Pharmacology, Anand Nagar, Krishnan Kovil, 629 190, Tamil Nadu. They were initially acclimatized for the study, housed in polypropylene cages and maintained at 24 ± 2°C with relative humidity of 45-50% and equal 12 hour light and 12 hour dark cycles. The animals were fed *ad libitum*

with standard pellet diet (Lipton India Ltd., Bombay) and had free access to water. The animals before test had 24 h fast but water was given to them *ad libitum* whenever required.

All the pharmacological and toxicological experimental protocols were approved by the Institutional Animal Ethics committee (IAEC) for the purpose of control and supervision on experimentation on Animals (CPCSEA), New Delhi, vide sanction on 12th March 2007.

3.3.4 Chemicals

Fine chemicals were purchased from Sigma-Aldrich, St. Louis, MO 63103, and S.D. Fine Chemicals, Mumbai, India, and other chemicals from SISCO Research Laboratories Pvt. Ltd., Mumbai, India.

3.3.5 Acute Toxicity Studies

The oral acute toxic class method (Roll *et al.,* 1986) was performed as per the Organization for Economic Co-operation and Development (OECD) 423 guidelines. Swiss albino female mice weighing 15-25 g were fasted overnight, provided with water *ad libitum* and divided into groups of six animals each. The test extracts such as hexane, chloroform, methanol and bergenin were administered orally at the initial dose of 5 mg/kg body weight by intragastric tube and observed for 1 week. The animals were observed continuously for 2 h and then at half hourly interval for the next 6 h, 24 h, for observing changes in gross general behavior and daily for 1 week for any possible drug-induced mortality. Since, there was no mortality with 5 mg/kg for 1 week the procedure was repeated for next higher doses such as 50, 500 and 2000 mg/kg for all the test extracts such as hexane, chloroform, methanol and bergenin.

3.3.6 Analgesic Activity

3.3.6.1 Hot-Plate Method (Eddy and Leimback, 1953; Turner, 1965)

Wistar albino mice (25–30 g) were divided into eight groups, each consists of six animals. Group 1 animals served as vehicle control and received only 1% w/v SCMC (10 ml/kg, p.o.). Group 2 animals received MO (5 mg/kg, S.C.). Groups 3 and 4 animals received hexane extract 100 & 200 mg/kg, p.o., respectively. Groups 5 and 6 animals received chloroform extract 100 and 200 mg/kg/p.o., respectively. Groups 7 and 8 animals received methanol extract 100 and 200 mg/kg, p.o., respectively. Morphine (MO) injection was used as the standard drug and the equipment was Eddy's hot plate (Techno).

The animals were placed on the hot plate which maintained at constant temperature 55°C ± 5°C. Basal reaction time was recorded by observing hind paw licking or jump response (whichever appeared first) in animals. Normally, the response was obtained within 6 – 8 seconds. A cut off period of 15 seconds was observed to avoid damage to the paws. The time of reaction to pain stimulus (interval between placing the mice in the hot plate and the lick or jump response) of the mice was recorded at every 15, 30, 60 and 120 min after drug administration. The percentage increase in reaction time was taken as the index of analgesia.

3.3.6.2 Acetic acid-induced Writhing in Mice (Kulkarni, 1999)

Wistar albino mice (25 – 30g) were divided into eight groups, each consists of six animals. Group 1 animals received only 1% w/v SCMC (10 ml/kg, p.o.). Group 2 animals received indomethacin 100 mg/kg, p.o. Groups 3 to 8 animals received the test extracts (as like that of/as mentioned in) hot plate method.

All the extracts and the standard drug were administered orally using intragastric tube, 15 min prior to the administration of acetic acid injection. All the

animals received intraperitoneal injection (i.p.) of 3% v/v of acetic acid (1 ml/100 g), 30 min after the administration of the test drugs and the standard drug indomethacin.

The number of writhings (the constriction of abdominal muscle together with stretching of the hind limbs) produced by each animal was observed individually under a glass jar for a period of 20 min and the same was counted.

A significant reduction in the number of writhes when compared with vehicle treated animals was considered as antinociceptive response. The % protection of analgesic activity was calculated using the formula C-T/C X 100 where C is the number of writhings in control group and T is the number of writhings in the treated group.

3.3.7 Anti-inflammatory Activity

Carrageenan–induced Paw Oedema Method (Winter *et al.*, 1962)

The anti-inflammatory effect of the various solvent extracts was studied using carrageenan-induced hind paw oedema model. The most widely used primary test to screen new anti-inflammatory agents measures the ability of a compound to reduce local oedema-induced in rat paw by injection of an irritant – carrageenan. Carrageenan is a sulphated polysaccharide obtained from seaweed (Rhodophyceae) and causes the release of histamine, 5 – HT, bradykinin and prostaglandins it thus produces inflammation and oedema.

The rats were divided into eight groups, each consists of six animals. Group–1 animals served as vehicle control and received only 1% w/v SCMC (10 ml/kg, p.o.). Group 2 animals received indomethacin (10 mg/kg, p.o.). Indomethacin was employed as the standard drug. Groups 3 and 4 animals received hexane extract 100 and 200 mg/kg, p.o., respectively. Groups 5 and 6 animals received chloroform

extract 100 and 200 mg/kg/p.o., respectively. Groups 7 and 8 animals received methanol extract 100 and 200 mg/kg/p.o., respectively.

A mark was made on both the hind paws (right and left) just beyond tibio-tarsal junction so that every time the paw was dipped in the mercury column up to the fixed mark to ensure constant paw volume. The initial paw volume (both right and left) of each rat was measured by mercury displacement. After 30 min of drug administration, 0.1 ml of 1% (w/v) carrageenan was injected in the right hind paw, sub-planar region of each rat. The left paw served as reference (non-inflammatory paw) for comparison. The paw volumes of both the legs of control and test extracts (drugs) treated rats were measured at 0 h, 1 h, 3 h, and 5 h after carrageenan administration.

The mean increase in paw volume and the percentage inhibition of inflammatory swelling were calculated. The percentage inhibition of paw volume of the different test extracts were compared with that of control. The percentage inhibition of inflammatory swelling was calculated using the formula C-T/C x 100 where C was the oedema rate of control group and T was treated group.

3.3.8 *In vitro* Antioxidant Activity

3.3.8.1 DPPH Radical Scavenging Assay by Spectrophotometric Method (Sree Jeyan and Rao, 1996).

Chemicals

1, 1 diphenyl–1–2–picryl hydrazyl (DPPH) was obtained from the Sigma-Aldrich, St. Louis, MO 63103, naphthylene diamine dichloride was obtained from S.D. Fine Chemicals, Mumbai, India. All other reagents used were of analytical grade.

Experimental Protocol

The free radical scavenging activity of 100 µg/ml each of hexane, chloroform and methanol extracts of the plant was examined using DPPH radical.

To an ethanolic solution of DPPH (200 µl), 0.05 ml each of the test extracts (100 µg/ml concentration) was added. An equal amount of ethanol was added to the control. After 30 min, decrease in the absorbance of the test mixture was read at 517 nm. The experiment was performed in triplicate and the percentage inhibition was calculated according to the eq.1.

$$\text{Inhibition percentage (\%)} = \frac{[AC(0) - AA(t)]}{AC(0)} \times 100 \quad \text{..... eq.1}$$

Where AC (0) is an absorbance of control DPPH solution at 0 minute and AA (t) is an absorbance of test sample at 30 min. The antioxidant activity was compared with vitamin C which was used as the standard antioxidant.

3.3.8.2 Nitric oxide Radical Scavenging Assay (Sree Jayan and Rao, 1997)

Chemicals

Sodium nitroprusside, naphthyl ethylene diamine and sulphanilamide were obtained from S.D. Fine Chemicals, Mumbai, India.

Experimental Protocol

Sodium nitroprusside (5 mM) in phosphate-buffered saline was incubated at 25°C for 150 min, with different test extracts of 100 µg/ml each of hexane, chloroform and methanol which were dissolved in standard phosphate buffer. After 150 min, 0.5 ml of incubated solution was removed and diluted with 0.5 ml of griess reagent (prepared by mixing equal volume of 1% w/v sulphanilamide in 2% v/v phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride in water) and

the absorbance of chromophore formed was read at 546 nm. The control experiment was also carried out in similar manner.

The experiment was performed in triplicate and the percentage reduction in absorbance was calculated using the following formula:

[(Control – Test)/Control] X 100

The activity was compared with vitamin C, which was used as standard antioxidant.

3.3.9 Wound Healing Activity

3.3.9.1 Excision Wound Model (Udupa *et al.*, 1994 a; Saha *et al.*, 1997).

This model was used to monitor wound contraction and formation of epithelization time.

Materials

Simple ointment B.P., 0.2% w/w nitrofurazone ointment (as the reference standard), 0.5 g of (5% w/w) each test extract ointment (where 5 g of each test extract such as hexane, chloroform and methanol extracts were incorporated in 100 g of simple ointment base B.P. (Anonymous, 1993) was applied once daily, till the wound was completely healed.

Five groups of animals (male Wistar albino rats 150 – 180 g) containing six animals in each group were anesthetized with ether. The rats were depilated on the back and a predetermined area of 500 mm^2 full thickness skin was excised in the dorsal intercapsular region. Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. Wounds of rats were left undressed to the open environment.

The group 1 animals served as control and treated only with simple ointment base B.P. The group 2 animals were served as reference standard and treated with

0.2% w/w nitrofurazon ointment. Animals of groups 3, 4 and 5 were treated with 0.5 g of the hexane, chloroform and methanol extract ointments respectively. The treatment was continued till the wound was completely healed.

The progressive changes in wound area were monitored planimetrically by tracing the wound margin on a graph paper every alternate day. The changes in healing of wound, i.e. the measurement of wound on graph paper, were expressed as unit mm^2. Wound contraction was expressed as percentage reduction of original wound size.

3.3.9.2 Incision Wound Model (Udupa *et al.,* 1994 b)

This model was employed to assess the breaking strength of skin in rats.

Materials

Black silk surgical thread (No. 000), curved needle (No. 11), simple ointment B.P, 0.2% w/w nitrofurazone ointment (as the reference standard), 0.5 g of 5% w/w test extracts such as hexane, chloroform and methanol extracts as ointments. The ointments were applied to the wound twice daily until complete recovery.

Five groups with six animals (male wistar albino rats 150–180 g) in each group were anesthetized under light ether anesthesia, by the open mask method. Two para vertebral long incisions were made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on each side of the depilated back of the rat. Full aseptic measures were not taken and no local or systemic antimicrobials were used throughout the experiment (Udupa *et al.,* 1994 b) After the incision was made the parted skin was kept together and stitched with black silk surgical thread (No 000) at 0.5 cm intervals using a curved needle (No.11). The continuous threads on both the wound edges were tightened for good adaptation of wound and the wound was left undressed.

All the groups were treated in the same manner as mentioned in the case of the excision wound model such as the group 1 animals (control) were treated only with simple ointment base B.P., group 2 animals were treated with 0.2% w/w nitrofurazon ointment and the animals of the groups 3, 4 and 5 were treated with 5% w/w, 0.5 g of the test extract ointments such as hexane, chloroform and methanol extracts respectively throughout the period twice daily for nine days. When wounds were cured thoroughly the sutures were removed on the ninth day and tensile strength was measured using a tensiometer.

3.3.10 Antidiabetic Activity

Streptozotocin (STZ)–induced Diabetes (Elfellah *et al.*, 1984; Sood *et al.*, 2000)

Materials

Hexane, chloroform and methanol extracts of the plant were prepared in the form of 1% w/v SCMC suspension and the same was administered orally to animals. For inducing diabetes STZ in a citrate buffer (pH 4.5) was employed. Glibenclamide 10 mg/kg was used as a standard drug.

Experimental Protocol

Induction of Diabetes

Diabetes was induced into the 16 h fasted rats by a single intravenous injection of 50 mg/kg STZ in a citrate buffer with pH 4.5 (Basnet *et al.*, 1994). Diabetes was confirmed by the presence of serum glucose levels higher than 300 mg/dl, 2 days after the STZ treatment (Elfellah *et al.*, 1984; Sood *et al.*, 2000).

Antihyperglycemic Screening

The rats were divided in to 8 groups of six animals each. Group 1 served as diabetic control and received only 1% w/v SCMC. Group 2 served as positive control and received glibenclamide 10 mg/kg. Groups 3 and 4 received hexane extract 100

mg, 200 mg/kg, p.o., respectively. Groups 5 and 6 received chloroform extract 100 mg, 200 mg/kg, p.o., respectively and Groups 7 and 8 received methanol extract 100, 200 mg/kg, p.o., respectively. The treatment was continued for 8 days by administering the respective extracts/drug or 1% w/v SCMC twice daily.

Collection and Processing of Blood for Estimation of Glucose and Biochemical Parameters

On the ninth day of the therapy, blood samples (1 ml) were collected from the tail vein under mild ether anesthesia in Eppendorf tubes containing 100 µl of anticoagulant (10% tri sodium citrate solution).

Plasma was separated by centrifuging the samples at 5000 rpm for 10 min and stored in a refrigerator until analysis. The plasma was analyzed for glucose, total protein, cholesterol, triglycerides, alkaline phosphatase (ALP), aspartate aminotransferase (ASAT) formerly called glutamic oxaloacetic transaminase (GOT) and alanine aminotransferase (ALAT) formerly called glutamic pyruvic transaminase (GPT) using standard procedures in an autoanalyzer, Microlab 200 using Ecoline kits (E. Merck, Shiv Sagar Estate A, Worli, Mumbai, India).

3.3.11 Antiarthritic Activity

3.3.11.1 Complete Freund's Adjuvant (CFA)-induced Arthritis (Pearson, 1956)

Materials

The various test extracts such as hexane, chloroform, methanol and bergenin, standard drug indomethacin were prepared in the form of 1% w/v SCMC suspension and the same was administered orally to animals.

Preparation of Complete Freund's Adjuvant (CFA)

Twenty five mg of heat killed *Mycobacterium tuberculosis* cells (being killed at 60°C in 5–20 min in an Autoclave) was finely grounded using mortar and pestle

with sufficient amount of liquid paraffin, referred to as complete freund's adjuvant. The liquid paraffin alone was referred in the study as incomplete freund's adjuvant.

Induction of Arthritis

Arthritis was induced by a single intra dermal injection of 0.1 ml of complete freund's adjuvant (CFA) containing 1 mg dry heat killed *Mycobacterium tuberculosis* per ml sterile paraffin oil into a foot pad of the left hind paw of male rats (Mizushima *et al.,* 1972). A glass syringe (1 ml) with locking hubs and a 26G needle were used for injection. The rats were anesthetized with ether inhalation prior to and during adjuvant injection as the very viscous nature of the adjuvant exerted difficulty while injecting. The swelling in hind paws was periodically examined in each paw from the ankle using Plethysmography (Winter *et al.,* 1962).

Experimental Setup

The animals (male wistar rats 200–250 g) were divided into ten groups of six animals in each group as follows:

Group 1 animals served as inflamed control group and received only normal saline. Group 2 animals served as non-inflamed control group, consisted of rats injected with 0.1 ml of liquid paraffin alone. Group 3 animals received 10 mg/kg/p.o. of the standard drug indomethacin. Group 4 animals treated with bergenin 50 mg/kg/p.o., Groups 5 and 6 animals treated with the hexane extract 100 and 200 mg/kg/p.o., respectively. Groups 7 and 8 animals administered with the chloroform extract 100 and 200 mg/kg/p.o., respectively. Groups 9 and 10 animals treated with the methanol extract 100 and 200 mg/kg/p.o., respectively. The test groups consisted of complete freund's adjuvant-injected rats challenged with doses of the test drugs administered orally 18 h and 2 h before induction of arthritis. The drug administrations were continued daily at the same time of the day for 19 more days.

Assessment of Arthritis

Arthritis was assessed by physical and biochemical measurements as well as by radiographic analysis. Initial and successive body weight changes and paw volume changes were measured and recorded. The body weight changes of the various groups were recorded at periodic intervals up to the day 19. Development of adjuvant-induced swelling in the paws of the injected and non-injected limbs of each rat was monitored daily as the percentage increase in paw volume.

Biochemical Studies

As there was dose-dependent inhibition observed in the hind paw swelling only the animals treated with the higher doses of the test extracts were selected for the biochemical studies such as hexane, chloroform, methanol, 200 mg/kg/p.o. respectively and bergenin 50mg/kg/p.o.

Rats were killed on day 19 and blood was collected from the various groups and serum was separated. The liver, kidney and spleen were dissected out from the body and washed with cold saline and their weights were recorded. Then, they were cut into small pieces and homogenized using buffer (P^H 7.4) to prepare a 10% homogenate. This was centrifuged at 12,000 g for 30 min (Latha *et al.*, 1998). The supernatant fluid was used for the assay of various enzymes. Estimations were done such as aminotransferases by King (1965 a) using sodium pyruvate as standard, acid phosphatase by King (1965 b) using disodium phenylphosphate as substrate, and Cathepsin–D by the modified method of Etherington (1972).

Radiographic Analysis

On the last day of experiment, before collecting blood and tissues for biochemical parameters, the rats were placed on a radiographic box at a distance of 90 cm from X-ray source. Radiographic analysis of arthritic hind paws was performed

by X-ray machine (Univet LX 160), Multimage, Cavaria, Italy) with a 40-kw exposition for 0.01S (Rojasa *et al.*, 2003).

3.3.12 Immunomodulatory Properties

Materials

Antigen (SRBC)

Fresh blood of a healthy sheep was collected from the local slaughter house in a mixture of 0.49% ethylene diamine tetraacetic acid (EDTA) and 0.9% sodium chloride solution. It was preserved at a temperature from 2-8°C. On the day of immunization, the blood sample was centrifuged at 5000 rpm for 10 min and then washed thrice to remove plasma with 0.9% sodium chloride solution and adjusted to a concentration of 0.1 ml containing 1 x 10^8 cells for immunization and challenge.

Culture

Candida albicans was purchased from the IMTECH, Chandigarh, India.

Standard Drug

The standard drug cyclophosphamide was obtained from Sigma-Aldrich St. Louis, MO 63103. The test extracts such as hexane, chloroform and methanol and the standard drug cyclophosphamide were prepared in the form of 1% w/v SCMC suspension.

Animals

Swiss albino mice weighing 18-25 g and albino rats (150 to 200 g) were used.

Methods

3.3.12.1 Delayed Type Hypersensitivity Reaction using SRBC as an Antigen (Gokhale *et al.,* 2003)

Mice were divided into eight groups of six in each group. Group 1 animals served as vehicle control and received 1% w/v of SCMC (10 ml/kg, p.o.). The Group 2 animals treated with the immunosuppressive drug cyclophosphamide 50 mg/kg/p.o.

Groups 3 and 4 animals were administered with the hexane extract 100 and 200 mg/kg/p.o., respectively. Groups 5 and 6 animals were treated with the chloroform extract 100 and 200 mg/kg/p.o., respectively. Groups 7 and 8 animals were received methanol extract 100 and 200 mg/kg/p.o., respectively. In all the groups ranging from 2 to 8, the respective drugs were administered on day 0 and have been lasted till the day of challenge. The mice were primed with 0.1 ml of SRBC suspension containing 1 x 10^8 cells, i.p., on day 7 and challenged on day 14 with 0.05 ml of 2 x 10^8 SRBC in the right hind foot pad. The contralateral paw received equal volume of saline. The paw volume was determined after injection of SRBC challenges and at after 72 h by Plethysmographic method.

3.3.12.2 Humoral Antibody Response to SRBC (Gokhale *et al.,* 2003)

The mice were divided into eight groups of six in each group. Group 1 animals served as vehicle control and received only 1% w/v of SCMC (10 ml/kg p.o.). Group 2 animals were treated with cyclophosphamide (50 mg/kg/p.o.) was administered 2 days before the experiment.

Groups 3 to 8 animals were treated with the test extracts (as like that of/as mentioned in) the previous test (delayed type hypersensitivity reaction). In all these groups from 3 to 8, the treatment was started on 0^{th} day and has been lasted till the day of experiment. On day 7, the mice were immunized with 0.1 ml of 1 x 10^8 SRBC

i.p.. Blood samples were collected from the orbital plexuses of individual animals on day 14 and the antibody titres were determined. After preparing sera from peripheral blood, aliquots (25 µl) of two-fold diluted sera in saline were challenged with 25 µl of 1% v/v SRBC suspension in microtitre plates. The plates were incubated at 37°C for 1 h and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titre.

3.3.12.3 Non-specific Immunity Determined by Survival Rate against Fungal Infection (Thakur *et al.*, 2007)

Wistar albino rats (150 to 200 g) were used. The rats were divided into 5 groups comprising of six animals each. The group 1 animals served as vehicle control and were administered with 1% w/v SCMC only. Group 2 animals received 200 mg/kg hexane extract and Groups 3 and 4 animals received 200 mg/kg of chloroform and methanol extracts, respectively. Treatments of all the four groups started 14 days before challenge. On the day of challenge, all the groups were injected with 5×10^7 viable *Candida albicans* cells and observed daily for mortality for a period of 10 days.

3.3.12.4 Macrophage phagocytosis by carbon clearance method (Jayathiratha and Mishra, 2004)

Swiss albino mice of either sex, weighing 20-25 g were divided into seven groups comprising six animals each. The group 1 animals served as vehicle control and were administered with 1% w/v SCMC only. Groups 2 and 3 animals received methanol extract of 200 and 100 mg/kg/p.o. respectively. Groups 4 and 5 animals received hexane extract of 200 and 100 mg/kg/p.o. respectively and groups 6 and 7 animals treated with chloroform extract of 200 and 100 mg/kg/p.o. respectively.

The treatment was continued for five days. At the end of five days, after 48 h, mice were injected via the tail vein with carbon ink suspension (10 µl/g body weight)

(Pelican AG. Germany). Blood samples were collected (in EDTA solution, 5 µl) from the retro–orbital vein at 0 and 15 min, and 25 µl sample was mixed with 0.1% sodium carbonate solution (2 ml) and its absorbance at 660 nm was determined. The phagocytic index K was calculated using the following equation:

$K = Log_e^{OD1} - Log_e^{OD2}/15$, where OD1 and OD2 were the optical densities at 0 and 15 min, respectively.

3.3.12.5 Cyclophosphamide–induced Myelosuppression Assay (Gokhale *et al.*, 2003; Jayathiratha and Mishra, 2004)

Swiss albino mice were divided into eight groups of six animals in each group. Group 1 animals served as vehicle control and received only 1% w/v of SCMC. Group 2 animals served as cyclophosphamide control. Groups 3 and 4 animals treated with hexane extract of 100 and 200 mg/kg/p.o. respectively. Groups 5 and 6 animals administered with chloroform extract of 100 and 200 mg/kg/p.o. respectively and groups 7 and 8 animals received methanol extract of 100 and 200 mg/kg/p.o. respectively. Animals treated with the test extracts (Groups 3 to 8), administration of extracts started 13 days prior to administration of cyclophosphamide. On 11^{th}, 12^{th} and 13^{th} days, all the animals except group 1 were administered with cyclophosphamide 30 mg/kg/p.o. 1 h after the administration of extract. Blood samples were collected on 14^{th} day from retro-orbital plexuses of individual animals and the total white blood cell (WBC) count was determined.

3.3.13 Hepatoprotective Studies

3.3.13.1 *In vitro* Inactivation of HBsAg

The assay was performed using ETI-MAK-4HBsAg Kit followed by modified version of Venkateswaran *et al.* method (1987). Serial dilutions of the methanol extract of the plant and bergenin were mixed with an equal volume of sera positive for

HBsAg and the mixture was incubated for 60 min at 37°C. The evaluation was carried out using the procedure as stated in the Kit insert. Briefly anti-HBs (sheep) coated microtitre wells were filled with 100 µl of serum drug suspension and incubated for 60 min at 37°C followed by anti-HBs conjugate addition. Each step was followed by washing and finally chromogen treatment was done. The reaction was stopped by addition of 0.5 M sulphuric acid. The results were read on Micro Elisa reader. The cut off value for each set of screening was calculated using the mean absorbance of negative controls. Samples with an absorbance either equal or greater than the cut off were considered as HBsAg positive. Inactivation of HBsAg activity was expressed as the decrease (in per cent) in the absorption of the test sample compared to that of control.

3.3.13.2 Isoniazid (INH) and Rifampicin (RMP)-induced Hepatic Injury in Rats

(Tasduq *et al.,* 2005 b; Pal *et al.,* 2006)

Materials

The various solvent test extracts such as hexane, chloroform, methanol, bergenin and the standard drug silymarin were prepared in the form of 1% w/v SCMC suspension and the same was administered orally to animals. INH and RMP solutions were prepared separately with sterile distilled water. The pH of rifampicin solution was adjusted to 3.0 with 0.1 mol/L HCl (Bahri *et al.,* 1981).

Isoniazid and rifampicin were procured from the Lupin Pharmaceuticals Ltd., Mumbai, and silymarin from the Silybon, Microlabs, Bangalore, was used as standard drug.

The serum marker enzymes and other biochemical markers were estimated such as aspartate transaminase (AST) and alanine transaminase (ALT) by Reitman & Frankel (1957), alkaline phosphatase (ALP) by Kind & King (1954), bilirubin (BILN)

by Malloy & Evelyn (1937), total proteins (TPN) by Lowry *et al.* (1951), triglycerides (TGL) by Rice (1970) and total cholesterol (CHL) by Pattabiraman (2004).

Experimental Protocol

The male Wistar albino rats weighing 150–200 g were randomly divided into 7 groups of six animals in each group. Group 1 served as vehicle control and received only 1% w/v SCMC (10 ml/kg, p.o.) for 9 days. Group 2 served as toxic control and received INH + RMP (50 mg/kg, p.o.) per day for 9 days. Group 3 animals served as standard drug control and received silymarin 100 mg/kg/p.o., along with INH+RMP 50 mg/kg/p.o. for nine days. Group 4 animals received bergenin 200 mg/kg/p.o., along with INH + RMP p.o. for nine days. Groups 5, 6 and 7 animals received 200 mg/kg each of hexane, chloroform and methanol extracts respectively along with the INH + RMP 50 mg/kg/p.o. for nine days respectively.

The test drugs were administered orally half-an-hour before the INH + RMP (50 mg/kg/p.o.) doses in groups 3, 4, 5, 6 and 7.

Biochemical Analysis

After the experimental period, the blood samples were collected by sinus orbital puncture using sterilized capillary tube under light ether anesthesia in sterile vials from all the groups. Coagulation was permitted and serum was separated from blood by centrifugation (3000 rpm for 15 min) and then the serum was subjected to the analysis of various biochemical parameters such as AST, ALT, ALP, BILN, TPN, TGL and CHL.

Histopathological Examination

On 10^{th} day, after withdrawal of the blood, the rats were sacrificed by the cervical dislocation. The liver was quickly dissected out from the animals and washed separately with normal saline and processed for dehydration, infiltration and

embedding. Initially, the materials were fixed in 10% buffered neutral formalin for 48 h and then with bovine solution for 6 h. They were infiltered and embedded with paraffin. The microtome sections were taken at 5 μ thickness, processed in alcohol–xylene series, stained with alum haematoxylene and eosin and examined under microscope for the evaluation of histopathological changes.

3.3.14 Statistical Analysis

All the data were statistically evaluated with SPSS 10 software for windows. The data were expressed as mean ± standard error for mean (SEM). The difference among means has been analyzed by Student's t-test (Woolson, 1987). A value of $P<0.05$ was considered statistically significant.

CHAPTER 4

RESULTS

4.1. PHYTOCHEMISTRY

4.1.1 Preliminary Phytochemical Screening

The preliminary phytochemical analysis of the whole plant revealed the presence of glycosides, steroids, triterpenes, flavones, phenols in all the three extracts and catechins, coumarins, sugars, saponins and tannins in methanol extract (Table 4).

4.1.2 Qualitative Analysis

4.1.2.1 Extractive Value

The results of the successive solvent plant extracts such as hexane, chloroform and methanol were found to be 2.30%, 3.60%, and 14.70% respectively.

4.1.2.2 Fluorescent Analysis

Characteristic color variations were recorded in ordinary and UV lights (254 nm) corresponding to pH when treated with different solvents and acidic and basic reagents (Table 5).

4.1.2.3 Ash Values

The total ash value was found to be 8.29%, which showed the presence of inorganic constituents. The low value of 1.25% was recorded for acid insoluble ash that indicated the presence of negligible amount of siliceous matter. The water soluble ash was found to be 1.40%.

4.1.2.4 Loss of Weight on Drying

The moisture content of the powdered drug was found to be 53.50%. It indicated total water content and volatile matter of the plant ingredients.

Table 4. Preliminary Phytochemical Screening of the Various Plant Extracts of *Phyllanthus wightianus*

S. No.	Test	Hexane	Chloroform	Methanol
1	Alkaloids	-	-	-
2	Catechins	-	-	+
3	Coumarins	-	-	+
4	Anthraquinones	-	-	-
5	Glycosides	+	+	+
6	Steroids	+	+	-
7	Triterpenes	+	+	+
8	Sugars	-	-	+
9	Flavones	+	+	+
10	Phenolic groups	+	+	+
11	Saponins	-	-	+
12	Tannins	-	-	+
13	Quinones	-	-	-
14	Oils	-	-	-

+ Presence; - Absence

Table 5. Fluorescence Analysis of the Whole Plant of *Phyllanthus wightianus*

S. No.	Treatment	Day light	UV light (254 nm)
1	Dry powder	Pale green	Green
2	Drug powder in aqueous 1 N NaOH	Dark greenish brown	Dark yellowish brown
3	Drug powder in alcoholic 1 N NaoH	Greenish brown	Yellowish brown
4	Drug powder in HCl	Green	Greenish brown
5	Powder in 1:1% H_2SO_4	Olive greenish brown	Yellowish brown
6	Powder in 1:1% HNO_3	Olive greenish brown	Yellowish brown
7	Powder with solvents		
	a) Hexane	Yellowish green	Green
	b) Benzene	Greenish brown	Yellowish brown
	c) Chloroform	Olive greenish brown	Yellowish brown
	d) Petroleum ether	Greenish brown	Yellowish brown
	e) Acetone	Green	Green
	d) Alcohol	Greenish brown	Dark brown
	f) Water	Brown	Yellowish brown

4.1.2.5 Tests for Inorganic Elements

The ash analyzed for the presence inorganic elements has revealed the presence of calcium, carbonate, iron, potassium, sodium and magnesium and copper (Table 6).

4.1.3 Quantitative Analysis of Inorganic Elements (Salts and Minerals)

Quantity of sodium and potassium present in 1 g plant material estimated using Flame Photometry is 2.960 mg and 1.200 mg respectively. Quantity of calcium, cobalt, copper, iron, magnesium and manganese present in 1 g of plant material analyzed using Atomic Absorption Spectrophotometer is 6.300 mg, 0.003 mg, 0.060 mg, 2.130 mg, 1.089 mg and 0.345 mg respectively.

4.1.4 Paper Chromatographic Analysis of Amino Acids

The Rf values of the standard amino acids detected as purple spots after the application of ninhydrin reagent from the chromatogram, compared with the Rf values of the standard amino acids (Table 7) and confirmed further by co-chromatography with authentic samples, has resulted in the identification of DL - Alanine (0.225), L - Arginine mono HCl (0.162), DL - Aspartic acid (0.347), L - Cystinine (0.120), L - Glutamic acid (0.160), Glysine (0.138), DL - Methieonine (0.154), DL - Tryptophan (0.624), and L - Tyrosine (0.375).

4.1.5 High Performance Thin Layer Chromatography

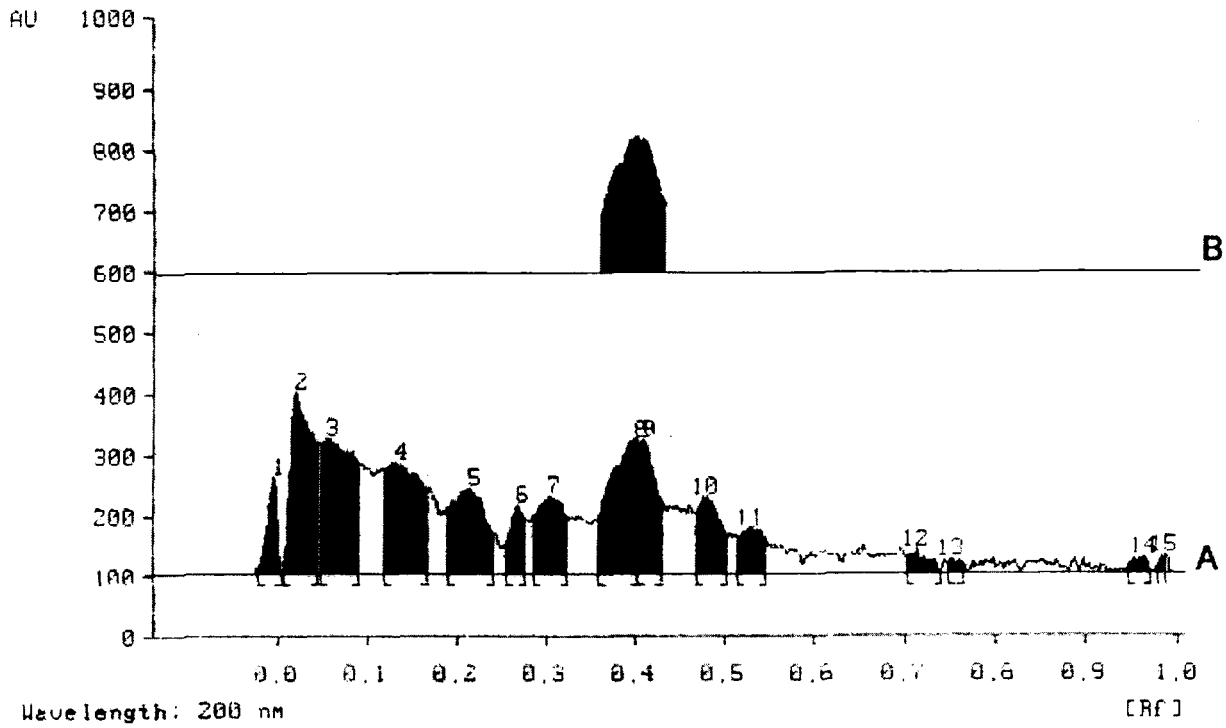
The HPTLC chromatogram of hexane extract showed 15 peaks (Fig. 1). The peak at Rf value 0.47 corresponded to the marker compound friedelin (Fig. 2). The superimposibility with the authentic compound is shown (Fig. 3). The HPTLC chromatogram of chloroform extract showed 18 peaks (Fig. 4). The peak 16 with Rf value 0.83 corresponded to the marker compound lupeol (Figs. 2 & 4.1). The superimposibility of this compound with the authentic sample is shown (Fig. 5).

Table 6. Tests for Inorganic Elements in *Phyllanthus wightianus*

S. No.	Chemical constituents	Test	Observation	Results
1	Arsenic	i) To the neutral solution Silver Nitrate added	No yellow precipitate	Absence of Arsenic
		ii) To the neutral solution, Copper Sulphate was added	No green precipitate	Absence of Arsenic
2	Borate	To the drug in solution, Sulphuric Acid and Alcohol 95% was added. The solution was exposed to blue flame of burner	No green colour	Absence of Borate
3	Copper	Excess of Ammonia was added to the solution	Presence of deep blue colour	Presence of Copper
4	Calcium	i) The solution was treated with Ammonium Carbonate solution	White precipitate formed	Presence of Calcium
		ii) The solution was treated with Ammonium Oxalate	White precipitate formed	Presence of Calcium
5	Carbonate	i) Treated with dilute acid with ash	Liberation of carbon di oxide	Presence of Carbonate
		ii) To the test solution added Mercuric Chloride solution	Brownish red precipitate formed	Presence of Carbonate
		iii) To the test solution added Magnesium Sulphate	White Precipitate formed	Presence of Carbonate
6	Lead	i) The solution was treated with HCl	No characteristic reaction	Absence of Lead
		ii) The solution was treated with dilute Sulphuric Acid	No characteristic reaction	Absence of Lead
7	Iron	i) The solution was treated with Potassium Ferrocynide	Dark blue colour produced	Presence of Iron
		ii) The solution was treated with Potassium Thiocyanate	Blood red colour produced	Presence of Iron
8	Potassium	i) Solution was treated with Perchloric Acid 60%	White precipitate formed	Presence of Potassium
		ii) Flame Test	Violet colour to the flame	Presence of Potassium
9	Mercury	Neutral solution of drug was treated with Potassium Iodide solution	No characteristic reaction	Absence of Mercury
10	Sodium	i) 10 ml ash extract + 2 ml of Potassium Pyroantimonate Pyroanthllollate	White precipitate formed	Presence of Sodium
		ii) Prepared thick paste of ash with concentrated HCl. Take paste on platinum wire loop and introduce into Bunsen flame	Golden yellow flame observed	Presence of Sodium
11	Magnesium	i) Treated with Ammonium Carbonate	White precipitate formed	Presence of Magnesium

Table 7. R_f values of Standard Amino Acids in the Whole Plant of *Phyllanthus wightianus*

S. No.	Name of the Amino Acid	R_f Value	
		Standard	Sample
1	DL – Alanine	0.227	0.225
2	DL-2-Amino-n-butyric acid	0.355	-
3	L– Arginine mono HCl	0.163	0.162
4	DL – Aspartic acid	0.348	0.347
5	L – Cystenine – HCl	0.420	-
6	L – Cystine	0.122	0.120
7	3 (3,4-dihydroxyl phenyl)- DL–Alanine	0.280	-
8	L – Glutamic acid	0.161	0.160
9	G - Lysine	0.139	0.138
10	L – Histidine mono HCl	0.102	-
11	L – Hydroxyproline	0.216	-
12	DL – Isoleucine	0.618	-
13	DL Nor – Leucine	0.693	-
14	L – Leucine	0.676	-
15	L – Lysine Mono HCl	0.141	-
16	DL – Methionine	0.157	0.156
17	L – Ornithine mono HCl	0.130	-
18	DL – B – Phenylalanine	0.586	-
19	L – Proline	0.379	-
20	DL – Serine	0.214	-
21	DL – Thereonine	0.237	-
22	DL – Tryptophan	0.626	0.624
23	L – Tyrosine	0.372	0.375
24	DL – Valine	0.467	-



Wavelength: 200 nm
Track: 2, noise level: 2.213AU,
U4.04 S/N:0312A012 CAMAG SOFTWARE (c) 1996 SCANNER 3: 030618

Track 2, Analysis b: H.Ext

Peak	start		max			end		area	
#	Rf	H	Rf	H	[%]	Rf	H	F	[%]
1	-0.02	5.7	-0.01	157.6	7.92	0.00	1.1	1489.3	3.28
2	0.01	2.2	0.02	295.9	14.87	0.04	211.3	6177.0	13.62
3	0.05	211.4	0.06	220.0	11.05	0.09	178.7	6920.8	15.26
4	0.12	168.9	0.13	180.8	9.08	0.17	139.1	6135.0	13.53
5	0.19	105.5	0.21	139.6	7.01	0.24	66.2	4470.7	9.86
6	0.26	52.3	0.27	112.4	5.65	0.28	91.4	1454.7	3.21
7	0.29	94.0	0.30	126.6	6.36	0.32	94.3	3357.2	7.40
8	0.36	93.5	0.40	218.7	10.99	0.40	218.2	5808.6	12.81
9	0.40	218.2	0.41	218.7	10.99	0.43	112.0	3872.3	8.54
10	0.47	103.3	0.48	125.0	6.28	0.50	56.8	2664.6	5.88
11	0.52	58.8	0.53	73.5	3.69	0.55	58.3	1565.3	3.45
12	0.70	24.7	0.71	37.3	1.87	0.74	4.2	668.8	1.47
13	0.75	15.5	0.75	25.1	1.26	0.77	15.6	240.8	0.53
14	0.95	10.7	0.97	28.2	1.42	0.97	6.9	375.5	0.83
15	0.98	12.0	0.99	31.0	1.56	0.99	31.0	149.5	0.33

Total height = 1990.3 total area = 45350.1

Developing system : Hexane: Toluene

Scanning wavelength : 200 nm

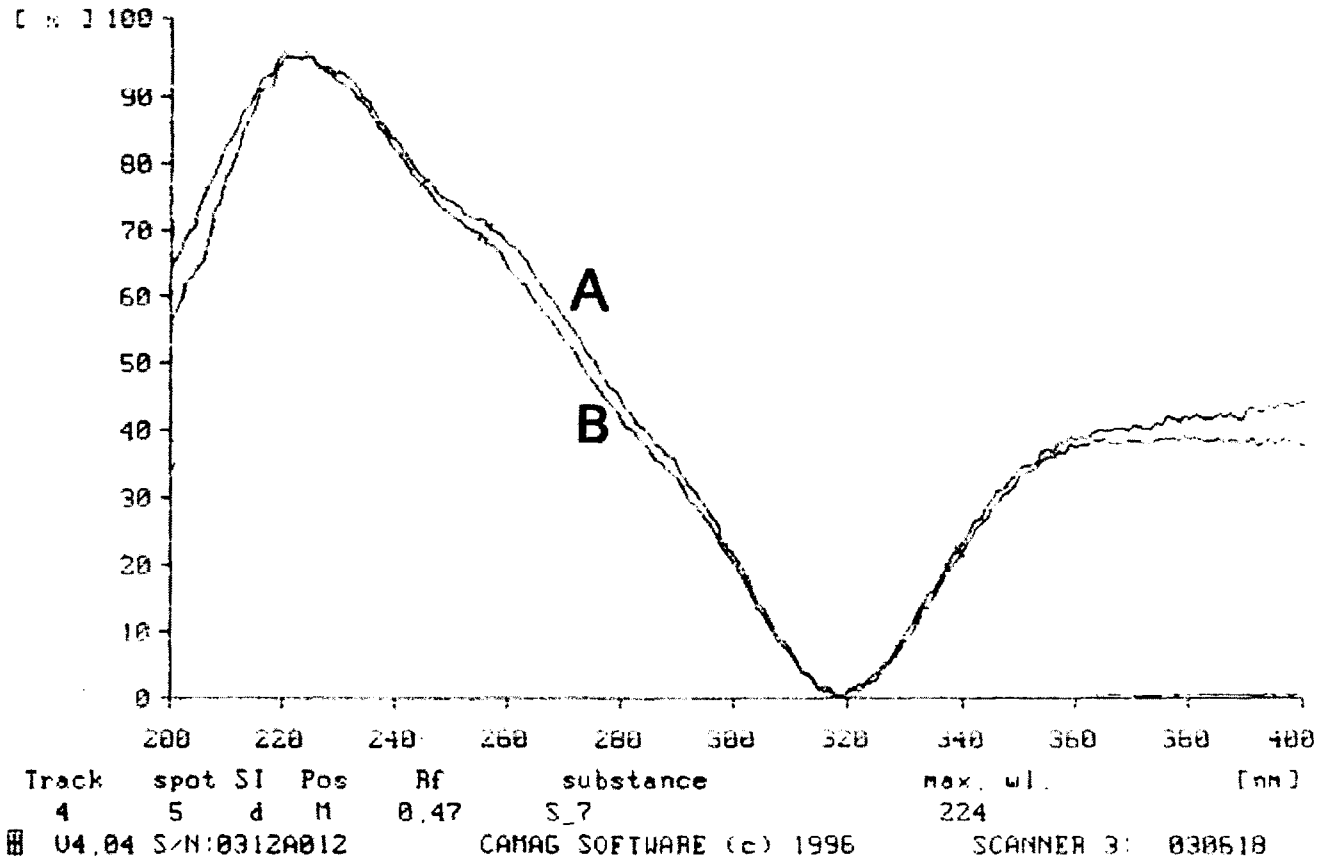
A - hexane extract

B - friedelin marker

Fig.1. HPTLC chromatogram of the hexane extract of *Phyllanthus wightianus*

Fig.2. HPTLC chromatogram of *Phyllanthus wightianus*

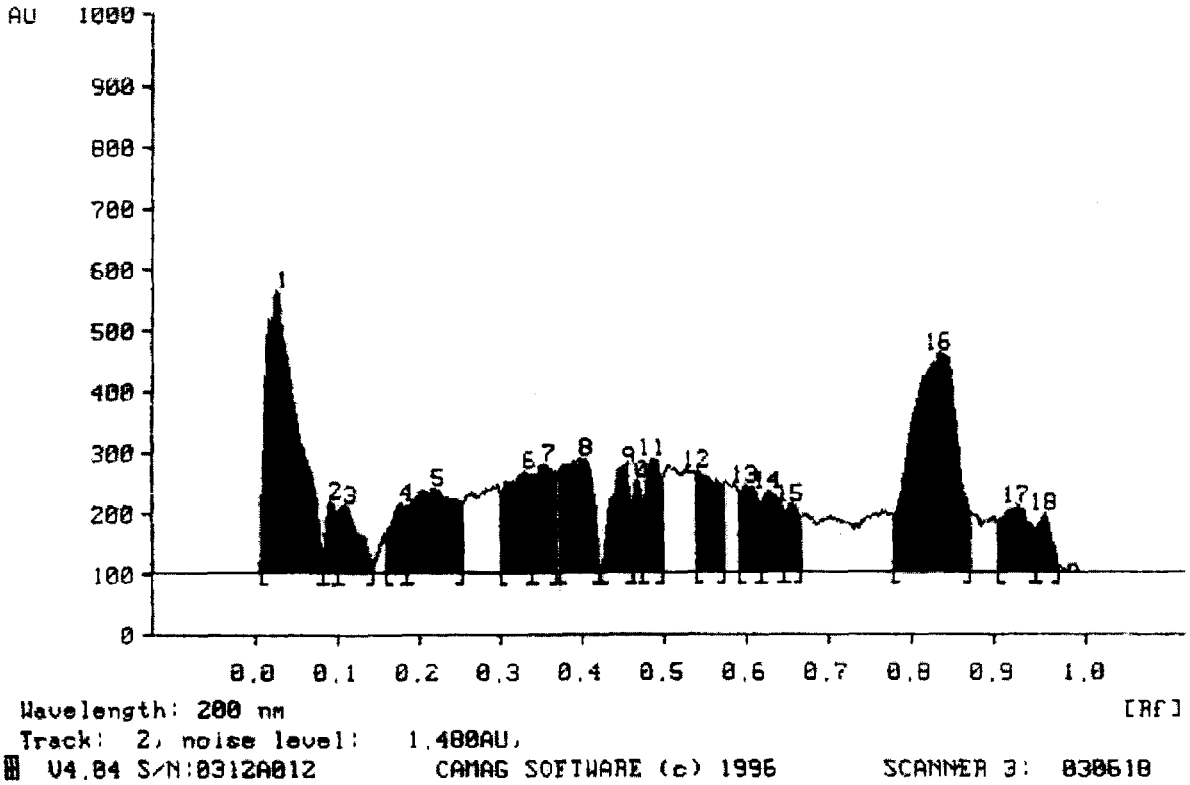
1. Hexane extract and marker compound friedelin
2. Chloroform extract and marker compound lupeol
3. Methanol extract and marker compounds gallic acid and bergenin



A - friedelin in the hexane extract of *Phyllanthus wightianus*

B - authentic marker

Fig.3. UV superimposibility of friedelin with the authentic marker



Track 2, Analysis b: spl

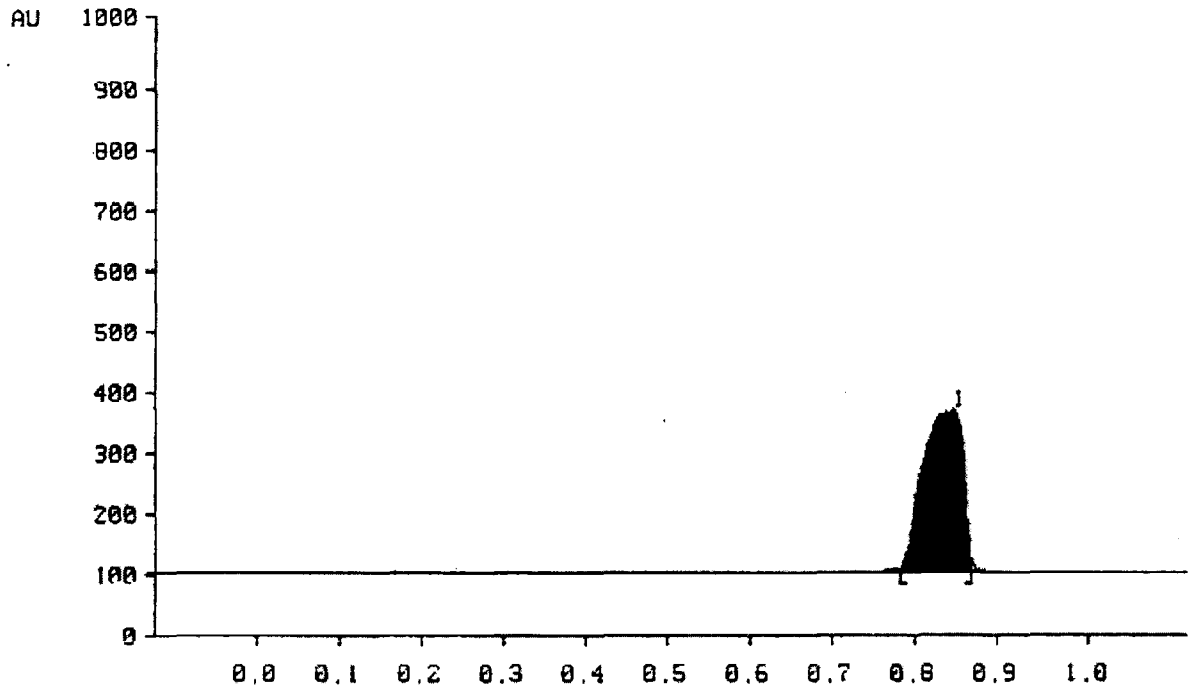
Peak	start		max			end		area	
#	Rf	H	Rf	H	[%]	Rf	H	F	[%]
1	0.01	38.3	0.03	456.1	14.90	0.08	34.2	15785.5	18.78
2	0.08	34.2	0.09	116.8	3.81	0.10	100.6	1413.4	1.68
3	0.10	100.6	0.11	109.8	3.59	0.14	16.7	2439.1	2.90
4	0.16	56.9	0.18	114.2	3.73	0.18	100.3	1824.6	2.17
5	0.19	105.8	0.22	138.0	4.51	0.25	114.4	6565.4	7.81
6	0.30	131.0	0.33	162.4	5.31	0.34	154.9	4641.7	5.52
7	0.34	154.9	0.35	176.4	5.76	0.37	160.6	3962.0	4.71
8	0.37	165.9	0.40	183.5	5.99	0.42	3.4	5768.3	6.86
9	0.42	9.6	0.45	170.5	5.57	0.46	108.8	3440.7	4.09
10	0.46	108.8	0.47	150.1	4.91	0.47	113.6	1331.6	1.58
11	0.47	113.6	0.49	183.1	5.98	0.50	150.5	3220.8	3.83
12	0.54	159.0	0.54	163.7	5.35	0.57	139.8	4057.6	4.83
13	0.59	129.6	0.60	139.3	4.55	0.62	112.7	2750.9	3.27
14	0.62	112.7	0.62	132.3	4.32	0.64	91.4	2721.8	3.24
15	0.64	91.4	0.65	111.0	3.63	0.66	89.3	1528.3	1.82
16	0.78	90.3	0.83	350.7	11.46	0.87	94.4	17893.6	21.28
17	0.90	81.9	0.92	108.6	3.55	0.95	68.1	3278.8	3.90
18	0.95	68.1	0.96	94.8	3.10	0.97	6.1	1443.5	1.72

Total height = 3061.0 total area = 84067.6

Developing system : Chloroform

Scanning wavelength : 200 nm

Fig.4. HPTLC chromatogram of the chloroform extract of *Phyllanthus wightianus*



Track 1, Analysis a: lupeol

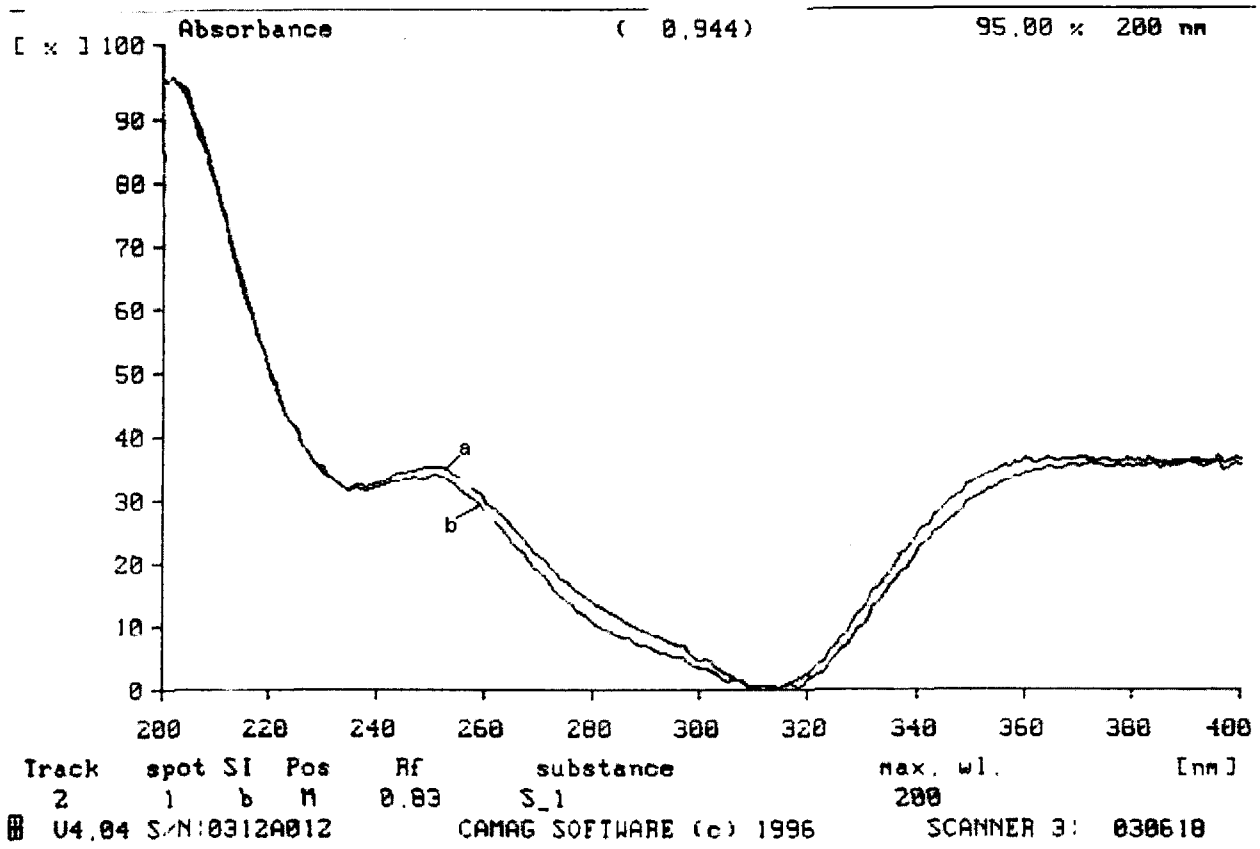
Peak	start		max			end		area	
#	Rf	H	Rf	H	[%]	Rf	H	F	[%]
1	0.78	5.3	0.84	260.9	100.00	0.87	13.1	11767.3	100.00

Total height = 260.9 total area = 11767.3

Developing system : Chloroform

Scanning wavelength : 200 nm

Fig.4.1. HPTLC chromatogram of the authentic marker lupeol



a - lupeol in the hexane extract of *Phyllanthus wightianus*

b - authentic marker

Fig.5. UV superimposibility of lupeol with the authentic marker

The HPTLC chromatogram of methanol extract showed 5 peaks (Fig. 6). The peak 5 with Rf value 0.85 corresponded to the marker compound gallic acid (Fig. 6.1) which is compared with the authentic sample by superimposibility (Fig. 7). The peak 2 with Rf value 0.63 corresponded to the marker compound bergenin (Figs. 2 & 6.2). Its superimposibility with the authentic sample is shown (Fig. 8).

4.1.6 HPLC Analysis and Estimation of Tannins and Lignans

4.1.6.1 Tannins

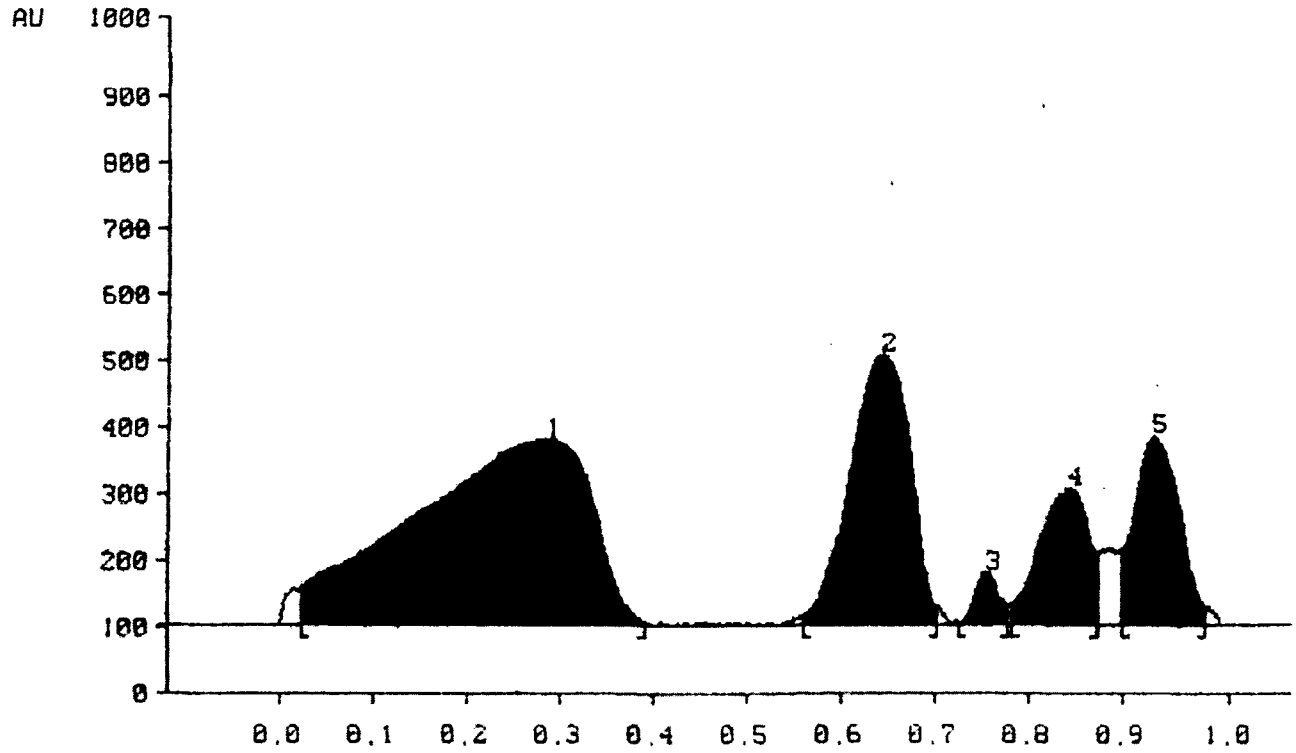
The chromatogram of methanol extract showed 36 peaks at 220 nm and 5 peaks at 254 nm with various retention times (Figs. 9 & 9.1). Standard tannins such as gallic acid (GA), corillagin (C), geraniin (G) and ellagic acid (EA) were also run (Figs. 10 & 10.1). It has resulted in the identification of tannins in comparison with the values of the standards given in the parentheses such as gallic acid (GA) - 5.158 (5.092), corillagin (C) - 18.900 (18.875), geraniin (G) - 20.292 (19.817) and ellagic acid (EA) - 27.617 (27.592). Quantification estimated by the area of peak is 3.89% for corillagin, the most abundant one, followed by 3.19% for geraniin, 0.68% for ellagic acid and 0.38% for gallic acid.

4.1.6.2 Lignans

Though the individual chromatograms showed 8 peaks with various retention times it did not show two peaks at 13.20 for hypophyllanthin and 16.50 for phyllanthin (Figs. 11 & 11.1). It indicated their absence in this plant.

4.1.7 Lipid Profile of a Fraction of Hexane Extract

The hexane extract was chromatographed over silica gel (100-200 mesh) packed with hexane. The increasing polarity of hexane: benzene 4:1 yielded a group of lipids. This fraction was subjected to GC-MS analysis which showed 23 peaks with



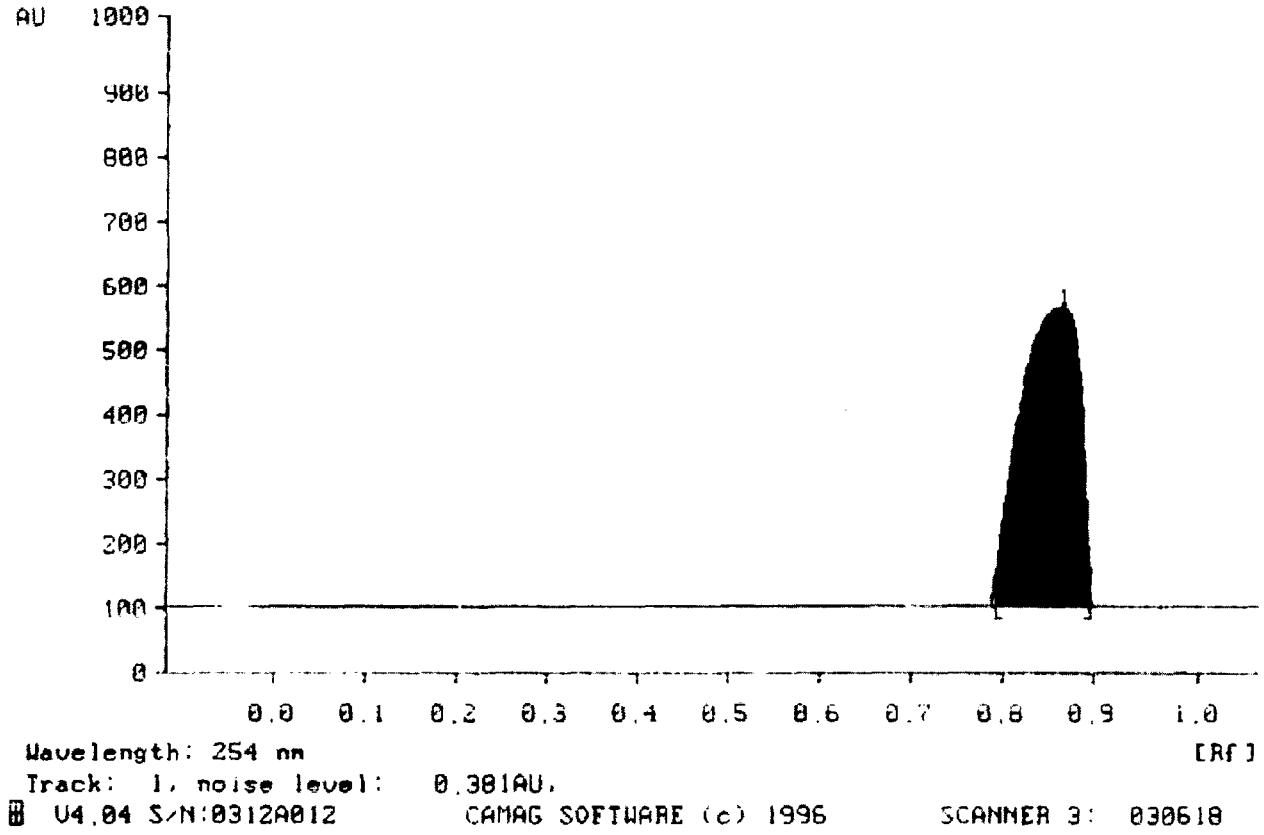
Peak	start		max			end		area	
#	Rf	H	Rf	H	[%]	Rf	H	F	[%]
1	0.02	55.1	0.28	275.1	22.34	0.39	4.1	50303.1	50.45
2	0.56	11.5	0.64	397.7	32.30	0.70	26.5	24330.1	24.40
3	0.72	0.4	0.75	78.3	6.36	0.77	29.3	1919.9	1.93
4	0.78	30.4	0.84	201.0	16.32	0.87	106.7	10213.8	10.24
5	0.89	109.4	0.92	279.3	22.68	0.98	24.4	12932.1	12.97

Total height = 1231.4 total area = 99699.1

Developing system : Ethyl acetate

Scanning wavelength : 254 nm

Fig. 6. HPTLC chromatogram of methanol extract of *Phyllanthus wightianus*



Track 1, Analysis a: Galic

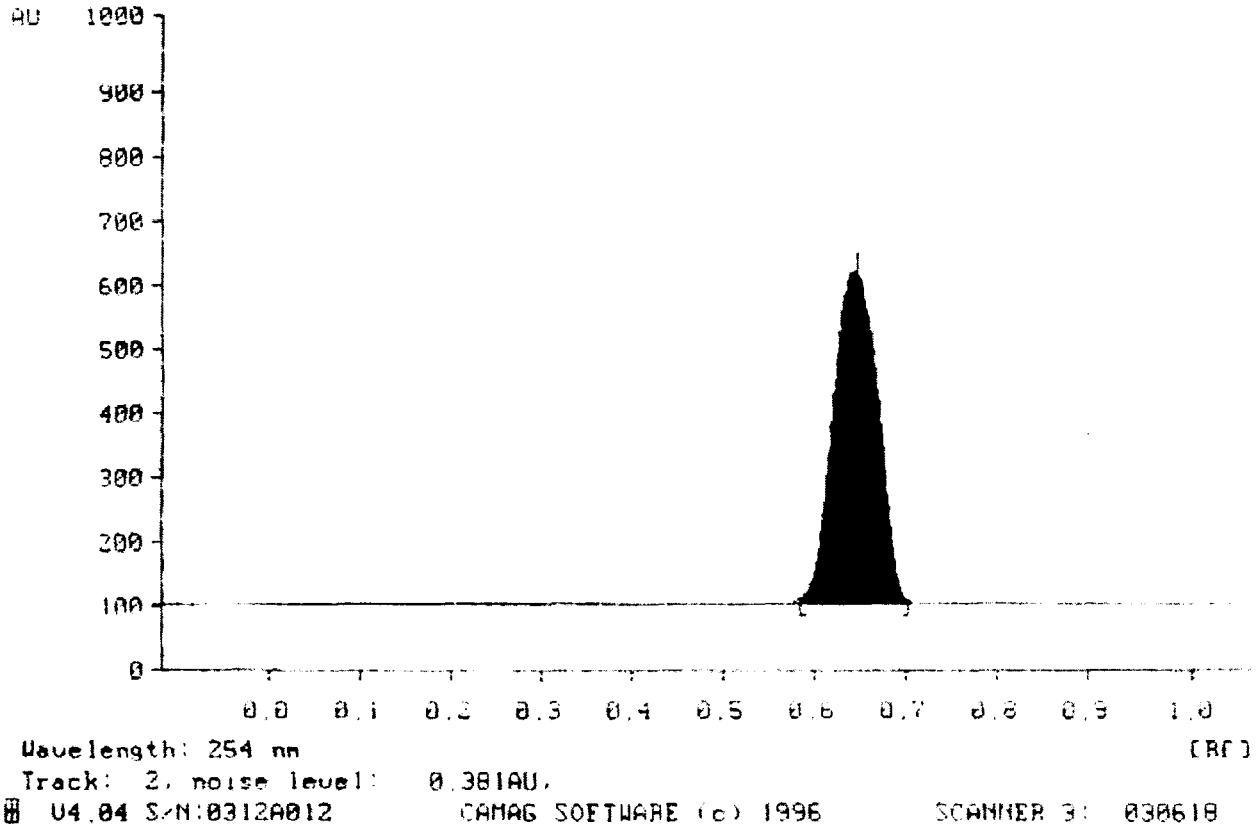
Peak	start		max			end		area	
#	Rf	H	Rf	H	[%]	Rf	H	F	[%]
1	0.78	53.0	0.85	453.5	100.00	0.89	20.5	28809.2	100.00

Total height = 453.5 total area = 28809.2

Developing system : Ethyl acetate

Scanning wavelength : 254 nm

Fig.6.1. HPTLC chromatogram of the authentic marker gallic acid



Track 2, Analysis b: Berg

Peak	start		max			end		area	
#	Rf	H	Rf	H	[%]	Rf	H	F	[%]
1	0.58	7.7	0.63	504.1	100.00	0.70	4.6	23240.6	100.00

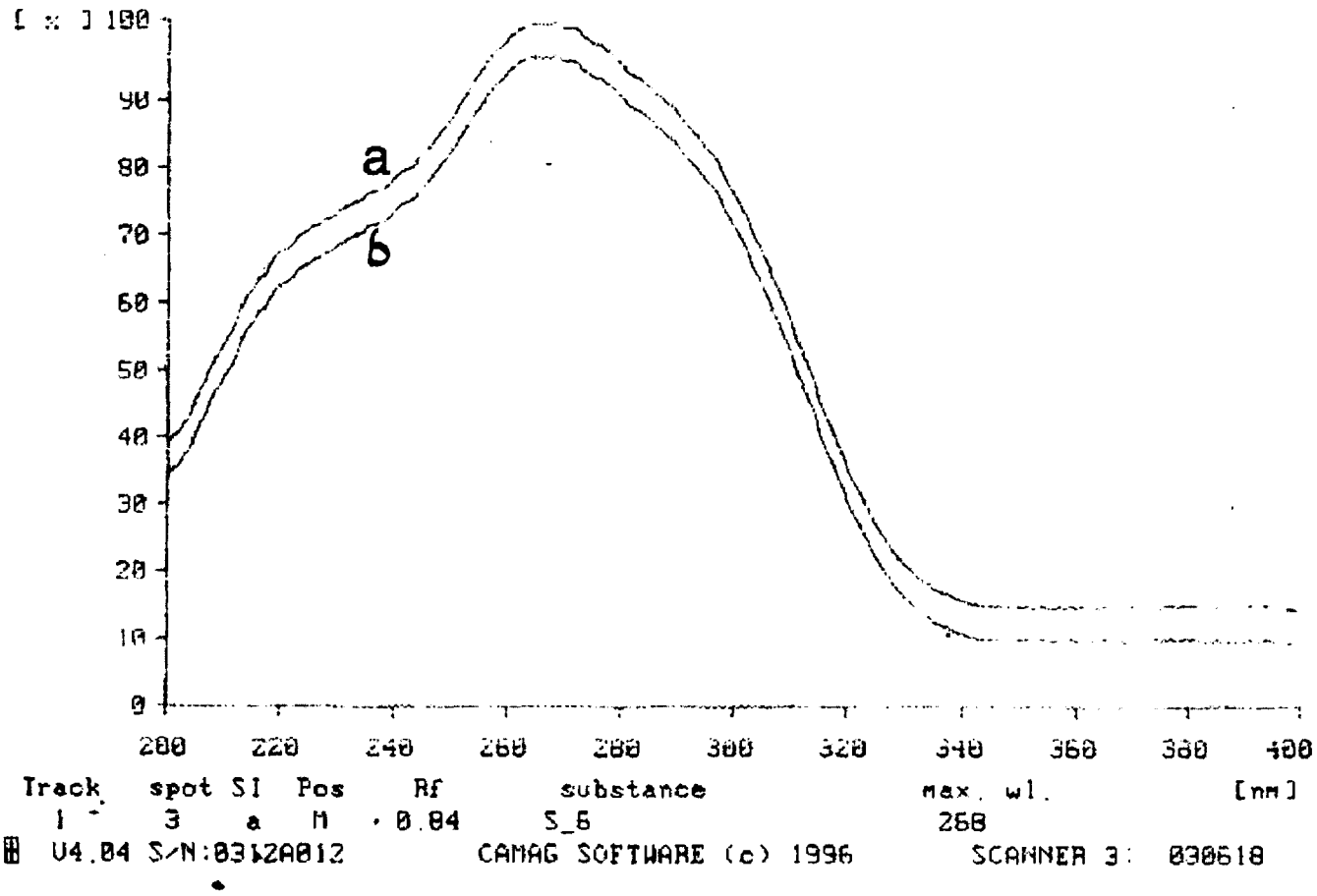
Total height = 504.1

total area = 23240.6

Developing system : Ethyl acetate

Scanning wavelength : 254 nm

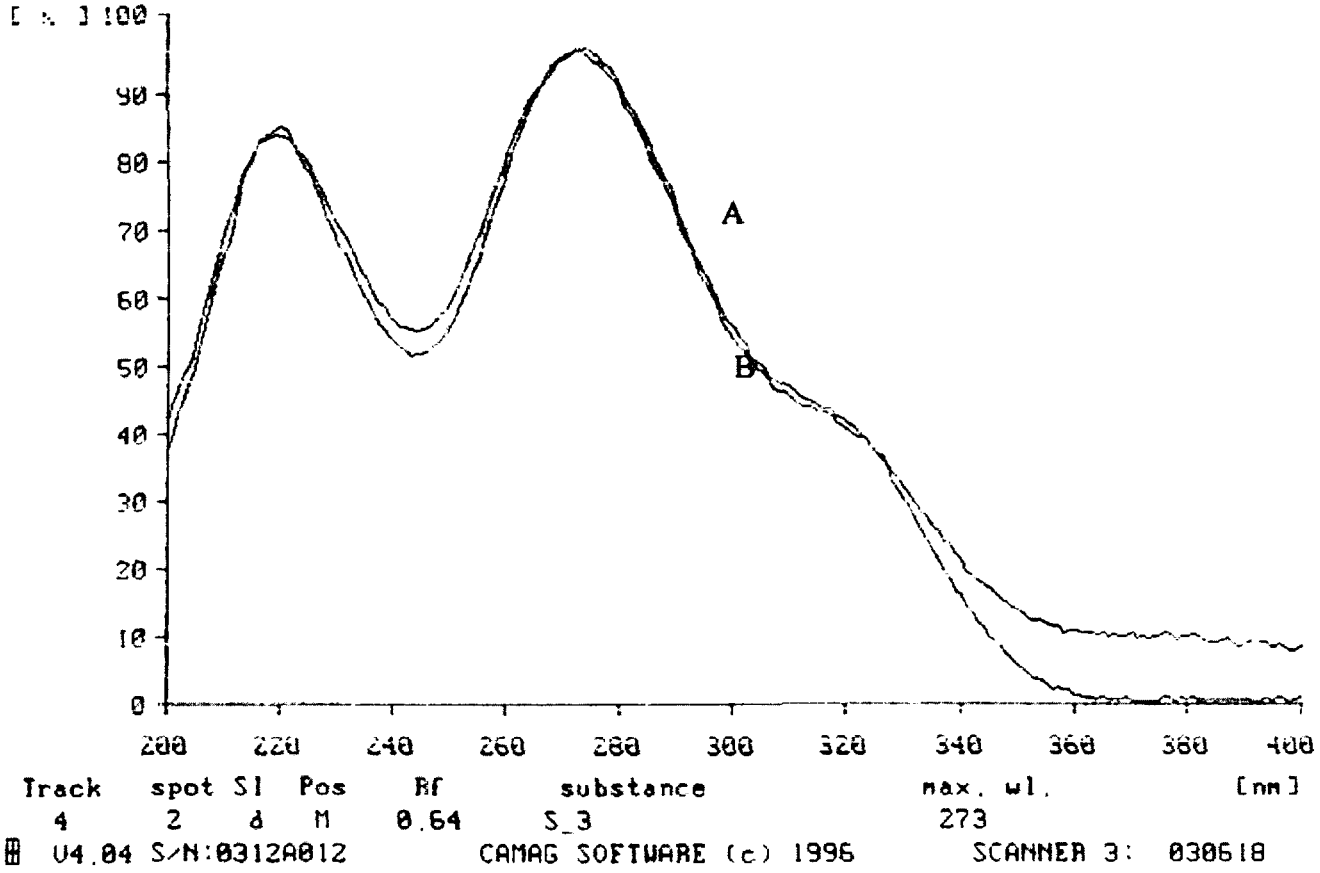
Fig. 6.2. HPTLC chromatogram of the authentic marker bergenin



a - gallic acid in the methanol extract of *Phyllanthus wightianus*

b - authentic marker

Fig.7. UV superimposibility of gallic acid with the authentic marker

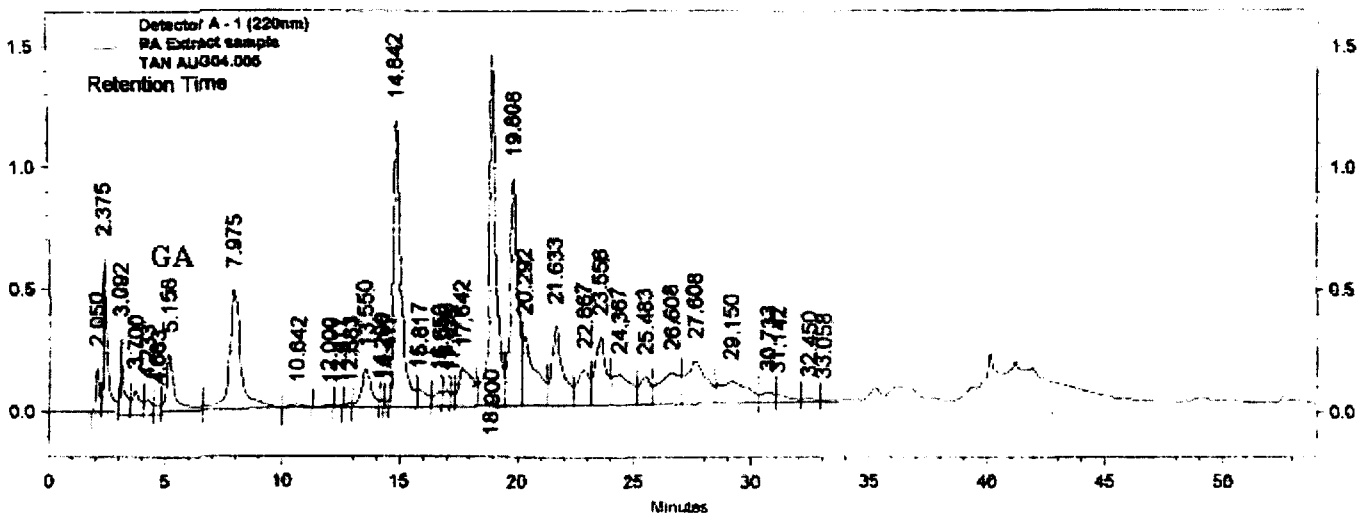


A - bergenin in the methanol extract of *Phyllanthus wightianus*

B - authentic marker

Fig.8. UV superimposibility of bergenin with the authentic marker

Method Name	**: E:\METHODS\tanin.met**
Data Name	**: E:\UV\AUG06\TAN AUG04.005**
Column	**: Lichrocart RP 18E(02/04)**
Mobile Phase	**: BINARY GRADIENT**
	PUMP- A:0.05% OPA IN CH3CN;PUMP-B:0.05% OPA IN WATER.
UV	**: 220nm**
Flow	**: 1.0ml/min**

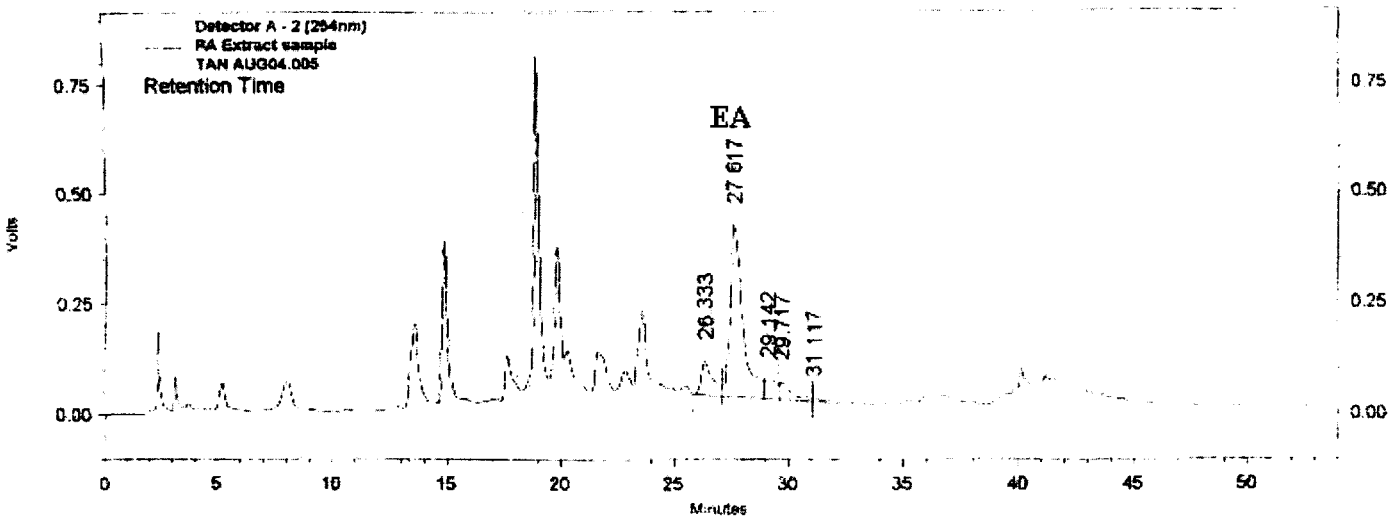


Detector A - 1 (220nm)

Pk #	Name	Retention Time	Area	Area Percent
1		2.050	1815608	0.86
2		2.375	6964547	3.30
3		3.092	2844222	1.35
4		3.700	1835494	0.87
5		4.233	1017353	0.48
6		4.683	439039	0.21
7	TANNIN-GA	5.158	5894485	2.79
8		7.975	17124283	8.12
9		10.642	662232	0.31
10		12.000	385010	0.18
11		12.417	250466	0.12
12		12.883	305387	0.14
13		13.550	4638058	2.20
14		14.200	338113	0.16
15		14.417	296213	0.14
16		14.842	26091706	12.37
17		15.817	2051150	0.97

Fig.9. HPLC chromatogram of methanol extract of *Phyllanthus wightianus* at 220 nm

Method Name	**: E:\METHODS\tanin.met**
Data Name	**: E:\UV\AUG06\TAN AUG04.005**
Column	**: Lichrocart RP 18E(02/04)**
Mobile Phase	**: BINARY GRADIENT**
	PUMP- A:0.05% OPA IN CH3CN;PUMP-B:0.05% OPA IN WATER.
UV	**: 254nm**
Flow	**: 1.0ml/min**



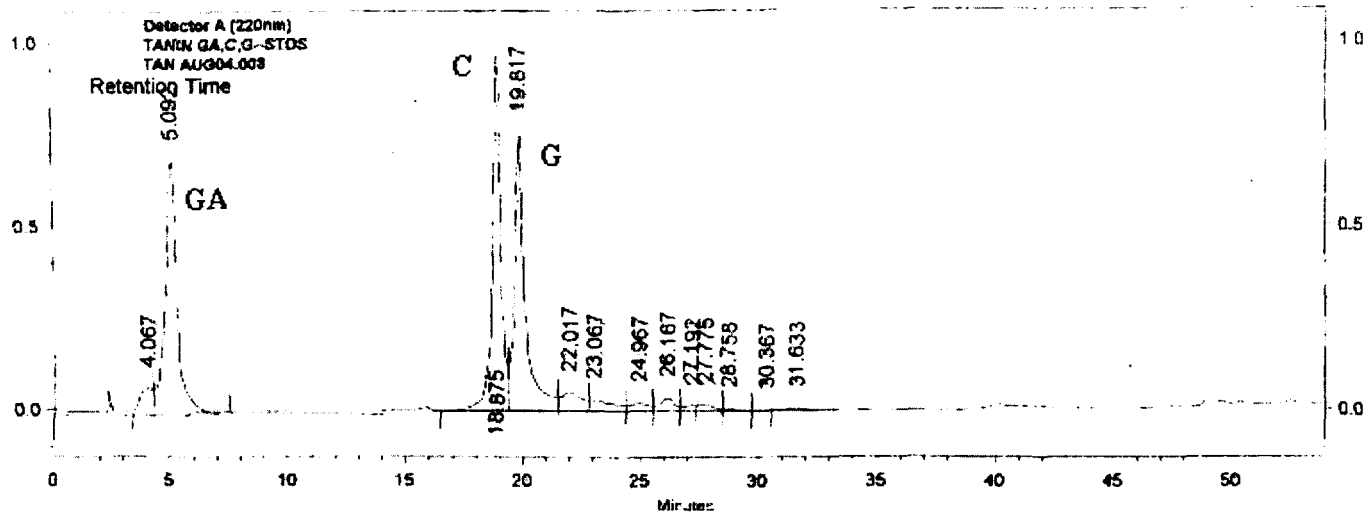
Detector A - 2 (254nm)

Pk #	Name	Retention Time	Area	Area Percent
1		26.333	2667831	12.73
2	TANNIN - EA	27.617	15085922	71.97
3		29.142	1550293	7.40
4		29.717	1451267	6.92
5		31.117	205372	0.98
Totals			**20960685**	**100.00**

EA-Ellagic acid

Fig.9.1. HPLC chromatogram of methanol extract of *Phyllanthus wightianus* at 254 nm

Method Name	: E:\METHODS\tanin.met
Data Name	: E:\UV\AUG06\TAN AUG04.003
Column	: Lichrocart RP 18E(02/04)
Mobile Phase	: BINARY GRADIENT PUMP- A:0.05% OPA IN CH3CN;PUMP-B:0.05% OPA IN WATER.
UV	: 220nm
Flow	: 1.0ml/min
Sample	: TANIN GA,C,G--STDS



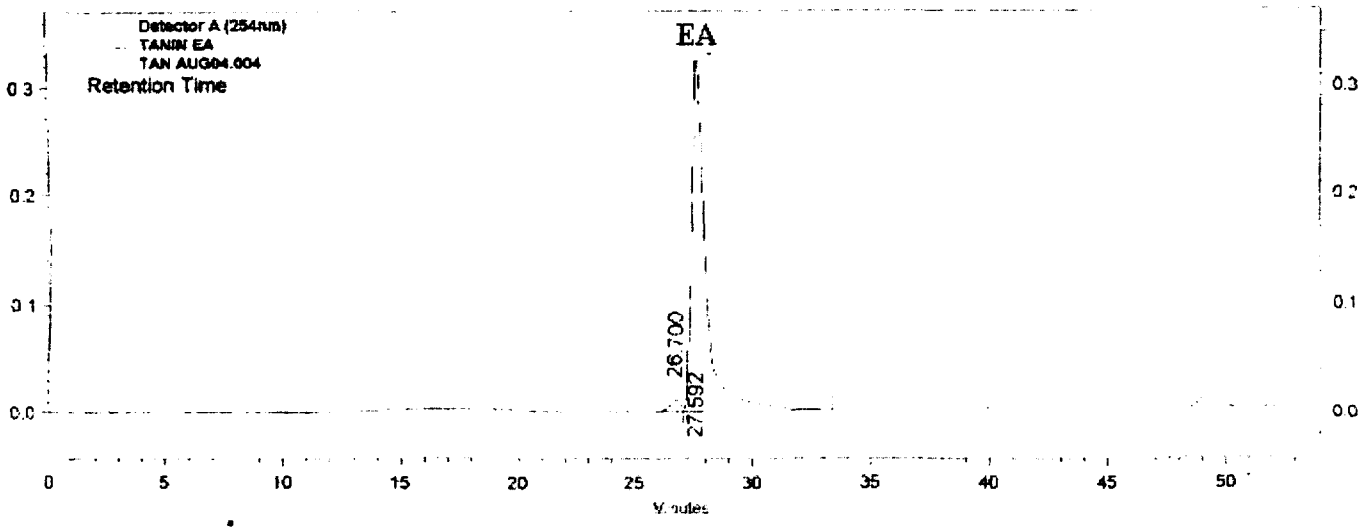
Detector A (220nm)

Pk #	Name	Retention Time	Area	Area Percent
1		4.067	2318690	2.71
2	TANNIN-GA	5.092	23758857	27.79
3	TANNIN-C	18.875	24686378	28.88
4	TANNIN-G	19.817	24498770	28.66
5		22.017	3121083	3.65
6		23.067	2235330	2.61
7		24.967	1156809	1.35
8		26.167	1527403	1.79
9		27.192	548018	0.64
10		27.775	828202	0.97
11		28.758	286239	0.33
12		30.367	101402	0.12
13		31.633	416746	0.49
Totals			85483927	100.00

C - Corillagin; GA-Gallic acid; G-Geraniin

Fig.10. HPLC chromatogram of standards at 220nm

Method Name	: E:\METHODS\tanin.met
Data Name	: E:\UV\AUG06\TAN AUG04.004
Column	: Lichrocart RP 18E(02/04)
Mobile Phase	: BINARY GRADIENT PUMP- A:0.05% OPA IN CH3CN;PUMP-B:0.05% OPA IN WATER.
UV	: 254nm
Flow	: 1.0ml/min
Sample	: TANIN EA



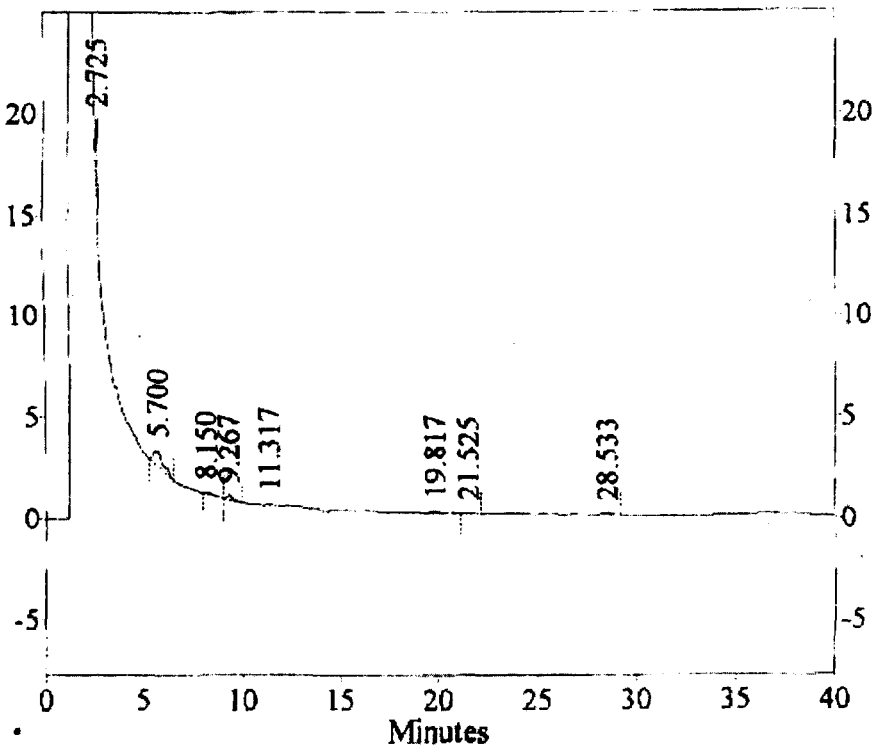
Detector A (254nm)

Pk #	Name	Retention Time	Area	Area Percent
1		26.700	379628	2.69
2	TANNIN-EA	27.592	13739848	97.31
Totals			14119476	100.00

EA-Ellagic acid

Fig.10.1. HPLC chromatogram of ellagic acid(standard) at 254 nm

Column : Spherisorb ODS-2
M.Phase : 65 CH3OH,35 H2O
UV : 230nm
Flow rate: 1.0 ml/min

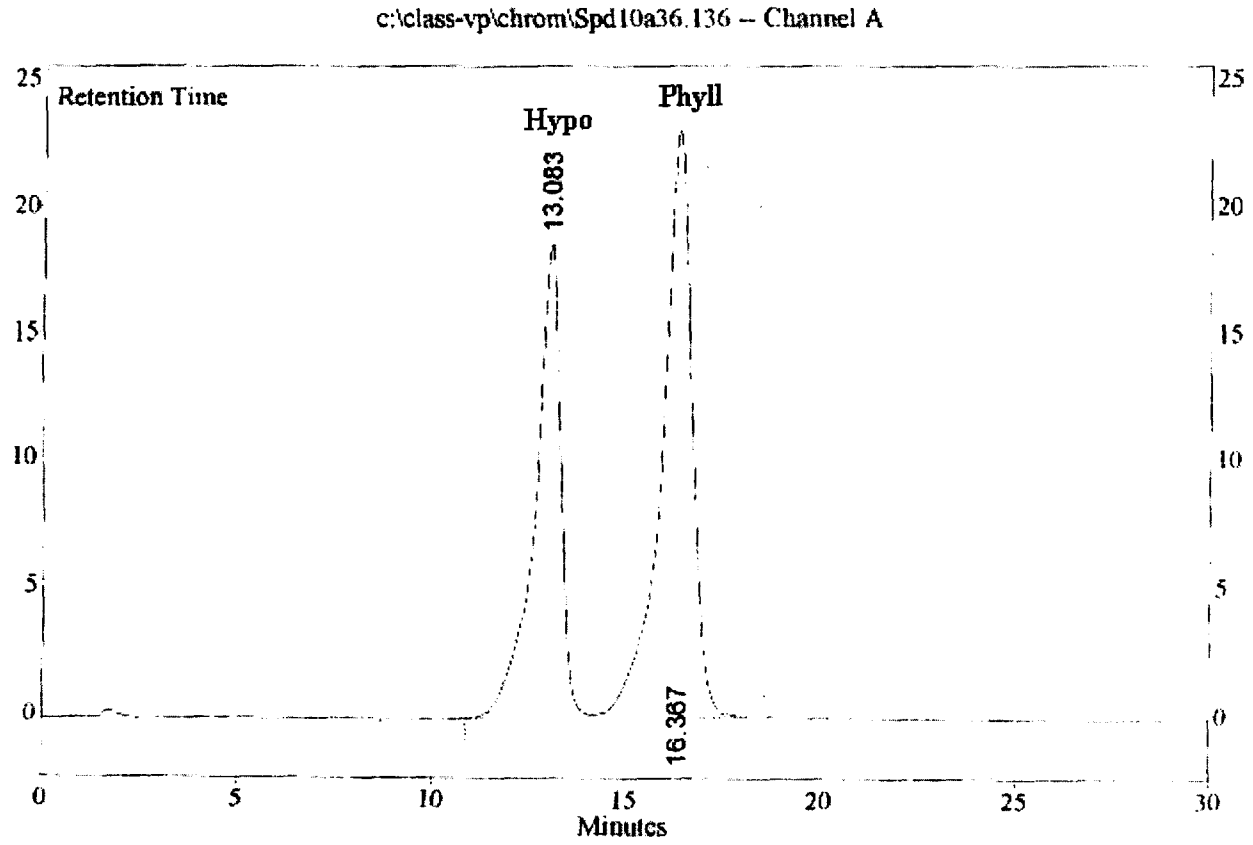


Channel A Results

Peak	Name	Time	Area	Area%
1		2.72	18525	30.85
2		5.70	25030	41.69
3		8.15	2967	4.94
4		9.27	4082	6.80
5		11.32	1797	2.99
6		19.82	3656	6.09
7		21.52	1547	2.58
8		28.53	2437	4.06
Totals			60041	100.00

Fig.11. HPLC chromatogram of methanol extract of *Phyllanthus wightianus*

Column :Spherisorb ODS-2
M.Phase :65 CH3OH,35 H2O
UV :230nm
F/R :1.3ml/min.
Sample :Hypo(0.256),Phyll(0.514)



Channel A Results

Peak	Name	Time	Area	Area %
1		13.08	864455	40.89
2		16.37	1249855	59.11
Totals :			2114310	100.00

Hypo- Hypophyllanthin

Phyll- Phyllanthin

Fig.11.1. HPLC chromatogram of hypophyllanthin and phyllanthin

various retention times and molecular weights wherein only seven compounds could be identified unequivocally (Table 8; Figs. 12, 12.1 – 12.8, 13-19).

4.1.8 Isolation of Compounds from Hexane and Chloroform Extracts

Hexane and chloroform extracts were combined and chromatographed over silica gel (100-200 mesh) built with petroleum ether and eluted with solvents of increasing polarity such as benzene, chloroform, ethyl acetate and methanol (Fig. 20).

4.1.8.1 Isolation and Characterization of PW1 (Friedelin)

Column eluted with hexane: benzene 1:1 yielded a colorless powder PW1 (20 mg, m.p. 263°C), which gave a positive Noller's test for triterpenoid (when warmed with tin and thionyl chloride, gave a pink color). It gave a single spot on TLC, with hexane: toluene 3:1 as the solvent system (Rf = 0.47). IR: $\nu_{Max}^{Kbr} cm^{-1}$ 2926, 2870 (CH stretching), 1715 (C = O stretching), 1453 (CH_2 bending), 1389 and 1325 (*gem* dimethyl). The ^1H NMR (δ $CDCl_3$, 400 MHz) spectrum showed seven tertiary methyl groups in this region δ 0.65 -1.1 including the secondary methyl (C-4 methyl) at δ 0.80 appearing as doublet J = 6.8 MHz and the remaining hydrogen as follows 0.80 (3H, d, J =6.8Hz, H-23), 0.65 (3H, S, H-24), 0.88 (3H, S, H-25), 0.94 (3H, S, H-26), 1.05 (3H, S, H-27), 1.11 (3H, S, H-28), 0.93 (3H, S, H-29), 0.98 (3H, S, H-30). ^{13}C NMR (δ $CDCl_3$, 100 MHz) 22.27 (C-1), 41.52 (C-2), 213.18 (C-3), 58.22 (C-4), 42.14 (C-5), 41.29 (C-6), 18.23 (C-7), 53.10 (C-8), 37.43 (C-9), 59.48 (C-10), 35.62 (C-11), 30.50 (C-12), 39.69 (C-13), 38.29 (C-14), 38.28 (C-15), 32.76 (C-16), 36.01 (C-17), 30.06 (C-18), 35.33 (C-19), 28.15 (C-20), 32.41 (C-21), 39.24 (C-22), 6.81 (C-23), 14.64 (C-24), 17.93 (C-25), 20.25 (C-26), 8.65 (C-27), 32.08 (C-28), 31.76 (C-29) and 35.01 (C-30).

Table 8. Lipid Profile of a Fraction of Hexane Extract of the Whole Plant of *Phyllanthus wightianus*

S. No.	Name of the Compound	Molecular Weight	Molecular Formula	Retention time (min)	Peak area (%)	Super-imposibility (%)	CAS No.	References
1	6,10,14-trimethyl-penta-decan-2-one	268	$C_{18}H_{36}O$	18.42	0.54	91	000-502–69-2	*
2.	Hexa-decanoic acid methyl ester	270	$C_{17}H_{34}O_2$	19.31	1.02	98	000-112–39-0	*
3	3,7,11,15-tetramethyl-1-hexadecen-3-ol (Isophytol)	296.52	$C_{20}H_{40}O$	19.57	0.14	90	000-505–32-8	Demole, 1956; Ali *et al.*, 2001
4	Phytol	278	$C_{20}H_{40}O$	21.29	0.71	80	000-150–86-7	Flanagan and Ferretti, 1973; Flanagan *et al.*, 1975
5	Octa-decanoic acid methyl ester	298.50	$C_{19}H_{38}O_2$	21.39	0.29	99	000-112 – 61-8	Goren *et al.*, 2003
6	9 – octa-decanoic acid methyl ester	296.4	$C_{19}H_{36}O_2$	21.15	0.85	99	001-937–62-8	Goren *et al.*, 2003
7	Di-n-octyl phthalate	390.56	$C_{24}H_{38}O_4$	25.18	0.38	86	000-117–84-0	Cho *et al.*, 2005

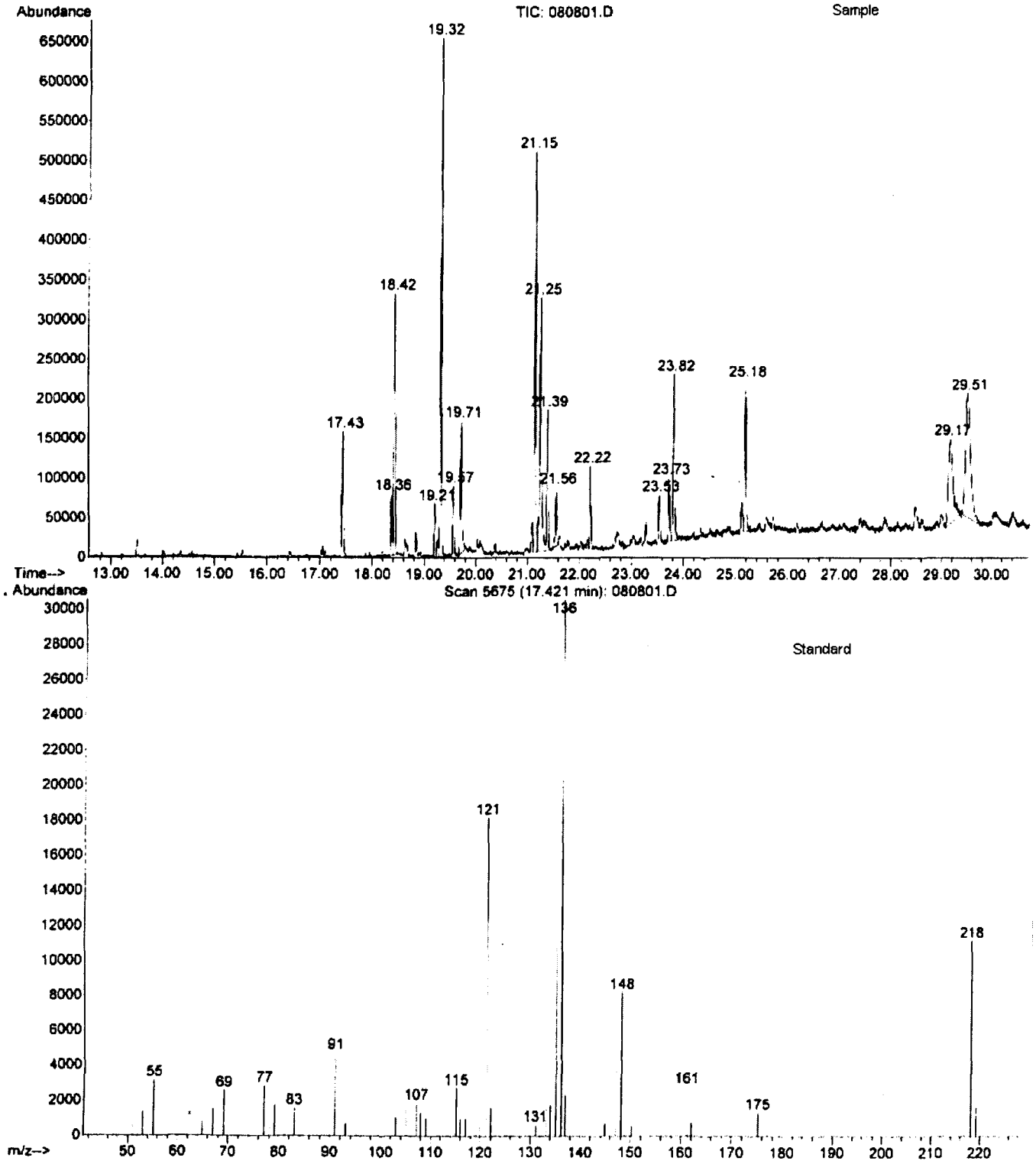


Fig.12.GC-MS spectrum of a fraction of hexane extract

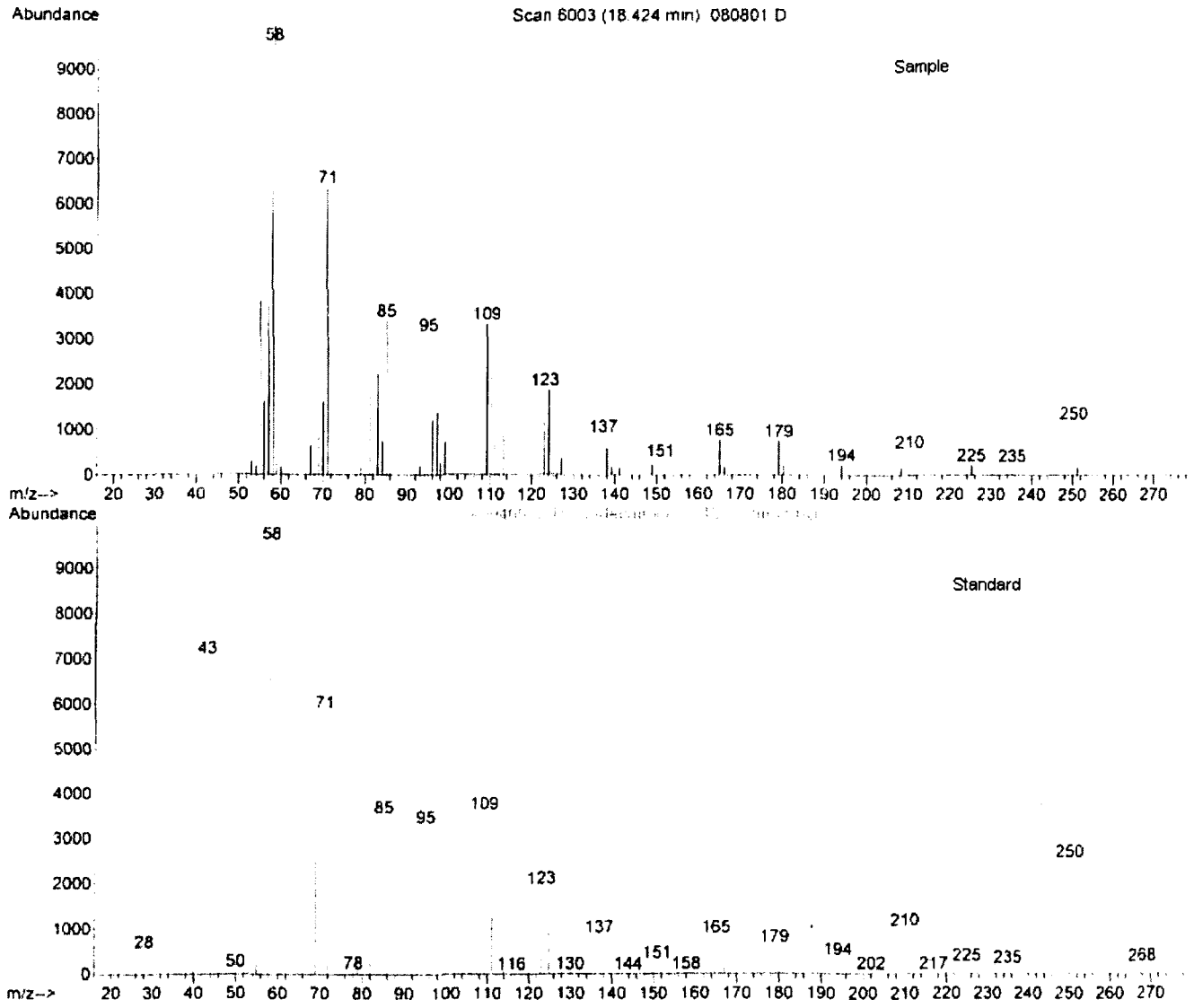


Fig.12.1. GC-MS spectrum of 6, 10, 14, trimethyl penta decan-2one

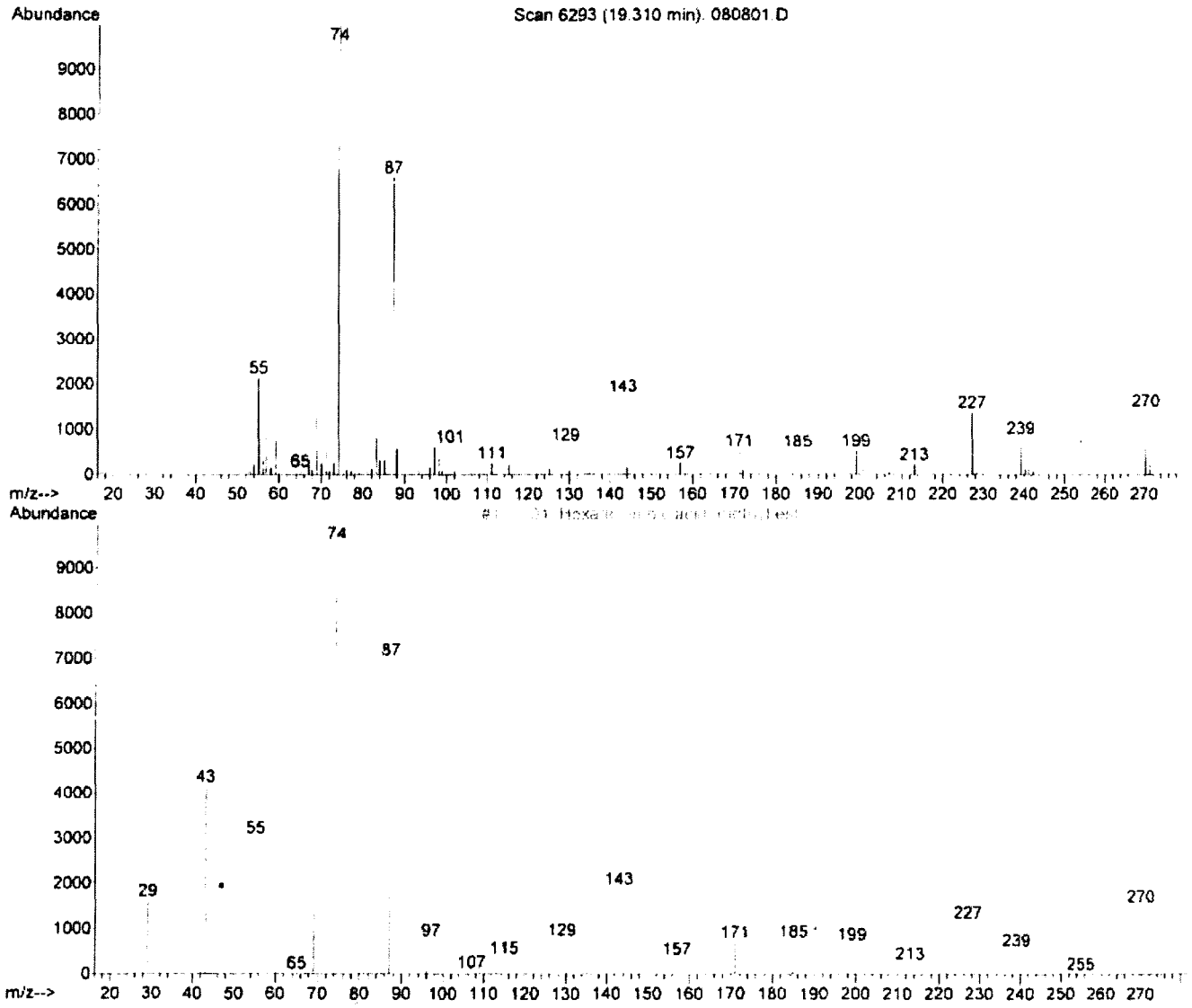


Fig.12.2. GC-MS spectrum of hexadecanoic acid methyl ester

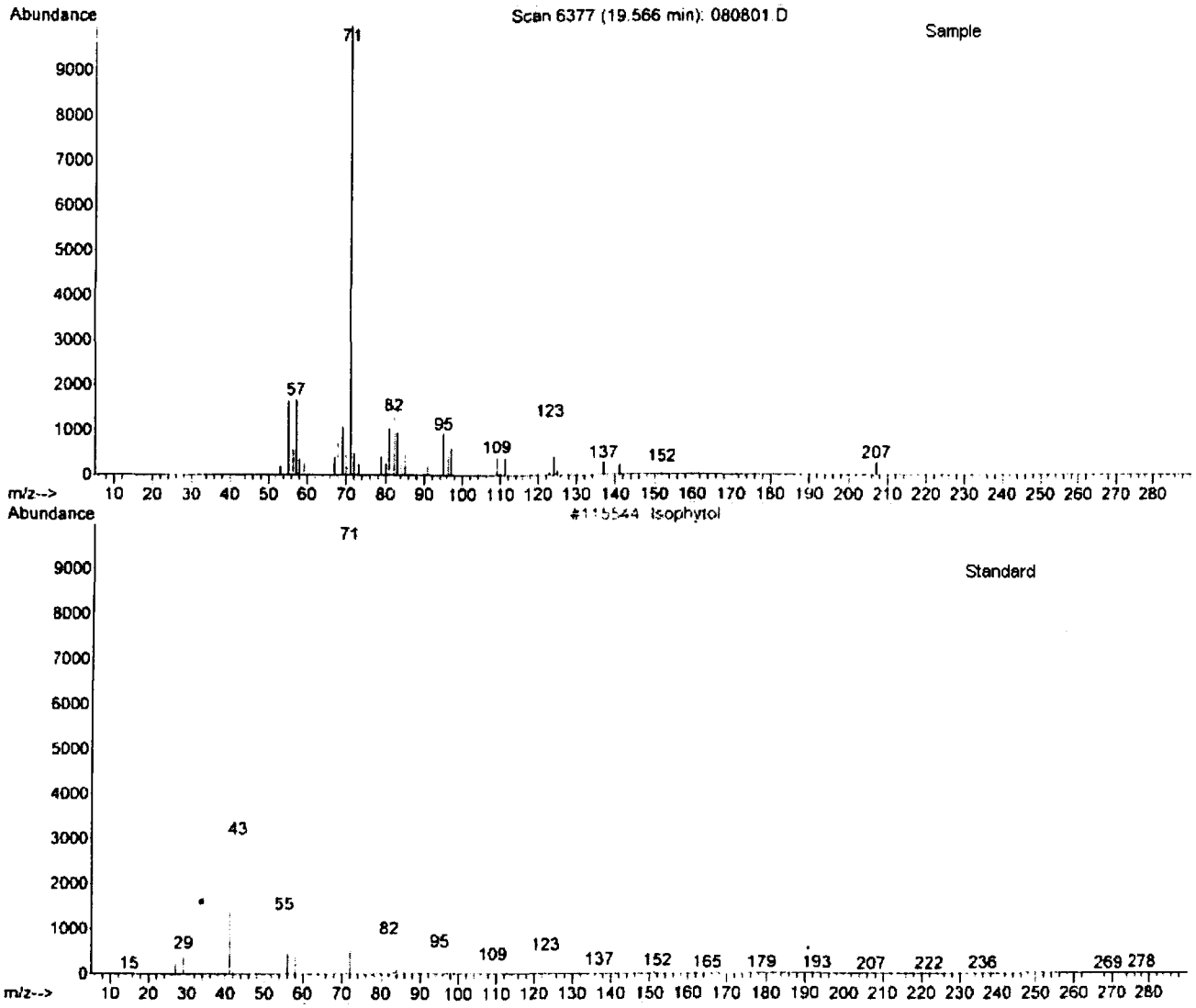


Fig.12.3. GC-MS spectrum of Isophytol

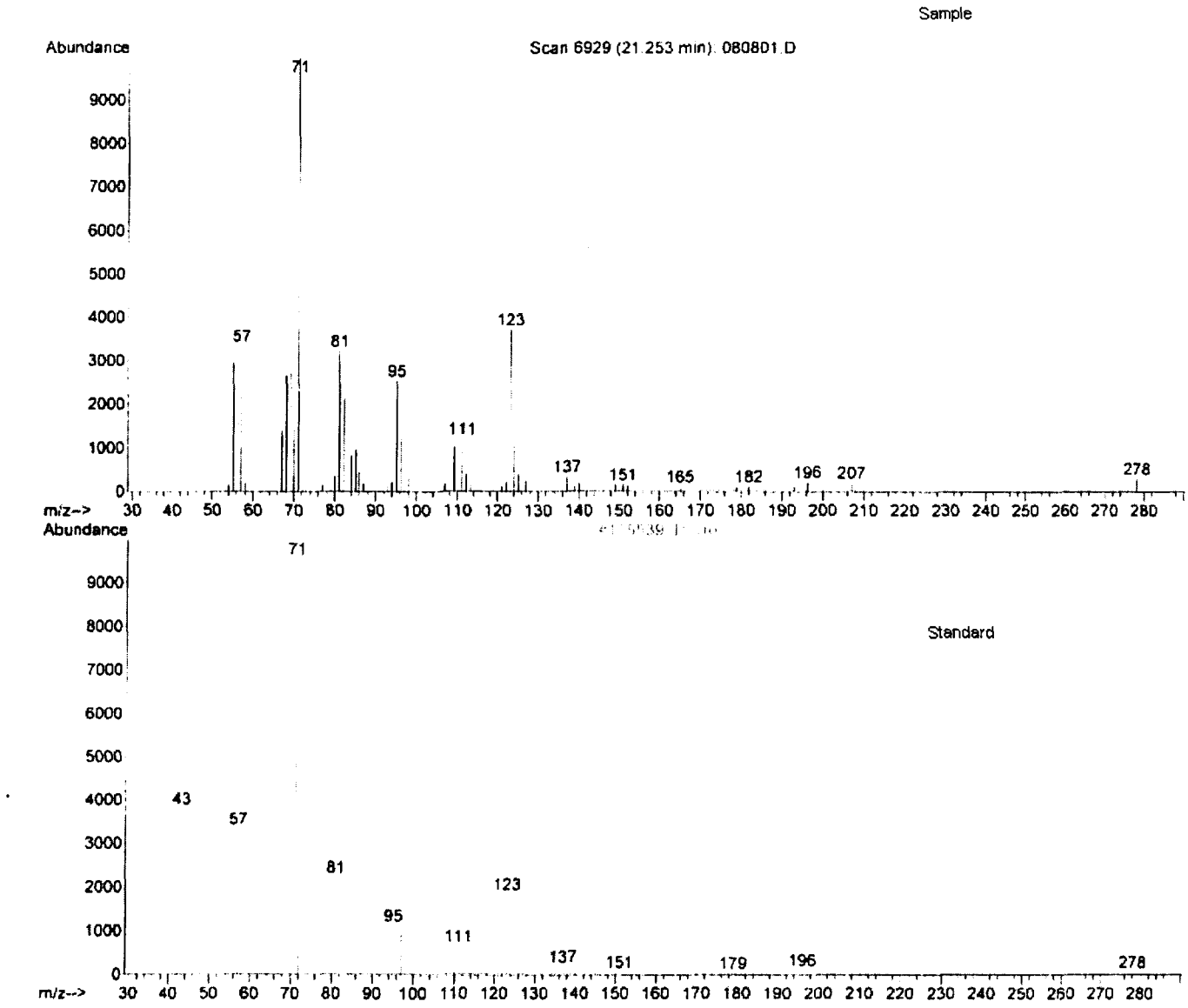


Fig.12.4. GC-MS spectrum of Phytol

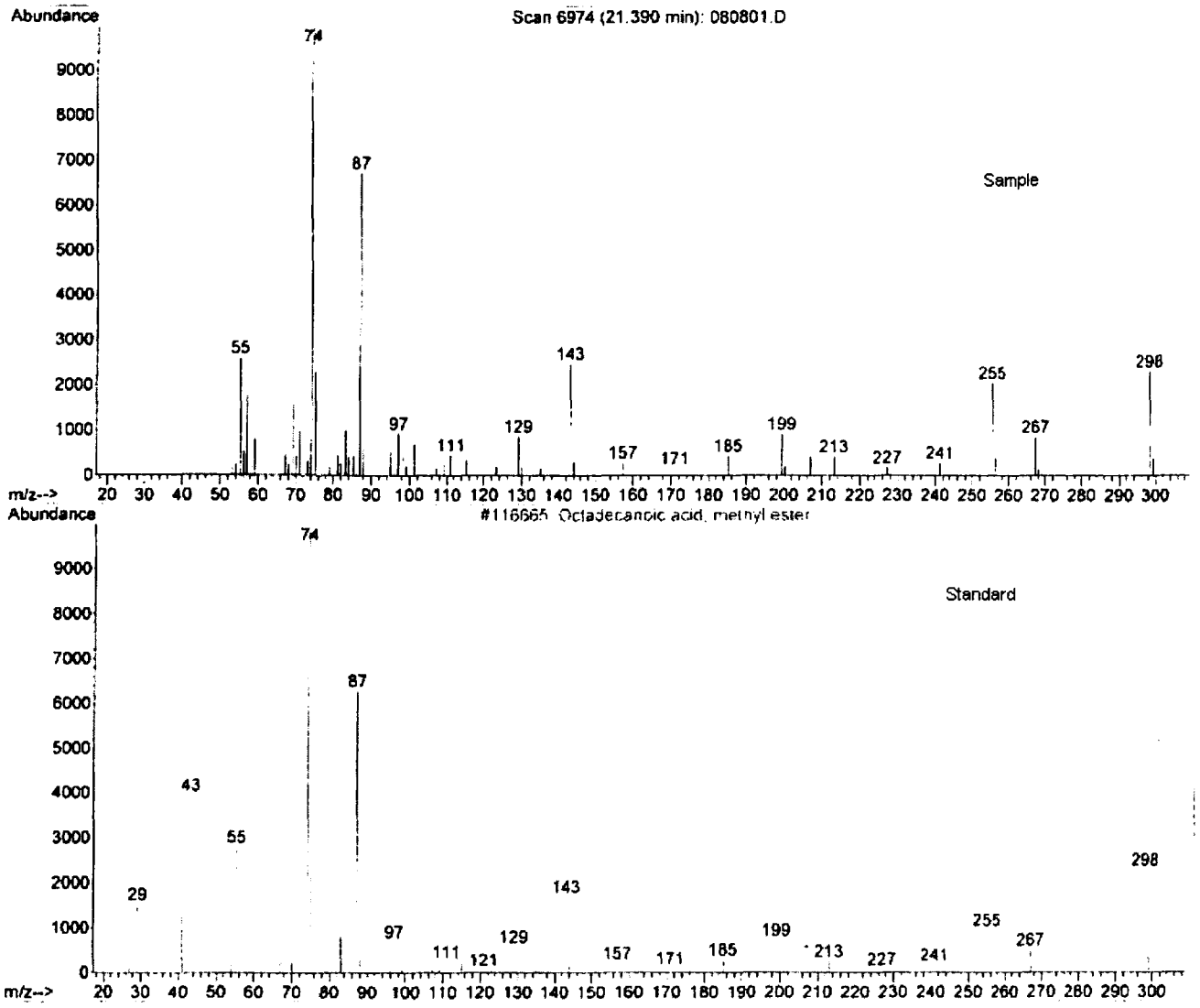


Fig.12.5. GC-MS spectrum of Octa decanoic acid methylester

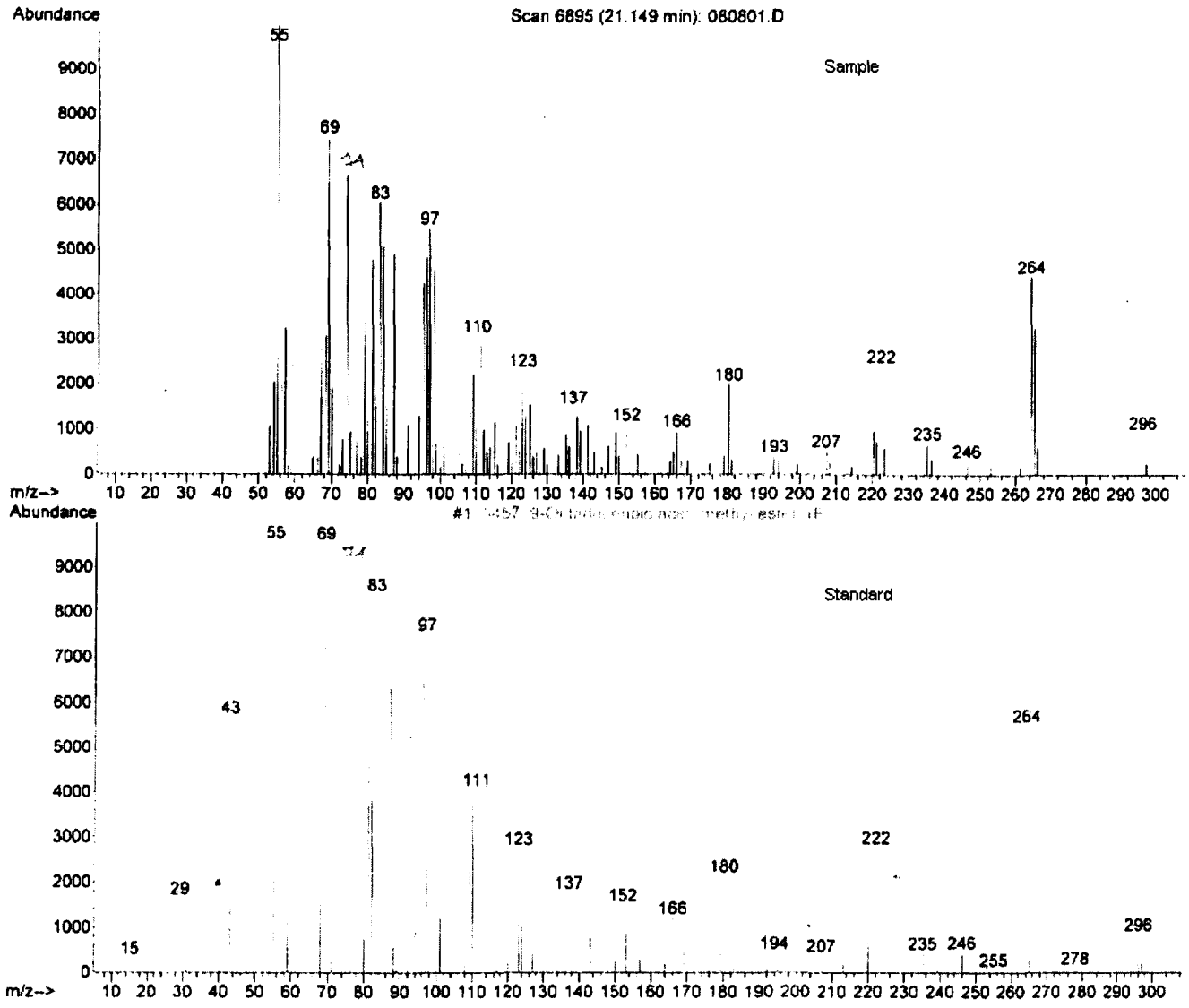


Fig.12.6. GC-MS spectrum of 9- Octa decanoic acid methylester

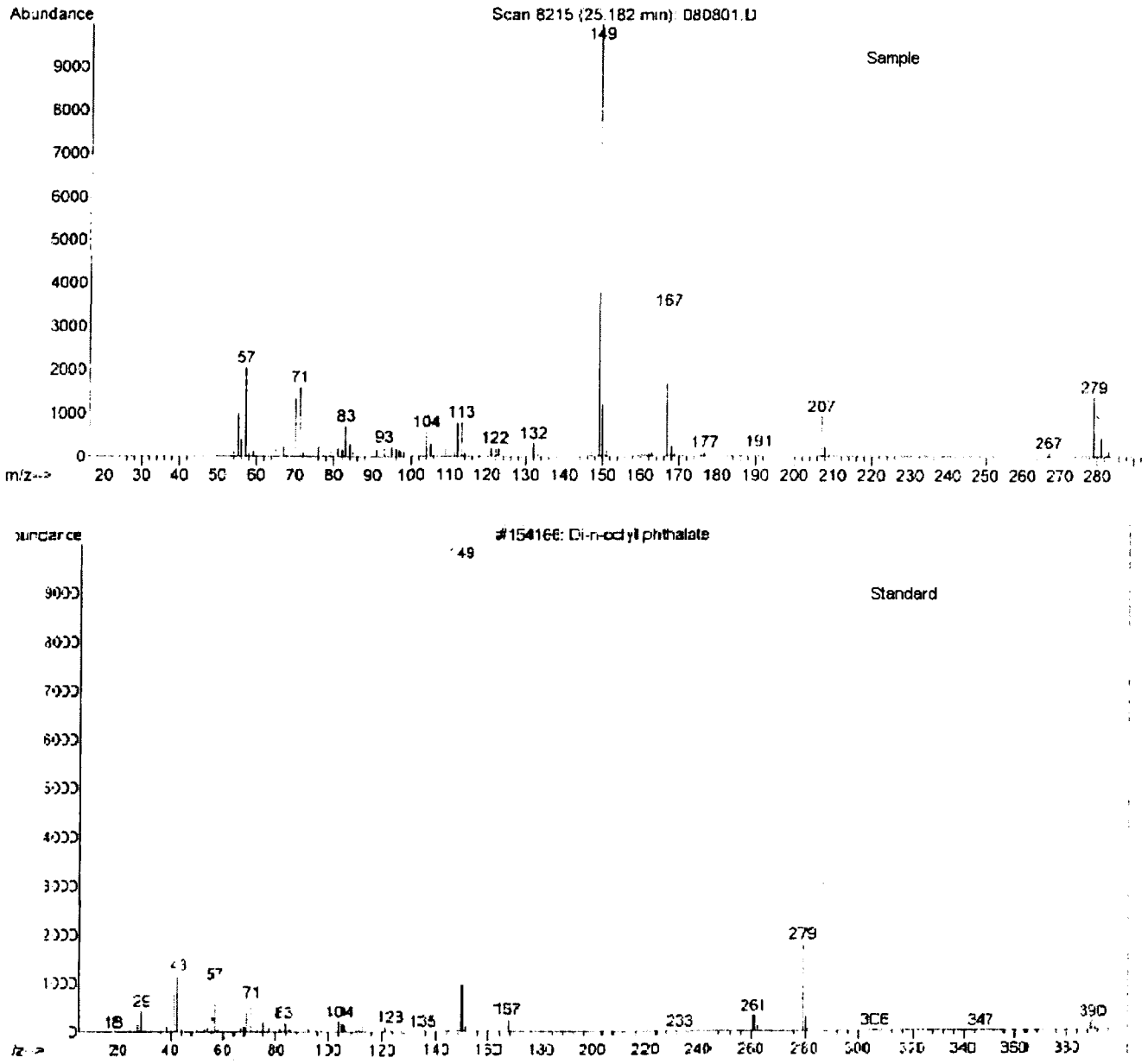
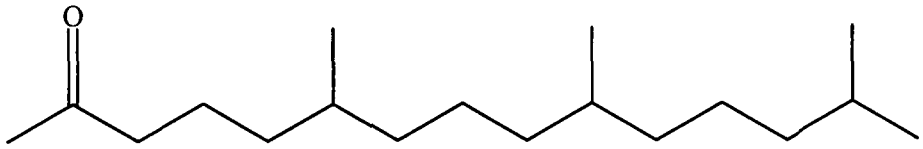
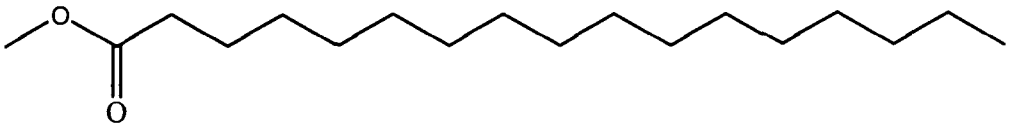


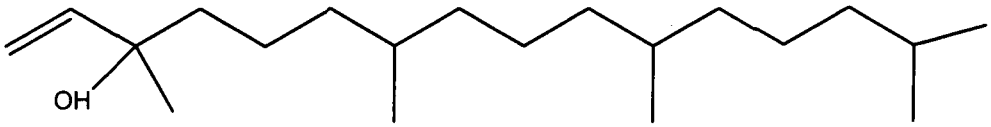
Fig.12.7. GC-MS spectrum of Di-n-octylpthalate



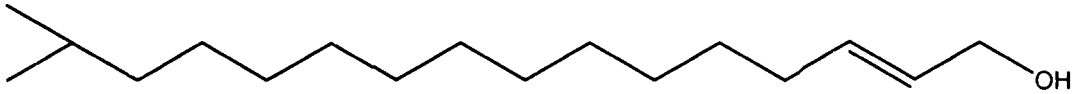
1. 6, 10, 14, trimethyl penta decan-2one



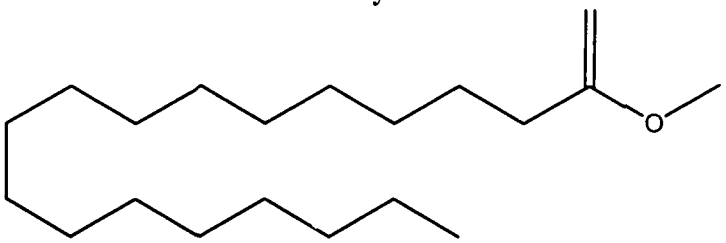
2. Hexadecanoic acid methyl ester



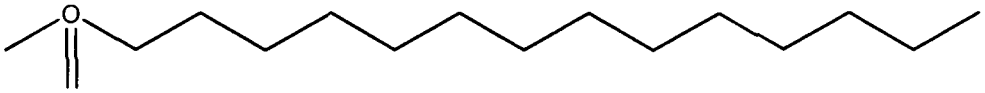
3. Isophytol



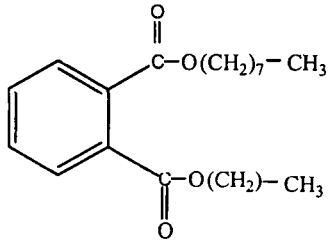
4. Phytol



5. Octa decanoic acid methylester



6. 9-Octa decanoic acid methyester

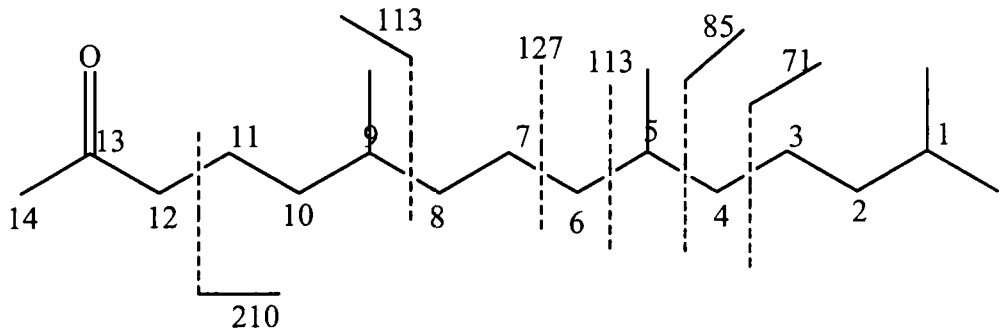


7. Di-n-octylpthalate

Fig. 12.8. Lipids isolated from hexane extract of *Phyllanthus wightianus*

Fig. 13. Mass Fragmentation Pattern of 6, 10, 14, trimethyl penta decan – 2 one

($C_{18}H_{36}O$) (M^+= 268)



$M^+ = 268$

$M^+ - H_2O = 250$

$M^+ - CH_3 (CO) CH_2 – H = 210$

$CH_3 – C = CH_2$ (McLafferty fragment) 58 (100%)

$(CH_3)_2 CH – CH_2 C^+ H_2 = 71$

$(CH_3)_2 CH – CH_2 CH_2 C H^+_2 = 85$

$(CH_3)_2 CH (CH_2)_3 C H^+ - CH_3 = 113$

$(CH_3)_2 CH (CH_2)_3 C H – (CH_3)^+ CH_2 = 127$

$M^+ - (CH_3)_2 CH (CH_2)_3 C H (CH_3) (CH_2)_3 - 113$

Fig. 14. Mass Fragmentation Pattern of Hexadecanoic acid methyl ester

($C_{17}H_{34}O_2$) (M^+= 270)

$M^+ = 270$

M^+ - OMe 239

$M^+ - CH_3 (CH_2)_2$ 227

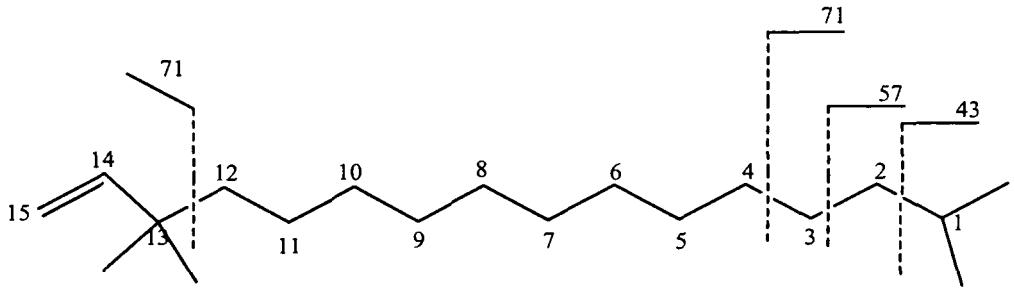
Peaks due to consecutive loss of fourteen

Mass units from m/z 227

213, 199, 185, 171, 157, 143, 129, 101, 87, 73 and 59

$CH_2 = C(OH^+)OCH_3$ (McLafferty frangment) 74 (100%)

Fig. 15. Mass Fragmentation Pattern of Isophytol ($C_{20}H_{40}O$) (M^+= 296)



$M^+ = 296$

$M^+ - H_2O$ 278 $\rightarrow$ $(CH_2)^+_2$ 71

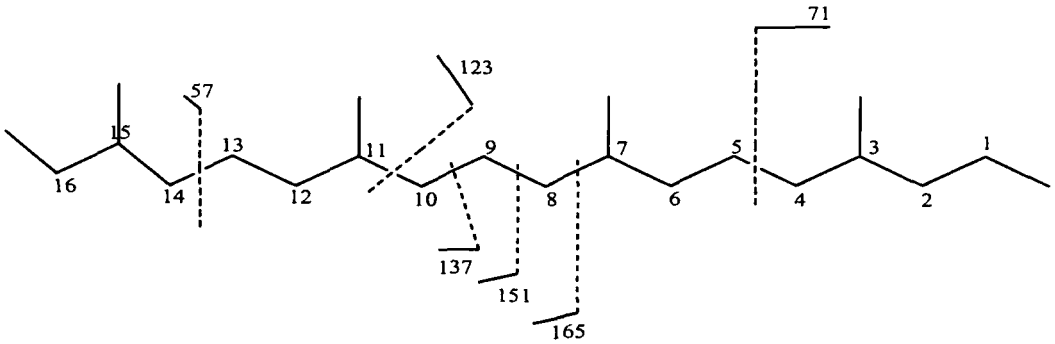
278 - $\rightarrow$ $(CH_2)_2$ 207

$CH_2 = CH - C^+(CH_3)(OH)$ 71 (base peak)

278 - $CH_2 = CH - C(CH_3)(OH)$ 207

278 - $\rightarrow$ $(CH_2)_3$ - CH – $(CH_2)_3$ – CH 95
(CH₃ and CH₃ branches)

Fig. 16. Mass Fragmentation Pattern of Phytol ($C_{20}H_{40}O$) (M^+ = 278)



$[M = H_2O]^+ = 278$

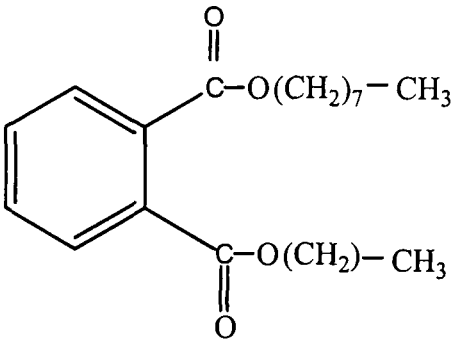
278 - $\rightarrow$ $(CH_2)_2 = 207$

$[M - H_2O]$ m/z = 278

$[M - (CH_3)_2 - CH_2 - CH_2] = 207$

Peaks at m/z 57, 71, 123, 137, 151, 165 are also found

Fig. 17. Mass Fragmentation Pattern of Di-n-octylpthalate ($C_{24}H_{38}O_4$) (M^+ = 390.56)

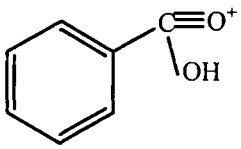


Molecular formula $C_{24}H_{38}O_4$; Molecular weight = 390

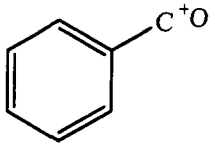
$CH3\ (CH2)_4^+$ = m/z 71

$CH3\ (CH2)_3$ = m/z 57

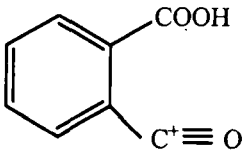
$CH3\ (CH2)^+$ = m/z 113



m/z 122



m/z 104



m/z 149

McLafferty fragment

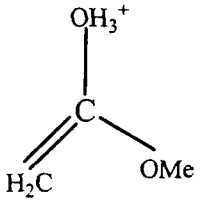


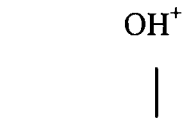
Fig. 18. Mass Fragmentation Pattern of Octadecanoic acid methyl ester (Stearic acid) ($C_{19}H_{38}O_2$) ($M^+ = 298.50$)

$M^+ = 298$

$M^+ - O\,CH_3$ 267

$M^+ - CH_3\,(CH_2)_2$ 255

Peaks due to successive loss of methylene groups from m/z 255; 241, 227, 199, 185, 171, 157, 143, 129, 101, 87, 73, and 59

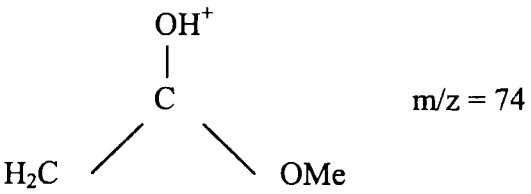


$CH_2 = C - OCH_3$ (McLafferty frangment) 74 (100%)

Fig. 19. Mass Fragmentation Pattern of 9 – Octa decenoic acid methyl ester (Olic acid) ($C_{19}H_{36}O_2$) ($M^+ = 296.40$)

$CH_3\,(CH_2)$, $CH = CH\,(CH_2)\,COOMe$

$C_{19}\,H_{36}\,O_2\,M^+ = 296$



m/z = 74

Mc Lafferty Fragment
$[296 -) 4]^+ = 222$

$[222 - CH_3]^+ = 207$

$[222 - CH_3 - CH_2]^+ = 193$

$M^+ - OMe - H = 262$

$M^+ - (CH_2)_6\,COOMe - H = 152$

$C^+H\,(CH_2)_7 = 97$

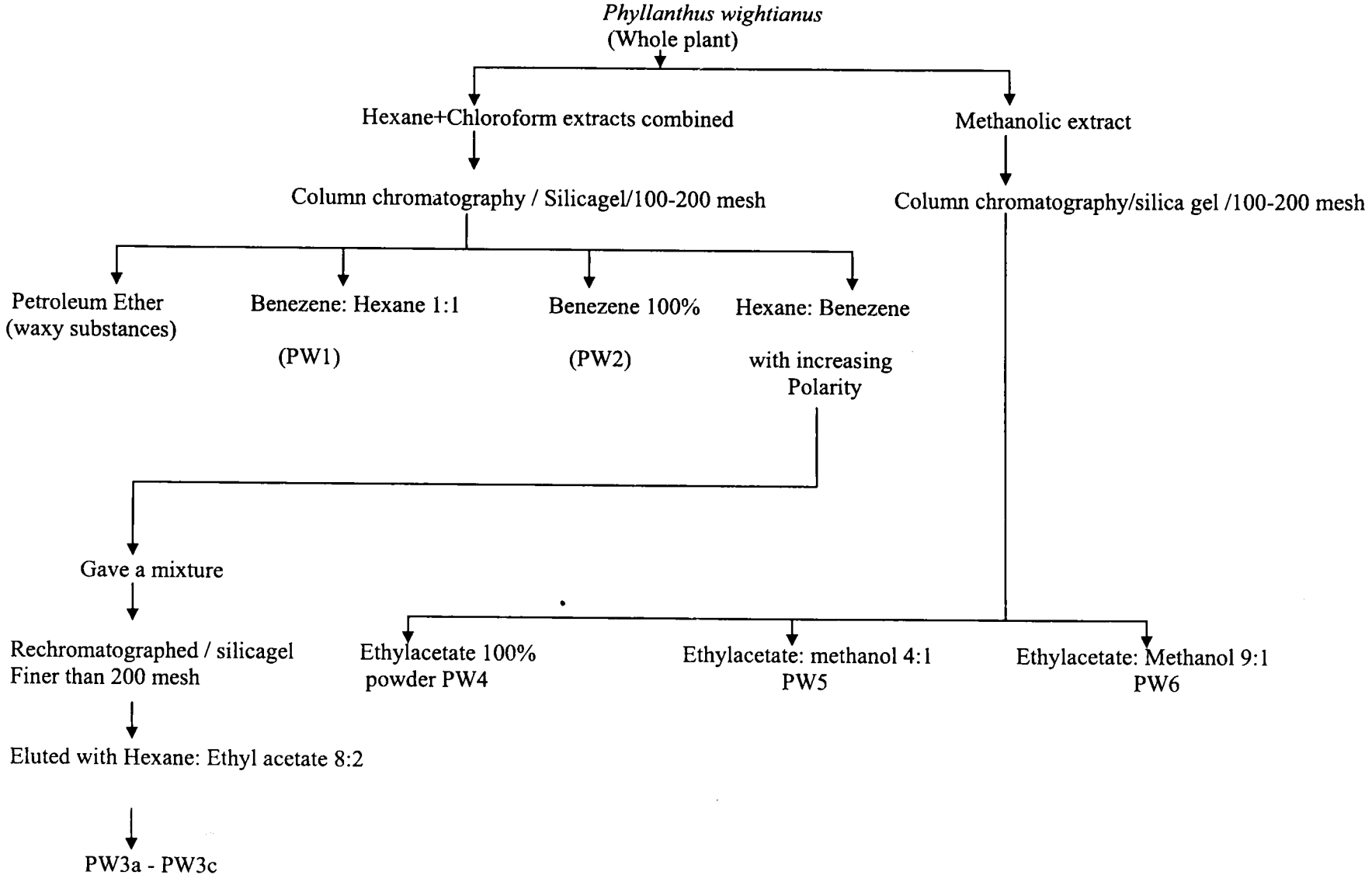


Fig. 20. Separation and Isolation of Compounds from *Phyllanthus wightianus*

4.1.8.2 Isolation and Characterization of PW2 (Lupeol)

Further elution of the column with benzene (100%) gave a colorless needle which was crystallized from acetone (18 mg, m.p. 213 – 215°C). It answered for Noller's test for triterpenoid and gave a single spot on TLC with benzene: ethyl acetate (19: 1) as the developing system (Rf = 0.6). IR: $\nu_{Max}^{Kbr} cm^{-1}$ 3360 (-OH), 2945, 2856, 1637 (> = CH_2), 1454, 1388 (- C Me), 1043 (- OH) , 879 (> = CH_2). ^{1}H NMR (δ, $CDCl_3$, 400 MHz), 0.76 (3H, S, H-24) , 0.79 (3H, S, H-28) , 0.83 (3H, S, H-25), 0.94 (3H, S, H-27) , 0.97 (3H, S, H-26), 1.68 (3H, S, H-30), 3.20 (1H, m, H-3), 2.40 (1H, m, H-19), 4.56, 4.68 (1H each, br S, H-29). ^{13}C NMR (δ, $CDCl_3$, 100 MHz) 38.84 (C-1), 27.97 (C-2), 78.99 (C-3) , 37.26 (C-4), 55.28 (C-5), 18.30 (C-6), 34.27 (C-7), 40.81 (C-8), 50.42 (C-9), 38.04 (C-10), 20.91 (C-11), 25.12 (C-12), 38.03 (C-13), 42.80 (C-14), 27.43 (C-15), 38.69 (C-16), 42.98 (C-17), 48.28 (C-18), 47.90 (C-19), 150.94 (C-20), 26.69 (C-21), 39.99 (C-22), 29.84 (C-23), 15.36 (C-24), 16.10 (C-25), 15.96 (C-26), 14.53 (C-27), 17.99 (C-28), 109.32 (C-29), and 19.30 (C-30).

4.1.8.3 Isolation and Characterization of PW3a - PW3c (Sterol Mixture)

The gas chromatogram of the sterol mixture showed the presence of three compounds with retention times of 13.75, 14.78 and 17.39 min with relative amounts of 18.09, 18.63 and 61.09%, respectively (Figs. 21, 21.1-21.3). Their mass spectra showed that they are structurally different sterols with a molecular ion peak at 400 (*m*/z), 385, 382, 367, 357, 315, 301, 273, 255, 231 and 213 (PW3a); 412 (*m*/z), 394, 379, 369, 273, 255 and 231 (PW3b) and 414 (*m*/z) 396, 381, 371, 329, 315, 329, 303, 273, 255, 231 and 213 (PW3c) (Figs. 22-24). IR: ν_{Max}^{Kbr} cm^{-1} 3431 (hydroxyl), 2960, 2865, 1638 (tri- substituted double bond) 1464, 1381, 1240, 1063 (hydroxide), 1022, 970 (trans di- substituted bond), 959, 838 (tri-substituted double bond) and

File :D:\200206\200212.D
Operator : SGS
Acquired : 16:54 using AcqMethod PEST2.M
Instrument : Instrument #1
Sample Name: 6110003897
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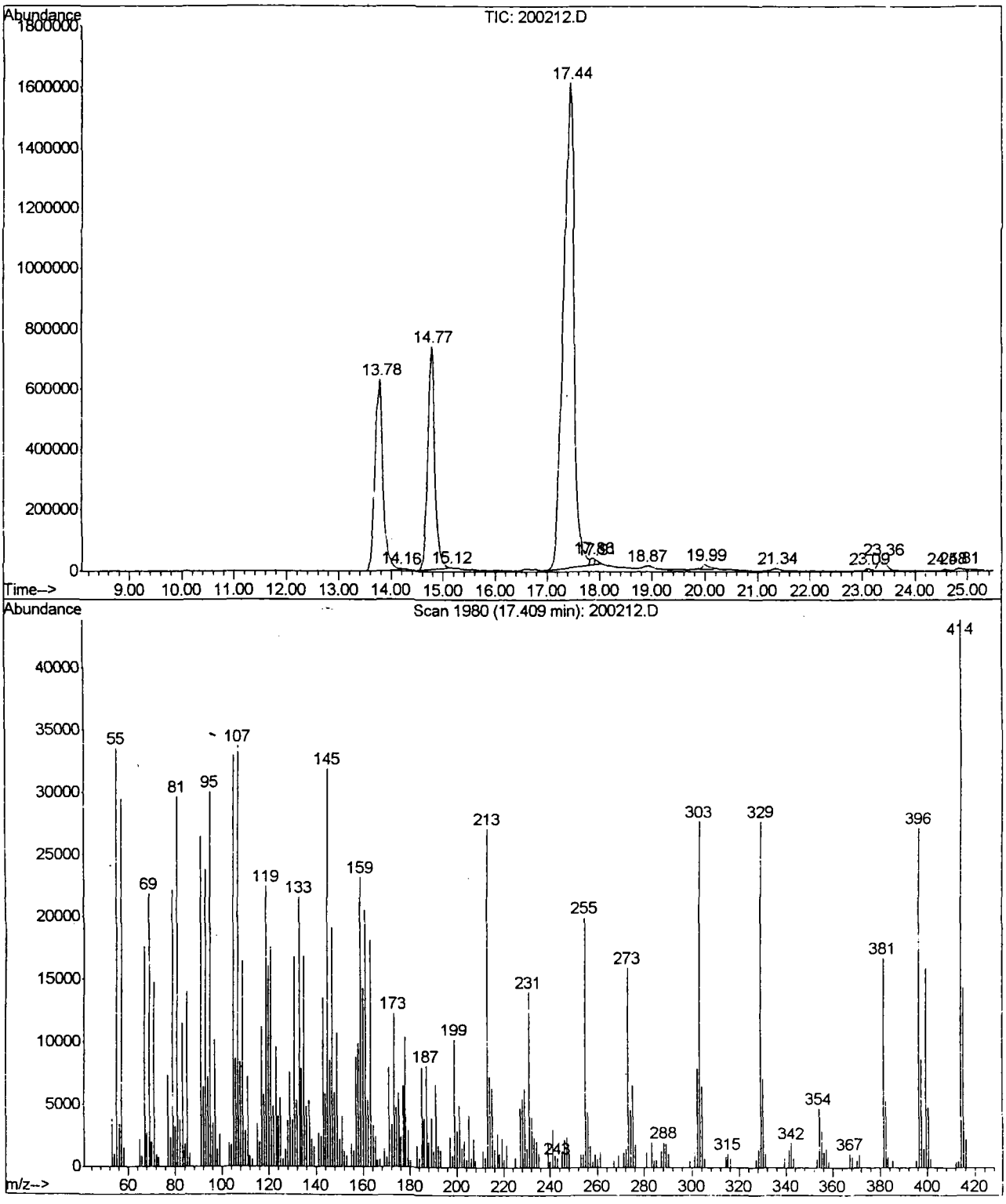


Fig.21. GC-MS Spectrum of Sterol mixture

Library Searched : C:\Database\NIST02.L
Quality : 99
ID : Campesterol

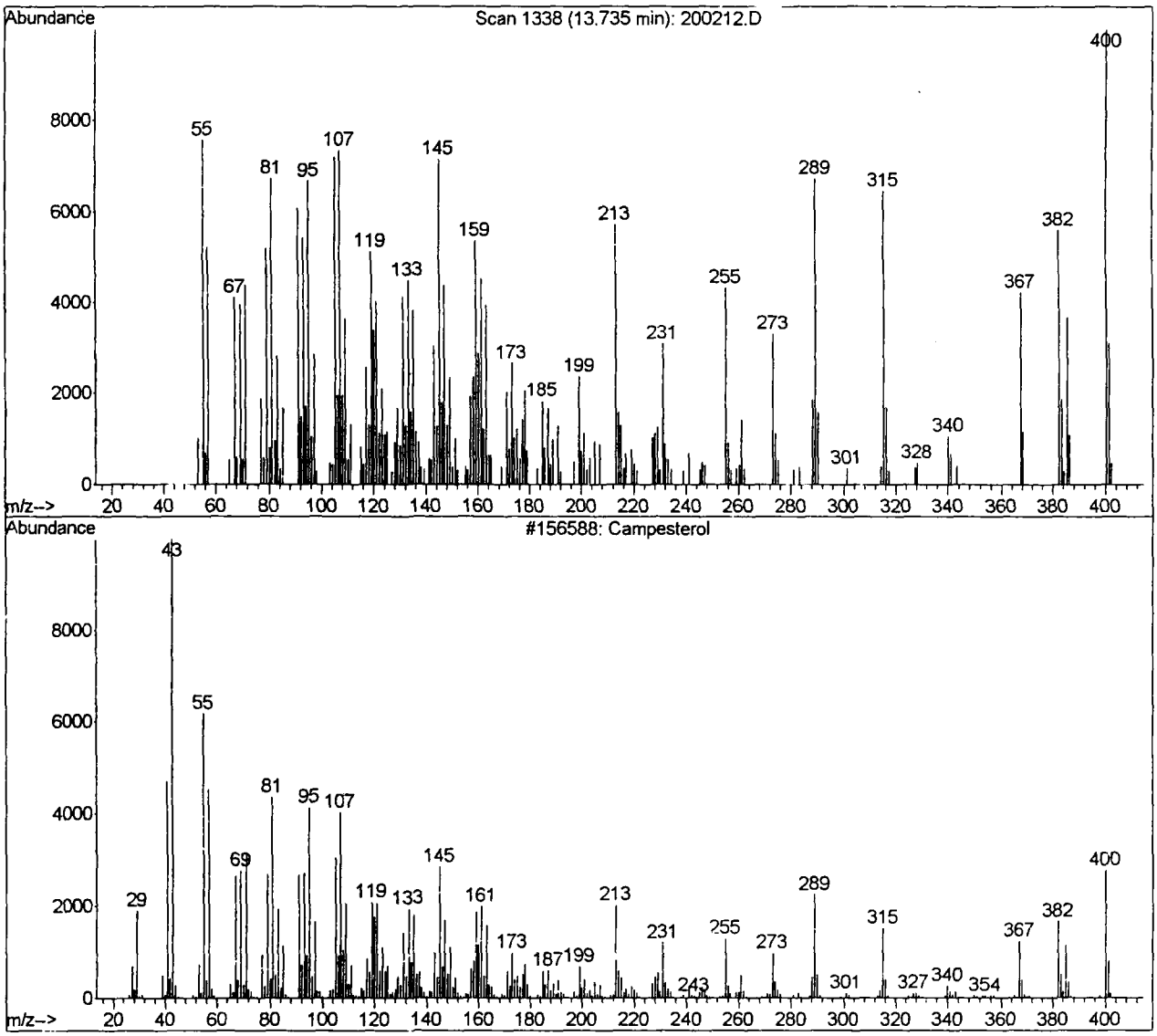


Fig.21.1. GC-MS Spectrum of Campesterol

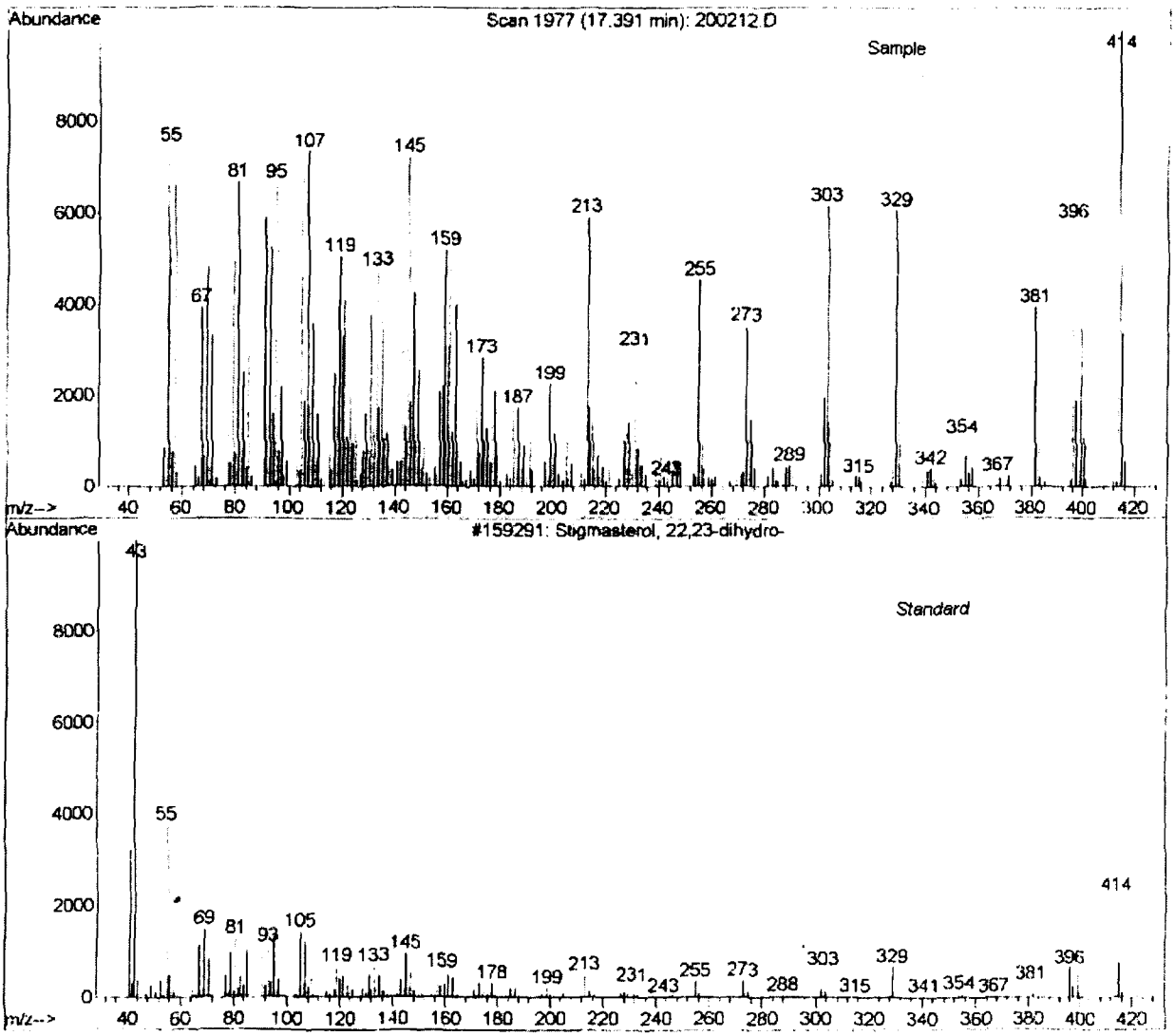


Fig.21.2. GC-MS spectrum of stigmasterol

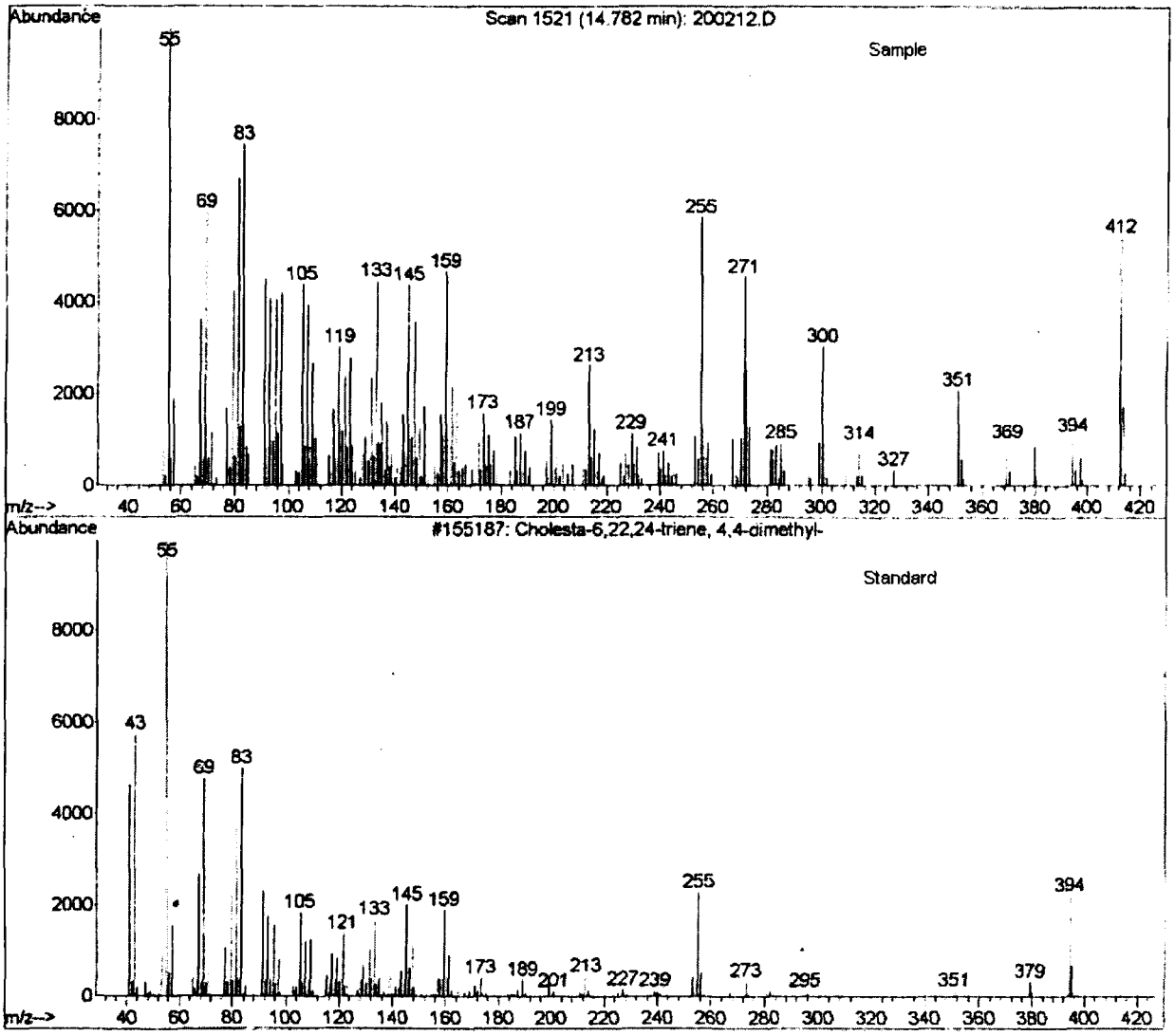
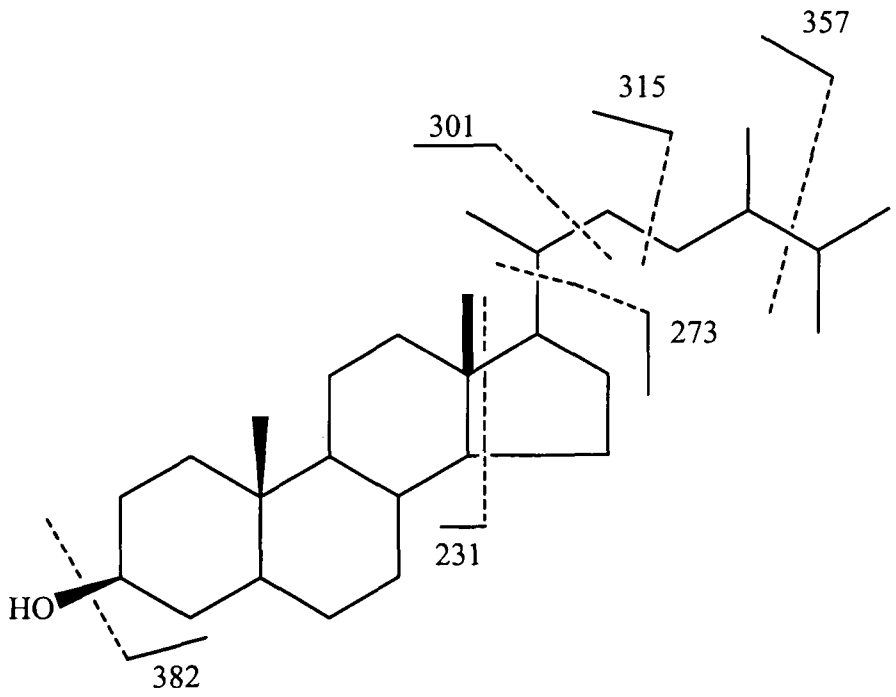


Fig.21.3. GC-MS spectrum of β-sitosterol

Fig. 22. Mass Fragmentation Pattern of Campesterol ($C_{28}H_{48}O$) ($M^+ = 400$)



$M^+ = 400$

$M^+ - CH_3 = 385$

$M^+ - H_2O = 382$

$M^+ - H_2O - CH_3 = 367$

M^+ - Side chain = 273

M^+ Side chain - H_2O = 255

M^+ Side chain – ring D = 231

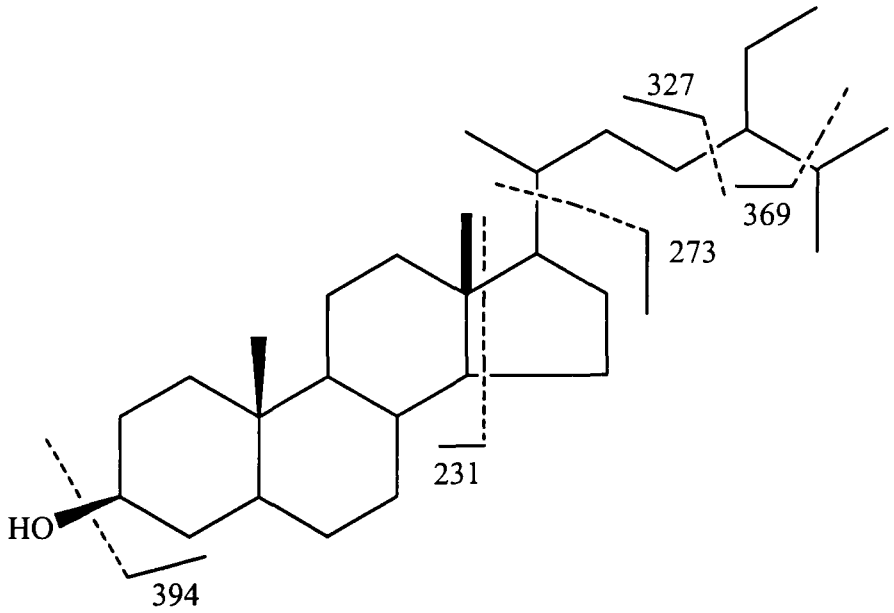
231 - H_2O = 213

M^+ - Cleave C22 – C23 bond = 315

M^+ - Cleave C20 – C22 bond = 301

M^+ - Cleave C24 – C25 bond = 357

Fig. 23. Mass Fragmentation Pattern of Stigmasterol ($C_{29}H_{48}O$) ($M^+ = 412$)



$M^+ = 412$

$M^+ - H_2O = 394$

$M^+ - H_2O - CH_3 = 379$

M^+ - Side chain = 273

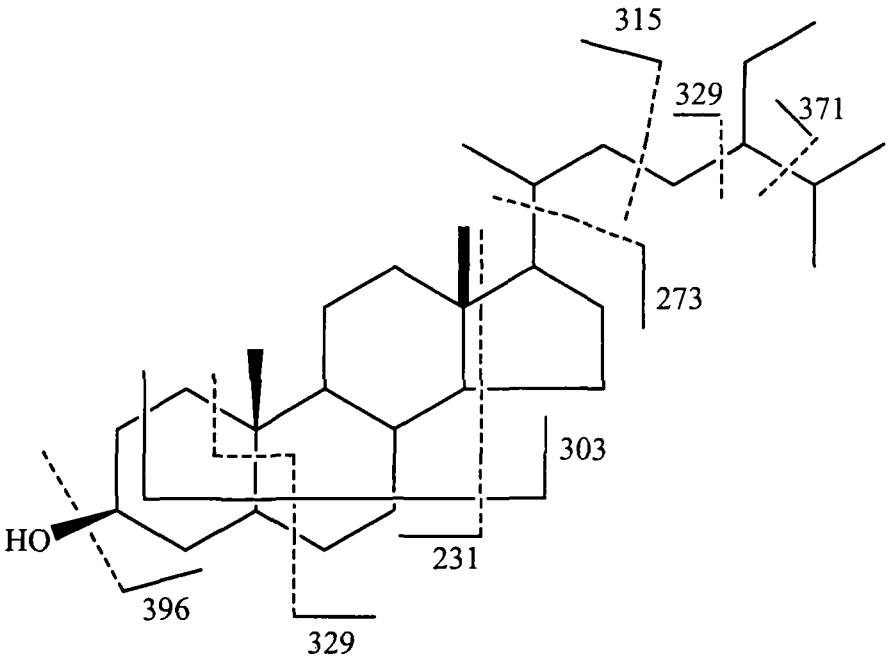
M^+ Side chain - H_2O = 255

M^+ Side isopropyl = 369

M^+ - Cleave C23 – C24 bond = 327

M^+ - Side chain – ring D = 231

Fig. 24. Mass Fragmentation Pattern of β - Sitosterol ($C_{29}H_{50}O$) ($M^+ = 414$)



$M^+ = 414$

$M^+ - H_2O = 396$

$M^+ - H_2O - CH_3 = 381$

M^+ - Side chain = 273

M^+ Side chain - H_2O = 255

M^+ Side chain – ring D = 231

231 - H_2O = 213

M^+ - Isoprophyl = 371

M^+ - Cleavage C23 – C24 bond = 329

M^+ - Cleavage C23 – C24 bond = 315

M^+ - ring A = 329

M^+ - ring A and partial ring B = 303

m/z 303 is more intense that m/z 300 charteristic of β- sitosterol

801. ^{1}H NMR (δ, $CDCl_3$, 400 MHz), 0.68-1.25 (sterol methyls), 0.84 (d, J = 6.4 MHz), 0.91 (d, J = 6.3 MHz), 5.01 (dd, J = 1.5 and 8.7 MHz) H-23 of stigmasterol, 5.15 (dd, J = 5.4 and 8.06 MHz) H- 22 of stigmasterol 5.35 (brd, H - 6), 3 .52 (m, H-3).

4.1.9 Isolation of Compounds from Methanol Extract

Methanol extract was chromatographed over silica gel (100-200 mesh) built with chloroform and eluted with solvents of increasing polarity such as chloroform, ethyl acetate and methanol (Fig. 20).

4.1.9.1 Isolation and Characterization of PW4 (Gallic Acid)

Elution with ethyl acetate (100%) yielded a white amorphous powder (21 mg, m.p. 250°C). It gave a single spot on TLC with ethyl acetate as the developing system (Rf = 0.85). On spraying with alcoholic ferric chloride, the spot turned into blue and answered for phenol. IR: ν_{Max}^{Kbr} cm^{-1} 3364, 3288, 1713, 1617, 1541, 1470, 1339, 1245, 1202, 1054, 1027, 866, 790, 763, 731, 701. ^{1}H NMR (δ, $CDCl_3$, 400 MHz) 6.92 (2H, s). ^{13}C NMR (δ, $CDCl_3$, 100 MHz) 108.88, 145.54, 138.13 and 167.63.

4.1.9.2 Isolation and characterization of PW5 (Ellagic Acid)

Further elution of the column with ethyl acetate: methanol 4:1 yielded a colorless crystalline solid on crystallization from acetone (19 mg, m.p. 360°C). It gave a positive ferric reaction (phenol) on adding alcoholic ferric chloride by producing bluish green color. IR: ν_{Max}^{Kbr} cm^{-1} 3143 (hydroxyl), 2863, 1720 (β - unsaturated, δ-lactone), 1610, 1509, 1457, 1265, 1118, 806 (aromatic).

4.1.9.3 Isolation and Characterization of PW6 (Bergenin)

Methanol extract elute of ethyl acetate - methanol 1:1 yielded acetate colourless prisms (57 mg, m.p. 140-141°C). Molecular formula: $C_{14}H_{18}O_{10}$; UV

λ_{Max}^{MeOH} 275, 310 nm; IR: ν_{Max}^{Kbr} cm^{-1} 3391 (hydroxyl), 2949, 2895, 1701 (coumarin lactone carboxyl), 1612, 1528, 1464, 1374, 1335, 1294, 1234, 1181, 1128, 1092, 1071, 1044, 1012, 965, 906, 860, 817, 766, 723 (aromatic); EIMS m/z: 679, 629, 610, 579, 525, 475, 423, 392, 383, 351, 329, 279, 233, 208, 177, 131. X-ray crystallography analysis of PW6 belonged to orthorhombic system, P2 (1) 2 (1) 2 (1) with a = 7.4907 (7) A alpha = 90 deg., b = 13.9314(13). A beta = 90 deg., c = 14.2744 (13) A gamma = 90 deg., wavelength 0.71073 A, volume 1489.6(2) A^3, Z, calculated density 4, 1.544 Mg/m^3.

4.2. ANTIMICROBIAL STUDIES

4.2.1 Antibacterial Activity

The antibacterial activity of the successive solvent extracts of *P. wightianus* such as hexane, chloroform and methanol against different disease causing gram-positive and gram-negative bacterial strains is given (Table 9 & 10; Figs. 25-27).

The test extracts are potent against almost all the gram-negative bacterial strains than the gram-positive bacterial strains at the tested concentrations. Methanol extract produced better inhibition followed by hexane extract whereas chloroform extract was moderate in action.

4.2.1.1 Antibacterial Activity of Various Solvent Extracts

1. *Bacillus cereus*

Hexane extract produced maximum inhibition of 18 mm at 100 mg/ml, minimum as 14 mm at 25 mg/ml and inactive at 12.5 mg/ml concentration. Chloroform extract did not show any activity to the tested concentrations. Methanol extract exhibited maximum activity as 28 mm at 100 mg/ml and its lower

Table 9. Antibacterial Activity of Various Solvent Extracts of *Phyllanthus wightianus*

Test Microorganisms	Hexane extract (mg/ml)				Chloroform extract (mg/ml)				Methanol extract (mg/ml)				Standard
	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5	
Bacillus cereus	18	16	14	-	-	-	-	-	28	16	-	-	33 (T)
Bacillus subtilis	23	10	-	-	-	-	-	-	28	25	13	10	30 (A)
Staphylococcus aureus	25	22	18	9	20	15	-	-	17	16	15	13	45 (M)
Staphylococcus epidermidis	18	16	14	-	16	14	-	-	20	17	13	10	40 (T)
Aeromonas hydrophila	20	18	15	9	16	14	-	-	25	20	13	-	20 (Tr)
Enterobacter aerogenes	26	20	15	10	16	14	12	-	20	19	16	15	22 (Tr)
Escherichia coli	30	26	18	10	21	18	-	-	33	30	13	10	30 (K)
Klebsiella pneumoniae	24	15	-	-	-	-	-	-	18	17	16	15	30 (K)
Pseudomonas aeruginosa	25	16	-	-	18	16	12	-	31	26	25	23	25 (E)
Proteus mirabilis	23	20	16	-	25	22	10	-	29	25	15	13	30 (T)
Proteus vulgaris	25	22	10	8	23	12	-	-	30	25	15	13	20 (K)
Salmonella paratyphi	17	13	-	-	-	-	-	-	31	26	24	20	35 (G)
Salmonella typhi	16	14	-	-	-	-	-	-	22	19	10	6	20 (Na)
Vibrio cholerae	24	20	-	-	-	-	-	-	30	27	15	13	31 (Tr)
Vibrio parahaemolyticus	-	-	-	-	-	-	-	-	27	24	23	9	14 (K)
Vibrio vulnificus	-	-	-	-	-	-	-	-	23	20	13	10	16 (K)

* Values (Mean of three replicates) are diameter of zone of inhibition in mm;

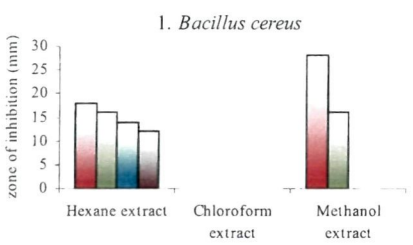
- Absence of inhibition; Ampicillin (A); Erythromycin (E); Kanamycin (K); Methicillin (M); Nalidixic acid (Na); Rifampicin (R); Trimethoprin (Tr); Tetracycline (T); Gentamicin (G).

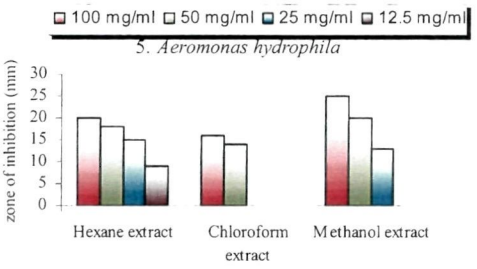
Table 10. MIC and MBC of Various Solvent Extracts of *Phyllanthus wightianus*

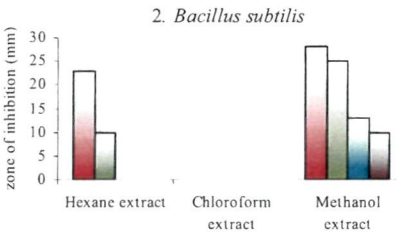
Test Microorganisms	Hexane extract (mg/ml)					Chloroform extract (mg/ml)					Methanol extract (mg/ml)				
	20	10	5	2.5	1.25	20	10	5	2.5	1.25	20	10	5	2.5	1.25
Bacillus cereus	**	-	-	-	-	-	-	-	-	-	**	-	-	-	-
Bacillus subtilis	**	-	-	-	-	**	-	-	-	-	+*	**	-	-	-
Staphylococcus aureus	**	**	-	-	-	**	-	-	-	-	**	**	-	-	-
Staphylococcus epidermidis	+*	**	-	-	-	**	-	-	-	-	+*	**	-	-	-
Aeromonas hydrophila	+*	**	-	-	-	**	**	-	-	-	+	+*@	**	-	-
Enterobacter aerogenes	+@	+*	-	-	-	+*@	**	-	-	-	+@	+*	**	-	-
Escherichia coli	+@	+*	**	-	-	+*	**	-	-	-	+	+@	+*	**	-
Klebsiella pneumoniae	+@	+*	-	-	-	-	-	-	-	-	+*	**	-	-	-
Pseudomonas aeruginosa	+*@	**	-	-	-	+*	-	-	-	-	+	+	+	+*@	**
Proteus mirabilis	+*@	-	-	-	-	+*	-	-	-	-	+	+@	+*	-	-
Proteus vulgaris	+*@	-	-	-	-	+*	-	-	-	-	+	+@	+*	-	-
Salmonella paratyphi	**	-	-	-	-	-	-	-	-	-	+@	+*	**	-	-
Salmonella typhi	**	-	-	-	-	-	-	-	-	-	+@	+*	**	-	-
Vibrio cholerae	+*	**	-	-	-	-	-	-	-	-	+@	+*	**	-	-
Vibrio parahaemolyticus	-	-	-	-	-	-	-	-	-	-	+	+@	+*	-	-
Vibrio vulnificus	-	-	-	-	-	-	-	-	-	-	+@	+*	**	-	-

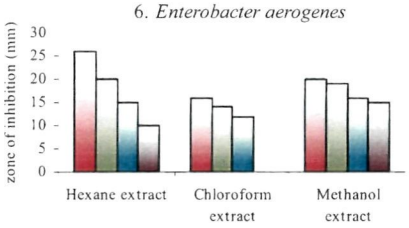
+ complete inhibition; +* MIC; ** Marked inhibition; - Absence of inhibition; @ MBC

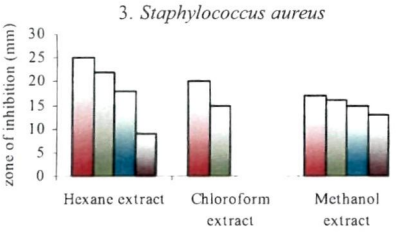
Fig. 25. Antibacterial activity of *Phyllanthus wightianus*

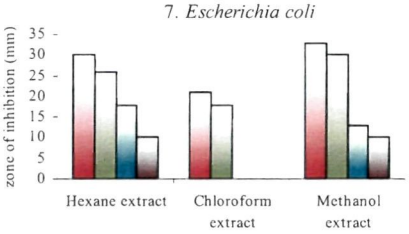


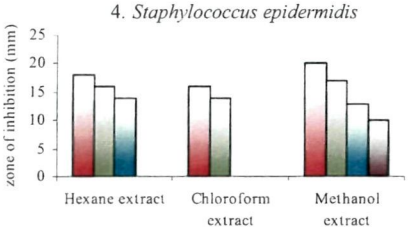


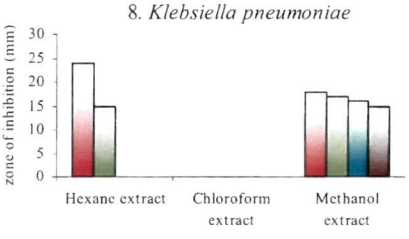


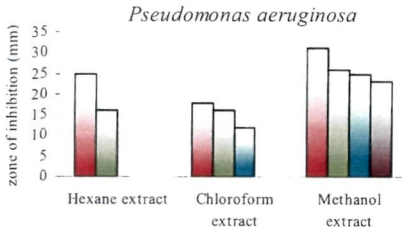


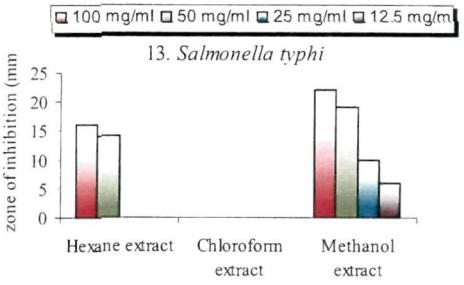


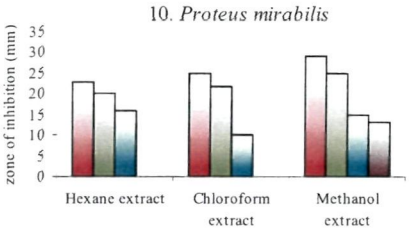


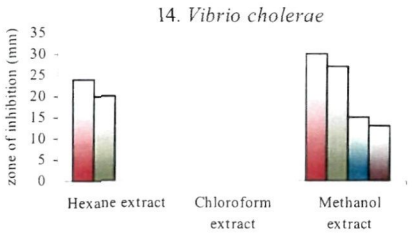


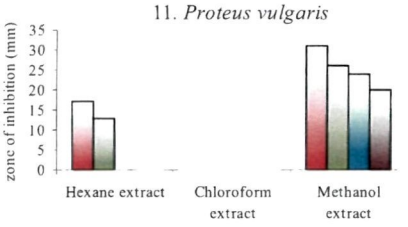


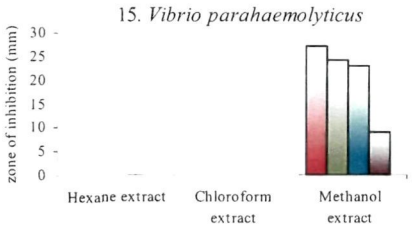

Pseudomonas aeruginosa
zone of inhibition (mm)
35
30
25
20
15
10
5
0
Hexane extract
Chloroform extract
Methanol extract

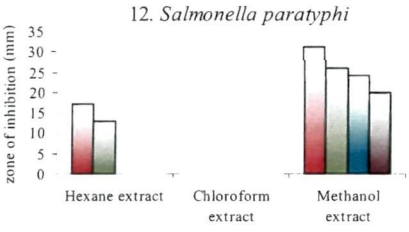

100 mg/ml
50 mg/ml
25 mg/ml
12.5 mg/ml
13. Salmonella typhi
zone of inhibition (mm
25
20
15
10
5
0
Hexane extract
Chloroform extract
Methanol extract


10. Proteus mirabilis
zone of inhibition (mm)
35
30
25
20
15
10
5
0
Hexane extract
Chloroform extract
Methanol extract


14. Vibrio cholerae
zone of inhibition (mm)
35
30
25
20
15
10
5
0
Hexane extract
Chloroform extract
Methanol extract


11. Proteus vulgaris
zone of inhibition (mm)
35
30
25
20
15
10
5
0
Hexane extract
Chloroform extract
Methanol extract


15. Vibrio parahaemolyticus
zone of inhibition (mm)
30
25
20
15
10
5
0
Hexane extract
Chloroform extract
Methanol extract


12. Salmonella paratyphi
zone of inhibition (mm)
35
30
25
20
15
10
5
0
Hexane extract
Chloroform extract
Methanol extract

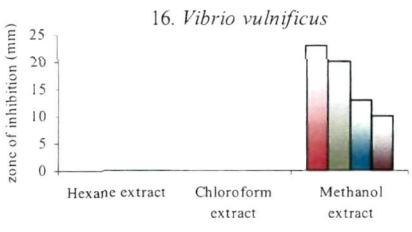
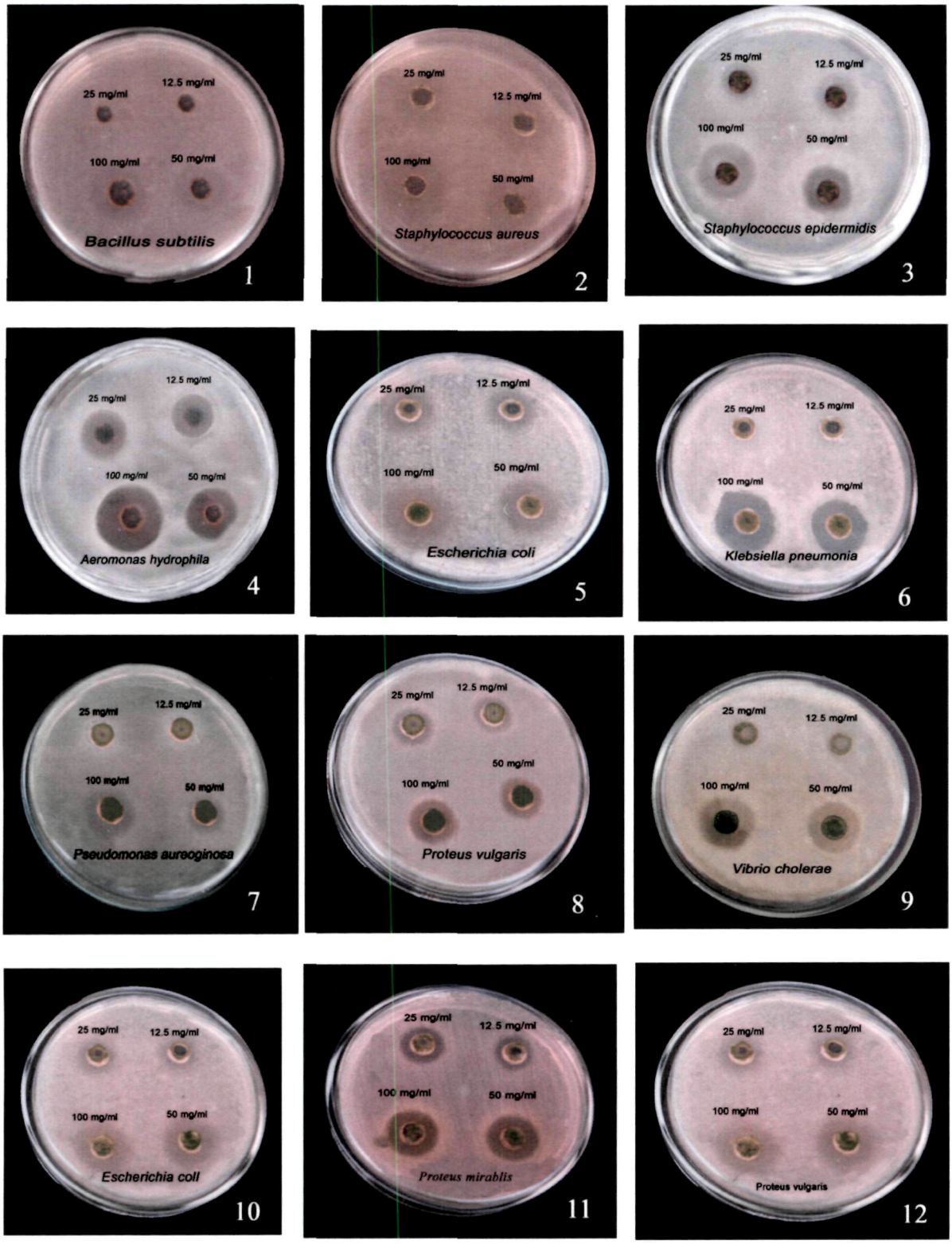

16. Vibrio vulnificus
zone of inhibition (mm)
25
20
15
10
5
0
Hexane extract
Chloroform extract
Methanol extract

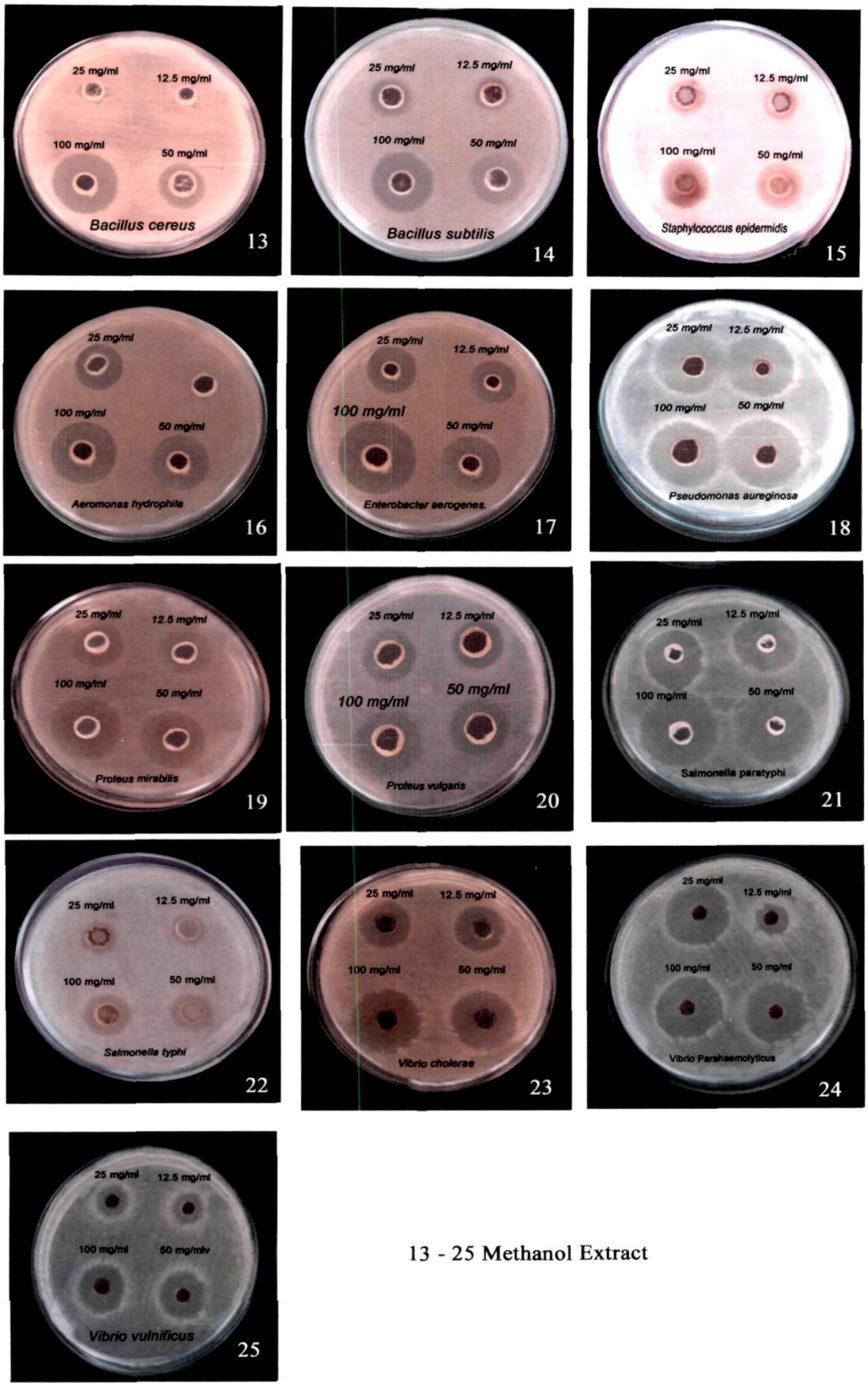
Fig. 26. Antibacterial activity of *Phyllanthus wightianus* extracts



1 - 9 Hexane extract

10 - 12 Chloroform extract

Fig.27. Antibacterial activity of *Phyllanthus wightianus* extracts



13 - 25 Methanol Extract

concentration was inactive. The MIC and MBC were not determined for all the test extracts at the tested concentrations.

2. *Bacillus subtilis*

The maximum inhibition was recorded at 100 mg/ml as 28 mm for methanol extract and 23 mm for hexane extract. Chloroform extract was inactive at all the tested concentrations. The MIC was determined as 20 mg/ml for both methanol and hexane extracts. The MBC was not determined at the tested concentrations.

3. *Staphylococcus aureus*

Hexane extract produced maximum zone of inhibition than methanol extract such as 25 mm at 100 mg/ml and minimum of 9 mm at 12.5 mg/ml. Methanol extract produced 17 mm inhibition at 100 mg/ml as maximum and 13 mm at 12.5 mg/ml as minimum. Though chloroform extract showed maximum activity as 20 mm at 100 mg/ml lower concentrations at 25 mg/ml and 12.5 mg/ml were inactive. The MIC was observed as 20 mg/ml for hexane extract. The MIC & MBC were not determined for the tested concentrations of methanol and chloroform extracts.

4. *Staphylococcus epidermidis*

Methanol extract exhibited maximum activity such as 20 mm at 100 mg/ml and 10 mm at 12.5 mg/ml as minimum. Hexane extract produced 18 mm inhibition at 100 mg/ml and 14 mm at 25 mg/ml. For chloroform extract, the maximum inhibition was 16 mm at 100 mg/ml and its lower concentrations at 25 and 12.5 mg/ml were inactive. The MIC for methanol and hexane extracts was 20 mg/ml. The MIC was not determined for chloroform extract to the tested concentrations. The MBC was not determined at the tested concentrations for all the test extracts.

5. *Aeromonas hydrophila*

Maximum zone of inhibition was observed as 25 mm, 20 mm and 16 mm at 100 mg/ml for methanol, hexane and chloroform extracts respectively. The MIC was determined as 10 mg/ml for methanol extract and 20 mg/ml for chloroform and hexane extracts. The MBC was recorded as 10 mg/ml for methanol extract which was not determined for chloroform and hexane extracts at the tested concentrations.

6. *Enterobacter aerogenes*

The maximum inhibition was observed as 26 mm, 20 mm and 16 mm at 100 mg/ml for hexane, methanol and chloroform extracts respectively. The MIC was recorded as 10 mg/ml for methanol and hexane extracts and 20 mg/ml for chloroform extract. The MBC noticed as 20 mg/ml for all the test extracts.

7. *Escherichia coli*

Maximum inhibition was observed as 33 mm, 30 mm and 21 mm at 100 mg/ml for methanol, hexane and chloroform extracts respectively. The MIC was noticed as 5 mg/ml for methanol extract, 10 mg/ml for hexane extract and 20 mg/ml for chloroform extract. The MBC was determined as 10 mg/ml for methanol extract and 20 mg/ml for hexane extract and it was not determined for chloroform extract at the tested concentrations.

8. *Klebsiella pneumoniae*

Hexane extract produced maximum inhibition as 24 mm at 100 mg/ml and 15 mm at 50 mg/ml. Its lower concentrations at 25 mg/ml and 12.5 mg/ml were inactive. Chloroform extract was inactive at all the tested concentrations. Methanol extract exhibited 18 mm of inhibition at 100 mg/ml and 15 mm at 12.5 mg/ml. The MIC was noticed as 20 mg/ml for both methanol and hexane extracts. The MBC was not determined for all the test extracts at the tested concentrations.

9. *Pseudomonas aureoginosa*

Though hexane extract exhibited maximum inhibition at 100 mg/ml as 25 mm the lower concentrations at 25 and 12.5 mg/ml were inactive. Chloroform extract produced 18 mm at 100 mg/ml and 12 mm inhibition at 25 mg/ml. Methanol extract exhibited potent activity than hexane and chloroform extracts at 100, 50, 25 and 12.5 mg/ml as 31 mm, 26 mm, 25 mm and 23 mm respectively. The MIC and MBC was determined as 2.5 mg/ml for methanol extract and 20 mg/ml for hexane extract. The MIC for chloroform extract was noticed as 20 mg/ml and the MBC was not determined at the tested concentrations for chloroform extract.

10. *Proteus mirabilis*

Methanol extract produced maximum inhibition at 100 mg/ml as 29 mm whereas it was 25 mm for chloroform extract and 23 mm for hexane extract. Minimum zone of inhibition was noticed at the lower concentration of 12.5 mg/ml as 13 mm for methanol extract whereas hexane and chloroform extracts at this concentration were inactive. However, theses two extracts produced 10 mm and 16 mm zone of inhibition at 25 mg/ml. The MIC was noticed as 5 mg/ml for methanol extract and 20 mg/ml for hexane extract and which was not determined for chloroform extract at the tested concentrations.

11. *Proteus vulgaris*

Maximum zone of inhibition at 100 mg/ml was observed as 30 mm for methanol extract, 25 mm for hexane extract and 23 mm for chloroform extract. Inhibitions at 12.5 mg/ml were 13 mm for methanol extract and 8 mm for hexane extract. Nevertheless, chloroform extract was inactive at this concentration and also at 25 mg/ml. The MIC was determined as 5 mg/ml for methanol extract and 20 mg/ml for chloroform and hexane extracts. The MBC was determined as 10 mg/ml

for methanol extract and 20 mg/ml for chloroform extract whereas it was not determined for hexane extract.

12. *Salmonella paratyphi*

Maximum inhibition was produced by methanol extract as 31 mm at 100 mg/ml and 20 mm at 12.5 mg/ml as minimum. Chloroform extract was completely inactive at the tested concentrations. Hexane extract produced 17 mm inhibition at 100 mg/ml but was relatively inactive at the lower concentrations such as 25 and 12.5 mg/ml. The MIC and MBC was determined only for methanol extract as 10 mg/ml and 20 mg/ml respectively.

13. *Salmonella typhi*

Methanol extract produced maximum inhibition at 100 mg/ml as 22 mm and minimum as 6 mm at 12.5 mg/ml. Chloroform extract was completely inactive at the tested concentrations. Hexane extract exhibited 16 mm inhibition at 100 mg/ml, 14 mm at 50 mg/ml and inactive at the lower concentrations of 25 and 12.5 mg/ml. The MIC and MBC was determined only for methanol extract as 10 mg/ml and 20 mg/ml respectively.

14. *Vibrio cholerae*

At 100 mg/ml concentration, methanol extract exhibited 30 mm inhibition and 24 mm inhibition for hexane extract. The former extract produced 13 mm inhibition at the lower concentration of 12.5 mg/ml whereas hexane extract was inactive at both the concentrations of 25 mg/ml and 12.5 mg/ml. Chloroform extract was completely inactive at all the tested concentrations. The MIC was noticed as 10 mg/ml and 20 mg/ml for methanol and hexane extracts respectively and the MBC for methanol extract was 20 mg/ml.

Table 11. Antifungal Activity of Various Extracts of *Phyllanthus wightianus*

Test Microorganisms	Hexane extract (mg/ml)				Chloroform extract (mg/ml)				Methanol extract (mg/ml)				Standard
	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5	
Fungi													
Aspergillus flavus	20	18	16	12	-	-	-	-	29	20	19	13	K (28)
Aspergillus fumigatus	18	16	14	12	12	10	-	-	23	19	17	13	K (30)
Aspergillus niger	22	18	16	12	12	13	12	10	30	20	18	12	K (28)
Candida albicans	28	25	22	21	18	16	14	12	30	29	27	26	K (24)
Dermatophytes													
Epidermophyton floccosum	22	18	14	12	-	-	-	-	28	20	18	12	K (32)
Microsporum canis	16	14	12	12	12	10	-	-	22	20	18	16	Ch (32)
Microsporum gypseum	22	16	14	12	-	-	-	-	40	38	36	20	Ch (30)
Trichophyton mentagrophytes	24	20	16	13	13	10	-	-	32	30	29	18	Ch (30)
Trichophyton rubram	18	16	12	9	16	14	12	10	22	20	18	16	K (32)

* Values (Mean of three replicates) are diameter of zone of inhibition (mm): - absence of inhibition; Ketoconazole (K); Chloromphenicol (Ch).

15. *Vibrio parahaemolyticus*

Only methanol extract was active as that of *Vibrio vulnificus* and produced 27 mm inhibition at 100 mg/ml and a minimum of 9 mm at 12.5 mg/ml. The MIC and MBC were determined as 5 mg/ml and 10 mg/ml respectively. Both chloroform and hexane extracts were inactive at the tested concentrations.

16. *Vibrio vulnificus*

Only methanol extract was active and produced 23 mm inhibition at 100 mg/ml and 10 mm at 12.5 mg/ml and its MIC and MBC were 10 mg/ml and 20 mg/ml respectively. Both chloroform and hexane extracts were inactive at the tested concentrations.

4.2.2 Antifungal Activity

Hexane, chloroform and methanol against showed very potent antifungal activity including dermatophytes, which was comparatively more than that of antibacterial activity (Tables 11 & 12; Figs. 28 & 29). All the test extracts exhibited more inhibition at higher concentrations. However, methanol extract exhibited better inhibition than hexane and chloroform extracts.

4.2.2.1 Activity of Various Solvent Extracts against Fungal Strains

1. *Aspergillus flavus*

Maximum zone of inhibition at 100 mg/ml was noticed as 29 mm and 20 mm for methanol and hexane extracts respectively. Chloroform extract was completely inactive at all the tested concentrations. The MIC and MFC were determined as 100 mg/ml and 20 mg/ml respectively for both methanol and hexane extracts.

2. *Aspergillus fumigatus*

Methanol extract exhibited maximum zone of inhibition at 100 mg/ml as 23 mm and 13 mm at 12.5 mg/ml as minimum. For hexane extract, it was noticed as 18

Table 12. MIC and MFC of Various Extracts of *Phyllanthus wightianus*

Test Microorganisms	Hexane extract (mg/ml)					Chloroform extract (mg/ml)					Methanol extract (mg/ml)				
	20	10	5	2.5	1.25	20	10	5	2.5	1.25	20	10	5	2.5	1.25
Fungi															
Aspergillus flavus	+@	+*	**	-	-	-	-	-	-	-	+@	+*	**	-	-
Aspergillus fumigatus	+*	**	-	-	-	**	-	-	-	-	+@	+*	**	-	-
Aspergillus niger	+@	+*	**	-	-	+*	**	-	-	-	+	+	+*@	**	-
Candida albicans	+	+@	+*	**	-	+*@	**	-	-	-	+	+@	+*	**	-
Dermatophytes															
Epidermophyton floccosum	+	+*@	**	-	-	-	-	-	-	-	+	+@	+*	**	-
Microsporum canis	+@	+*	**	-	-	**	-	-	-	-	+@	+*	**	-	-
Microsporum gypseum	+@	+*	**	-	-	-	-	-	-	-	+	+@	+*	**	-
Trichophyton mentagrophytes	+@	+*	**	-	-	**	-	-	-	-	+@	+	+*	**	-
Trichophyton rubram	+*@	**	-	-	-	**	-	-	-	-	+@	+*	**	-	-

+ complete inhibition; +* MIC; ** Marked inhibition; - Absence of inhibition; @ MFC

Fig. 28. Antifungal activity of *Phyllanthus wightianus*

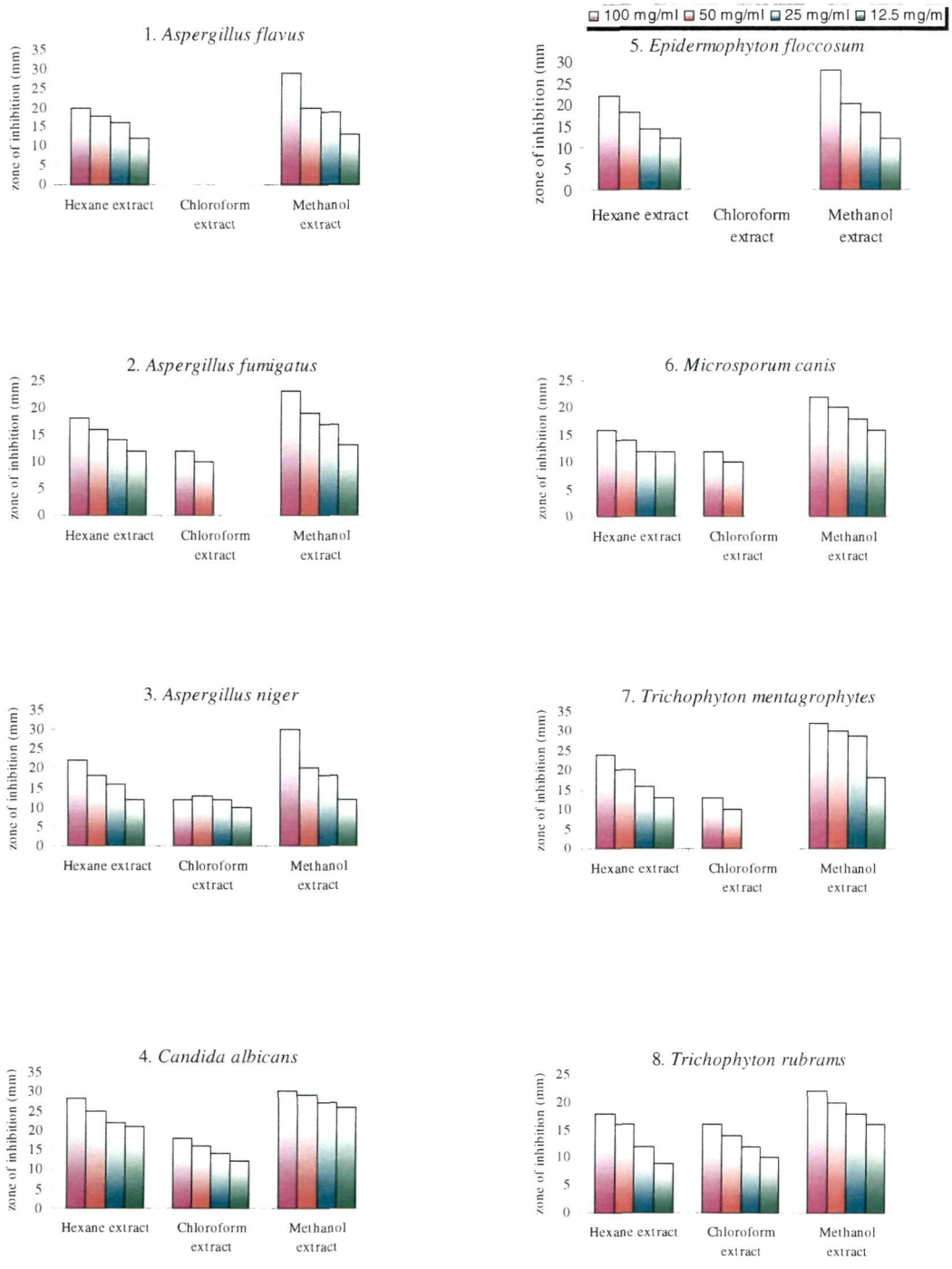
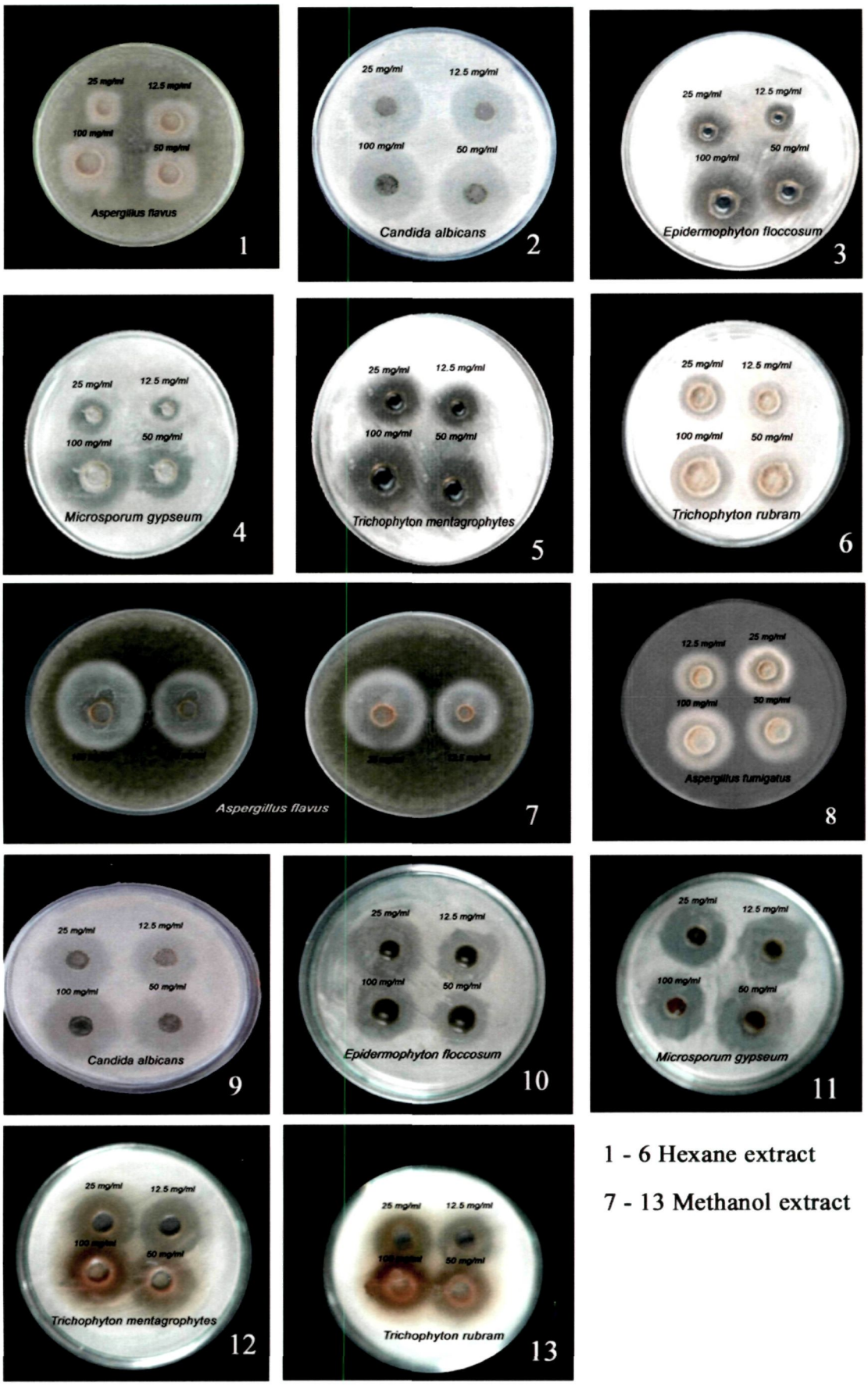


Fig.29. Antifungal activity of *Phyllanthus wightianus* extracts



1 - 6 Hexane extract

7 - 13 Methanol extract

mm at 100 mg/ml and 12 mm at 12.5 mg/ml. Chloroform extract produced maximum inhibition as 12 mm at 100 mg/ml which was inactive at the lower concentrations such as 25 and 12.5 mg/ml. The MIC and MFC for methanol extract was noticed as 10 mg/ml and 20 mg/ml respectively. The MIC for hexane extract was 20 mg/ml and the MIC and MFC for chloroform extract was not determined at the tested concentrations.

3. *Aspergillus niger*

At 100 mg/ml, methanol, hexane and chloroform extracts exhibited 30 mm, 22 mm and 12 mm inhibition respectively. Minimum inhibition was noticed at 12.5 mg/ml as 12 mm for methanol and hexane extracts and 10 mm for chloroform extract. The MIC and MBC for methanol extract was noticed as 5 mg/ml, and the MIC and MFC was 10 mg/ml, 20 mg/ml respectively for hexane extract. For chloroform extract, the MIC was noticed as 20 mg/ml and the MFC was not determined at the tested concentrations.

4. *Candida albicans*

Zone of inhibition for hexane extract was observed as 30 mm, 29 mm, 27 mm and 26 mm at 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml respectively. Next to it, methanol extract produced better inhibition such as 28 mm, 25 mm, 22 mm and 21 mm respectively at these concentrations. Chloroform extract was moderate in action and produced 18 mm as maximum inhibition at 100 mg/ml. The MIC was noticed as 5 mg/ml for methanol and hexane extracts respectively and 20 mg/ml for chloroform extract. The MFC was determined as 10 mg/ml for both hexane and methanol extracts and 20 mg/ml for chloroform extract.

5. *Epidermophyton floccosum*

Maximum zone of inhibition was observed at 100 mg/ml for methanol extract as 28 mm and minimum as 12 mm at 12.5 mg/ml. Hexane extract exhibited maximum inhibition at 100 mg/ml as 22 mm and minimum at 12.5 mg/ml as 12 mm. Chloroform extract was completely inactive at all the tested concentrations. The MIC for methanol extract was noticed as 5 mg/ml and 10 mg/ml for hexane extract. For both methanol and hexane extracts, the MFC was 10 mg/ml.

6. *Microsporum canis*

Methanol extract produced maximum inhibition at 100 mg/ml as 22 mm and minimum as 16 mm at 12.5 mg/ml. Hexane extract exhibited maximum inhibition at 100 mg/ml as 16 mm and minimum as 12 mm at 12.5 mg/ml. Chloroform extract was comparatively weak and it produced maximum zone of inhibition as 12 mm at 100 mg/ml which was inactive at the lower concentrations such as 25 mg/ml and 12.5 mg/ml. The MIC and MFC was determined respectively as 10 mg/ml and 20 mg/ml for both methanol and hexane extracts.

7. *Microsporum gypseum*

Methanol extract was more potent and produced maximum zone at 100 mg/ml as 40 mm and minimum as 20 mm at 12.5 mg/ml, which was followed by hexane extract such as 22 mm as maximum inhibition at 100 mg/ml and 12 mm as minimum inhibition at 12.5 mg/ml whereas the chloroform extract was completely inactive at all the tested concentrations. The MIC was noticed as 5 mg/ml for methanol extract and 10 mg/ml for hexane extract. The MFC for methanol and hexane extracts was recorded as 10 mg/ml and 20 mg/ml respectively.

8. *Trichophyton mentagrophytes*

Maximum inhibition was exhibited by methanol extract as 32 mm which was followed by hexane extract as 24 mm and then chloroform extract as 13 mm at 100 mg/ml. Methanol extract produced minimum inhibition as 18 mm at 12.5 mg/ml, which was followed by hexane extract as 13 mm, whereas chloroform extract was inactive at its lower concentrations at 25 mg/ml and 12.5 mg/ml. The MIC was recorded as 5 mg/ml and 10 mg/ml for methanol and hexane extracts respectively. The MFC was determined as 20 mg/ml for methanol and hexane extracts, which was not determined for chloroform extract at the tested concentrations.

9. *Trichophyton rubram*

Methanol extract produced maximum zone of inhibition at 100 mg/ml as 22 mm, which was followed by hexane and chloroform extracts as 18 mm and 16 mm respectively. Minimum zone of inhibition produced at 12.5 mg/ml by methanol, chloroform and hexane extracts was 16 mm, 10 mm and 9 mm respectively. The MIC for methanol extract was noticed as 10 mg/ml whereas it was 20 mg/ml for chloroform and hexane extracts. The MFC was noticed as 20 mg/ml for hexane and methanol extracts and was not determined for chloroform extract at the tested concentrations.

4.3 PHARMACOLOGY

4.3.1 Acute Toxicity Studies

Hexane, chloroform and methanol of *P. wightianus* and bergenin were found to be safe up to 2000 mg/kg per body weight. There were no signs of CNS, ANS and CVS toxicities with all the extracts and bergenin. As no mortality was observed the LD_{50} for the test extracts was unclassified according to the classification of globally harmonized system (GHS).

4.3.2 Analgesic Activity

4.3.2.1 Hot Plate Method

In this method, the standard drug morphine (5 mg/kg, s.c.) showed significant protection (at 60 min 12.1±0.5 and 120 min 18.3±0.63); P<0.001) of analgesia (Table 13; Fig. 30). Hexane, chloroform and methanol extracts did not show significant protection of analgesia at tested dose levels 100 mg and 200 mg/kg/b.w. p.o.

4.3.2.2 Acetic Acid-induced Writhing Response in Mice

Hexane and chloroform extracts and the standard drug indomethacin showed significant and dose-related reduction in the number of writhing in mice (Table 14; Fig. 31). Hexane extract (100 and 200 mg/kg/b.w.) produced 37.35 and 45.67% protection (P<0.001) respectively while chloroform extract shown 35.18 and 43.59% protection (P<0.001) respectively. Methanol extract exhibited 52.40 and 59.11% protection (P<0.001) respectively.

4.3.3 Anti-inflammatory Activity

It was observed that carrageenan-induced acute paw oedema model, indomethacin 10 mg/kg/p.o. and hexane, chloroform and methanol extracts at 100 mg/kg/p.o. and 200 mg/kg/p.o. exhibited significant and dose-dependent reduction (P<0.001) in the volume of paw oedema in rats (Table 15; Fig. 32). All the test extracts showed maximum inhibition at the end of 3 h. Their % inhibition of oedema is given in parentheses. Indomethacin exhibited a maximum of 70.31% inhibition of oedema. Among the test extracts, methanol extract showed maximum protection such as 54.68 and 60.93%, followed by hexane extract such as 43.75 and 50.00% and by chloroform extract such as 35.93 and 42.18%, respectively at the tested concentrations of 100 and 200 mg/kg/p.o..

Table 13. Analgesic Activity of Solvent Extracts of *Phyllanthus wightianus* on Hot Plate method

Treatment	Reaction in Seconds			
	15 min	**30 min**	**60 min**	**120 min**
Control 1% w/v SCMC (10 ml/kg/bw)	4.20± 0.1	4.26± 0.37	4.26± 0.70	4.33± 0.71
Standard (Morphine)	4.28± 0.22	8.20± 0.45[a]	12.1± 0.54 [a]	18.3± 0.63 [a]
Hexane extract (100 mg/kg)	3.16± 0.22	3.43± 0.57	4.30± 0.63	4.43± 0.77
Hexane extract (200 mg/kg)	4.06± 0.67	4.27± 0.97	4.30± 0.95	4.26± 0.48
Chloroform extract (100 mg/kg)	4.08± 0.88	4.13± 0.43	3.90± 0.87	4.28± 0.76
Chloroform extract (200 mg/kg)	3.90± 0.33	4.06± 0.61	4.26± 1.60	4.36± 0.76
Methanol extract (100 mg/kg)	3.86± 0.76	4.01± 1.05	4.23± 1.30	4.28± 0.79
Methanol extract (200 mg/kg)	4.03± 0.73	4.26± 0.67	4.28± 0.95	4.36± 0.87

Values are mean± SEM (n=6); One way ANOVA followed by Post - hoc Dunnett's test; [a]P<0.001 compared to control group

Fig. 30. Analgesic activity of solvent extracts of *Phyllanthus wightianus* on hot plate method

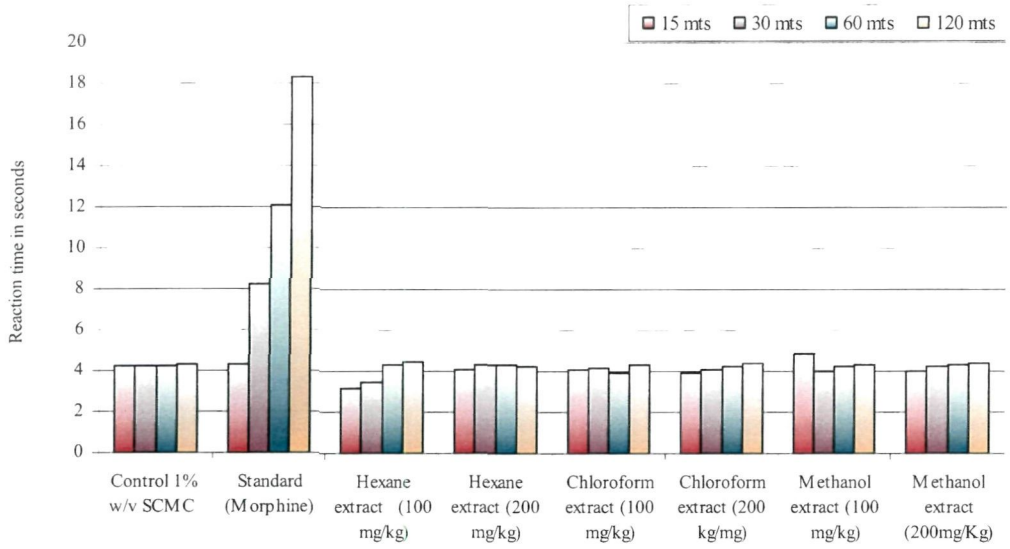


Fig. 31. Analgesic activity of solvent extracts of *Phyllanthus wightianus* on acetic acid induced writhing in mice

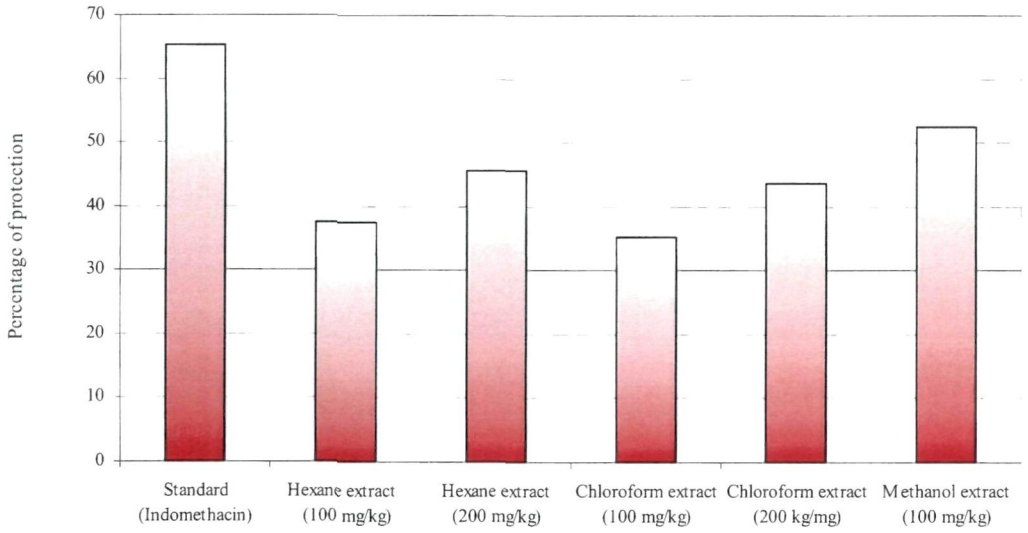


Table 14. Analgesic Activity of Solvent Extracts of *Phyllanthus wightianus* on Acetic Acid Induced Writhing in Mice

S. No.	Treatment	No. of writhings (Mean± SEM)	Percentage of Protection
1	Control 1% w/v SCMC (10 ml/kg/bw)	46.50± 0.73	-
2	Standard Indomethacin (10 mg/kg/bw)	16.13± 0.47	65.31[a]
3	Hexane extract (100 mg/kg)	29.13± 0.03	37.35[a]
4	Hexane extract (200 mg/kg)	25.26± 0.16	45.67[a]
5	Chloroform extract (100 mg/kg)	30.14± 1.32	35.18[a]
6	Chloroform extract (200 mg/kg)	26.23 ± 0.08	43.59[a]
7	Methanol extract (100 mg/kg)	22.13 ± 1.30	52.4[a]
8	Methanol extract (200 mg/kg)	19.01 ± 0.62	59.11[a]

Values are mean± SEM (n=6); One way ANOVA followed by Post - hoc Dunnett's test

[a]$P<0.001$ compared to control group

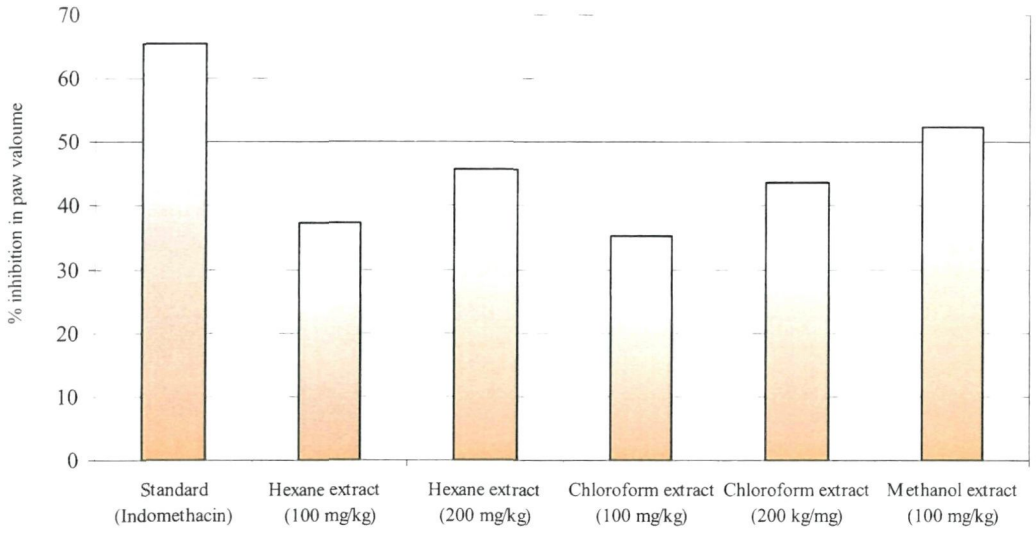
Table 15. Anti-inflammatory activity of *Phyllanthus wightianus* Against Carrageenan – induced Paw Oedema in Rats

Group	Treatment	Increase in Paw Volume ± SEM (ml)		
		1 h	3 h	5 h
1	1% w/v SCMC (10 ml/kg/bw)	0.56± 0.01	0.64± 0.02	0.68± 0.01
2	Standard Indomethacin (10 mg/kg/bw)	0.22± 0.02[a] (60.71)	0.19± 0.01[a] (70.31)	0.25± 0.02[a] (63.23)
3	Hexane extract (100 mg/kg)	0.35± 0.03[a] (37.5)	0.36± 0.06[a] (43.75)	0.40± 0.07[a] (41.17)
4	Hexane extract (200 mg/kg)	0.33± 0.04[a] (41.07)	0.32± 0.01[a] (50.00)	0.36± 0.06[a] (47.05)
5	Chloroform extract (100 mg/kg)	0.41± 0.06[a] (26.78)	0.42± 0.07[a] (35.93)	0.44± 0.09[a] (32.35)
6	Chloroform extract (200 mg/kg)	0.36± 0.03[a] (35.71)	0.37± 0.06[a] (42.18)	0.43± 0.07[a] (38.23)
7	Methanol extract (100 mg/kg)	0.31± 0.02[a] (44.64)	0.29± 0.01[a] (54.68)	0.33± 0.06[a] (51.47)
8	Methanol extract (200 mg/kg)	0.28± 0.01[a] (50.00)	0.25± 0.03[a] (60.93)	0.28± 0.04[a] (58.82)

Values are mean± SEM (n=6); percentage of protection against carrageenan induced paw oedema are in parenthesis. The statistical difference between control and treated groups were tested by one way ANOVA followed by post - hoc Dunnett's test

[a] $P<0.001$ compared to control group

Fig. 32. Anti-inflammatory activity of *Phyllanthus wightianus* against carrageenan–induced paw oedema in rats



4.3.4 *In-vitro* Antioxidant Activity

4.3.4.1 DPPH Assay

Hexane, chloroform and methanol extracts (100 µg/ml) were studied for *in-vitro* antioxidant activity by DPPH assay (Table 16). Vitamin C was used as the reference standard and exhibited maximum inhibition of 72.07%. It was observed that all the extracts had very good action on DPPH free radical. Maximum inhibition was exhibited by methanol extract (48.60%), followed by hexane extract (41.05%) and chloroform extract (25.54%) at 100 µg/ml.

4.3.4.2 Nitric Oxide Radical Scavenging Assay

Except chloroform extract, hexane and methanol extracts were effectively inhibited nitric oxide radical generation at 100 µg/ml (Table 16). Methanol extract exhibited maximum % of inhibition (70.09%), followed by hexane extract (54.59%). Standard vitamin C showed maximum of 77.07% inhibition.

4.3.5 Wound Healing Activity

4.3.5.1 Excision Wound Model

The results revealed the significant wound contracting ability of the test extract ointments compared to control (Table 17; Figs. 33, 34). Standard drug ointment was 2% w/w nirofurazone. Methanol extract-ointment produced a potent activity, almost similar as that of standard. The time of wound closure for the ointments of standard drug and methanol extract was observed as 14±2 days. This was followed by 16±2 days for hexane extract and 18±2 days for chloroform extract.

4.3.5.2 Incision Wound Model

There was a significant increase in tensile strength on 10 day–old wound treated with nitrofurazone ointment and ointments of the test extracts compared to

Table 16. ***In-vitro*** **Free Radical Scavenging Effect of Solvent Extracts of** ***Phyllanthus wightianus***

S. No.	Test samples	DPPH method		Nitric Oxide method	
		Mean Absorbance values	% inhibitions	Mean Absorbance values	% inhibitions
1	Reagent control	0.967± 0.019	-	0.903± 0.021	-
2	Standard (Vit–C) (100 µg/ml)	0.270± 0.168[a]	72.07	0.207± 0.006[a]	77.07
3	Hexane extract (100 µg/ml)	0.570± 0.025	41.05	0.410± 0.071	54.59
4	Chloroform extract (100 µg/ml)	0.720± 0.012	25.54	Not active	-
5	Methanol extract (100 µg/ml)	0.497± 0.010[a]	48.60	0.270± 0.005[a]	70.09

Results are expressed as ± SEM; [a] - $p<0.001$ compared to reagent blank by Student's t-test

Table 17. Effect of Test Extract Ointments of *Phyllanthus wightianus* and Nitrofurazone Ointment on Wound Contraction by Excision Wound Model

Post wounding days	Wound area (mm^2) ±SEM and percentage of wound contraction				
	Simple ointment (Control)	Nitrofurazone ointment (0.2% w/w)	Hexane extract ointment (5% w/w)	Chloroform extract ointment (5% w/w)	Methanol extract ointment (5% w/w)
0	520± 18.4 (0.0)	516± 16.5 (0.0)	514± 18.6 (0.0)	513± 21.2 (0.0)	524± 17.4 (0.0)
2	460± 19.3 (11.53)	412± 21.6 (20.15)	480± 16.4 (6.61)	485± 18.3 (5.45)	452± 14.4 (13.74)
4	403± 20.6 (22.5)	294± 28.6[a] (43.02)	443± 14.8 (13.81)	443± 17.2 (13.64)	340± 31.3 (35.11)
6	372± 13.5 (28.46)	206± 23.4[b] (60.07)	346± 12.2 (32.68)	366± 20.6 (28.65)	248± 25.4[b] (52.67)
8	312± 12.8 (40.0)	102± 11.3[b] (80.23)	262± 11.3[a] (49.02)	312± 18.6[a] (39.18)	151± 20.1[b] (71.18)
10	296± 13.6 (43.07)	34± 10.6[b] (93.41)	200± 10.6[b] (61.08)	226± 16.4[b] (55.94)	62± 13.6[b] (88.16)
12	275± 10.6 (47.11)	5± 11.6[b] (99.3)	112± 12.1[b] (78.21)	114± 18.9[b] (77.77)	12± 13.1[b] (97.70)
14	258± 13.4 (50.38)	0.0[b] (100)	40± 8.6[b] (92.21)	82± 14.0[b] (84.01)	0.0 [b] (100)
16	220± 15.6 (57.69)	-	0.00[b] (100)	31± 12.6[b] (93.95)	-
18	210± 13.4 (59.61)	-	-	0.0 [b] (100)	-

p values Vs Control by Student's t-test

[a]$p < 0.01$; [b] $p < 0.001$. Figures in parentheses represent percentage of wound contraction

Fig. 33. Effect of Test extract ointments of *Phyllanthus wightianus* and Nitrofurazone ointment on wound contraction by excision wound model

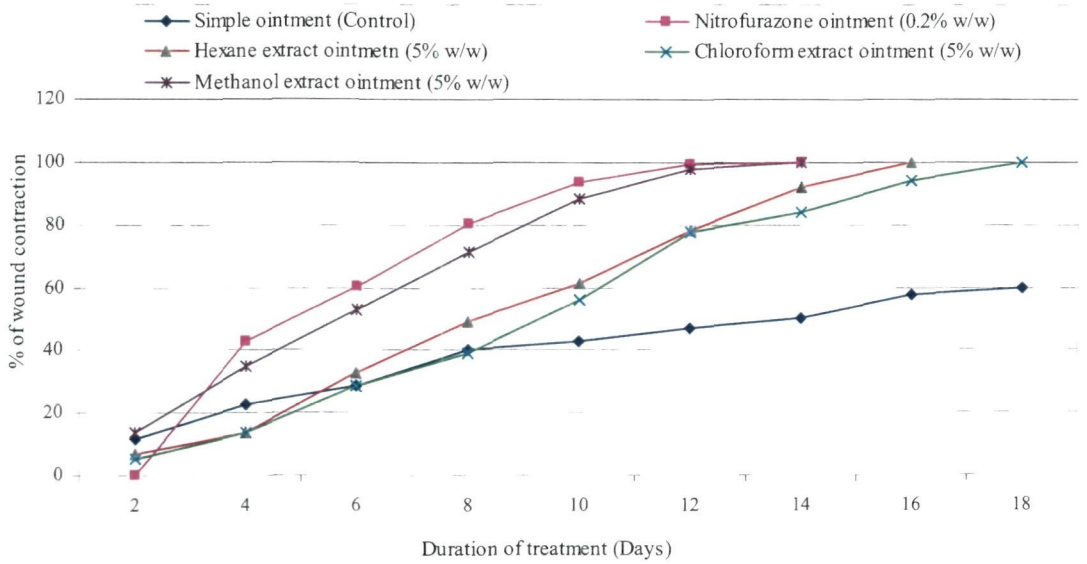


Fig. 35. Effect of test extracts ointments of *Phyllanthus wightianus* and Nitrofurazone ointment on wound contraction by incision wound model

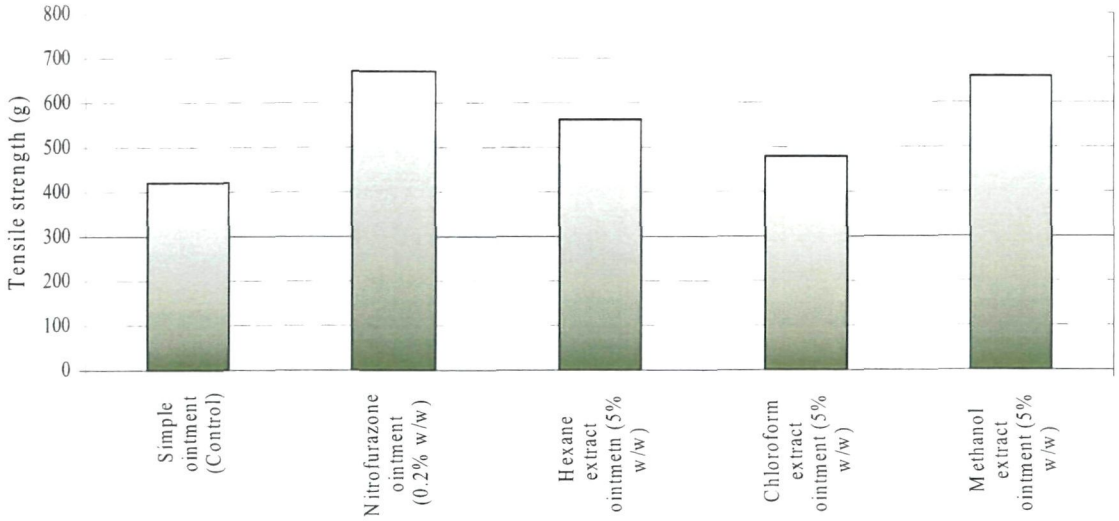
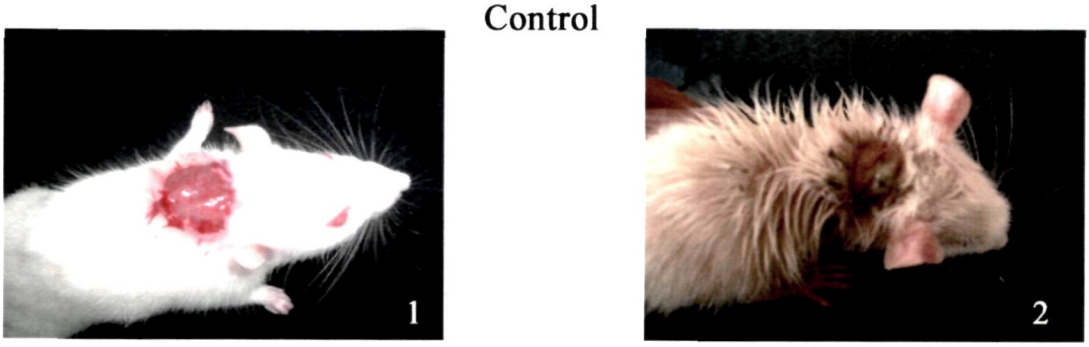
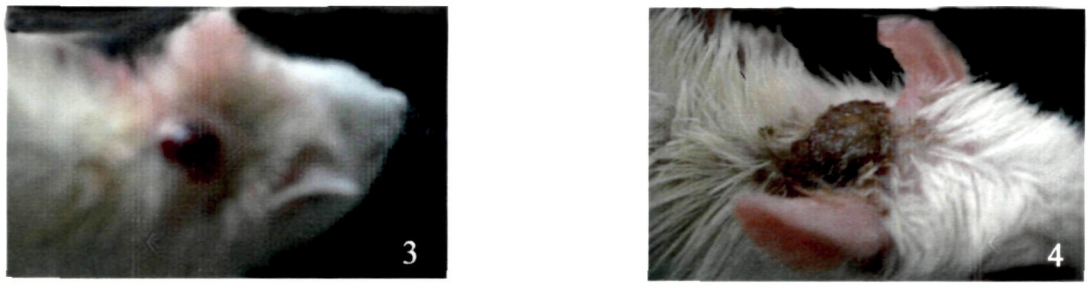


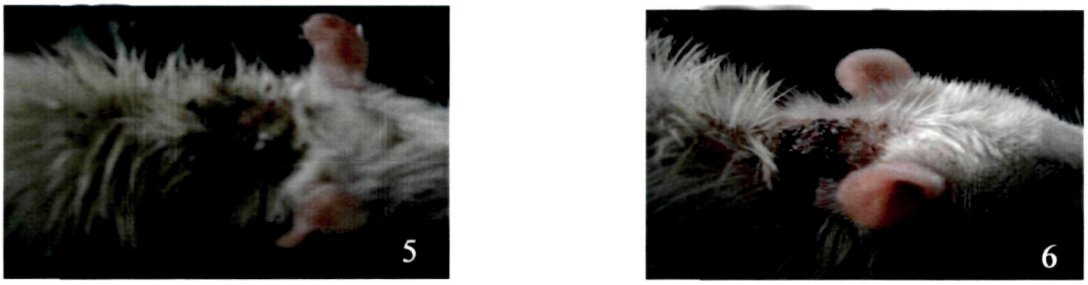
Fig.34. Wound healing activity of solvent extracts of *Phyllanthus wightianus*
Excision wound model

Control

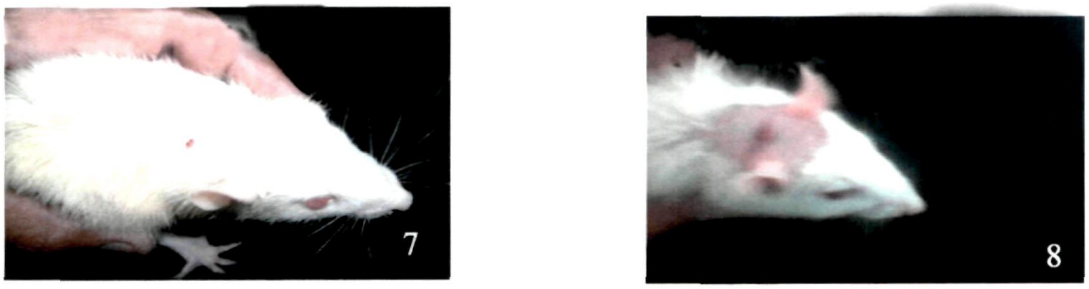


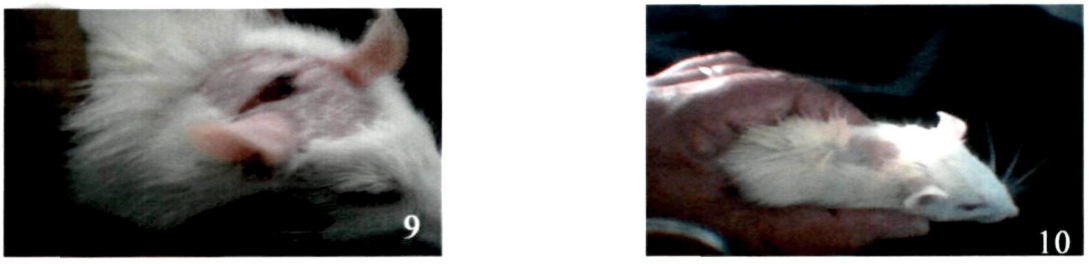
Wound contraction on 6th day





Wound contraction on 10th day





1.On day 0; 2.On day6; 3.Standard - Nitrofurazone ointment; 4.Hexane extract ointment; 5.Chloroform extarct ointment; 6.Methanol extract ointment; 7.Standard - Nitrofurazone ointment; 8.Hexane extract ointment; 9.Chloroform extract ointment; and 10.Methanol extarct ointment

control (Table 18; Fig. 35). Among the test extracts, methanol extract exhibited potent activity. Tensile strength of the standard and methanol extract found to be more or less similar such as 670±12.3 g and 659±13.1 g respectively. In the case of hexane extract, it was 562±12.6 g while chloroform extract exhibited relatively low such as 480±11.6 g.

4.3.6 Antidiabetic Activity

4.3.6.1 Effect on Blood Glucose

STZ (50 mg/kg, IV)-elevated blood glucose levels along with significant decrease in body weight of rats (15.02%). Administration of the test extracts and glibenclamide had partially restored or improved body weight (Table 19). There was a significant reduction in the blood glucose level for all the test extracts and glibenclamide-treated animals compared to non-treated diabetic rats in dose-dependent manner. Hexane extract (100 and 200 mg/kg) produced reduction of blood glucose levels such as 36.73 and 42.49% respectively. Similar concentrations exhibited reduction of 32.38 and 41.29% for chloroform extract and 43.50 and 52.00% for methanol extract respectively. Glibenclamide (10 mg/kg) produced a maximum of 61.80%.

4.3.6.2 Effects on Biochemical Parameters

4.3.6.2.1 Effect on Plasma Protein

A marked decrease in the plasma protein content of untreated diabetic group such as 3.9±1.5 g/dl was observed when compared to control group (6.90±0.40 g/dl) and treatment with the test extracts and the standard drug attenuated this alteration (Table 20; Fig. 36). Methanol extract exhibited better protection by normalizing/increasing total proteins followed by hexane and chloroform extracts.

Table 18. Effect of Test Extracts Ointments of *Phyllanthus wightianus* and Nitrofurazone Ointment on Wound Contraction by Incision Wound Model

S. No.	Treatment	Tensile Strength (g) (SEM)
1	Simple ointment (Control)	420±10.1
2	Nitrofurazone ointment (0.2% w/w)	670± 12.3[a]
3	Hexane extract ointment	562±12.6[a]
4	Chloroform extract ointment	480± 11.6
5	Methanol extract ointment	659±13.1[a]

Results were compared to control and p values were calculated by Student's t-test; [a]p< 0.001

Table 19. Effect of Solvent Extracts of *Phyllanthus wightianus* on Body Weight and Blood Glucose Level in STZ- induced Diabetic Rats

Group	Body Weight (g)			Blood glucose Level (mg/dl)			% of activity
	Before	After STZ	After drug Treatment	Normal	After STZ	After drug Treatment	
Normal Control 1% w/v SCMC	193.3± 2.6	164.3± 2.2	164.6± 1.8	85.5± 6.7	359.5± 34.8	368.6± 11.3	
Positive control Glibenclamide (10 mg /kg)	189.6± 2.8	173.6± 2.9	178.5± 3.8	86.1± 5.0	398.3± 40.6	152.6± 31.2	61.80[a]
Hexane extract (100 mg/kg)	200.0± 6.3	178.6± 2.8	179.6± 6.3	84.6± 3.3	392.2± 21.3	248.9± 20.6	36.73[a]
Hexane extract (200 mg/kg)	185.6± 3.8	172.8± 2.8	177.8± 3.3	86.4± 5.0	393.5± 18.3	226.13± 16.3	42.49[a]
Chloroform extract (100 mg/kg)	197.3± 1.6	167.8± 6.3	178.12± 6.3	86.5± 4.9	402.8± 19.6	272.6± 18.3	32.28[a]
Chloroform extract (200 mg/kg)	202.0± 1.8	177.3± 1.5	182.3± 3.9	87.7± 3.3	402.8± 14.9	236.8± 19.8	41.29[a]
Methanol extract (100 mg/kg)	186.5± 2.2	166.5± 2.1	178.6± 6.8	88.9± 1.5	400.6± 31.6	226.9± 15.6	43.5[a]
Methanol extract (200 mg/kg)	196.4± 6.3	173.5± 2.9	186.9± 3.9	88.8± 6.7	400.5± 11.3	192.8± 12.6	52.0[a]

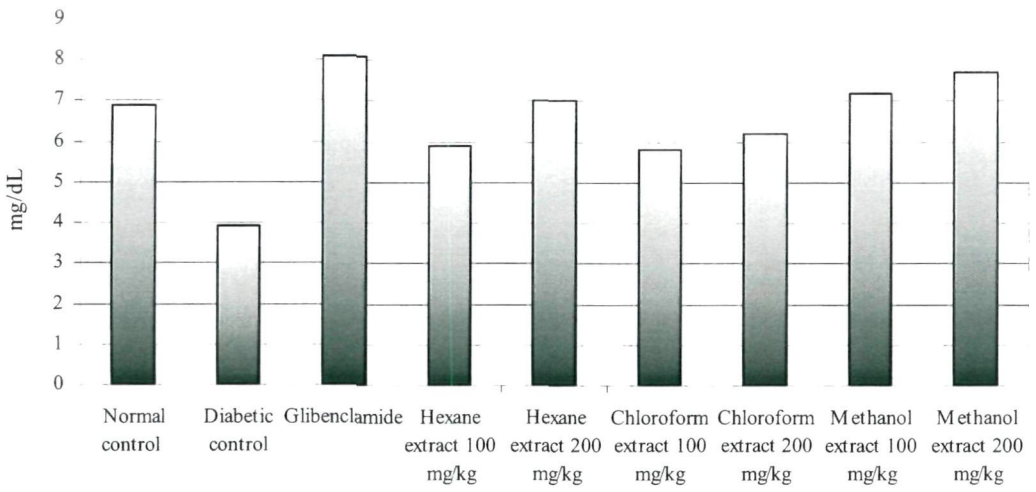
[a] $P<0.01$ Vs Control; values are in ± SEM (n=6)

Table 20. Effects of Solvent Extracts of *Phyllanthus wightianus* on Biochemical Parameters

Treatment	Cholesterol (mg/dl)	Triglycerides (mg/dl)	Total Protein (g/dl)	Alkaline Phosphatase (U/l)	ASAT (U/l)	ALAT (U/l)
Normal Control (Non-diabetic)	68.4± 3.5	97.6± 9.8	6.9± 0.04	168.6± 11.3	64.8± 7.9	45.5± 6.7
Diabetic Control (Untreated)	181.5± 3.2 [1]	183.6± 4.8 [2]	3.9± 1.5 [3]	279.2± 12.6 [3]	126.5± 1.2 [2]	81.6± 3.3 [4]
Glibenclamide (10 mg/kg)	73.5± 2.4 [a]	106.8± 12.3 [b]	8.1± 0.4 [b]	169.8± 13.0 [b]	72.4± 1.9 [b]	48.7± 3.2 [d]
Hexane extract (100 mg/kg)	99.2± 7.4 [a]	121.2± 2.6 [c]	5.9± 0.2 [c]	202.8± 12.4 [b]	99.7± 1.8 [b]	59.6± 1.6 [d]
Hexane extract (200 mg/kg)	81.8± 2.2 [a]	113.8± 2.9 [b]	7.0± 0.2 [b]	189.2± 12.8 [c]	94.7± 6.3 [b]	53.7± 9.1 [d]
Chloroform extract (100 mg/kg)	102.3± 6.3 [a]	130.6± 6.3 [c]	5.8± 0.4 [c]	221.2± 12.6 [c]	113.5± 7.2 [c]	62.6± 1.3 [d]
Chloroform extract (200 mg/kg)	85.5± 2.5 [a]	122.7± 3.9 [b]	6.2± 0.8 [b]	201.6± 13.3 [b]	102.3±6.8 [c]	61.5± 5.0 [d]
Methanol extract (100 mg/kg)	81.4± 4.7 [a]	110.5± 9.3 [b]	7.2± 0.2 [b]	181.3± 12.3 [b]	93.9± 3.4 [b]	53.1± 1.4 [d]
Methanol extract (200 mg/kg)	75.5± 2.6 [a]	108.7± 4.7 [b]	7.7± 0.6 [b]	171.1± 11.6 [b]	84.3± 7.1 [b]	48.7± 1.6 [d]

Superscripts 1, 2, 3 and 4 indicate statistical significance compared to normal control group at $P<0.001$, $P<0.01$, $P<0.02$, $P<0.05$ respectively. Superscripts a, b, c and d indicate statistical significance compared to diabetic control group at $P<0.001$, $P<0.001$, $P<0.002$ and $P<0.05$.

Fig. 36. Effect of solvent extracts of *Phyllanthus wightianus* on total protein levels in diabetic rats



4.3.6.2.2 Effects on Lipids

A significant increase in cholesterol (181.50±3.20 mg/dl) and triglycerides levels (183.60±4.80 mg/dl) in diabetic group was observed when compared to normal control such as 68.40±3.50 mg/dl and 97.60±9.80 mg/dl, respectively (Table 20; Fig. 37). The test extracts and standard drug significantly controlled elevation of cholesterol and triglycerides in comparison with diabetic rats. Methanol extract significantly reduced elevated levels of cholesterol ($P<0.001$) and triglycerides ($P<0.001$), followed by hexane and chloroform extracts.

4.3.6.2.3 Effect on ALP, ASAT (GOT) and ALAT (GPT)

ALP, ASAT (GOT) and ALAT (GPT) levels were found to be increased significantly such as 279.20±12.30 U/L, 126.50 ± 1.20 U/L and 81.60 ± 3.30 U/L in STZ-treated diabetic rats when compared to normal animals such as 168.60 ± 11.30 U/L, 64.80 ±7.90 U/L and 45.50 ± 6.70 U/L, respectively (Table 20; Fig. 38). The test extracts and standard drug significantly decreased elevated ALP, ASAT (GOT) and ALAT (GPT) levels in diabetic rats. Methanol extract significantly decreased elevated ALP ($P<0.001$) and ASAT ($P<0.001$) and ALAT ($P<0.05$) levels in diabetic rats, followed by hexane and chloroform extracts respectively in dose-dependent manner. Glibenclamide exhibited maximum protection by decreasing elevated ALP ($P<0.001$), ASAT ($P<0.001$) and ALAT ($P<0.05$).

4.3.7 Antiarthritic Activity

4.3.7.1 Effects on Body Weight Changes

In arthritic rats (control – 1 inflamed), retardation of growth was observed when compared to control rats (control – 2 non- inflamed) (Table 21). Test extracts and bergenin-treated animals, there was no retardation of growth. However, restoration/slight increase in body weights was observed in treated groups.

Fig. 38. Effect of solvent extracts of *Phyllanthus wightianus* on ASP, ASAT and ALAT levels in diabetic rats

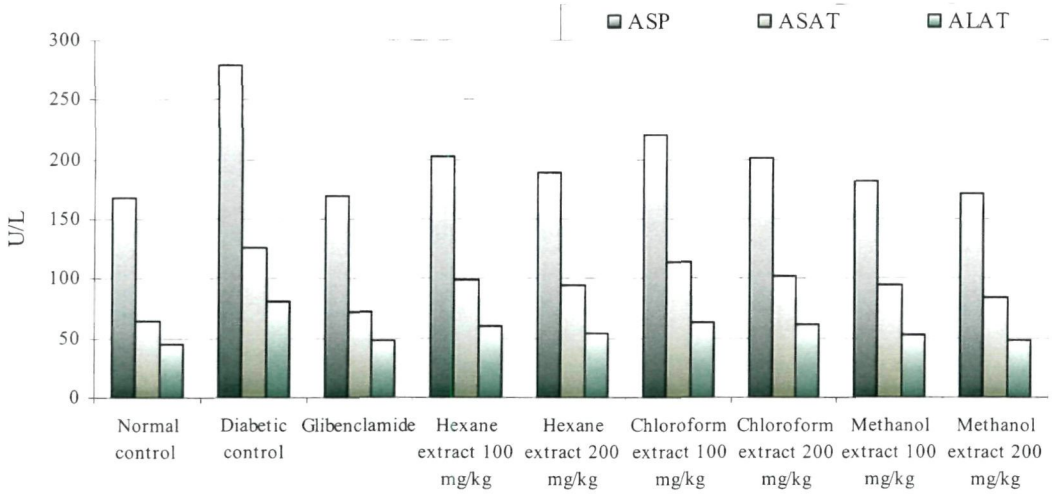


Fig. 37. Effect of solvent extracts of *Phyllanthus wightianus* on cholesterol and triglycerides levels in diabetic rats

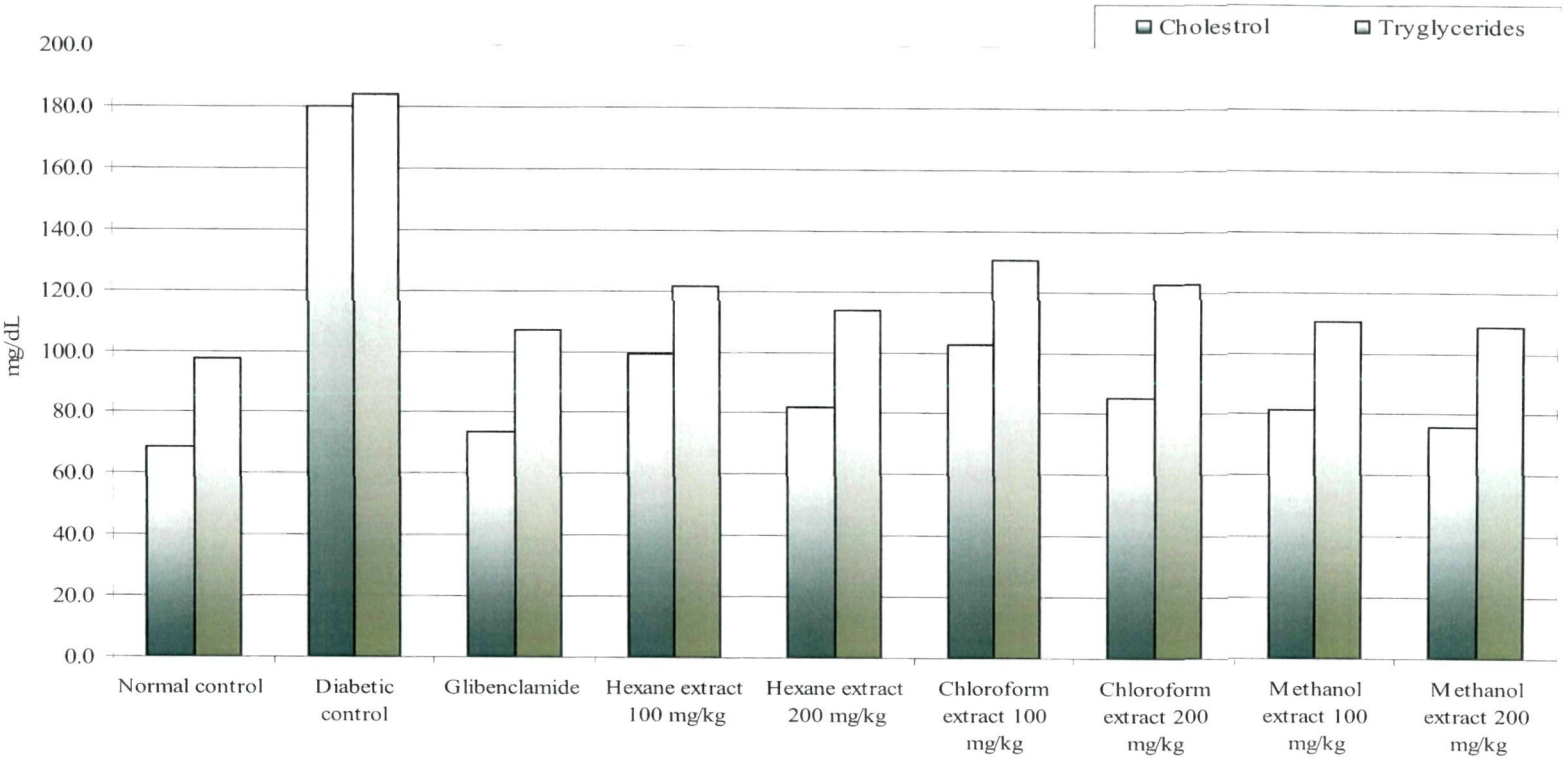


Table 21. Body Weight Changes in Control and Arthritic Animals Treated with Test Extracts of *Phyllanthus wightianus* and Bergenin

Treatment	Body weight changes (g)									
No. of days	1	3	5	7	9	11	13	15	17	19
Control (Inflamed)	200.3± 0.12	198.3± 0.6	196.6± 0.1	196.2± 0.1	195.6± 0.5	195.3± 0.13	193.6± 0.13	192.2± 0.01	192.2± 0.6	190.6± 0.11
Non-inflamed	198.1± 0.6	198.3± 0.12	197.3± 0.13	197.2± 0.6	197.6± 0.11	197.3± 0.1	197.6± 0.1	196.1± 0.1	196.2± 0.1	197.1± 0.6
Indomethacin (10 mg/kg)	200.6± 0.11	200.7± 0.13	199.6± 0.11	199.3± 0.6	200.1± 0.7	200.9± 0.12	201.6± 0.13	201.9± 0.14	202.0± 0.1	202.0± 0.3
Bergenin (50 mg/kg)	198.3± 0.15	198.0± 0.11	199.3± 0.11	199.6± 0.16	200.6± 0.11	200.3± 0.6	201.3± 0.16	201.6± 0.11	203.1± 0.6	203.3± 0.12
Hexane extract (100 mg/kg)	200.3± 0.8	200.6± 0.11	199.3± 0.16	199.3± 0.22	199.9± 0.22	200.6± 0.11	200.9± 0.23	201.3± 0.11	201.8± 0.28	201.6± 0.31
Hexane extract (200 mg/kg)	192.3± 0.6	192.4± 0.7	191.6± 0.8	192.7± 0.9	192.6± 0.8	192.6± 0.6	193.3± 0.11	193.8± 0.12	193.6± 0.12	193.9± 0.11
Chloroform extract (100 mg/kg)	198.01± 0.1	198.0± 0.13	197.6± 0.16	197.7± 0.18	197.6± 0.17	198.3± 0.63	198.4± 0.11	199.1± 0.11	199.3± 0.23	199.3± 0.15
Chloroform extract (200 mg/kg)	192.2± 0.6	192.3± 0.6	191.3± 0.13	191.06± 0.1	192.3± 0.11	192.6± 0.26	192.6± 0.36	193.1± 0.11	193.3± 0.13	193.3± 0.11
Methanol extract (100 mg/kg)	190.1± 0.1	190.6± 0.2	190.06± 0.1	189.06± 0.1	189.03± 0.1	190.06± 0.1	191.03± 0.1	191.06± 0.1	191.07± 0.1	191.1± 0.13
Methanol extract (200 mg/kg)	189.3± 0.13	189.6± 0.3	188.03± 0.1	190.3± 0.12	191.0± 0.12	191.06± 0.1	191.08± 0.1	192.03± 0.1	192.6± 0.16	192.8± 0.11

4.3.7.2 Effects on Tissue Weight Changes

There was no significant change in the weight of liver and kidney of arthritic rats. However, spleen weight was significantly increased ($p < 0.001$) (Table 22). Test extracts and bergenin showed significant decrease in spleen weight when compared to arthritic rats.

4.3.7.3 Effects on Percentage Increase in Paw Volume

In arthritic animals (control – 1 inflamed), there was a significant increase in the % of paw volume. Bergenin and test extracts showed significant ($p<0.001$) reduction in the % increase in paw volume in dose-dependent manner (Table 23; Fig. 39). The cordial signs of the chronic inflammatory reactions such as redness, swelling, arthralgia and immobility of affected joints were significantly less in drug-treated animals than that of control (Fig. 40). Among the test drugs, bergenin exhibited maximum protection ($p<0.001$) compared to indomethacin. Methanol and hexane extracts at 100 and 200 mg/kg also exhibited better inhibition of increase in paw volume ($P<0.001$). In all the test extracts, animals treated with higher doses offered maximum protection when compared to that of lower doses. Among the test extracts, chloroform extract exhibited less degree of protection ($p<0.01$) when compared to methanol and hexane extracts.

On swelling in non-injected foot pad, a significant inhibition was observed when compared to control group (Table 24; Fig. 41). A red patch on ears and an inflammatory node on tails were found simultaneously which were disappeared at the end of treatment. In the non-injected foot pad inhibition, the protection was exhibited in a dose-dependent manner as that of injected foot pad such as bergenin >methanol >hexane >chloroform. Compared with control, there was a significant inhibition of

Fig. 39. Changes in the injected paw volume of control and various test extracts *Phyllanthus wightianus* and bergenin treated animals

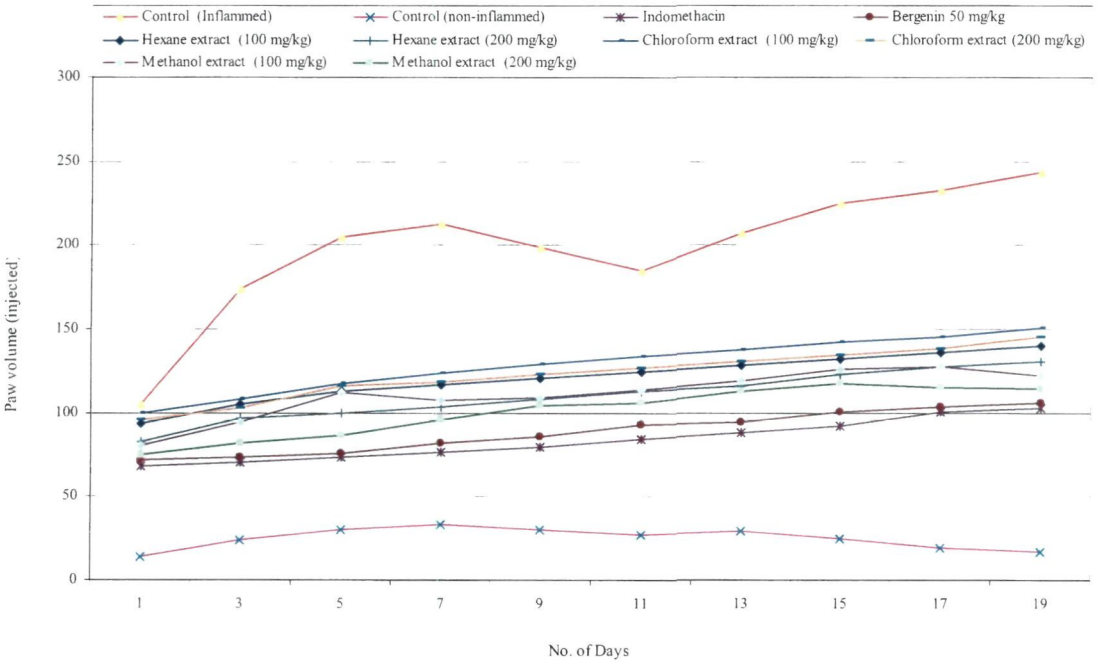


Fig. 41. Changes in the non-injected paw volume of control and various test extracts of *Phyllanthus wightianus* and bergenin treated animals

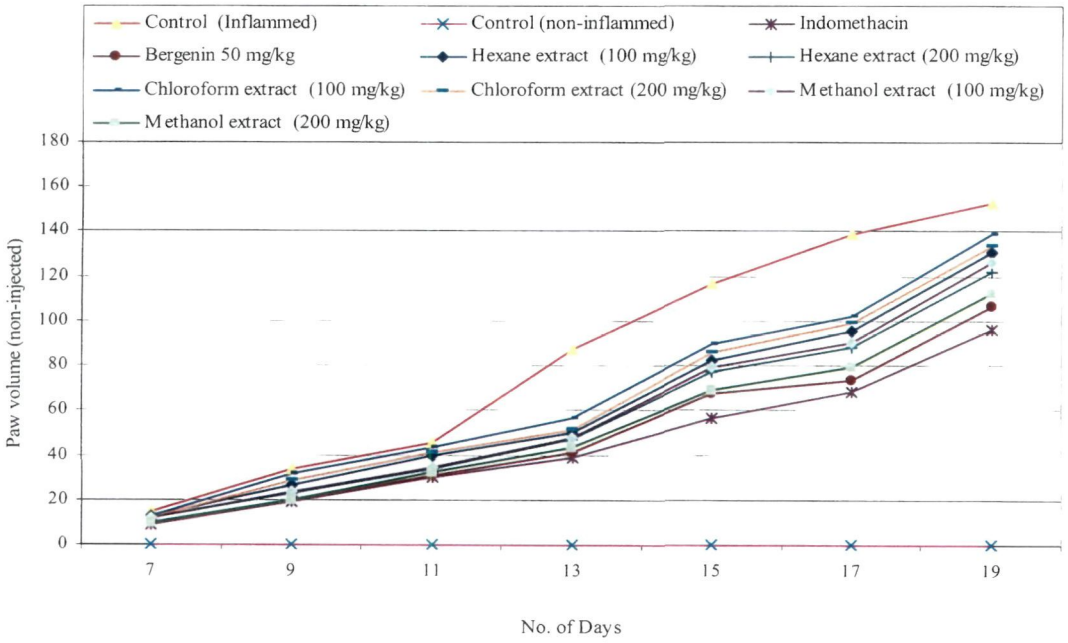
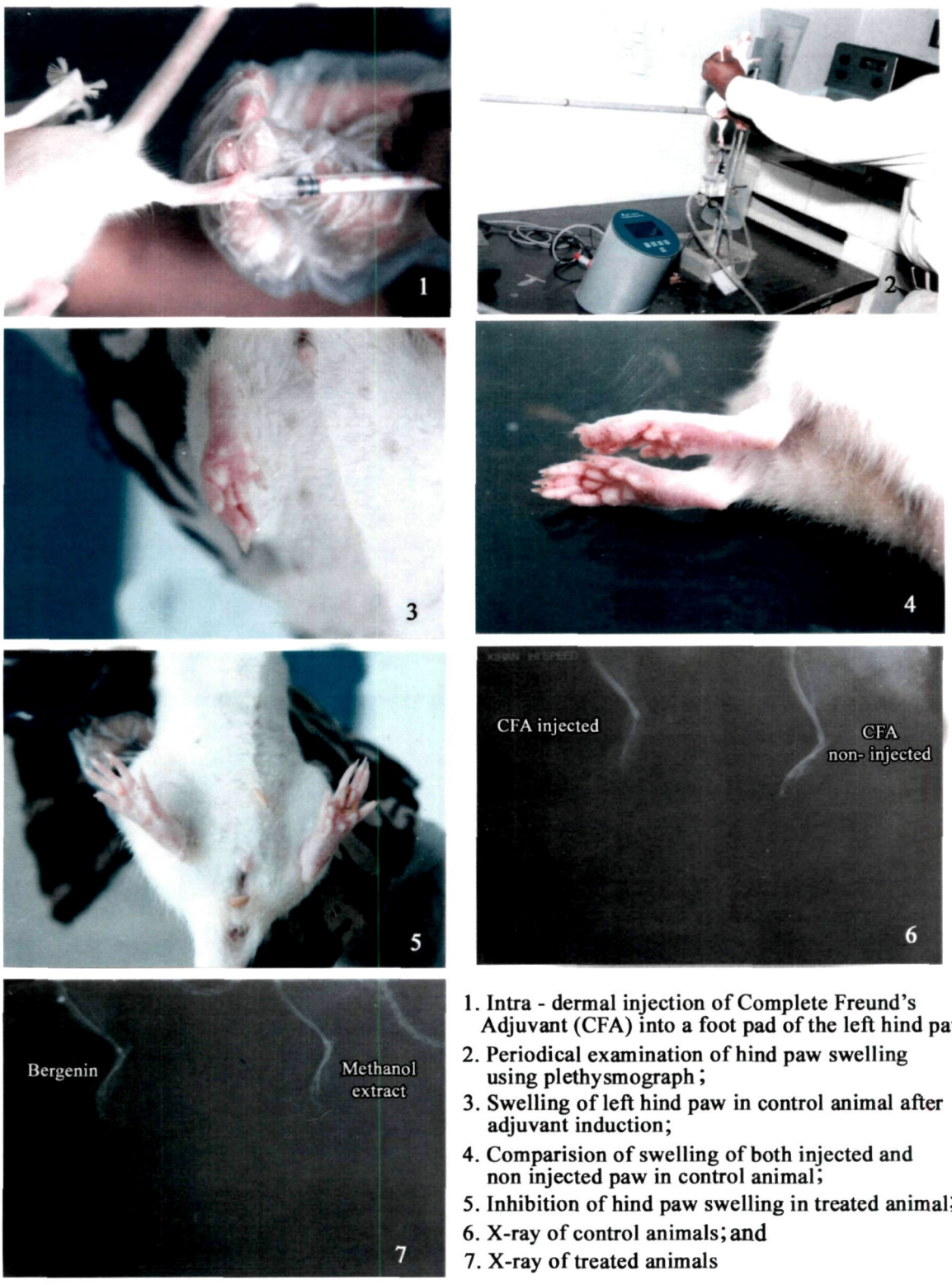


Fig.40. Antiarthritic activity of solvent extracts and bergenin from ***Phyllanthus wightianus***



1. Intra - dermal injection of Complete Freund's Adjuvant (CFA) into a foot pad of the left hind paw;
2. Periodical examination of hind paw swelling using plethysmograph;
3. Swelling of left hind paw in control animal after adjuvant induction;
4. Comparision of swelling of both injected and non injected paw in control animal;
5. Inhibition of hind paw swelling in treated animal;
6. X-ray of control animals; and
7. X-ray of treated animals

Table 22. Effect of Solvent Extracts of *Phyllanthus wightianus* and Bergenin on Tissue Weight Changes

Treatment	Tissue Weight Changes (g % body weight)		
	Liver	Kidney	Spleen
Control (Inflamed)	3.69± 0.13	0.81± 0.01	0.48± 0.01
Control (Non-inflamed)	4.12± 0.05[a]	0.88± 0.04[a]	0.30± 0.03[a]
Indomethacin (10 mg/kg)	4.21± 0.01[a]	0.93± 0.01[a]	0.29± 0.02[a]
Bergenin (50 mg/kg)	4.19± 0.13[a]	0.91± 0.02[a]	0.31± 0.01[a]
Hexane extract (200 mg/kg)	4.01± 0.02[b]	0.88± 0.06[a]	0.35± 0.01[a]
Chloroform extract (200 mg/kg)	3.92± 0.06[b]	0.85± 0.07[b]	0.37± 0.03[b]
Methanol extract (200 mg/kg)	4.15± 0.02[a]	0.89± 0.03[a]	0.32± 0.02[a]

Values represent mean (g % body weight ± SD, n = 6; [a]P<0.001; [b]P<0.01; When compared to Control (Inflamed)

Table 23. Changes in the Paw Volume of Control, Solvent Extracts of *Phyllanthus wightianus* and Bergenin Treated Animals

Treatment	% Increase in paw volume on injected paw Mean ± S.E. (n=6)									
	Post insult time of assay in days									
	1	3	5	7	9	11	13	15	17	19
Control (Inflamed)	105.0 3± 8.5	174.08± 12.5	204.6± 15.3	212.6± 17.6	198.4± 12.6	184.6± 15.4	207.4± 10.3	225.3± 13.9	232.6± 17.6	243.6± 12.1
Control (Non-inflamed)	14.2± 0.93	23.6± 1.5	30.3± 1.7	33.5± 2.4	30.5± 1.8[a]	27.3 ± 1.6[a]	29.5 ± 1.7[a]	24.5 ± 1.6[a]	19.3 ± 1.27[a]	17.2± 1.2[a]
Indomethacin (10 mg/kg)	68.03± 4.8	70.06± 5.5	73.6± 6.3	76.9 ± 4.6	79.8± 5.0[b]	84.3 ±6.7[b]	88.07 ± 2.8[b]	92.03 ± 8.6[b]	100.8 ± 7.3[b]	102.9± 6.3[b]
Bergenin (50 mg/kg)	71.8± 6.6	73.5± 8.3	75.8± 7.2	81.6 ± 4.6	85.6 ±8.2[b]	92.6 ±8.4[b]	94.2 ± 9.3[b]	100.3± 9.7[b]	103.6± 8.5[b]	105.6± 9.3[b]
Hexane extract (100 mg/kg)	93.2± 9.2	105.3± 8.5	112.6± 9.2	116.4± 10.1	120.4± 11.2[a]	124.5± 12.2[a]	128.6 ± 11.3[a]	132.5 ± 12.6[a]	136.3 ± 13.2[a]	140.2± 13.8[a]
Hexane extract (200 mg/kg)	82.6± 8.1	96.3± 8.2	99.5± 8.6	103.6 ± 9.2	108.2± 9.8[b]	112.6± 10.6[b]	116.3 ± 10.5[b]	122.6 ± 11.5[b]	127.4 ±12.8[b]	130.5± 12.7[b]
Chloroform extract (100 mg/kg)	99.8± 4.7	108.6± 3.6	117.8± 4.7	123.6± 4.7	128.9± 5.7[a]	133.6± 6.3[a]	137.8 ± 5.6[a]	142.3± 3.9[a]	145.2 ± 4.9[a]	150.8± 7.6[a]
Chloroform extract (200 mg/kg)	95.6± 3.6	102.7± 6.3	116.3± 4.3	118.6± 1.2	122.8± 3.8[a]	126.8± 5.6[a]	130.5 ± 7.6[a]	134.6 ± 1.3[a]	138.7± 4.8[a]	145.0± 5.7[a]
Methanol extract (100 mg/kg)	80.6± 7.2	94.6± 8.3	112.3± 10.2	107.5± 9.2	109.3 ± 9.6[b]	113.4 ± 11.2[b]	119.3± 10.5[b]	125.9± 12.5[b]	127.4± 11.6[b]	122.5± 10.5[b]
Methanol extract (200 mg/kg)	75.3± 6.3	81.6± 7.2	86.3± 7.5	96.2± 8.5	104.5± 9.6[b]	106.3± 8.9[b]	112.7± 10.5[b]	117.4± 10.8[b]	115.2± 10.6[b]	114.6± 10.5[b]

Table 24. Changes in the Paw Volume of Control and Solvent Extracts of *Phyllanthus wightianus* and Bergenin Treated Animals

Treatment	% increase in paw volume on non-injected paw ± SEM (n=6)						
	Post insult time of assay in days						
	7	9	11	13	15	17	19
Control (Inflamed)	14.5± 2.7	33.6 ± 2.48	45.7± 4.4	87.6± 7.5	116.6 ± 10.6	138.5± 10.3	152.6 ±12.75
Control (Non-inflamed)	-	-	-	-	-	-	-
Indomethacin (10 mg/kg)`	9.0± 0.3	19.2± 1.0	30.0± 1.6	38.6± 2.3 [b]	56.3± 6.7[b]	68.4± 3.8[b]	96.3 ± 4.6[b]
Bergenin (50 mg/kg)	9.3± 1.0	19.9± 1.5	30.6± 2.8	41.5± 4.15[b]	67.5 ± 5.4[b]	73.6± 6.9[b]	106.3± 10.2[b]
Hexane extract (100 mg/kg)	12.3± 1.1	26.3± 2.1	39.6± 3.4	50.3 ± 4.6[a]	82.5±7.7[a]	95.5± 8.6[a]	130.5 ± 12.6[a]
Hexane extract (200 mg/kg)	11.6± 1.0	22.6± 1.9	33.6± 3.4	46.8 ± 4.2[a]	76.8 ±4.2[a]	87.8± 8.2[a]	121.9 ±11.6[a]
Chloroform extract (100 mg/kg)	12.5±3.1	31.9± 4.3	43.6± 4.8	56.3± 3.8[a]	89.6±8.1[a]	102.3± 3.9[a]	138.7 ± 8.6[a]
Chloroform extract (200 mg/kg)	12.0±2.1	28.8± 3.8	41.3± 3.4	51.6± 6.3*	86.3± 3.4*	99.3 ± 6.8*	133.6 ± 9.6*
Methanol extract (100 mg/kg)	11.6± 1.9	23.6± 1.7	34.7± 3.2	47.6± 4.3*	79.3 ± 6.3*	90.6± 8.5*	126.3 ± 11.6*
Methanol extract (200 mg/kg)	9.6± 0.9	19.7± 1.5	32.3± 2.5	43.3± 3.5[b]	69.4± 6.1[b]	79.4 ± 72 [b]	112.6± 10.6[b]

* $P<0.01$ Vs Control; [b] $P<0.001$ Vs Control by Student's t-test

paw swelling was observed both in injected and non-injected foot pad of the treated animals.

4.3.7.4 Effect on Biochemical Changes

Changes in AST and ALT in various experimental groups are given (Table 25). A marked increase in serum and tissue enzyme levels was observed in arthritic rats ($p < 0.001$). The treatment with test extracts and bergenin brought back enzyme levels nearly normal. The changes in levels of lysosomal enzymes such as acid phosphatase (ACP) and cathepsin–D are given. A significant increase in ACP levels was recorded in the sera and tissues of the arthritic group (control - 1 inflamed) ($p<0.001$) whereas in the treated animals, near normalization of these enzymes was observed (Table 26).

4.3.7.5 Radiographic Analysis

Radiography of the tibiotarsal joint of the inflammed (control – 1) group showed severe inflammation, soft tissue swelling and bone resorption when compared to non-injected animals (Fig. 40.6). In the case of treated animals, there was a very mild inflammatory reaction with normal bone structure (Fig. 40.7)

4.3.8 Immunomodulatory Activity

4.3.8.1 Delayed Type Hypersensitivity Reaction using SRBC as an Antigen

There was a significant and dose-dependent inhibition of DTH response observed to the test extracts whereas cyclophosphamide (group 2) exhibited elevation of inflammatory response (Table 27; Fig. 42). Among the test extracts, methanol extract exhibited a pronounced inhibition ($p<0.001$) to both doses at 200 (0.83 ± 0.01) and 100 (0.96 ± 0.06) mg/kg followed by hexane extract such as 1.19 ± 0.03 and 1.32 ± 0.01 respectively. Chloroform extract showed comparatively less inhibition ($p<0.05$) than methanol and hexane extracts such as 1.43 ± 0.02 at both concentrations.

Table 25. Effect of Solvent Extracts of *Phyllanthus wightianus* and Bergenin on Serum and Tissue Amino Transferases

Treatment	Alanine amino transferase (ALT)				Aspartate amino transferase (AST)			
	Serum	Liver	Kidney	Spleen	Serum	Liver	Kidney	Spleen
Control (Inflamed)	0.99± 0.03	6.3± 0.01	0.046± 0.001	0.040± 0.002	0.98± 0.07	1.4± 0.01	0.28±0.03	0.007± 0.001
Control (Non-inflamed)	0.59± 0.02	2.13± 0.05	0.027± 0.002	0.020± 0.003	0.51± 0. 01	0.17± 0.01	0.13± 0.08	0.003± 0.001
Indomethacin (10 mg/kg)`	0.63± 0.06[b]	2.51 ± 0.01[b]	0.023 ±0.003[b]	0.019± 0.00[b]	0.49± 0.06[b]	0.18± 0.01[b]	0.13± 0.07[b]	0.003±0.002[b]
Bergenin (50 mg/kg)	0.68± 0.06[b]	3.12 ± 0.12[b]	0.029± 0.006[b]	0.021± 0.006[b]	0.48 ± 0.07[b]	0.20± 0.04[b]	0.14 ± 0.07[b]	0.0031±0.004[b]
Hexane extract (200 mg/kg)	0.73 ± 0.08[b]	3.26 ± 0.11[b]	0.033± 0.006[b]	0.025± 0.003[b]	0.58± 0.07[b]	0.31± 0.018[b]	0.18 ± 0.07[b]	0.004± 0.003[b]
Chloroform extract (200 mg/kg)	0.75±0.06[a]	4.16±0.21[a]	0.034± 0.007[a]	0.027 ± 0.004[a]	0.69 ± 0.01[a]	0.46± 0.02[a]	0.19± 0.08[a]	0.0045± 0.003[a]
Methanol extract (200 mg/kg)	0.69 ± 0.0[b]	3.18±0.013[b]	0.031± 0.00[b]	0.023±0.001[b]	0.53± 0.06[b]	0.26± 0.03[b]	0.16 ± 0.06[b]	0.0038± 0.004[b]

Value represent: Tissue, micromoles of pyruvate liberated/min per milligram of protein, serum; micromoles x 10^{-3} of pyruvate liberated/min per mg

[a]P<0.01 Vs Control; [b] P<0.001 Vs Control by Student's t-test

Table 26. Effect of Solvent Extracts of *Phyllanthus wightianus* and Bergenin on Serum and Tissue Lysosomal Enzyme Levels

Treatment	Acid Phosphatase (ACP)				Cathepsin – D			
	Serum	Kidney	Liver	Spleen	Serum	Liver	Kidney	Spleen
Control (Inflamed)	0.04± 0.001	0.26± 0.01	0.52± 0.06	0.49± 0.01	0.008± 0.0001	0.70± 0.12	0.93± 0.07	0.75± 0.01
Control (Non-inflamed)	0.02± 0.001	0.15± 0.02	0.24± 0.01	0.30± 0.02	0.0029± 0.0002	0.18± 0.03	0.30±0.01	0.38± 0.02
Indomethacin (10 mg/kg)	0.02 ± 0.003[b]	0.14± 0.01[b]	0.031± 0.02[b]	0.31± 0.03[b]	0.0028± 0.001[b]	0.19 ± 0.01[b]	0.29±0.02[b]	0.39± 0.08[b]
Bergenin (50 mg/kg)	0.018± 0.002[b]	0.15± 0.01[b]	0.30± 0.04[b]	0.31± 0.01[b]	0.003± 0.004[b]	0.19± 0.02[b]	0.28±0.01[b]	0.36± 0.06[b]
Hexane extract (200 mg/kg)	0.022± 0.002[b]	0.17± 0.01[b]	0.35± 0.06[b]	0.34± 0.06[b]	0.004± 0.006[b]	0.34± 0.03[b]	0.41±0.07[b]	0.43± 0.06[b]
Chloroform extract (200 mg/kg)	0.025±0.001[b]	0.18±0.03[b]	0.36± 0.01[a]	0.35± 0.07[a]	0.005± 0.006[a]	0.46±0.02[a]	0.45±0.06[a]	0.48± 0.04[a]
Methanol extract (200 mg/kg)	0.021± 0.001[b]	0.16± 0.03[b]	0.33± 0.07	0.33 ± 0.03[b]	0.0035± 0.003[b]	0.22± 0.01[b]	0.38±0.06[b]	0.40± 0.01[b]

Values represent: Acid phosphatase; micromoles x 10^{-1} of phenol liberated/mg protein per minute at 37°C; Cathepsin D; micromoles x 10^{-2} of tyrosine liberated/mg protein per minute

[a] - $P<0.01$ Vs Control; [b] - $P<0.001$ Vs Control by Student's t-test

Table 27. Effect of Solvent Extracts of *Phyllanthus wightianus* on SRBC induced Delayed Type Hypersensitivity Reaction (DTH)

S. No.	Treatment	Inflammation in Paw (mm)
1	1% Control w/v SCMC (10 ml/kg)	0.49± 0.02
2	Cyclophosphamide (50 mg/kg/p.o.)	3.12± 0.03
3	Hexane extract (100 mg/kg)	1.32± 0.01[a]
4	Hexane extract (200 mg/kg)	1.19± 0.03[a]
5	Chloroform extract (100 mg/kg)	1.62± 0.02[b]
6	Chloroform extract (200 mg/kg)	1.43± 0.02[b]
7	Methanol extract (100 mg/kg)	0.96± 0.06[a]
8	Methanol extract (200 mg/kg)	0.83± 0.01[a]

Values are expressed as Mean ±SEM (n = 6); a - $p < 0.001$ Vs Control; b - $p < 0.05$ Vs Control by Student's t-test

Fig. 42. Effect of solvent extracts of *Phyllanthus wightianus* on SRBC induced delayed type hypersensitivity reaction (DTH)

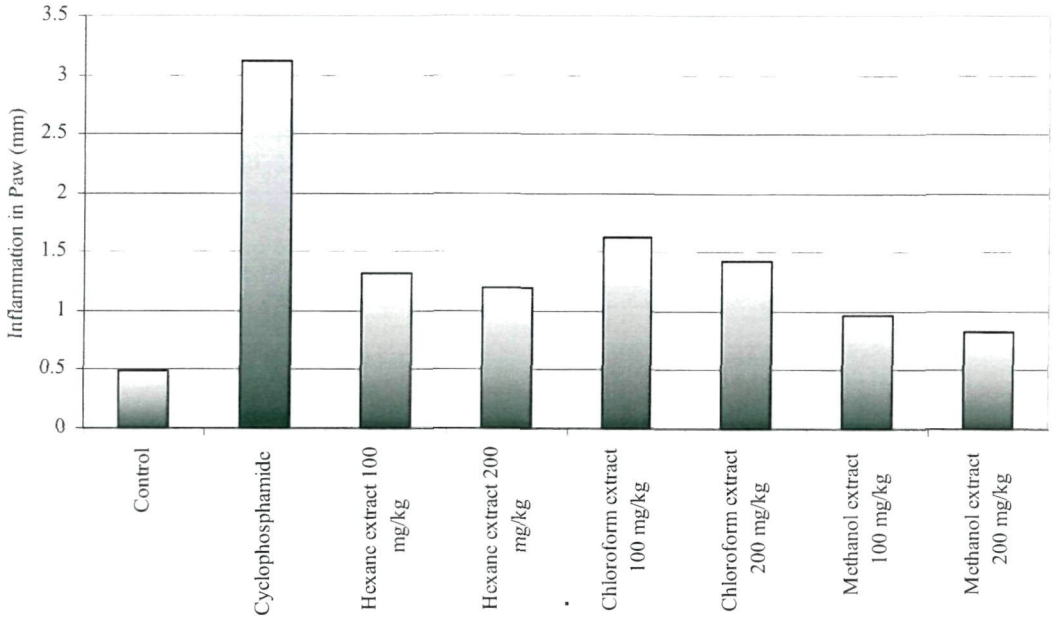
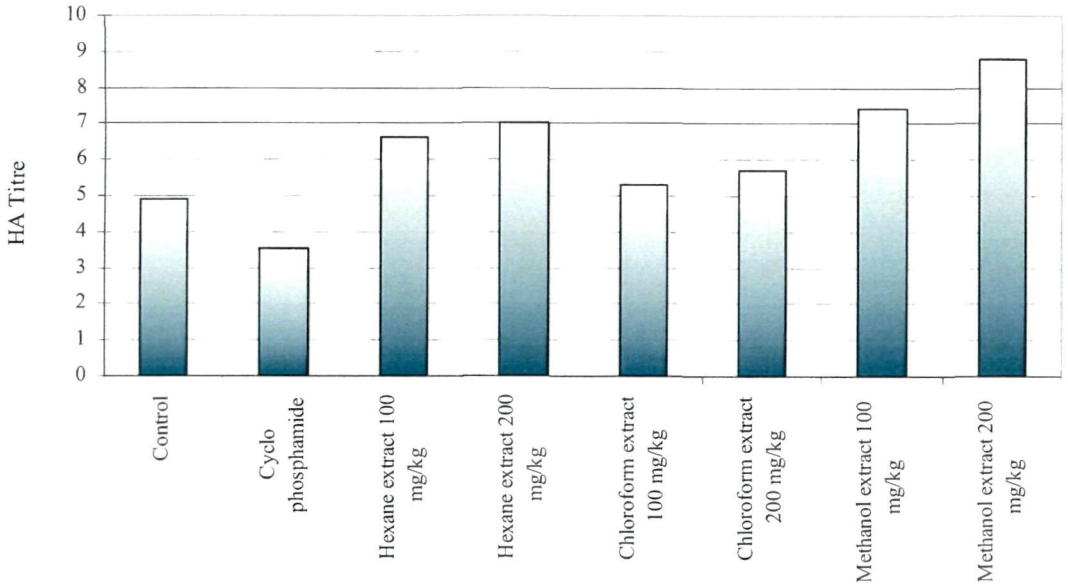


Fig. 43. Effect of solvent extracts of *Phyllanthus wightianus* on humoral antibody response to SRBC



4.3.8.2 Humoral Antibody Response to SRBC

A significant dose-dependent increase in humoral antibody titres was observed to the test extracts (Table 28; Fig. 43). Methanol extract exhibited maximum increase in HA titer ($p<0.05$) when compared to control. It was 8.80±0.62, 7.40±0.32 for 200 and 100 mg/kg respectively. Similar concentrations of hexane extract showed 7.01±0.16 and 6.6 ± 0.31 respectively whereas chloroform extract showed less significant levels such as 5.70 ± 0.31 and 5.30 ± 0.26 respectively.

4.3.8.3 Non-specific Immunity Determined by Survival Rate against Fungal Infection

Survival rate of the animals was considerably enhanced after treatment with the test extracts expressing the potential of non-specific immune response (Table 29; Fig. 44). Methanol, hexane and chloroform extracts exhibited 96.35±6.43, 90.42±3.33 and 82.36±5.26% survival after infection with *Candida albicans*. Reduction in mortality rate was more effective such as 59.95% - methanol extract, 54.02% - hexane extract and 45.96% - chloroform extract when compared to control.

4.3.8.4 Macrophage Phagocytosis by Carbon Clearance Method

Phagocytic index was increased dose-dependently in all the test extracts (Table 30; Fig. 45). However, increase was not highly significant. Moderately significant increase ($p<0.05$) was observed to methanol extract such as 0.116±0.012 and 0.110± 0.012, followed by hexane extract as 0.106±0.014 and 0.102±0.011 and chloroform extract as 0.95±0.014 and 0.093±0.012 at 200 and 100 mg/kg respectively. The results revealed no significant potential.

4.3.8.5 Cyclophosphamide-induced Myelosuppression Assay

All the test extracts significantly increased WBC count in a dose-dependent manner ($p<0.05$; Table 31; Fig. 46). Methanol extract exhibited maximum increase in

Table 28. Effect of Solvent Extracts of *Phyllanthus wightianus* on Humoral Antibody Response to SRBC

S. No.	Treatment	HA Titer
1.	Control 1%w/v SCMC (10 ml/kg)	4.89± 0.63
2.	Cyclophosphamide (50 mg/kg/p.o.)	3.53± 0.41
3.	Hexane extract (100 mg/kg)	6.6± 0.31
4.	Hexane extract (200 mg/kg)	7.01± 0.16 [a]
5.	Chloroform extract (100 mg/kg)	5.3± 0.26
6.	Chloroform extract (200 mg/kg)	5.7± 0.31
7.	Methanol extract (100 mg/kg)	7.40± 0.32 [a]
8.	Methanol extract (200 mg/kg)	8.80± 0.62 [a]

Values are expressed as ± SEM (n = 6) ; [a] - $p < 0.05$ as compared to Control

Table 29. Effect of Solvent Extracts of *Phyllanthus wightianus* on Survival Rate Against Fungal Infection

S. No.	Treatment	Survival Rate Percentage
1	Control 1%w/v SCMC (10 ml/kg)	37.2± 3.362
2	Hexane extract (200 mg/kg)	90.42± 3.33 [a]
3	Chloroform extract (200 mg/kg	82.36± 5.26 [a]
4	Methanol extract (200 mg/kg)	96.35± 6.431 [a]

Values are expressed as ± SEM (n = 6); [a] - $p < 0.05$ Vs Control

Fig. 44. Effect of solvent extracts of *Phyllanthus wightianus* on survival rate against fungal infection

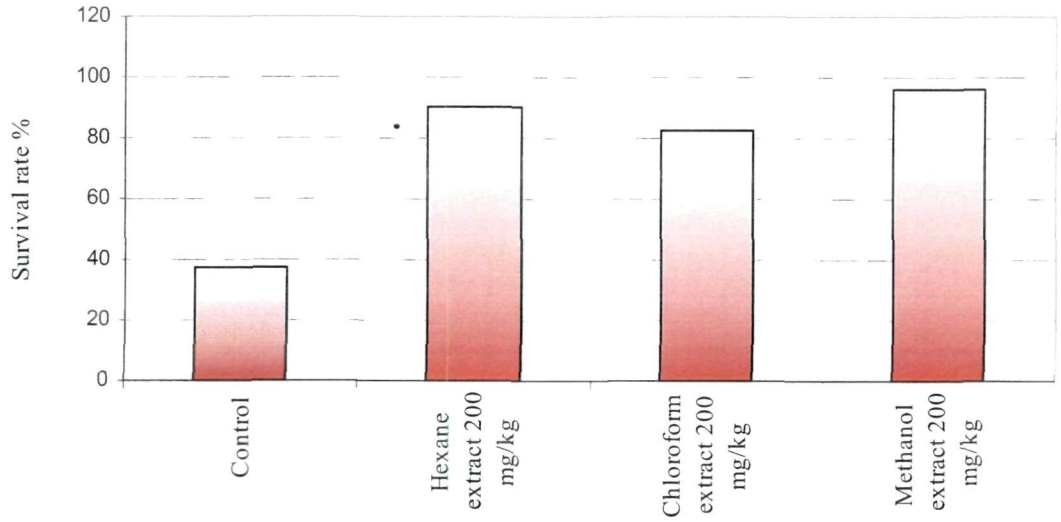


Fig. 45. Effect of solvent extracts of *Phyllanthus wightianus* on phagocytic index

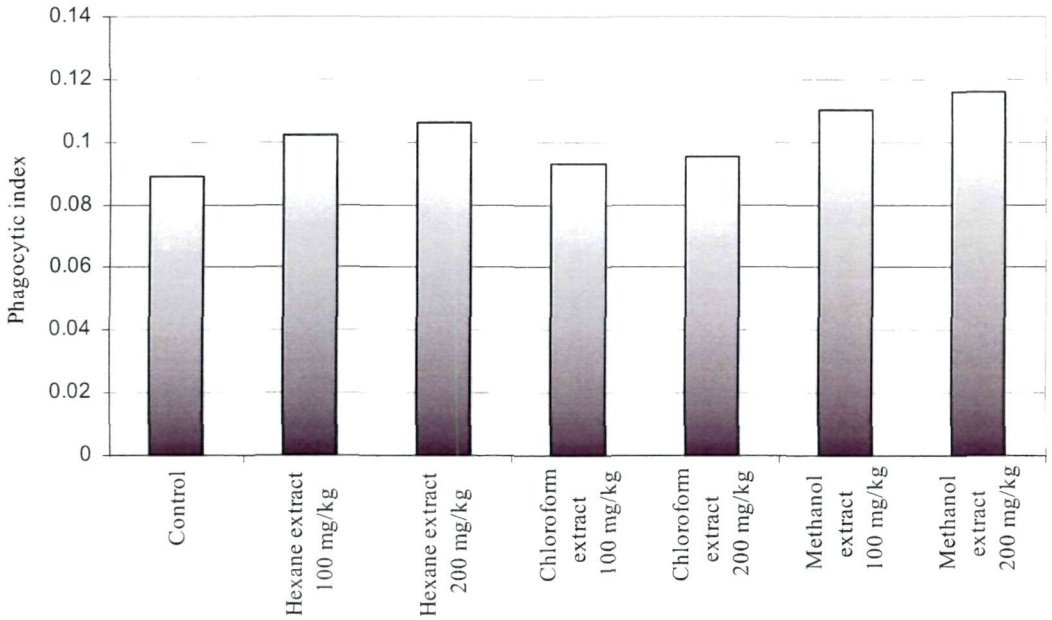


Table 30. Effect of Solvent Extracts of *Phyllanthus wightianus* on Phagocytic Index

S. No.	Treatment	Phagocytic Index
1	Control 1%w/v SCMC (10 ml/kg)	0.089± 0.012
2	Hexane extract (100 mg/kg)	0.102± 0.011
3	Hexane extract (200 mg/kg)	0.106± 0.014 [a]
4	Chloroform extract (100 mg/kg)	0.093± 0.012
5	Chloroform extract (200 mg/kg)	0.095± 0.014
6	Methanol extract (100 mg/kg)	0.110± 0.013 [a]
7	Methanol extract (200 mg/kg)	0.116± 0.012 [a]

Values are expressed as ± SEM (n = 6); [a] - $p < 0.05$ Vs Control

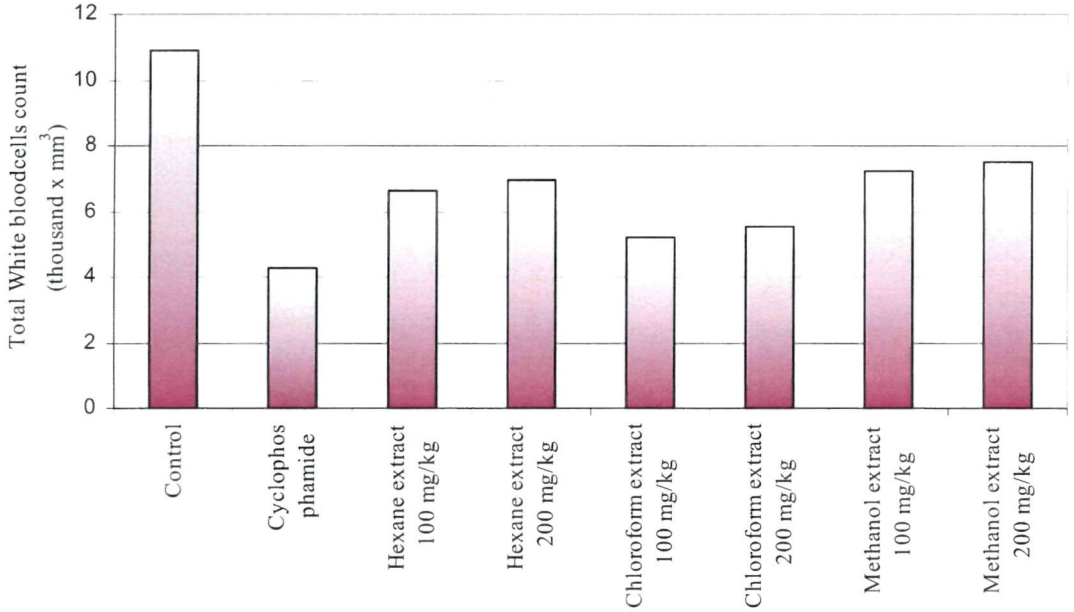
Table 31. Effect of Solvent Extracts of *Phyllanthus wightianus* on Cyclophosphamide Induced Myelosuppression

S. No.	Treatment	Total White Blood Cell Count (Thousand x mm^3)
1	Control 1% w/v SCMC (10 ml/kg)	10.93± 0.11
2	Cyclophosphamide (50 mg/kg/p.o.)	4.3± 0.16[a]
3	Hexane extract (100 mg/kg)	6.63± 0.21[a]
4	Hexane extract (200 mg/kg)	6.96± 0.23[b]
5	Chloroform extract (100 mg/kg)	5.23± 0.19[a]
6	Chloroform extract (200 mg/kg)	5.56± 0.28[a]
7	Methanol extract (100 mg/kg)	7.23± 0.11 [b]
8	Methanol extract (200 mg/kg)	7.52± 0.21[b]

Values are expressed as Mean± SEM (n = 6) ; [a] - $p < 0.05$ as compared to Control

[b] - $p < 0.05$ as compared to Cyclophosphamide Control

Fig. 46. Effect of solvent extracts of *Phyllanthus wightianus* on cyclophosphamide induced myelosuppression



WBC count such as 7.52±0.21 and 7.23±0.11 at 200 and 100 mg/kg respectively. Similar concentrations exhibited 6.96±0.23 and 6.63±0.21 to hexane extract and 5.56±0.28 and 5.23±0.19 to chloroform extract respectively.

4.3.9 Hepatoprotective Activity

4.3.9.1 *In vitro* Inactivation of HBsAg

There was a dose-dependent HBs Ag inactivation observed for the methanol extract and bergenin.

A 12% of inhibition was observed for the methanol extract at 1 mg/ml concentration and the maximum inhibition was observed at 8 mg/ml as 56% (Table 32). At 50 mg/ml concentration, bergenin exhibited 28% of inactivation of HBs Ag and the maximum inactivation of 59% was observed at 200 mg/ml and there was a decline in inhibition as 50% at 400 mg/ml.

4.3.9.2 Isoniazid (INH) and Rifampicin (RMP)-induced Hepatotoxicity in Rats

4.3.9.2.1 Effect on Body Weight and Liver Weight Changes

The body weight and relative liver weights of the experimental animals calculated at the end of the study had no significant difference statistically when compared to control (Table 33).

4.3.9.3 Effect on Changes in Biochemical Parameters

In rats treated with INH + RMP (group - 2), there was a significant liver damage observed from elevated serum levels of hepatic marker enzymes (AST, ALT and ALP) and changes in other biochemical parameters (Table 33; Figs. 47 & 48). Serum bilirubin level was drastically increased in group-2 whereas levels of total proteins were significantly decreased ($p<0.001$) when compared to control (Figs. 49 & 50). Serum triglycerides and serum total cholesterol levels in group-2 were

Table 32. Effect of Methanol Extract of *Phyllanthus wightianus* and Bergenin on *in vitro* Inactivation of HBsAg

S. No.	Methanol extract (mg/ml)	Bergenin (mg/ml)	% inhibition of anti-HBs binding	
			Methanol extract	Bergenin
1	0.25	12.5	0	0
2	0.50	25.0	0	0
3	1.00	50.0	12	28
4	2.00	100.0	30	50
5	4.00	200.0	42	59
6	8.00	400.0	56	50

Table 33. Hepatoprotective Activity of Solvent extracts of *Phyllanthus wightianus* and Bergenin on INH + rifampicin induced Hepatotoxcicity in Rats

S. No.	Treatment	Body weight (g)	Relative liver weight (g)	Serum Biochemical parameters						
				SGOT/ AST (Units/ml)	SGPT/ALT (Units/ml)	ALP (KA Units)	Bilirubin (mg/dl)	Total protein (g/dl)	Serum triglycerides (mg/dl)	Serum total cholesterol (mg/dl)
1	Control 1% w/v SCMC (10 ml/kg)	154± 18	4.12±0.23	41.80± 1.9	31.69± 1.92	13.13± 0.5	0.88± 0.03	9.23± 0.14	53.5± 1.6	104.9± 9.2
2	Toxicant & INH+ rifampicin (50 mg/kg)	153± 12	4.15±0.21	200.41± 5.3[a]	212.92± 3.2[a]	40.55± 1.6[a]	2.63± 0.34[a]	4.52± 0.12[a]	112.6± 0.6[a]	140.3± 4.6[a]
3	Silymarin & INH + rifampicin (100 mg/kg + 50 mg/kg)	151± 13	4.31±0.40	77.53± 3.3[c]	43.41± 3.8[a]	15.63± 0.2[a]	0.96± 0.6[a]	8.63± 0.11[a]	66.7± 1.3[a]	108.27± 3.8[a]
4	Bergenin & INH + rifampicin (200 mg/kg + 50 mg/kg)	152± 12	4.20±0.29	82.15± 6.8[c]	48.32± 6.3[c]	17.02± 1.8[c]	0.99± 0.4[c]	8.70± 0.11[c]	68.6± 1.6[c]	109.6± 5.3[c]
5	Hexane extract & INH + rifampicin (200 mg/kg + 50 mg/kg)	154± 15	4.23±0.10	98.71± 2.3[c]	69.14± 6.4[c]	23.14± 6.3[c]	1.41± 0.26[c]	6.7± 0.16[c]	80.13± 6.3[c]	120.46± 6.3[c]
6	Chloroform extract & INH + rifampicin (200 mg/kg + 50 mg/kg)	153± 13	4.21±0.18	101.72±3.0[b]	88.05± 1.5[b]	28.59± 1.5[b]	1.56± 0.34[b]	5.81± 0.13[b]	97.16± 7.6[b]	130.57± 6.4[b]
7	Methanol extract & INH + rifampicin (200 mg/kg + 50 mg/kg)	153± 16	4.27±0.11	89.61± 4.6[c]	53.51± 3.9[c]	19.16± 1.7[c]	1.12± 0.3[c]	7.57± 0.13[c]	72.7± 3.5[c]	112.39± 5.4[c]

P Values [a]<0.001 when compared to normal control group; [b] <0.05 when compared to INH + rifampicin treated groups; [c]<0.001 when compared to INH + rifampicin treated groups

Fig. 47. Level of SGOT and SGPT in hepatoprotective activity of solvent extracts of *Phyllanthus wightianus* and bergenin

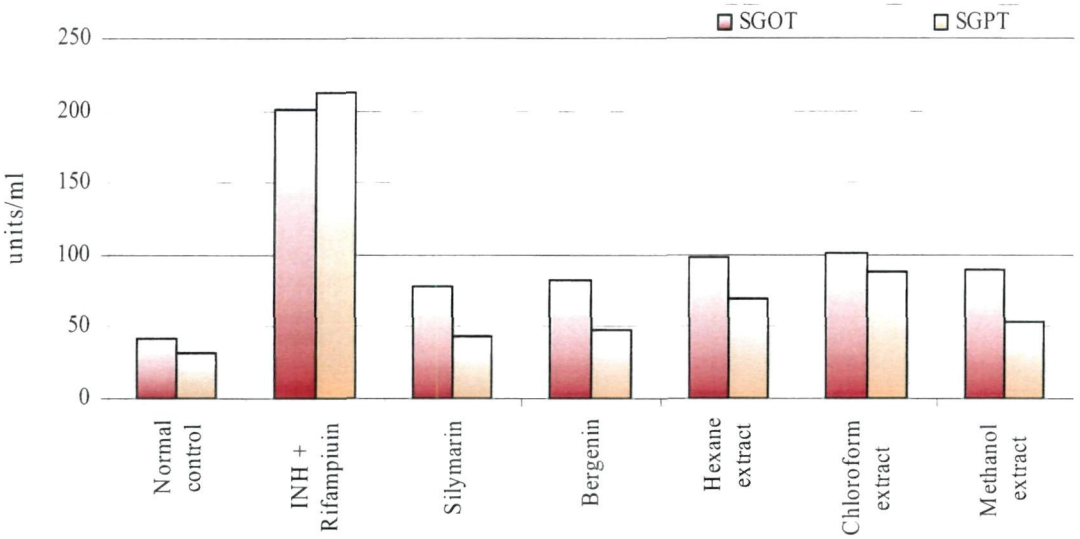


Fig. 48. Level of ALP in hepatoprotective activity of solvent extracts of *Phyllanthus wightianus* and bergenin

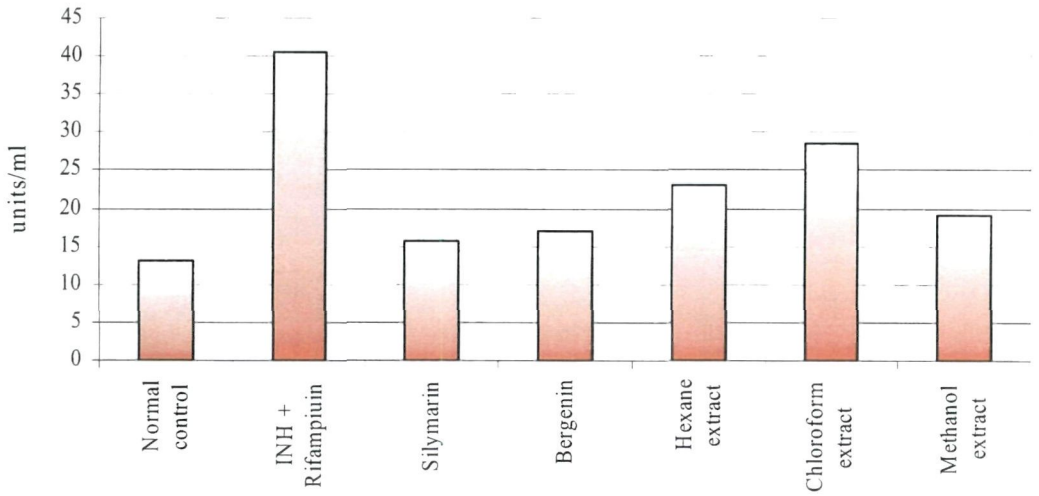


Fig. 49. Level of total protein in hepatoprotective activity of solvent extracts of *Phyllanthus wightianus* and bergenin

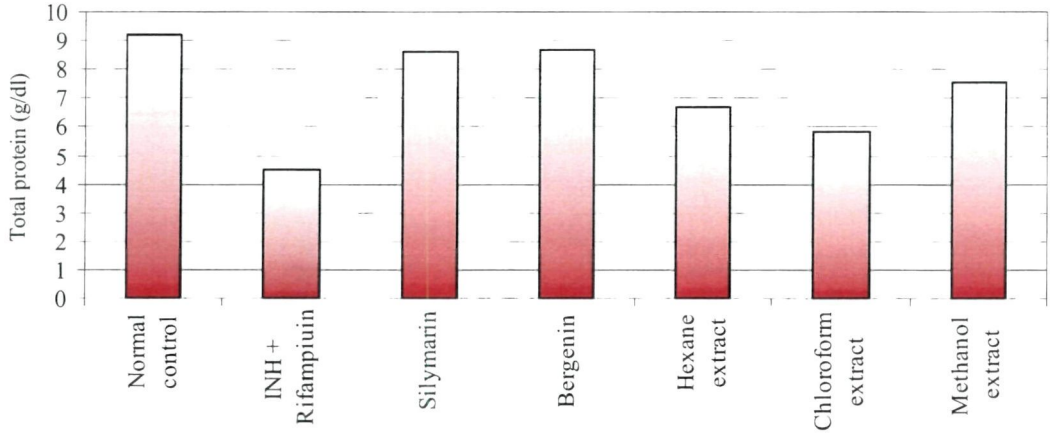
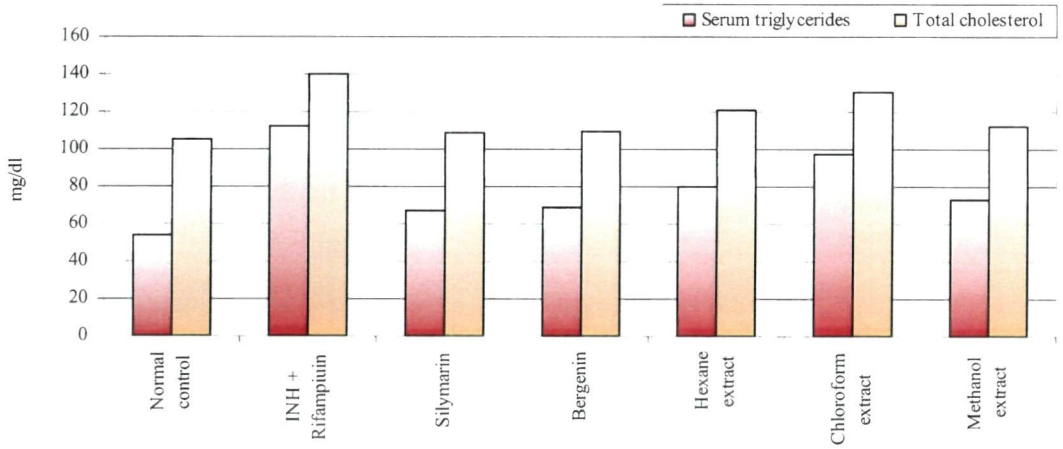


Fig. 50. Level of serum triglycerides and serum total cholesterol in hepatoprotective activity of solvent extracts of *Phyllanthus wightianus* and bergenin



significantly enhanced by INH+RMP administration. Histopathological changes also confirmed this observation when compared to normal animal liver tissue (Fig. 51.1) with centrilobular necrosis and severe fatty changes of liver (Fig. 51.2) Oral administration of silymarin ($p<0.001$), bergenin ($p<0.001$) and methanol ($p<0.001$), hexane ($p<0.001$) and chloroform ($p<0.05$) extracts significantly decreased elevated levels of serum AST, ALT and ALP. Drug treatment significantly reduced elevated serum bilirubin level and raised total protein levels significantly. Treatment with test extracts, standard drug and bergenin significantly brought back increased levels of serum triglycerides and serum total cholesterol to near normality.

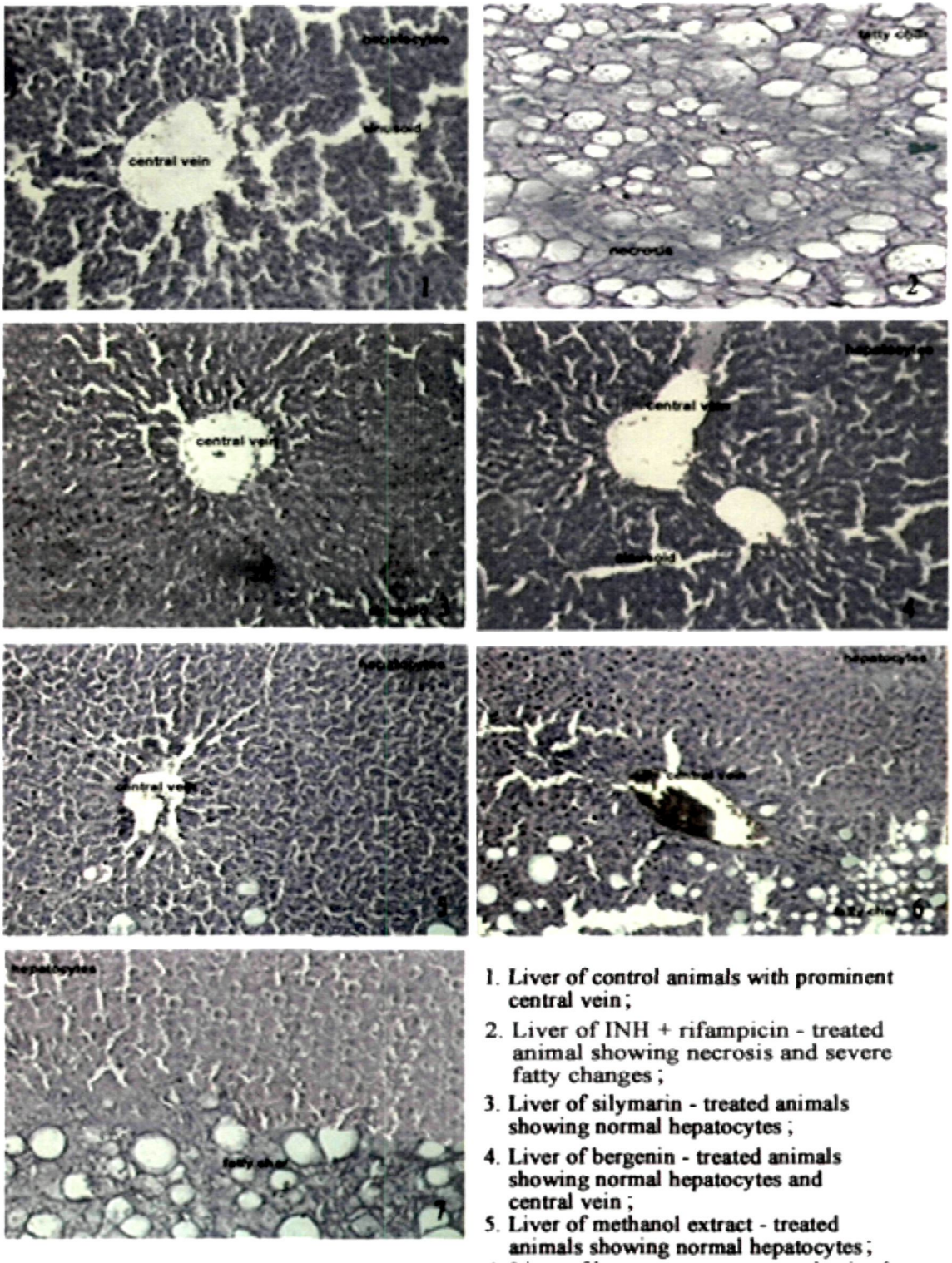
Maximum degree of protection was observed to silymarin-treated rats. This group showed serum hepatic marker enzymes such as AST, ALT and ALP (77.53±3.30, 43.41± 3.80, 15.63±0.20 respectively) almost comparable to AST, ALT and ALP levels of 82.15 ±6.80, 48.32±6.30 and 17.02±1.80 respectively for bergenin and 89.61±4.60, 53.51±3.90 and 19.16±1.70 respectively for methanol extract (Table 33; Figs. 47 & 48). This observation was followed by hexane (98.71±2.30, 69.14±6.40, 23.14±6.30) and chloroform (101.72±2.96, 88.05±1.50 and 28.59±1.50) extracts respectively. Chloroform extract exhibited moderately significant protection when compared to that of silymarin.

Among the test drugs, bergenin and methanol extract exhibited a high degree of protection whereas hexane extract exhibited a moderate level of protection and less degree of protection in the case of chloroform extract in terms of recovery of serum bilirubin, total proteins, serum triglycerides and serum cholesterol.

4.3.9.4 Histopathology

Histological observations also supported biochemical parameters. Normal rat liver (group-1) showed (Fig. 51.1) liver with rows of hepatocytes separated by

Fig.51. Histopathology of solvent extracts and bergenin treated animals in comparison with control



1. Liver of control animals with prominent central vein;
2. Liver of INH + rifampicin - treated animal showing necrosis and severe fatty changes;
3. Liver of silymarin - treated animals showing normal hepatocytes;
4. Liver of bergenin - treated animals showing normal hepatocytes and central vein;
5. Liver of methanol extract - treated animals showing normal hepatocytes;
6. Liver of hexane extract - treated animals showing moderate fatty lobules and central vein; and
7. Liver of chloroform extract - treated animals showing increased accumulation of fatty lobules

sinusoids radiated from central vein. Group-2 animals exhibited hepatocytic necrosis and severe fatty changes (Fig. 51.2). Simultaneous administration of toxic substances with silymarin (group-3), bergenin (group-4) and methanol extract (group-5) prevented introduction of histopathological injuries. There is no evidence of necrotic areas. showing significant signs of hepatoprotection revealed by the presence of normal hepatic cords, absence of necrosis and fatty infiltration (Figs. 51.3-51.5) whereas animals co-treated with hexane and chloroform extracts showed larger areas of normal hepatocytes with a moderate accumulation of fatty lobules (Figs. 51.6 & 51.7).

CHAPTER 5

DISCUSSION

5.1 PHYTOCHEMISTRY

5.1.1 Qualitative Analysis

The extractive values of *P. wightianus* which positively correlate the nature of the constituents and to evaluate the total content of the secondary metabolites present in the crude extracts are useful to fix pharmacopoeial standard. It was higher in methanol extract such as 14.70% whereas lower values were registered for chloroform and hexane extracts such as 3.60% and 2.30%.

The total ash value was found to be 8.29%, which showed the presence of inorganic constituents. The low value of 1.25% for acid insoluble ash indicated the presence of negligible amount of siliceous matter. The water soluble ash was found to be 1.40%. These studies form the basis for judging the identity and purity of the crude drugs.

5.1.2 Quantitative Analysis of Inorganic Elements (Salts and Minerals)

Determination of elements in the medicinal plant drugs and its extracts is of special importance. Therapeutic applications introduce these minerals into the human body. The favorable effect of the extract is presumable considering the combined effect of organic and inorganic compounds such as metal iron complexes of organic constituents.

There are fourteen trace minerals thought to be essential. They are chromium, cobalt, copper, fluorine, iodine, iron, manganese, molybdenum, nickel, selenium, silicon, tin, vanadium and zinc. Barthakur *et al.* (2005) and Gennaro (2004) have documented the importance of minerals in human nutrition and health. Even though

they are needed in minute quantities they play a bigger role for the normal and better health of a person. Such minerals include calcium, chlorine, magnesium, phosphorus, potassium, sodium and sulphur. According to Recommended Dietary Allowances (RDA, 1989), per day intake-need of an adult is 2000 mg potassium, 800 mg calcium, 350 mg magnesium, 15 mg zinc, 2.5 mg manganese and 1.5 – 3.0 mg copper. In the present study, a one g plant material contained high amounts of sodium (2.960 mg), potassium (1.200 mg), calcium (6.300 mg), iron (2.130 mg) and magnesium (1.089 mg) while other minerals such as manganese (0.345 mg), copper (0.060 mg) and cobalt (0.003 mg) were present in trace quantities. So, it is concluded that the plant extract is a good source of calcium, potassium and sodium and iron.

5.1.3 HPLC Analysis and Estimation of Tannins and Lignans

5.1.3.4 Tannins

The HPLC fingerprint of the methanol extract of *P. wightianus* (Fig. 9) showed 36 peaks with various retention times (quantified by the area of peak) along with standard tannins. The following tannins were identified in comparison with retention time of the standards such as gallic acid (GA) - 5.158 (standard 5.092), corillagin (C) - 18.900 (18.875), geraniin (G) - 20.292 (19.817) and ellagic acid (EA) - 27.617 (27.592). Corillagin was present most abundantly (3.89%), followed by geraniin (3.19%). Ellagic acid (0.68%) and gallic acid (0.38%) were present in minute quantities.

5.1.3.5 Lignans

Analysis of the HPLC fingerprint of the methanol extract of *P. wightianus* showed 8 peaks with various retention times wherein absence of peaks at R_t = 13.20 for hypophyllanthin and 16.50 for phyllanthin indicated their absence (Fig. 11).

5.1.4 GC-MS Analysis of Lipids

GC-MS analysis of hexane: benzene (4:1) fraction of the hexane extract showed 23 peaks (Fig.12) wherein 7 compounds could be identified unequivocally (Table 8).

5.1.5 Isolation and Characterization of Compounds from Various Extracts (Figs. 52-70)

The compound PW1 eluted with hexane: benzene (1:1) gave a colourless material, which was crystallized from acetone (m.p. 263°C). IR spectrum showed no absorption for hydroxyl group. It showed absorption at 1716 cm^{-1} assignable to cyclohexanone and 1389 cm^{-1} assignable to gem-dimethyl group (Fig. 52). The ^{1}H NMR spectrum showed seven tertiary methyl groups in this region δ 0.65 –1.1 including the secondary methyl at δ0.80 appearing as doublet J = 6.8 Hz (Fig. 53). The ^{13}C NMR spectrum also showed the relevant signals for friedelin (Ali *et al.*, 1999). The three keto carbonyl carbon appeared at δ 213.18 (Fig. 54). The IR, ^{1}H and ^{13}C NMR spectral data determined the structure of the compound as friedelin. The compound PW1 further confirmed by superimposibility of HPTLC chromatogram of the authentic compound (Fig. 3).

The compound PW2 eluted with benzene gave an amorphous powder, which was crystallized from acetone as colourless needles (m.p.213 – 215°C). The IR spectrum showed an intense band at 3360 and 1643 cm^{-1} corresponding to hydroxyl group and vinylidine group at 1637 and 790 cm^{-1} (Fig. 55). The ^{1}H NMR spectrum showed the presence of 6 tertiary methyl groups at δ 0.76 - 1.68 (Fig. 56). The vinylic methyl group attached to C – 20 position. The H – 3 axial protons appeared at δ 3.20 as a multiplet. H-29 methylene protons appeared as broad singlet at δ 4.56 and 4.68. H – 19 appeared as multiplet at δ 2.40. The ^{13}C NMR spectrum (Fig. 57) also

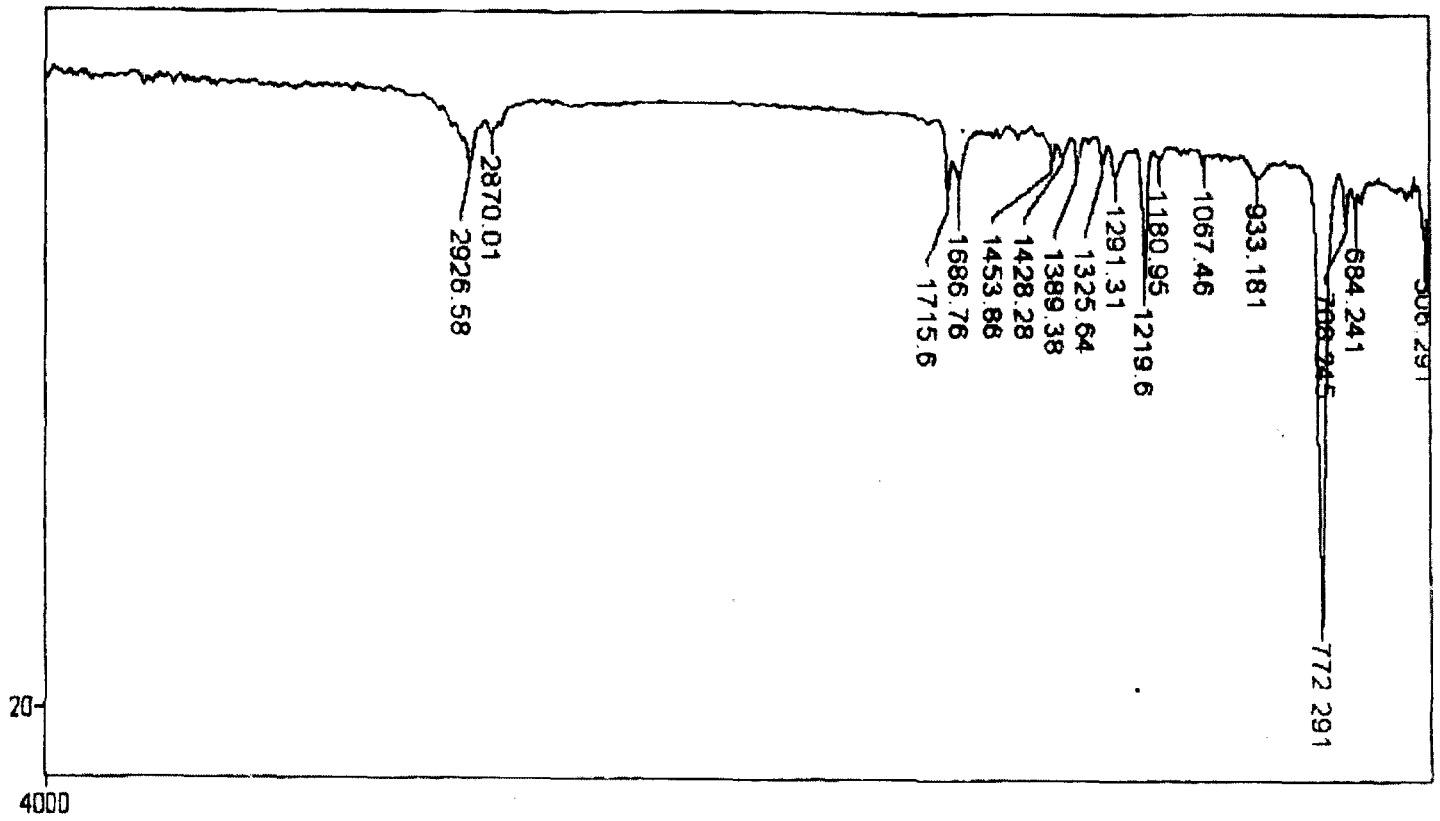
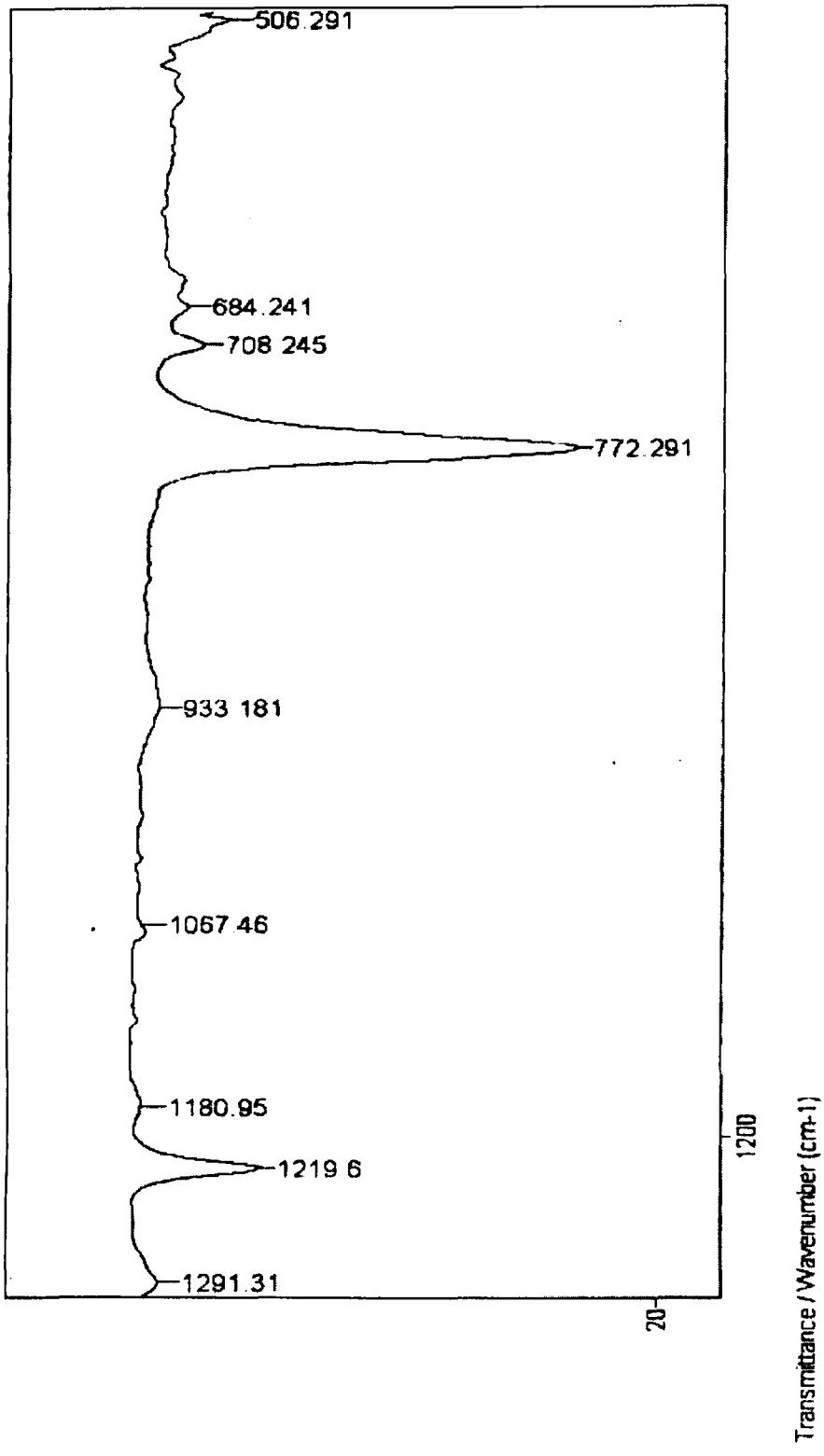


Fig.52. IR spectrum of friedelin


506.291
684.241
708 245
772.291
933 181
1067.46
1180.95
1219 6
1291.31
1200
20
Transmittance / Wavenumber (cm-1)

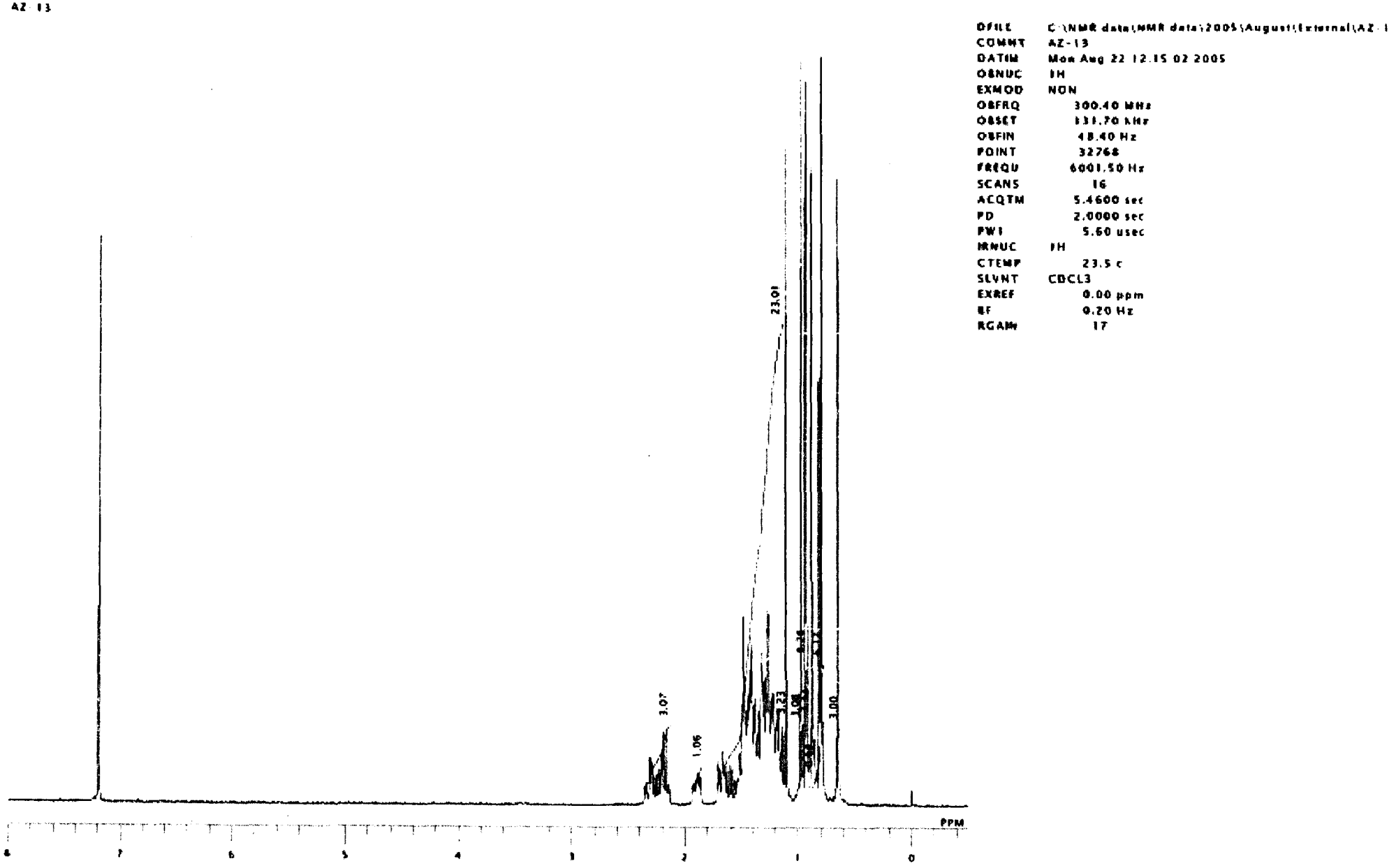
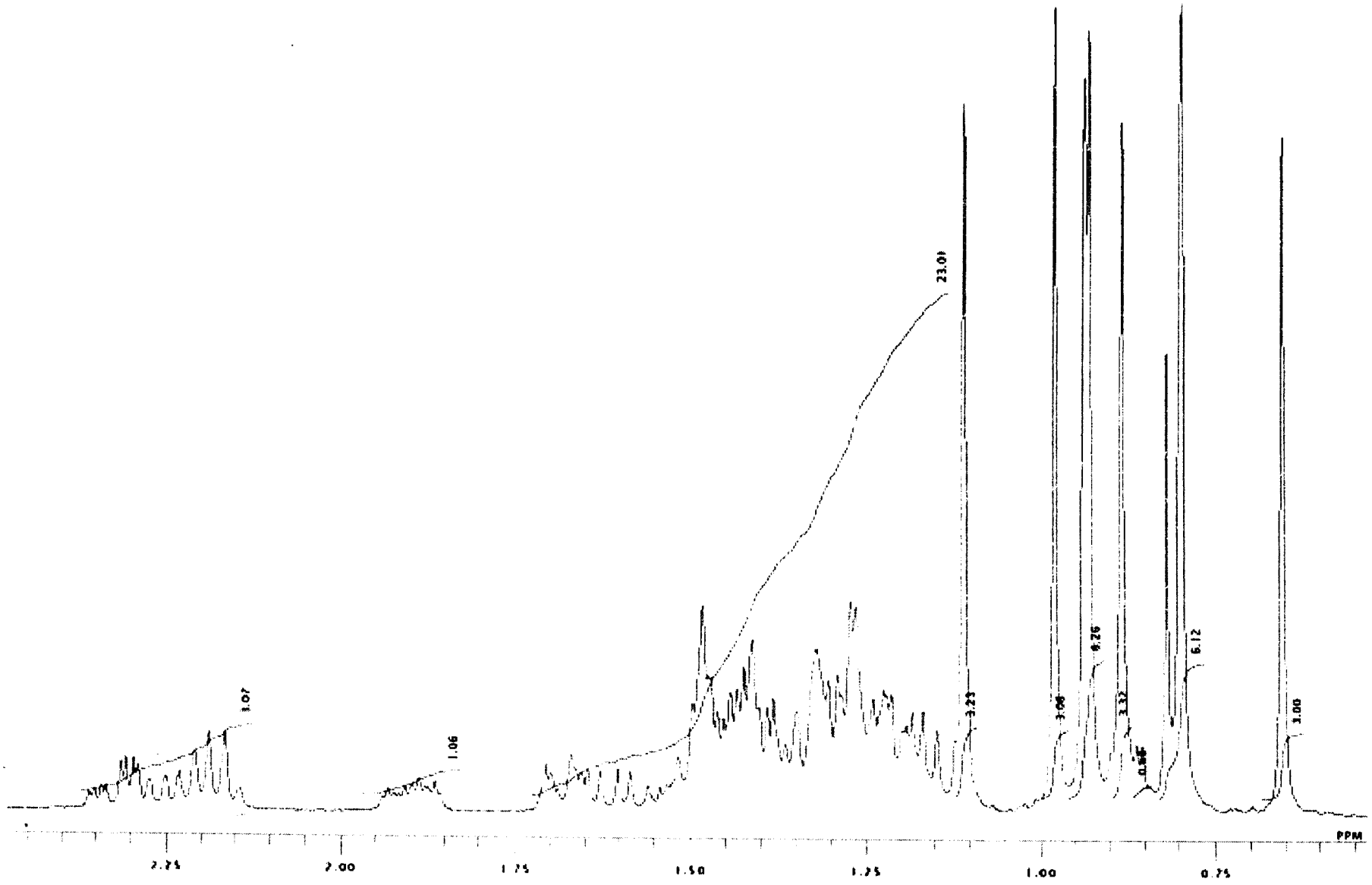


Fig.53. ^{1}H NMR spectrum of friedelin

AZ-13


3.07
1.06
23.01
3.23
3.08
6.26
3.32
6.12
3.00
PPM
2.25
2.00
1.75
1.50
1.25
1.00
0.75

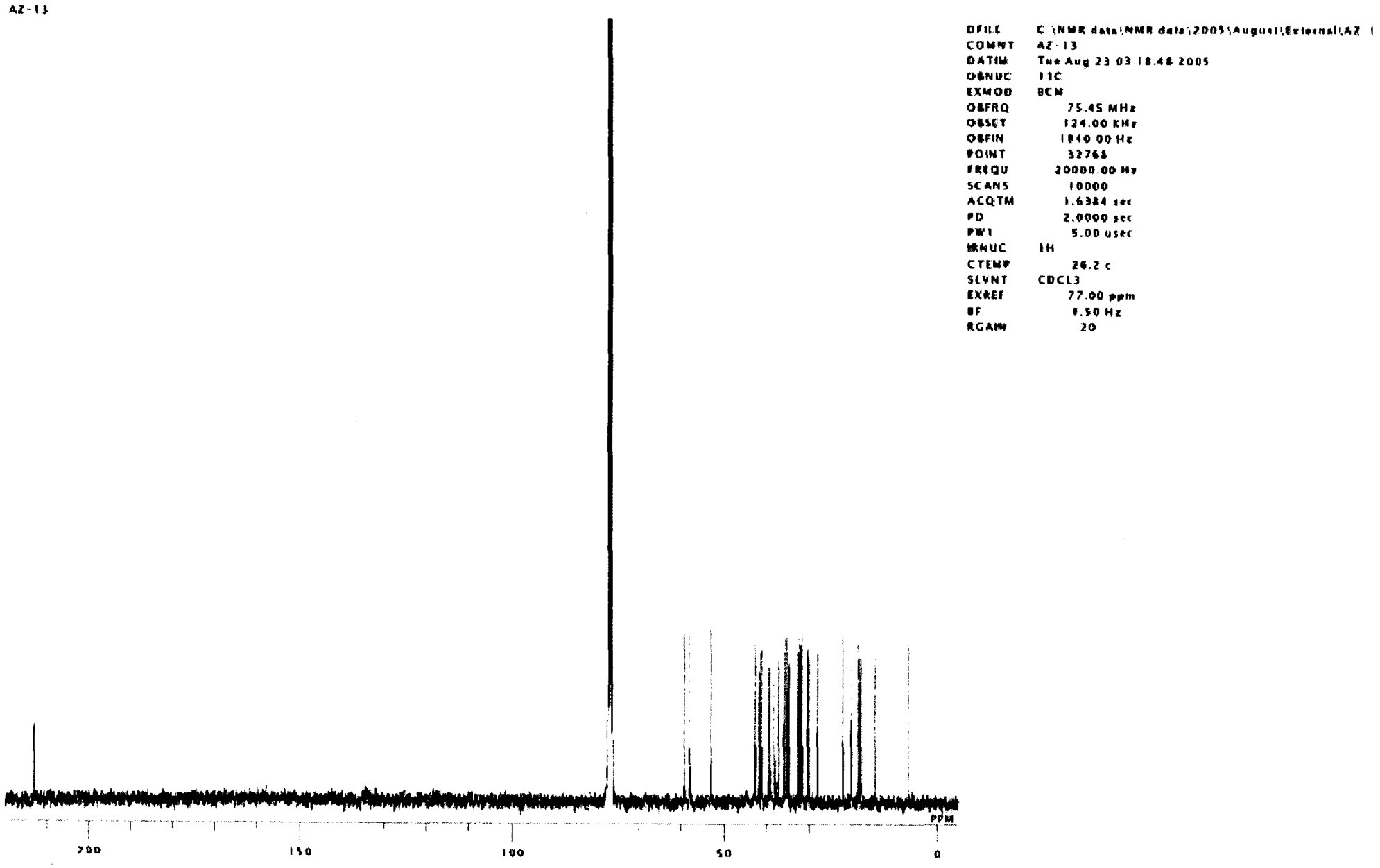
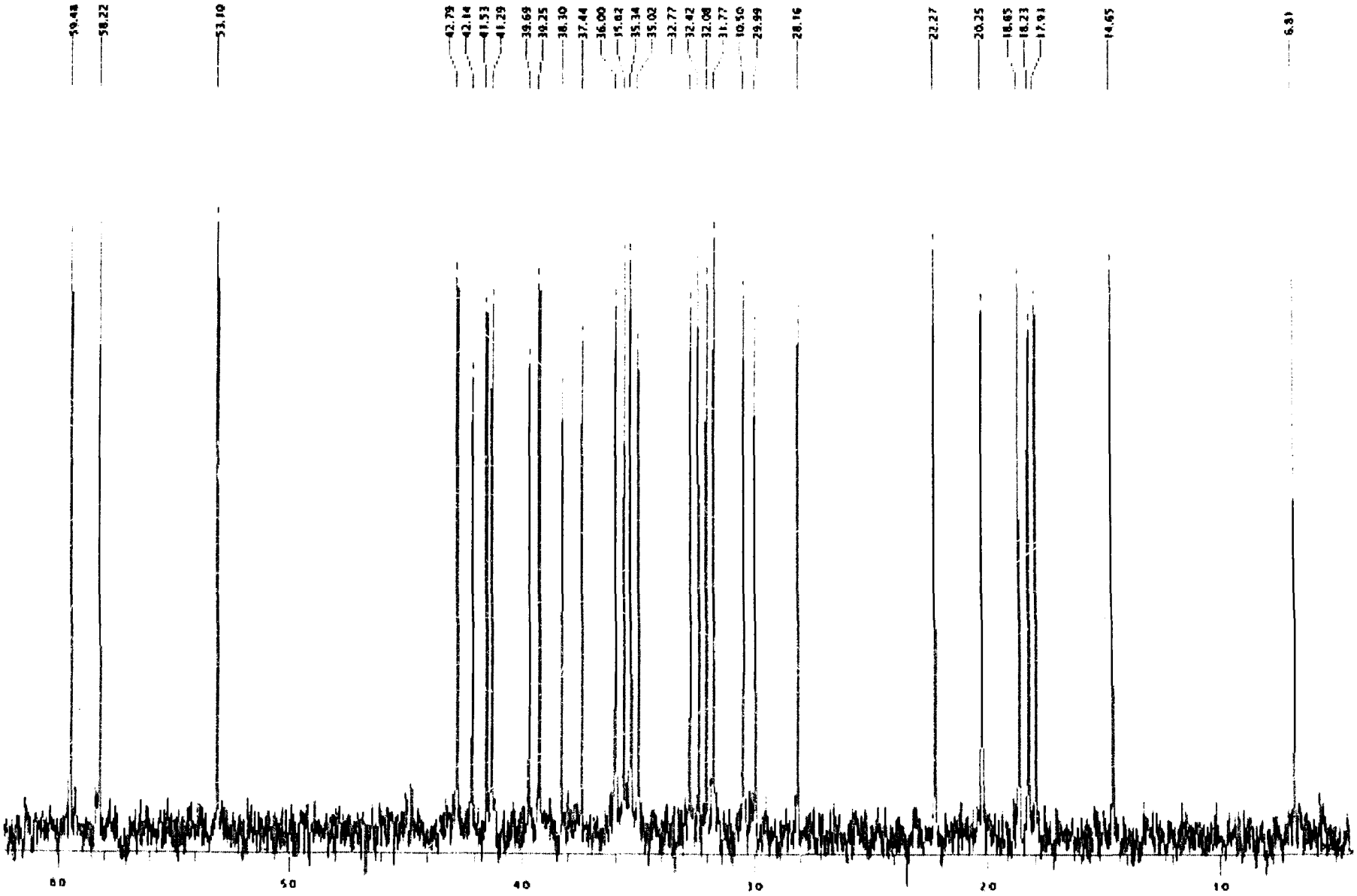


Fig.54. ^{13}C NMR spectrum of friedelin

AZ 13


59.48
58.22
53.10
42.79
42.14
41.53
41.29
39.69
39.25
38.30
37.44
36.00
35.34
35.02
32.77
32.42
32.08
31.77
30.50
29.99
28.16
22.27
20.25
18.65
18.23
14.65
80
60
40
30
20
10

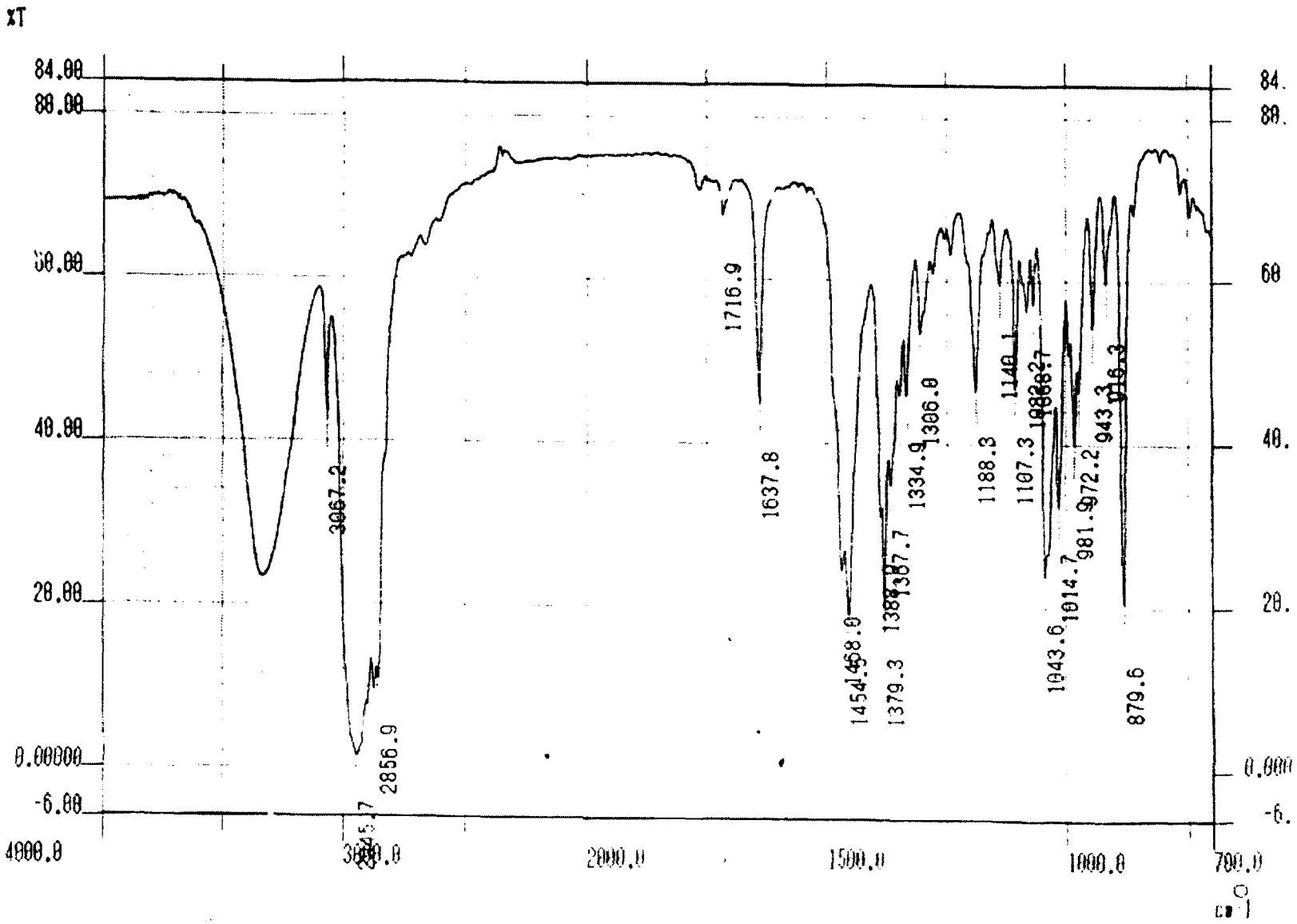
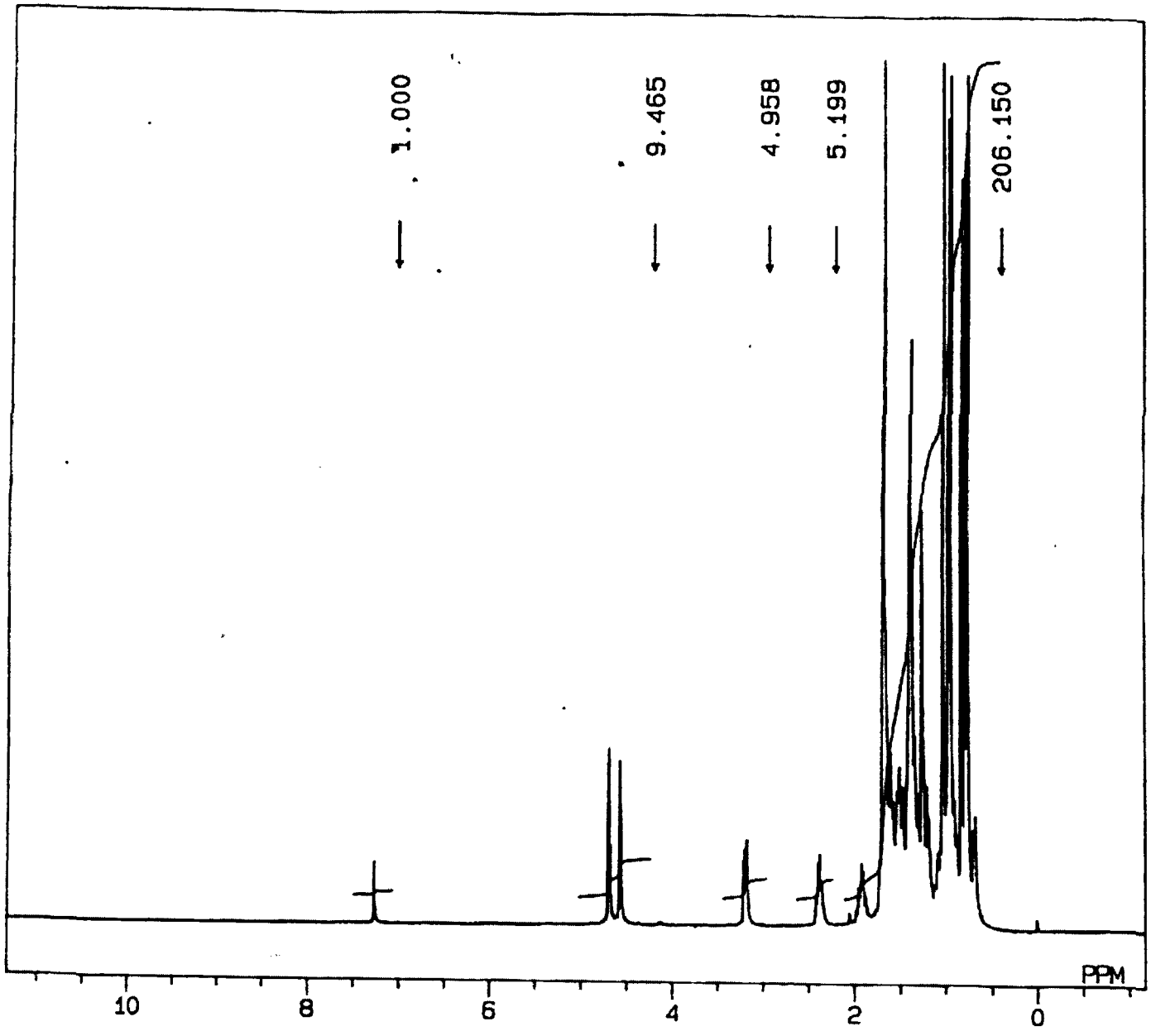
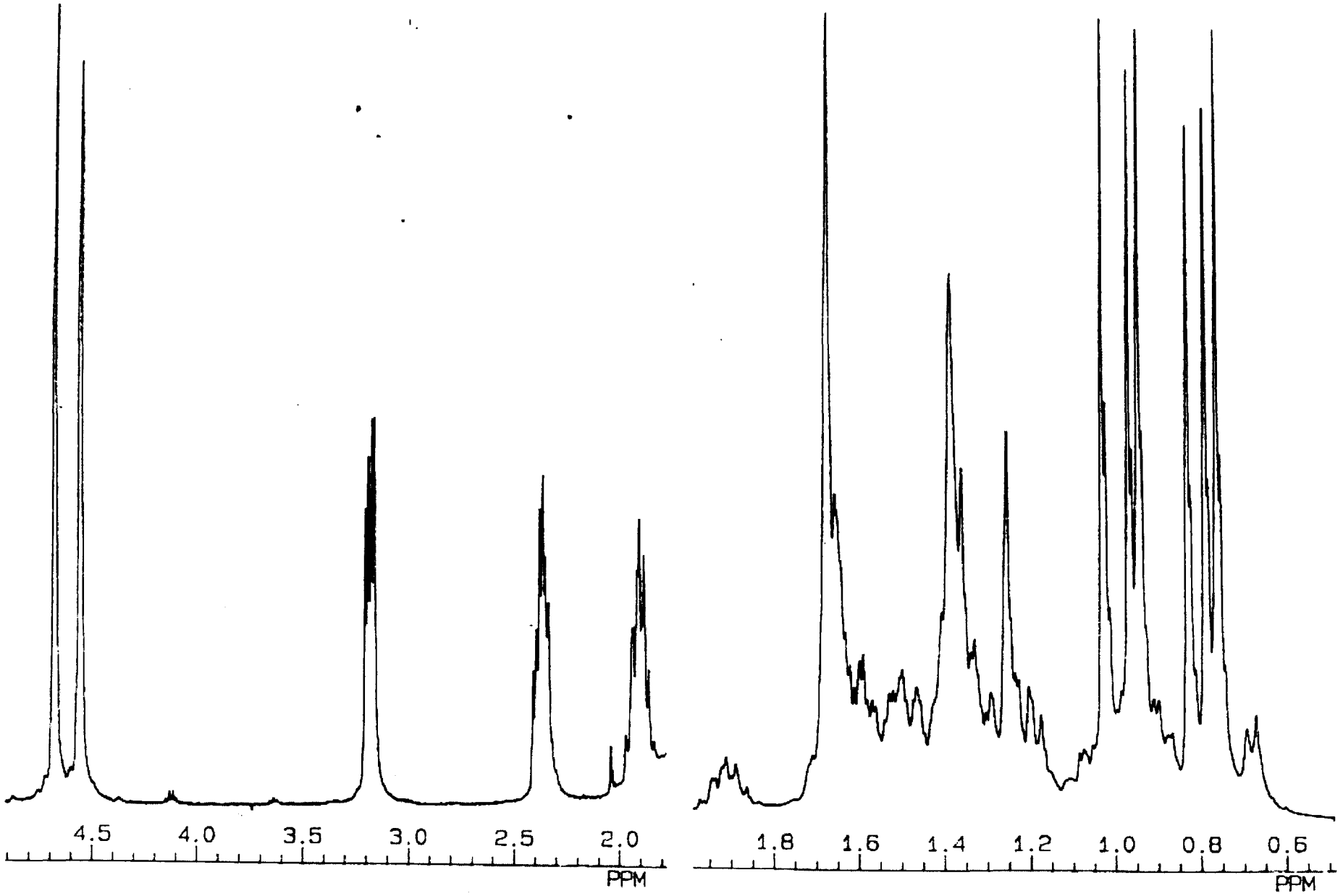


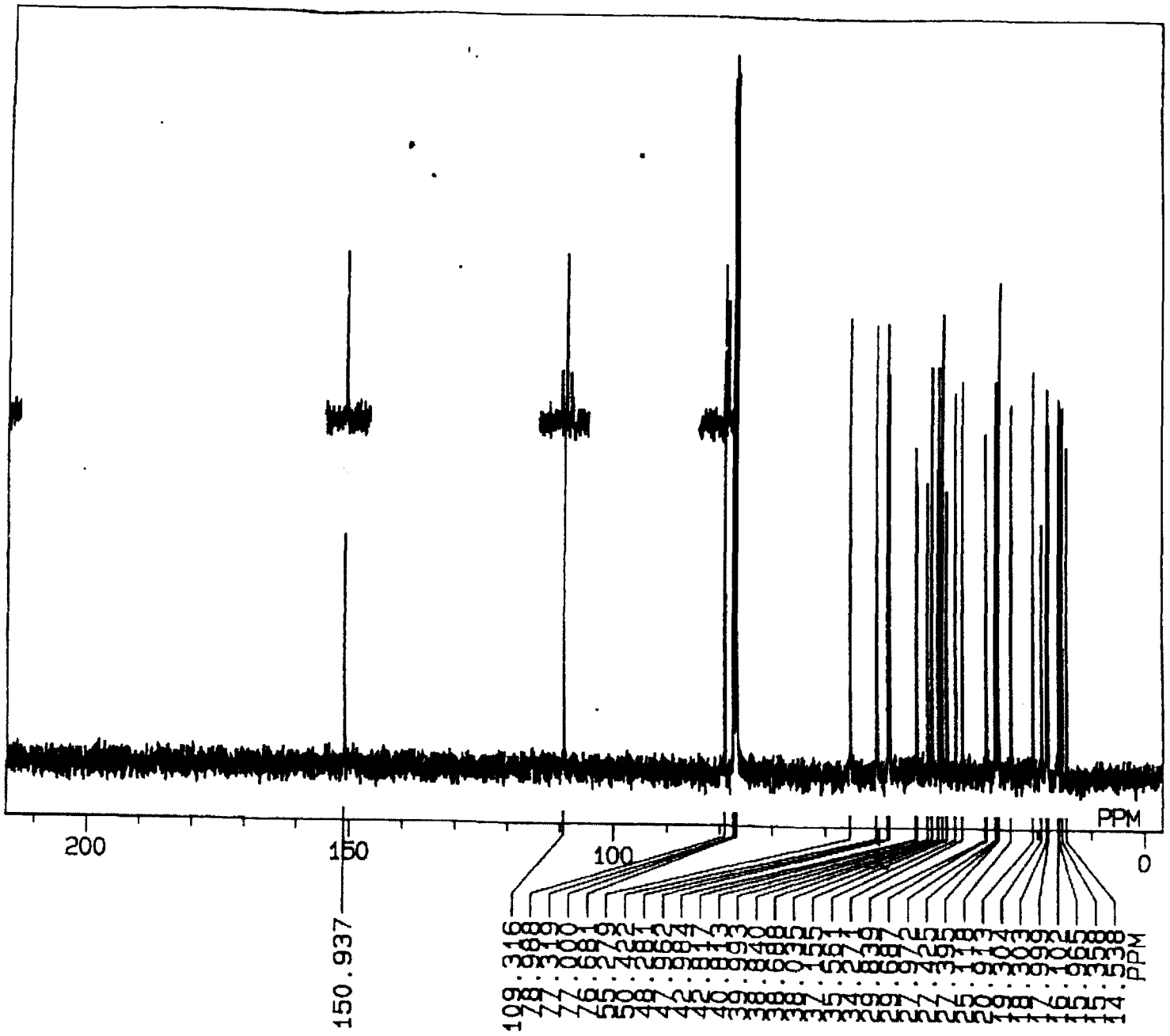
Fig.55 . IR spectrum of lupeol



EXMOD [70, 11] SGNON
OBFRQ 399.65
OBNUC 1H
SCANS 128
ACQTM 2.048
PD 3.270
PW1 8.0
RESOL 0.49
TEMP. 23.0 c
SPEED 15
SLVNT CDCL3
YG 3.42
YG2 9.1265
RGAIN 13
XE 5000.0000
XS 0.0000
Hz/cm
VALUE 250.0000
DFILE PBLU01
INSTRUMENT: JEOL
MODEL: GSX 400

Fig.56. ^{1}H NMR spectrum of lupeol


4.5
4.0
3.5
3.0
2.5
2.0
PPM
1.8
1.6
1.4
1.2
1.0
0.8
0.6
PPM



EXMOD SGCOM
OBFRQ 100.40
OBNUC 13C
SCANS 400
ACQTM 0.655
PD 1.689
PW1 4.0
RESOL 1.53
TEMP. 23.0 c
SPEED 15
SLVNT CDCL3
YG 2.77
YG2 2.7726
RGAIN 28
XE 22000.0000
XS 1200.0000
Hz/cm
VALUE 1100.0000
DFILE PBLU02
INSTRUMENT: JEOL
MODEL: GSX 400

Fig.57. ^{13}C NMR spectrum of lupeol

SAIF/IIT/B/
PPM
79.579 78.578.0
78.988 PPM
55.279
50.422
48.281
47.962
42.817
42.984
40.813
37.155
35.561
34.271
29.839
29.687
27.395
27.372
25.118
20.913
19.304
17.999
15.102
PPM
RPM
20
30
40
50
60

confirmed the structure of lupeol (Wang *et al.,* 1992). The compound PW2 further confirmed by superimposibility of HPTLC chromatogram of the authentic compound (Fig. 5).

The compounds PW3a – PW3c were eluted with benzene: chloroform (1:1) as sterol mixture. The IR spectrum of the sterol mixture showed the presence of hydroxyl (3431 and 1063 cm^{-1}) tri-substituted double bond (1638 and 838 cm^{-1}) and trans di-substituted double bond (970 cm^{-1}; Fig. 58) . The tri-substituted double bond peak arises due to - Δ^5 double bond. The trans di-substituted double bond peak arises due to $\Delta^{22\,(23)}$ double bond of stigmasterol. In the ^1H NMR spectrum of the sterol mixture, the methyls of the sterols appeared in the region δ 0.68 – 1.25 methyl doublets appeared at δ 0.84 and 0.91 (J ≈ 6.4 Hz) due to secondary methyls in the side chain (Fig. 59). H - 22 and H – 23 of stigmasterol appeared as dd at δ 5.15 and 5.01, the coupling contacts being 5.4, 8.6, 1.5 and 8.7 Hz respectively (Hung and Yen, 2001). The broad multiplet at δ 5.35 corresponds to H – 6 of the sterols. The multiplet at δ 3.52 corresponds to H – 3 of the sterols. The ^{13}C NMR also showed the presence of three sterols. C – 3 being the hydroxyl in all the sterols appeared at δ 71.8. C – 5 and C - 6 representing the tri-substituted double bond appeared at δ 140.7 and 121.7 (Fig. 60). The side chain trans di-substituted double bond of stigmasterol gave C -22 and C -23 at δ 129.2 and 138.3, C -18 and C -19 appeared at δ 21.0 and 23.0 and C-2 appeared at δ 19.8. The C – 29 methyl groups appeared at δ 11.9.

The compound PW4 eluted with ethyl acetate (100%) yielded a white amorphous powder (m.p. 250°C). The IR spectrum showed peaks for phenol (3364 and 3288 cm^{-1}), aromatic carboxylic acid (1713 cm^{-1}) and an aromatic peaks (1617, 1541, 1470, 1339, 1245, 1202, 1054, 1027, 866, 790, 763, 731 and 701 cm^{-1}; Fig. 61).

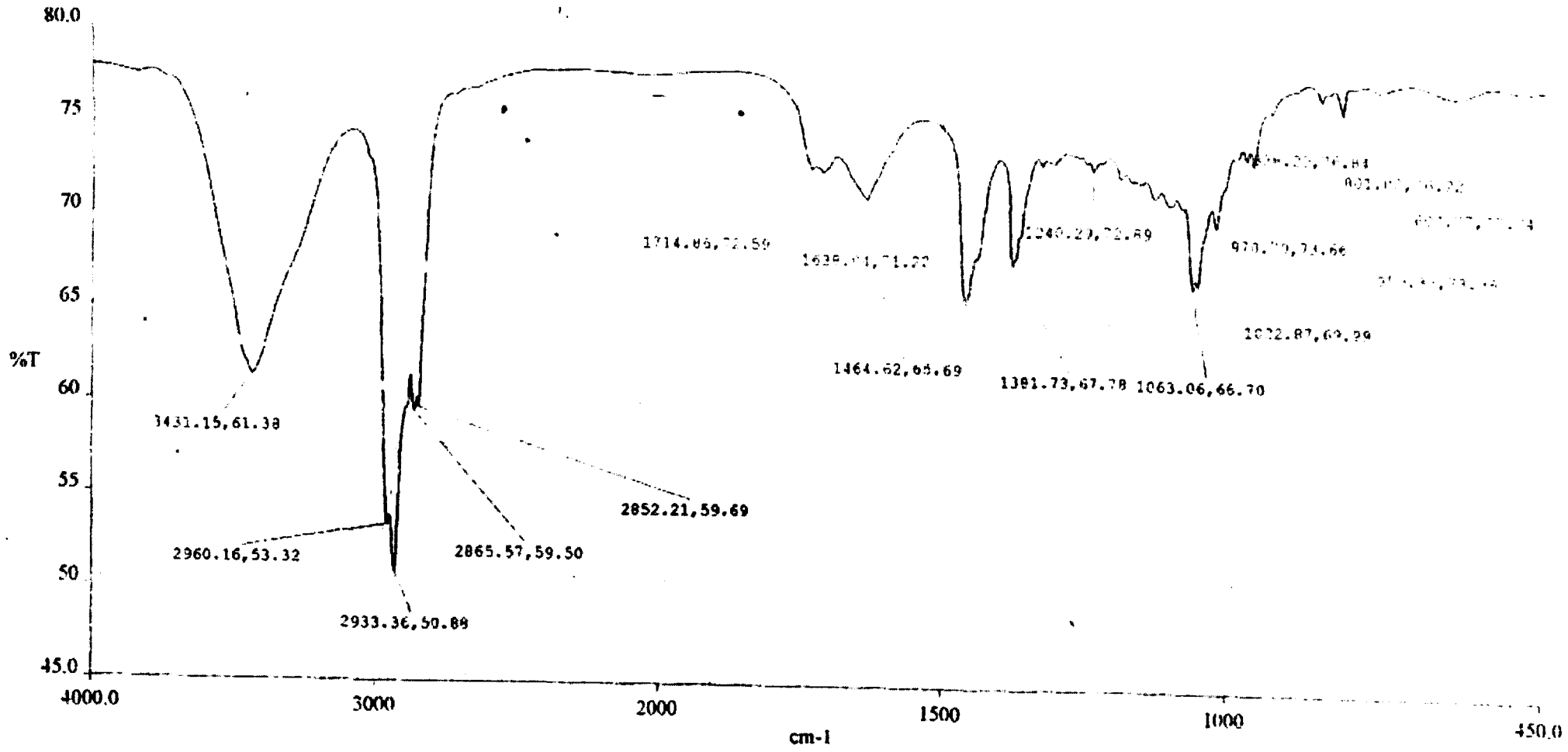
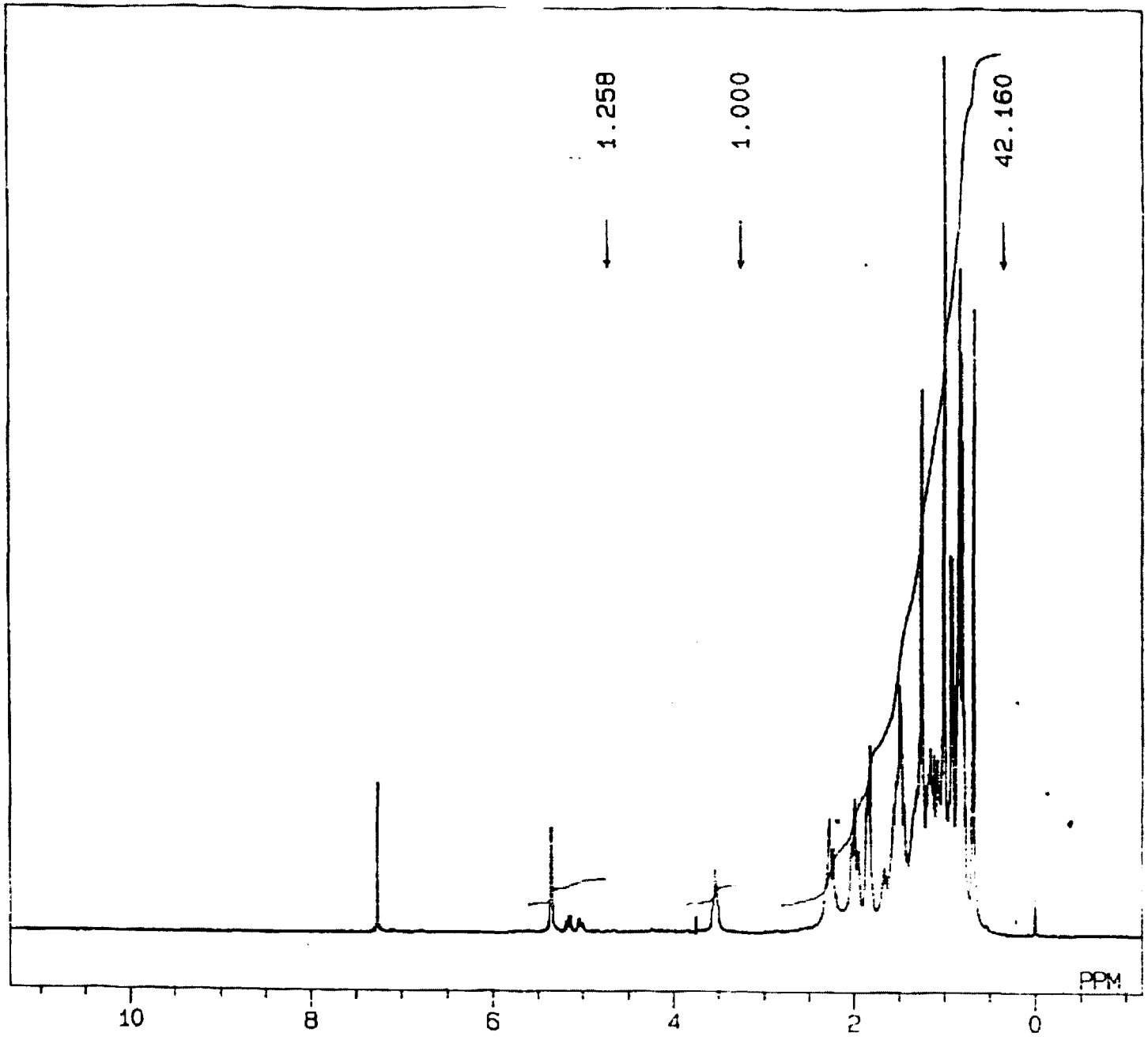


Fig.58. IR spectrum of sterol mixture

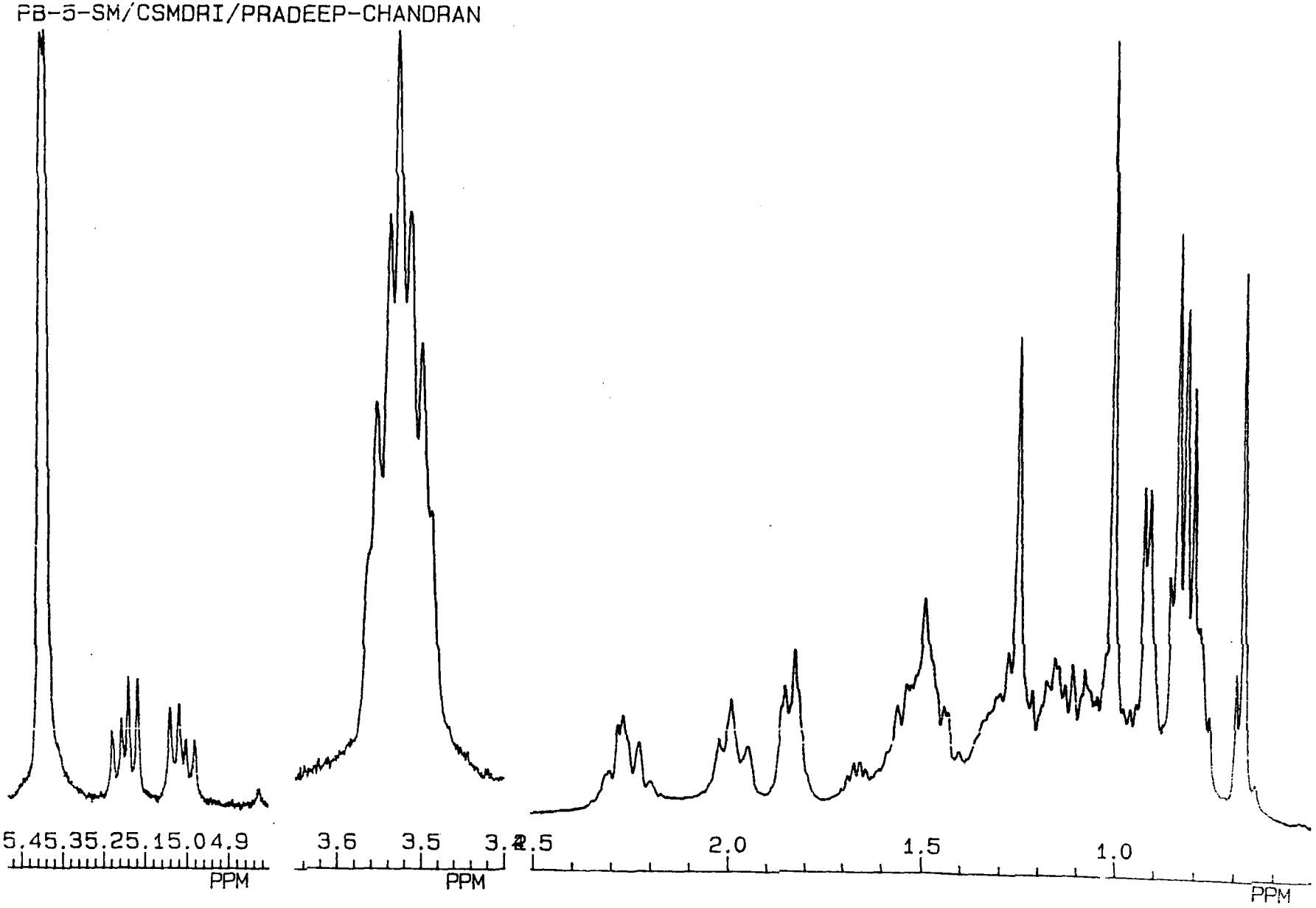
PEAK	22	
MAIN	570673700	
RESOL	0.4[illegible]	Hz
RESUL	0.0012257	PPM
CXREF	0.0000000	PPM
OBS	-2029.79	Hz
ABOBS	399[illegible]84.5000000	KHz
NGAIN	12	

NO.	PPM	INT(%)	FREQ(Hz)	POSITION	BAR GRAPH
1	7.2647	16.96506	2904.30	6401	+++
2	5.3447	12.00188	2136.72	7973	++
3	3.5249	7.46363	1409.18	9463	+
4	2.2717	13.20695	908.20	10489	+++
5	2.2314	9.81243	892.09	10522	++
6	1.9945	15.40756	797.36	10716	+++
7	1.8308	21.55671	731.93	10850	++++
8	1.4937	28.25770	597.17	11126	++++++
9	1.2519	61.98685	500.49	11324	++++++++++++
10	1.1591	21.11647	463.38	11400	++++
11	1.1127	20.34958	444.82	11438	++++
12	1.0809	19.79156	432.13	11464	++++
13	1.0076	100.00000	402.83	11524	++++++++++++++++++++
14	0.928[illegible]	43.07040	371.09	11589	+++++++++
15	0.91[illegible]	42.8232[illegible]	364.7[illegible]	11602	+++++++++
16	[illegible]	41.76400	343.75	11645	++++++
17	[illegible]8440	45.7073[illegible]	[illegible]	11658	+++++++++++++++
[illegible]	[illegible]	[illegible]	[illegible]	11676	++++++++++++
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	+++++++++++
[illegible]	[illegible]	[illegible]	[illegible]	11778	++++
[illegible]	[illegible]	41.07110	[illegible]	11793	++++++++++++++
[illegible]	[illegible]	4.[illegible]	[illegible]	12349	+



EXMOD [70, 11] SGNON
OBFRQ 399.65
OBNUC 1H
SCANS 64
ACQTM 2.048
PD 3.270
PW1 8.0
RESOL 0.49
TEMP. 23.0 c
SPEED 15
SLVNT CDCL3
YG 3.57
YG2 27.5636
RGAIN 17
XE 5000.0000
XS 0.0000
Hz/cm
VALUE 250.0000
DFILE PB5SMH
INSTRUMENT: JEOL
MODEL: GSX 400

Fig.59. ^{1}H NMR spectrum of sterol mixture


PB-5-SM/CSMDRI/PRADEEP-CHANDRAN
5.4 5.3 5.2 5.1 5.0 4.9
PPM
3.6
3.5
3.4
PPM
2.5
2.0
1.5
1.0
PPM

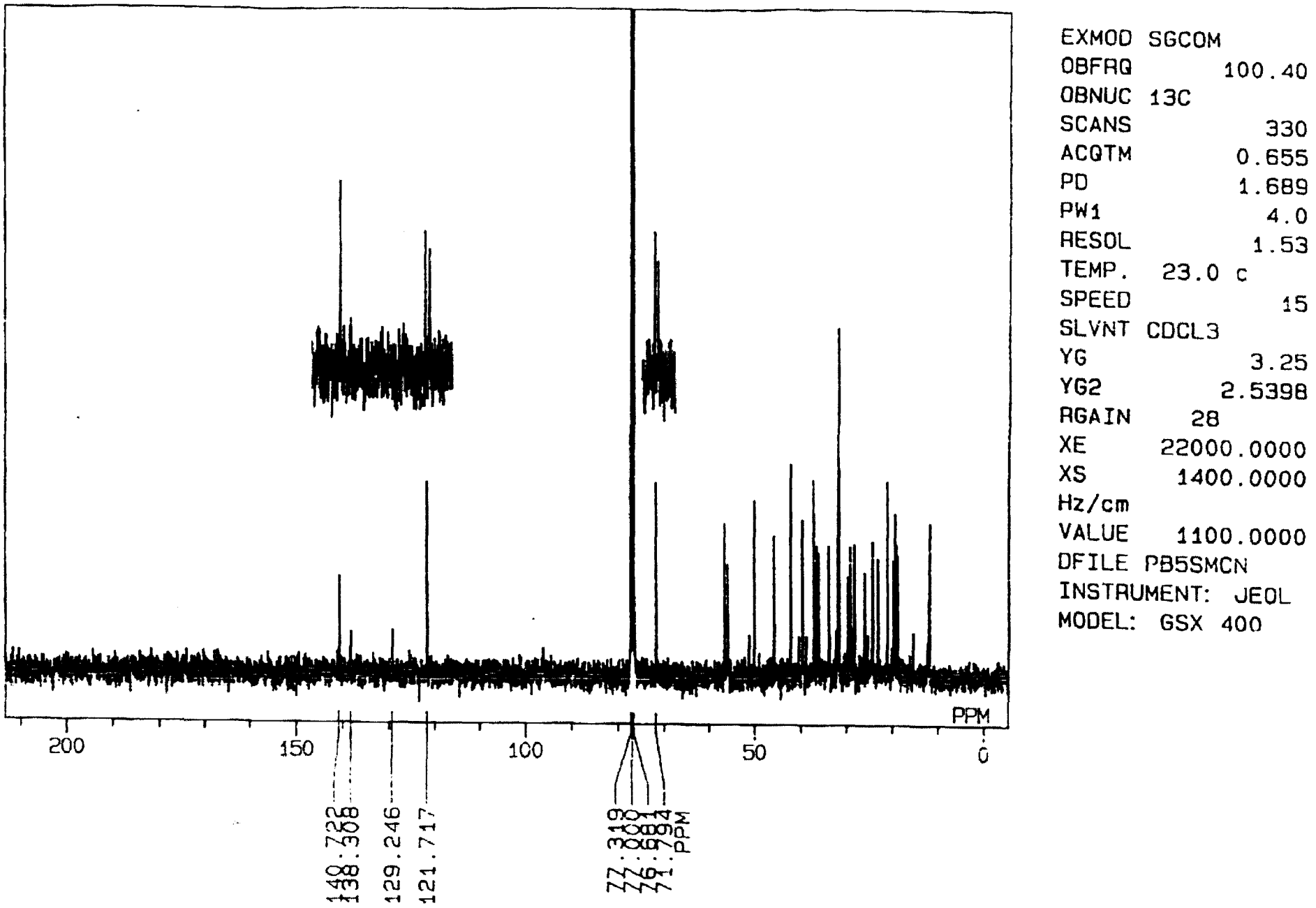


Fig.60. ^{13}C NMR spectrum of sterol mixture

PPM
11.852
11.973
15.358
18.773
19.014
19.356
19.821
21.065
23.039
24.283
25.408
26.029
28.245
28.913
29.125
29.702
31.630
32.088
32.404
33.694
33.922
36.138
36.487
37.231
38.810
39.751
40.494
42.301
42.740
45.807
50.103
51.225
55.931
56.092
56.754
PPM

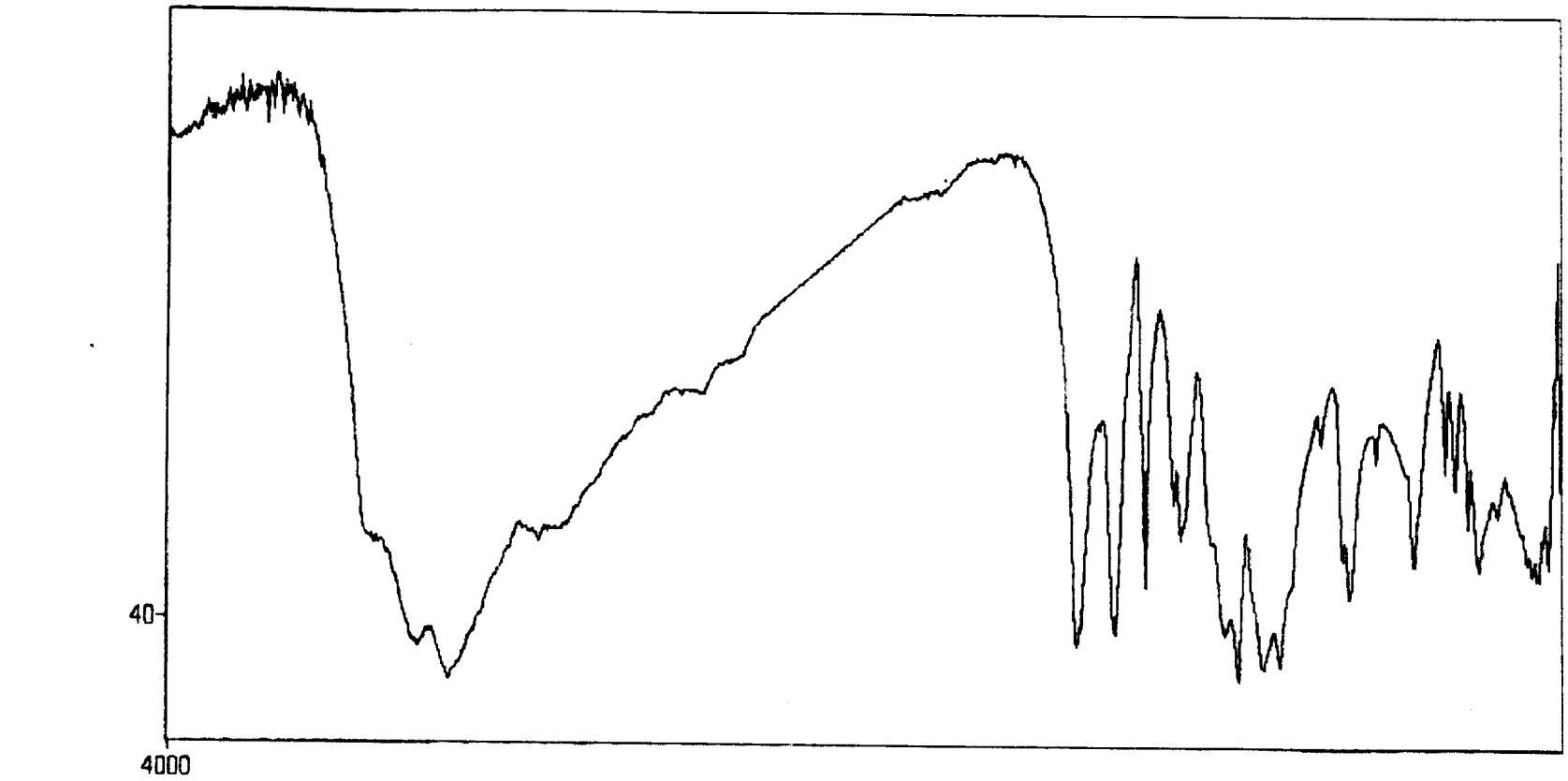
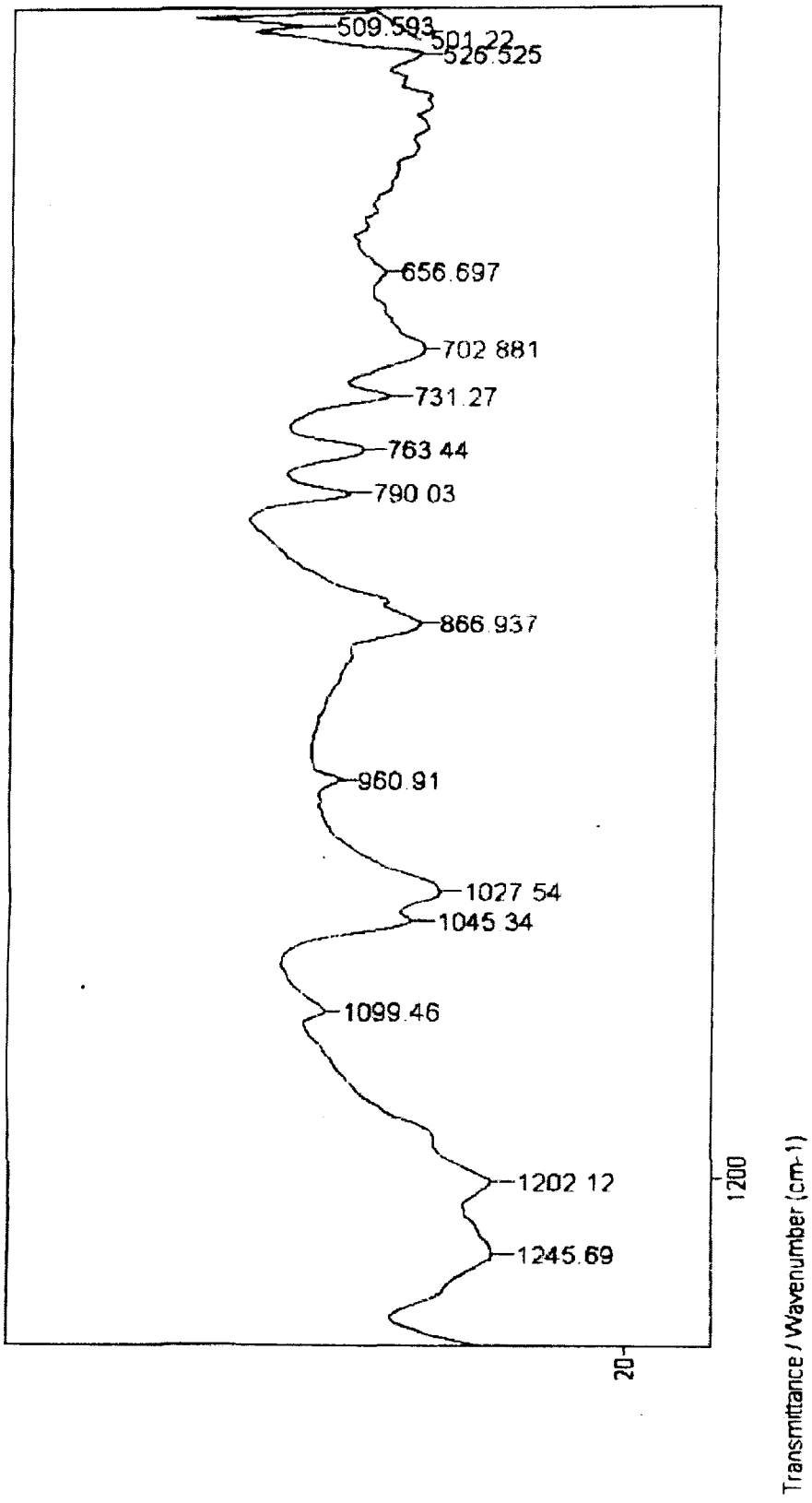
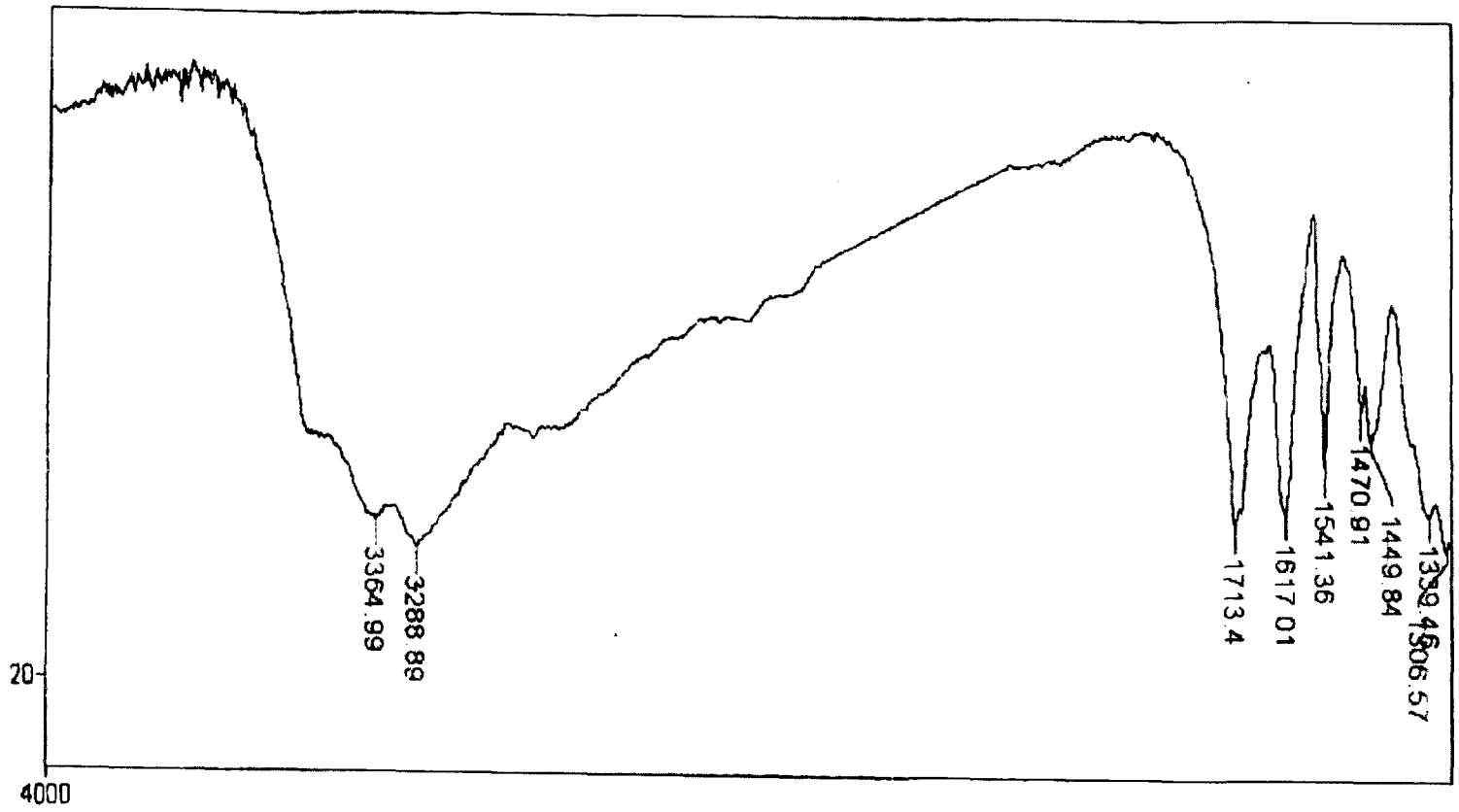


Fig. 61. IR spectrum of gallic acid


509.593
501.22
526.525
656.697
702.881
731.27
763.44
790.03
866.937
960.91
1027.54
1045.34
1099.46
1202.12
1245.69
20
1200
Transmittance / Wavenumber (cm-1)

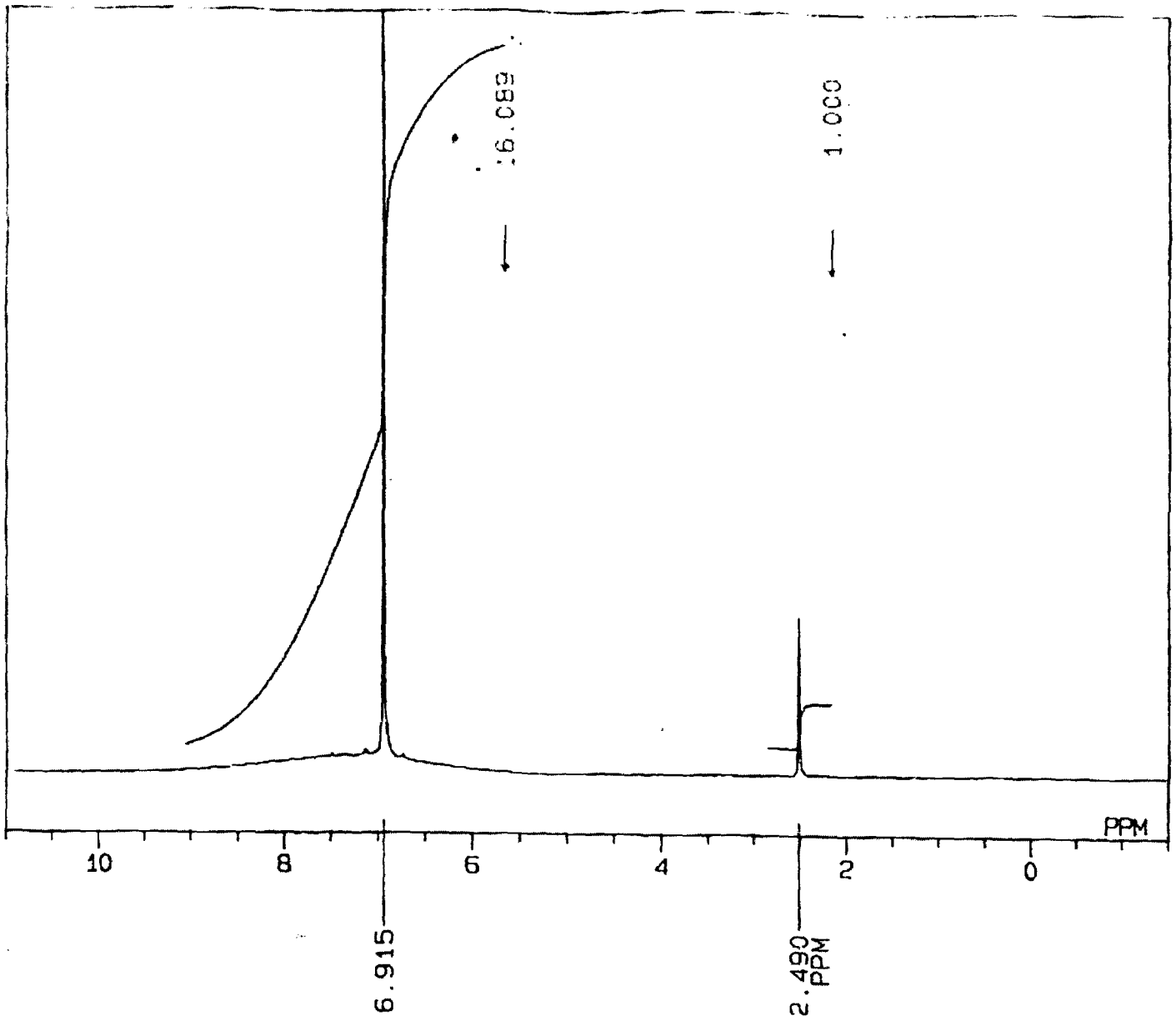


Transmittance / Wavenumber (cm-1)

^1H NMR spectrum showed only a singlet at δ 6.92 corresponding to the two equivalent aromatic protons of gallic acid (Fig. 62). The ^{13}C NMR also confirmed the compound to be gallic acid. The carboxylic acid carbon appeared at δ 167.63 (Fig. 63). The Para carbon to the carboxylic acid appeared at 138.13. The two adjacent carbons being equivalent to appeared at δ145.54. The two carbon atoms, unsubstituted and equivalent appeared at δ 108.88.

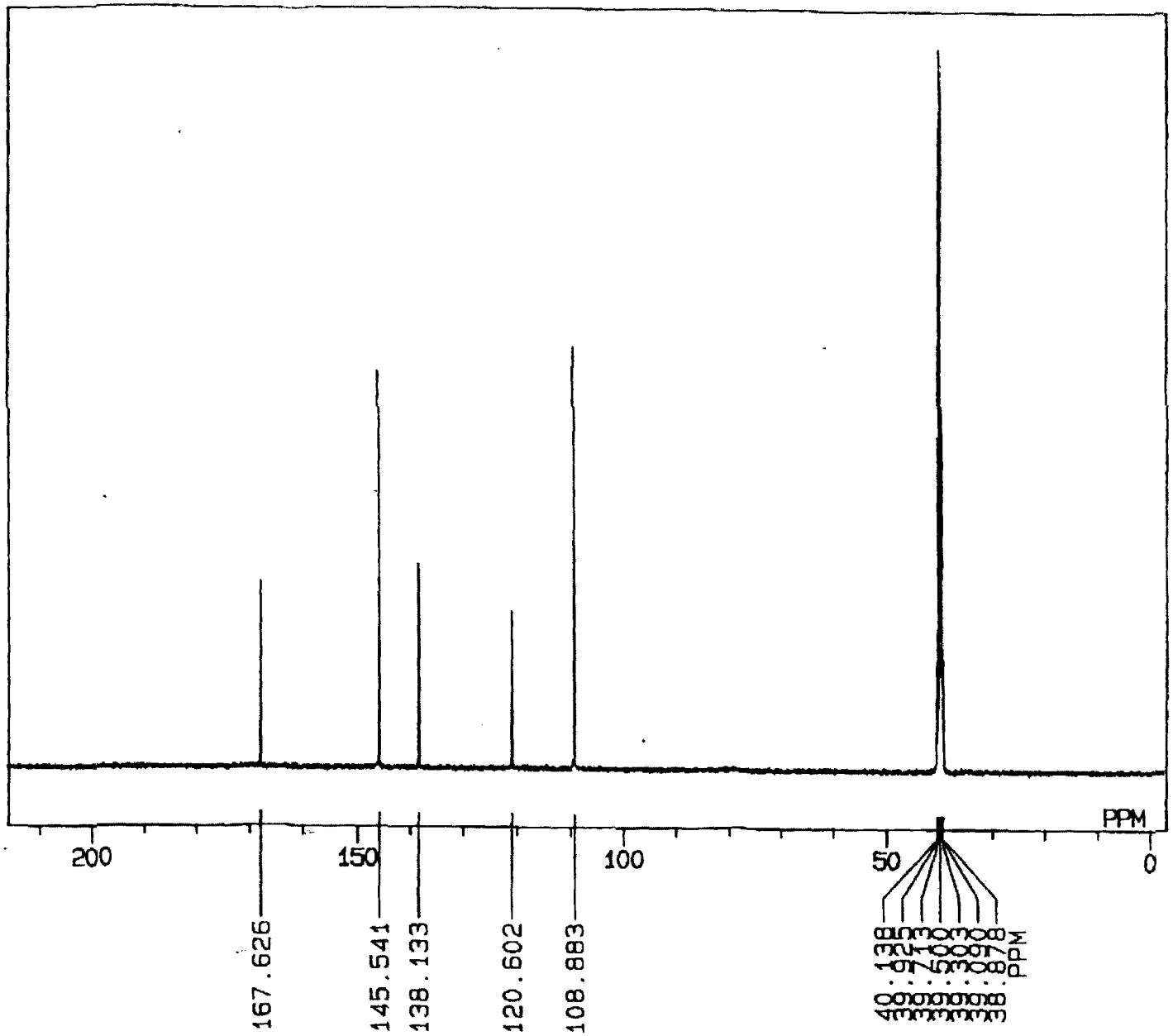
The compound PW5 eluted with ethyl acetate: methanol (4:1) gave a colourless crystalline solid on crystallization from acetone (m.p. 360°C). It gave a positive ferric reaction on adding alcoholic ferric chloride by producing bluish green colour. The IR spectrum showed peaks for hydroxyl (3143 cm^{-1}; Fig. 64), α-β-unsaturated-δ- lactone (1720 cm^{-1}) and aromatic systems (1610, 1509, 1457 and 806 cm^{-1}). The compound was identified as ellagic acid in comparison with authentic sample (m.p., m.m.p. and superimposible IR.

The compound PW6 eluted with ethyl acetate: methanol (9:1) gave acetate crystals (m.p. 140 – 141°C) on several recrystallizations from methanol extract yielded pale yellow prisms, and confirmed by TLC using an ethyl acetate: methanol: water (88: 12: 5) as the mobile phase (Rf = 0.7). Mass spectrum showed molecular ion peak of *m/z* 346.28 having molecular formula $C_{14}H_{18}O_{10}$. The UV spectrum showed maxima at 275 and 310 nm (Fig. 65). The IR spectrum showed peaks for hydroxyl at 3391 cm^{-1}, coumarin lactone carboxyl group at 2949, 2895 and 1701 cm^{-1} and an aromatic system at 1612, 1528, 1464 cm^{-1} (Fig. 66). All the above spectral data confirmed compound PW6 to be bergenin. The structure of the compound was further confirmed by X-ray crystallographic data (Tables 34 & 35) and spectrum (Fig. 67).



EXMOD [70, 11] SGNON
OBFRQ 399.65
OBNUC 1H
SCANS 120
ACQTM 2.048
PD 3.270
PW1 8.0
RESOL 0.49
TEMP. 23.0 c
SPEED 15
SLVNT DMSO
YG 9.00
YG2 2.2021
RGAIN 16
XE 5000.0000
XS 0.0000
Hz/cm
VALUE 250.0000
DFILE DU1: [100, 130
INSTRUMENT: JEOL
MODEL: GSX 400

Fig. 62. ^{1}H NMR spectrum of gallic acid



EXMOD SGCOM
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OBNUC 13C
SCANS 1024
ACQTM 0.655
PD 1.689
PW1 4.0
RESOL 1.53
TEMP. 23.0 c
SPEED 15
SLVNT DMSO
YG 3.46
YG2 3.4594
RGAIN 27
XE 22000.0000
XS 1100.0000
Hz/cm .
VALUE 1100.0000
DFILE DU1: [100, 130
INSTRUMENT: JEOL
MODEL: GSX 400

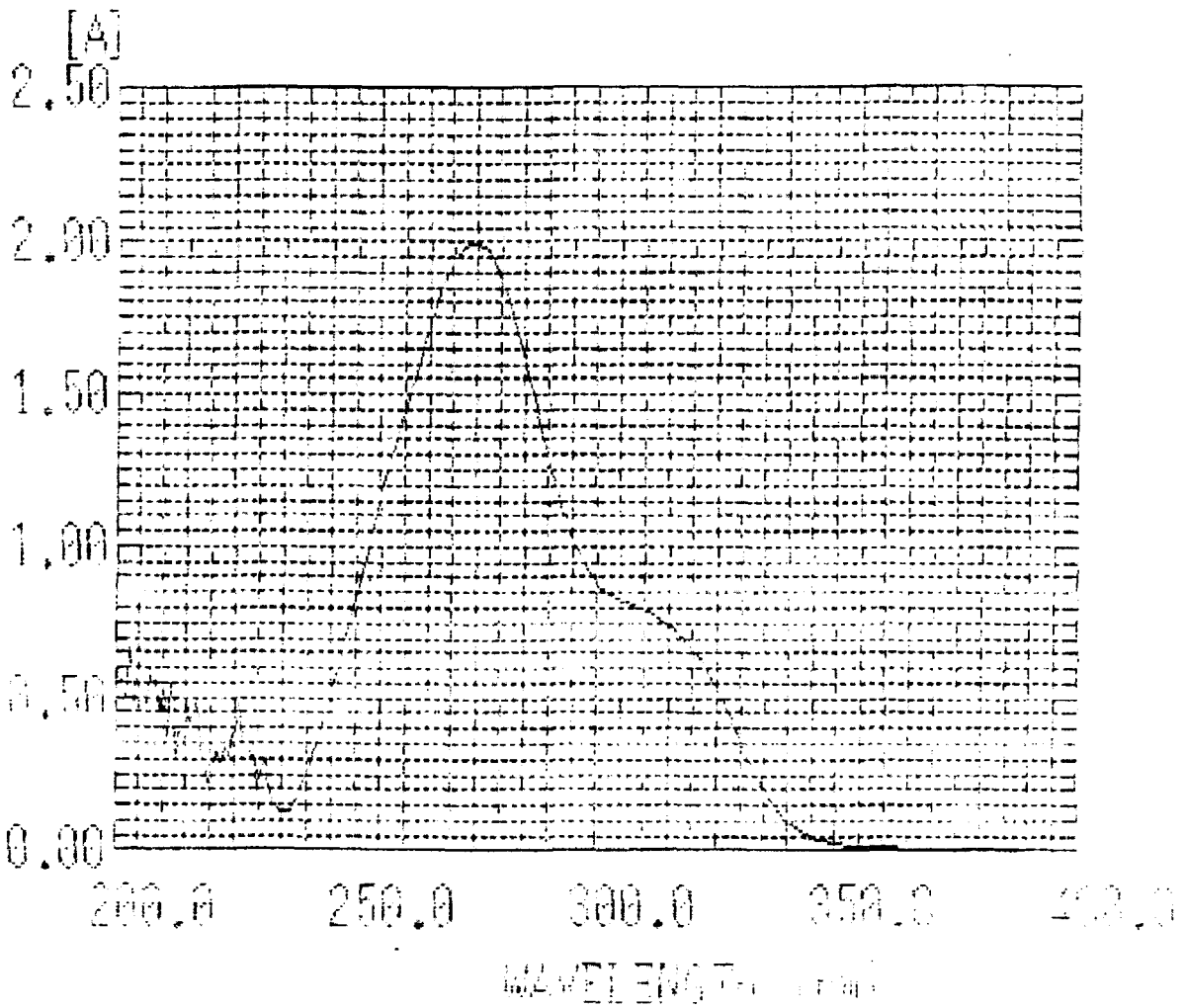


Fig.65.UV-VIS spectrum of bergenin

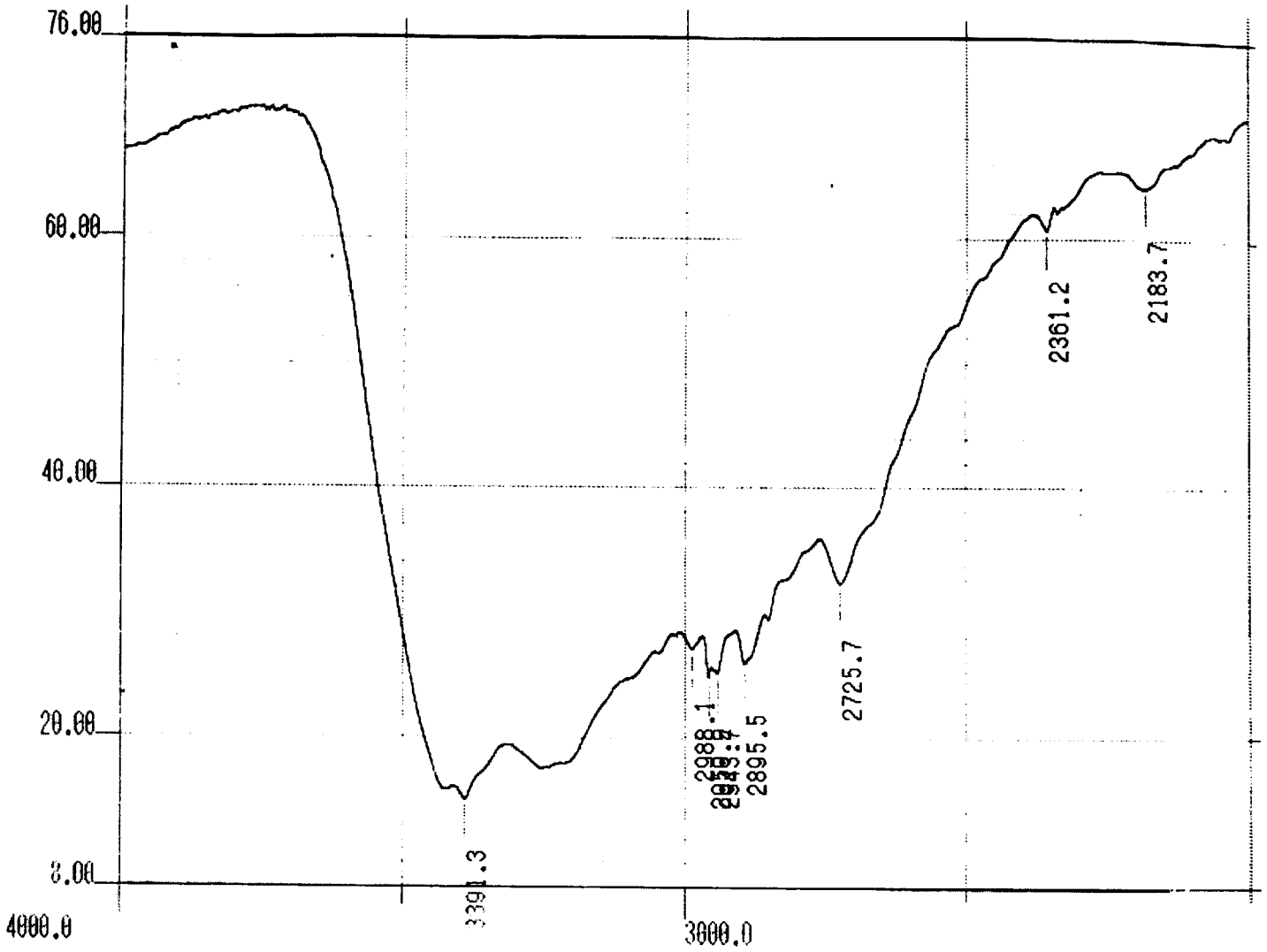


Fig. 66. IR spectrum of bergenin

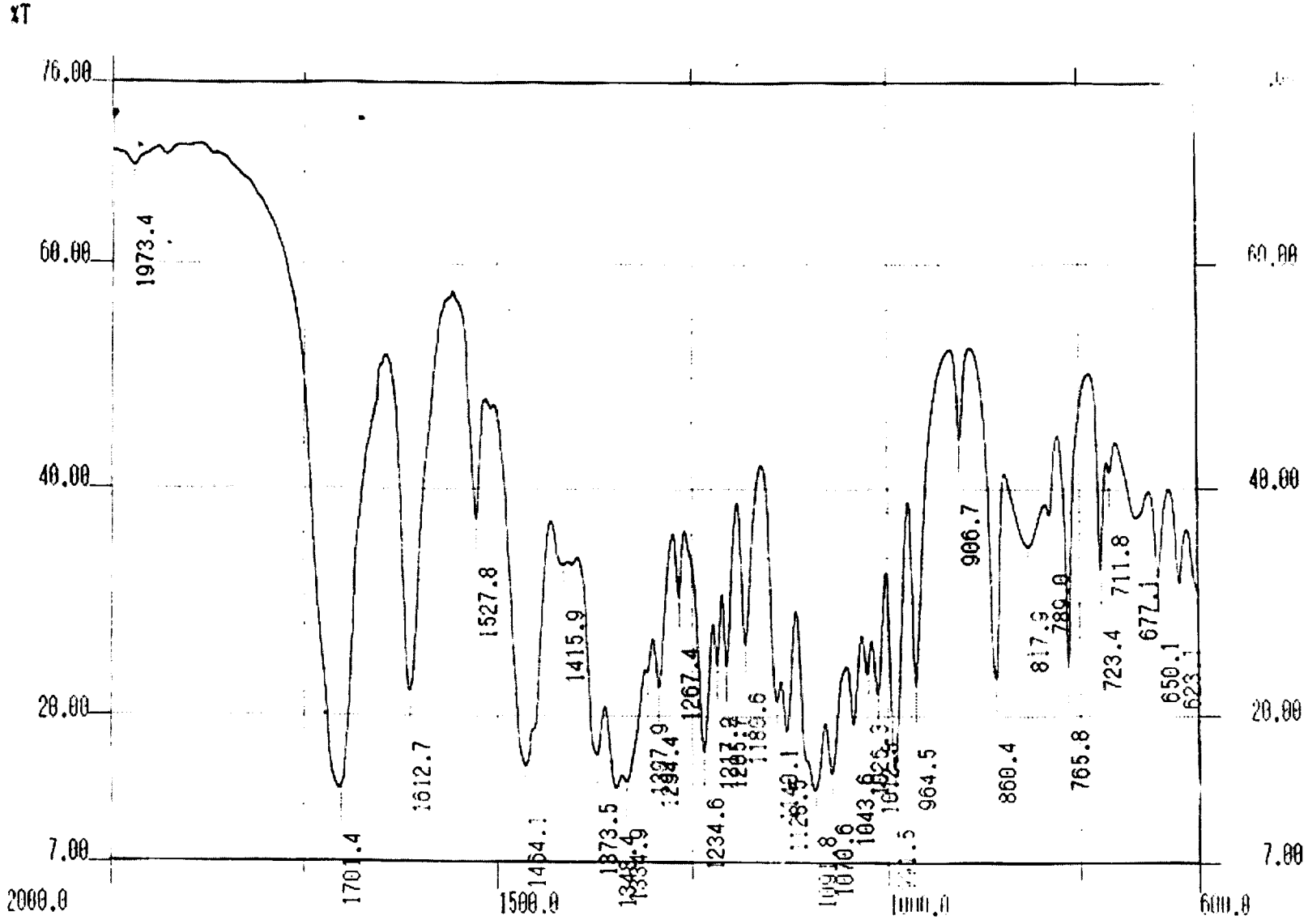

%T
80.00
60.00
40.00
20.00
7.00
2000.0
1500.0
1000.0
600.0
1973.4
1612.7
1527.8
1415.9
1267.4
1234.6
1043.6
964.5
906.7
860.4
817.3
765.8
723.4
711.8
677.1
650.1

Table 34. Atomic Coordinates x10^4) and Equivalent Isotropic Displacement Parameters (A^2 x 10^3) for newlx12m. U(eq) is defined as one third of the trace of the orthogonalized Uij tensor

	x	y	z	U(eq)
C(1)	-2815 (2)	5411 (1)	6492(1)	34(1)
C(3)	-1448(2)	4613(1)	5190(1)	27(1)
C(4)	-1248(2)	3586(1)	4854(1)	27(1)
C(5)	167(2)	3529(1)	4086(1)	27(1)
C(6)	1883(2)	4064(1)	4353(1)	28(1)
O(9)	1459(1)	5026(1)	4652(1)	29(1)
C(8)	355(2)	5010(1)	5472(1)	26(1)
C(9)	102(2)	6001(1)	5864(1)	27(1)
C(10)	-1461(2)	6176(1)	63751)	30(1)
C(11)	-1774(2)	7056(1)	6802(1)	33(1)
C(12)	-538(2)	7786(1)	6699(1)	32(1)
C(13)	1012(2)	7636(1)	6180(1)	29(1)
C(14)	1352(2)	6737(1)	5777(1)	29(1)
C(15)	3150(2)	4144(1)	3528(1)	34(1)
C(16)	3754(3)	8314(2)	6576(2)	42(1)
O(6)	4829(1)	4529(1)	3795(1)	61(1)
O(7)	2919(2)	6636(1)	5309(1)	44(1)
O(8)	-756(2)	8679(1)	7069(1)	45(1)
O(10)	-2652(1)	4601(1)	5983(1)	36(1)
O(1)	-4053(2)	5451(1)	7033(1)	51(1)
O(2)	568(1)	2553(1)	3875(1)	34(1)
O(3)	2159(2)	8388(1)	6046(1)	36(1)
O(4)	-2902(1)	3191(1)	4546(1)	33(1)
O(1W)	6557(2)	8924(1)	8223(1)	41(1)

Table 35. Hydrogen Coordinates (x 10^4) and Isotropic Displacement Parameters (A^2 x 10^3) for newlx12m

U(eq)	x	y	z	U (eq)
H(3)	-1957	5008	4689	32
H(4)	-830	3201	5385	32
H(5)	-321	3828	3520	32
H(6)	2478	3723	4865	33
H(8)	896	4594	5948	32
H(11)	-2803	7154	7154	39
H(15A)	3326	3513	3256	41
H(15B)	2619	4552	3053	41
H(16A)	3465	8268	7230	91
H(16B)	4478	8873	6471	91
H(16C)	4400	7752	6387	91
H(6A)	4941	5068	3573	63
H(7)	2905	6133	5010	66
H(8A)	-1672	8693	7384	68
H(2)	980	2290	4342	51
H(4A)	-3448	3594	4241	50
H(2W)	5780(40)	9389(18)	8113(18)	69(7)
H(1W)	5970(40)	8463(18)	8407(17)	65(7)

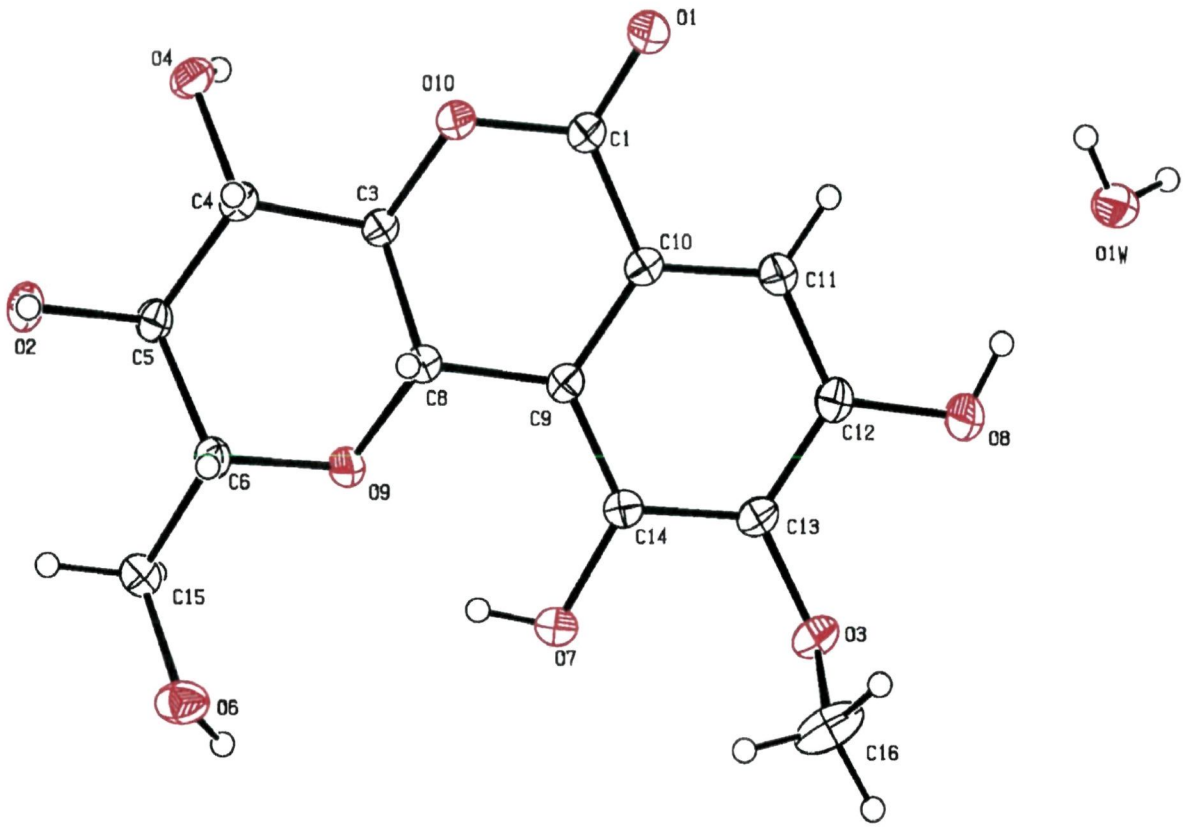
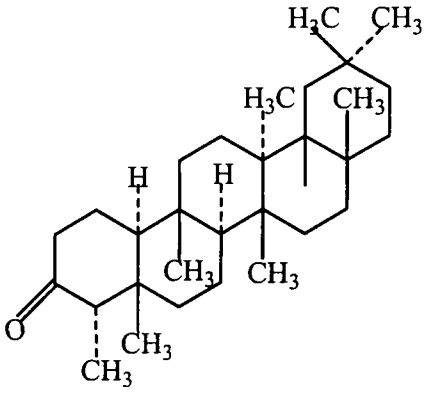
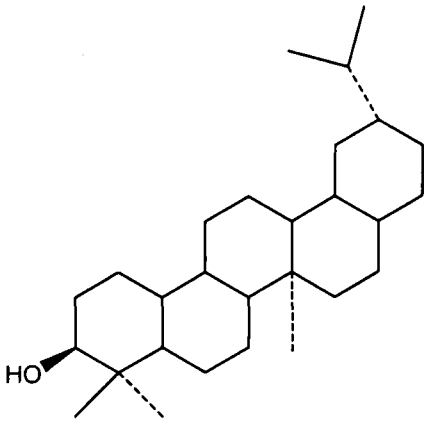


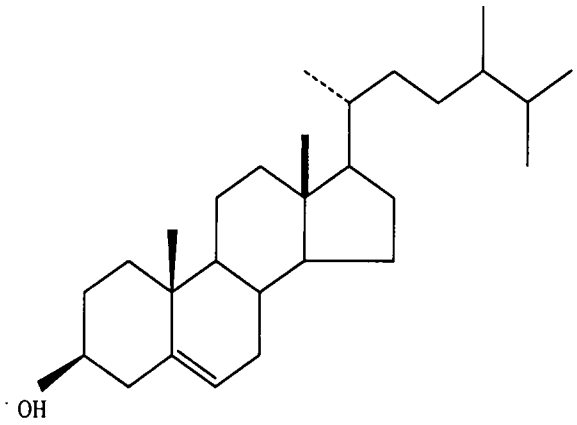
Fig. 67. Perspective view of the molecular structure of bergenin monohydrate



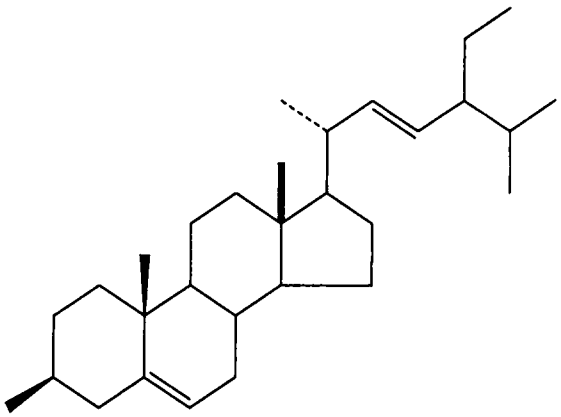
1. Friedelin (PW1)



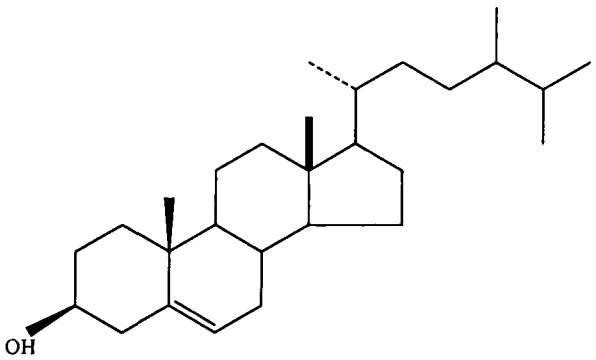
2. Lupeol (PW2)



3. Campesterol (PW3a)

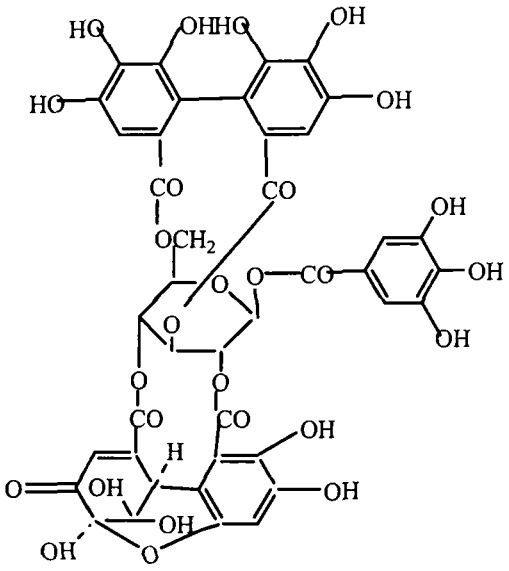


4. Stigmasterol (PW3b)

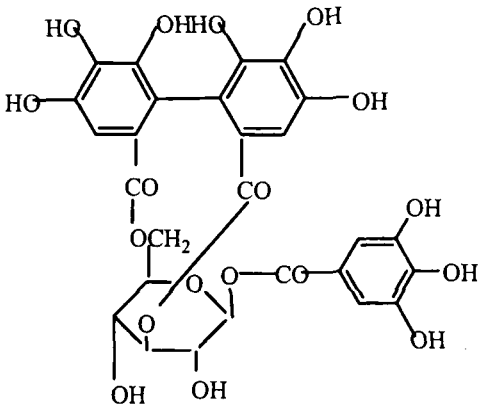


5. β-sitosterol (PW3c)

Fig. 68. Compounds isolated from hexane and chloroform extracts of *Phyllanthus wightianus*

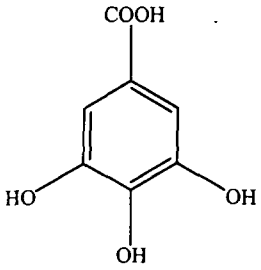


1. Geraniin

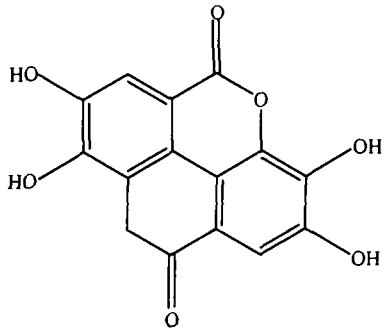


2. Corillagin

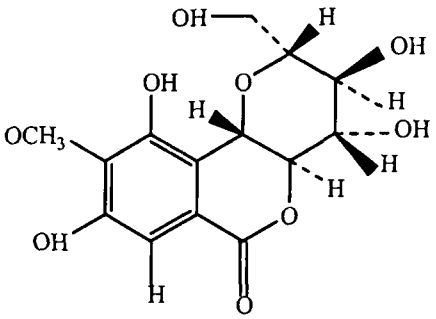
Fig. 69. HPLC identification of compounds from methanol extract of *Phyllanthus wightianus*



1. Gallic acid (PW4)



2. Ellagic acid (PW5)



3. Bergenin (PW6)

Fig. 70. Compounds isolated from methanol extract of *Phyllanthus wightianus*

5.2 ANTIMICROBIAL STUDIES

The screening of plant extracts and plant products for antimicrobial activity has shown that the higher plants represent a potential source of novel antibiotic prototypes (Afolayan, 2003). There are many published reports on the effectiveness of traditional herbs against gram-positive and gram-negative microorganisms, and as a result, plants are still recognized as the bedrock for modern medicine to treat infectious diseases (Evans *et al.,* 2002).

In the present study, the various test extracts of *P. wightianus* such as hexane, chloroform and methanol were evaluated for their antibacterial, antifungal and antidermatophytic properties.

The presence of antibacterial substance in the higher plants is well established (Srinivasan *et al.,* 2001). Several workers have reported that gram-positive bacteria are more susceptible towards plant extracts compared to gram negative bacteria (Mc Cutcheon *et al.,* 1992; Lin *et al.,* 1999; Rios and Recio, 2005; Parekh and Chanda, 2006, 2007). In the present study, the test extracts of *P. wightianus* were active against both gram-positive and gram-negative bacteria. The potency was higher in gram-negative bacteria such as *Escherichia coli* (33 mm, 30 mm, 13 mm, 10 mm), *Pseudomonas aureoginosa* (31 mm, 26 mm, 25 mm, 23 mm), *Proteus mirabilis* (29 mm, 25 mm, 15 mm, 13 mm), *Proteus vulgaris* (30 mm, 25 mm, 15 mm, 13 mm), *Salmonella paratyphi* (31 mm, 26 mm, 24 mm, 20 mm), *Salmonella typhi* (31 mm, 26 mm, 24 mm, 20 mm), *Vibrio cholerae* (30 mm, 27 mm, 15 mm, 13 mm) and *Vibrio parahaemolyticus* (27 mm, 24 mm, 23 mm, 9 mm) for methanol extract than that of gram-positive bacteria such as *Bacillus cereus* (28 mm, 16 mm, 0, 0), *Bacillus subtilis* (28 mm, 25 mm, 13 mm, 10 mm), *Staphylococcus aureus* (17 mm, 16 mm, 15 mm, 13 mm), *Staphylococcus epidermidis* (20 mm, 17 mm, 13 mm, 10 mm), *Aeromonas*

hydrophila (25 mm, 20 mm, 13 mm, 0), *Enterobacter aerogenes* (20 mm, 19 mm, 16 mm, 15 mm) at 100, 50, 25, 12.5 mg/ml respectively (Tables 9 & 10; Figs. 24 - 26).

According to Mc Cutcheon *et al.* (1992), the activity against both gram-positive and gram-negative bacteria indicates the presence of broad spectrum of antibiotic compounds or simply general metabolic toxins. Action of the test extracts against almost all the gram-positive and gram-negative bacteria also corroborate the same. Further, the test extracts were highly potent against fungal strains that strongly support the broad spectrum of the test extracts (Tables 11 & 12; Figs. 27 - 29).

Verpoorte and Dihal (1987) reported the antimicrobial activity of *P. amarus* and *P. urinaria.* Ethanol extract of *P. amarus* showed activity of <15 mm at 50 mg/ml concentration against *B. subtilis* and *Staph. aureus*. *P. urinaria* was active only against *Staph. aureus* and produced <15 mm activity at 50 mg/ml. In the present study, methanol extract at 50 mg/ml exhibited 16 mm inhibition against *Staph. aureus*, and chloroform and hexane extracts produced 15 and 22 mm inhibition, respectively. In the case of *B. subtilis*, methanol extract produced 25 mm inhibition and hexane extract exhibited 10 mm and the chloroform extract was inactive at 50 mg/ml. Comparisons reveal that action against *Staph. aureus* is more or less (16 mm) similar as that of *P. amarus* (< 15 mm) whereas against *B. subtilis*, the activity is superior (25 mm) that of *P. amarus* (< 15 mm). Variation in activity may be due to the presence or absence of different types of active constituents in them. *P. amarus* and *P. discoideus* did not exhibit any kind of activity against *E. coli, Ps. aeruginosa, A. niger* and *C. albicans*. In turn, all the test extracts in the present study were active against them. The results proved its high potency than that of *P. amarus*, *P. urinaria* and *P. discoideus*.

Olukoya *et al.* (1993) reported potent activity of ethanol extract of *P. discoideus* against *Staph. aureus, E. coli* and moderate activity against *Streptococcus* group D strains.

In the present study, methanol extract of *P. wightianus* exhibited potent inhibition against most of the bacterial strains which was more than that of the standard drugs (given in parentheses), such as 31 mm for *P. aeruginosa* (25 mm), 33 mm for *E. coli* (30 mm), 30 mm for *Pr. vulgaris* (20 mm), 25 mm for *Aeromonas hydrophila* (20 mm), 22 mm for *S. typhi* (20 mm), 23 mm for *V. vulnificus* (16 mm) and 27 mm for *V. parahaemolyticus* (14 mm). In the case of fungal strains, it was more potent such as 40 mm against *M. gypseum* (30 mm), 32 mm against *T. metagrophytes* (30 mm), 30 mm against *A. niger* (28 mm) and 30 mm against *C. albicans* (24 mm; Table 11). However, the MIC and MBC of the methanol extract for most of the bacterial strains and MFC of the fungal strains were recorded as 10 mg/ml and 20 mg/ml, respectively (Tables 10 & 12). This is followed by hexane extract exhibiting maximum activity and chloroform extract expressed moderate activity against almost all the bacterial and fungal strains.

Among the fungal strains, the test extracts were most active against dermatophytes which cause skin infections. Antidermatophytic activity has been reported by Agrawal *et al.* (2004) for chloroform fraction of *P. amarus* against *M. gypseum* and Ahmad *et al.* (1998) and Rani and Khullar (2004) for alcohol and aqueous extracts of *Emblica officinalis*.

Antimicrobial properties of the secondary metabolites in plants have been reported by several researchers such as tannins by Chung *et al.* (1988), Machado *et al.* (2003), Singh *et al.* (2005), Lim *et al.* (2006) and Chattopadhyay *et al.* (2007), phenols by Jurd *et al.* (1971), Alberto *et al.* (2006) and Rao *et al.* (2006), flavonoids

by Pepeljnjak *et al.* (2005) and Singh *et al.* (2006), coumarins by Cowan *et al.* (1999), triterpenoids by Rojas *et al.* (1992), saponins by Tyler (1993), Wang *et al.* (1998), Turker and Camper (2002) and Wallace (2004) and catechins by Toda *et al.* (1989), Alberto *et al.* (2004) and Bendini *et al.* (2006). In the present study, the preliminary phytochemical screening of the *P. wightianus* revealed the presence of glycosides, steroids, triterpenes, flavones and phenols in the hexane, chloroform and methanol extracts and catechins, coumarins, sugars, saponins and tannins in the methanol extract alone. Furthermore, the phytochemical evaluation (Section 4.1.8) also revealed the presence of lupeol, friedelin, β-sitosterol, campesterol and stigmasterol in the hexane and chloroform extracts and bergenin, gallic acid, ellagic acid, corillagin and geranin in the methanol extract.

Goyal and Rani (1989) reported *in vitro* antimicrobial activity of lupeol against both gram-positive bacteria such as *S. albus, S. aureus* and *B. subtilis* and gram-negative bacteria such as *E. coli, K. pneumoniae, Shigella dysenteriae, P. vulgaris* and *P. pyocyanea.* β-sitosterol has been shown to have a variety of antibacterial activity against *Staphylococcus, Streptococcus* and *E. coli* antiviral and antifungal activities (www. enerex.ca) and against *E. coli* by Singh and Singh (2003).

Prithiviraj *et al.* (1997) reported antifungal activity of bergenin against some plant pathogenic fungi and proved its monosodium salt was effective against the plant pathogenic fungi. In the present study also, the test extracts, especially the methanol extract had a potent activity against all the tested human pathogenic fungi. It may be due to the presence of bergenin in it.

Tannins serve as a natural defense mechanism against microbial infections. They inhibited the growth of many bacteria, fungi, yeasts and viruses (Chattopadhyay *et al.,* 2007). They have been traditionally in use for protection of inflamed surfaces

of the mouth and treatment of catarrh, wounds, haemorrhoiods and diarrhea and as an antidote in heavy metal poisoning (Ogunleye and Ibitoye, 2003). Medicinal plants containing phenolic compounds including tannins as major constituents are used topically for care and repair of skin wounds (Dweck, 2002). Their mode of antimicrobial action may be related to their ability to inactive microbial adhesions, enzymes, cell envelope transport of proteins, etc. (Cowan, 1999). They are complex with polysaccharide (Ya *et al.,* 1988) and can also directly inactivate microorganisms. Condensed tannins have been determined to bind cell walls of ruminal bacteria, preventing growth and protease activity (Jones *et al.,* 1994). A high concentration of tannins coagulates the bacterial cell wall protein resulting in bactericidal activity while in low concentration it is bacteriostatic (Robinson, 1975; Trease and Evans, 1996). According to Scalbert (1991), tannins can be toxic to filamentous fungi, yeast and bacteria. Thus, in the present study, the high content of tannins in the methanol extract may be responsible for its better antimicrobial action.

Ndukwe and Zhao (2007) reported antibacterial activity of 2, 3, 8 – tri – O-methyl ellagic acid against *S. pneumoniae* (19 mm), *V. cholerae* (24 mm), *Staph. aureus* (25 mm), *K. pneumoniae* (20 mm), *Ps. aeruginosa* (20 mm), *B. cereus* (21 mm), *E. coli* (25 mm) and *S. typhi* (22 mm). In the case of present study, methanol extract (containing ellagic acid) showed more activity such as 28 mm against *B. cereus,* 17 mm against *Staph. aureus,* 33 mm against *E. coli,* 18 mm against *K. pneumoniae,* 31 mm each against *Ps. aeruginosa* and *S. typhi* and 30 mm against *V. cholerae* at 100 mg/ml. The results also corroborate these findings.

Reddy *et al.* (2007) reported potent antibacterial activity of ellagic acid and gallic acid against *E. coli* and *Ps. aeruginosa.* However, these compounds were inactive against fungi such as *A. fumigatus* and *C. albicans* at the tested

concentrations. Alberto *et al.* (2001, 2004) reported inhibition of microbial growth by gallic acid whereas Cowan (1999) reported its activity against *Staph. aureus.*

The phenolic compounds are generally toxic to microorganisms due to the enzymatic inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more non-specific interactions with the proteins (Mason and Wasserman, 1987). Polyphenols are known to form with proteins soluble complexes of high molecular weight. Thus after being adsorbed, the polyphenols will react with the protein moiety of cell enzymes (oxidoreductases) in the cytoplasm and cell wall. They may also bind to bacterial adhesions and so, interfering with the availability of receptors on the cell surface (Machado *et al.,* 2003). Flavones, flavonoids and catechins have the ability to complex with extra-cellular and soluble proteins and also with bacterial cell walls, thus disrupt the microbes. Some of them disrupt and damage microbial membranes (Tsuchiya *et al.,* 1996). Generally, flavonoids are phenolic in nature (disinfectant) as they cause cell membrane damage, thus release cell contents and cause lysis, mostly bactericidal. Probable targets in the microbial cell for the coumarins are surface-exposed adhesions, cell wall polypeptides and membrane-bound enzymes (Cowan, 1999). Antibacterial (Fernandez *et al.,* 1996) especially gram-positive and antifungal (Hoult *et al.,* 1996) nature of coumarins were well-studied.

Saponins also are surface agents which lead to the lysis of cell surface (Robinson, 1975; Trease and Evans, 1996) and are high in antimicrobial action than flavonoids and tannins. Wang *et al.* (1998) and Wallace (2004) have reported antimicrobial activity of saponins against bacteria and fungi. Saponins are also effective in removing protozoa from the rumen (Wallace, 2004). Numerous studies have demonstrated that saponins and saponin – containing plants have toxic effects on

protozoa (Navas- Camacho *et al.,* 1993; Diaz *et al.,* 1994; Newbold *et al.,* 1997; Odenyo *et al.,* 1997). So, it can be possibly implied for the role of saponins present in the methanol extract against the antimicrobial activity.

According to Irobi *et al.* (1994) and Tereschuk *et al.* (1997), plant extract with antioxidant activity frequently display antimicrobial activity. In the present study, the test extracts of *P. wightianus* exhibited potent *in vitro* antioxidant activity. In DPPH assay at 100 µg/ml methanol extract exhibited 48.60%, followed by hexane extract 41.05% and chloroform extract exhibited 25.54% scavenging activity. In nitric oxide radical scavenging assay, methanol extract exhibited maximum % of inhibition (70.09%), followed by hexane extract (54.59%). Chloroform extract was inactive to scavenge the nitric oxide radical generation at 100 µg/ml. This may also be responsible for the observed antimicrobial activity of the respective test extracts.

In the present study, the polarity of the solvent seems to be played an important role in exhibiting potential antibacterial activity. Methanol extract of *P. wightianus* showed remarkable activity against most of the bacterial and fungal strains. This view has been supported by Rabc and van Staden (1997), Grierson and Afolayan (1999) and Parekh and Chanda (2006, 2007).

It was observed from the present study that the methanol extract was potent against almost all the bacterial and fungal strains tested, however, it was more active against bacterial strains such as *Ps. aeruginosa, E. coli, S. paratyphi, V. cholerae, Pr. mirabilis, Pr. vulgaris, B. cereus* and *B. subtilis*. Among the fungal strains, it was potent against *M. gypseum, T. mentagrophytes, C. albicans* and *Aspergillus* species. These agents commonly cause skin infections, especially *Staph. aureus* and *Ps. aeruginosa* are predominant organisms in both leg ulcers and superficial wounds and showed increased resistance to commonly used antibiotics (Valencia *et al.,* 2004).

The potential of *P. wightianus* extract against the standard strains of *Staph. aureus* and *Ps. aeruginosa* may be explored in order to develop a topical antimicrobial therapy to promote skin wound healing. In addition, the anti-pseudomonial and anti-staphylococcal activities of the plant extracts are considered important because the test extract has expressed activity against in nosocomial infections. Further, the anti-dermatophytic and anti-candidal roles of the test extracts strongly support the efficacy of the test extracts against the agents of both bacterial and fungal organisms causing skin diseases.

The group of fungi causing skin, hair and nail infections are collectively called dermatophytes. The superficial mycotic infection is the series of infection of the skin caused by dermatophytic organisms and its related fungi. They invade the keratinized portion of the skin, hair and nail and use keratin as nitrogen source (Joklik *et al.,* 1992). Dermatophytosis is one of the most prevalent infections in the world. Though they are extremely annoying and millions of dollars are spent annually in their treatment with few exceptions they are not debilitating or life threatening. The genus *Epidermophyton* infects skin and nail only, the genus *Trichophyton* infects skin, nail and hair and the genus *Microsporum* infects skin and hair (Jagdish Chander, 1996). The clinical forms of dermatophytosis were erroneously termed tinea or ring worm, depending on their anatomical site involved. For example, Tinea corporis affects body and Tinea pedis affects foot called athlet's foot (Chakrabarti, 2001.) For all dermatophytes, the first step in infection is colonization of the horny layer of tissue, and then the fungus spreads in a centrifugal pattern forming the ring that gives the infection to the common name "Ring Worm" (Fisher and Cook, 1998).

Trichophyton mentagrophytes, Trichophyton rubrum and *Microsporum canis* were frequently isolated from the arms and legs of the clinical form Tinea corporis

whereas *Trichophyton mentagrophytes* and *Trichophyton rubrum* are also responsible for the clinical form Tinea pedis, which affect feet. *Epidermophyton floccosum* mainly produces the clinical form Tinea cruris (Forbes *et al.*, 1998).

In the present study, it was clearly observed that the test extracts have potential activity against the all the tested dermatophytes which strongly support the use of test extracts against skin infection causing agents.

The causative agents of urinary tract infections are *Ps. aeruginosa, E. coli, Pr. mirabilis, Pr. vulgaris* and *K. pneumoniae*. The potent inhibition of the test extracts against them is an important finding in the present study expressing the possibility to use *P. wightianus* for developing drugs against urinary tract infections. *Ps. aeruginosa* is the commonest and most serious cause of infection in burns (Ananthanarayan and Jayaram Paniker, 1996). *E. coli* is also responsible for pyogenic infections apart from its role in urinary tract infections and diarrhea. *Vibrio vulnificus* is responsible for wound infection and septicemia. In the present study, a strong and potent inhibition recorded against *Ps. aeruginosa* (31 mm), *E. coli* (33 mm), *V. vulnifius* (23 mm) at 100 mg/ml for methanol extract, organisms reveal the potentiality of the test extracts against the respective skin infection causing agents. Moreover, the extracts exhibited activity against the enteric agents such as *E. coli* - 30 mm (hexane), 21 mm (chloroform) and 33 mm (methanol), *S. typhi* - 16 mm (hexane) and 22 mm (methanol), *S. paratyphi* - 17 mm (hexane) and 31 mm (methanol), *V. cholerae* - 24 mm (hexane) and 30 mm (methanol) and *V. parahaemolyticus* - 27 mm (methanol) at 100 mg/ml (Table 9). Thus, the results in the present study validate the ethnotherapeutic claim of the plant as an antidiarrheal agent.

5.3 PHARMACOLOGY

5.3.1 Analgesic Activity

Analgesia is a state of reduced awareness to pain and analgesics are substances which decrease pain sensation by increasing threshold to painful stimuli.

There are two types of analgesic drugs such as narcotic and non-narcotic agents. Narcotic analgesic drugs act through their interaction with opioid receptors and at spinal level, they inhibit the transmission nociceptive impulses through the dorsal horn and suppress nociceptive spinal reflexes. The non-narcotic analgesic agents (NSAIDs-Non steroidal anti-inflammatory drugs) act through the inhibition of arachidonate cyclooxygenase, which in turn lead to the decreased production of prostaglandins, sensitize nociceptive nerve endings to inflammatory mediators such as bradykinin and 5 hydroxy tryptamine. In order to distinguish between the central and peripheral analgesic action of the test drugs both hot plate method (Thermal stimulation by radiant heat) as well as acetic acid-induced (chemical stimulation) writhing reflex were carried out. The acetic acid- induced writhing is a non-specific model, i.e., it will not distinguish between opioid and non-opioid type activities while pain induced by thermal methods indicates narcotic involvement (Turner, 1965; Besra *et al.,* 1996). Acetic acid-induced writhing assay is used for detecting both central and peripheral analgesics whereas the hot plate and other thermal methods are more sensitive in centrally acting analgesics (Dewey *et al.,* 1970; Fukawa *et al.,* 1980).

Acetic acid causes inflammatory pain by inducing capillary permeability (Amico-Roxas *et al.,* 1984) and creates increase in peritoneal fluids of prostaglandin E_2 (PGE_2) and prostaglandin F_{2a} (PGF_{2a}) (Deraedlt *et al.,* 1980; Bentley *et al.,* 1983). Several mediators such as kinin substance (Chapman and Dickenson, 1992; Correa *et al.,* 1996; De Campos *et al.,* 1996), acetylcholine and prostaglandins (Chapman and

Dickenson, 1992) also take part in the visceral pain model nociception (Vogel and Vogel, 1997) and transmission of nociception from the viscera (Cervero and Laird, 1999).

The hot plate method in the present study, hexane (4.30±0.63 at 60 min and 4.43 ±0.77 at 120 min; 4.30±0.95 at 60 min and 4.26±0.48 at 120 min), chloroform (3.90±0.87 at 60 min and 4.28 ±0.76 at 120 min; 4.26±1.60 at 60 min and 4.36±0.76 at 120 min) and methanol (4.23±1.30 at 60 min and 4.28±0.79 at 120 min; 4.28±0.95 at 60 min and 4.36±0.87 at 120 min) extracts of *P. wightianus* failed to increase the latency at the dose levels of 100 and 200 mg/kg/bw respectively when compared to the standard drug morphine (12.1±0.5 at 60 min and 18.3± 0.63 at 120 min; $P<0.001$) at dose level 5 mg/kg s.c., respectively (Table 13; Fig. 30). Almost all the species of *Phyllanthus* have shown very similar results so far (Calixto *et al.,* 1998) and have been virtually inactive against tail flick and hot plate tests (Gorski *et al.,* 1993; Santos *et al.,* 1994, 1995 a, b, 1999; Perianayagam *et al.,* 2004).

The extracts of several species of *Phyllanthus,* including *P. corcovadensis* (Gorski *et al.,* 1993), methanolic extract of callus culture of some species of *Phyllanthus* (Santos *et al.,* 1994), *P. niruri, P. sellowianus, P. tenellus* and *P. urinaria* (Santos *et al.,* 1995 c) and *P. carolinensis* (Filho *et al.,* 1996), *P. amarus, P. fraternus, P. orbiculatus* and *P. stipulatus* (Santos *et al.,* 2000) were all effective in preventing the pain response induced by acetic acid, formalin and capsaicin-induced neurogenic pain in mice. The results in the present study also support the same.

In acetic acid writhing test, all the extracts of *P. wightianus* exhibited significant and dose-dependent reduction in writhings (Table 14; Fig. 31). All the test drugs showed superior activity at the dose level of 200 than 100 mg/kg/b.w. Methanol extract recorded maximum % protection of analgesia such as 59.11% and 52.40% at

200 and 100 mg/kg/b.w. respectively. This is followed by hexane extract such as 45.59% and 37.35% and chloroform such as 43.59% and 35.18% at similar doses. The analgesic effect produced by these extracts may be peripheral in nature and may not involve any central analgesic action. The findings of Gorski *et al.* (1993) and Santos *et al.* (1995 a, b, 1999) also support this observation. The effect could be due to the inhibition of capillary permeability (Amico – Roxas *et al.,* 1984) by decreasing prostaglandin E_2 (PGE_2) and prostaglandin F_{2a} (PGF_{2a}) (Deraedlt *et al.,* 1980; Bentley *et al.,* 1983) in peritoneal fluids and inhibition of histamine. Many classes of naturally – occurring secondary metabolites have been isolated and characterized in *Phyllanthus* species such as steroids, flavonoids, alkaloids, terpenes, lignans, tannins and phenols (Ueno *et al.,* 1988; Bachmann *et al.,* 1993; Miguel *et al.,* 1995 a, b, 1996; Calixto *et al.,* 1998). Some of them include β-sitosterol, stigmasterol, geraniin, furosin and quercetin, which produce significant and dose-related antinociception when assessed in several chemical models of nociception in mice (Miguel *et al.,* 1995 a, b, 1996; Santos *et al.,* 1995 c; Filho *et al.,* 1996). Further, the presence of ellagic acid, gallic acid and rutin in this genus could also be responsible for antinociceptive properties (Ihantola – Vormisto *et al.,* 1997). Gorski *et al.* (1993) and Santos *et al.* (1995 b) have reported antinociceptive property of the hydro-alcoholic extract of *P. carolinensis* and other species of *Phyllanthus* and postulated that antinociception could be contributed by phytosterols, quercetin, gallic acid ethyl ester, geraniin and the flavonoid mixture.

Ellagitannin and geraniin have been reported in several species of *Phyllanthus* such as *P. urinaria* (Okuda *et al.,* 1980), *P. niruri* (Ueno *et al.,* 1988), *P. amarus* (Foo, 1993 a, b) and *P. sellowianus* (Miguel *et al.,* 1995 a). Ellagitannin is known for its analgesic effect (Miguel *et al.,* 1996). In addition, it has the capacity to reduce

systemic blood pressure by inhibiting nor-adrenaline release (Cheng *et al.*, 1994). Geraniin also inhibits the formation of 5 – lipooxygenase and cyclooxygenase products derived from arachidonic acid path way in rat peritoneal polymorpho nuclear leukocytes (Kimura *et al.*, 1986).

Miguel *et al.* (1996) reported the antinociceptive effect of geraniin isolated from *P. sellowianus*. Geraniin expressed approximately eight times more active than acetaminophen and aspirin when tested against abdominal constriction induced by acetic acid. Perianayagam *et al.* (2004) reported antipyretic and analgesic activities and the presence of alkaloids, tannins, phenolic compounds, carbohydrates and amino acids in *Emblica officinalis*. This observation provides support to the presence of active constituents and their analgesic effect.

The results in the present study strongly provide the basis to *P. wightianus* to the presence of different classes of constituents such as β-sitosterol, campesterol and stigmasterol under the group of sterols in the hexane and chloroform extracts and tannins such as ellagic acid, gallic acid and geraniin in the methanol extract and their synergistic antinociceptive action. However, the plant may contain many other constituents that would have contributed analgesic activity.

5.3.2 Anti-inflammatory Activity

The test extracts produced significant inhibition of carrageenan-induced rat paw inflammation, the test that has significant predictive value for anti-inflammatory agents acting by inhibiting the mediators of acute inflammation (Olajide *et al.*, 1999).

Carrageenan induces an inflammatory reaction in two different phases. The initial phase, which occurs between 0 and 24 h after injection of carrageenan, has been attributed to the release of histamine, serotonin and bradykinin on vascular permeability (Vinegar *et al.*, 1987). The oedema maintained during the plateau phase

is presumed to be due to kinin-like substances (van Arman *et al.,* 1965; Di Rosa and Sorrentino, 1968). Inflammation volume reaches its maximum approximately 3 h post-treatment after which it begins to decline (Garcia *et al.,* 2004). The late phase, which is also a complement- dependent reaction, has been shown to be due to over production of prostaglandins in tissues (Di Rosa *et al.,* 1971; Malpure *et al.,* 2006).

In the present study at 100 and 200 mg/kg/p.o., methanol extract showed maximum protection such as 54.68 and 60.93%, followed by hexane extract such as 43.75 and 50.00% and by chloroform extract such as 35.93 and 42.18%, respectively.

Anti-inflammatory activities of various species of *Phyllanthus* have been studied by several researchers following different models such as inhibitory activity of PMNs and platelets by the leaves of *P. emblica* (Ihantola-Vormisto *et al.*, 1997), COX-1 and COX-2 assays by *P. amarus* (Chukwujekwu *et al.*, 2005), carrageenan-induced paw oedema and neutrophils influx model in *P.amarus* (Kassuya *et al.,* 2005), carrageenan-induced paw oedema in *P. amarus* (Mahat and Patil, 2007), in *P. debilis* (Chandrasekhar *et al.*, 2005) and in *P. singampattiyana* (Maridass *et al.,* 2005) with significant protection. The present findings also corroborate the same.

In the present study, the inhibition of the oedema by the test extracts may be acting on late phases (Table 15) and suggested that anti-inflammatory activity of the test extracts may be mediated by inhibiting the over production of prostaglandins, i.e. PGE2 and nitric oxide.

Most currently used anti-inflammatory agents inhibit cyclooxygenase and therefore synthesize prostaglandins. Free radical scavenging agents also play a role in the treatment of inflammation because reactive oxygen radicals produced by neutrophils and macrophages are implicated in tissue damage during the inflammatory process in some conditions.

Traditional medicinal preparations are in wide use for inflammatory disease (Crellin and Philpott, 1990; Bohlin, 1995; Miller and Murray, 1998). Eight out of 16 species used for anti-inflammatory or anti-histamine in nature belong to the subgenus *Phyllanthus* (Unander *et al.,* 1995). Methanol extract of *Phyllanthus amarus* significantly inhibited acute, sub-acute and chronic pain produced in different models of inflammatory pain (Mahat and Patil, 2007). *In vitro* and *in vivo* studies have reported inhibition of induction of cytokines, NOS and COX – 2 (Kiemer *et al.,* 2003). COX-2 is responsible for the production of the prostanoid mediators of inflammation (Vane and Botting, 1996). Most NSAIDs in current use are inhibitors of both iso enzymes (COX-1, COX-2) even though they vary in their degree of inhibition (Griswold and Adams, 1996).

During the inflammatory phase from the macrophages, the reactive free radical nitric oxide is synthesized by inducible NO syntheses iNOS (Rao *et al.*, 2006). Excessive production of NO plays a pathogenic role in both acute and chronic inflammation (Clancy *et al.,* 1998). NO is responsible for the vasodilatation, increase in vascular permeability, oedema formation and synthesis of prostaglandins at the site of inflammation (Moncada *et al.,* 1991; Grisham, 1999). Selvemini *et al.* (1996) reported the role of NO in relation to carrageenin-induced paw oedema. Manipulation of NO free radical can be a potential and promising therapeutic area to treat inflammations. Approaches being currently used for inflammatory disorders include NO scavengers as well as NO inhibition (Mittal *et al.,* 2003). In the present study, the test extracts of *P. wightianus* exhibited significant *in vitro* – NO free radical scavenging activity such as hexane extract 54.59%, chloroform extract – inactive and methanol extract 70.09% at 100 µg/ml respectively (Table 16). NO free radical scavenging activity of the test extracts might be in part attributed to the observation of

anti-inflammatory effect. A large number of plant-derived compounds are principally phenolics and terpenes which have anti-inflammatory effects (Polya *et al.,* 2003 a) wherein a number of them are variously used as antioxidants and analgesics (Harborne and Baxter, 1993).

Swarnalakshmi *et al.* (1984) reported the anti-inflammatory activity of bergenin against carrageenin-induced rat paw oedema. Its potency is dose-dependent and maximum at higher doses. Li *et al.* (2004) reported racemosic acid from the bioassay–guided fraction of the ethanol extract of *Ficus racemosa* and its potential inhibitory activity against COX-1 and 5-COX *in vitro*. In addition, they isolated bergenin along with racemosic acid from the active fractions. Lupeol has been shown to possess anti-arthritic activity (Chronic inflammatory disorder) by its possible suppression of the T-lymphocyte (Bani *et al.,* 2006). Hasmeda *et al.* (1999) reported the inhibition of protein kinase in the anti-inflammatory effects of triterpenes such as lupeol and other ones.

In the present study, in chronic tests using adjuvant-induced polyarthritis model, the test extracts and bergenin demonstrated superior activity compared to the acute test model. Calixto *et al.* (1998) reported anti-inflammatory activity of β-sitosterol isolated from *Phyllanthus flexuous*. As lipooxygenase inhibitors possess significant anti-inflammatory effect (Singh and Majumdar, 1997). Geraniin may be in part attributed to the anti-inflammatory effect of the methanol extract. Therefore, it has the potential to inhibit both the cyclooxygenase and lipooxygenase pathways of arachidonate metabolism (dual inhibition property).

The presence of lupeol and sterols (β-sitosterol, campesterol and stigmasterol) in the hexane and chloroform extracts and bergenin and geraniin in the methanol extract may be either individually or synergistically responsible for the anti-

inflammatory activity. The results seem to support the traditional use of this plant in relieving inflammation.

5.3.3. *In vitro* Antioxidant Activity

Many antioxidant compounds, naturally occurring in plant sources, have been identified as a free radical or active oxygen scavengers (Zheng and Wang, 2001). These plant products exert antioxidative effects by quenching various free radicals and the singlet form of molecular oxygen. Higher levels of the antioxidant enzymes have been correlated with decreased susceptibility to cell damage (Kinsella *et al.*, 1993; Khan *et al.*, 1997; Aruoma, 1998; Lai *et al.*, 2001).

Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity (Ito *et al.*, 1983). In addition, natural antioxidants have the capacity to improve food quality and stability and can also act as nutraceuticals to terminate free radical chain reactions in biological systems and thus may provide additional health benefits to consumers (Kumaran and Karunakaran, 2007).

Effect on DPPH

In the present study, all the extracts had very good action on DPPH free radical and maximum inhibition was exhibited by methanol extract (48.60%), followed by hexane extract (41.05%) and chloroform extract (25.54%) at 100 µg/ml. Vitamin C was used as the reference standard and exhibited maximum inhibition of 72.07%.

DPPH is relatively a stable free radical and the assay determines the ability of the test extract to reduce DPPH radical to the corresponding electrons to paired ones. Antioxidants can act by converting the unpaired electrons to paired ones. The

reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. The extracts are able to reduce the stable radical DPPH to the yellow-coloured diphenyl picryl hydrazine. DPPH react stoichometrically with antioxidants which are good hydrogen donors (Blois, 1958; Gardner *et al.,* 1998; Schwarz *et al.,* 2001). Thus, the inhibition of DPPH radical indicates that the test extracts cause reduction of DPPH radical in a stoichometric manner. This observation has already been reported by Vani *et al.* (1997), Sanchez-Moreno *et al.* (1999) and Sanchez-Moreno (2002). The experimental data reveal that all the extracts of *P. wightianus* are likely to have the effect of scavenging free radical (Table 16).

The high potent DPPH radical –scavenging activity of extracts of *P. niruri* (IC_{50} values at 10-30 µg/ml) has been reported by Harish and Shivanandappa (2006). Kumaran and Karunakaran (2007) reported the antioxidant potentials of methanol extract and standard with the DPPH radical effect of some of the *Phyllanthus* species in the following order: *P.debilis* (87.24%) >Ascorbic acid (77.75%) >*P.urinaria* (73.18%) >BHT (72.20%) >*P. virgatus* (63.21%) >*P. maderaspatensis* (48.9%) >*P. amarus* (38.67%) at the dose of 25 µg/ml. In the present study, the methanol extract exhibited better activity of 48.60% at 100 µg/ml.

Effect on Inhibition of Nitric Oxide Radical Generation

In the present study, methanol extract exhibited better NO scavenging activity (70.09%) compared with the standard Vitamin C (77.07%), followed by hexane extract (54.59%). This observation reveals that these two extracts competed with oxygen to react with nitric oxide and thus inhibited the generation of the anions. Chloroform extract was inactive to scavenge the nitric oxide radical generation at 100 µg/ml (Table 16).

A substance may act as an antioxidant due to its ability to reduce ROS by donating hydrogen atom (Jayaprakash *et al.,* 2001; Khanam *et al.,* 2004). The reducing property of the test extracts implies that it is capable of donating hydrogen atom. The presence of phenolic compounds in the test extracts may be a contributing factor towards antioxidant activity because the phenolic compounds are known to have direct antioxidant property due to the presence of hydroxyl groups, which can function as hydrogen donor (Duh *et al.,* 1999; Dreosti, 2000).

The preliminary phytochemical analysis reveals the presence of flavonoids in the test extracts and tannins such as gallic acid, ellagic acid, corillagin and geraniin in the methanol extract. Polyphenols particularly flavonoids and tannins are well-known natural antioxidants (Arnason *et al.,* 1981; Duh *et al.,* 1999; Dreosti, 2000). Flavonoids are highly effective scavengers of all types of oxidizing radicals (Halliwell, 1996 a, b; Bors *et al.,* 1997). The concentration of hydrogen peroxide in water may vary according to the phenolic compounds. Since phenolic compounds present in the extract are good electron donors, they may accelerate the conversion of $H_2O_2 - H_2O$ (Ruch *et al.*, 1984). Nakagawa and Yokozawa (2002) reported that green tea can directly scavenge NO and O_2 and that its action is attributable to its phenolic components. Phenolic compounds of plant materials have been shown to neutralize free radicals in various model systems (Zhang *et al.,* 1996). The potent antioxidant activity exhibited by Amala is due to its polyphenols such as ellagic acid, gallic acid, tannin, etc. (Ihantola - Vormisto *et al.,* 1997; Bhattacharya *et al.,* 1999; Santos *et al.,* 1999). Kumaran and Karunakaran (2006 a) reported the potent activity of polyphenols such as geraniin, corillagin, furosin and gallic acid and rutin from *Phyllanthus debilis* against the DPPH radical scavenging antioxidant activity. When up to 1.0 g is daily ingested from a diet rich in fruits and vegetables (Tanaka *et al.,*

1988 b, c) polyphenolic compounds present in them inhibit mutagenesis and carcinogenesis in humans. The DPPH radical scavenging activity of *Phyllanthus* extracts may be mostly related to the hydroxyl group in phenolics (Kumaran and Karunakaran, 2007). In the present study, the methanol extract of *P. wightianus* showed strong antioxidant activity in both the models of assays such as DPPH (48.60%) and nitric oxide (70.09%) scavenging. It is concluded that the superior activity exhibited by methanol extract might be due to the presence of the phenolic compounds.

It is found out that the antioxidant property of the plants possesses hepatoprotective activity (Gupta *et al.,* 2004; Mondal *et al.,* 2005; Pal *et al.*, 2006) and hypoglycemic property (Garg and Bansal, 2000; McCune and Johns, 2002; Mazumder *et al.,* 2005; Mondal *et al.,* 2006). In the present study, the various test extracts of *P. wightianus* possess antidiabetic (Section 4.3.6) and hepatoprotective (Section 4.3.9) activities. It explains the antioxidant potential of the test extracts for the hepatoprotective and antidiabetic activities.

5.3.4 Wound Healing Activity

The term "wound" has been defined as a disruption of normal anatomical structure and more importantly function. Therefore, "healing" is the complex and dynamic process that results in the restoration of anatomical continuity and function (Lazarus *et al.,* 1994). Acute wounds normally heal in a very orderly and efficient manner characterized by four distinct but overlapping phases such as hemostasis, inflammation, proliferation and remodeling (Diegelmann and Evans, 2004). These steps are orchestrated in a controlled manner by a variety of bioactive molecules like growth factors, cytokines, their receptors and matrix molecules (Shukla *et al.,* 1999). Such a controlled phenomenon can be disrupted in diseases like diabetes, immuno-

compromised persons, ischemia, etc., thus leading to the development of a chronic wound. Prolonged or incomplete wound healing is then a troublesome complication (Ingold, 1993).

Apart from that, secondary infections by microbes in the wounds may further aggravate the conditions. Some of the important organisms include *Staph. aureus, Str. pyogenes, Corynebacterium* species, *E. coli* and *Ps. aeruginosa* wherein the most common are *Staph. aureus* and β-hemolytic *Str.* species (Mertz and Ovingotn, 1993) which are considered "transient flora" of the skin (Bikowski, 1999). *P. aeruginosa* is the predominant organism, which causes air born infection and its frequency of infection is more in burn patients. Infected wounds heal more slowly and have an increased incidence of scarring (Robson, 1997). Mycotic infections are also an important etiology of these infections, most of them are caused by dermatophytes and other related fungi. A wide range of antibiotics are being used at present for healing wounds and for treating wound infections but they are now proved to have adverse effects in the human body. In view of these developments, so much of attention has been paid recently to extracts of biologically active compounds isolated from plant species used in herbal medicinal system (Essawi and Srour, 2000).

The results in the present study suggest that topical application of the test drugs in animals significantly enhanced the rate of wound healing as assessed by the wound contraction (The time of wound closure for the ointments of standard drug and methanol extract was observed as 14±2 days and 16±2 days for hexane and 18±2 days for chloroform extract) and tensile strength (420±10.1 g, 670±12.3 g, 659±13.1 g, 562±12.6 g, 480±11.6 g - for control, standard, methanol, hexane and chloroform extracts respectively).

Treated excision wounds showed an increased rate of wound contraction, leading to faster healing as confirmed by the increased healed area when compared to the control group. The increased tensile strength in the test extract ointment-treated groups (659± 13.1 g, 562±12.6 g and 480±11.6 g - for methanol, hexane and chloroform extracts respectively) and the standard ointment treated (670±12.3 g) group in the incision model also confirms this observation (Table 17; Figs. 33 & 34).

It is well-accepted that several local growth factors help in the wound healing process. It is possible that the test extracts may have a growth factor like activity or have the ability to stimulate the expression of growth factors like the basic fibroblast growth factor (bFGF). The bFGF has the broadest range of target cells such as endothelial cells, fibroblasts, myoblasts, etc. (Schweigerer, 1988). Wound contraction is mediated by specialized myofibroblasts found in the granulated tissue (Moulin *et al.,* 2000). The increase in tensile strength of treated wounds may be due to increase in collagen concentration and stabilization of the fibers (Udupa, 1994 a; Udupa *et al.,* 1995).

Pro-inflammatory cytokines have been implicated to stimulate the synthesis of platelet activating factors by the recruited monocytes which in turn induce several angiogenic factors and chemokines (Lupia *et al.,* 1996). Moon *et al.* (1999) reported the pronounced improvement of type – I collagen material invasion by β-sitosterol which acts as an angiogenic factor in wound healing. Angiogenesis is the growth of new vascular capillary channels from pre-existing vessels and is of fundamental importance in a number of physiological processes such as embryonic development, reproduction, wound healing and bone repair (Maheswari *et al.,* 2006).

When wounding occurs it is accompanied within quite a short time by pain, reddening and oedema of the surrounding tissue. These are all classical symptoms of

inflammation and are caused by the release of the eicosanoids, prostaglandins and leutkotrienes and of reactive oxygen species (ROS).

The release of other factors such as the cytokines is also important. The release of these substances is caused by neutrophils aggregating at the wound site and producing proteolytic enzymes and ROS as antimicrobial defenses and as aids to the debridement of dead tissue. As the test extracts of *P. wightianus* exhibited potent anti-inflammatory (Section 4.3.3) and antioxidant (Section 4.3.4) activities these properties could have contributed wound healing in part.

Apart from the role of antioxidants in removing products of inflammation, they are also beneficial in wound healing in some other means. Antioxidants counter the excess proteases and ROS often formed by neutrophils accumulation in the wound area and protect protease inhibitors from oxidative damage. Fibroblasts and other cells may be killed by excess ROS and skin lipids will be made less flexible. So, antioxidant substances will reduce the possibility of occurrence of these adverse events. Because of these factors, overall antioxidant effects appear to be important in the successful treatment of wounds (Houghton *et al.*, 2005). In the present study, the test extracts were shown to be strongly antioxidant due to the presence of flavonoids and polyphenols.

Open wounds are particularly prone to infection, especially by bacteria and superficial mycotic agents and also provide an entry point for systemic infections. Infected wounds heal less rapidly and also often result in the formation of unpleasant exudates and toxins that will be produced with concomitant killing of regenerating cells (Houghton *et al.*, 2005). *Staph. aureus, Str. pyogenes,* and *Ps. aeruginosa* are the most common wound pathogens with $\geq 10^3$ CFU/g tissue which has been classified as infection (Bergstrom *et al.*, 1994).

In vitro antimicrobial studies (Section 4.2) of the present study showed that the test extracts were active against both gram-positive and gram-negative bacteria and *Candida albicans*. They are especially active against the superficial skin infection causing keratinophilic fungi dermatophytes. The presence of saponins, flavonoids and other phenolics in the test extracts could contribute to wound healing because of their detergent ability to remove grease, dirt and bacteria from tissue and act as antimicrobials (Houghton and Mensah, 1997).

The potent antioxidant and antimicrobial activities exhibited by the test extracts also contribute for the wound healing activity. This observation agrees with the opinion expressed by Singh *et al.* (2006).

The results in the present study reveal that the test extracts demonstrated polyvalent activity due to their anti-inflammatory, antioxidant and antimicrobial properties to heal wounds.

Among the test extracts, the methanol extract exhibited better activity than other extracts. It may be due to the presence of several active constituents and their synergistic activity. The significant burn wound healing activity (ED_{50} =190μg/wound; Kimura *et al.,* 2006) and antiulcer activity (In aspirin-induced gastric ulcers in rats bergenin reduced the ulcer index as 6.3±2.1 at 50 mg/kg when compared to control-18.3±3.3) (Goel and Maiti, 1997). These reports on bergenin also support the present findings.

5.3.5 Antidiabetic Activity

The ethnopharmacological use of herbal remedies for the treatment of diabetes mellitus is an area of study ripe with potential as a starting point in the development of alternative, inexpensive therapies for treating the disease. Several plants are used in folk medicine as antidiabetic agents (Bailey and Day, 1989; Ivorra *et al.,* 1989;

Swanston- Flatt *et al.,* 1990; Alarcon-Aguilar *et al.,* 1998; Bnouham *et al.,* 2006). Despite the availability of different types of oral hypoglycemic agents, there is a growing trend towards using natural products as adjuncts to conventional therapies (WHO, 1980). Species of *Phyllanthus* reported for their potent and promising antidiabetic activity include *P. amarus* (Srividya and Periwal, 1995; Adeneye *et al.,* 2006; Ali *et al.,* 2006), *P. niruri* (Chopra *et al.,* 1956; Perry, 1980), *P. sellowianus* (Hnatyszyn *et al.,* 1997, 2002), *P. urinaria* (Higashino *et al.,* 1992), *P. fraternus* (Hukeri *et al.,* 1988), *P. maderaspatensis* (Prashanth *et al.,* 2001), *P. emblica* (*Emblica officinalis*) (Anila and Vijayalakshmi, 2000; Sabu and Kuttan, 2002; Babu and Stanely Mainzen Prince, 2004; Rao *et al.,* 2005).

The results in the present study showed that the plasma glucose levels in diabetic- treated animals were significantly decreased (36.73 and 42.49% for hexane extract; 32.38 and 41.29% for chloroform extract; 43.50 and 52.00% for methanol extract at 100 and 200 mg/kg respectively; and glibenclamide (10 mg/kg) produced a maximum of 61.80%) in comparison with diabetic control animals (Table 19). The body weight of the treated animals was also restored. A pronounced reduction of cholesterol, triglycerides, alkaline phosphatase, ASAT and ALAT was recorded in the extract-treated animals and glibenclamide-treated animals (Table 20). It is known that glibenclamide stimulates insulin secretion but also reduces glucose levels acting at the liver and other tissues (Luzi and Pozza, 1997). The drug reduces the potassium permeability of β-cells by blocking the ATP sensitive potassium channels, causing depolarization, Ca^{2+} entry and hence insulin secretion (Rang *et al.,* 1999). The results in the present study indicate that the hypoglycemic activity of the test extracts may be due to a stimulating effect on the remnant β-cells, an improvement in insulin action or due to insulin-like effect. This view is in agreement with Hnatyszyn *et al.* (2002).

Diabetes mellitus has been shown to be associated with atherosclerotic and cardiovascular disease and cholesterol is involved in atherosclerosis (Kannel and McGee, 1979).

Insulin deficiency leads to various metabolic aberrations in the animals such as increased levels of blood glucose, cholesterol, alkaline phosphatase and transaminase and decreased level of protein content (Felig *et al.,* 1970; Begum and Shanmugasundaram, 1978; Shanmugasundaram *et al.,* 1983 a, b). A marked increase in serum TGL (183.6±4.8 mg/dl) and serum cholesterol (181.5±3.20 mg/dl) was observed in diabetic rats in the present study. This is in agreement with the findings of Nikkhila and Kekki (1973) and Dhanabal *et al.* (2004). Elevation of plasma lipid concentration in diabetic rats is well-documented. In the present study, the test extracts-treated groups of *P. wightianus* reduced cholesterol (99.2±7.4 mg/dl and 81.8 ±2.2 mg/dl for hexane extract; 102.3±6.3 mg/dl and 85.5±2.5 mg/dl for chloroform extract; 81.4±4.7 mg/dl and 75.5±2.6 mg/dl for methanol extract at 100 and 200 mg/kg respectively) and TGL levels (121.2±2.6 mg/dl and 113.8±2.9 mg/dl for hexane extract; 130.6±6.3 mg/dl and 122.7±3.9 mg/dl for chloroform extract; 110.5±9.3 mg/dl and 108.7±4.7 mg/dl for methanol extract at 100 and 200 mg/kg respectively). Several investigators have demonstrated that near normalization of blood glucose level resulted in the significant reduction of plasma cholesterol, free fatty acids and plasma apoprotein (Anila and Vijayalakshmi, 2000).

It is known that hypercholesterolemia, diabetes mellitus and obesity are closely associated to hypertension and stroke. Adeneye *et al.* (2006) reported dose–dependent decrease in the fasting plasma glucose and cholesterol and hypolipidemic potential of *P. amarus*. Jahromi *et al.* (1992) reported hypolipidemic and significant decrease in atherogenic index by bergenin isolated from the leaves of *Flueggea*

microcarpa. Anila and Vijayalakshmi (2000) reported the significant reduction of cholesterol, triglycerides, phospholipids and fatty acids by flavonoids isolated from *Emblica officinalis* and its inversely related action of hyperglycemia and hyperlipidemia. Thakur and Mandal (1984) reported the anticholesterolemic and antiatherogenic effect of the fruits of *Emblica officinalis.*

In diabetic animals, the change in the levels of serum enzymes is directly related to changes in the metabolism in which the enzyme is involved (Dhanabal *et al.,* 2004). Several researchers have reported increase in transaminase activity in the liver and serum of diabetic animals which is active in the absence of insulin because of increased availability of amino acids in diabetes. This mechanism is responsible for the increased gluconeogenesis and ketogenesis observed in diabetes (Felig *et al.,* 1970). In the present study, the test extracts significantly decreased ASAT (99.7±1.8 U/L and 94.7±6.38 U/L for hexane extract; 113.5±7.2 U/L and102.3±6.8 U/L for chloroform extract; 93.9±3.4 U/L and 84.3±7.1 U/L for methanol extract at 100 and 200 mg/kg respectively) and ALAT (59.6±1.6 U/L and 53.7±9.1 U/L for hexane extract; 62.6±1.3 U/L and 61.5±5.0 U/L for chloroform extract; 53.1±1.4 U/L and 48.7±1.6 U/L for methanol extract at 100 and 200 mg/kg respectively) enzyme activities. Hence, the improvements recorded in the levels of ASAT and ALAT are as a consequence of improvement in the carbohydrate, fat and protein metabolism due to the treatment by the test extracts. Restoration of ASAT and ALAT to their normal levels after the treatment also indicates revival of insulin secretion to near normal levels.

Among the parameters of protein metabolism, the present study showed an overall reduction in serum total protein (3.9±1.5 g/dl) in diabetic rats and the test extracts showed an elevation (5.9±0.2 g/dl and 7.0±0.2 g/dl for hexane extract;

5.8±0.4 g/dl and 6.2±0.8 g/dl for chloroform extract; 7.2±0.2 g/dl and 7.7±0.6 g/dl for methanol extract at 100 and 200 mg/kg respectively) of total protein. The increased levels of ALP in diabetic rats (279.2±12.6 U/L) were found to be significantly reversed by the action of the test extracts (202.8±12.4 U/L and 189.2±12.8 U/L for hexane extract; 221.2±12.6 U/L and 201.6±13.3 U/L for chloroform extract; 181.3±12.3 U/L and 171.1±11.6 U/L for methanol extract at 100 and 200 mg/kg respectively) (Table 20).

Treatment of the test extracts in the present study exhibited a significant increase in the body weight of the diabetic animals. This may be due to the improvement in glycemic control.

There are several reports explaining hypoglycemic property of ellagic acid. Shimizu *et al.* (1989) reported the aldose reductase inhibitory activity of it isolated from *P. niruri.* Sabu and Kuttan (2002) studied the anti diabetic and anti-oxidant activities of *Emblica officinalis*, *Terminalia bellerica* and *Terminalia chebula* and reported the presence of gallic acid or gallic acid-derivatives and ellagic acid responsible for their observed antidiabetic and antioxidant activities. Mankil *et al.* (2006) also reported ellagic acid as an anti diabetic agent. Their observations support the present study that the test extracts may decrease the effect of inflammatory cytokine release in diabetics which in turn might reduce insulin resistance.

Huang *et al.* (2005) reported that there is an improved sensitivity of the insulin receptor exhibited by *Punica granatum* flower and attributed responsibility for the presence of gallic acid in it. Steroid containing plants are known to exhibit antidiabetic activity. Oral administration of β-sitosterol dramatically brought down high blood sugar levels. The antihyperglycemic effect of β-sitosterol is believed to be by the increase of insulin level which is attributable to a stimulation of insulin

secretion from pancreatic β-cells. β-sitosterol given to diabetic rats orally improved diamine oxidase (DAO) levels. The results in the present study indicate a possible role of antihyperglycemic use for it in the prevention and treatment of diabetes (*www.enerex.ca*). It also helps to lower the cholesterol level and is believed to reduce the serum cholesterol by inhibiting the intestinal re-absorption of circulating cholesterol which is secreted in the bile. Human liver microsome studies show that it inhibits cholesterol absorption and people given by it were lowered their cholesterol and triglyceride levels (*www.enerex.ca*).

A study showed that vegetarians to be protected from fat loading diets by their high intake of β-sitosterol from plants. It is known that certain flavonoids exhibit hypoglycemic activity (Hukeri *et al.,* 1988; Pathak *et al.,* 1991; Geetha *et al.,* 1994; Ahmed *et al.,* 2000; Anila and Vijayalakshmi, 2000).

Other phytochemical constituents such as triterpenoids and glycosides (Mankil *et al.,* 2006), polyphenols (Orhan *et al.,* 2006; Aslan *et al.,* 2007), tannins (Teotia and Singh, 1997) and saponins (Sui *et al.,* 1994; Murakami *et al.,* 1996) have been reported as antidiabetic agents. Ali *et al.* (2006) reported α-amylase inhibitory property of *P. amarus* and attributed credit to the presence of three pure pentacyclic triterpenoids such as oleanolic acid, ursolic acid and lupeol. Prashanth *et al.* (2001) reported *in vitro* α-amylase inhibition by *P. maderaspatensis* and explained its therapeutic use to control obesity and diabetes.

Oliver (1980) and Mankil *et al.* (2006) have enumerated glycosides, alkaloids, flavonoids, terpenoids, phenolics and steroidal compounds as active ingredients in the plants reported for hypoglycemic property.

There is no doubt that herbs may be effective due to their fiber, vitamin and mineral content. Diet is the basic control of diabetic disorders. Addition of natural

fiber in the diet is widely encouraged. Vitamins and minerals are helpful to exacerbate the formation of insulin resistance due to working as co-factors in the signaling of insulin action and/or the glucose metabolism (Polya *et al.,* 2003 b). Plants may provide certain necessary elements like calcium, zinc, magnesium, manganese and copper to the β-cells (Akhtar and Iqbal, 1991). Supplement of minerals such as manganese, selenium and zinc has been used for long time. Due to the presence of such minerals in *P. wightianus*, it could be possible to achieve collective antidiabetic effect. So, it is concluded that the presence of different types of phytochemical constituents in the test extracts is synergistically responsible for the hypoglycemic activity. However, contribution of other unidentified constituents can not be ruled out for the activity. In future, the extracts of *P. wightianus* can be used to find out the antimicrobial activity of the diabetes mellitus - II patients against microbial diseases as people with diabetes are more prone to microbial infections than the normal healthy persons.

5.3.5.1 Diabetes Prone to Microbial Infections

Diabetes is associated with increased susceptibility to a number of infections but it manifests only when it is uncontrolled. It is attributable to impaired functioning of the polymorphonuclear leucocytes, defective chemotaxis, phagocytic uptake and probably intracellular killing also. Infections known to be associated with diabetes mellitus include those of the female urinary tract infections, staphylococcal infections, skin sepsis, perinephric abscess, tuberculosis, pulmonary infections, malignant otitis externa due to *Ps. aeruginosa* and a variety of fungal infections such as athlete's foot, ringworm and vaginal infections. The IDDM group has more of congenital anomalies and viral infections prone to ketosis (Abraham and Geevarghese, 1990).

Acute pyelonephritis, asymptomatic bacteriuria (Harding *et al.,* 2002), acute cellulitis and lymphangitis (Lee *et al.,* 2003), *Staph. aureus* bacteremia (Akbar *et al.,* 2000), osteomyelitis, gangrenous infection and anaerobic cellulitis (Lipsky *et al.,* 2005), bacterial pneumonia caused by *Staph. aureus* (Boyko *et al.,* 1989) and gram-negative organisms such as *K. pneumoniae, E. coli, Enterobacter* species, *Pseudomonas* species and *Acinetobacter* species (Johanson *et al.,* 1979), *H. influenzae* (Levinson and Kaye, 1985), pulmonary tuberculosis (Koziel and Koziel, 1995; Bashar *et al.,* 2001; Perez – Guzman *et al.,* 2001), pulmonary infections caused by the mycotic agents include Mucor mycosis (Bigby *et al.,* 1986), *A. fumigatus, A. niger, A. flavus* (Bouter *et al.,* 1991), *Cryptococcus neoformans* and *Coccidioides immitis* (Baker *et al.,* 1992). Parasitic infections include *Pneumocystis carinii* (Kovacs *et al.,* 1988) and viral infections such as influenza (Bouter *et al.,* 1991), diabetes and human immunodeficiency virus (Murray and Lumpkin, 1997) and polio virus types 1 – 3.

5.3.6 Antiarthritic Activity

Adjuvant-induced arthritis in rat model leads to a severe inflammatory joint disease primarily affecting synovial membrane of affected joints, with clinical and laboratory features representing a valid model for human rheumatoid arthritis (Pearson, 1956; Pearson and Wood, 1963, 1964; Barbier *et al.,* 1984; Taurog *et al.,* 1988; Billingham *et al.,* 1990). Rheumatoid arthritis, one of the commonest autoimmune diseases, is a chronic, progressive, systemic inflammatory disorder affecting the synovial joints and typically producing symmetrical arthritis that leads to joint destruction, which is responsible for the deformity and disability. Experimental adjuvant-induced arthritis model develops swelling, warmth, erythema and tenderness

in the distal joints and tendons of the animals with a maximum severity between 16 and 21 days (Escandell *et al.,* 2006).

As in the human pathology, the process is mediated by various inflammatory cells and mediators. Neutrophils produce reactive oxygen species and the activated macrophages generate reactive oxygen and nitrogen species (Eicosanoids through cyclooxygenase – 2 and nitric oxide through nitric oxide synthase – 2). Along with cytokines such as interleukin – 1 β and tumor necrosis factor - α (Park *et al.,* 2004 a, b), they have destructive effects on the cartilage.

Although non-steroidal anti-inflammatory drugs (NSAIDs), steroidal agents and immuno-suppressants have been developed and used in the treatment of rheumatoid arthritis over the past few decades there remains an ideal strategy to alleviate the symptoms for the longer term because of their side effects including gastrointestinal disorders, immunodeficiency, humoral disturbances, etc. Therefore, therapeutic agents that could be used for long-term administration (Agnello *et al.,* 2002) with lower side effects (Badger and Lee, 1997) are highly desirable.

The present study shows that the adverse physical, biochemical and radiological changes in arthritic animals were reversed to a considerable extent by oral administration of the test extracts of *P. wightianus* and its bergenin. Changes in body weight in arthritic animals were found to occur in response to the incidence and severity of arthritis. Less in body weight has also been reported in arthritic rats (Walz *et al.,* 1971; Besson and Guilbaud, 1988).

Increase in serum aminotransferase is due to liver impairment which is a feature of adjuvant arthritis (Whitehouse *et al.,* 1974). In the present study also, there was a significant increase in serum aminotransferase of the arthritic rats (0.99±0.03) which was brought back to near normalization after treatment with the test extracts

and bergenin (0.73±0.08, 0.75±0.06, 0.69±0.01and 0.68±0.06 for hexane, chloroform, methanol extracts at 200 mg/kg and bergenin at 50 mg/kg respectively; Table 25). There is a correlation between the development of inflammatory process and the release of lysosomal enzymes into the extra–cellular compartment in arthritic rats (Weissman, 1972). Acid phosphatase also seems to be important index for the examination of the integrity of the lysosomal membrane and is responsible for the tissue damage and necrosis of hepatic tissue (Yasuda *et al.,* 2000). Increased activities of cathepsin-D and acid phosphates (Olsen *et al.,* 1990; Geetha, 1993) have been observed in arthritic rats. Hydrolytic enzymes are released by the rupture of the lysosomal membrane which in turn initiates the synthesis of inflammatory mediators such as thromboxanes, prostaglandins and leucotrienes. This may be attributed towards persistent inflammation. These changes are in agreement with the decreased lysosomal stability in adjuvant-induced arthritis. Drugs capable of stabilizing the lysosomal membrane can reduce inflammation (Agha and Gad, 1995).

Denaturation of proteins as one of the causes of rheumatoid arthritis is well-documented (Singhal and Patterson, 1993). Igdoura *et al.* (1995) reported the role of cathepsin-D in the intracellular degradation of exogenous and endogenous proteins and its proteolytic activity is increased during various pathogenic processes leading to injury of lysosomes (Kominami *et al.,* 1991). In the present study, the activity of lysosomal enzymes in both serum and body tissues was markedly increased in the adjuvant-induced arthritic rats and significantly reduced after treatment with the test extracts and bergenin (Table 26). The marked decrease in serum and tissue lysosomal enzyme activity in the treated group indicated that the test extracts and bergenin may have an enhancing effect in membrane stabilization. An important

mechanism of anti-inflammatory activity has been found to be the membrane stability modulating effect (Subrata *et al.*, 1994).

Inhibition of paw oedema in adjuvant arthritic rat is a hallmark of anti-inflammatory drug action (Ramprasath *et al.*, 2005). Treatment of adjuvant arthritic rats with bergenin and the test extracts showed decline in inflammation. The oral administration of the test extracts showed a remarkable inhibition on both primary and secondary inflammations of adjuvant-induced arthritis in rats. The inhibition of inflammation by bergenin and methanol extract at 200 mg/kg/p.o. was higher than other test extracts and comparable to indomethacin-treated group (Tables 23 & 24).

It has been accepted that the adjuvant injection may not only cause the rat arthritic inflammation in the injected site as two phases of primary and secondary swellings but also in non-injected hind paw as well as other diarthrodial joints and tail synarthroses (Jiang *et al.*, 1997). The initial reduction of oedema and soft tissue thickening at the depot site is probably due to the effect of adjuvant whereas the late occurring disseminated arthritis and flare in the injected foot are presumably immunological events (Ward and Sidney Cloud, 1966).

The pathogenesis of rheumatoid arthritis is perpetuated by the activity of a complex network of cytokines (Choy and Panayi, 2001). As a consequence of the inflammatory process a large number of cytokines and growth factors with overlapping biological effects are found in the synovium. A marked hyperplasia of synoviocytes and blood vessels in the synovium, and a mononuclear cellular infiltrate consisting of macrophages, T and B cells are found. CD_4^+ T- helper cells (Th) can differentiate into two distinct subsets designated Th_1 and Th_2 type cells, which are characterized by different cytokine production profiles and effector functions.

Th_1 cells produce interleukin – 2 (IL-2) and interferon gamma (IFN -γ), support macrophage activation and are involved in delayed type hypersensitivity responses (van der Graaff *et al.,* 1999). Th_2 cells, on the other hand, secrete IL-4, IL-5 and IL-13 and provide efficient help for β-cell activation, antibody production and down modulate the production of pro-inflammatory cytokines by macrophages. From animal experiments, it has become clear that balance between Th_1 and Th_2 cells or their cytokines is important in the induction or prevention of organ specific autoimmune disease (Liblau *et al.,* 1995). Several reports have been published on the detection of Th_1 and Th_2 cytokines in rheumatoid arthritis (Milterburg *et al.,* 1992; Chen *et al.,* 1993; Quayle *et al.,* 1993; Simon *et al.,* 1994). Biological agents that specifically inhibit the effects of TNF-2 or IL-1 represent a major advancement in the treatment of rheumatoid arthritis (Shanahan *et al.,* 2003).

In the present study, the immunomodulatory activity of the test extracts (Section 4.3.8) has also confirmed the strong inhibition by SRBC – induced DTH reaction. The test extracts suppressed the DTH reaction mainly against the effector phase of DTH without inhibition of SRBC – induced humoral immune response. The suppression of DTH reaction positively correlates the suppression of Th_1- inhibition. At the same time, the test extracts also showed a remarkable inhibition on the carrageenin-induced paw edema (Section 4.3.3) Therefore, the inhibition of the test extracts against adjuvant-induced arthritis might include both direct anti-inflammatory and anti-DTH mechanisms. This view is in agreement with Jiang *et al.* (1997). Further, Swarnalakshmi *et al.* (1984) evaluated the anti-inflammatory activity of bergenin. Nazir *et al.* (2007) also reported the immunomodulatory effect of bergenin in the adjuvant – induced arthritis rats using Flow cytometric study. They have proved the mechanism of action of bergenin and norbergenin as Th_1 – inhibition as

well as Th_2 induction/production, i.e. the possible modulation of Th_1/Th_2 cytokine balance – to support the anti-arthritic activity of bergenin and norbergenin. Bani *et al.* (2006) also reported the anti-arthritic activity of lupeol through possible suppression of the immune system, i.e. the cytokines IL-2, IFN (γ) (Th_1) and IL4-(Th_2).

β-sitosterol is also known to boost the function of the T-cells and "prime" the immune system to function and operate more efficiently. If the immune system is over reacting as in the rheumatoid arthritis it can return to normal by decreasing the inflammatory response while helping to control the B-cell activity or antibody production (www.enerex.ca).

The human skeleton consists of 80% cortical bone and 20% trabecular bone. Trabecular bone having a large surface area is metabolically more active and more affected by factors that lead to bone loss. The main bone minerals are calcium and phosphates. More than 99% of the calcium in the body are in the skeleton, mostly as crystalline hydroxyapatite, but some as non-crystalline phosphates and carbonates; together, these make up half the bone mass phosphates which are also a major constituent of bone and are important in modifying the calcium concentration in bone and other tissues, in part by an effect on the synthesis of calcitriol (Rang *et al.,* 1999). In the present study also, qualitative analysis of the test extracts and estimation of calcium, cobalt and other metals by atomic absorption spectroscopy revealed the presence of calcium in larger amounts (6.300 mg in 1 g plant material; Section 4.1.3). Radiological analysis in arthritic rats strongly supports the ethnobotanical efficacy such as the topical application of the crude plant paste on fractured bones by the Malayali tribes for bone setting.

The role of free radicals in inflammatory diseases like rheumatoid arthritis can not be ruled out. The increase in lipid peroxidation can be attributed to weakening or

failure of the antioxidant defense system in rheumatoid arthritis (Barbar and Harris, 1994). In the present study also, severe tissue damages and increased biochemical markers were observed in the serum and tissues of the arthritic rats which was brought back to the near normalization level with the treatment of test extracts and bergenin. So, we can not rule out the role of the antioxidant nature of the test extracts and bergenin for the observed antiarthritic effects (Section 4.3.4).

In all aspects of the present study right from physical, biochemical, immunological to radiological aspects, the improvement of the arthritic animals treated with the test extracts and bergenin strongly supports the ethnobotanical use of the plant as an antiarthritic and an agent for using bone fractures. The results suggest that the test extracts and bergenin might be useful for the treatment of clinical rheumatoid arthritis because of the similarities of this model to human rheumatoid arthritis and the sensitivity of this model to anti-inflammatory and immune suppressing agents (Walz *et al.,* 1971; Baimgartner *et al.,* 1974) as suggested by several authors.

5.3.7 Immunomodulatory Activity

Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases. Several *Phyllanthus* species have been reported for their Immunomodulation potentials such as *P. debilis* (Thabrew *et al.,* 1991), *P. emblica* (Suresh and Vasudevan, 1994), *P. tenellus* (Ignacio *et al.,* 2001) and *P. sellowianus* (Fernandez *et al.,* 2002).

In the present study, the test extracts of *P. wightianus* showed significant inhibition on the delayed type hypersensitivity reactions induced by SRBC with an anti- inflammatory action but without inhibiting the humoral immune response which shows the selective suppression of cellular immune response (CIR) by the test

extracts. This is in agreement with the view of Xu *et al.* (1993). Among the test extracts, methanol extract exhibited better inhibition than hexane and chloroform extracts. Bergenin, one of the active constituents of methanol extract was reported for its potent immuno modulating activity in chronic immuno inflammatory disease (adjuvant arthritis) by Nazir *et al.* (2007). They reported the balancing potentials of bergenin and norbergenin in Th_1/Th_2 cytokines. Th_1 cells produce interleukin - 2 (IL-2) and interferon gamma (IFN-γ), which support macrophage activation are involved in delayed type hypersensitivity responses (van der Graaff *et al.*, 1999). Th_2 cells, on the other hand, secrete IL-4, IL-5 and IL-13 and provide efficient help of B cell activation, antibody production and down modulate the production of pro-inflammatory cytokines by macrophages. According to Nazir *et al.* (2007), bergenin effectively suppresses the production of Th_1 cells and promotes the production of Th_2 cells as an anti-inflammatory agent.

In the present study, better inhibition showed by the methanol extract may be due to the presence of bergenin in it. However, it can not be ruled out the role of other active constituent(s) present in the methanol extract for its immuno modulating potential. Further, the phytochemical evaluation revealed the presence of lupeol in hexane and chloroform extracts and the preliminary phytochemical screening reveals the presence of flavonoids in the test extracts. The immuno suppressive role of lupeol in chronic immune inflammatory reaction was also supported by Bani *et al.* (2006). Okoli *et al.* (2003) reported lupeol as an anti-inflammatory agent. The presence of lupeol in the hexane and chloroform extracts may be responsible for its anti-inflammatory action in DTH response. In the humoral antibody response to SRBC, there was a dose-dependent increase in HA titers perhaps showing the stimulation of B-lymphocytes for the production of antibodies. The methanol and hexane extracts

exhibited significant increase in HA titer such as 8.80±0.62, 7.40 ±0.32 for methanol and 7.01±0.16, 6.6±0.31 for hexane at 200 and 100 mg/kg respectively, whereas in the chloroform extract such as 5.70±0.31, 5.30±0.26 at 200 and 100 mg/kg respectively at both dose levels, it was less, which showed the ineffectiveness of the stimulation of B-lymphocytes (Table 28). Increase in HA titer by methanol and hexane extracts indicates enhanced responsiveness of macrophages and T and B lymphocyte subsets involved in antibody synthesis (Benacerraf, 1978). According to Makare *et al.* (2001) and Dash *et al.* (2006), this could be due to the presence of flavonoids which augment the humoral response by stimulating the macrophages and B-lymphocytes subsets involved in antibody synthesis.

In the non-specific immunity determined by survival rate against fungal infection, there was a significant increase in survival rate of all the test extracts. The increase in survival rate is a general marker exhibiting potency of the test extracts to overcome infectious condition. Better inhibition of methanol extract (59.95%) indicates its high potency to overcome infectious condition than hexane (54.02%) and chloroform extracts (45.96%).

The process of phagocytosis by macrophages includes opsonisation of the foreign particulate matter with antibodies and complement C_3b, leading to more rapid clearance of foreign particulate matter from the blood (Furthvan and Bergvanden, 1991). Increased carbon clearance is an indicator of enhanced *in vivo* phagocytic activity and competency of granulopoetic system in removal of foreign particle, thereby an indicator of enhanced immunological response (Thakur *et al.,* 2007). Phagocytic defects are associated with varied pathological condition in humans (Jo White and Gallin, 1986). When the carbon particles are injected intravenously the rate of clearance of carbon from blood by macrophages is governed by an exponential

equation. This observation seems to be the general way in which inert particulate matter is cleared from the blood (Gokhale *et al.,* 2003). However, in the present study, the test extracts were found to be moderately stimulating the phagocytic activity of the macrophages as evidenced by the slight increase in the rate of carbon clearance when compared to the control animals.

In the cyclophosphamide-induced myelosuppression assay, the test extracts significantly increased the total white blood cell count such as 7.52±0.21 and 7.23±0.11 to methanol extract; 5.56±0.28 and 5.23±0.19 to chloroform extract; and 6.96±0.23 and 6.63±0.21 to hexane extract at 200 and 100 mg/kg respectively (Table 31) and also restored the myelosuppressive effects induced by cyclophosphamide. The total WBC count was significantly increased in all test extracts and among them, methanol and hexane extracts exhibited better increase in WBC count. According to Hartwell (1969), plant phenolics having anti-inflammatory potential showed B-cell stimulation to a significant extent. On the whole, the results indicated that the test extracts of *P. wightianus* hold promising immunomodulatory potential.

5.3.8 Hepatoprotective Activity

5.3.8.1 *In vitro* Inactivation of HBsAg

More than 2 billion people have been infected with HBV worldwide and about 350 million people are chronically infected carriers of the virus (Merle *et al.*, 2001). HBV contains numerous antigenic components including hepatitis surface antigen (HBsAg). HBV Infected cells secrete HBsAg and complete virion particles. Although HBsAg is not infectious complete particles are infectious (Dane particles). When HBsAg is in the blood the complete virus is also present (Brind *et al.,* 1997). Therefore, compounds inhibiting the production or secretion of HBsAg may be used as therapeutic agents against HBV (Kwon *et al.*, 2005).

Apart from the development of specific hepatoprotective drugs, many of *Phyllanthus* species have been reported for the activities of HBsAg inhibition such as *P. amarus* (Thyagarajan *et al.*, 1988, 1990; Blumberg *et al.*, 1989; Jayaram *et al.*, 1990, 1997; Jayaram and Thyagarajan, 1996; Mehrotra *et al.*, 1991; Liu *et al.*, 2001), *P. chamaecristoides* (Alonso *et al.*, 1995), *P. niruri* (Thyagarajan *et al.*, 1982; Jayaram *et al.*, 1987; Venkateswaran *et al.*, 1987; Mehrotra *et al.*, 1990).

In the present study both the methanol extract and bergenin exhibited potent inhibition of anti-HBs binding activity and the activity was dose-dependent. At the dose level of 200µg/ml, bergenin showed its maximum activity as 59% inhibition while it was 50% at 400µg/ml. This showed that maximum activity could be realized at 200µg/ml itself therefore further increase in concentration reduced the percentage of activity. In the case of methanol extract, increase in concentration such as 8 mg/ml exhibited maximum inhibition up to 56%.

The dose-dependent inhibition of HBsAg activity by the plant extracts has been reported by Thyagarajan *et al.* (1982), Venkateswaran *et al.* (1987), Mehrotra *et al.* (1990, 1991), Alonso *et al.* (1995), Jayaram and Thyagarajan (1996) and Kwon *et al.* (2005).

Alonso *et al.* (1995) reported *in vitro* inactivation of HBsAg in three species of *Phyllanthus* and attributed its inactivation in *P. chamaecristoides* to the presence of flavonoids. Lim *et al.* (2000 c) reported *in vitro* effect of bergenin (100µ M) isolated from *Mallotus japonicus* in galactosamine-intoxicated primary cultured rat hepatocytes showing the inhibition by the release of glutamic pyruvic transaminase and sorbitol dehydrogenase and the increase of RNA synthesis. Kim *et al.* (2000) also reported similar activity by the model of CCl_4 - intoxicated primary cultured rat

hepatocytes and proved its antihepatotoxcicity through glutathione-mediated detoxification and free radical suppressing activity.

However, there has been no published literature available for *in vitro* inactivation of HBsAg by bergenin so far. The present study proved the efficacy of bergenin against *in vitro* inactivation of HBsAg for the first time in science. Further, detailed study with different incubation timings at different temperatures may be useful to fix the exact dose level to treat the clinical conditions. So, it can be concluded that the potent inhibiting activity of methanol extract against HBsAg is due to the presence of bergenin in it.

5.3.8.2 Isoniazid (INH) and Rifampicin (RMP)-induced Hepatic Injury in Rats

Isoniazid (INH) was the first effective bactericidal drug used to treat tuberculosis and is an important part of most anti-tubercular drug regimens till far. Rifampicin (RMP), which is another effective bactericidal drug added to the regimen in 1962 has remained the most effective combination along with isoniazid (Snider *et al.,* 1984). However, these drugs are also well known as hepatotoxic agents (Steele *et al.,* 1991). Hepatitis has been reported to occur in 0.46% of patients receiving these anti-tubercular drugs (Alexander *et al.,* 1982). Toxic neuropathy and hepatitis are the most common adverse reactions to isoniazid (Nolan *et al.,* 1999; Blumberg *et al.,* 2003; Yee *et al.,* 2003; Shakya *et al.,* 2004). Rifampicin has produced severe immuno allergic reactions along with hepatocellular carcinoma (Blumberg *et al.,* 2003; Shakya *et al.,* 2004) and the rate of hepatotoxic reaction reported was much higher in the Indian patients (Ramachandran, 1980) compared to that of the developed countries at similar doses (O'Brien *et al.,* 1983; Mindie and Gabriel, 2002). Nelson *et al.* (1976) postulated that one of the isoniazid metabolites acts as an acetylating agent causing injury to the macromolecules of hepatocytes. Santhosh *et al.* (2006) reported that

rifampicin, a powerful inducer of drug metabolizing enzymes in man and rats, contributes to the hepatotoxcicity of isoniazid by enhancing the rate of the production of toxic metabolites.

So, management of hepatic disorders has become a matter of serious concern worldwide and there is a great lack in modern medicines to treat hepatitis, cirrhosis, liver damage and hepatic carcinoma produced by toxins or for biliary tract disorders. However, from time immemorial, physicians practicing different principles including herbs have been trying various plant products to alleviate these disorders and diseases, e.g. silymarin, a natural flavonoid derived from *Silybum marianum*. The drug is recommended in the management of clinical cases of hepatic disorders. It is well-known that species of *Phyllanthus* (Euphorbiaceae) have been in wider use by the traditional medical practitioners to treat liver disorders with promising results (Syamsundar *et al.*, 1985; Venkateswaran *et al.*, 1987; Thyagarajan *et al.*, 1988).

Hepatoprotective nature of bergenin isolated from the cortex of *Mallotus japonicus* was reported both *in vivo* and *in vitro* against the toxic substances such as CCl_4 by Lim *et al.* (2000 b) and Kim *et al.* (2000) and D-galactosamine by Lim *et al.* (2000 c, 2001).

Hepatotoxcicity of INH is thought to be initiated by cytochrome P450 (CYP)-mediated metabolism of INH to acetylhydrazine and hydrazine (Jenner and Timbrel, 1994; Sarich *et al.*, 1996, 1999; Chowdhury *et al.*, 2006).

Rifampicin generally co-administered with INH in the treatment of tuberculosis enhances hydrazine production by enzyme induction (Pessayre *et al.*, 1997). The high reactivity of hydrazine with sulfhydryl groups results in glutathione (GSH) depletion within the hepatocytes, which leads to cell death (van den Dobbelsteen *et al.*, 1996; Macho *et al.*, 1997; Tasduq *et al.*, 2005 b). Sodhi *et al.*

(1997) and Attri *et al.* (2000) have also demonstrated the critical role of GSH in anti-tubercular drugs-induced hepatotoxcicity. According to Tasduq *et al.* (2005 b), the hepatic injury by anti- tubercular drugs is due to membrane damage (as indicated by increased serum markers), suppression of antioxidant defense mechanisms accompanied by enhanced lipid peroxidation and stimulation of metabolic activation by CYP 2E1 and modulation of [Ca_2+] ions. Chowdhury *et al.* (2001) demonstrated oxidative stress in patients having anti-tubercular drugs-induced hepatotoxcicity. Oxidative damage by these drugs is generally attributed to the formation of highly reactive oxygen species, which acts as stimulator of lipid peroxidation and source for destruction and damage to the cell membrane (Georgieva *et al.*, 2004). Apart from increased oxidative stress in the liver, reported that occurrence of increased oxidative stress in the liver mitochondria, associated with mitochondrial permeability alterations and increased apoptosis of the hepatocytes was an important mode of liver injury (Chowdhury *et al.*, 2006). Increased GSH depletion as well as oxidation in the liver of mice co-treated with INH + RMP could be either due to its consumptive utilization by the drugs metabolites or an inability of the GSH synthetic machinery in the liver to cope up with the increase demand of synthesis or both resulting in the imbalance in GSH homeostasis.

Aminotransferases are an important class of enzymes linking carbohydrate with amino acid metabolism thus they clearly establish the relationship between the intermediates of the citric acid and amino acids. Alanine aminotransferase and asparate aminotransferase are well-known diagnostic indicators of liver diseases. In case of liver damage with hepatocellular lesions and parenchymal cell necrosis, these marker enzymes are released from the damaged tissues into the blood stream. Elevated levels of these enzymes in serum are presumptive markers of drug-induced

necrotic lesions in the hepatocytes (Amr and Alaa, 2005). In turn, the damage in hepatocytes increased the bilirubin release (Man–Fung *et al.,* 2003). Increased level of AST, ALT, ALP and bilirubin has been reported in INH+RMP-induced hepatotoxcicity in rats (Tasduq *et al.,* 2005 b; Pal *et al.,* 2006; Santhosh *et al.*, 2006). Enhanced susceptibility of hepatocytes in cell membrane might have resulted in increased release of these diagnostic marker enzymes in systemic circulation (Santhosh *et al.,* 2006). In the present study also, it was observed that the administration of INH + RMP elevated the levels of serum marker enzymes such as AST (200.41±5.3 U/L), ALT (212.92±3.2 U/L), ALP (40.55±1.6 KA units) and bilirubin (2.63±0.34 mg/dl) in group–2 animals.

The co-treatment with the test extracts and bergenin 200 mg/kg attenuated the condition and elevated serum levels of AST (98.71±2.3, 101.72±2.96, 89.61±4.682 and 15±6.8 U/L to hexane, chloroform, methanol and bergenin at 200mg/kg), ALT (69.14± 6.4, 88.05±1.5, 53.51±3.9, 48.32±6.3 U/L to hexane, chloroform, methanol and bergenin at 200 mg/kg) and ALP (23.14±6.3, 28.59±1.5, 19.16±1.7, 17.02±1.8 KA units to hexane, chloroform, methanol and bergenin at 200 mg/kg), bilirubin (1.41±0.26, 1.56± 0.34, 1.12±0.3, 0.99±0.4 mg/dl to hexane, chloroform, methanol and bergenin at 200 mg/kg) were significantly decreased towards normalization (Table 33). This was in agreement with the reports of Lim *et al.* (2000 b). The stabilization of serum bilirubin, AST, ALT and ALP by the drug treatment is a clear indication of the improvement of the functional status of the liver cells (Table 33). Histopathological studies also support these findings.

The improved histology of liver co-treated with the test extracts and bergenin (Fig 51) as compared to that observed in animals administered with only INH + RMP indicates the possibility of the test extracts being able to induce accelerated

regeneration of liver cells by reducing the leakage of AST, ALT and ALP in the blood, and thereby lowering their values in the serum. Serum transaminase returns to normal with the healing of liver parenchyma and regeneration of liver cells.

Alterations of protein metabolism have been considered for decades to be one of the conditions associated with hepatic dysfunction. In the present study also there was a significant decline in protein of animals treated with INH + RMP alone (4.52±0.12 g/dl) when compared to that of control animals (9.23±0.14 g/dl). The disaggregation of polyribosomal profiles induced by anti-tubercular drugs is also associated with the inhibition of protein synthesis, which may be partially responsible for the fatty liver, probably not necrosis, although it contributed to disabling of the cells. Co-treatment with bergenin and the test extracts brought back the protein levels towards normalization (6.7 ±0.16, 5.81±0.13, 7.57±0.13, 8.70±0.11 g/dl to hexane, chloroform, methanol and bergenin at 200 mg/kg; Table 33) that is parallel with the report of Santhosh *et al.* (2006).

The major disorder encountered in anti-tubercular drugs induced hepatitis is fatty accumulation in the liver which develops either due to excessive supply of lipids to the liver or interference with lipid deposition (Santhosh *et al.*, 2006). In the present study also the levels of total cholesterol and triglycerides were significantly higher in group–2 INH+RMP administered rats (Table 33) to that of normal control animals indicating anti-tubercular drugs induced hypercholesterolemic condition, which also indicates that hepatic injury related alterations in lipid composition of liver tissue appears to occur due to destruction of hepatocytes. Co-administration with bergenin and the test extracts of *P. wightianus* significantly reduced the anti-tubercular drugs-induced elevation in the levels of total cholesterol and triglycerides compared to that of group–2 animals indicating hypolipidemic nature of bergenin and the test extracts.

Jahromi *et al.* (1992) reported that oral administration of bergenin isolated from *Flueggea microcarpa* significantly decreased (lowered) the serum total cholesterol and triglycerides. Cholesterol lowering effect of *Phyllanthus* species has been reported by Mishra *et al.* (1981), Thakur (1985), Jacob *et al.* (1988), Mathur *et al.* (1996) and Adeneye *et al.* (2006).

The phytochemical evaluation of the present study reveals the presence of sterols in the hexane and chloroform extracts. Although β-sitosterol is very similar in its chemical composition to serum blood cholesterol it is completely different in its biological function. It interferes with cholesterol absorption, prevents the rise in serum cholesterol and inhibits the intestinal re-absorption of circulating cholesterol which is secreted in the bile. Therefore, hepatoprotective effect of bergenin and the test extracts of *P. wightianus* is probably related to its ability to inhibit lipid accumulation in the liver tissue by its antilipidemic property. The findings of the histopathological evidences are positive and support the hepatoprotective effect.

Combination of INH+RMP treatment in experimental animals enhanced lipid peroxidation indicating increased oxidative stress in liver (Skakun and Slivaka, 1992; Chowdhury *et al.,* 2001). It is well-known that drugs with antioxidant activity are effective in the treatment of hepatotoxicity (Roy *et al.,* 2006).

Antioxidants can interfere with the oxidation process by reacting with the free radicals, checking the free catalytic metals and also by acting as oxygen scavengers (Gulcin *et al.,* 2002). All the test extracts exhibited significant *in vitro* antioxidant activity against DPPH and nitric oxide free radical assay (Section 4.3.4). The antioxidant capacity of bergenin has been well-documented (Takahashi *et al.,* 2003; Rana *et al.,* 2005). The antioxidant capacity of the test extracts and bergenin would have contributed hepatoprotection.

Hepatoprotective activity of the extracts of *Phyllanthus* species has been reported by Gulati *et al.* (1995), Prakash *et al.* (1995), Unander *et al.* (1995), Asha *et al.* (2004), Harish and Shivanandappa (2006) and Pramyothin *et al.* (2006).

Tasduq *et al.* (2005 a) reported the protective effect of 50% hydro-alcoholic extract of *Emblica officinalis* against anti-tubercular drug-induced liver toxicity and the activity was found to be due to its membrane stabilizing, antioxidative and CYP 2E1 inhibitory effects.

Mankani *et al.* (2006) and Prasad *et al.* (2006) have reported lupeol as a hepatoprotective agent whereas the latter commented that it might be due to its combating effect on oxidative stress.

Shin *et al.* (2005) reported that ellagic acid isolated from *P. urinaria* showed strong hepatoprotection by inhibiting HB e Ag secretion in the HePG 22.2.15 cell line. Lim *et al.* (2000 b, 2001) studied CCl_4 and D-galactosamine-induced toxicity *in vivo* to assess hepatoprotection of bergenin. Similarly, in the present study, bergenin demonstrated hepatoprotective activity *in vivo* against liver injury induced by INH + RMP (Table 33; Fig. 51).

Bergenin has shown consistent and better hepatoprotection. However, methanol extract of *P. wightianus* offered more or less related or comparable protection. This could be due to the presence of bergenin in it or the synergistic activity of other polyphenols in the methanol extract such as gallic acid and or ellagic acid or a combination of other compound(s) in it. The overall hepatoprotective effect may probably due to a counteraction of free radicals by its antioxidant nature/or to its ability to inhibit lipid accumulation by its antilipidemic property.

CHAPTER 6

CONCLUSION

Since antiquity, man has used plants traditionally as medicines to treat diseases including common infectious ones wherein some of them are still included as part of the habitual treatment of various maladies (Rios and Recio, 2005). The most notable discoveries include the *Ipecacuanha* root from Brazil which yields a chemical called emetine, that kills *Entamoeba histolytica*, the cause of amoebic dysentery, and the Cinchona bark from Peru, which yields quinine, an alkaloid that kills *Plasmodium*, the protozoa that causes malaria (Modi, 1995).

The phytochemical screening of species of *Phyllanthus* has yielded to identify 299 compounds wherein biological activity has been reported for about 28 compounds only. In the case of antimicrobial activity, 4 species each have been reported for antibacterial activity, antifungal activity and 7 species for both antibacterial and antifungal activities.

The plant, *P. wightianus*, chosen for the present study is one such species. Though the Malayali tribals in the Vellore District of Tamil Nadu State in Peninsular India traditionally use to set bones (bone fractures) and to treat diarrhoea it has been not been investigated by phytochemical, antimicrobial and pharmacological studies prior to the present study. The name bears its efficacy such as Elumbotti in Tamil language combining two words such as Elumbu means bone and otti means pastes or sets right fractured bones. Therefore, the present study is aimed to prove the efficacy scientifically by isolating the bioactive principles/compounds responsible for efficacy and test them *in vivo*. Further screening of extracts and its compounds isolated from them for other biological activities could also prove useful. Keeping these objectives

in mind, the extracts of the plant and an isolated compound such as bergenin have been scientifically investigated.

The preliminary phytochemical analysis of the plant revealed the presence of glycosides, steroids, triterpenes, flavones, phenols in all the extracts such as hexane, chloroform and methanol extracts and catechins, coumarins, sugars, saponins and tannins in methanol extract.

The results of the fluorescent analysis were quite helpful to fix parameters in assessing quality and standardization. Analysis of ash for inorganic elements revealed the presence of 2.960 mg sodium and 1.200 mg potassium and 6.300 mg calcium, 0.003 mg cobalt, 0.060 mg copper, 2.130 mg iron, 1.089 mg magnesium and 0.345 mg manganese.

The compounds were isolated applying various chromatographic techniques (TLC, CC, HPLC, HPTLC and GC - MS) and identified by the analysis of spectral data (^{1}H and ^{13}C NMR, IR, UV - VIS, MS) and X-ray crystallographic analysis in specific cases. Confirmation was done by direct comparison with authentic sample (m.p., m.m.p. and superimposible IR and HPTLC).

The paper chromatographic analysis led to the identification of amino acids such as DL - Alanine (0.225), L - Arginine mono HCl (0.162), DL - Aspartic acid (0.347), L – Cystine (0.120), L - Glutamic acid (0.160), G - Lysine (0.138), DL - Methionine (0.154), DL - Tryptophan (0.624) and L - Tyrosine (0.375).

When the spectra were compared by superimposable methods the HPTLC chromatograms performed with marker compounds revealed the presence of friedelin in hexane extract, lupeol in chloroform extract, and gallic acid and bergenin in methanol extract.

The HPLC analysis done in comparison with the values of standards given in parentheses helped to identify and estimate tannins such as gallic acid (GA) - 5.158 (5.092), corillagin (C) - 18.900 (18.875), geraniin (G) - 20.292 (19.817) and ellagic acid (EA) - 27.617 (27.592). Estimation of area of peak facilitated to quantify corillagin - 3.89%, (the most abundant one), geraniin - 3.19%, followed by meager quantities of ellagic acid - 0.68% and gallic acid - 0.38%. The HPLC analysis of the methanol extract carried out along with marker compounds of lignans such as hypophyllanthin and phyllanthin indicated their absence.

A fraction of hexane extract subjected to GC - MS analysis showed 23 peaks with various retention times and molecular weights wherein only seven compounds such as 6,10,14-trimethyl-penta-decan-2-one, hexa-decanoic acid methyl ester, 3,7,11,15-tetramethyl-1-hexadecen-3-ol (isophytol), phytol, octa-decanoic acid methyl ester, 9–octa-decanoic acid methyl ester and di-n-octyl phthalate could be identified unequivocally.

GC - MS analysis of the hexane and chloroform extracts resulted to isolate and identify friedelin and lupeol and sterols such as stigmasterol, campesterol and β-sitosterol in a mixture form. Bergenin, corillagin, ellagic acid, gallic acid and geraniin were isolated and identified from the methanol extract.

Agar-well diffusion method followed to assess antimicrobial activity. Gram-positive, gram-negative and pathogenic fungi including dermatophytes were tested. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were estimated. When similar concentrations were compared it is revealed that the extracts were more potent against almost all the gram-negative bacterial strains than that of gram-positive bacterial strains. Out of three extracts, the methanol extract produced better inhibition with

stronger and broader spectrum. It was followed by the hexane extract. Moderate activity recorded for the chloroform extract. Dose-dependent inhibition and increased inhibitions at higher concentrations were observed. Inhibiting activity of the extracts might be due to the presence of different classes of compounds or their synergistic action. The observation of strong and potential activity against both bacterial and fungal agents including those of skin and wound infections causing microorganisms supports not only the traditional efficacy of bone setting but also provides the possibility of developing a good therapeutic agent for skin and wound infections.

The present study has revealed the possibilities of the test extracts to develop drugs for different diseases such as the potential inhibition against *Ps. aeruginosa, E. coli, Pr. mirabilis* and *Pr. vulgaris* gives scientific evidence to the test extracts for their ability to act against the agents causing urinary tract infections; the activity against the enteric agents such as *E. coli, V. cholerae* and *V. parahaemolyticus* provides scientific evidence to the test extracts as an effective antidiarrheal agent; antipseudomonial and antistaphylococcal activities of the test extracts are considered important against nosocomial infections; and anti-dermatophytic and anti-candidal role of the test extracts strongly supports the efficacy of the test extracts against the agents of bacteria and fungi causing skin infections.

Pharmacological screening such as oral acute toxicity studies, analgesic, anti-inflammatory, *in vitro* antioxidant, wound healing, antidiabetic, antiarthritic, immunomodulatory and hepatoprotective activities (*in vitro* and *in vivo*) for various test extracts was done based on the ethnobotanical claim, qualitative analysis of various test extracts and the presence of active compounds. Scrutiny of published literature facilitated to test bergenin, an isolated compound from methanol extract, for antiarthritic and hepatoprotective activities (*in vitro* and *in vivo*).

In the case of analgesic activity, hexane, chloroform and methanol extracts did not show significant increase in latency to hot plate method. Significant % of protection was recorded in the order of methanol, hexane and chloroform extracts against acetic acid - induced writhing in mice. The findings suggest that the analgesic activity of the test extracts might be due to peripheral analgesic mechanism rather than central analgesic mechanism and it appears to occur by mechanisms independent of activation of opoid receptors as there was a complete lack of analgesic effect to radiant heat in the hot plate test, a very sensitive opoid assay, where morphine caused a graded increase in paw latency. The presence of β-sitosterol, campesterol and stigmasterol in the hexane and chloroform extracts and gallic acid, ellagic acid and geraniin in the methanol extract might have contributed for analgesic activity.

Significant protection of oedema in the anti-inflammatory activity for all the test extracts might be due to significant *in vitro* free radical scavenging activity of the different classes of compounds present in them.

In the case of *in vitro* antioxidant activity, all the test extracts expressed DPPH and NO radical scavenging activity (except chloroform extract). Of which, the methanol extract exhibited strong free radical scavenging activity due to the presence of phenolics in it. It could be correlated to the significant hepatoprotective, antidiabetic and anti-inflammatory activities (both acute and chronic).

All the test extracts exhibited significant wound healing activity. Among the ointments prepared with test extracts, methanol extract ointment produced a highly significant activity to both the models of wound healing, i.e., more or less similar to that of standard drug, nitrofurazone (2% w/w). It may due to the presence of active constituents such as phenols, polyphenols, flavonoids, tannins, saponin and coumarins and their individualistic or synergistic action. The polyvalent activities of the test

extracts such as anti-inflammatory, antioxidant and antimicrobial activities would have hastened the wound healing activity. Thus, the present study confirms the promising wound healing activity and provides scientific validation to the ethnotherapeutic efficacy of the plant as a potential wound healing agent.

All the test extracts exhibited significant hypoglycemic activity. Among all, the methanol extract expressed better activity which might be due to the presence of flavonoids, β-sitosterol, lupeol, gallic acid, ellagic acid, saponins and tannins and it could be correlated to the synergistic relationship in healing with antioxidant and free radical scavenging activities.

The plant chosen for the present study has not been scientifically investigated for arthritic and rheumatoid diseases. The findings against adjuvant - induced arthritis in rats revealed the significant protection of the test extracts. Among all, the methanol extract and its isolate bergenin exhibited better protection. Better protection exhibited by the methanol extract could mostly be due to the presence of bergenin in it as there was only a slight difference between the activity of methanol extract at 200 mg/kg/p.o. and bergenin at 50 mg/ kg/p.o.. Further, the activity of bergenin was comparable to that of standard, indomethacin. The significant protection might be due to the presence of β-sitosterol in the hexane extract and lupeol in the chloroform extract. However, it is impossible to rule out the role of other active constituents for their antiarthritic activity. Invariably, all the extracts exhibited better protection at higher doses than lower doses. The results of the present study provide strong evidence for the possibility to develop antiarthritic and antirheumatic drugs and also suggest that the test extract and compound might be useful for the treatment of clinical rheumatoid arthritis while considering the similarities of this model to human rheumatoid arthritis.

In the case of immunomodulating activity, the methanolic extract is superior over hexane and chloroform extracts. The test extracts express not only potential non-specific immune response but also effective in improving humoral and cell-mediated immunity. The test extracts selectively suppress the cell-mediated immune response without inhibiting the humoral immune response that showed its worth as an anti-inflammatory agent against immunologically induced chronic inflammatory diseases such as arthritis.

The increase in survival rate is a general marker exhibiting potency of the test extracts to overcome infectious conditions. The active constituents such as bergenin in methanol extract, friedelin in the hexane extract and lupeol in the chloroform extract might be responsible for their potential immunomodulating activity. However, the role of other constituents present in them can not be ruled out for the immunomodulating potential.

As far as hepatoprotective activity is concerned the test extracts were active to both *in vitro* and *in vivo* models. Both methanol extract and bergenin exhibited dose - dependent inhibition of HBsAg at the tested temperature and incubation periods proving their antiviral capacity. As that of other *Phyllanthus* species, popularly known for antiviral properties, the plant chosen for the present study also has similar property. The findings of *in vivo* model revealed that all the test extracts and bergenin exhibited significant hepatoprotection against INH + RMP - induced hepatic damage. The hepatoprotective activity of bergenin is so comparable to that of standard silymarin. The methanol extract had been effective in offering protection which is more or less related to the influence of bergenin. Among all, the methanol extract exhibited superior activity than hexane and chloroform extracts. It may be due to the presence of bergenin or other polyphenols such as ellagic acid, gallic acid, corillagin

and geraniin and or their synergistic activity. The high potency of the methanol extract in hepatoprotection could be associated with its high medicinal value. The presence of different active constituents such as β-sitosterol in the hexane extract and lupeol in the chloroform extract or other active constituents may be responsible for hepatoprotection. The overall hepatoprotective effect of the test extracts and bergenin is probably due to a counteraction of free radicals by its antioxidant nature/or to its ability to inhibit lipid accumulation by its antilipidemic property.

Salient findings of the present study include

- Reporting of friedelin, lupeol, β-sitosterol, campesterol, stigmasterol, corillagin, ellagic acid, gallic acid, geraniin, 6,10,14,trimethyl penta decan-2 one, hexa decanoic acid methyl ester, isophytol, phytol, octa decanoic acid methyl ester, 9- octa decanoic acid methyl ester, and di-n-octyl phthalate from *P. wightianus* **for the first time in science**.
- Among all the *Phyllanthus* species, bergenin was reported only in *Phyllanthus flexuosus* (Tanaka and Matsunaga, 1988). **Next, it is reported here.**
- Significant activity against skin infection causing agents such as dermatophytes, *Staph. aureus, Staph. epidermis* and *Ps.* species is reported to *P. wightianus* here for the first time.
- Screening of plant extracts and bergenin to treat arthritis provides scientific evidence for the ethnobotanical claim for treating bone disorders and diseases.
- The potent activity recorded to wound healing models substantiates the efficacy of the ethnobotanical claim in the treatment of skin infections.
- Scientific evidence generated through screening against almost all the enteric bacteria by recording significant activity and ability of the plant to treat diarrhea.

- The absence of lignans such as phyllanthin and hypophyllanthin reported as hepatoprotective agents (Thyagarajan *et al.*, 1988) does not make any difference in hepatoprotection. The potent hepatoprotective activity may be attributed for the presence of other bioactive compounds. Hepatoprotective activity has been reported to bergenin and ellagic acid by Shin *et al.* (2005) and to bergenin isolated from *Mallotus japonicus* by Kim *et al.* (2000) and Lim *et al.* (2000 b) *in vivo* and *in vitro*. Ellagic acid may also be responsible for hepatoprotective activity.
- Hepatoprotective activity of the bergenin is reported here for the first time from *P. wightianus* by *in vivo* and *in vitro* models and those models that have not been tested so far were **tested for the first time here to bergenin.**

Suggested perspectives

As perspective of this work could suggest that in the antimicrobial studies the enteric bacteria can be analyzed individually for recognition of the largest prevalence antimicrobial in relation to the individualized bacterial species. For the anti-inflammatory activity, in the future it can be used culture of PBMCs and human macrophages activated with LPS of bacteria and molecules of protozoa-recognized inflammatory agents of several human pathologies – and like this to test the anti-inflammatory action that it can be analyzed by nitric oxide dosage and different pro- inflammatory proteins (interleukins). In the increase in humoral antibody (immunomodulatory activities) it should be defined the profile of the subclass of the antibody and in the delayed type hypersensitivity reaction (DTH) to determine the dosage of lymphocytes T helper and suppressor, since the immunological recognition of the immunomodulation demands the determination of this profile. Important, also, in the antiarthritic activity using Freund's adjuvant

of this profile. Important, also, in the antiarthritic activity using Freund's adjuvant it is the dosage of markers as protein C reactive (PCR), rheumatoid factor and latex, among others. In the hepatoprotective studies using INH + rifampicin, to test also with anesthetics that are pathological agents recognized of the hepatic cells and that are used with great frequency in practice medicine, where it would generate in the future a study control pre-anesthetic and post-anesthetic. In the antidiabetic activity using STZ could be used as safe marker the glycosylate hemoglobin and tests in vitro with cortisol – a recognized hyperglycemia agent. Finally, in the activity against skin infection it could be used *S. aureus* beta-lactamase positive, since that exists a high incidence in the world of these infections for this etiological agent without easy control for antibiotic.

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ANNEXURE

PAPERS TO BE COMMUNICATED FOR PUBLICATION

1. Antimicrobial activity of *Phyllanthus wightianus*. **Fitoterapia.**
2. Biological activity of Bergenin - a review. **Journal of Ethnopharmacolgy.**
3. *In vitro* antioxidant and Anti-inflammatory activity of *Phyllanthus wightianus* extracts on carrageenan induced paw edema in rats. **International Journal of Pharmacology.**
4. Anti-hyperglycemic effect of *Phyllanthus wightianus* in streptozotocin-induced diabetic rats. **Pharmazie.**
5. Immunomodulatory properties of *Phyllanthus wightianus*. **Immunology Today.**
6. Wound healing activity of *Phyllanthus wightianus*. **Phytotherapy Research.**
7. Analgesic effect of *Phyllanthus wightianus*. **Phytotherapy Research.**
8. Effect of *Phyllanthus wightianus* (whole plant) and bergenin on adjuvant-induced arthritis in rats**. Journal of Ethnopharmacolgy.**
9. Effect of *Phyllanthus wightianus* (whole plant) and bergenin on isoniazid an rifampicin-induced hepatic injury in rats. **Phytomedicine.**