SCREENING OF SOME MEDICINAL PLANTS FOR ANTIMICROBIAL PROPERTIES - PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF A SELECTED MEDICINAL PLANT

A thesis submitted to Saurashtra University for the degree of Doctor of Philosophy in Biosciences (Ethnopharmacology)

Saurashtra University

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CERTIFICATE

"This is to certify that the Ph.D. thesis entitled 'Screening of some medicinal plants for antimicrobial properties–phytochemical and pharmacological studies of a selected medicinal plant' embodies the original results of bonafide experimental work carried out by Mr. Yogeshkumar Vaghasiya under my guidance and supervision at the Department of Biosciences, Saurashtra University, Rajkot."

"It is further certified that he has put eight terms for research work and that this work has not been submitted to any other University / Institution for the award of Ph.D. degree. His thesis is recommended for acceptance for the award of the Ph.D. degree by the Saurashtra University."

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Dedicated

to my

Family

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Review of Literature

CHAPTER 1: REVIEW OF LITERATURE

1.1. INTRODUCTION OF HERBAL MEDICINE

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been derived from natural sources, many of these isolations were based on the uses of the agents in traditional medicine (Cragg and Newman 2001).

Herbal Medicine is defined as a branch of science in which plant based formulations are used to alleviate diseases. It is also known as botanical medicine or phytomedicine. Lately phytotherapy has been introduced as more accurate synonym of herbal or botanical medicine. In the early twentieth century herbal medicine was prime healthcare system as antibiotics or analgesics were not as yet discovered. With the advent of allopathic system of medicine, herbal medicine gradually lost its popularity among people, which is based on the fast therapeutic actions of synthetic drugs (Singh 2007).

Recently there has been a shift in universal trend from synthetic to herbal medicine, which can be said ‘Return to Nature’. Medicinal plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of diseases and ailments (Sharma et al. 2008). The search for eternal health and longevity and for remedies to relieve pain and discomfort drove early man to explore his immediate natural surroundings and led to the use of many plants, animal products, minerals etc. and the development of a variety of therapeutic agents (Nair and Chanda 2007).

Plants have been used as medicines throughout history. Indeed, studies of wild animals show that they also instinctively eat certain plants to treat themselves for certain illnesses. In Asia, the practice of herbal medicine is extremely well established and documented; as a result, most of the medicinal plants that have international recognition come from China and India. In

Europe and North America, the use of herbal medicine is increasing fast, especially for correcting imbalances caused by modern diets and lifestyles. Many people now take medicinal plant products on a daily basis, to maintain good health as much as to treat illness.

The importance of medicinal plants and traditional health systems in solving the health care problems of the world is gaining increasing attention. Because of this resurgence of interest, the research on plants of medicinal importance is growing phenomenally at the international level, often to the detriment of natural habitats and mother populations in the countries of origin. Most of the developing countries have adopted traditional medical practice as an integral part of their culture. Historically, all medicinal preparations are derived from plants, whether in the simple form of raw plant materials or in the refined form of crude extracts, mixtures, etc (Krishnaraju et al. 2005).

In the early development of modern medicine, biologically active compounds from higher plants have played a vital role in providing medicines to combat pain and diseases. For example, in The British Pharmacopoeia (1932), over 70% of organic monographs are on plant-derived products. However, with the advent of synthetic medicines, and subsequently of antibiotics, the role of plant derived therapeutic agents significantly declined (mostly) in the economically developed nations. Thus, in The British Pharmacopoeia (1980), the share of plant-based monographs fell to approximately 20%. In terms of new chemical entities introduced as medicinal agents over the past several decades, the share of plant-based drugs has been no more than 2% (Dev 1997).

This recent resurgence of interest in plant remedies has been spurred on by several factors (WHO 2002; WHO 2005; Calixto 2000; Kong et al. 2003):

- The effectiveness of plant medicines
- Source of direct therapeutic agents
- Affordable by the people

- Raw material base for the elaboration of more complex semi-synthetic chemical compounds
- Models for new synthetic compounds
- Taxonomic markers for the discovery of new compounds
- The production, consumption and international trade in medicinal plants and phytomedicines are growing and expected to grow in future quite significantly
- Renewable source
- The preference of consumers for natural therapies, a greater interest in alternative medicines and a commonly held belief that herbal products are superior to manufactured products
- A dissatisfaction with the results from synthetic drugs and the belief that herbal medicines may be effective in the treatment of certain diseases where conventional therapies and medicines have proven to be inadequate
- The high cost and side effects of most modern drugs
- Improvements in the quality, efficacy, and safety of herbal medicines with the development of science and technology
- Patients' belief that their physicians have not properly identified the problem; hence they feel that herbal remedies are another option
- A movement towards self-medication

Investigation of the chemical and biological activities of plants during the past two centuries have yielded compounds for the development of modern synthetic organic chemistry as a major route for discovery of novel and more effective therapeutic agents (Nair et al. 2007).

1.2. INDIAN SYSTEM OF MEDICINE

Ayurveda, meaning the "science of life," is said to be the oldest and most complete medical system in the world and dates back to 5000 B. C. There is no denying the benefits of Ayurvedic treatments that several Indians and others across the globe have experienced. The diagnostic and treatment procedures used are unique and are still valid today as are its foundational

principles of panchamahabhutha (five basic elements of nature), tridosha (three humours) and prakrithi (individual constitution) (Venkatasubramanian 2007).

Ayurveda has vast literature in Sanskrit and various Indian languages, covering various aspects of diseases, therapeutics, and pharmacy (Dev 1999). The original source of Ayurveda is the Vedas and the texts known as the Samhitas, which are treatises on health care and describe medical procedures, including surgery and a form of massage of vital energy points (Ebadi 2007). The earliest references to such plants are found in the Rig Veda and the Atharva Veda, dating back to the second millennium B.C. The Charaka Samhita (900 B.C.) is the first recorded treatise fully devoted to the concepts and practice of Ayurveda; its primary focus was therapeutics (Charaka Samhita 1949; Sharma 1981). This text sets out all the fundamental principles of Ayurveda but concentrates most of its attention on digestion (described as internal fire, or agni). Another early classic, the Susruta Samhita, focuses on surgical techniques (Majumdar 1971; Krishnamurthy 1991). The Astanga Hridayam, written in about 500 A.D., sets out most of the detailed principles of Ayurveda, including the dosha and subdosha (Grade 1954; Sharma 1979). The Madhava Nidana (800-900 A.D.) was the next important milestone; it is the most famous Ayurvedic work on the diagnosis of diseases. As per Ayurveda, every material (dravya) is a manifestation of five elements (earth, water, fire, air and space) in different proportions. The material could be living as well as non-living things. Depending on the predominant combination of the elements, nature can be categorized into three doshas, namely vata, pitta and kapha:

- Vata, linked to the wind, the force that controls movement and the functioning of the nervous system in the body
- Pitta, the force of heat and energy, linked with the sun, that controls digestion and all biochemical processes in the body
- Kapha, the force of water and tides, influenced by the moon, the stabilizing influence that controls fluid metabolism in the body

When balanced these three forces ensure that the body is healthy, but when they are “abnormal” or unbalanced, disease follows (Thomas 1997).

India has a rich cultural heritage of traditional medicines which chiefly comprised the two widely flourishing systems of treatments i.e. Ayurvedic and Unani systems since ancient times (Surana et al. 2008). Ayurveda is considered not just as an ethnomedicine but also as a complete medical system that takes in to consideration physical, psychological, philosophical, ethical and spiritual well being of mankind. It lays great importance on living in harmony with the Universe and harmony of nature and science. This universal and holistic approach makes it a unique and distinct medical system. This system emphasizes the importance of maintenance of proper life style for maintaining positive health (Ravishankar and Shukla 2007).

Nature has bestowed upon us a very rich botanical wealth and a large number of diverse types of plants grow wild in different parts of our country. In India, the use of different parts of several medicinal plants to cure specific ailments has been in vogue from ancient times (Bhattacharjee 1998). India is one of the 12-mega biodiversity centers having about 10% of the world’s biodiversity wealth, which is distributed across 16 agro-climatic zones (Shiva 1996). In India around 20,000 medicinal plant species have been recorded recently (Dev 1997), but more than 500 traditional communities use only about 800 plant species for curing different diseases (Kamboj 2000). With a view to strengthen the medicinal plants sector all over the country as well as to conserve the wild stock, the NMPB (National Medicinal Plants Board) was set up by the Government of India in 2000. The prime objective of setting up the board was to establish an agency which would be responsible for coordination of all matters with respect to the medicinal plants sector, including drawing up policies and strategies for *in situ* conservation, cultivation, harvesting, marketing, processing, drug development, etc. (Kala and Sajwan 2007). In India, several steps have been taken to improve the quality of Ayurvedic medicines. Good manufacturing practice (GMP) guidelines have been introduced so as to ensure quality control. Medicinal plant boards have been constituted at state and central level to inspire people particularly the farmers

for adopting cultivation of medicinal plants. Herbal gardens have been developed to make common man conversant with the rich heritage of Indian system of medicine. Various institutes like National Institute of Pharmaceutical Education and Research (NIPER), National Botanical Research Institute (NBRI), Central Institute of Medicinal and Aromatic Plants (CIMAP) and Central Research Drug Institute (CDRI) are playing pivotal role in laying down standards for Ayurvedic system of medicine (Singh 2007).

1.3. THE RECENT DEVELOPMENT OF NATURAL DRUGS

A total of 122 biologically active compounds have been identified, derived only from 94 species of plants. A conservative estimate of the number of flowering plants occurring on the planet is 2,50,000. Of these, only about 6% have been screened for biological activity and a reported 15% have been evaluated phytochemically. Consistent findings should be carried out to discover a probable abundance of medicinal extracts in these plants (Turker and Usta 2008).

The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed (UNESCO 1996). Furthermore, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies (UNESCO 1998).

The Pharmaceutical Research and Development Committee report of Ministry of Chemicals, Government of India also underscores the importance of traditional knowledge (Mashelkar 1999). The increasing use of traditional therapies demands more scientifically sound evidence for the principles behind such therapies and for effectiveness of medicines. Recent advance in the analytical and biological sciences, along with innovations in genomics and proteomics can play an important role in validation of these therapies. Western scientific community views traditional medicines cautiously and

stresses the concerns related to research, development and quality (Patwardhan et al. 2003; Fabricant and Farnsworth 2001).

A large proportion of such medicinal compounds have been discovered with the aid of ethnobotanical knowledge of their traditional uses. The rich knowledge base of countries like India and China in medicinal plants and health care has led to the keen interest by pharmaceutical companies to use this knowledge as a resource for research and development programs in the pursuit of discovering novel drugs (Krishnaraju et al. 2005).

The rapid pace of research and development in herbal medicine has made it an interdisciplinary science. If any scientific monograph of a medicinal plant is seen, it can be concluded that knowledge of Alternative and Complementary Systems of Medicines like Ayurveda, botany, pharmacognosy and phytochemistry, biochemistry, ethnopharmacology and toxicology is integral part of herbal medicine. There has been an explosive growth of herbal drug industry recently. Data analysis has shown that more and more people are consulting the herbal medicine practitioners. World Health Organization has also identified the importance of herbal medicines. According to a study from U.S., 60-70% patients living in rural areas are dependent on herbal medicine for their day to day diseases. Several authors have reported favorable results with herbal drugs (mostly in the form of extracts) either in animal or in human studies (Padma 2005).

1.4. STANDARDIZATION OF PHYTOMEDICINE

Herbs are natural products and their chemical composition varies depending on several factors, such ac botanical species, used chemotypes, the anatomical part of the plant used (seed, flower, root, leaf, fruit rind, etc.), also storage, sun, humidity, type of ground, time of harvest, geographic area etc. This variability can result in significant differences in pharmacological activity: involving both pharmacodynamics and phamacokinetics issues (Park 2008).

It is very important that a system of standardization is established for every plant medicine in the market because the scope for variation in different batches of medicine is enormous (Ekka et al. 2008). Herbal medicines are very different from well-defined synthetic drugs. For example, the availability and quality of the raw materials are frequently problematic; the active principles are frequently unknown; and standardization, stability and quality control are feasible but not easy. Strict guidelines have to be followed for the successful production of a quality herbal drug. The medicinal plants should be authentic and free from harmful materials like pesticides, heavy metals, microbial and radioactive contamination. The source and quality of raw materials, good agricultural practices and manufacturing processes are certainly essential steps for the quality control of herbal medicines and play a pivotal role in guaranteeing the quality and stability of herbal preparations. The herbal extract should be checked for biological activity in experimental animal models. The bioactive extract should be standardized on the basis of active compound. The bioactive extract should undergo limited safety studies (De Smet 1997; Blumenthal et al. 1998; EMEA 2002; WHO 2004; Ahmad et al. 2006; Samy and Gopalakrishnakone 2007).

1.5. MODERN MEDICINE FROM HIGHER PLANTS

Natural products play an important role in the field of new drugs research and development, but it was not until the 19th century that man began to isolate the active principles of medicinal plants and the landmark discovery of quinine from Cinchona bark was made by the French scientists Caventou and Pelletier. Prior to World War II, a series of natural products isolated from higher plants became clinical agents and a number of them are still in use today (Kong et al. 2003).

Table 1.1 Some examples of plant derived drugs and their clinical applications

Name of the plants	Drugs	Clinical application
Rauwolfia serpentina	Serpentine	Hypertension and lowering of blood pressure
Catharanthus rosesus	Vinblastine	Hodgkins, choriocarcinoma, non-hodgkins lymphomas, leukemia in children, testicular and neck cancer
Phodophyllum emodi	Phophyllotoxin	Testicular, small cell lung cancer and lymphomas
Papaver somniferum	Codeine, morphin	Headaches, arthritis and inducing sleep.
Ephedra sinica	Ephedrine	Respiratory ailments
Cinchona spp.	Quinine	Fevers
Cephaelis spp.	Emetine	To induce vomiting and cure dysentery.
Digitalis leaves	Digoxin	Heart therapy

Natural products have served as a major source of drugs for centuries, and about half of the pharmaceuticals in use today are derived from natural products. Quinine, theophylline, penicillin G, morphine, paclitaxel, digoxin, vincristine, doxorubicin, cyclosporin, and vitamin A all share two important characteristics: they are cornerstones of modern pharmaceutical care, and they are all natural products (Ebadi 2007).

In fact, most of the major anticancer drugs are derived from plants or microorganisms. Important examples include bleomycin, doxorubicin, daunorubicin, vincristine, vinblastine, mitomycin, streptozocin, and most recently, paclitaxel, ironotecan (a camptothecin derivative), and etoposide and tenoposide (podophyllotoxin derivatives) (Ebadi 2007).

1.6. MARKET POTENTIAL OF PHYTOMEDICINE

Herbal medicines are readily available in the market from health food stores without prescriptions and are widely used all over the world. The utilization of herbal drugs is on the flow and the market is growing step by step. The annual turnover of the Indian herbal medicinal industry is about Rs. 2,300 crore as against the pharmaceutical industry's turnover of Rs. 14,500 crores with a growth rate of 15 percent (Krishnan 1998). The major pharmaceuticals exported from India in the recent years are isabgol, opium alkaloids, senna derivatives, vinca extract, cinchona alkaloids, ipecac root alkaloids, solasodine, diosgenine/16DPA, menthol, gudmar herb, papian, rauwolfia guar gum, jasmine oil, agar wood oil, sandal wood oil, etc (Kokate et al. 2005). The global market for herbal medicines currently stands at over $60 billion annually. The sale of herbal medicines is expected to get higher at 6.4% an average annual growth rate (Inamdar et al. 2008).

Herbal drugs are marketed in various forms. They are available in both classical forms (tablets, powder, decoction, medicated oil, medicated ghee, fermented products) and modern drug presentation forms like capsules, lotions, syrups, ointments, creams, granules etc. There are more than 8500 manufacturers of herbal drugs in the country (Jain 2001).

Thus, plant-based therapeutic agents continue to have scientific, social, and commercial significance and appear to be gathering a momentum in health-relevant areas. A study of the process by which the traditional or more recent plant-based molecular drugs or the new breed of herbal drugs came to be used in present-day medicine reveals that, in over 70% of the cases, the starting point has been some reference to the use of that plant as an indigenous cure in a folklore or traditional system of medicine of one culture or other.

1.7. ROLE OF WORLD HEALTH ORGANIZATION IN PHYTOMEDICINE

The legal process of regulation of herbal medicines changes from country to country. The reason for this involves mainly cultural aspects and also the fact that herbal medicines are rarely studied scientifically. World Health Organization (WHO) has published guidelines in order to define basic criteria for evaluating the quality, safety and efficacy of herbal medicines aimed at assisting national regulatory authorities, scientific organizations and manufacturers in this particular area (Akerele 1993). The salient features of WHO guidelines were: 1). Quality assessment: Crude plant materials or plant extract preparation and finished product. 2). Stability: Shelf life. 3). Safety assessment: Documentation of safety based on experience and toxicological studies. 4). Assessment of efficacy: Documented evidence of traditional use and activity determination (Animals and human).

Since ages, literature has revealed plants to be the most important source of medicines for human health (Balandrin et al. 1993). According to the findings of the World Health Organization, up to 80% of the world's population relies on plants for their primary health care (Farnsworth et al. 1985; WHO 2002; Dash et al. 2005).

1.8. NEED OF THE HOUR

Herbal medicines are an essential and growing part of the international pharmacopeia. Knowledge of their medicinal properties is growing as a result of research and testing, which will make them an increasingly safe alternative or a preferred option to allopathic medicine. Today, there is a renewed interest in traditional medicine and an increasing demand for more drugs from plant sources. This revival of interest in plant-derived drugs is mainly due to the current widespread belief that "green medicine" is safe and more dependable than the costly synthetic drugs, many of which have adverse side effects (Parekh and Chanda 2006). There is a growing interest in correlating phytochemical constituents of a plant with its pharmacological activity (Gupta 1994; Vaidya 1994). Scientists have even started correlating the botanical

properties of plants with their pharmacological activity (Rawat et al. 1997). In future, more co-ordinated multidimensional research aimed at correlating botanical and phytochemical properties to specific pharmacological activities is expected (Dahanukar et al. 2000).

Thus, determining the biological activities of plants used in traditional medicine is helpful to the rural communities and informal settlements. Several studies are currently being undertaken to isolate the active compound(s) by bioassay-guided fractionation from the species that show high biological activity during screening. With the availability of primary information, further studies can be carried out like standardization of the extracts, identification and isolation of active principles and pharmacological studies of isolated compound. Therefore, these scientific investigations may be utilized to develop drugs for diseases. Further research is desired to isolate the compounds responsible for the observed biological activity.

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Antimicrobial Screening

CHAPTER 2: SCREENING OF SOME MEDICINAL PLANTS FOR ANTIMICROBIAL PROPERTY

2.1. INTRODUCTION

The discovery and development of antibiotics are among the most powerful and successful achievements of modern science and technology for the control of infectious diseases. However, the rate of resistance of pathogenic microorganisms to conventionally used antimicrobial agents is increasing with an alarming frequency (Ge et al. 2002; Nair and Chanda 2005; Neogi et al. 2008). Isolation of microbial agents less susceptible to regular antibiotics and recovery of resistant isolates during antibacterial therapy is increasing throughout the world (Cohen 2002; Hancock 2005). In addition to this problem antibiotics are sometimes associated with adverse side effects on the host, which include hypersensitivity, depletion of beneficial gut and mucosal microorganisms, immunosuppression and allergic reactions (Al-Jabri 2005). The number of multi-drug resistant microbial strains and the appearance of strains with reduced susceptibility to antibiotics are continuously increasing. This increase has been attributed to indiscriminate use of broad-spectrum antibiotics, immunosuppressive agent, intravenous catheters, organ transplantation and ongoing epidemics of HIV infection (Ng 1994; Dean and Burchard, 1996; Gonzalez et al, 1996). Examples include methicillin-resistant staphylococci, pneumococci resistant to penicillin and macrolides, vancomycin-resistant Enterococci as well as multi-drug resistant gram-negative organisms (Norrby et al. 2005). There is an urgent need to control antimicrobial resistance by improved antibiotic usage and reduction of hospital cross-infection (Voravuthikunchai and Kitpipit 2005; Sung and Lee 2007), however, the development of new antibiotics should be continued as they are of primary importance to maintain the effectiveness of antimicrobial treatment (van der Waaij and Nord 2000; Marchese and Shito 2001).

The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against

microbes; as a result, plants are one of the bedrocks for modern medicine to attain new principles (Evans et al. 2002). Plant based antimicrobials represent a vast untapped source of medicine. Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials. Further continued exploration of plant derived antimicrobials is needed today (Hussain and Gorsi 2004).

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being (Iwu et al. 1999). Medicinal plants constitute an effective source of both traditional and modern medicines. Herbal medicine has been shown to have genuine utility and about 80% of rural population depends on it as primary health care (Farnsworth et al. 1985; Akinyemi et al. 2005). Over the years, the World Health Organization advocated that countries should encourage traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origins (WHO 1978). In recent years, pharmaceutical companies have spent a lot of time and money in developing natural products extracted from plants, to produce more cost effective remedies that are affordable to the population (Doughari 2006).

Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19th century (Zaika 1975). It is estimated that today, plant materials are present in, or have provided the models for 50% Western drugs (Robbers et al. 1996). Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, or for other purposes that suggested potentially useful biological activity. The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment.

The medicinal plants around the world contain many compounds with antibacterial activity (Marjorie 1999). Many efforts have been made to discover new antimicrobial compounds from various sources such as micro-organisms, animals, and plants. Systematic screening of them may result in the discovery of novel effective antimicrobial compounds (Tomoko et al. 2002). The use of botanical medicines is generally on the rise in many parts of the world (Bbosa et al. 2007). The screening of plant extracts and plant products for antimicrobial activity has shown that plants represent a potential source of new anti-infective agents (Amani et al. 1998; Salvat et al. 2001; Costa et al. 2008). Numerous experiments have been carried out to screen natural products for antimicrobial property (Martinez et al. 1996; Ateb and Erdourul 2003; Nair and Chanda 2006; Nair et al. 2007a; Ndhlala et al. 2009).

Considering the above, it can be stated that plants are valuable sources for new compounds and should receive special attention in research strategies to develop new antimicrobials urgently required in the near future (Shahidi Bonjar et al. 2004; Aslim and Yucel 2008).

In the present study, methanol and acetone extracts of 53 medicinal plants were screened for antimicrobial activity against 15 different standard strains of microorganisms. Further, the most potent plant extracts (4) were screened for antimicrobial activity against 24 standard strains of microorganisms and 74 clinically isolated microorganisms.

2.2. MATERIALS AND METHODS

2.2.1. Plant materials

Fresh plant/plant parts were collected randomly from Gujarat region, India (Anand Agricultural University, Anand; Junagadh Agricultural University, Junagadh and Saurashtra University campus, Rajkot). The details of the plant/plant parts screened, their families, voucher number, vernacular names and their therapeutic uses are given in Table 2.1 (Anjaria et al. 2002; Sriram et al. 2004; Khare 2007). Plants were compared with voucher specimens

deposited by Dr. P. S. Nagar at Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. Fresh plant materials were washed in tap water, air dried and then homogenized to fine powder and stored in airtight bottles.

2.2.2. Extraction

10 g of air-dried powder was taken in 100 ml of petroleum ether in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 190-220 rpm for 24 h. After 24 h, the supernatant was discarded and petroleum ether was evaporated from the powder. This dry powder was then taken in 100 ml of solvent (methanol or acetone) in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 190-220 rpm for 24 h. After 24 h, the extracts were centrifuged at 5000 g for 10 min, the supernatant was collected, solvents were evaporated, and the dry extract was stored at $4^{o}C$ in airtight bottles. The extraction was done at least three times for each plant and the mean values of extractive yields are presented (Vaghasiya and Chanda 2007).

Table 2.1 Ethnobotanical information of the plants screened for antimicrobial activity

No.	Name of the plant (Family, Vernacular name)	Parts used (Voucher no.)	Ethnomedicinal use
1	*Abutilon glaucum* (Cav.) (Malvaceae, Balbij)	Leaf + stem (PSN42)	Pectoral, demulcent, diuretic
2	*Adenanthera pavonina* L. (Mimosaceae, Raktchandan/Ratanjali)	Leaf	Boils, inflammation, ophthalmia, chronic rheumatism, gout, haematuria
3	*Agave vera* Cruz, Mill. (Agavaceae, Agave)	Leaf	-
4	*Alangium salvifolium* (Linn. f.) Wang. (Alangiaceae, Ankola)	Leaf (PSN 346)	Rheumatism, leprosy, inflammation and for external and internal application in case of bites of rabid dog
5	*Alpinia speciosa* (Wendl.) K.Schum. (Zingiberaceae, Sthulgranthi)	Leaf + stem	Diuretic, hypertension, antiulcerative, spasmolytic.
6	*Argemone mexicana* L. (Papaveraceae, Darudi)	Leaf + stem (PSN 11)	Infestation, skin disease, leprosy, pruritus, blennorrhagia, inflammations, all type of poisoning
7	*Argyreia speciosa* Sweet (Convolvulaceae, Samudra Shokha/ Bidhaaraa)	Leaf + stem (PSN492)	Tonic, aphrodisiac, nervine, diuretic, antirheumatic, hypotensive, spasmolytic, skin diseases, rubefacient, topically stimulant

No.	Name of the plant (Family, Vernacular name)	Parts used (Voucher no.)	Ethnomedicinal use
8	*Aristolochia bracteolata* Lam. (Aristolochiaceae, Kidamari)	Whole plant (PSN662)	Constipation, inflammation, foul ulcers, boils, syphilis, gonorrhoea, eczema and intermittent fevers
9	*Aristolochia indica L.* (Aristolochiaceae, Nodvel/ Isarmuula)	Leaf	In venomous insect bites and internally in intermittent fevers, blood complains, Oxytocic, abortifacient, emmenagogue.
10	*Asphodelus tenuifolius* Cav. (Liliaceae, Dungro)	Leaf + stem (PSN726)	Diuretic, inflamed part, antiulcer
11	*Baliospermum montanum* (Euphorbiaceae, Danti)	Leaf + stem	Purgative, antiasthmatic, ache and pain of joints, cathartic, antidropsical.
12	*Boerhavia diffusa* L. (Nyctaginaceae, Satodi/ Punarnavaa)	Leaf + stem (PSN 631)	Asthma, anemia, inflammation, hepatic disorder, rheumatic and gouty complains, chronic peritoneal conditions, heart disease and kidney ailments
13	*Bombax ceiba* L. (Bombacaceae, Simlo)	Leaf (PSN 74)	Dysentery, influenza, menorrhagia, blood impurities, healing

No.	Name of the plant (Family, Vernacular name)	Parts used (Voucher no.)	Ethnomedicinal use
14	*Carissa carandas* L. var. (Apocynaceae, Karamda)	Leaf + stem (PSN440)	Antiscorbutic, stomachic, refrigerant, digestive, astringent, anthelmintic
15	*Cassia occidentalis* L. (Caesalpiniaceae, Kasundro)	Leaf + stem (PSN254)	Inflammation, diabetes, elephantiasis, leprosy, ulcers, constipation, hicough and fever, purgative, diuretic, febrifugal, expectorant, stomachic, skin diseases
16	*Casuarina equisetifolia* L. (Casuarinaceae, Sharu)	Leaf + stem + fruit (PSN713)	Astringent, antidiarrhoeal, antispasmodic, used in colic, hypoglycaemic.
17	*Celastrus paniculatus* Willd. (Celastraceae, Malkangni)	Leaf (PSN117)	Painful joints, hemiflegia ulcers, skin disease, piles, brain tonic, diaphoretic, febrifugal, mental depression, hysteria, scabies, eczema, wounds, rheumatic pains, paralysis.
18	*Citrus medica* L. (Rutaceae, Bijoru)	Leaf (PSN 106)	Bilious fever, dyspepsia, inflammation, antiscorbutic, astringent, carminative, stomachic, antibacterial, dyspepsia, cold.

No.	Name of the plant (Family, Vernacular name)	Parts used (Voucher no.)	Ethnomedicinal use
19	*Clerodendrum phlomidis* (Verbenaceae, Arani, Bhaandira)	Leaf + stem (PSN602)	Convalescence of measles, neglected syphilitic complains, obesity, diarrhoea, worms.
20	*Datura innoxia* Mill. (Solanaceae, Daturo)	Leaf (PSN 530)	Dysmenorrhoea, neuralgia, sciatic, breast inflammation.
21	*Desmodium gangeticum* L. DC (Fabaceae, Saliparni)	Leaf (PSN 182)	Anorexia, dysentery, dyspepsia, hemorrhoids, fever, gout, cough, inflammation, asthma, bronchitis, cardiopathy, debility
22	*Drypetes roxburghii* (Wall.) Hurasawa (Euphorbiaceae, Putranjiva)	Leaf	Burning sensation, hyperdipsia, constipation, elephantiasis, inflammations, strangury, habitual abortion, sterility
23	*Ficus carica* L. (Moraceae, Anjir)	Leaf (PSN 704)	Constipation, renal and vesicle calculi, visceral obstruction, piles, gout and externally ulcers, gum, boils, asthma.
24	*Gymnema sylvestre* (Retz.) R. Br. (Asclepiadaceae, Madhunasini)	Leaf + stem	In snake bites, swallon glands and visceral enlargement, internally in cough, diabetes, fever.

No.	Name of the plant (Family, Vernacular name)	Parts used (Voucher no.)	Ethnomedicinal use
25	*Hemidesmus indicus* (L.) R.Br. (Asclepiadaceae, Anantmul)	Leaf (PSN465)	Chronic cough, syphilitic, cachexia, lecorrhoea, strangury dyspeptic, nutritional disorders
26	*Holoptelea integrifolia* (Roxb.) Planch. (Urticaceae, Chibil/Charel)	Leaf (PSN 711)	Internally and externally used in rheumatism, scabies, ringworm.
27	*Jasminum auriculatum* Vahl. (Oleaceae, Juuhi)	Leaf	Astringent, cardiac tonic, ringworm, chronic fistulas
28	*Leptadenia reticulata (Retz.) Wight & Arn.* (Asclepidaceae, Dodishaak)	Leaf + stem (PSN454)	Stimulant and restorative, habitual abortion, skin diseases.
29	*Leucas aspera* (Willd.) Link. (Labiatae, Chotahalkusa/Kubo)	Leaf + stem (PSN 617)	Colic, dyspepsia, verminosis, chronic skin eruption, cough, intermittent fevers, ulcers
30	*Madhuca indica* Gmelin. (Sapotaceae, Mahudo)	Leaf (PSN 426)	Verminosis, gastropathy, Dipsia, bronchitis, consumption, dermatopathy, rheumatism, cephalgia and hemorrhoids
31	*Mangifera indica* L. (Anacardiaceae, Keri)	Seed	Inflammation, constipation, hyperpiesia, hemorrhages, wounds, ulcers, dysentery

No.	Name of the plant (Family, Vernacular name)	Parts used (Voucher no.)	Ethnomedicinal use
32	*Maranta arundinacea* L. (Marantaceae, Kookaineer)	Leaf + stem	Nutritive, demulcent, acute diarrhea, gastroenteritis
33	*Merremia turpethum* (L.) Shah and Bhatt (Convolvulaceae, Nashottar)	Leaf	Leucoderma, itch, ulcers, constipation, abdominal troubles, inflammation, anaemia, fever, jaundice
34	*Murraya paniculata (L.) Jack* (Rutaceae, Kamini)	Leaf + stem (PSN110)	regulate fertility
35	*Origanum marjoram* L. (Lamiaceae, Dawanam)	Leaf + stem	Antiseptic, Antispasmodic, Cholagogue, Tonic, Emmenagogue, Stimulant
36	*Phyla nodiflora* (L.) Greene (Verbenaceae, Ratvelio)	Leaf + stem	Burning sensation, fever, ulcers, asthma, bronchitis, gonorrhoea
37	*Phyllanthus reticulatus* Poir (Euphorbiaceae, Kamboi)	Leaf + stem	Burning sensation, strangury, sores, diarrhea, skin eruption
38	*Pluchea arguta* Boiss (Asteraceae, Rashna)	Leaf + stem (PSN402)	Arthritis, constipation, respiratory disease, inflammation
39	*Randia dumetorum* Lam. (Rubiaceae, Gangeda)	Leaf + stem (PSN364)	Nervine, calmative, antispasmodic, emetic, anthelmintic, abortifacient

No.	Name of the plant (Family, Vernacular name)	Parts used (Voucher no.)	Ethnomedicinal use
40	*Rauvolfia tetraphylla* L. (Apocynaceae, Bada Chand)	Leaf + stem	Sedative, hypotensive, skin diseases, to destroy parasites.
41	*Scaevola koenigii* Vahl. (Goodeniaceae, Bhadraaksha)	Leaf (PSN421)	Digestive, carminative, applied externally on tumours and swollen legs, dysentery.
42	*Scindapsus officinalis* (Roxb.) Schott (Araceae, Gajapipal)	Leaf	Pruritus, adentitis, ripening boils, ostalgia, arthralgia, malaria, inflammations, ulcers, Anthelmintic, purgative and tonic.
43	*Strychnos nux-vomica* L (Loganiaceae, Zerchochlu)	Leaf + stem	Cholera, chronic wounds, ulcers, paralytic complains, diabetes, asthma, malaria
44	*Tamarindus indica* L. (Caesalpiniaceae, Amli)	Leaf + stem (PSN 263)	Acidity, dyspepsia, constipation, intoxication from spirituous, liquors, scurvy, bleeding piles, dysentery
45	*Tecomella undulata* (Sm.) Seem. (Bignoniaceae, Ragatrohido)	Leaf + stem (PSN 566)	Remedy for syphilis, spleen diseases
46	*Tephrosia purpurea* (L.) Pers. (Fabaceae, Sarpankho)	Leaf (PSN223)	Jaundice, skin disease, elephantiasis, dyspepsia, asthma, pimples, syphilis, gonorrhea

No.	Name of the plant (Family, Vernacular name)	Parts used (Voucher no.)	Ethnomedicinal use
47	*Thespesia populnea* (L.) Sol ex Correa (Malvaceae, Paraspipal)	Leaf (PSN 71)	Cough, psoriasis, skin disease, dysentery, diabetes, gonorrhea, indigestion, ulcers.
48	*Tinospora cordifolia* (Willd.) Miers Hook. F & Th (Menispermaceae, Galo)	Leaf + stem (PSN 8)	Malaria, rheumatism, dyspepsia, antipyretic, antiperiodic, anti-inflammatory, antirheumatic, spasmolytic, hypoglycaemic, hepatoprotective
49	*Trienthema monogyna* L. (Aizoaceae, Satodo)	Leaf + stem (PSN 308)	Alexiteric, analgesic, diuretic, bronchitis, inflammation, ulcers, itching, night blindness
50	*Trigonella foenum-graecum* L. (Fabaceae, Methi)	Leaf + stem (PSN 230)	Antipemic, anticholestremic, demulcent, diuretic, carminative aphroedisiac
51	*Vitex negundo* L. (Verbenaceae, Nagod)	Leaf + stem (PSN611)	Catarrhal dengue, puerperal fever, splenic enlargement, irritable bladder, rheumatism, dyspepsia, colic worms, liver disease,
52	*Woodfordia fruticosa* Kurz. (Lythraceae, Dhavdi)	Leaf (PSN303)	Astringent, acrid, refrigerant, stimulant, depurative, styptic, uterine sedative, constipating, antibacterial, skin disease
53	*Woodfordia fruticosa* Kurz. (Lythraceae, Dhavdi)	Stem (PSN303)	

2.2.3. Microorganisms

The standard microorganisms (American Type Culture Collection) were obtained from National Chemical Laboratory, Pune, India and clinically isolated microorganisms were obtained from Department of Microbiology, Smt.N.H.L. Municipal Medical College, Sheth V.S. General Hospital, Ahmedabad and Spandan Diagnostics, Rajkot. The bacterial strains were grown in the nutrient broth and maintained on nutrient agar slants at 4^{o}C, while fugal strains were grown in sabourad broth and maintained on MGYP slants at 4^{o}C.

2.2.4. Preparation of the extract for antimicrobial assay

Plant extracts were dissolved in 100% dimethylsulphoxide (DMSO) for antimicrobial study. Concentration of methanol and acetone extracts of 53 plants was 600 µg/disc. Concentrations of methanol extracts of four selected plants were 600 µg/disc and 1200 µg/disc.

2.2.5. Antibiotics

Fifteen antibiotics were used for antibiotic susceptibility study against standard and clinically isolated microorganisms. All antibiotic discs were purchased from Hi-Media, Bombay, India. The names and concentration of the antibiotics and antifungal are as follows: Amikacin (10 mcg), Azithromycin (15 mcg), Carbenicillin (100 mcg), Cefaclor (30 mcg), Ceftazidime (30 mcg), Chloramphenicol (30 mcg), Ciprofloxacin (10 mcg), Imipenem (10 mcg), Methicillin (5 mcg), Piperacillin (100 mcg), Tetracycline (10 mcg), Amphotericin-B (100 units), Fluconazole (25 mcg), Ketoconazole (10 mcg), Nystatin (100 units).

2.2.6. Antimicrobial assay

The antimicrobial assay was performed by agar disc diffusion method (Bauer et al. 1966, NCCLS 2003, Parekh and Chanda 2007). The molten Mueller Hinton Agar (HiMedia) was inoculated with 200 µl of the inoculum (1 x 10^{8}

Cfu) and poured into the sterile Petri plates (Hi-media). The disc (7 mm in diameter, Hi-Media) was saturated with 20 µl of the extract and discs were air dried. Thereafter the discs were introduced on the upper layer of the seeded agar plate. Paper discs loaded with 20 µl of DMSO served as negative control. 11 standard antibiotics and 4 standard antifungal were used as positive controls. The plates were incubated at 37^{o}C for all the bacterial strains while that of fungal strains were incubated at 28^{o}C for 48 h. The experiment was carried out three times and the mean values are presented. The antimicrobial activity was evaluated by measuring the diameter of zone of inhibition in mm.

2.3. RESULTS AND DISCUSSION

The use of antimicrobial agents is critical to the successful treatment of infectious diseases. Although there are numerous classes of drugs that are routinely used to treat infections in humans, pathogenic microorganisms are constantly developing resistance to these drugs (Al-Bari et al. 2006) because of indiscriminate use of antibiotics (Gibbons 1992; Rahman et al. 2001).

The use of higher plants and preparations made from them to treat infections is a longstanding practice in a large part of the population, especially in the developing countries, where there is dependence on traditional medicine for a variety of ailments (Ahmad and Mohammad 1998). Interest in plants with antimicrobial properties increased because of current problems associated with the antibiotics (Emori and Gaynes 1993; Pannuti and Grinbaum 1995). Recently, the antimicrobial effects of various plant extracts against certain pathogens have been reported by a number of researchers (Ahmed and Beg 2001; Erasto et al. 2004; Nair et al. 2007b; Carneiro et al. 2008; Liasu and Ayandele 2008; Parekh and Chanda 2008; Chanda et al. 2009).

Disc diffusion method is the most widely used procedure for testing antimicrobial susceptibility (Sambath Kumar et al. 2006). The disc diffusion procedure (Kirby-Bauer method) has been accepted by the Food and Drug

Administration (FDA) and as a standard by the National Committee for Clinical Laboratory Standards (Barry and Thornsberry 1985; NCCLS 2003).

In the present study, methanol and acetone extracts of 53 plants were studied against 15 standard strains of microorganisms (Table 2.2). Methanol extracts of 4 selected plants were studied against 41 clinically isolated bacterial strains, 33 clinically isolated fungal strains, 20 standard bacterial strains and 4 standard fungal strains. 11 antibiotics were used as positive controls against all bacterial strains and 4 antibiotics were used as positive controls against all fungal strains.

2.3.1. Antimicrobial screening of methanol and acetone extracts of 53 plants

Methanol and acetone extracts of 53 plants were screened for its antimicrobial potency against 15 medically important pathogenic microorganisms. The results of the antimicrobial screening are shown in Table 2.2. Antibacterial activity of plant extracts were compared with standard antibiotics (Table 2.6).

22.64% of methanol extracts were active against *S. aureus* while, only 8.49% of acetone extracts showed antibacterial activity against *S. aureus*. Maximum activity against S. aureus was shown by acetone extract of *Aristolochia indica* followed by methanol extract of *Abutilon glaucum*. The activity of acetone extract of *Aristolochia indica* was more than seven antibiotics studied.

S. epidermidis was inhibited by 10.38% methanol extracts and 7.55% acetone extracts of plants screened. Methanol extract of *Mangifera indica* showed maximum activity followed by acetone extract of *Woodfordia fruticosa* (stem) against *S. epidermidis*, which was comparable to Piperacillin and Azithromycin.

Table 2.2 Evaluation of antimicrobial activity of methanol and acetone extracts of some medicinal plants and its extractive yield

No.	Plants	Ext.	Yield (%)	Microbial strains														
				Gram positive bacteria					Gram negative bacteria							Fungus		
				SA	SE	BC	BS	MF	PA	EC	KP	PM	PV	ST	CF	CA	CT	CL
1	*Abutilon glaucum*	ME	5.37	16	-	-	-	-	-	-	12	8	-	-	-	8	10	11
		AE	1.49	-	-	9	-	-	-	-	12	-	-	-	-	-	-	15
2	*Adenanthera pavonina*	ME	3.9	-	-	-	-	10	-	-	10	-	-	-	-	-	-	-
		AE	2.18	-	-	11	-	-	-	-	10	-	-	-	-	-	-	-
3	*Agave vera*	ME	9.41	-	-	13	15	-	-	-	-	-	-	-	-	14	14	17
		AE	2.07	-	-	-	-	-	-	-	12	-	-	-	-	-	-	-
4	*Alangium salviifolicum*	ME	8.42	-	-	-	-	13	-	-	18	-	-	-	-	-	-	-
		AE	1.55	-	-	-	-	10	-	-	11	-	-	-	-	-	-	-
5	*Alpinia speciosa*	ME	7.9	10	-	11	-	19	-	-	12	-	-	-	-	-	11	-
		AE	2.01	-	-	12	-	11	-	-	13	9	-	-	-	-	-	-
6	*Argemone mexicana*	ME	4.6	10	9	10	9	21	-	8	11	8	-	-	-	-	11	17
		AE	0.72	-	11	10	9	25		8	-	8	-	-	-	10	19	15
7	*Argyreia speciosa*	ME	2.92	-	-	10	-	-	-	-	9	-	-	-	-	-	-	-
		AE	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	*Aristolochia bracteolata*	ME	9.63	-	-	-	-	-	-	-	16	11	-	-	-	-	11	-
		AE	1.98	15	-	14	11	-	-	-	13	13	-	-	11	-	-	-
9	*Aristolochia indica*	ME	11.46	10	11	15	11	-	-	-	13	11	-	-	-	-	10	-
		AE	1.47	19	-	16	13	-	-	-	13	13	-	-	-	-	-	13
10	*Asphodelus tenuifolius*	ME	11.36	9	-	13	-	-	-	-	-	-	-	-	10	-	11	-
		AE	3.63	-	-	-	-	-	-	-	17	-	-	-	-	-	11	20

No.	Plants	Ext.	Yield (%)	Microbial strains														
				Gram positive bacteria					Gram negative bacteria							Fungus		
				SA	SE	BC	BS	MF	PA	EC	KP	PM	PV	ST	CF	CA	CT	CL
11	*Baliospermum montanum*	ME	13.01	9	-	13	-	12	-	-	12	-	-	-	-	-	-	-
		AE	5.13	-	-	12	-	12	-	-	14	10	-	-	-	-	-	-
12	*Boerhavia diffusa*	ME	4.38	-	11	-	-	-	-	-	-	-	-	-	-	-	-	-
		AE	1.2	-	-	-	-	-	-	-	11	-	-	-	-	-	-	-
13	*Bombax ceiba*	ME	4.3	-	-	-	-	-	-	-	12	-	-	-	-	-	-	-
		AE	1.09	9	-	11	-	-	-	-	-	-	-	-	-	-	-	-
14	*Calastrus paniculatus*	ME	3.25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		AE	1.71	-	-	10	-	-	-	-	12	-	-	-	-	-	-	-
15	*Carissa carandas*	ME	8.53	10	-	12	12	-	-	-	11	10	-	-	-	-	13	15
		AE	4.08	-	-	10	-	-	-	-	-	-	-	-	-	10	-	12
16	*Cassia occidentalis*	ME	8.8	11	-	12	-	-	-	-	-	9	-	-	-	-	12	10
		AE	1.78	-	-	-	-	-	-	-	11	-	-	-	-	-	11	-
17	*Casuarina equissetifolia*	ME	12.1	10	-	14	-	14	-	-	11	10	11	12	14	-	-	-
		AE	2.46	-	-	12	-	11	-	-	10	-	10	9	11	-	-	-
18	*Citrus medica*	ME	4.08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		AE	1.71	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	*Clerodendron phlomidis*	ME	2.2	-	13	-	-	-	-	-	-	-	-	-	11	-	-	-
		AE	0.78	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	*Datura innoxia*	ME	14.4	-	-	10	9	-	-	-	11	10	-	-	-	8	-	12
		AE	2.26	10	-	15	11	11	-	-	18	11	-	-	-	-	-	-
21	*Desmodium gangeticum*	ME	4.37	-	-	-	-	-	-	-	13	-	-	-	-	-	-	-
		AE	1.17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

No.	Plants	Ext.	Yield (%)	Microbial strains														
				Gram positive bacteria					Gram negative bacteria							Fungus		
				SA	SE	BC	BS	MF	PA	EC	KP	PM	PV	ST	CF	CA	CT	CL
22	*Drypetes roxburghii*	ME	10.5	11	11	-	-	13	-	-	-	-	-	-	-	-	-	-
		AE	2.6	-	9	10	-	11	-	-	13	-	-	-	-	-	-	-
23	*Ficus carica*	ME	7.62	-	-	-	-	-	-	-	10	-	-	-	-	-	-	-
		AE	1.58	-	-	-	-	-	-	-	-	-	-	-	-	8	9	11
24	*Gymnema sylvestre*	ME	9.87	-	-	10	-	-	-	-	11	-	-	-	-	10	-	-
		AE	2.24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	*Hemidesmus indicus*	ME	11.29	10	-	12	-	14	-	-	11	-	-	-	-	8	-	-
		AE	4.48	-	-	11	-	11	-	-	-	8	-	-	-	-	-	-
26	*Holoptelea integrifolia*	ME	3.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		AE	0.91	-	-	-	-	-	-	-	11	-	-	-	-	-	-	-
27	*Jasminum auriculatum*	ME	1.38	-	-	11	-	-	-	-	13	-	-	-	-	-	13	-
		AE	1.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	*Leptadenia reticulate*	ME	9.9	9	11	-	-	-	-	-	13	12	-	-	-	-	9	-
		AE	2.52	-	-	-	-	-	-	-	11	15	-	-	11	-	10	-
29	*Leucas aspera*	ME	7.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		AE	2.5	-	-	-	-	-	-	-	8	-	-	-	-	-	-	-
30	*Madhuca indica*	ME	6.98	9	-	10	-	-	-	-	-	-	-	-	-	-	-	-
		AE	2.2	10	-	10	-	11	-	-	-	-	-	-	-	-	-	-
31	*Mangifera indica*	ME	8.24	14	15	16	9	12	13	15	11	14	14	18	17	-	-	-
		AE	3	11	12	15	-	16	13	12	11	10	12	15	13	-	-	-
32	*Maranta arundinacea*	ME	1.94	12	-	13	-	-	-	-	-	9	-	-	-	-	12	11
		AE	1.68	-	-	-	-	-	-	-	13	8	-	-	-	-	10	-
33	*Merremia turpethum*	ME	9.12	10	-	11	-	-	-	-	-	-	-	-	-	-	15	-
		AE	2.58	-	-	-	-	-	-	-	11	-	-	-	-	-	-	-

No.	Plants	Ext.	Yield (%)	Microbial strains														
				Gram positive bacteria					Gram negative bacteria							Fungus		
				SA	SE	BC	BS	MF	PA	EC	KP	PM	PV	ST	CF	CA	CT	CL
34	*Murraya paniculata*	ME	16.69	-	-	10	-	-	-	-	12	-	-	-	-	9	9	12
		AE	4.72	-	-	10	-	-	-	-	13	-	-	-	-	-	-	-
35	*Origanum marjoram*	ME	11.75	-	9	-	-	-	-	-	10	-	-	-	-	-	-	-
		AE	4.8		12	10	9				10	-	-	-	-	-	-	-
36	*Phyla nodiflora*	ME	10.82	-	-	15	-	-	-	-	-	-	-	-	14	-	-	-
		AE	2.5	-	-	12	-	-	-	-	-	-	-	-	-	-	-	-
37	*Phyllanthus reticulatus*	ME	7.66	9	-	13	-	23	-	-	-	-	-	-	15	-	-	11
		AE	2.49	-	-	-	-	15	-	-	12	-	-	-	-	-	-	-
38	*Pluchea arguta*	ME	9.9	10	-	15	-	20	-	-	14	-	-	-	-	-	9	10
		AE	2.08	-	-	10	-	-	-	-	9	-	-	-	-	-	-	20
39	*Randia dumetorum*	ME	9.02	11	-	11	-	15	-	-	-	9	-	-	-	-	9	-
		AE	1.64	-	-	-	-	12	-	-	-	9	-	-	-	-	-	-
40	*Rauvolifera tetraphylla*	ME	13.48	-	-	12	-	10	-	-	20	9	-	-	-	-	8	-
		AE	4.35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41	*Scaevola koenigii*	ME	11.78	11	-	10	-	-	-	-	12	-	-	-	-	-	9	-
		AE	1.69	-	-	-	-	-	-	-	15	-	-	-	-	-	-	-
42	*Scindapsus officinalis*	ME	7.7	-	-	14	-	-	-	-	16	-	-	-	11	-	-	11
		AE	2.12	-	-	-	-	-	-	-	12	-	-	-	-	-	10	16
43	*Strychnos nux-vomica*	ME	8.7	14	14	13	-	14	-	-	13	-	-	-	-	-	-	-
		AE	3.73	-	13	11	-	-	8	10	-	-	-	15	-	-	-	-
44	*Tamarindus indica*	ME	10.41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		AE	4	-	-	-	-	-	-	-	11	-	-	-	-	-	-	-
45	*Tecomella undulate*	ME	7.91	-	-	10	-	-	-	-	9	8	-	-	-	-	-	-
		AE	2.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

No.	Plants	Ext.	Yield (%)	Microbial strains														
				Gram positive bacteria					Gram negative bacteria							Fungus		
				SA	SE	BC	BS	MF	PA	EC	KP	PM	PV	ST	CF	CA	CT	CL
46	*Tephrosia purpurea*	ME	8.25	12	-	13	-	-	-	-	20	-	-	-	-	-	8	12
		AE	1.98	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
47	*Thespesia populnea*	ME	9.89	-	-	9	10	-	-	-	11	-	-	-	-	10	-	-
		AE	2.59	12	14	12	12	10	-	-	14	13	-	-	-	-	-	-
48	*Tinospora cordifolia*	ME	5.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		AE	1.59	-	-	-	-	10	-	-	-	-	-	-	-	-	-	-
49	*Trienthema monogyna*	ME	5.58	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		AE	1.34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50	*Trigonella foenumgraecum*	ME	9.7	-	-	-	-	-	-	-	9	-	-	-	-	-	-	-
		AE	1.5	-	-	-	-	-	-	-	8	10	-	-	-	-	-	-
51	*Vitex negundo.*	ME	9.3	-	-	11	-	-	-	-	12	8	-	-	-	10	10	-
		AE	3.14	-	-	12	-	-	-	-	10	9	-	-	-	-	-	-
52	*Woodfordia fruticosa* (Leaf)	ME	16.41	12	12	13	9	12	12	12	13	15	13	14	16	-	-	-
		AE	8.39	11	13	14	10	15	12	11	11	13	12	14	13	-	-	-
53	*Woodfordia fruticosa* (Stem)	ME	12.24	10	13	15	-	10	11	12	-	11	13	15	14	-	-	-
		AE	5.03	12	14	16	-	15	12	12	-	12	12	14	13	-	-	-

SA-*Staphylococcus aureus* ATCC25923; SE-*Staphylococcus epidermidis* ATCC12228; BC-*Bacillus cereus* ATCC11778; BS-*Bacillus subtilis* ATCC6633; MF-*Micrococcus flavus* ATCC10240; PA-*Pseudomonas aeruginosa* ATCC27853; EC- *E. coli* ATCC25922; KP-*Klebsiella pneumoniae* NCIM2719; PM-*Proteus mirabilis* NCIM2241; PV-*Proteus vulgaris* NCTC8313; ST-*Salmonella typhimurium* ATCC23564; CF-*Citrobacter freundii* ATCC10787; CA-*Candida albicans* ATCC2091; CT-*Candida tropicalis* ATCC4563; CL-*Cryptococcus luteolus* ATCC32044; ME- Methanol extract; AE- Acetone extract; - means no activity; Data showed diameter of zone of inhibition in mm

31.13% of methanol extracts and 23.58% of acetone extracts showed activity against *B. cereus.* Maximum activity against *B. cereus* was shown by acetone extracts of *Aristolochia indica* and *Woodfordia fruticosa* and methanol extract of *Mangifera indica*, which was also comparable to Piperacillin. Only 7.55% methanol extracts and 6.6% acetone extracts showed antibacterial activity against *B. subtilis*. Maximum activity was shown by methanol extract of *Agave vera*, which was also comparable to Piperacillin (Table 2.2).

M. flavus was inhibited by 15.09% of both methanol and acetone extracts. The maximum activity was shown by acetone extract of *Argemone mexicana* which was also comparable with standard antibiotics. Only 2.83% methanol extracts and 3.77% acetone extracts showed antibacterial activty against *P. aeruginosa*. Methanol and acetone extracts of *Mangifera indica* showed maximum activity compared with the other extracts. *E. coli* was inhibited by 3.77% methanol extracts and 4.72% acetone extracts. Methanol extract of *Mangifera indica* showed maximum zone of inhibition against *E. coli*, which was comparable to standard antibiotics Piperacillin and Amikacin (Table 2.2).

Methanol and acetone extracts of the plants screened showed 31.13% and 30.19% inhibition against *K. pneumoniae* respectively. Both the extracts showed almost similar antibacterial activity against *K. pneumoniae*. Methanol extracts of *Rauvolifera tetraphylla* and *Tephrosia purpurea* showed maximum inhibition against *K. pneumoniae. P. mirabilis* was inhibited by 16.04% methanol extracts and 15.09% acetone extracts of the plants screened. Acetone extract of *Leptadenia reticulata* and methanol extract of *Woodfordia fruticosa* showed maximum inhibition zone compared with other plant extracts.

Only 3.77% methanol and acetone extracts showed antibacterial activity against *P. vulgaris*. Methanol extract of *Mangifera indica* showed maximum inhibition against *P. vulgaris* which was similar to the standard antibiotic Amikacin.

Similar to *P. vulgaris*, *S. typhimurium* was also inhibited by 3.77% of methanol extracts and 4.72% of acetone extracts. Here also methanol extract of *Mangifera indica* showed maximum inhibition, comparable to standard antibiotics. *C. freundii* was inhibited by 8.49% methanol extracts and 5.66% acetone extracts. Methanol extracts of *Mangifera indica* and *Woodfordia fruticosa* (leaf) showed maximum inhibition against *C. freundii*, which was more than standard antibiotic Amikacin and comparable to Piperacillin (Table 2.2).

Antifungal activity was investigated against *C. albicans*, *C. tropicalis* and *C. luteolus*. Methanol extracts of the plants screened showed 7.55% antifungal activity against *C. albicans*, 19.81% against *C. tropicalis* and 11.32% against *C. luteolus*. Acetone extracts showed 2.83% antifungal activity against *C. albicans*, 5.66% against *C. tropicalis* and 7.55% against *C. luteolus* (Table 2.2).

From the above results, it can be stated that methanol extracts showed 34.72% antibacterial activity against Gram positive bacteria, 19.95% against Gram negative bacteria and 25.79% against fungal strains studied. Acetone extracts showed 24.53% antibacterial activity against Gram positive bacteria, 19.41% against Gram negative bacteria and 10.69% against fungal strains. Thus, methanol and acetone extracts showed 29.62% inhibition against Gram positive bacteria, 19.68% inhibition against Gram negative bacteria and against 18.24% inhibition against fungal strains.

Methanol extracts of *Mangifera indica* and *Woodfordia fruticosa* (leaf) showed higher antibacterial activity and acetone extract of *Argemone mexicana* showed higher antifungal activity.

2.3.2. Antibacterial screening of 4 selected plants against 41 clinically isolated bacteria

From results of the antimicrobial study of methanol and acetone extracts of 53 plants screened, methanol extracts of *Aristolochia indica* (AIM), *Argemone Mexicana* (AMM), *Mangifera indica* (MIM) and *Woodfordia fruticosa* (WFM) were selected for antimicrobial activity against 41 clinically isolated bacterial strains (Table 2.3).

AIM showed 94.12% antibacterial activity against 17 Gram positive bacteria at both the concentrations, while AIM-600 and AIM-1200 showed 54.12% and 58.13% antibacterial activity against 24 Gram negative organisms respectively. *Enterococci* species were the most susceptible strains amongst all the bacterial strains studied, which was comparable to standard antibiotics.

AMM-600 and AMM-1200 showed 41.18% and 47.06% antibacterial activity against Gram positive bacteria respectively. AMM did not show any activity against Gram negative bacteria. AMM showed poor antibacterial activity when compared with other 3 plants studied.

MIM showed 100% antibacterial activity against all the bacterial strains studied. MIM was the most potent extract which was comparable to standard antibiotics. *Enterococci* species were the most susceptible strains which was also comparable to standard antibiotics. MIM showed potent antibacterial activity against one of the urine samples of *E. coli* (E. coli-16), which was higher than all the antibiotics studied.

WFM-600 and WFM-1200 showed 82.35% and 88.24% antibacterial activity against 17 Gram positive bacteria respectively, while WFM did not show antibacterial activity against Gram negative bacteria.

Table 2.3 Antibacterial activity of methanol extracts of 4 selected plants against some clinical isolates

Gram negative Strains (Samples)	Extracts (µg/disc)							
	AIM-600	AIM-1200	AMM-600	AMM-1200	MIM-600	MIM-1200	WFM-600	WFM-1200
E. coli-1(F)			-	-	10	11	-	-
E. coli-2 (P)	9	8	-	-	9	10	-	-
E. coli-3 (P)	9	10	-	-	10	11	-	-
E. coli-4 (St)	-	-	-	-	9	10	-	-
E. coli-5 (St)	-	-	-		10	11	-	-
E. coli-6 (St)	-	-	-	-	10	11	-	-
E. coli-7 (St)	9	9	-	-	11	10	-	-
E. coli-8 (St)	-	-	-	-	9	9	-	-
E. coli-9 (St)	-	-	-	-	9	10	-	-
E. coli-10 (St)	10	12	-	-	12	15	-	-
E. coli-11 (Sw)	9	-	-	-	9	10	-	-
E. coli-12 (Sw)	-	-	-	-	9	10	-	-
E. coli-13 (U)		-	-	-	10	10	-	-
E. coli-14 (U)	9	8	-	-	10	11	-	-
E. coli-15 (U)	-	-	-	-	8	10	-	-
E. coli-16 (U)	11	11	-	-	15	17	-	-
Kleb-1 (B)	10	9	-	-	9	10	-	-
Kleb-2 (B)	-	8	-	-	9	10	-	-
Kleb-3 (B)	8	9	-	-	9	9	-	-
Kleb-4 (C)	9	10	-	-	10	11	-	-
Kleb-5 (P)	8	10	-	-	9	11	-	-
Kleb-6 (Sw)	9	10	-	-	10	11	-	-
Kleb-7 (U)	9	10	-	-	10	11	-	-
Prot-1 (Sw)	-	9	-	-	9	10	-	-

Gram positive strains (Samples)	Extracts (µg/disc)							
	AIM-600	AIM-1200	AMM-600	AMM-1200	MIM-600	MIM-1200	WFM-600	WFM-1200
Ent-1 (B)	13	17	-	-	19	21	12	14
Ent-2 (Sw)	13	18	10	11	16	16	12	12
S. alb-1 (B)	11	-	-	12	9	14	10	12
S. alb-2 (B)	9	11	-	-	11	13	8	11
S. alb-3 (B)	10	11	-	-	13	13	-	-
S. alb-4 (P)	-	12	-	-	10	14	8	11
S. aur-1 (P)	9	11	8	12	11	13	9	10
S. aur-2 (P)	10	11	9	12	11	13	8	11
S. aur-3 (St)	10	12	10	12	12	14	9	12
S. cit-1 (P)	8	12	-	-	12	18	-	11
S. cit-2 (P)	10	11	-	-	11	14	9	10
S. cit-3 (U)	8	10	-	-	13	15	8	10
S. cit-4 (U)	9	12	-	-	11	15	8	11
Staph-1 (B)	12	14	-	-	14	14	-	-
Staph-2 (P)	10	10	10	11	12	13	10	10
Staph-3 (St)	10	12	8	11	13	14	8	10
Staph-4 (St)	9	11	9	10	12	14	8	9

n=3; Values indicate diameter of zone of inhibition in mm; - means no activity; B- Blood; C- Catheter; P- Pus; St- Stool; Sw- Swab; U- Urine; Ent- *Enterococci* species; S. alb- *Staphylococcus albus*; S. aur- *Staphylococcus aureus*; S. cit- *Staphylococcus citrous;* Staph- *Staphylococcus* species; E. coli- *Escherichia coli*; Kleb- *Klebsiella* species; Prot- *Proteus* species; AIM- Methanol extract of *Aristolochia indica*; AMM- Methanol extract of *Argemone mexicana*; MIM- Methanol extract of *Mangifera indica*; WFM- Methanol extract of *Woodfordia fruticosa*

Overall MIM-600 and MIM-1200 showed 100% antibacterial activity against 41 clinically isolated Gram positive and Gram negative bacterial strains followed by AIM-1200 (73.17%), AIM-600 (70.73%). WFM-1200 (36.59%), WFM-600 (34.15%), AIM-1200 (19.51%) respectively and AIM-600 showed poor antibacterial activity (17.07%) against clinically isolated bacterial strains.

Thus, methanol extract of *Mangifera indica* showed higher antibacterial activity followed by *Aristolochia indica. Woodfordia fruticosa* and *Argemone mexicana* showed poor antibacterial activity against clinically isolated bacterial strains.

2.3.3. Antibacterial screening of 4 selected plants against standard bacterial strains

Screening of methanol extracts of *Aristolochia indica* (AIM), *Argemone mexicana* (AMM), *Mangifera indica* (MIM) and *Woodfordia fruticosa* (WFM) was carried out against 20 standard bacterial strains (Table 2.4).

AIM-600, AIM-1200 and MIM-600 showed 100% antibacterial activity against 8 standard Gram positive bacterial strains followed by AMM-1200 (87.5%), MIM-1200 (87.5%), WFM-600 (87.5%), WFM (87.5%) and AMM-600 (75%) respectively.

MIM-600, MIM-1200 and WFM-1200 showed 81.82% antibacterial activity against 11 standard Gram negative strains followed by WFM-600 (72.73%), AIM-1200 (63.64%) and AIM-600 (45.45%). AMM-600 showed only 9.09% antibacterial activity, while AMM-1200 did not show antibacterial activity against 11 standard Gram positive strains.

Overall results showed that MIM-600 showed 85% antibacterial activity against all the standard strains of Gram positive and Gram negative bacteria followed by MIM-1200 (80%), WFM-1200 (80%), AIM-1200 (75%), WFM-600 (75%), AIM-600 (65%), AMM-600 (35%) and AMM-1200 (35%), respectively.

Thus, methanol extract of *Mangifera indica* showed higher antibacterial activity followed by *Woodfordia fruticosa* and *Aristolochia indica* against standard bacterial strains. *Argemone maxicana* showed poor antibacterial activity against standard bacterial strains.

Table 2.4 Antibacterial activity of methanol extracts of 4 selected plants against 20 standard bacterial strains

Standard strains	Extracts (µg/disc)							
	AIM-600	AIM-1200	AMM-600	AMM-1200	MIM-600	MIM-1200	WFM-600	WFM-1200
Gram positive strains								
Bc	13	14	10	10	15	15	10	12
Bm	9	8	11	13	8	-	9	9
Bs	11	12	9	10	9	10	-	-
Cr	10	11	-	-	14	15	9	10
Mf	12	14	20	21	13	12	12	12
Sa-1	10	12	10	10	13	14	9	9
Sa-2	11	13	10	10	13	15	10	12
Se	12	12	-	10	14	15	10	11
Ss	10	10	-	8	10	10	8	10
Gram negative strains								
Cf	-	-	-	-	15	16	14	15
Ea	-	-	-	-	-	-	-	-
Ka	11	14	-	-	13	14	10	12
Pmira	9	11	-	-	13	13	8	10
Pmorg	10	12	-	-	11	14	10	11
Pv	-	10	-		14	15	12	12
Pp	12	12	-	-	12	11	14	15
Pstut		11	-	-	14	15		10
Psyr	10	10	10	-	11	11	9	9
Pt	-	-	-	-	-	-	-	-
St	-	-	-	-	16	17	12	13

n=3; Values indicate diameter of zone of inhibition in mm; - means no activity; Bc- *Bacillus cereus* ATCC11778; Bm- *Bacillus megaterium* ATCC9885; Bs- *Bacillus subtilis* ATCC6633; Cr- *Cornybacterium rubrum* ATCC 14898; Mf- *Micrococcus flavus* ATCC10240; Sa-1- *Staphylococcus aureus* ATCC25923, Sa-2- *Staphylococcus aureus* ATCC29737; Se- *Staphylococcus epidermidis* ATCC12228; Ss- *Staphylococcus subflava* NCIM2178; Cf- *Citrobacter freundii* ATCC10787; Ea- *Enterobacter aerogenes* ATCC13048; Ka- *Klebsiella aerogenes* NCTC418; Pmira- *Proteus mirabilis* NCIM2241; Pmorg- *Proteus morganii* NCIM2040; Pv- *Proteus vulgaris* NCTC8313; Pp- *Pseudomonas pictoruim* NCIB9152; Pstut- *Pseudomonas stutgeri* ATCC17588; Psyr- *Pseudomonas syringae* NCIM5102; Pt- *Pseudomonas testosteroni* NCIM5098; St- *Salmonella typhimurium* ATCC23564; AIM- Methanol extract of *Aristolochia indica*; AMM- Methanol extract of *Argemone mexicana*; MIM- Methanol extract of *Mangifera indica*; WFM- Methanol extract of *Woodfordia fruticosa*

2.3.4. Antifungal activity of methanol extracts of 4 selected plants against 33 clinically isolated fungal strains and 4 standard fungal strains

AIM, AMM, MIM and WFM did not show antifungal activity against clinical isolates of *Candida* species and standard fungal strains (Data not presented). AMM showed antifungal activity against three standard fungal strains, *C. neoformans*, *C. tropicalis* and *T. beigelli* (Table 2.7).

2.3.5. Antibiotic susceptibility study of clinical isolates and standard microorganisms

Clinically, *in vitro* antimicrobial susceptibility tests are useful as a guide for determining antimicrobial chemotherapy whenever the susceptibility of a pathogen is unpredictable or when an infection has not responded to therapy that otherwise appears appropriate.

Eleven antibiotics were studied against clinically isolated and standard Gram positive and Gram negative microorganisms and the results are presented in Table 2.5 and Table 2.6 respectively. The results of antibiotic susceptibility study against Gram positive bacteria (clinical isolates) were as follows: Imipenem (100%) < Tetracycline and Amikacin (94.12%) < Chloramphenicol (82.35%) < Cefaclor (70.59%) < Carbenicillin and Ciprofloxacin (64.71%) < Ceftazidime (58.82%) < Azithromycin and Piperacillin (52.94%) < Methicillin (35.29%). The results of antibiotic susceptibility study against Gram positive bacteria (standard strains) were as follows: Amikacin, Azithromycin, Tetracycline, Imipenem, Chloramphenicol, Cefaclor and Ciprofloxacin (100%) < Carbenicillin, Ceftazidime, Piperacillin and Methicillin (87.5%).

Antibiotic susceptibility study against Gram negative bacteria (clinical isolates) was as following manner: Imipenem (100%) < Amikacin (87.5%) < Tetracycline (70.83%) < Chloramphenicol (66.67%) < Ceftazidime (33.33%) < Carbenicillin and Cefaclor (16.67%) < Azithromycin and Ciprofloxacin (12.5%) < Piperacillin (8.33%) < Methicillin (0%). Antibiotic susceptibility

study against Gram negative bacteria (standard strains) was as following manner: Tetracycline, Imipenem and Chloramphenicol (100%) < Piperacillin (92.86%) < Amikacin and Ciprofloxacin (85.71%) < Ceftazidime and Carbenicillin (71.43%) < Azithromycin (64.29%) < Cefaclor (57.14%) < Methicillin (14.29%).

Table 2.5 Antibiotic susceptibility study of 41 clinically isolated bacteria

Gram positive strains (Samples)	Antibiotics										
	M	**At**	**Pc**	**Ak**	**T**	**I**	**Cb**	**Ca**	**C**	**Cj**	**Cf**
Ent-1 (B)	-	-	13	25	31	30	17	-	30	16	35
Ent-2 (Sw)	-	-	11	16	32	21	12	10	21	16	20
S. alb-1 (B)	-	16	-	16	11	18	-	10	20	12	-
S. alb-2 (B)	-	-	-	19	10	40	-	-	-	-	-
S. alb-3 (B)	-	-	-	10	8	15	-	-	12	-	-
S. alb-4 (P)	-	-	-	10	-	16	-	-	20	-	-
S. aur-1 (P)	15	12	-	10	20	30	-	-	-	-	-
S. aur-2 (P)	15	15	-	10	24	32	-	-	-	-	-
S. aur-3 (St)	12	15	10	-	12	24	15	16	20	19	8
S. cit-1 (P)	-	-	35	21	21	40	35	25	20	26	20
S. cit-2 (P)	-	-	25	16	20	30	30	20	25	30	23
S. cit-3 (U)	-	15		14	20	30	30	18	25	22	20
S. cit-4 (U)	-	16	25	20	20	35	30	20	15	25	-
Staph-1 (B)	-	-	20	13	28	15	19	-	16	10	15
Staph-2 (P)	17	10	28	10	20	42	10	15	19	20	-
Staph-3 (St)	12	11	10	11	20	30	8	16	16	13	9
Staph-4 (St)	12	13	-	17	16	21	10	15	15	16	-
Gram negative strains (Samples)	**Antibiotics**										
	M	**At**	**Pc**	**Ak**	**T**	**I**	**Cb**	**Ca**	**C**	**Cj**	**Cf**
E. coli-1(F)	-	-	-	-	18	11	-		15	-	-
E. coli-2 (P)	-	-	-	13	-	20	-	-		-	-
E. coli-3 (P)	-	-	-	15	-	19	-	-	15	-	-
E. coli-4 (St)	-	24	-	15	17	30	21	16	28	9	26
E. coli-5 (St)	-	-	-	13	9	14	-	-	21	-	-
E. coli-6 (St)	-	25	11	14	15	15	17	13	25	-	21
E. coli-7 (St)	-	-	-	16	-	19	-	8	14	-	-
E. coli-8 (St)	-	-	-	18	10	20	-	9	20	-	-
E. coli-9 (St)	-	-	-	15	-	20	-	-		-	-
E. coli-10 (St)	-	-	-	15	-	20	-	-	13	-	-
E. coli-11 (Sw)	-	-	-	13	9	17	-	-	17	-	-
E. coli-12 (Sw)	-	-	-	17	8	20	-	-	-	-	-

Gram negative strains (Samples)	Antibiotics										
	M	**At**	**Pc**	**Ak**	**T**	**I**	**Cb**	**Ca**	**C**	**Cj**	**Cf**
E. coli-13 (U)	-	30	30	25	11	20	28	26	30	13	12
E. coli-14 (U)	-	-	-	20	24	16	15	15	20	11	-
E. coli-15 (U)	-	-	-	18	9	25	-	-	25	-	-
E. coli-16 (U)	-	-	-	15	-	16	-	11	-	-	-
Kleb-1 (B)	-	-	-		10	19	-	-	10	-	-
Kleb-2 (B)	-	-	-	15	13	15	-	-	15	-	-
Kleb-3 (B)	-	-	-	-	15	20	-	-	-	-	-
Kleb-4 (C)	-	-	-	20	9	21	-	-	-	-	-
Kleb-5 (P)	-	-	-	8	10	11	-	-	8	-	-
Kleb-6 (Sw)	-	-	-	13	12	16	-	-	-	-	-
Kleb-7 (U)	-	-	-	15	-	20	-	12	14	10	-
Prot-1 (Sw)	-	-	-	9	10	9	-	-	-	-	-

n=3; Values indicate diameter of zone of inhibition in mm; - means no activity; B- Blood; C- Catheter; P- Pus; St- Stool; Sw- Swab; U- Urine; Ent- *Enterococci* species; S. alb- *Staphylococcus albus*; S. aur- *Staphylococcus aureus*; S. cit- *Staphylococcus citrous;* Staph- *Staphylococcus* species; E. coli- *Escherichia coli*; Kleb- *Klebsiella* species; Prot- *Proteus* species; Ak- Amikacin, At- Azithromycin, T- Tetracycline, I- Imipenem, C- Chloramphenicol, Cj- Cefaclor, Cf- Ciprofloxacin, Cb- Carbenicillin, Ca- Ceftazidime, Pc- Piperacillin, M- Methicillin

Imipenem was the most active antibiotic against all the clinically isolated Gram positive and Gram negative microorganisms followed by Amikacin and Tetracycline, while Methicillin showed less activity against Gram positive organisms as compared with the other antibiotics. Methicillin did not show antibacterial activity against clinically isolated Gram negative organisms.

The standard Gram positive strains were highly susceptible to all the antibiotics in the range of 87.5 to 100% while the susceptibility of standard Gram negative strains to antibiotics was only in the range of 14.29 to 100%.

Table 2.6 Antibiotic susceptibility study of 20 standard bacterial strains

Standard strains	Antibiotics										
	M	**At**	**Pc**	**Ak**	**T**	**I**	**Cb**	**Ca**	**C**	**Cl**	**Cf**
Gram positive strains											
Bc	-	11	16	11	17	36	10	18	10	20	19
Bm	14	12		23	19	41	-	-	14	12	27
Bs	16	14	17	15	21	37	20	25	16	35	30
Cr	15	14	19	16	20	24	22	19	15	27	16
Mf	10	27	27	20	26	37	32	23	26	30	21
Sa-1	20	15	18	12	15	25	21	18	20	25	15
Sa-2	16	15	21	13	19	25	22	19	15	20	14
Se	18	14	11	16	25	38	17	24	23	22	24
Gram negative strains											
Cf	-	-	18	8	15	25	20	15	15	8	13
Ea	-	-	12	16	11	19	17	16	13	-	11
Ec	-	12	14	24	16	24	13	12	24	-	35
Ka	-	-	11	-	10	30	-	-	13	16	10
Kp	-	-	30	20	12	27	-	17	34	17	28
Pmira	10	20	16	18	24	31	23	-	10	12	-
Pmorg	-	-	10	10	15	30	-	-	15	21	11
Pv	-	13	15	11	15	15	11	14	18	-	18
Pa	-	12	-	22	15	27	12	17	34	-	28
Pp	14	18	15	20	25	27	20	12	25	23	23
Pstut	-	32	22	28	25	38	28	30	22	-	30
Psyr	-	17	18	-	20	30	12	-	16	21	-
Pt	-	20	10	12	15	11	-	10	16	-	25
St	-	11	14	15	18	16	21	18	16	17	15

Bc- *Bacillus cereus* ATCC11778; Bm- *Bacillus megaterium* ATCC9885; Bs- *Bacillus subtilis* ATCC6633; Cr- *Cornybacterium rubrum* ATCC 14898; Mf- *Micrococcus flavus* ATCC10240; Sa-1- *Staphylococcus aureus* ATCC25923, Sa-2- *Staphylococcus aureus* ATCC29737; Se- *Staphylococcus epidermidis* ATCC12228; Ss- *Staphylococcus subflava* NCIM2178; Cf- *Citrobacter freundii* ATCC10787; Ea- *Enterobacter aerogenes* ATCC13048; Ka- *Klebsiella aerogenes* NCTC418; Pmira- *Proteus mirabilis* NCIM2241; Pmorg- *Proteus morganii* NCIM2040; Pv- *Proteus vulgaris* NCTC8313; Pp- *Pseudomonas pictoruim* NCIB9152; Pstut- *Pseudomonas stutgeri* ATCC17588; Psyr- *Pseudomonas syringae* NCIM5102; Pt- *Pseudomonas testosteroni* NCIM5098; St- *Salmonella typhimurium* ATCC23564; Ak- Amikacin, At- Azithromycin, T- Tetracycline, I- Imipenem, C- Chloramphenicol, Cj- Cefaclor, Cf- Ciprofloxacin, Cb- Carbenicillin, Ca- Ceftazidime, Pc- Piperacillin, M- Methicillin

Table 2.7 Antifungal activity of methanol extract of *Argemone mexicana* and four standard antifungal against 33 clinical isolates and 4 standard fungal strains

Fungal strains	Samples	Antifungal drugs				Extracts (µg/disc)	
		Ap	Fu	Kt	Ns	AMM-600	AMM-1200
Standard strains							
C. albicans	ATCC2091	13	-	20	18	-	-
C. neoformans	NCIM3542	17	27	23	21	9	9
C. tropicalis	ATCC4563	14	-	-	19	11	12
T. beigelli	NCIM3404	15	-	-	17	9	10
Clinical isolates							
***Candida* spp.**	Blood	-	-	-	-	-	-
***Candida* spp.**	Vaginal swab	10	-	-	17	-	-
***Candida* spp.**	Vaginal swab	10	-	-	17	-	-
***Candida* spp.**	Vaginal swab	15	14	12	20	-	-
***Candida* spp.**	Vaginal swab	14	-	-	18	-	-
***Candida* spp.**	Vaginal swab	12	-	-	15	-	-
***Candida* spp.**	Vaginal swab	13	-	-	15	-	-
***Candida* spp.**	Vaginal swab	17	-	-	20	-	-
***Candida* spp.**	Vaginal swab	12	-	-	15	-	-
***Candida* spp.**	Vaginal swab	12	-	-	14	-	-
***Candida* spp.**	Vaginal swab	12	-	-	15	-	-
***Candida* spp.**	Vaginal swab	13	-	-	15	-	-
***Candida* spp.**	Vaginal swab	14	-	-	17	-	-
***Candida* spp.**	Vaginal swab	-	-	-	-	-	-
***Candida* spp.**	Vaginal swab	12	-	-	17	-	-
***Candida* spp.**	Vaginal swab	12	-	-	15	-	-
***Candida* spp.**	Vaginal swab	14	-	-	20	-	-
***Candida* spp.**	Vaginal swab	15	-	-	17	-	-
***Candida* spp.**	Vaginal swab	13	-	-	15	-	-
***Candida* spp.**	Vaginal swab	13	-	-	18	-	-
***Candida* spp.**	Vaginal swab	12	-	-	17	-	-
***Candida* spp.**	Vaginal swab	16	-	-	21	-	-
***Candida* spp.**	Vaginal swab	15	-	-	18	-	-
***Candida* spp.**	Vaginal swab	11	-	-	15	-	-
***Candida* spp.**	Vaginal swab	13	-	-	15	-	-
***Candida* spp.**	Vaginal swab	13	-	-	18	-	-
***Candida* spp.**	Vaginal swab	15	-	-	20	-	-

Fungal strains	Samples	Antifungal drugs				Extracts (µg/disc)	
		Ap	Fu	Kt	Ns	AMM-600	AMM-1200
***Candida* spp.**	Vaginal swab	12	-	-	20	-	-
***Candida* spp.**	Vaginal swab	14	-	-	20	-	-
***Candida* spp.**	Vaginal swab	15	-	-	20	-	-
***Candida* spp.**	Urine	13	-	-	13	-	-
***Candida* spp.**	Urine	12	-	-	12	-	-
***Candida* spp.**	Urine	12	-	-	12	-	-

n=3; Values indicate diameter of zone of inhibition in mm; - means no activity; Ap- Amphotericin-B; Fu- Fluconazole; Kt- Ketoconazole; Ns- Nystatin

Amphotericin-B, Fluconazole, Ketoconazole and Nystatin were studied against 33 clinically isolated *Candida* species and 4 standard fungal strains for antifungal activity (Table 2.7). Amphotericin-B and Nystatin showed 100% antifungal activity against standard strains, while Amphotericin-B and Nystatin showed 93.94% antifungal activity against clinically isolated *Candida* species. Fluconazole showed antifungal activity against *C. neoformans* and only one clinically isolated *Candida* species. Ketoconazole showed antifungal activity against *C. albicans, C. neoformans* and only one clinically isolated *Candida* species. Thus, Amphotericin-B and Nystatin were more potent antifungal agents than Fluconazole and Ketoconazole against fungal strains studied.

2.4. CONCLUSION

Antimicrobial activities of various herbs and spices in plant leaves, flowers, stems, roots or fruits have been reported by many workers in different solvent extracts (Mau et al. 2001; Uz-Zaman et al. 2006; Al-Bayati and Sulaiman 2008; Nair et al. 2009).

From the results it can be concluded that methanol extracts of the plants screened gave better yield than acetone extracts. It is evident that the Gram-positive microorganisms were more sensitive to the plant extracts than the Gram-negative microorganisms. These findings are in agreement with other researchers (Oboh et al. 2007; Nair and Chanda 2007; Costa et al. 2008; Khan et al. 2008). The susceptibility of Gram-positive bacteria may be due to

their cell wall structure which is of a single layer while the Gram-negative cell wall is a multi-layered structure and quite complex (Essawi and Srour 2000). All the extracts showed varying degrees of antimicrobial activity on the microorganisms tested. Some of these plant extracts were more effective than antibiotics to combat the pathogenic microorganisms studied. The antimicrobial activity was more apparent in methanol than acetone extracts of the same plants. These plants may be a source of new antibiotic compounds. This *in vitro* study demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. *Argemone mexicana, Aristolochia indica, Mangifera indica* and *Woodfordia fruticosa* were more potent plants than other plants screened for antimicrobial activity against standard strains of the microorganisms. *Mangifera indica* was the most potent plant than the other three plants screened against clinically isolated bacterial strains, while methanol extracts of 4 selected plants did not show antifungal activity against standard and clinically isolated fungal strains. This study gives an indication of the efficacy of the plants obtained from the traditional healers. The results from this study form a basis for further studies of the potent plants so as to isolate the compounds responsible for the antimicrobial activity.

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Phytochemical Analysis

CHAPTER 3: PHYTOCHEMICAL ANALYSIS OF SOME MEDICINAL PLANTS

3.1. INTRODUCTION

Various medicinal properties have been attributed to natural herbs. Medicinal plants constitute the main source of new pharmaceuticals and healthcare products (Ivanova et al. 2005). The history of plants being used for medicinal purpose is probably as old as the history of mankind. Extraction and characterization of several active phytocompounds from these green factories have given birth to some high activity profile drugs (Mandal et al. 2007). A growing body of evidence indicates that secondary plant metabolites play critical roles in human health and may be nutritionally important (Hertog et al. 1993). Phytochemical screening of plants has revealed the presence of numerous chemicals including alkaloids, tannins, flavonoids, steroids, glycosides, saponins etc. Many plant extracts and phytochemicals show antioxidant/free radical scavenging properties (Larson 1988; Nair et al. 2007; Parekh and Chanda 2007a). Secondary metabolites of plants serve as defense mechanisms against predation by many microorganisms, insects and herbivores (Lutterodt et al. 1999; Marjorie 1999).

Normally free radicals of different forms are generated at a low level in cells to help in the modulation of several physiological functions and are quenched by an integrated antioxidant system in the body. However, if free radicals are produced in excess amount they can be destructive leading to inflammation, ischemia, lung damage and other degenerative diseases (Halliwell et al. 1992; Hadi et al. 2000; Cavalcanti et al. 2006). Free radical reactions, especially with participation of oxidative radicals, have been shown to be involved in many biological processes that cause damage to lipids, proteins, membranes and nucleic acids, thus giving rise to a variety of diseases (Lee et al. 2005; Campos et al. 2006).

The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites that possess an aromatic ring bearing one or more hydroxyl constituents (Singh et al. 2007). Phenolic compounds are widely found in the secondary products of medicinal plants, as well as in many edible plants (Hagerman et al. 1998). A number of studies have focused on the biological activities of phenolic compounds, which are potential antioxidants and free radical-scavengers (Rice-Evans et al. 1995; Kahkonen et al. 1999; Sugihara et al. 1999; Cespedes et al. 2008; Reddy et al. 2008). Several studies have described the antioxidant properties of medicinal plants, foods, and beverages which are rich in phenolic compounds (Brown and Rice-Evans 1998; Krings and Berger 2001).

Flavonoids are a broad class of plant phenolics that are known to possess a well established protective ability against membrane lipoperoxidative damages (Sen et al. 2005).

Plant products have been part of phytomedicines since time immemorial. These can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc (Gordon and David 2001) i.e. any part of the plant may contain active components. Knowledge of the chemical constituents of plants is desirable because such information will be of value for the synthesis of complex chemical substances. Such phytochemical screening of various plants is reported by many workers (Mojab et al. 2003; Parekh and Chanda 2007b; Parekh and Chanda 2008).

In the present work, qualitative and quantitative phytochemical analysis was carried out in the 53 plants screened.

3.2. MATERIALS AND METHODS

3.2.1. Plant collection and extraction

Plant collection and extraction was done as described in Chapter 2.2.1 and Chapter 2.2.2. The preliminary qualitative phytochemical analysis was carried out in crude dry powder of 53 plants; while total phenol and flavonoid content was estimated in methanol and acetone extracts of 53 plants.

3.2.2. Preliminary qualitative phytochemical screening (Harbone 1998; Parekh and Chanda 2007b)

3.2.2.1. Alkaloids

The methanolic extract of the crude dry powder of each plant was evaporated to dryness in a boiling water bath. The residue was dissolved in 2 N HCl. The mixture was filtered and the filtrate was divided into 3 equal portions. One portion was treated with a few drops of Mayer's reagent; one portion was treated with equal amount of Dragondroff’s reagent and the other portion was treated with equal amount of Wagner's reagent. The creamish precipitate, orange precipitate and brown precipitate, indicated the presence of respective alkaloids (Salehi-Surmaghi et al. 1992).

3.2.2.2. Flavonoids

The presence of flavonoids was estimated by Shinoda test. The alcoholic extract of the crude dry powder of each plant was treated with a few drops of concentrated HCl and magnesium ribbon. The appearance of pink or tomato red colour within a few minutes indicated the presence of flavonoids (Somolenski et al. 1972).

3.2.2.3. Tannins

The water extract of the crude dry powder of each plant was treated with alcoholic $FeCl_3$ reagent. Blue color indicated the presence of tannins (Segelman et al. 1969).

3.2.2.4. Cardiac glycosides

Keller-kiliani test was performed to assess the presence of cardiac glycosides. The crude dry powder of each plant was treated with 1 ml of $FeCl_3$ reagent (mixture of 1 volume of 5% $FeCl_3$ solution and 99 volumes of glacial acetic acid). To this solution a few drops of concentrated H_2SO_4 was added. Appearance of greenish blue color within a few minutes indicated the presence of cardiac glycosides (Ajaiyeobu 2002).

3.2.2.5. Steroids

Liebermann-Burchard reaction was performed to assess the presence of steroids. A chloroform solution of the crude dry powder of each plant was treated with acetic anhydride and a few drops of concentrated H_2SO_4 were added down the sides of the test tube. A blue green ring indicated the presence of terpenoids.

3.2.2.6. Saponins

The presence of saponins was determined by Frothing test. The crude dry powder of each plant was vigorously shaken with distilled water and was allowed to stand for 10 minutes and classified for saponin content as follows: no froth indicate absence of saponins and stable froth more than 1.5 cm indicated the presence of saponins (Kapoor et al. 1969).

3.2.3. Quantitative phytochemical screening

3.2.3.1. Total phenol determination:

Total phenolic content of the extracts was determined by Folin Ciocalteu reagent method (Mc Donald et al. 2001**)** with some modifications. Plant extract (1 ml) was mixed with Ciocalteu reagent (0.1 ml, 1 N), and allowed to stand for 15 min. Then 5 ml of saturated Na_2CO_3 was added. The mixtures were allowed to stand for 30 min at room temperature and the total phenols were determined spectrophotometrically at 760 nm. Gallic acid was used as a standard. Total phenol values are expressed in terms of gallic acid equivalent (mg g^{-1} of extracted compound).

3.2.3.2. Flavonoid determination:

Aluminium chloride colorimetric method (Chang et al. 2002) with some modifications was used to determine flavonoid content. Plant extract (1ml) in methanol was mixed with 1ml of methanol, 0.5 ml aluminium chloride (1.2 %) and 0.5 ml potassium acetate (120 mM). The mixture was allowed to stand for 30 min at room temperature; then the absorbance was measured at 415 nm. Quercetin was used as standard. Flavonoid content is expressed in terms of quercetin equivalent (mg g^{-1} of extracted compound).

3.3. RESULTS AND DISCUSSION

3.3.1. Preliminary qualitative phytochemical screening

In recent years, secondary plant metabolites (phytochemicals) with antibacterial potency have been actively investigated as alternatives to and／or in combination with antibiotics in the therapy of bacterial infections (Sato et al. 1995; Liu et al. 2001).

The preliminary qualitative phytochemical screening of the crude powder of 53 plants was done to assess the presence of bioactive components. The

presence of alkaloids (Dragendroff, Mayer, Wagner), flavonoids, tannins, steroids, saponins and cardiac glycosides was determined (Table 3.1).

Table 3.1 Preliminary qualitative phytochemical analysis of crude powder of 53 plants

No.	Name of the plants	Alkaloids			TN	CG	ST	FL	SP
		DR	MR	WR					
1	*Abutilon glaucum*	+	-	+	-	+++	+	-	-
2	*Adenanthera pavonina*	+	-	-	+	+++	+++	-	-
3	*Agave Vera*	+	-	+	++	-	-	-	+++
4	*Alangium salvifolium*	-	-	+	+	++	++	++	+
5	*Alpinia speciosa*	-	-	+	+++	-	-	+	-
6	*Argemone maxicana*	-	-	++	+++	+++	++	-	+
7	*Argyreia speciosa*	-	-	-	-	-	-	-	+
8	*Aristolochia bracteolata*	-	-	-	+++	++	++	-	+
9	*Aristolochia indica*	-	+	+	+	+	++	-	+
10	*Asphodelus tenuifolius*	-	-	+	-	-	-	-	-
11	*Baliospermum montanum*	+	+	-	+++	-	-	+	++
12	*Boerhavia diffusa*	-	-	-	+	++	+++	-	-
13	*Bombax ceiba*	+	-	-	+++	+	++	-	-
14	*Carissa carandas*	+	-	-	+++	-	+		+
15	*Cassia occidentalis*	+	-	-	++	+	+	+	-
16	*Casuarina equissetifolia*	+	-	-	+++	+	+	+	-
17	*Celastrus paniculata*	-	-	-	-	-	++	-	-
18	*Citrus medica*	+	-	-	-	+	++	-	-
19	*Clerodendron phlomidis*	-	-	-	-	-	-	-	-
20	*Datura innoxia*	-	-	+	-	+++	+++	+	-
21	*Desmodium gangeticum*	-	-	-	-	+++	+++	-	-
22	*Drypetes roxburghii*	+	-	-	+++	+++	++	-	+
23	*Ficus carica*	-	-	-	++	++	+	-	-
24	*Gymnema sylvestre*	+	-	-	+	+++	+	-	+++
25	*Hemidesmus indicus*	-	-	+	+++	+	-	-	+
26	*Holoptelea integrifolia*	+	-	-	+	++	+++	+	+
27	*Jasminum auriculatum*	-	-	+	++	++	++	-	+

No.	Name of the plants	Alkaloids			TN	CG	ST	FL	SP
		DR	MR	WR					
28	*Leptadenia reticulata*	-	-	-	+	+++	++	-	-
29	*Leucas aspera*	-	-	-	+	++	+++	-	-
30	*Madhuca indica*	+++	+	-	++	-	++	-	-
31	*Mangifera indica*	-	-	-	+++	-	+	-	-
32	*Maranta arundinacea*	-	-	-	-	-	-	-	-
33	*Merremia turpethum*	+		+	+	++	++	-	-
34	*Murraya paniculata*	-	-	+	++	++	-	-	+
35	*Origanum marjoram*	-	-	-	++	-	-	-	-
36	*Phyla nodiflora*	-	-	+	+++	-	-	+	-
37	*Phyllanthus reticulatus*	+	-	-	+++	+	++	-	+
38	*Pluchea arguta*	+	++	++	+++	+++	-	-	+
39	*Randia dumetorum*	++	+		++		+	-	+
40	*Rauvofia tetraphylla*	-	-	+	+++	++	++	-	+
41	*Scaevola koenigii*	-	-	+	-	-	-	+	-
42	*Scindapsus officinalis*	-	-	+	+	++	++	-	-
43	*Strychnos nux-vomica*	-	-	-	+++	+	+++	-	-
44	*Tamarindus indica*	+	+	-	-	+	-	-	-
45	*Tecomella undulata*	+	+	+	+	+	-	-	-
46	*Tephrosia purpurea*	-	-		+++	++		-	-
47	*Thespesia populnea*	+	-	-	-	-	-	-	-
48	*Tinospora cordifolia*	-	-	-	-	+	++	-	-
49	*Trienthema monogyna*	-	-	-	-	-	+	-	-
50	*Trigonella foenum-graecum*	-	-	-	-	+	+++	-	-
51	*Vitex negundo*	-	-	+	+++	+++	-	-	+
52	*Woodfordia fruticosa* (Leaf)	+++	+	+	+++	-	-	-	+
53	*Woodfordia fruticosa* (Stem)	++	-	-	+++	+	++	-	+

DR- Dragondroff's reagent; MR- Mayer's reagent; WR- Wagner's reagent; TN- Tannins; FL- Flavonoids; CG- Cardiac glycosides; ST- Steroids; SP- Saponins

Alkaloids were present in lower amount (+) in 32.08% of plants with Dragondroff test, in 13.21% with Mayer test and in 33.96% with Wagner test. Moderate amount (++) of alkaloids were present in 3.77% of the plants with Dragondroff test and Wagner test, 1.89% with Mayer test. Only 3.77% of plants contained higher amount (+++) of alkaloids with Dragondroff test.

Tannins were present in 20.75% of plants in lower amount, 13.21% in moderate amount and 33.96% in higher amount. Cardiac glycosides were present in 22.64% of plants in lower amount, in 20.75% in moderate amount and in18.87% in higher amount. Steroids were present in 13.21% of plants in lower amount, 32.08% in moderate amount and 15.09% in higher amount. Flavonoids were present in 13.21% of plants in lower amount and 1.89% in moderate amount. Saponins were present in 33.96% of plants in lower amount, 1.89% in moderate amount and 3.77% in higher amount.

3.3.2. Quantitative phytochemical screening

Antioxidants and antimicrobial properties of various extracts from many plants have recently been of great interest in both research and the food industry, because their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidants and antimicrobials with natural ones (Deba et al. 2008).

The results of total phenolic content and flavonoid content of methanol and acetone extracts of 53 plants are presented in Table 3.2.

Total phenolic content was higher in methanol extracts than acetone extracts. Only 11.32% acetone extracts showed higher total phenolic content than methanol extracts, while 88.68% methanol extracts showed higher total phenolic content than acetone extracts. 35.85% acetone extracts showed higher flavonoid content than methanol extracts, while 64.15% methanol extracts showed higher flavonoid content than acetone extracts.

Out of 53 acetone extracts of the plants studied, 9 plants contained higher total phenolic content (>70 mg/g), which were in the following order: *Mangifera indica* > *Strychnos nux-vomica* > *Phyllanthus reticulates* > *Casuarina equissetifolia* > *Woodfordia fruticosa* (Stem) > *Drypetes roxburghii* > *Woodfordia fruticosa* (Leaf) > *Madhuca indica* > *Carissa carandas* (Table 3.2.).

Table 3.2 Total phenolic and flavonoids content of acetone and methanol extracts of 53 plants

Plants	**Total phenol (mg/g)**		**Flavonoids (mg/g)**	
	AE	**ME**	**AE**	**ME**
Abutilon glaucum	12.85 ± 0.11	17.89 ± 0.52	14.68 ± 0.38	18.25 ± 0.07
Adenanthera pavonina	22.48 ± 0.35	57.43 ± 1.41	19.86 ± 0.46	32.50 ± 0.17
Agave vera	27.51 ± 0.13	25.54 ± 0.46	4.16 ± 0.14	15.85 ± 7.89
Alangium salviifolicum	15.48 ± 0.14	65.71 ± 1.93	0.83 ± 0.05	44.74 ± 0.11
Alpinia speciosa	27.41 ± 0.19	120.54 ± 3.47	35.21 ± 0.73	64.65 ± 2.23
Argemone mexicana	17.49 ± 0.26	29.75 ± 0.31	28.32 ± 0.30	79.54 ± 0.62
Argyreia speciosa	58.43 ± 0.58	81.78 ± 0.19	33.87 ± 0.15	27.08 ± 3.97
Aristolochia bracteolate	39.67 ± 0.92	59.22 ± 0.65	130.93 ± 2.58	36.06 ± 0.17
Aristolochia indica	25.11 ± 0.18	56.66 ± 1.68	30.41 ± 0.19	27.22 ± 0.1
Asphodelus tenuifolius	17.23 ± 0.53	23.88 ± 0.86	34.02 ± 1.20	8.79 ± 0.31
Baliospermum montanum	16.87 ± 0.41	71.48 ± 1.98	52.55 ± 0.32	42.67 ± 8.17
Boerhavia diffusa	28.07 ± 0.21	84.05 ± 0.11	1.23 ± 0.01	29.77 ± 0.20
Bombax ceiba	31.11 ± 0.77	45.64 ± 0.24	25.74 ± 0.14	76.94 ± 0.60
Calastrus paniculatus	7.68 ± 0.04	14.81 ± 0.11	29.62 ± 0.65	31.49 ± 0.19
Carissa carandas	77.98 ± 0.60	84.70 ± 0.20	27.42 ± 5.20	28.65 ± 0.52
Cassia occidentalis	65.82 ± 0.90	71.28 ± 0,84	21.90 ± 0.48	45.76 ± 0.51
Casuarina equissetifolia	146.33 ± 0.73	248.15 ± 3.55	42.28 ± 0.74	33.38 ± 0.39
Citrus medica	20.05 ± 0.41	40.77 ± 0.29	30.67 ± 0.53	22.62 ± 0.18
Clerodendron phlomidis	9.05 ± 0.10	29.78 ± 0.32	35.88 ± 0.30	17.98 ± 0.55
Datura innoxia	3.65 ± 0.05	29.53 ± 0.38	19.35 ± 0.20	33.00 ± 0.20
Desmodium gangeticum	6.66 ± 0.16	49.19 ± 0.96	10.61 ± 0.47	37.02 ± 0.15
Drypetes roxburghii	136.15 ± 2.18	83.04 ± 1.25	26.83 ± 0.31	42.32 ± 0.07

Plants	Total phenol (mg/g)		Flavonoids (mg/g)	
	AE	ME	AE	ME
Ficus carica	4.02 ±0.08	46.45 ± 0.68	24.26 ± 0.24	65.44 ± 0.60
Gymnema sylvestre	26.41 ± 0.32	40.22 ± 0.74	70.99 ± 0.89	17.98 ± 0.55
Hemidesmus indicus	11.97 ± 0.14	85.45 ± 0.82	31.03 ± 0.42	26.29 ± 0.29
Holoptelea integrifolia	10.65 ± 0.17	29.62 ± 0.30	43.59 ± 0.94	51.37 ± 0.37
Jasminum auriculatum	68.74 ± 1.05	41.97 ± 0.68	26.14 ± 0.28	22.79 ± 0.12
Leptadenia reticulate	24.45 ± 0.03	47.15 ± 0.21	24.9 ± 0.92	54.88 ± 9.42
Leucas aspera	23.58 ± 0.46	50.90 ± 0.35	1.56 ± 0.02	48.19 ± 0.32
Madhuca indica	83.02 ± 1.98	59.44 ± 0.26	15.15 ± 0.10	26.99 ± 0.15
Mangifera indica	219.31 ± 3.69	427.28 ± 10.5	9.86 ± 0.7	6.36 ± 0.3
Maranta arundinacea	5.96 ± 0.13	25.92 ± 0.86	13.69 ± 0.19	17.63 ± 0.38
Merremia turpethum	12.34 ± 0.03	45.75 ± 0.14	15.81± 0.69	30.16 ± 0.09
Murraya paniculata	14.83 ± 0.12	53.00 ± 0.90	25.3 ± 0.08	41.92 ± 13.92
Origanum marjoram	33.75 ± 1.02	274.95 ± 2.63	27.65 ± 0.08	45.01 ± 0.92
Phyla nodiflora	10.79 ± 0.26	64.84 ± 1.04	39.79 ± 0.26	31.64 ± 0.18
Phyllanthus reticulatus	191.73 ± 6.62	172.27 ± 2.90	20.31 ± 0.30	49.84 ± 0.13
Pluchea arguta	59.50 ± 0.79	78.82 ± 1.28	88.47 ± 0.91	53.67 ± 0.44
Randia dumetorum	23.65 ± 0.08	66.72 ± 0.13	49.16 ± 0.86	22.98 ± 0.08
Rauvolifera tetraphylla	25.54 ± 0.59	59.3 ± 0.80	20.21 ± 0.61	65.50 ± 0.60
Scaevola koenigii	18.69 ± 0.14	36.31 ± 0.30	16.03 ± 0.15	14.36 ± 0.18
Scindapsus officinalis	7.81 ± 0.13	54.35 ± 0.52	63.12 ± 0.19	33.11 ± 0.18
Strychnos nux-vomica	208.18 ± 2.73	337.82 ± 2.80	19.15 ± 0.10	56.76 ± 0.29
Tamarindus indica	17.84 ± 0.28	29.26 ± 0.17	3.84 ± 0.05	18.77 ± 0.02
Tecomella undulate	31.46 ± 0.07	65.01 ± 1.24	14.77 ± 0.08	34.14 ± 0.16
Tephrosia purpurea	16.52 ± 0.46	58.74 ± 0.06	15.89 ± 0.11	31.84 ± 0.03
Thespesia populnea	6.78 ± 0.32	37.77 ± 0.45	28.64 ± 0.13	27.41 ± 0.07
Tinospora cordifolia	15.9 ± 0.32	38.86 ± 0.32	17.76 ± 0.10	28.09 ± 0.08
Trienthema monogyna	11.69 ± 0.01	26.85 ± 0.46	11.83 ± 0.09	14.82 ± 0.43
Trigonella foenumgraecum	15.79 ± 0.11	46.03 ± 0.51	14.49 ± 0.11	30.97 ± 0.24
Vitex negundo	23.81 ± 0.47	62.38 ± 0.55	16.37 ± 0.51	21.8 ± 0.69
Woodfordia fruticosa (Stem)	140.28 ± 5.73	130.43 ± 2.13	14.87 ± 0.85	9.10 ± 0.14
Woodfordia fruticosa (Leaf)	118.31 ± 6.19	387.84 ± 7.73	63.74 ± 0.71	38.42 ± 0.71

n=3; Data is presented as Mean ± SEM; AE- Acetone extract; ME- Methanol extract

Of the methanol extracts of the 53 plants studied, 16 plants contained higher total phenolic content (>70 mg/g), which were in the following order: *Mangifera indica* > *Woodfordia fruticosa* (Leaf) > *Strychnos nux-vomica* > *Origanum marjoram* > *Casuarina equissetifolia* > *Phyllanthus reticulatus* > *Woodfordia fruticosa* (Stem) > *Alpinia speciosa* > *Hemidesmus indicus* > *Carissa carandas* > *Boerhavia diffusa* > *Drypetes roxburghii* > *Argyreia speciosa* > *Pluchea arguta* > *Baliospermum montanum* > *Cassia occidentalis* (Table 3.2.).

Of the acetone extracts of the 53 plants studied, only 3 plants contained higher amount of flavonoids (>70 mg/g), which were in the following order: *Aristolochia bracteolate* > *Pluchea arguta* > *Gymnema sylvestre*. Out of 53 methanol extracts of the plants studied, only two plants contained higher amount of flavonoids, which were *Argemone mexicana* and *Bombax ceiba*.

The antioxidant properties of plant extracts is attributed to their polyphenolic contents (Lu and Foo 2001; Murthy et al. 2002). As such plants containing a high level of polyphenols have a greater importance as natural antimicrobics (Baravalia et al. 2009).

From the results of antimicrobial screening, methanol extract of *Mangifera indica* showed potent antimicrobial activity, which might be due to the higher content of total phenolics. Similar results were reported by Salah et al. (2006). Acetone extract of *Aristolochia bracteolate* showed potent antimicrobial activity, which might be due to the higher content of flavonoids. *Alpinia speciosa, Carissa carandas*, *Strychnos nux-vomica, Casuarina equissetifolia, Phyllanthus reticulatus, Pluchea arguta, Woodfordia fruticosa* (Leaf) and *Woodfordia fruticosa* (Stem) showed good antimicrobial activity and higher total phenolic content. *Argemone mexicana* and *Pluchea arguta* showed good antimicrobial activity and higher amounts of flavonoids.

3.4. CONCLUSION

The results of preliminary qualitative phytochemical study of the crude powder of 53 plants showed the presence of alkaloids (30.82%), tannins (67.92%), cardiac glycosides (62.26%), steroids (60.38%), flavonoids (15.09%) and saponins (39.62%). Tannins were present in more number of plants followed by cardiac glycosides and steroids.

The results of total phenolic and flavonoid content showed that the methanol extract of *Mangifera indica* showed highest total phenolic content, while acetone extract of *Aristolochia bracteolate* showed highest flavonoid content than other plant extracts. Thus, the plants studied here can be seen as a potential source of new useful drugs. The phytochemical characterization of the extracts, the identification of responsible bioactive compounds and quality standards are necessary for future study.

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Antiinflammatory Screening

CHAPTER 4: SCREENING OF SOME PLANTS FOR ANTI-INFLAMMATORY ACTIVITY IN CARRAGEENAN INDUCED PAW EDEMA MODEL

4.1. INTRODUCTION

There are hundreds of medicinal plants that have a long history of curative properties against various diseases and ailments. Alternative medicine for treatment of various diseases is getting more and more popular. Many medicinal plants provide relief comparable to that obtained from allopathic medicines. The majority of clinically important medicines belong to steroidal and non-steroidal anti-inflammatory chemical therapeutics for treatment of various inflammatory diseases (Choi and Hwang 2003).

The greatest disadvantage in presently available potent synthetic drugs is their toxicity and reappearance of symptoms after discontinuation. Therefore, screening and development of drugs for anti-inflammatory activity is still in progress and there is much hope for finding anti-inflammatory drugs from indigenous medicinal plants (Verpoorte 1999; Amresha et al. 2007; Juneja et al. 2007).

A number of animal models of inflammation such as paw edema, air pouch granuloma, sponge implantation and pleurisy have been developed using a variety of agents such as carrageenan (Fukuhara and Tsurufuji 1969), Freund's adjuvant (Ohuchi et al. 1982), turpentine (Spector and Willougby 1957), zymosan (Konno and Tsurufuji 1983), formalin (Northover and Subramanian 1961), dextran (Winter and Porter 1957) and monosodium urate crystals (Gordon et al. 1985).

The presence of edema is one of the prime signs of inflammation (Sur et al. 2002). It has been documented that carrageenan induced rat paw edema is a suitable *in vivo* model to predict the value of anti-inflammatory agents, which act by inhibiting the mediators of acute inflammation (Morebise et al. 2002).

The method was chosen for this study since edema induced by carrageenan has been widely used to induce experimental acute footpad edema in laboratory animals to screen new anti-inflammatory drugs (Niemegeers et al. 1964; El-Shenawy et al. 2002; Badilla et al. 2003; Ratheesh and Helen 2007). This method was first introduced by Winter et al. (1962) and remains an acceptable preliminary screening test for anti-inflammatory activity.

In the present study, six plants were screened for acute anti-inflammatory study by carrageenan induced paw edema. The plants were selected on the basis of traditional use of the plant or pharmacological activity reported in the literature.

4.2. MATERIALS AND METHOD

4.2.1. Collection and extraction:

Aristolochia indica, Argemone mexicana, Alpinia speciosa, Causarina equissetifolia, Gymnema sylvestre and *Pluchea arguta* were screened for acute anti-inflammatory study. Collection and extraction of the plants is described in the Chapter 2.2.1 and Chapter 2.2.2. Family, part used and traditional uses of the plants are given in Table 2.1.

4.2.2. Animals

Wistar albino rats of either sex (200-300 g) were used for the anti-inflammatory studies. The animals were obtained from Sarabhai Research Center (SRC), Baroda. All the rats were housed in standard plastic rat cages with stainless steel coverlids and wheat straw was used as bedding material. The animals were kept at the animal house of Department of Biosciences, Saurashtra University, Rajkot. The animals were kept in a group of 6-8 animals per cage and facilitated with standard environmental condition of photoperiod (12:12 h dark: light cycle) and temperature (25 ± 2ºC). They were provided with commercial rat and mice feed (Pranav Agro Industries Ltd., Amruth Brand rat & mice pellet feed) and water given *ad libitum*. The use of

these animals and the study protocols were approved by CPCSEA approved local ethical committee.

4.2.3. Preparation of test drug and standard drug

The test drug (Methanol extract of all the plants) and standard drug (Indomethacin) were prepared as a suspension in distilled water using mortal and pestle.

4.2.4. Animal Grouping

The animals were divided in to fourteen groups for acute anti-inflammatory study. Each group consisted of six animals of either sex. The groups were:

Group I: Vehicle treated control (Distilled water)
Group II: Indomethacin (2.5 mg/kg)
Group III: Methanol extract of *Aristolochia indica* (200 mg/kg)
Group IV: Methanol extract of *Aristolochia indica* (400 mg/kg)
Group V: Methanol extract of *Argemone mexicana* (200 mg/kg)
Group VI: Methanol extract of *Argemone mexicana* (400 mg/kg)
Group VII: Methanol extract of *Alpinia speciosa* (200 mg/kg)
Group VIII: Methanol extract of *Alpinia speciosa* (400 mg/kg)
Group IX: Methanol extract of *Causarina equissetifolia* (200 mg/kg)
Group X: Methanol extract of *Causarina equissetifolia* (400 mg/kg)
Group XI: Methanol extract of *Gymnema sylvestre* (200 mg/kg)
Group XII: Methanol extract of *Gymnema sylvestre* (400 mg/kg)
Group XIII: Methanol extract of *Pluchea arguta* (200 mg/kg)
Group XIV: Methanol extract of *Pluchea arguta* (400 mg/kg)

4.2.5. Carrageenan induced rat paw edema (Winter et al. 1962)

After grouping, the animals initial paw volume was measured using a plethysmograph (Bhatt et al. 1977). The plethysmograph employed, consisted of 10 ml glass vessel (25 mm x 65 mm) fixed 2 ml glass syringe through

pressure tubing. About 4 ml of mercury was filled in the syringe and the mercury level was adjusted to zero mark on the micropipette. The space between the zero mark and the fixed mark on the glass vessel was filled with the water. The initial level of the water was adjusted and set at zero. The paw was immersed in water exactly up to tibio tarsal articulation. The increased level of water in the glass vessel was adjusted to the prefixed mark by releasing the pressure of connected syringe. The level where water and mercury interface in the micropipette was recorded as paw volume.

Thereafter, distilled water or indomethacin (2.5 mg/kg) or methanolic extract of the plants (200, 400 mg/kg; p.o.) were orally administered to the respective group of the animals. After drug administration hydration was given to all the animals (4 ml/100 g body weight). One hour after drug administration, oedema was induced by subplantar injection of freshly prepared 0.1 ml of 1% carrageenan (Hi-media) in normal saline. After injection of carrageenan, the paw volume was measured at 1 h, 2 h and 3 h. Increase in the paw volume (%) was calculated by following formula:

$$\text{Increase in paw volume (\%)} = \frac{\text{Final paw volume} - \text{Initial paw volume}}{\text{Initial paw volume}} \times 100$$

4.3. RESULTS AND DISCUSSION

Carrageenan injection into the rat paw provokes a local, acute inflammatory reaction that is a suitable criterion for evaluation of anti-inflammatory agents (Segura et al. 1998). The inflammation consists of two phases, early phase which is related to the production of histamine, 5- hydroxytryptamin, bradykinins and cyclooxygenase products and delayed phase which is linked to neutrophil infiltration, as well as production of arachidonic acid metabolites (Dawson et al. 1991; Salvemini et al. 1996; Boughton-Smith et al. 1999). The results of the present study showed that the extracts of all the plants showed decrease in paw volume in carrageenan induced inflammation in rats at the

dose level of 200 mg/kg and 400 mg/kg as compared to the control group (Table 4.1).

A. indica showed significant decrease in paw volume at 1 h (51.9%, P≤ 0.05) and 2 h (47.57%, P≤ 0.01) at 400 mg/kg dose level. *A. indica* did not show significant decrease in paw volume at 200 mg/kg dose level.

Like *A. indica*, *A. mexicana* also did not show any significant decrease in paw volume at 200 mg/kg dose level. However, *A. mexicana* showed significant decrease in paw volume at 1h (58.58%, P≤ 0.01), 2 h (42.43%, P≤ 0.05) and 3 h (42.80, P≤ 0.05) at 400 mg/kg dose level.

A. speciosa showed significant decrease in paw volume at 1 h (50.63%, P≤ 0.01) and 2 h (48.15%, P≤ 0.05) at 200 mg/kg dose level and also showed decrease at 400 mg/kg (59.24%, P≤ 0.01 at 1 h and 54.46%, P≤ 0.001 at 2 h). It did not show any significant anti-inflammatory activity at 3 h.

C. equissetifolia showed significant decrease in paw volume at 1 h (55.32%, P≤ 0.01), 2 h (48.63%, P≤ 0.01) and 3 h (39.78%, P≤ 0.05) at 200 mg/kg dose level. At 400 mg/kg dose level, it showed significant decrease in paw volume at 1 h (62%, P≤ 0.01) and 2 h (50.82%, P≤ 0.01) only.

G. sylvestre did not show any significant anti-inflammatory activity at 200 mg/kg dose level like *A. indica* and *A. mexicana*. While it showed significant decrease in paw volume at 2 h (27.22%, P≤ 0.05) at 400 mg/kg dose level only.

P. arguta showed significant decrease in paw volume at both the dose levels. It showed significant decrease in paw volume at 1 h (58.54%, P≤ 0.01), 2 h (54.22%, P≤ 0.001) and 3 h (48.71%, P≤ 0.05) at 200 mg/kg dose level, and at 400 mg/kg dose level, the decrease in paw volume at 1 h was (71.61%, P≤ 0.001), 2 h (56.68%, P≤ 0.001) and 3 h (44.3%, P≤ 0.05) at. Anti-

Table 4.1 Anti-inflammatory screening of six medicinal plants by carrageenan induced rat paw edema

Group No.	Groups	Dose	1h		2h		3h	
			% Increase In paw Volume	% Change	% Increase In paw volume	% Change	% Increase In paw volume	% Change
I	Control	D/W (5ml/kg)	28.45 ± 2.88	-	38.98 ± 2.45	-	38.94 ± 5.33	-
II	Indomethacin	2.5 mg/kg	9.26 ± 1.25***#	67.45↓	13.05 ± 3.17***#	66.53↓	10.67 ± 3.39**#	72.61↓
III	*A. indica*	200 mg/kg	17.39 ± 4.13#	38.89↓	31.16 ± 5.49#	20.05↓	28.02 ± 3.91#	28.04↓
IV		400 mg/kg	13.69 ± 3.69*#	51.90↓	20.44 ± 4.2**#	47.57↓	24.88 ± 3.98#	36.10↓
V	*A. mexicana*	200 mg/kg	21.16 ± 2.58#	25.64↓	35.54 ± 4.10#	8.82↓	27.80 ± 4.11#	28.61↓
VI		400 mg/kg	11.78 ± 1.44**#	58.58↓	22.44 ± 4.60*#	42.43↓	22.27 ± 2.65*#	42.80↓
VII	*A. speciosa*	200 mg/kg	14.05 ± 2.55**#	50.63↓	20.21 ± 5.66*#	48.15↓	25.67 ± 3.35#	34.09↓
VIII		400 mg/kg	11.60 ± 2.26**#	59.24↓	17.75 ± 1.90***#	54.46↓	27.74 ± 4.02#	28.76↓
IX	*C. equissetifolia*	200 mg/kg	12.71 ± 2.50**#	55.32↓	20.02 ± 2.38**#	48.63↓	23.45 ± 3.43*#	39.78↓
X		400 mg/kg	10.81 ± 3.05**#	62.00↓	19.17 ± 3.88**#	50.82↓	29.88 ± 4.55#	23.28↓
XI	*G. sylvestre*	200 mg/kg	21.80 ± 4.56#	23.38↓	25.88 ± 5.62#	33.60↓	24.12 ± 3.78#	38.06↓
XII		400 mg/kg	22.93 ± 2.86#	19.41↓	28.37 ± 1.90*#	27.22↓	23.80 ± 3.28#	38.87↓
XIII	*P. arguta*	200 mg/kg	11.80 ± 2.65**#	58.54↓	17.85 ± 2.32***#	54.22↓	19.97 ± 4.93*#	48.71↓
XIV		400 mg/kg	8.08 ± 1.59***#	71.61↓	16.89 ± 2.77***#	56.68↓	21.69 ± 3.95*#	44.30↓

Data are expressed as mean ± SEM, P≤ (0.05*, 0.01**, 0.001***), F ≤ 0.05

inflammatory activity of *P. arguta* was comparable with the standard indomethacin at the 1 h at 400 mg/kg dose level.

Anti-inflammatory activity of 6 plants studied was as following manner at 1h: *P. arguta* (400 mg/kg) > *C. equissetifolia* (400 mg/kg) > *A. speciosa* (400 mg/kg) > *A. mexicana* (400 mg/kg) > *P. arguta* (200 mg/kg) > *C. equissetifolia* (200 mg/kg) > *A. indica* (400 mg/kg) > *A. speciosa* (200 mg/kg) > *A. indica* (200 mg/kg) > *A. mexicana* (200 mg/kg) > *G. sylvestre* (200 mg/kg) > *G. sylvestre* (400 mg/kg) (Table 4.1).

Anti-inflammatory activity of 6 plants studied was as following manner at 2 h: *P. arguta* (400 mg/kg) > *A. speciosa* (400 mg/kg) > *P. arguta* (200 mg/kg) > *C. equissetifolia* (400 mg/kg) > *C. equissetifolia* (200 mg/kg) > *A. speciosa* (200 mg/kg) > *A. indica* (400 mg/kg) > *A. mexicana* (400 mg/kg) > *G. sylvestre* (200 mg/kg) > *G. sylvestre* (400 mg/kg) > *A. indica* (200 mg/kg) > *A. mexicana* (200 mg/kg) (Table 4.1).

Anti-inflammatory activity of 6 plants studied was as following manner at 3 h: *P. arguta* (200 mg/kg) > *P. arguta* (400 mg/kg) > *A. mexicana* (400 mg/kg) > *C. equissetifolia* (200 mg/kg) > *G. sylvestre* (400 mg/kg) > *G. sylvestre* (200 mg/kg) > *A. indica* (400 mg/kg) > *A. speciosa* (200 mg/kg) > *A. speciosa* (400 mg/kg) > *A. mexicana* (200 mg/kg) > *A. indica* (200 mg/kg) > *C. equissetifolia* (400 mg/kg) (Table 4.1).

4.4. CONCLUSION

The results of the present study revealed that all the plants showed anti-inflammatory activity as compared to the control group. *A. indica* and *A. mexicana* showed anti-inflammatory activity in dose dependent manner, while *A. speciosa*, *C. equissetifolia* and *P. arguta* showed higher activity in lower dose (200 mg/kg) than higher dose (400 mg/kg) at 3 h. *G. sylvestre* showed higher activity in lower dose (200 mg/kg) than at higher dose (400 mg/kg) at 1 h and 2 h. Methanol extracts of *A. indica, A. speciosa*, *C. equissetifolia* and *P. arguta* showed higher anti-inflammatory activity at 1 h than at 3 h at both the doses. Methanol extract of *A. mexicana* showed higher activity at 3 h than at 1 h in lower dose, while it showed higher activity at 1 h than at 3 h in higher dose. Methanol extract of *G. sylvestre* showed higher anti-inflammatory activity at 3 h than at 1 h.

Thus, methanol extracts of *A. indica* (400 mg/kg), *A. mexicana* (400 mg/kg), *A. speciosa* (200 and 400 mg/kg), *C. equissetifolia* (200 and 400 mg/kg) and *P. arguta* (200 and 400 mg/kg) significantly inhibited early phase of the inflammation induced by carrageenan, while *A. mexicana* (400 mg/kg), *C. equissetifolia* (200

mg/kg) and *P. arguta* (200 and 400 mg/kg) significantly inhibited late phase of inflammation induced by carrageenan.

Out of six plant extracts, the methanol extract of *P. arguta* posses a potent anti-inflammatory activity followed by *C. equissetifolia* and *A. speciosa,* while *G. sylvestre* showed less anti-inflammatory activity. Thus, on the bases of availability of plant material and literature search, *P. arguta* (Leaf + stem) was selected for further pharmacognostic, toxicological and pharmacological studies.

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Pharmacognostic Study

CHAPTER 5: PHARMACOGNOSTIC STUDY OF *PLUCHEA ARGUTA* BOISS.

5.1. INTRODUCTION

Pharmacognosy is the scientific study of structural, physical, chemical and sensory characters of drugs. As late as the beginning of the 20th century, the subject had developed mainly on botanical side being concerned with history, identification, collection, preparation and storage of botanical drugs (Shinde and Dhalwal 2007).

Evaluation of plant materials and their derived products has always been an important part of the professional expertise of a pharmacognosist (Brain and Turner 1975). However, over the years the nature and degree of this evaluation have changed. Initially it was considered sufficient to authenticate the plant material by comparison with a standard botanical description or monograph. Later it was realized that, for detection of adulterants, this practice must be supplemented with other important procedures like microscopy, chemical tests and advanced analytical techniques. Currently plant based drugs are researched, dispensed, formulated and manufactured in modern framework. Hence, it has becomes an important interface among various branches of pharmaceutical sciences. It is now emerging as interdisciplinary science that incorporates inputs from chemistry, biology and biotechnology directed towards natural products based drug discovery (Kinghorn 2002).

Medicinal plant materials are characterized according sensory microscopic and macroscopic characteristics. Taking into consideration the variation in sources of crude drugs and their chemical nature, they are standardized by using different techniques including the method of estimation of chief active constituent (Soni et al. 2008).

Several problems not applicable to synthetic drugs influence the quality of herbal drugs (Ahmed et al. 2006):

- Herbal drugs are usually mixtures of many constituents.
- The active principle(s) is (are), in most cases unknown.
- Selective analytical methods or reference compounds may not be available commercially.
- Plant materials are chemically and naturally variable.
- Chemo-varieties and chemo cultivars exist.
- The source and quality of the raw material are variable.
- The methods of harvesting, drying, storage, transportation and processing for example, mode of extraction and polarity of the extracting solvent, instability of constituents etc. have an effect.

Strict guidelines have to be followed for the successful production of a quality herbal drug. Among them are proper botanical identification, phytochemical screening and standardization. Quality control and the standardization of herbal medicines involve several steps. The source and quality of raw materials, good agricultural practices and manufacturing processes are certainly essential steps for the quality control of herbal medicines and play a pivotal role in guaranteeing the quality and stability of herbal preparations (De Smet 1997; Roberts and Tyler 1997; Blumenthal et al. 1998; EMEA 2002; WHO 2003; WHO 2004).

The objective of the present study was to evaluate various pharmacognostic standards like botanical description, microscopy, ash values, extractive values, microscopical characteristics of powder, heavy metals, pH, solubility and preliminary phytochemical analysis of *Pluchea arguta* Boiss. (Leaf + stem).

5.2. MATERIAL AND METHODS

5.2.1. Collection and identification of the plant material

Fresh Leaves and stem of *Pluchea arguta* Boiss. were collected in the month of December 2006, from Junagadh Agriculture University, Junagadh, Gujarat, India. It was identified by Dr. P. S. Nagar, Department of Botany, M.S. University, Baroda, Gujarat, India (Voucher specimen number PSN 402). Fresh plant material was washed with tap water, air dried and then homogenized to fine powder and stored in airtight bottles.

5.2.2. Botanical description

Pluchea arguta Boiss. is a member of family Asteraceae, it is a short branched shrub, shrubby and with thin leaves, the upper parts glandular pubescent. Leaves sessile, 1-3 inch long, varying much in breadth, obovate oblong or oblanceolate, sometimes inciso pinnetified, glandular pubescent acutely serrate or dentate, narrowed at the base. Heads peduncled, subsolitary 1/3 – 1/2 inch in diameter. Involucre bracts rigid, ∞ seriate, the outer very small, narrowly lanceolate subulate, pubescent and ciliate, those of intermediate rows similar but longer, the innermost elongate, linear, acute, pubescent and ciliate at the tip. Receptacle naked. Pappus hairs slander, shortly barbellate. Achenes with a few appressed hairs, cylindric, slightly ribbed (Figure 5.1).

5.2.3. Microscopic characteristics

Free hand sections of the fresh leaf and stem samples of *Pluchea arguta* were taken. Sections were cleared with chloral hydrate and then stained with phloroglucinol and hydrochloric acid and mounted with glycerin. Same procedure was followed for microscopic characteristics of powdered material of leaf and stem of *Pluchea arguta.*

5.2.4. Determination of physicochemical parameters

Following physicochemical parameters was carried out: (The Ayurvedic Pharmacopoeia of India 2008; Vaghasiya et al. 2008)

- Total ash
- Acid insoluble ash
- Water soluble ash
- Alcohol soluble extractive
- Water soluble extractive
- Methanol soluble extractive
- Petroleum ether soluble extractive
- Solubility test
- pH
- Heavy metal analysis
- Determination of microorganisms

5.2.4.1. Loss on drying

2 g of crude powder of *Pluchea arguta* (Leaf + stem) was taken in an evaporating dish and then dried in an oven at 105ºC till constant weight was obtained. The weight after drying was noted and loss on drying was calculated. The percentage was calculated on the basis of sample taken initially.

5.2.4.2. Total ash

2 g powder of *Pluchea arguta* (leaf + stem) was taken in a silica crucible and ignited it by gradually increasing the heat to 500°C until it was white, indicating the absence of carbon. Ash was cooled in a desiccator and weighed without delay. Total ash value was calculated in mg per g of air-dried material.

5.2.4.2.1. Acid insoluble ash

To the crucible containing total ash, 25 ml of hydrochloric acid (~70g/l) was added; it was covered with a watch-glass and boiled gently for 5 minutes. The watch-glass was rinsed with 5 ml of hot water and this liquid was added to the crucible. The insoluble matter was collected on an ashless filter paper and it was washed with hot water until the filtrate was neutral. The filter paper containing the insoluble matter was transferred to the original crucible; it was dried on a hot plate and ignited to constant weight. The residue was allowed to cool in a desiccator for 30 minutes and then weighed without delay. Acid insoluble ash was calculated in mg per g of air dried material.

5.2.4.2.2 Water soluble ash

To the crucible containing the total ash, 25 ml of water was added and boiled for 5 minutes. The insoluble matter was collected on an ashless filter paper. It was washed with hot water and ignited in a crucible for 15 minutes. Weight of insoluble matter was subtracted from the weight of total ash. The content of water soluble ash was calculated in mg per g of air dried material.

5.2.4.3. Determination of alcohol soluble extractive

Four grams of crude powder of *Pluchea arguta* was macerated with 100 ml of alcohol in a closed flask and was kept on a rotary shaker for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105ºC till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

5.2.4.4. Determination of water soluble extractive

Four grams of crude powder of *Pluchea arguta* was macerated with 100 ml of water in a closed flask and was kept on a rotary shaker for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105ºC till constant

weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

5.2.4.5. Determination of methanol soluble extractive

Four grams of crude powder of *Pluchea arguta* was macerated with 100 ml of methanol in a closed flask and was kept on a rotary shaker for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105ºC till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

5.2.4.6. Determination of petroleum ether soluble extractive

Four grams of crude powder of *Pluchea arguta* was macerated with 100 ml of petroleum ether in a closed flask and was kept on a rotary shaker for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105ºC till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

5.2.4.7. Solubility

The quantitative solubility test of methanol extract of *Pluchea arguta* was determined in different solvents. 50 mg of extract was weighed for solubility test for different solvents. The extract was added in each solvent until saturated solution developed. Solubility was calculated in mg/ml.

5.2.4.8. Determination of pH

The methanol extract of *Pluchea arguta* was dissolved in distilled water and was kept in a water bath for 20 min. It was then filtered and the pH of the filtrate was noted with the help of a Systronic pH meter (pH system 361).

5.2.4.9. Determination of heavy metals

The analysis for heavy metals like arsenic, chromium, cobalt, lead, mercury and nickel for crude powder and methanol extract of *Pluchea arguta* were done at Choksi Laboratries Limited, Vadodara.

5.2.4.10. Determination of microorganisms

Total bacterial and total fungal counts as well as specific count for *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* for crude powder and methanol extract of *Pluchea arguta* (Leaf + stem) was carried out using reported methods (The Ayurvedic Pharmacopoeia of India 2008).

5.2.5. Phytochemical study

Preliminary qualitative phytochemical study for methanol extract of *Pluchea arguta* was carried out. Methods of alkaloids, flavonoids, tannins, cardiac glycosides, steroids and saponins were as described in Chapter 3.2.2.

5.2.6. HPTLC fingerprinting

The HPTLC fingerprinting of the methanol extract of *Pluchea arguta* was carried out at Anchrom Enterprises (I) Pvt. Ltd., Mulund, Mumbai. Analysis report was given by Anchrom Test Lab Pvt. Ltd.

Table 5.1 Information about HPTLC analysis given by Anchrom Test Lab Pvt. Ltd.

Information	
Application position	8 mm
Solvent front position	85 mm
Instrument (CAMAG TLC Scanner 3)	
Executed by	Anchrom
Number of tracks	12
Position of first track X	20.7 mm
Distance between tracks	14.5 mm
Scan start position Y	5 mm
Scan end position Y	85 mm
Slit dimensions	6 X 0.45 mm, Micro
Optimize optical system	Light
Scanning speed	20 mm/s
Data resolution	100 µm/step
Measurement Table	
Wavelength	230/290/580
Lamp	D_2
Measurement type	Remission
Measurement mode	Absorption
Optical filter	Second order
Detector mode	Automatic
PM high voltage	342 V
Detector properties	
Y-position for 0 adjust	5 mm
Track # for 0 adjust	0
Analog offset	10%
Sensitivity	Automatic
Integration	
Properties	
Data filtering	Savitsky-Golay-7
Baseline correction	Lowest slope
Peak threshold min. slope	5
Peak threshold min. height	10 AU
Peak threshold min. area	50
Peak threshold mix. height	990 AU
Track start position	5 mm
Track end position	75 mm
Display scaling	1000

5.3. RESULTS AND DISCUSSION

5.3.1. Microscopic characteristics

5.3.1.1. Transverse section (T.S.) of Stem

The T.S. of stem is shown in Figure 5.2. The epidermis is single layered and contains multi-cellular and glandular trichomes. The cork cambium originated from the outer layer of collenchyma with tightly packed cells. The cortex is made up of collenchymatous cells and is multilayered; endodermis is present. A single layer pericycle is present. The vascular bundles are of collateral type. They are numerous, distinct and arranged in a ring. The medullary rays clearly separate the vascular bundles. The pith is large and parenchymatous.

5.3.1.2. Longitudinal section (L.S.) of Leaf

The L.S. of leaf is as shown in the Figure 5.3. The upper epidermis is single layered, closely packed with a thick, waxy cuticle. There are many multicellular trichomes present on upper epidermis. The lower epidermis is also single layered but with a thin cuticle. Multi-cellular trichomes are also present on the lower epidermis. The mesophyll tissue is made up of palisade parenchyma and spongy parenchyma. The palisade tissue contains numerous oil glands. The spongy parenchymatous cells are irregular and more on lower side as compared to upper side. The vascular bundles are of collateral type. The xylem is exarch..

5.3.1.3. Powder characteristics

The microscopic study of the powder of leaf and stem is presented in Figure 5.4 and Figure 5.5. The powder study of stem showed starch grains, cork cells, annular/spiral vessel, sclerenchymatous cells and glandular trichomes. The powder study of leaf showed phloem fibers, multi-cellular trichome, spiral/annular xylem vessel, and anisocytic stomata with wavy surface, .

5.3.2. Determination of physicochemical parameters

The results of physicochemical parameters are presented in Table 5.2 and Table 5.3.

Table 5.2 Determination of proximate parameters of crude powder of *Pluchea arguta*

Parameters	Value
Loss on drying	6.21% (w/w)
Total ash	14.94% (w/w)
Acid insoluble ash	2.43% (w/w)
Water soluble ash	10.7% (w/w)
Water soluble extractive	12.57% (w/w)
Alcohol soluble extractive	10.42% (w/w)
Methanol soluble extractive	10.05% (w/w)
Petroleum ether soluble extractive	3.23% (w/w)
pH	5.42

The physical constant evaluation of the drugs is an important parameter in detecting adulteration or improper handling of drugs. The moisture content (6.21%) of the drug is not too high, thus it could discourage bacteria, fungi or yeast growth. Equally important in the evaluation of crude drugs, is the ash value and acid insoluble ash value determination. The total ash is particularly important in the evaluation of purity of drugs, i.e. the presence or absence of foreign inorganic matter such as metallic salts and/or silica (Musa et al. 2006). Total ash of crude powder of *Pluchea arguta* was 14.94%, acid insoluble ash was 2.43% and water soluble ash was 10.7%. Extractive yield of crude powder of *Pluchea arguta* was higher in water (12.57%) followed by alcohol (10.42%) and methanol (10.05%). Petroleum ether soluble extractive value (3.23%) was very less than other solvents studied. pH value showed the crude powder of *Pluchea arguta* was acidic (5.42) in nature.

Table 5.3 Determination of solubility of methanol extract of *Pluchea arguta*

Solvent	Solubility (mg/ml)
Acetone	8
Chloroform	1
Dimethylformamide	24
Dimethylsulphoxide	31
Dioxan	3
Distilled water	20
Ethyl acetate	3
Hexane	-
Methanol	29
Petroleum ether	-
Toluene	-

Methanol extract of *Pluchea arguta* was highly soluble in dimethylsulphoxide and methanol, while it was not soluble in hexane, petroleum ether and toluene.

Contamination by toxic metals can either be accidental or intentional. Contamination by heavy metals such as mercury, lead, copper, cadmium, and arsenic in herbal remedies can be attributed to many causes, including environmental pollution, and can pose clinically relevant dangers for the health of the user and should therefore be limited (De Smet et al. 1992; Lazarowych and Pekos 1998). Arsenic, chromium, cobalt, lead, mercury and nickel were not present in crude powder and methanol extract of *Pluchea arguta*.

Medicinal plant materials normally carry a great number of bacteria and moulds, often originating in soil. While a large range of bacteria and fungi form the naturally occurring micro flora of herbs, aerobic spore-forming bacteria frequently predominate. Current practices of harvesting, handling and

production may cause additional contamination and microbial growth. The determination of *Escherichia coli* and moulds may indicate the quality of production and harvesting practices (WHO 1998). In the present study cruder and methanol extract of *Pluchea arguta* were free from microbial contamination.

5.3.3. Phytochemical study

Qualitative phytochemical study of methanol extract of *Pluchea arguta* showed the presence of alkaloids, tannins and steroids in higher amount. Saponins were present in lower amount. Flavonoids were absent in methanol extract of *Pluchea arguta*.

5.3.4. HPTLC fingerprinting

High Performance Thin Layer Chromatography (HPTLC) technique is most simple and fastest separation technique available today which gives better precision and accuracy with extreme flexibility for various steps. The methanol extract of *Pluchea arguta* was subjected to HPTLC fingerprinting. The results showing number of peaks, start height, maximum Rf value, maximum height, maximum %, end Rf value, end height, total area and % area (Table 5.4 – Table 5.6). HPTLC spectral analysis was done in three different wavelengths 230, 290 and 580 nm (Figure 5.6). The results showed 10 peaks at 230 nm (Table 5.4), 11 peaks at 290 nm (Table 5.5) and 13 peaks at 580 nm wavelengths (Table 5.6). Peak number 7 covered maximum % of area at 230 nm with 0.49 Rf value. Peak number 7 covered maximum % of area at 290 nm with 0.48 Rf value. Peak number 4 covered maximum % of area at 580 nm with 0.34 Rf value. HPTLC plates showing different bands for methanol extract of *Pluchea arguta* at different wavelength (Figure 5.7).

Table 5.4 HPTLC spectral analysis of methanol extract of *Pluchea arguta* at 230 nm wavelength

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.01	43.5	0.00	373.5	18.25	0.05	95.4	8034.4	9.66
2	0.05	95.8	0.07	173.3	8.47	0.10	100.0	4837.7	5.82
3	0.11	98.2	0.15	155.1	7.58	0.19	116.7	8370.6	10.06
4	0.24	133.2	0.27	165.9	8.11	0.29	159.3	6028.8	7.25
5	0.29	159.6	0.34	290.8	14.21	0.36	213.3	13624.3	16.38
6	0.36	213.3	0.38	218.8	10.69	0.40	205.3	6421.9	7.72
7	0.44	193.4	0.49	287.8	14.06	0.56	123.6	20329.5	24.44
8	0.56	123.7	0.59	134.2	6.56	0.60	127.6	4158.5	5.00
9	0.60	127.7	0.62	132.0	6.45	0.67	64.2	5728.9	6.89
10	0.72	51.8	0.76	115.4	5.64	0.84	7.6	5657.2	6.80

Table 5.5 HPTLC spectral analysis of methanol extract of *Pluchea arguta* at 290 nm wavelength

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.02	1.1	0.01	469.1	15.41	0.05	148.4	10648.6	7.60
2	0.05	148.4	0.07	253.1	8.31	0.10	155.3	7812.9	5.58
3	0.10	155.5	0.15	252.5	8.29	0.20	199.9	16640.5	11.88
4	0.22	206.0	0.27	271.0	8.90	0.28	264.9	12916.1	9.22
5	0.29	265.1	0.34	419.0	13.76	0.36	346.7	21636.5	15.45
6	0.37	346.8	0.38	351.6	11.55	0.42	338.4	14893.7	10.63
7	0.44	337.1	0.48	402.0	13.20	0.56	186.0	30258.1	21.60
8	0.56	186.4	0.59	217.0	7.13	0.61	207.3	7846.2	5.60
9	0.61	207.3	0.62	208.2	6.84	0.67	98.4	7883.5	5.63
10	0.67	98.5	0.70	111.3	3.66	0.73	80.1	4602.6	3.29
11	0.74	80.0	0.76	90.2	2.96	0.87	0.9	4939.1	3.53

Table 5.6 HPTLC spectral analysis of methanol extract of P *Pluchea* arguta at 580 nm wavelength

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.03	0.1	-0.01	122.2	8.53	0.03	29.9	2659.1	4.77
2	0.04	29.6	0.06	56.3	3.93	0.09	23.2	1344.3	2.41
3	0.09	23.3	0.17	61.2	4.27	0.19	59.4	3455.1	6.20
4	0.19	59.2	0.34	203.9	14.24	0.37	100.1	17076.1	30.65
5	0.38	100.1	0.38	101.4	7.08	0.42	90.4	3159.7	5.67
6	0.44	89.7	0.48	130.0	9.08	0.48	127.0	4110.2	7.38
7	0.48	127.9	0.49	138.5	9.67	0.52	102.4	3073.9	5.52
8	0.52	102.8	0.53	109.3	7.63	0.55	87.8	2353.1	4.22
9	0.55	89.2	0.55	114.9	8.02	0.58	89.6	2728.9	4.90
10	0.58	89.5	0.63	133.2	9.30	0.70	61.2	8600.6	15.44
11	0.70	62.1	0.71	96.4	6.73	0.74	43.4	2061.5	3.70
12	0.74	43.8	0.77	94.6	6.60	0.81	47.9	3732.5	6.70
13	0.81	48.2	0.82	70.2	4.90	0.87	0.4	1365.0	2.45

5.4. CONCLUSION

The main goal of pharmacognosy is to assess the value of raw materials and to ensure that the final product is of the required standard. Strict standardization procedures and pharmacognostical studies of medicinal plants would reduce drastically much of the accidents in wrong prescriptions of traditional herbal medicines (Dinesh Kumar 2007). Standardization and quality control of plants, is of growing concern over ensuring purity of raw material before processing. Yet alternative medicines based on plant substances are extremely popular, even though their safety and efficacy have not been scientifically proven. Now a days routine pharmacognosy has changed demanding interdisciplinary research. From the present study it can be concluded that, contamination of heavy metals and microorganisms did not observed in dry powder of *Pluchea arguta*. Various pharmacognostic standards like botanical description, microscopy, ash values, extractive values, microscopic characteristics of powder, heavy metals, pH, solubility, HPTLC and preliminary phytochemical study of *Pluchea arguta* could be useful for the compilation of a suitable monograph for its proper identification.

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Toxicological Study

CHAPTER 6: ACUTE TOXICITY STUDY OF *PLUCHEA ARGUTA* BOISS. IN MICE

6.1. INTRODUCTION

Toxicology is an aspect of pharmacology that deals with the adverse effect of bioactive substance on living organisms. In order to establish the safety and efficiency of a new drug, toxicological studies are very essential experiments in animals like mice, rat etc. No drug substance is used clinically without its laboratory safety assessment at preclinical phase. Toxicological studies help to make a decision whether a new drug should be adopted for clinical use or not (Anisuzzaman et al. 2001; Alam et al. 2006).

In order to support an application for a clinical trial or for the registration of a new drug, it is necessary to satisfy legislation that requires that certain data should be produced from a variety of toxicological investigations that show the safety profile of the compound to which humans may be exposed. Therefore, in the majority of cases of evaluation of the toxicity of most substances, rodents and non-human primates are first used in preclinical animal safety studies before further studies are done in humans. These animals are mainly used because of their biological similarity to humans that allows them to be regarded as the suitable metabolic models for humans in a broad range of investigations (Pascoe 1983; Loomis and Hayes 1996).

Toxicity testing in animals is carried out on new drugs to identify potential hazards before administrating them to humans. It involves a wide range of tests in different species, with long term administration of the drug, regular monitoring for physiological or biochemical abnormalities, and a detailed postmortem examination at the end of the trial, to detect any gross or histological abnormalities (Rang et al. 2003).

Plants have always been an important source of drugs. From ASPIRIN to TAXOL, modern pharmaceutical industries largely take profit of the diversity of

secondary metabolites from plants for new drug research. In developing countries, a substantial part of the population uses folk medicine for its daily health care. Some of the most common practices involve the use of crude plant extracts, which may contain a broad diversity of molecules with often unknown biological effects (Konan et al. 2007).

The evaluation of the toxic action of plant extracts is indispensable in order to consider a treatment safe; it enables the definition of the intrinsic toxicity of the plant and the effects of acute overdose. Laboratory mice are sensitive to toxic substances occurring in plants. The administration of the extracts in increasing amounts enables the evaluation of the toxicity limits, and the test should be carried out in two ways, for three doses, and for both sexes, taking into account such factors as age, sex, weight, species, diet, and environmental conditions (Lagarto Parra et al. 2001).

6.1.1. Toxicity aspects of use of herbal preparations

The World Health Organization estimated that perhaps eighty percent of the inhabitants of the world rely chiefly on traditional medicines. It, therefore, approved the use of herbal products for national policies and drug regulatory measures in order to strengthen research and evaluation of the safety and efficacy of these products (Saxena 2001). Despite widespread use, few scientific studies have been undertaken to ascertain the safety and efficacy of traditional remedies (Veerappan et al. 2007). An increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used herbal remedies (UNESCO 1998).

In the absence of an efficient primary health care system, traditional medicine occupies a central place in the provision of health care, especially among rural communities of developing countries. The strong historical bond between

plants and human health is well substantiated by plant species diversity and related knowledge of their use as herbal medicines (Tabuti et al. 2003)

The use of plants for healing purpose is getting increasingly popular as they are believed as being beneficial and free of side effects (Leonardo et al. 2000). Currently, there is an ongoing world-wide “green” revolution which is mainly premised on the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs (Williamson et al. 1996; Parekh and Chanda 2006).

Although many medicinal plant products are used as relief for many ailments in humans, very little is known about their toxicity. Safety should be the overriding criterion in the selection of medicinal plants for use in healthcare systems (Tomlinson and Akerele 1998).

6.1.2. Route of administration

To evaluate toxicity of a compound in animals various routes may be used, but two most commonly used modes of administration for animals studies are intraperitoneal injection or the oral route (Poole and Leslie 1989).

The oral route is probably one of the most common means by which a chemical enters the body. In short, the oral administration is the form of administration involving the gastrointestinal tract, which may be viewed as a tube going through the body, starting at the mouth and ending at the anus. Although it is within the body, its contents are essentially exterior to the body fluids. Most orally administered chemicals can otherwise have a systemic effect on the organism only after absorption has occurred from the mouth or the gastrointestinal tract. Oral administration of chemicals that are rapidly absorbed from the gastrointestinal tract would theoretically expose the liver to concentrations of the agent that would not be obtained if other routes of administration were used (Loomis and Hayes 1996).

The acute toxicity defined by the Organization of Economic Cooperation and Development (OECD) Guidelines as the adverse effects occurring within a short time after oral administration of a single dose of a substance or multiple doses given within 24 h (Chan and Hayes 1994).

Usually acute (single dose) toxicity study is carried out on laboratory animals by using high dose (sufficient to produce death or morbidity) of the substance in question and/or based on previous report on its toxicity or toxicity of structurally related compounds (Demma et al. 2007). There was no previous report on toxicity of *Pluchea arguta*.

The present study was undertaken to contribute data on the safety of the methanol extract of *Pluchea arguta*, focusing on its acute toxicity study and its effect on gross behaviour changes, food and water intake, body weight changes, different organ weight changes and haematological parameters.

6.2. MATERIAL AND METHODS

6.2.1. Experimental animals

Swiss albino mice of either sex (20-25g) were used for acute toxicity studies. The animals were obtained from Sarabhai Research Centre (SRC), Baroda. All the mice were housed in standard plastic cages with stainless steel coverlids and wheat straw as bedding material at the animal house of Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. The animals were kept in a group of 5-6 per cage and facilitated with standard environmental condition of photoperiod (12:12 h dark: light cycle) and temperature (25 ± 2^{o}C). They were provided with commercial rat and mice feed (Pranav Agro Industries Ltd., Amrut brand rat and mice pellet feed) and water given *ad libitum*. The use of these animals and the study protocols were approved by CPCSEA local ethical committee.

6.2.2. Plant material

Collection and identification of plant material is described in Chapter 5.2.1.

6.2.3. Extraction

The dried crude powder of *Pluchea arguta* Boiss was extracted by soxhlet extraction method (Vaghasiya et al. 2008). Plant material was defatted by petroleum ether. Defatted plant material was extracted in methanol. The methanol was evaporated from the extract, and the dried extract was collected, weighed and stored in air-tight bottles at $4^{O}C$ for further experiments.

6.2.4. Acute toxicity study

6.2.4.1. Experimental groups and drug administration

In order to study any possible toxic effect or changes in normal behaviour 4 groups of 10 mice (five male and five female) were used in this experiment. Group I served as control group (D/W) and the other groups II, III and IV were treated with the extract of *Pluchea arguta* (1000, 3000 and 6000 mg/kg respectively). All the animals were fasted overnight prior to dosing. Before commencing the experiment, the body weight of mice was recorded. All the animals except group I were administered a single oral dose of methanol extract of *Pluchea arguta* at 1000, 3000, and 6000 mg/kg body weight. The control animals received only vehicle (D/W).

6.2.4.2. Behavioural study

The mice were placed one by one at the centre of three concentric circles drawn on a rubber sheet with diameter of 7 cm, 14 cm and 21 cm. After dosing all animals were observed for gross behaviour parameters like CNS depression (Hypoactivity, passivity, relaxation, narcosis and ataxia), CNS stimulation (Hyperactivity, irritability, stereotypy, tremors, convulsions, straub

tail and analgesia) and ANS stimulation (Ptosis and exophthalmia) at 1 h, 2 h, 3 h, 24 h and 48 h (Morpugo 1971). The observed results were recorded as sign of toxicity/number of animals studied. Signs of toxicity and mortality were observed daily for 7 days and were monitored daily for changes in body weight.

6.2.4.3. Food and water consumption

The amount of feed and water consumption was evaluated daily as known amount of food and water was given to each animal in each cage. After 24 h, the remaining food and water was taken from the cage and weighed. To find out the food and water consumption, remaining amount of food and water was deducted from the total amount.

6.2.4.4. Absolute and relative organ weight

The position, shape, size and color of internal organs such as liver, kidney, lung, heart, spleen, brain, adrenal, thymus, testis and uterus was visually observed for any sign of gross lesions. These organs were collected, weighted to determine the relative organ weight. The relative organ weight was calculated using the following formula:

Relative organ weight (g) = [Organ weight (g) / body weight (g)] X 100

Relative organ weight was compared with the control group.

6.2.4.5. Haematological parameters

On 8^{th} day, the animals were made to fast overnight and sacrificed by decapitation and blood was collected. The haematological parameters like haemoglobin, total red blood cell (RBC), leukocyte (WBC), PCV, MCV, MCH, MCHC, neutrophils, lymphocytes, eosinophils and platelet counts were determined using an autoanalyzer (System H1, Bayer Diagnostics).

6.2.5. Statistical analysis

All values are expressed as the mean ± SEM. The statistical comparisons were made by means of the student's t test and one way ANOVA. Values are considered statistically significant at $p<0.05$ for t-test and $F<0.05$ for ANOVA.

6.3. RESULTS AND DISCUSSION

Phytotheraputic products are many times, mistakenly regarded as safe because they are natural (Gesler 1992). Nevertheless, these products contain bioactive principles with potential to cause adverse effect (Bent and Ko 2004). Therefore, all the natural products used in therapeutics must be submitted to efficacy and safety test by the same methods used for new drugs (Talalay and Talalay 2001). Usually acute (single dose) toxicity study is carried out on laboratory animals by using high dose of the substance in question and/or based on previous report on its toxicity or toxicity of structurally related compounds. However, sometimes the acute toxicity test is criticized as a parameter for assessing toxicity but there are still occasions when some useful information could be obtained from such studies. Apart from giving a clue on the range of doses that could be used in subsequent toxicity testing, it could equally reveal the possible clinical signs elicited by the substance under investigation. It is also a useful parameter to investigate therapeutic index (i.e. LD_{50}/ED_{50}) of drugs and xenobiotics (Rang et al. 2001).

6.3.1. Gross behaviour study

No mortality was observed after the administration of methanol extract of *Pluchea arguta* at the doses of 1000, 3000 and 6000 mg/kg body weight. This is an indication that the extract has negligible level of toxicity when administered orally. According to Kennedy et al. (1986), substances with LD_{50} higher than 5 g/kg by oral route are regarded as being safe or practically non-toxic. The behavioral signs of toxicity such as CNS depression (Hypoactivity, passivity, relaxation, narcosis and ataxia), CNS stimulation (Hyperactivity, irritability, stereotypy, tremors, convulsions, straub tail and analgesia) and

ANS stimulation (Ptosis and exophthalmia) were also observed (Table 6.1 and Table 6.2). No signs of toxicity were observed, in eighter sex, in the control or treated groups. In addition, gross necropsy findings did not show any adverse effects in male and female mice of any organs in treated groups as compared to control group. Diarrhoea was observed in male and female mice at the dose of 6000 mg/kg at 1 h, 2 h and 3 h. After 24 h all the animals were normal. Anti-analgesic effect was seen at the doses of 1000, 3000 and 6000 mg/kg (Table 6.1 and Table 6.2).

6.3.2. Food and water consumption

Determination of food consumption is important in the study of safety of a product with therapeutic purpose, as proper intake of nutrients is essential to the physiological status of the animal and to the accomplishment of the proper response to the drug tested instead of a false response due to improper nutritional conditions (Stevens and Mylecraine 1994; Iversen and Nicolayen 2003).

In the present study, food and water consumption values were measured in male and female mice of both control and treated groups (Table 6.3 and Table 6.4). Statistically no significant difference was observed in *Pluchea arguta* methanol extract treated groups when compared with control group.

Table 6.1 Gross behavior study of methanol extract of *Pluchea arguta* at different doses in mice (Males)

Groups	Doses		Mortality	Parameters of gross behaviour														
				CNS Depression					CNS stimulation							ANS stimulation		Others
				HP	PS	RL	NC	AT	HR	IR	ST	TR	CN	SR	AN	PT	EX	DI
Group-I	0 (Vehicle)	1h	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		2h	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		3h	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		24h	0/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		48h	0/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Group-II	1000 mg/kg	1h	0/5	1/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5	0/5	0/5
		2h	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		3h	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		24h	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		48h	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Group-III	3000 mg/kg	1h	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
		2h	0/5	2/5	2/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
		3h	0/5	3/5	3/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5	0/5	0/5
		24h	0/5	3/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
		48h	0/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Group-IV	6000 mg/kg	1h	0/5	1/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	4/5
		2h	0/5	1/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	2/5	0/5	0/5	4/5
		3h	0/5	1/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	4/5
		24h	0/5	1/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5	0/5	0/5
		48h	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	3/5	0/5	0/5	0/5

Data is presented as response / number of animals; HP- Hypoactivity; PS- Passivity; RL- Relaxation; NC- Narcosis; AT- Ataxia; HR- Hyperactivity; IR- Irritability; ST- Stereotypy; TR- Tremors; CN- Convulsions; SR- Straub tail; AN- Analgesia; PT- Ptosis; EX- Exophthalmia; DI- Diarrhea

Table 6.2 Gross behavior study of methanol extract of *Pluchea arguta* at different doses in mice (Females)

Groups	Doses		Mortality	Parameters of gross behaviour														
				CNS Depression					CNS stimulation							ANS stimulation		Others
				HP	PS	RL	NC	AT	HR	IR	ST	TR	CN	SR	AN	PT	EX	DI
Group-I	0 (Vehicle)	1h	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		2h	0/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		3h	0/5	2/5	0/5	0/5	0/5	0/5	0/5	1/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		24h	0/5	1/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		48h	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Group-II	1000 mg/kg	1h	0/5	1/5	0/5	1/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	3/5	0/5	0/5	0/5
		2h	0/5	1/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
		3h	0/5	2/5	0/5	1/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
		24h	0/5	2/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		48h	0/5	2/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Group-III	3000 mg/kg	1h	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		2h	0/5	1/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		3h	0/5	2/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
		24h	0/5	1/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
		48h	0/5	2/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
Group-IV	6000 mg/kg	1h	0/5	0/5	1/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5
		2h	0/5	1/5	1/5	1/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5
		3h	0/5	1/5	1/5	1/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5
		24h	0/5	0/5	0/5	1/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
		48h	0/5	0/5	0/5	1/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5

Data is presented as response / number of animals; HP- Hypoactivity; PS- Passivity; RL- Relaxation; NC- Narcosis; AT- Ataxia; HR- Hyperactivity; IR- Irritability; ST- Stereotypy; TR- Tremors; CN- Convulsions; SR- Straub tail; AN- Analgesia; PT- Ptosis; EX- Exophthalmia; DI- Diarrhea

Table 6.3 Food consumption values of acute toxicity study of methanol extract of *Pluchea arguta* extract in male and female mice

Food consumption (g/animal/day)				
Groups	**Control**	**PAM-1000**	**PAM-3000**	**PAM-6000**
Males	11.40 ± 0.46	10.45 ± 0.26	10.70 ± 0.67	9.75 ± 0.58
Females	10.47 ± 0.45	10.03 ± 0.44	12.07 ± 0.52	8.67 ± 0.68

Data is presented as mean ± SEM (Average value of seven days), PAM-methanol extract of *P. arguta*

Table 6.4 Water consumption values of acute toxicity study of methanol extract of *Pluchea arguta* extract in male and female mice

Water consumption (ml/animal/day)				
Groups	**Control**	**PAM-1000**	**PAM-3000**	**PAM-6000**
Males	11.40 ± 0.46	10.12 ± 0.33	10.70 ± 0.67	9.75 ± 0.58
Females	10.27 ± 0.44	10.03 ± 0.44	12.07 ± 0.52	8.67 ± 0.68

Data is presented as mean ± SEM (Average value of seven days), PAM-methanol extract of *Pluchea arguta*

Body weight changes are indication of adverse effects of drugs and chemicals and it will be significant if the body weight loss is more than 10% from the initial body weight occurred (Tofovic and Jackson 1999; Raza et al. 2002; Teo et al. 2002).

Figure 6.1 and Figure 6.2 show the effects of *P. arguta* extract on body weight of male and female mice, respectively. Statistically no significant difference on body weights of the male and female mice receiving *Pluchea arguta* extract were noticed, as compared to the control group, throughout the experiment.

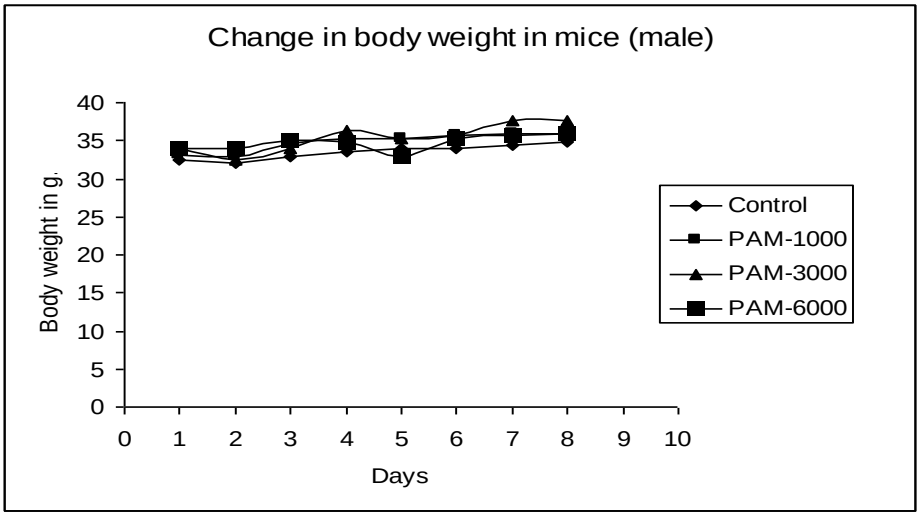

Fig. 6.1 Effect of methanol extract of *Pluchea arguta* (PAM) on body weight changes in male mice

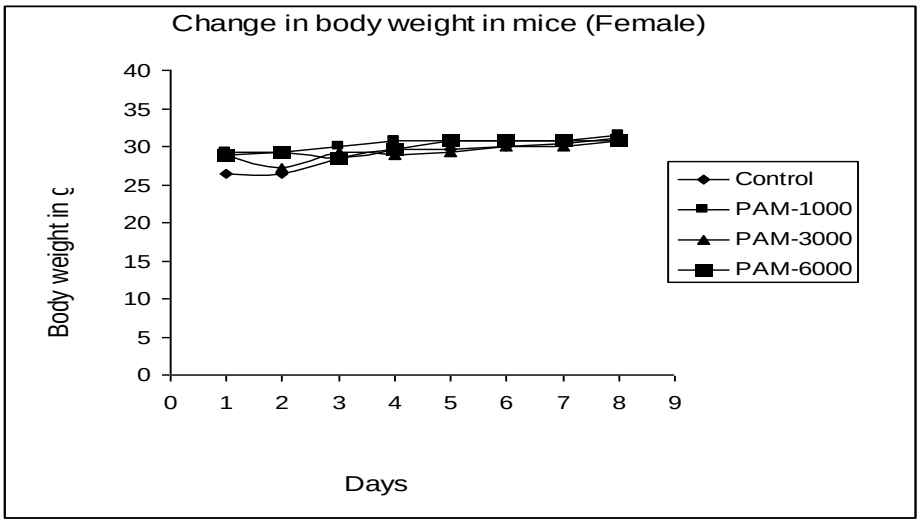

Fig. 6.2 Effect of methanol extract of *Pluchea arguta* (PAM) on body weight changes in female mice

6.3.3. Absolute and relative organ weight

Organ weight also is an important index of physiological and pathological status in man and animals. Table 6.5 and Table 6.6 depict the effect of methanol extract of *Pluchea arguta* on the weight of some vital organs in male and female mice. *Pluchea arguta* extract administration did not cause statistical difference in organ weight of male and female mice as compared to

the control group. Moreover, gross examination of internal organs of all the mice revealed no detectable abnormalities. Thus, it can be suggested that *Pluchea arguta* methanol extract of leaf is nontoxic at single dos level.

6.3.4. Haematological parameters

Blood parameters analysis is relevant to risk evaluation as the hematological system has a higher predictive value for toxicity in humans (91%) when assay involve rodents and non-rodents (Olson et al. 2000).

Hematological analyses (haemoglobin, total red blood cell (RBC), leukocyte (WBC), PCV, MCV, MCH, MCHC, neutrophils, lymphocytes, eosinophils and platelet counts) all the parameters were statistically not significant when compared to the control group, discarding the possibility of anemia or disturbances in the erythrocytes or hemoglobin production.

Table 6.5 Absolute organ weight (g) and relative organ weight values (g/100g body weight) of acute toxicity study of methanol extract of *Pluchea arguta* in male mice

Organs	Control		PAM-1000		PAM-3000		PAM-6000	
	AOW	ROW	AOW	ROW	AOW	ROW	AOW	ROW
Heart	0.18 ± 0.02	0.50 ± 0.03	0.18 ± 0.02	0.51 ± 0.04	0.19 ± 0.01	0.51 ± 0.02	0.18 ± 0.004	0.50 ± 0.02
Liver	1.79 ± 0.09	5.16 ± 0.19	1.96 ± 0.09	5.43 ± 0.2	2.02 ± 0.07	5.39 ± 0.25	1.96 ± 0.18	5.41 ± 0.32
Brain	0.5 ± 0.02	1.45 ± 0.09	0.54 ± 0.02	1.51 ± 0.04	0.53 ± 0.02	1.41 ± 0.05	0.51 ± 0.03	1.42 ± 0.09
Lung	0.3 ± 0.03	0.90 ± 0.14	0.36 ± 0.02	0.99 ± 0.04	0.33 ± 0.03	0.87 ± 0.07	0.38 ± 0.03	1.07 ± 0.07
Spleen	0.13 ± 0.02	0.37 ± 0.05	0.18 ± 0.03	0.49 ± 0.08	0.16 ± 0.02	0.43 ± 0.06	0.17 ± 0.04	0.47 ± 0.1
Kidney	0.33 ± 0.3	0.94 ± 0.09	0.36 ± 0.02	1.01 ± 0.04	0.37 ± 0.01	0.99 ± 0.03	0.35 ± 0.02	1.00 ± 0.11
Testis	0.10 ± 0.01	0.29 ± 0.01	0.12 ± 0.01	0.33 ± 0.02	0.11 ± 0.004	0.31 ± 0.02	0.10 ± 0.01	0.26 ± 0.04
Thymus	0.11 ± 0.01	0.62 ± 0.06	0.11 ± 0.01	0.58 ± 0.067	0.13 ± 0.01	0.67 ± 0.051	0.13 ± 0.01	0.74 ± 0.065

Data is presented as mean ± SEM; PAM- methanol extract of *Pluchea arguta*; AOW- Absolute organ weight; ROW- Relative organ weight

Table 6.6 Absolute organ weight (g) and relative organ weight (g/100g body weight) of acute toxicity study of methanol extract of *Pluchea arguta* in female mice

Organs	Control		PAM-1000		PAM-3000		PAM-6000	
	AOW	ROW	AOW	ROW	AOW	ROW	AOW	ROW
Heart	0.15 ± 0.01	0.47 ± 0.03	0.16 ± 0.01	0.50 ± 0.03	0.16 ± 0.01	0.50 ± 0.02	0.17 ± 0.01	0.55 ± 0.02
Liver	1.59 ± 0.12	5.08 ± 0.32	1.76 ± 0.05	5.64 ± 0.21	1.70 ± 0.08	5.52 ± 0.34	1.74 ± 0.05	5.66 ± 0.15
Brain	0.52 ± 0.02	1.65 ± 0.07	0.52 ± 0.01	1.64 ± 0.08	0.53 ± 0.01	1.73 ± 0.03	0.54 ± 0.01	1.75 ± 0.06
Lung	0.27 ± 0.02	0.86 ± 0.06	0.34 ± 0.03	1.09 ± 0.13	0.35 ± 0.03	1.12 ± 0.08	0.4 ± 0.02	1.30 ± 0.08
Spleen	0.11 ± 0.01	0.34 ± 0.03	0.14 ± 0.02	0.44 ± 0.06	0.15 ± 0.02	0.50 ± 0.06	0.13 ± 0.02	0.41 ± 0.04
Kidney	0.23 ± 0.01	0.73 ± 0.05	0.23 ± 0.01	0.71 ± 0.03	0.24 ± 0.02	0.77 ± 0.06	0.24 ± 0.01	0.79 ± 0.05
Uterus	0.20 ± 0.03	0.66 ± 0.11	0.23 ± 0.03	0.74 ± 0.08	0.24 ± 0.02	0.78 ± 0.06	0.26 ± 0.03	0.85 ± 0.1
Thymus	0.08 ± 0.02	0.53 ± 0.05	0.10 ± 0.02	0.62 ± 0.06	0.11 ± 0.01	0.70 ± 0.05	0.11 ± 0.02	0.73 ± 0.06

Data is presented as mean ± SEM, PAM- methanol extract of *Pluchea arguta*; AOW- Absolute organ weight; ROW- Relative organ weight

Table 6.7 Hematological parameters of acute toxicity study of *Pluchea arguta* extract in male mice

Hematological parameters	Control	PAM-1000	PAM-3000	PAM-6000
Hemoglobin (gms/100ml)	12.40 ± 1.49	14.45 ± 0.96	15.08 ± 0.21	14 ± 0.58
Total RBC (ml/cu. mm.)	7.70 ± 0.92	9.22 ± 0.73	9.60 ± 0.18	8.73 ± 0.36
PCV (%)	38.02 ± 4.92	44.98 ± 3.15	45.74 ± 0.64	43.08 ± 2.01
MCV (cu. micron)	49.06 ± 0.70	48.97± 0.89	47.76 ± 0.59	49.29 ± 0.48
MCH (Pico gram)	16.10 ± 0.13	15.87 ± 0.21	15.72 ± 0.16	16.03 ± 0.11
MCHC (%)	32.83 ± 0.51	32.43 ± 0.43	32.91 ± 0.14	32.54 ± 0.22
Neutrophils (%)	26.80 ± 4	40.40 ± 6.78	27.60 ± 3.33	36.40 ± 5.69
Lymphocytes (%)	72.40 ± 4.08	58.40 ± 6.93	71.20 ± 3.68	62.20 ± 6.29
Eosinophils (%)	0.80 ± 0.37	1.20 ± 0.73	1.20 ± 0.58	1.40 ± 0.68

Data is presented as mean ± SEM; PAM- methanol extract of *P. arguta*

Table 6.8 Hematological parameters of acute toxicity study of *Pluchea arguta* extract in female mice

Hematological parameters	Control	PAM-1000	PAM-3000	PAM-6000
Hemoglobin (gms/100ml)	15.50 ± 0.32	15.10 ± 0.24	14.68 ± 0.13	15.56 ± 0.25
Total RBC (ml/cu. mm.)	9.68 ± 0.1	9.54 ± 0.15	9.19 ± 0.12	9.51 ± 0.19
PCV (%)	47.08 ± 0.89	45.50 ± 1.02	44.34 ± 0.76	46.34 ± 0.73
MCV (cu. micron)	48.63 ± 0.55	47.70 ± 0.70	48.26 ± 0.60	48.47 ± 0.47
MCH (Pico gram)	16.01 ± 0.24	15.84 ± 0.30	15.99 ± 0.21	16.28 ± 0.16
MCHC (%)	32.93 ± 0.40	33.21 ± 0.45	33.13 ± 0.39	33.59 ± 0.56
Neutrophils (%)	27.60 ± 2.18	30.40 ± 2.77	24.40 ± 3.54	24.40 ± 3.36
Lymphocytes (%)	72 ± 2.45	69 ± 2.97	75.20 ± 3.53	75.40 ± 3.47
Eosinophils (%)	0.40 ± 0.40	0.60 ± 0.40	0.40 ± 0.40	0.20 ± 0.20

Data is presented as mean ± SEM, PAM- methanol extract of *Pluchea arguta*

6.4. CONCLUSION

In conclusion, *Pluchea arguta* extract was found to be fairly nontoxic when oral acute toxicity study in mice was performed at single dose level. Detailed experimental analysis on chronic toxicity is essential for further support of this drug.

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Pharmacological Study

CHAPTER 7: ANTI-INFLAMMATORY AND ANALGESIC EFFECTS OF *PLUCHEA ARGUTA* BOISS

7.1. INTRODUCTION

7.1.1. Inflammation

Inflammation, which was recognized as a simple allergic reaction for decades, is currently being considered to underline pathophysiology of a much broader spectrum of diseases than previously expected. The complex interplay of cellular and humoral mediators during inflammation is unfolding but our understanding of the inflammatory reaction is still incomplete (Sadowski-Debbing et al. 2002; Plytycz and Seljelid 2003). All inflammatory processes develop along a known sequence: locally increased blood supply, leakage of fluid, small molecules and proteins, and infiltration of cells (Roitt 1997).

Based on visual observation, the ancients characterized inflammation by five cardinal signs, viz. redness, swelling, heat, pain and loss of function (Hurley 1972). The swelling (oedema) is the result of increased passage of fluid from dilated and permeable blood vessels into the surrounding tissues, infiltration of cells into the damaged area, and in prolonged inflammatory responses deposition of connective tissue. Pain is due to the direct effects of mediators, either from initial damage or that resulting from the inflammatory response itself, and the stretching of sensory nerves due to oedema. The loss of function refers to either simple loss of mobility in a joint, due to the oedema and pain, or to the replacement of functional cells with scar tissue (Punchard et al. 2004).

The inflammatory process initiated in response to a pathogen or an injury is maintained at a certain, adequate level till the offending stimulus is neutralized, after which the reaction resolves on its own. In an auto-immune disorder, a harmless antigen is mistaken by the immune system as being foreign, thus initiating the inflammatory reaction. The persistence of a stimulus that physiologically resides in the organism prevents the natural, resolving

mechanisms from prevailing (Levy and Serhan 2003). As a result one's own defense mechanism can turn into a perpetuator of a persistent injury which although not fatal, can lead to loss of function of the organs involved. Additionally, many of the mediators can leak from the local region and initiate inflammatory reactions elsewhere (Cleeland et al. 2003). Animal experiments have been extremely useful in understanding the entire inflammatory reaction since they demonstrate a complete window of events, from the time when the stimulus is given till the reaction naturally resolves (Dawson et al. 1991; Finlay-Jones et al. 1999).

Inflammation is an essential and beneficial process in the protection against pathogens. Occasionally the inflammatory process is directed against autologous antigens, or the process does not alleviate after its onset and escalates into long-term persistence of inflammation, i.e., chronic inflammation (Robbins et al. 1999). The excessive release of proteases and ROS by the activated leukocytes and endothelial cells in such conditions can ultimately result in severe tissue damage (Conner and Grisham 1996). In the pathogenesis of several diseases such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, multiple sclerosis, Alzheimer's disease, and transplant rejection, inflammation- mediated tissue injury plays an important role (Cotran and Mayadas-Norton 1998).

Inflammation is a pathophysiological response of mammalian tissues to a variety of hostile agents including infectious organisms, toxic chemical substances, physical injury or tumor growth leading to local accumulation of plasmic fluid and blood cells (Sobota et al. 2000). Although inflammation is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can induce, maintain and aggravate many disorders. Hence, the employment of anti-inflammatory agents would be helpful in the therapeutic treatment of those pathologies associated with inflammatory reactions (Sosa et al. 2002).

There are several mediators involved in inflammation. Histamine, serotonine, bradykinin and prostaglandins (PG) are involved in the increased vascular

permeability (Posadas et al. 2004). Histamine, bradikinin and serotonin are released in the early phase (Di Rosa et al. 1971; Hori et al. 1988) while prostaglandins (PGs) are involved in both early and late phases of inflammation (Kikuchi et al. 1996). Prostaglandins (PGs) elicit a variety of important biological responses. Among the properties of PGs are their abilities to induce pain, fever and symptoms associated with inflammatory responses. Most of nonsteroidal anti-inflammatory drugs (NSAIDs), which are clinically used to date, inhibit the production of PGs by inhibition of COX enzymes and are the main drugs used to reduce the untoward consequences of inflammation (Albert et al. 2002). They are also involved in maintaining normal physiological processes (Moon et al. 1999). There are two isoforms of COX: COX-1 and COX-2. COX-1 is detectable, but COX-2 is not detectable in most normal tissues, however, COX-2 can be induced by many factors such as pro-inflammatory cytokines, phlogistic factors, etc. Studies indicated that COX-2 plays an important role in inflammation (Subbaramaiah et al. 1996). Thus, those agents that could suppress the activity or protein expression of COX-2 are likely to be valuable medicine for anti-inflammation and easing of pain (Shu et al. 2006). Recent development of selective COX-2 inhibitors as anti-inflammatory agents is considered a major therapeutic breakthrough in the treatment of inflammatory diseases since the inhibition of COX-2-derived PGs is thought to be the major pharmacodynamic action responsible for their anti-inflammatory effects (Abad et al. 2006).

Acute and chronic inflammatory diseases are still one of the most important health problems in the world. Various agents are adapted to treat inflammatory disorders; their prolonged use often leads to serious adverse reactions such as gastric intolerance, bone marrow depression, water and salt retention. Consequently, development of new anti-inflammatory drugs with low side-effects is still necessary (Xiao et al. 2005; Yonathan et al. 2006; Kupeli et al. 2007).

Acute inflammation is characterized by rapid onset and is of short duration. It is characterized by the exudation of fluids and plasma proteins and the migration of leukocytes, most notably neutrophils into the injured area. This

acute inflammatory response is believed to be a defense mechanism aimed at killing of bacteria, viruses and parasites while still facilitating wound repair (Iwalewa et al. 2007). Inflammatory response is triggered by tissue mast cells and resident macrophages whose degranulation and activation sequentially release a battery of inflammatory mediators, including bioactive amines (Histamine and 5-HT), cytokines, chemokines as well as lipid mediators that collectively recruit and activate inflammatory cells as well as bring about oedema formation (Lawrence and Gilroy 2007).

Chronic inflammation is the reaction arising when the acute response is insufficient to eliminate the pro-inflammatory agents. Chronic inflammation includes a proliferation of fibroblasts and infiltration of neutrophils with exudation of fluid. It occurs by means of development of proliferative cells which can either spread or form granuloma. Efficacy of anti-inflammatory agents in chronic inflammatory states is indicated by their ability to inhibit the increase in the number of fibroblasts during granular tissue formation (Recio et al. 1995; Gupta et al. 2003). The persistent chronic inflammation increases the development of the degenerative diseases such as rheumatoid arthritis, atherosclerosis, heart disease, Alzheimer's, asthma, acquired immunodeficiency disorder (AIDS), cancer, congestive heart failure, multiple sclerosis, diabetes, infections (bacterial, fungal, parasitic), gout, inflammatory bowel disease, aging and neurodegenerative CNS depression, all of which are associated with immunopathology that appears to play a key role in the onset of the condition (Dalgleish and O'Byrne 2002; Barnes 2004).

7.1.2. Analgesia

Pain is the most common symptomatic reason for seeking a medical consultation. Everyone is affected by pain at some point in their lives, whether it is from headaches, cuts and bruises or more severe pain resulting from surgery, which would be pre-controlled in anticipation of the event. Although chronic types of pain may generally appear to have no purpose, acute pain acts as an important warning mechanism to the person by instructing the brain to isolate the individual from that particular pain stimulus. The treatment

of pain, a major problem in medicine, is complicated by many factors. Pain is not a uniform sensation, as illustrated by its many common descriptions, e.g. sharp, dull, aching, burning, shooting, cramping, stabbing and throbbing. There are several ways to classify pain, but the first distinction usually made is that between acute and chronic pain. Pain is a subjective sensation which cannot be measured objectively (Buschmann et al. 2002).

The management and treatment of pain is probably one of the most common and yet difficult aspects of medicinal practice. Analgesic therapy is domain by two major classes of analgesic drugs; viz. opioids and non steroidal anti-inflammatory drugs (NSAIDs). Both classes of analgesic drugs produce serious side effects, such as gastrointestinal disturbance, renal damage (with NSAIDs drugs), etc. (Domaj et al. 1999; Dahl and Reader 2000).

7.1.3. Standard drugs as anti-inflammatory agents and their side effects

Inflammatory diseases are very common throughout the world and non-steroidal anti-inflammatory drugs are the most prescribed drugs for the treatment of inflammatory diseases. However, prolonged use of both steroidal and non-steroidal anti-inflammatory drugs is well known to be associated with gastric injury and ulceration, renal damage and bronchospasm due to their non-selective inhibition of both isoforms of the COX enzymes (Tapiero et al. 2002). The use of steroidal drugs as anti-inflammatory agents is also becoming highly controversial due to their multiple side effects (Van den Worm et al. 2001).

Therapy of inflammatory diseases is usually directed at the inflammatory processes. Through years of ingenious syntheses and structural modifications, which usually accompany design and development of new drug substances, many non-steroidal anti-inflammatory drugs (NSAIDS) have been prepared and marketed (Osadebe and Okoye 2003). NSAIDs are the most common medications taken worldwide for the treatment of pain, inflammation and fever. Although chemically disparate they produce therapeutic effects by

the common ability to inhibit the activity of cyclooxygenase (COX) enzymes (Ferreira et al. 1971; Smith and Willis 1971; Vane 1971).

The nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin and ibuprofen inhibit early steps in the biosynthesis pathway of prostaglandins by inhibition of COX enzymes and are the main drugs used to reduce the untoward consequences of inflammation (Albert et al. 2002). However, the side effects of the currently available anti-inflammatory drugs pose a major problem in their clinical use (Mattison et al. 1998). They provide sympathetic relief, but they do not modify the pathogenesis of inflammation and do not reduce the disabling bone and cartilage damage (Ford-Hutchinson et al. 1981). Therefore, people suffering from chronic inflammatory conditions have turned to the use of complementary and alternative medicines including herbal products to restore their health (Kaboli et al. 2001). As a result the search for other alternatives seems necessary and beneficial (Vasudevan et al. 2007).

7.1.4. Use of medicinal plants

Herbal drugs are being proved as effective as synthetic drugs with lesser side effects. So, there is a continuous search for indigenous drugs, which can provide relief from inflammation (Balasubramanian et al. 2005). Medicinal herbs have been used as a form of therapy for the relief of pain throughout history (Almeida et al. 2001). Medicinal plants are believed to be an important source of new chemical substances with potential therapeutics (Farnsworth 1989; Eisner 1990). The search for plants with reported folkloric use as pain relievers, anti-inflammatory agents, should therefore be viewed as a fruitful and logical research strategy in the search for new anti-inflammatory drugs.

Plants have been used by human beings since ages in traditional medicine due to their therapeutic potential and search for medicinal plants has led the discovery of novel drugs (Orhan et al. 2006). Plant extracts have been used for centuries as a popular method for treating several health disorders. Over the last ten years the study of plant extracts has attracted attention in different

fields of the biological sciences (Penna et al. 2003; Ratheesh and Helen 2007). The use of botanical and herbal medicines as a complementary approach for the treatment of inflammatory diseases has been steadily increasing, possibly because of the adverse effects associated with the use of non steroidal anti-inflammatory drugs. One approach to discover newer anti-inflammatory agents is to search from natural sources (Mazura et al. 2007).

Natural products from some plants, fungi, bacteria and other organisms continue to be used in pharmaceutical preparations either as pure compounds or as extracts. There is a great variety of compounds that can be extracted and characterized from plants, which can provide a wide spectrum of medicinal properties. The herbal products seems to be a potential source, having different substances to be investigated, including alkaloids, curcuminoids, terpenoids, flavonoids, etc, which have been commonly used in folk medicine and have efficacy against many diseases.

A number of natural products are used in the traditional medical systems in many countries. Alternative medicine for treatment of various diseases is getting more and more popular. Many medicinal plants provide relief from symptoms comparable to that obtained from allopathic medicines. The majority of clinically important medicines belong to steroidal or non-steroidal anti-inflammatory chemical therapeutics for treatment of various inflammatory diseases. Though these drugs have potent properties, they have various and severe adverse effects. Therefore, agents of natural origin with very little side effects are required as substitute chemical therapeutics (Ahmadiani et al. 1998; Verpoorte 1999).

Hence, search for new anti-inflammatory agents that retain therapeutic efficacy and yet are devoid of side effects is the need of the hour. Thus, study of plant species that traditionally have been used as pain killers should still be seen as a logical research strategy, in search for new anti-inflammatory and analgesic drugs (Mantri and Witiak 1994; Elisabetsky et al. 1995). There are many reports of natural products which show anti-inflammatory and analgesic

activity (Vaghasiya et al. 2007; Rao et al. 2008; Owoyele et al. 2009; Tanna et al. 2009).

7.1.5. *Pluchea arguta*

Pluchea arguta Boiss is a member of family Asteraceae and about 1000 genera of this family are well known in the world (Jaffri 1966). Different species of *Pluchea* are used in Ayurvedic system of medicine in various diseases. Pharmacological studies demonstrated anti-inflammatory and antioxidant activities of different *Pluchea* species (Barros et al. 2006; Fernandez and Torres 2006). In view of the therapeutic properties attributed by people to different species of this plant, anti-inflammatory and analgesic effects of methanol extract of *Pluchea arguta* Boiss in rats and mice was studied using various inflammatory and analgesic models.

7.1.6. Models for anti-inflammatory study

Rat paw edema is the most commonly used model for acute inflammation while subcutaneous implantation of biomaterial is usually used for the chronic inflammatory model. There are various models of acute inflammation which is induced by formalin, dextran, histamine, serotonin, bradykinin, prostaglandin and carrageenan and they are commonly used to investigate anti-inflammatory effects of drugs (Maling et al. 1974; Suleyman and Buyukokuroolu 2001). The cotton pellet method is widely used to evaluate the transudative and proliferative components of the chronic inflammation. The dry weight of the pellets correlates with the amount of the granulomatous tissue (Suleyman et al. 2002).

Considering the above, the aim of the present study was to evaluate the anti-inflammatory and analgesic potency of methanol extract of *Pluchea arguta* Boiss. (leaf + stem)

7.2. MATERIAL AND METHODS

7.2.1 Plant material and extraction

The collection of the plant material and the extraction of *Pluchea arguta* was done by soxhlet extraction method as described in Chapter 6.2.1. and Chapter 6.2.2.

7.2.2. Drug dose

The doses considered for the experiment on rat and mice were obtained from conversion of human dose of *Pluchea arguta* (3 g kg^{-1}). The conversion factor of human dose to rat (Per 200 g body weight) dose is 0.018 (Paget and Barnes 1964) and the conversion factor of human dose to mice (Per 20g body weight) dose is 0.0026. Hence the calculated dose for the rat is 270 mg/kg and for the mouse is 390 mg/kg. From this calculation, all the anti-inflammatory and analgesic experiments were carried out in two different doses of 200 and 400 mg/kg body weight.

7.2.3. Preparation of test drug and standard drug

Test drug (Methanol extract of *Pluchea arguta*) and standard drug (Indomethacin) were prepared as a suspension in distilled water using mortal and pestle.

7.2.4. Animal grouping

The animals were divided into four groups for anti-inflammatory and analgesic studies. Each group consisted of six animals of either sex. The groups were:

Group I: Negative control - Distilled water

Group II: Test drug- Methanol extract of *Pluchea arguta*- 200 mg/kg body weight (PAM-200)

Group III: Test drug- Methanol extract of *Pluchea arguta*- 400 mg/kg body weight (PAM-400)

Group IV: Positive control- Standard drug indomethacin- 2.5 mg/kg body weight (Indo-2.5)

7.2.5. Carrageenan induced rat paw edema (**Winter et al. 1962**)

Acute inflammation was produced by subplantar injection of 0.1 ml of 1% 0.1 ml of 1% carrageenan in normal saline in the hind paw of rats 1 h after the administration of the test drug as well as positive and negative controls. The paw volume was measured at 1 h, 2 h and 3 h after carrageenan injection, using plethysmograph as described in Chapter 4.2.5.

7.2.6. Dextran induced rat paw edema (Winter and Porter 1957)

The animals were treated in a manner similar to that of carrageenan induced paw edema model. 0.1 ml 1% dextran (60,000-90,000 m.w., HiMedia, India) was used for the study. Paw volume was measured as mentioned in carrageenan induced paw edema model at 1 h, 2 h and 3 h.

7.2.7. Histamine induced rat paw edema (Suleyman et al. 1991)

In this model paw edema of a rat was induced by subplantar injection of 0.1 ml of 1% freshly prepared histamine in normal saline and the paw oedema was measured as mentioned in carrageenan induced paw edema model. The paw volume was measured at 0.5 h, 1 h, 2 h and 3 h.

7.2.8. Formalin induced paw edema (Brownlee 1950)

The test drug was administered once daily for seven consecutive days to all the groups. On seventh day, initial paw volume was measured before drug administration. After 1 h of drug administration, paw edema of the rat was induced by subplantar injection of 0.1 ml of 3% formalin solution in normal

saline. Paw volumes were measured at 3 h, 24 h and 48 h after formalin injection as described earlier in carrageenan model.

7.2.9. Cotton pellet induced granuloma in rats (Swingle and Shideman 1972)

Cotton pellet induced granuloma formation in rats was performed for chronic anti-inflammatory study. This model represents the exudative and proliferative phases of inflammation. The cotton pellets weighing 100 mg were made by rolling of cotton piece and sterilizing by autoclaving. The rats were anaesthetized with ether; dorsum was shaved clear and swabbed with 70% (v/v) alcohol. Midline incision of 1 cm was made in the intrascapular region. A small tunnel was made on either side of the incision with the help of a small blunt forceps. Sterile cotton pellet (100 mg) was implanted in each tunnel. Air was removed from the tunnel and then incision was closed with sutures. The test drugs were administered for 7 consecutive days starting from the day of implantation. The rats were sacrificed on the 8^{th} day, cotton pellets were removed and cleaned of extraneous tissue and dried by placing them in a hot air oven overnight at $80^{O}C$ and then weighed. The difference between the initial weight and the final weight of the pellet after drying was taken as the granuloma tissue weight. The results were expressed as mg granulation tissue formed per 100 g body weight. The adrenal gland, spleen, thymus and lymph nodes were dissected carefully and weight of these organs was noted. Further, these organs were placed in 4% formalin solution and sent to the pathological laboratory for histopathological investigations. Blood samples were collected of all the animals and serum was separated. From the serum sample, following biochemical parameters were estimated:

7.2.9.1. Serum total protein:

The total serum protein was estimated by modified Biuret method (Vatzidis 1977) using the total protein test kit (Span Diagnostics Ltd.).

7.2.9.1.1. Principle

Peptide bonds of proteins react with cupric ions in alkaline solution to form a colored chelate, the absorbance of which is measured at 578 nm. The biuret reagent contains sodium-potassium tartrate, which helps in maintaining solubility of this complex at alkaline pH. The absorbance of final colour is proportional to the concentration of total protein in the samples.

Protein + Cu^{++} $\xrightarrow{\text{Alkaline pH}}$ Cu-protein complex

7.2.9.1.2. Reagents

Reagent I: Biuret Reagent (Copper sulphate, 7 mM; sodium hydroxide 200 mM; sodium potassium tartrate 20 mM; surfactant)

Reagent II: Protein Standard (BSA, 6.5 g/dl; preservative)

7.2.9.1.3. Procedure

3 ml of Reagent I was added to all the test tubes. Thereafter, 30 µl serum was added for the test and 30µl Reagent II was added for the standard, while in blank 30µl of D/W was added. They were then mixed well and incubated at 37 ^{o}C for 5 minutes. The absorbance was read at 578 nm.

7.2.9.1.4. Calculation:

$$\text{Total protein concentration (g/dL)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ X } 6.5$$

7.2.9.2. Serum albumin:

The serum albumin was estimated by the method given by Corcoran and Durnan (1977) using albumin test kit (Span Diagnostics Ltd.).

7.2.9.2.1. Principle

Determination of albumin in serum or plasma is based on the binding behaviour of the protein with the dye bromocresol green. At pH 3.68, albumin acts as a cation and binds to the anionic dye, forming a green complex, the absorbance of which is measured at 630 nm.

7.2.9.2.2. Reagents

Reagent I: Albumin reagent (Bromocresol green; Buffer pH 3.68; preservative; surfactant).

Reagent II: Albumin standard (BSA, 4 g/dl; Buffer, preservatives).

7.2.9.2.3. Procedure

3 ml of albumin reagent (Reagent I) was added to all the test tubes. Thereafter, 30 µl serum was added for the test and 30µl Reagent II was added for the standard, while in blank 30µl of D/W was added. They were then mixed well and incubated at room temperature for 1 min.

7.2.9.2.4. Calculation:

$$\text{Albumin (g/dL)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 4$$

7.2.9.3. Serum aspartate transaminase (AST):

Serum aspartate transaminase was estimated by the method of Reitman and Frankel (1957) using AST test kit (Span Diagnostics Ltd.).

7.2.9.3.1. Principle

α ketoglutarate and L- aspartate react to form L-glutmate and oxaloacetate. This reaction is catalysed by aspartate transaminase. Oxaloacetate so formed

is coupled with 2,4-dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydrazone, which gives brown color in alkaline medium and this can be measured colorimetrically.

7.2.9.3.2. Reagents

Reagent I: Buffered Aspartate α-KG substrate, pH 7.4
Reagent II: DNPH colour reagent
Reagent III: Sodium Hydroxide, 4 N
Reagent IV: Working Pyruvate Standard, 2 mM
Solution I: Dilute 1 ml of Reagent III up to 10 ml with D/W.

7.2.9.3.3 Procedure

0.25 ml of Reagent I was added in clean test tubes and incubated at 37 ºC for 5 minutes. 0.05 ml of serum was added in the test, 0.05 ml Reagent IV was added in standard and 0.05 ml distilled water was added in the blank. They were mixed well and incubated at 37ºC for 60 minutes. Thereafter, 0.25 ml of Reagent II was added to all the tubes, mixed well and allowed to stand at room temperature (15º-30ºC) for 20 minutes. Then 2.5 ml of Solution I was added to all the tubes, mixed well and allowed to stand at room temperature (15º-30ºC) for 10 min. The absorbance of blank, standard and test were read at 505 nm.

7.2.9.3.4. Calculation

Standard curve of O.D. of test on Y-axis against enzyme activity on X-axis was drawn. O.D. of test was marked on the Y-axis of the standard curve and extrapolated into the corresponding enzyme activity curve. Serum aspartate transaminase activity is expressed as IU/L.

7.2.9.4. Serum alanine transaminase (ALT):

Serum alanine transaminase was estimated by the method of Reitman and Frankel (1957) using ALT test kit (Span Diagnostics Ltd.).

7.2.9.4.1. Principle

α ketoglutarate and L- alanine react to form L-glutmate and pyruvate. This reaction is catalysed by alanine transaminase. Pyruvate so formed is coupled with 2,4-dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydrazone, which gives brown color in alkaline medium and this can be measured colorimetrically.

7.2.9.4.2. Reagents

Reagent I: Buffered Alanine α-KG substrate, pH 7.4
Reagent II: DNPH colour reagent
Reagent III: Sodium Hydroxide, 4 N
Reagent IV: Working Pyruvate Std., 2 mM
Solution I: Dilute 1 ml of Reagent III up to 10 ml with D/W.

7.2.9.4.3. Procedure

0.25 ml of Reagent I was added in clean test tubes and incubated at 37 ºC for 5 minutes. 0.05 ml of serum was added in the test, 0.05 ml Reagent IV was added in the standard and 0.05 ml distilled water was added in the blank. They were mixed well and incubated at 37ºC for 30 minutes. Thereafter, 0.25 ml of Reagent II was added to all the tubes, mixed well and allowed to stand at room temperature (15º-30ºC) for 20 minutes. Then 2.5 ml of Solution I was added to all the tubes, mixed well and allowed it to stand at room temperature (15º-30ºC) for 10 min. The absorbance of blank, standard and test were read at 505 nm. Serum alanine transaminase activity is expressed as IU/L.

7.2.9.4.4. Calculation

Standard curve of O.D. of test on Y-axis against enzyme activity on X-axis was drawn. O.D. of test was marked on the Y-axis of the standard curve and extrapolated in to the corresponding enzyme activity curve. Serum alanine transaminase activity is expressed as IU/L.

7.2.9.5. Alkaline Phosphatase (ALP)

Alkaline phosphatase activity was estimated by the method of Kind and King (1954) using ALP test kit (Span Diagnostics Ltd.).

7.2.9.5.1. Principle

Alkaline phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4-aminoantipyrine in the presence of oxidizing agent potassium ferricyanide and forms an orange-red coloured complex, which is measured colorimetrically at 510 nm. The color intensity is proportional to the enzyme activity.

7.2.9.5.2. Reagents

Reagent I: Buffered Substrate, pH 10.0
Reagent II: Chromogen Reagent
Reagent III: Phenol Standard, 10 mg%
Working solution: Reconstitute one vial of reagent I, buffered substrate with 2.2 ml of purified water.

7.2.9.5.3. Procedure

All the test tubes were marked properly as blank (B), standard (S), control (C), and test (T). 0.5 ml of working buffered substrate was added in clean tubes. 1.5 ml of purified water was added in all the tubes. They were mixed well and

incubated at 37^{o}C for 3 min. 0.05 ml of serum was added in test (T), 0.05 ml of reagent III (Phenol standard) was added in standard (S) and 0.05 ml of purified water was added in blank (B) tubes. All the tubes were mixed well and incubated at 37^{o} for 15 min. 1 ml of reagent II was added in all the tubes. 0.05 ml of serum was added in control (C). All the tubes were mixed well and O.D. was measured at 510 nm. Serum alkaline phosphatase activity is expressed as KA units.

7.2.9.5.4. Calculation

$$\text{Alkaline phosphatase activity (KA units)} = \frac{\text{O.D. of test - O.D. of control}}{\text{O.D. of standard - O.D. of blank}} \times 10$$

7.2.9.6. Acid phosphatase (ACP):

Acid phosphatase activity was estimated by the method of King and Jagatheesan (1959) using ACP test kit (Span Diagnostics Ltd.).

7.2.9.6.1. Principle

Acid phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 4.9. Phenol so formed reacts in alkaline medium with aminoantipyrine in the presence of oxidizing agent potassium ferricyanide and forms an orange red colored complex, which is measured colorimetrically. The color intensity is proportional to the enzyme activity.

7.2.9.6.2. Reagents

Reagent I: Buffered Substrate, pH 4.9
Reagent II: Sodium Hydroxide, 0.5 N
Reagent III: Sodium Bicarbonate, 0.5 N
Reagent IV: 4-aminoantipyrine, 0.6%
Reagent V: Potassium Ferricyanide, 2.4%
Reagent VI: Tartrate, 1 M

Reagent VII: Stock phenol Standard, 10 mg%

7.2.9.6.3. Working solutions

Solution I: Reconstitute one vial of reagent I, buffered substrate with 2 ml of purified water.

Solution II: Dissolve reagent 4 in 25 ml of purified water

Solution III: Dissolve reagent 5 in 25 ml of purified water

Working standard: Dilute stock phenol standard 0.5 to 5 with purified water

7.2.9.6.4. Procedure

All the test tubes were marked properly as blank (B), standard (S), control (C) and test (T). 0.5 ml of solution I was added in control (C) and test (T). 0.5 ml of purified water was added in control (C) and test (T). 0.6 ml of purified water was added in standard (S) and 1.1 ml of purified water was added in blank (B). All the tubes were mixed well and incubated at 37^{o} C for 3 min. 0.1 ml of serum was added in test (T), 0.5 ml of working standard was added in standard (S). All the tubes were mixed well and incubated at 37^{o} for 60 min. 0.5 ml of reagent II was added in all the tubes. 0.1 ml of serum was added in control (C). 0.5 ml of reagent III, 0.5 ml of solution II and 0.5 ml of solution III was added in all the tubes. All the tubes were mixed well and O.D. was measured at 510 nm.

7.2.9.6.5. Calculation

$$\text{Acid phosphatase activity (KA units)} = \frac{\text{O.D. of test - O.D. of control}}{\text{O.D. of standard - O.D. of blank}} \times 5$$

7.2.9.7. Ascorbic acid content in adrenal gland:

The adrenal glands collected at the end of cotton pellet granuloma test were processed to estimate the adrenal ascorbic acid content as per Roe and Kuether (1943).

7.2.9.7.1. Reagents

1. Trichloroacetic Acid (TCA) 6% w/v
2. 2,4-dinitrophenylhydrazine (DNPH) (2%) in 9 N H_2SO_4
3. Thiourea (10%)
4. H_2SO_4 (85%)
5. Activated Charcoal
6. Ascorbic Acid Standard (100 mg/dL) in D/W

7.2.9.7.2. Procedure

Adrenal glands were homogenized with 4 ml of 6% TCA by grinding in a mortar kept on ice blocks. The solutions were transferred to centrifuging tubes and approximately 10 mg (from a calibrated scoop) of acid washed norit was added and mixed thoroughly and centrifuged at 3000 rpm for 10 min.

Two ml of the supernatant was taken in labeled test tubes and 2ml of 6% TCA in blank test tube. 1 drop of thiourea was added to all the tubes followed by 0.5ml of DNPH except to blank. The tubes were transferred to a water bath maintained at $37^{O}C$ and incubated for 3 hours. The tubes were removed and placed in an ice water bath and 2.5ml of 85 % H_2SO_4 was added drop by drop while stirring in the ice-cold condition. Next 0.5 ml of DNPH was added to the blank tube. The tubes were maintained at room temperature for 30 min. After removing them from the ice bath, optical density of the colour developed was measured spectrophotometrically (Systronic) at 540 nm. Ascorbic acid content is expressed as µg/mg adrenal gland.

7.2.9.8. Histology of studied organs

7.2.9.8.1. Adrenal gland

The adrenal gland is reported to be the most common endocrine organ associated with chemically induced lesions (Ribelin 1984). It is especially important to understand the structure and function of the adrenal gland to correctly interpret the significance and mechanisms of drug induced lesions. The adrenal medulla is the central core of the adrenal gland, surrounded by the adrenal cortex. The adrenal cortex is composed of 3 distinct zones. The outer zone is the zona glomerulosa and is composed of a thin region of columnar cells arranged in an arched or arcuate pattern. This zone is also called the zona multiformis in animals because of its different patterns of arrangement of secretory cells. The zona glomerulosa produces the steroid hormone aldosterone, which is responsible for increasing sodium reabsorption and stimulating potassium excretion by the kidneys and thereby indirectly regulating extracellular fluid volume. Loss of this zone or the inability to secrete aldosterone may result in death due to retention of high levels of potassium with excess loss of sodium, chloride, and water. The zona fasciculate is the thickest zone (70% of the cortex) and is composed of columns of secretary cells separated by prominent capillaries. The cells are polyhedral and have many intracellular lipid droplets. This zone produces glucocorticoids. The zona reticularis is also composed of polyhedral cells, whose arrangement is less linear and more as round nests or clumps of cells. The zona reticularis produces glucocorticoids and in some species small amounts of sex steroids, namely, androgens, estrogens, and progestins (Rosol et al. 2001).

7.2.9.8.2. Lymph node

Pathogens can set up infections anywhere in the body. However, lymphocytes will meet the antigens in the peripheral lymphoid organs, which include lymph nodes. Lymph is derived from interstitial fluid and originates in the interstitial spaces of most of the body’s tissues. A vast system of converging lymphatic

vessels funnels lymph to the thorax where it is returned to the circulation via the thoracic duct. When foreign antigens invade the body, antigenic material, antigen presenting cells known as dendritic cells and inflammatory mediators generated by local immunological activity at the site of infection are all picked up by the lymphatic vessels and swept along in the flow of lymph. The system of lymphatic vessels has been called an “information superhighway” because lymph contains a wealth of information about local inflammatory conditions in upstream drainage fields (von Andrian and Mempel 2003).

The lymph node is surrounded by a fibrous capsule, and inside the lymph node the fibrous capsule extends to form trabeculae. The substance of the lymph node is divided into the outer cortex and the inner medulla surrounded by the former all around except for at the hilum, where the medulla comes in direct contact with the surface.

Thin reticular fibers, elastin and reticular fibers form a supporting meshwork called reticular network inside the node, within which the white blood cells (WBCs), most prominently, lymphocytes are tightly packed as follicles in the cortex. Elsewhere, there are only occasional WBCs (Kaldjian et al. 2001).

7.2.9.8.3. Spleen

The spleen is a dark red to blue-black organ located in the left cranial abdomen. It is adjacent to the greater curvature of the stomach and within the omentum. It is an elongated organ, roughly triangular in cross section. The gross appearance and size of the spleen are variable, depending on the species and the degree of distension; nonetheless, spleen weights can be important in its evaluation. The ratio of spleen weight to body weight remains fairly constant regardless of age and, in rats, is typically around 0.2% (Losco 1992). The spleen is the site of direct and indirect toxicity and a target for some carcinogens and also a site for metastasis of malignant neoplasms arising in other sites.

The functions of the spleen are centered on the systemic circulation. It is comprised of 2 functionally and morphologically distinct compartments, the red pulp and the white pulp. The red pulp is a blood filter that removes foreign material and damaged and effete erythrocytes. It is also a storage site for iron, erythrocytes, and platelets. In rodents, it is a site of hematopoiesis, particularly in fetal and neonatal animals. The spleen is also the largest secondary lymphoid organ containing about one-fourth of the body's lymphocytes and initiates immune responses to blood-borne antigens (Nolte et al. 2002; Balogh et al. 2004). This function is charged to the white pulp which surrounds the central arterioles. The white pulp is composed of three subcompartments: the periarteriolar lymphoid sheath, the follicles and the marginal zone. The spleen is surrounded by a capsule composed of dense fibrous tissue, elastic fibers, and smooth muscle.

The red pulp is a soft mass of a dark reddish-brown color, resembling grumous blood. It consists of a fine reticulum of fibers, continuous with those of the splenic trabeculae, to which are applied flat, branching cells.

7.2.9.8.4. Thymus

The thymus is the first of the lymphoid organs to be formed and grows considerably immediately after birth in response to postnatal antigen stimulation and the demand for large numbers of mature T cells. Genetic factors also influence the age of onset, rate and magnitude of thymus dependant immunological function. In rats and mice, the thymus reaches maximal size by sexual maturity and then gradually involutes (Pearse 2006).

A thin connective tissue capsule surrounds each lobe and, in most species, gives rise to septae, that partially subdivide the thymus into interconnecting lobules of variable size and orientation. The capsule is composed of an outer and inner layer of collagen and reticular fibers between which are occasional clusters of lymphocytes. This inner layer invaginates to form the septae. The bulk of the supporting framework in the thymus is composed of the network of epithelial reticular cells.

7.2.9.9. Methods for histopathological studies:

The organs were transferred to 4% formalin solution for fixation and later on processed for histopathological studies following the standard procedure described by Raghuramulu et al. (1983). The microtome sections were cut processed and stained with haematoxylin and eosin. The section thus obtained was scanned in Trinocular Carl-Zeiss microscope (Germany) under different magnifications. Changes if any in the cytoarchitecture were noticed.

7.2.9.9.1. Fixation

Fixation is the process of preserving, hardening and preventing postmortem changes of the tissues. The tissues were excised out immediately after sacrificing, cleaned of extraneous matter, cut in to pieces of such thickness that the fixative readily penetrated throughout the tissue to be fixed. Tissue was transferred to the 4% formalin solution and allowed to remain in it till they were taken up for processing.

7.2.9.9.2. Tissue processing

Tissue processing involves dehydration, clearing and infiltration of the tissue with paraffin. The usual dehydrating agent is ethyl alcohol; acetone and isopropyl alcohol can also be used. Following dehydration, the tissue was transferred to a paraffin solvent, which is miscible with the dehydrating agent as well. These are known as clearing agents such as chloroform and xylene. Tissue were thoroughly washed by placing them under running tap water and then conveyed through a series of the following solvents as per schedule for dehydration, clearing and paraffin infiltration.

Alcohol 70%	-	20 minutes
Alcohol 80%	-	20 minutes
Alcohol 90%	-	20 minutes
Alcohol 95% (2 changes)	-	20 minutes each

Isopropyl alcohol	-	20 minutes
Acetone (2 changes)	-	20 minutes each
Chloroform (3 changes)	-	20 minutes each
Melted paraffin wax (60^{O}C) (3 changes)	-	30 minutes each

Next the tissues were embedded in paraffin wax to prepare tissue blocks, which were oriented so that sections could be cut in desired plane of the tissue. Tissues were then fixed to metal object holder after trimming them to suitable size.

7.2.9.9.3. Section cutting

A smear of 5% Mayer's egg albumin was prepared and smeared onto the slide and dried. The tissue sections of 6 µm thickness were cut with the help of Spencer type rotating microtome. The tissue sections were put on slide and then section were floated in water on slide at 55-60^{O}C, water drained off and slide dried on hot plate at about 50^{O}C for 30 minutes. This section was ready for staining.

7.2.9.9.4. Staining procedure

Reagents:
1). Mayer's heamotoxyline stain
2). Eosin stain, 2% w/v in alcohol

After fixing the sections on slides, they were stained by serially placing them in the following reagents:

Xylol (2 changes)	-	3 minutes
Acetone	-	3 minutes
Alcohol 95%	-	3 minutes
Haematoxyline stain	-	20 minutes
Running water	-	20 minutes
Eosin stain	-	5 minutes

Alcohol 95% (3 changes)	-	3 minutes each
Acetone (2 changes)	-	3 minutes each
Xylol (2 changes)	-	3 minutes each

After passing through all the above reagents and stains, the slides were mounted with D.P.X. (Diphenyl Phthalate Xylene) and cover slip was placed. Care was taken to avoid air bubbles while mounting the slide. The slides were viewed under Trinocular research Carl Zeiss microscope (Germany) at various magnifications to note down the changes in the microscopic features of the tissues studied.

7.2.10. Analgesic studies

Analgesic study was determined in two different models:

7.2.10.1. **Acetic acid induced writhing in mice** (Hernandez-Perez and Rabanal 2002)

Intraperitoneal injection of diluted solution of acetic acid is a well established animal model for tonic visceral pain in rodents (Martinez et al. 1999). Swiss albino mice were used for acetic acid induced writhing test. Mice were divided in to four groups as described earlier in Chapter 7.2.4. Test drug or standard drug or distilled water was administered orally, 1 h prior to the injection of acetic acid. Writhing was induced by administrating of 3% aqueous solution of acetic acid (10ml/kg body weight) intraperitoneally. Immediately after the acetic acid injection, each animal was placed in a transparent observation cage and the number of writhes per mouse was counted for 30 min. Writhing movement is accepted as contraction of the abdominal muscles accompanied by stretching of hind limbs. The percentage inhibition was calculated using the following ratio:

[(Control mean - treated mean)/control mean] X 100

7.2.10.2. Formalin induced paw licking response in rats

The effect of methanol extract of *Pluchea arguta* Boiss upon formalin induced paw licking response was evaluated by adopting the procedure of Magali et al. (2000).

The procedure is same as that followed for formalin induced hind paw edema, which is mentioned above (7.2.8.). After the injection of formalin, the animals were kept under observation for half an hour. The amount of time spent licking the injected paw was noted, and was considered to be indicative of pain. The time taken for the onset of paw licking was initially measured. The first of the nociceptive responses normally peaked 5 min after formalin injection and the second phase 15-30 min after formalin injection, representing the neurogenic and inflammatory pain (Hunskaar and Hole 1987). Therefore the frequency of paw licking was measured in five intervals at 0-5 min., 6-10 min., 11-15 min., 16-20 min. and 21-30 min.

7.3. RESULTS AND DISCUSSION

7.3.1. Anti-inflammatory activities

From the results of screening of anti-inflammatory activity of 6 plants in carrageenan induced rat paw edema (Chapter 4), *Pluchea arguta* was selected for further pharmacological studies. Carrageenan-induced edema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1-2 h) of the carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase (3 h) is associated with neutrophil originated free radicals, such as hydrogen peroxide, superoxide and hydroxyl radicals, as well as prostaglandin release (Mazzon et al. 2001; Ogonowski et al. 1997; Vineger et al. 1969; Brito and Antonio1998)

7.3.1.1. Carrageenan induced paw edema

The results of anti-inflammatory activity of methanol extract of *Pluchea arguta* in carrageenan induced paw edema is shown in Table 7.1. PAM-200 group showed potent anti-inflammatory activity at 1 h (71.47%, P≤0.001), 2 h (45.25%, P≤0.001) and 3 h (39.06%, P≤0.05). PAM-400 group showed significant decrease in paw volume at 1 h (72.97%, P≤0.001), 2 h (68.45%, P≤0.001) and 3 h (49.08%, P≤0.05). Indomethacin group showed significant decrease in paw volume at 1 h (67.45%, P≤0.001), 2 h (66.53%, P≤0.001) and 3 h (72.61%, P≤0.01). PAM-200 showed more anti-inflammatory activity than reference drug indomethacin at 1 h, while PAM-400 group showed more anti-inflammatory activity than reference drug indomethacin at 1 h and 2 h. Thus it can be concluded that methanol extract of *Pluchea arguta* has potent anti-inflammatory activity in carrageenan induced rat paw edema in early phase (1 h) and also in later phase (3 h).

Hence, further anti-inflammatory studies were carried out on methanol extract of *Pluchea arguta* in different anti-inflammatory models.

Table 7.1 Anti-inflammatory activity of methanol extract of *Pluchea arguta* in carrageenan induced paw edema

Groups	After 1 h		After 2 h		After 3 h	
	Increase in paw volume (%)	% Change	Increase in paw volume (%)	% Change	Increase in paw volume (%)	% Change
Control	28.45 ± 2.88	-	38.98 ± 2.45	-	38.94 ± 5.33	-
PAM-200	8.12 ± 2.65**	71.47↓	19.90 ± 2.20**	45.25↓	23.73 ± 2.05*	39.06↓
PAM-400	7.69 ± 1.64***	72.97↓	12.30 ± 1.87***	68.45↓	19.83 ± 1.55*	49.08↓
Indo-2.5	9.26 ± 1.25***	67.45↓	13.05 ± 3.17***	66.53↓	10.67 ± 3.39**	72.61↓

Data is expressed as mean ± SEM; P≤ (0.05*, 0.01**, 0.001***); F ≤ 0.05; PAM- methanol extract of *Pluchea arguta,* ↓ decrease

7.3.1.2. Dextran induced paw edema

It is well established that carrageenan and dextran induce rat paw oedema by different mechanisms. Dextran is a polysaccharide of high molecular weight that induces anaphylactic reaction after injection in rat's extremities, which is characterized by extravasation and oedema formation, as a consequence of liberation of histamine and serotonin from mast cells (Rowley and Benditt 1956; Van Wauve and Goosens 1989).

The results of anti-inflammatory activity of methanol extract of *Pluchea arguta* in dextran induced paw edema is shown in Table 7.2. PAM-200 group and PAM-400 group showed significant anti-inflammatory activity in dextran induced paw edema model when compared with the control group. PAM-200 group showed significant decrease in paw volume at 1 h (31.97%, $P \leq 0.05$), 2 h (53.70%, $P \leq 0.01$) and 3 h (72.04%, $P \leq 0.01$). PAM-400 group showed significant decrease in paw volume at 1 h (32.26%, $P \leq 0.01$), 2 h (55.64%, $P \leq 0.001$) and 3 h (79.31%, $P \leq 0.01$). Indomethacin group showed significant decrease in paw volume at 1 h (49.39%, $P \leq 0.001$), 2 h (38.85%, $P \leq 0.05$) and 3 h (65.61%, $P \leq 0.01$). Methanol extract of *Pluchea arguta* showed anti-inflammatory activity in dose dependant manner. PAM-200 and PAM-400 groups showed almost similar anti-inflammatory activity at 1 h and 2 h, while at PAM-400 group showed higher anti-inflammatory activity than PAM-200 group at 3 h. PAM-200 and PAM-400 groups showed higher anti-inflammatory activity than reference drug indomethacin group at 2 h and 3 h, while it was lower at 1 h. The results tend to suggest that the anti-inflammatory activity of the methanol extract of *Pluchea arguta* is possibly backed by its anti-histamine or anti-serotonin activity.

Table 7.2 Anti-inflammatory activity of methanol extract of *Pluchea arguta* in dextran induced paw edema

Groups	After 1 h		After 2 h		After 3 h	
	Increase in paw volume (%)	% Change	Increase in paw volume (%)	% Change	Increase in paw volume (%)	% Change
Control	67.73 ± 2.87	-	51.19 ± 4.28	-	42.59 ± 5.69	-
PAM-200	46.08 ± 5.85*	31.97↓	23.70 ± 3.30**	53.70↓	11.91 ± 2.79**	72.04↓
PAM-400	45.88 ± 4.96**	32.26↓	22.71 ± 1.50***	55.64↓	8.81 ± 2.61**	79.31↓
Indo-2.5	34.28 ± 3.53***	49.39↓	31.30 ± 3.41*	38.85↓	14.65 ± 2.11**	65.61↓

Data is expressed as mean ± SEM, P≤ (0.05*, 0.01**, 0.001***), F ≤ 0.05, PAM- methanol extract of *Pluchea arguta*, ↓ decrease

7.3.1.3. Histamine induced paw edema

The histamine is a basic amine related with inflammatory and allergic process causing, among several effects, both vasodilatation and increase of vascular permeability (Cuman et al. 2001; Linardi et al. 2002). The results of anti-inflammatory activity of methanol extract of *Pluchea arguta* in histamine induced paw edema is shown in Table 7.3. Anti-inflammatory activity of PAM-200 and PAM-400 groups was statistically significant at 0.5 h, 1 h, 2 h and 3 h as compared with the control group. PAM-200 group showed significant decrease in paw volume at 0.5 h (24.39%, P≤ 0.05), 1 h (25.99%, P≤ 0.01), 2 h (36.40%, P≤ 0.05) and 3 h (39.27%, P≤ 0.05). PAM-400 group showed significant decrease in paw volume at 0.5 h (26.10%, P≤ 0.05), 1 h (26.16%, P≤ 0.01) and highly significant at 2 h (42.72%, P≤ 0.001) and 3 h (46.42%, P≤ 0.001). Reference drug indomethacin group showed highly significant decrease in paw volume at 0.5 h (51.15%, P≤ 0.001), 1 h (57.08%, P≤ 0.001), 2 h (66.45%, P≤ 0.001) and 3 h (66.34%, P≤ 0.001) as compared with the control group. Thus PAM-200 and PAM-400 groups did not show higher anti-inflammatory activity than reference drug indomethacin group.

Table 7.3 Anti-inflammatory activity of methanol extract of *Pluchea arguta* in histamine induced paw edema

Groups	After 0.5 h		After 1 h		After 2 h		After 3 h	
	Increase in paw volume	% Change	Increase in paw volume	% Change	Increase in paw volume	% Change	Increase in paw volume	% Change
Control	60.65 ± 3.33	-	58.42 ± 3.15	-	45.23 ± 2.76	-	35.98 ± 2.52	-
PAM-200	45.85 ± 4.15*	24.39↓	43.24 ± 2.48**	25.99↓	28.77 ± 3.53*	36.40↓	21.85 ± 3.30*	39.27↓
PAM-400	44.82 ± 3.36*	26.10↓	43.14 ± 2.46**	26.16↓	25.91 ± 1.64***	42.72↓	19.28 ± 1.11***	46.42↓
Indo-2.5	29.63 ± 3.42***	51.15↓	25.07 ± 2.72***	57.08↓	15.18 ± 2.80***	66.45↓	12.11 ± 2.20***	66.34↓

Data is expressed as mean ± SEM, P≤ (0.05*, 0.01**, 0.001***), F ≤ 0.05, PAM- methanol extract of *Pluchea arguta*, ↓ decrease

7.3.1.4. Formalin induced paw edema

The results of anti-inflammatory activity of methanol extract of *Pluchea arguta* in formalin induced paw edema is shown in Table 7.4. It is well known that inhibition of formalin induced paw edema in rats is one of the most suitable test procedures to screen anti-arthritic and anti-inflammatory agents as it closely resembles human arthritis (Greenwald 1991). Thus formalin-induced paw edema is a model used for the evaluation of an agent with anti-proliferative activity (Banerjee et al. 2000). Injection of formalin subcutaneously into hind paw of rats produces localized inflammation. The administration of PAM-200, PAM-400 and indomethacin daily for 7 days successfully inhibited edema induced by formalin (Table 7.4). PAM-200 group showed decrease in paw volume at 3 h (40.79%, P≤0.05), 24 h (34.98%, P≤0.05) and 48 h (35.96%). PAM-400 group showed decrease in paw volume at 3 h (42.96%, P≤0.05), 24 h (36.50%, P≤0.05) and at 48 h (39.67%). Indomethacin group showed decrease in paw volume at 3 h (30.98%, P≤0.05), 24 h (42.64%, P≤0.01) and 48 h (21.67%). PAM-200 and PAM-400 groups showed almost similar anti-inflammatory activity at 3 h, 24 h and 48 h.

PAM-200 and PAM-400 groups showed higher anti-inflammatory activity than reference drug indomethacin at 3 h and 48 h. Thus, from the results, it can be concluded, that methanol extract of *P. arguta* has higher anti-inflammatory activity in formalin induced paw edema test. PAM showed significant decrease in paw volume till 48 h with both doses, which suggests its long duration of action.

Table 7.4 Anti-inflammatory activity of methanol extract of *Pluchea arguta* in formalin induced paw edema

Groups	% Increase in Paw volume					
	After 3 h		After 24 h		After 48 h	
	Increase In paw volume	% Change	Increase In paw volume	% Change	Increase In paw volume	% Change
Control	53.03 ± 4.98	-	54.03 ± 4.97	-	38.57 ± 4.23	-
PAM-200	31.40 ± 3.76*	40.79↓	35.13 ± 2.12*	34.98↓	24.70 ± 4.31	35.96↓
PAM-400	30.25 ± 4.76*	42.96↓	34.31 ± 3.45*	36.50↓	23.27 ± 4.71	39.67↓
Indo-2.5	36.60 ± 3.73*	30.98↓	30.99 ± 2.52**	42.64↓	30.21 ± 3.89	21.67↓

Data is expressed as mean ± SEM, P≤ (0.05*, 0.01**), F ≤ 0.05, PAM- methanol extract of *Pluchea arguta*, ↓ decrease

7.3.1.5. Cotton pellet induced granuloma

Cotton pellet granuloma test is a chronic inflammation model commonly used to evaluate the anti-proliferative activities of drugs (Panthong et al. 2004; Gupta et al. 2005). Tissue granulation, one of the distinctive features of chronic inflammation, which is composed of marked infiltration macrophages and neovascularization, was induced by subcutaneous implantation of biomaterials. The implanted material induces a host's inflammatory response and modulates the release of inflammatory mediators which finally leads to tissue proliferation and granular formation (Tang and Eaton 1995; Hu et al. 2001).

The results of anti-inflammatory activity of methanol extract of *Pluchea arguta* in cotton pellet induced granuloma is shown in Table 7.5. In the present study, PAM-200 and PAM-400 groups showed dose dependent activity and markedly inhibited granuloma formation surrounding the pellets compared with the control group. PAM-200 group showed significant decrease in granuloma formation with 24.01% (P≤ 0.05), while PAM-400 group showed significant decrease in granuloma formation with 28.68% (P≤ 0.05) which was almost near to the reference drug indomethacin (33.97%, P≤ 0.01) group. Thus, the results showed that methanol extract of *Pluchea arguta* has potent anti-inflammatory activity in chronic inflammatory model.

Table 7.5 Anti-inflammatory activity of methanol extract of *Pluchea arguta* in cotton pellet induced granuloma formation

Groups	Pellet weight (g / 100g body weight)	% Change
Control	0.157 ± 0.011	-
PAM-200	0.119 ± 0.006*	24.2↓
PAM-400	0.112 ± 0.009*	28.66↓
Indo-2.5	0.104 ± 0.007**	33.76↓

Data is expressed as mean ± SEM, P≤ (0.05*, 0.01**), F ≤ 0.05, PAM- methanol extract of *Pluchea arguta*, ↓ decrease

7.3.1.5.1. Organ weight

7.3.1.5.1.1. Adrenal

The results of organ weight for the adrenal gland in cotton pellet induced granuloma rats are shown in Table 7.8. Increase in the absolute weight of the adrenal for PAM-200, PAM-400 and indomethacin groups was 3.7%, 7.41% and 29.63% respectively as compared with the control group. Increase in the relative weight of the adrenal for PAM-200, PAM-400 and indomethacin groups was 3.15%, 14.94% and 40.42% respectively as compared with the

control group. Indomethacin group showed higher increase in absolute and relative weight of the adrenal than PAM-200 and PAM-400 groups.

Table 7.6 Effect of methanol extract of *Pluchea arguta* on weight of adrenal in cotton pellet induced granuloma rats

Groups	Absolute weight	% Change	Relative organ weight	% Change
Control (D/W)	0.027 ± 0.002	-	0.01 ± 0.001	-
PAM-200	0.028 ± 0.002	3.7↑	0.011 ± 0.001	3.15↑
PAM-400	0.029 ± 0.002	7.41↑	0.012 ± 0.001	14.94↑
Indo-2.5	0.035 ± 0.003	29.63↑	0.015 ± 0.002	40.42↑

Data is expressed as mean ± SEM, PAM- methanol extract of *Pluchea arguta*, ↑ Increase

7.3.1.5.1.2. Lymph node

The results of organ weight for the lymph node in cotton pellet induced granuloma rats are shown in Table 7.7. The absolute weight of the lymph node for PAM-200 group was 33.33% less as compared with the control group, while it was similar for PAM-400 group and reference drug indomethacin group. The relative weight of the lymph node for PAM-200 group was 14.57% less and for PAM-400 it was 28.16% more as compared with the control group.

Table 7.7 Effect of methanol extract of *Pluchea arguta* on weight of lymph node in cotton pellet induced granuloma rats

Groups	Absolute weight	% Change	Relative organ weight	% Change
Control	0.03 ± 0.005	-	0.011 ± 0.002	-
PAM-200	0.02 ± 0.004	33.33↓	0.009 ± 0.002	14.57↓
PAM-400	0.03 ± 0.004	-	0.014 ± 0.002	28.16↑
Indo-2.5	0.03 ± 0.003	-	0.011 ± 0.002	-

Data is expressed as mean ± SEM, PAM- methanol extract of *Pluchea arguta*, ↑ Increase, ↓ decrease

7.3.1.5.1.3. Spleen

The results of organ weight for the spleen in cotton pellet induced granuloma rats are shown in Table 7.6. The absolute weight of the spleen for PAM-200 group was 1.61% more as compared with the control group, while it was similar for PAM-400 group. The absolute weight of the spleen for standard drug indomethacin group was 29.03% more as compared with the control group. The relative weight of the spleen for PAM-200 group was similar to the control group, while it was 5.16% more in PAM-400 group and much more with 36.8% (P≤ 0.05) in indomethacin group as compared with the control group.

Table 7.8 Effect of methanol extract of *Pluchea arguta* on weight of spleen in cotton pellet induced granuloma rats

Groups	Absolute weight	% Change	Relative weight	% Change
Control	0.62 ± 0.02	-	0.24 ± 0.01	-
PAM-200	0.63 ± 0.04	1.61↑	0.24 ± 0.01	-
PAM-400	0.62 ± 0.03	-	0.25 ± 0.01	5.16↑
Indo-2.5	0.80 ± 0.08	29.03↑	0.32 ± 0.03*	36.80↑

Data is expressed as mean ± SEM, P≤ (0.05*), PAM- methanol extract of *Pluchea arguta*, ↑ Increase

7.3.1.5.1.4. Thymus

The results of organ weight for the thymus in cotton pellet induced granuloma rats are shown in Table 7.9. The absolute weight of the thymus for PAM-200 group showed 8.51% increase, PAM-400 group showed 11.35% decrease and indomethacin group showed 4.61% decrease as compared with the control group. The relative weight of the thymus in PAM-200 group showed 8.85% increase, PAM-400 group showed 7.78% decrease and indomethacin group showed 2.59% increase as compared with the control group. PAM-400 group

showed more decrease in absolute and relative weight of the thymus than in other two groups, while PAM-200 showed more increase in absolute and relative weight of the thymus than in other two groups.

Table 7.9 Effect of methanol extract of *Pluchea arguta* on weight of thymus in cotton pellet induced granuloma rats

Groups	Absolute weight	% Change	Relative organ weight	% Change
Control (D/W)	0.564 ± 0.044	-	0.21 ± 0.01	-
PAM-200	0.612 ± 0.026	8.51↑	0.23 ± 0.01	8.85↑
PAM-400	0.500 ± 0.052	11.35↓	0.20 ± 0.01	7.78↓
Indo-2.5	0.538 ± 0.029	4.61↓	0.22 ± 0.01	2.59↑

Data is expressed as mean ± SEM, PAM- methanol extract of *Pluchea arguta*, ↑ Increase, ↓ decrease

7.3.1.5.2. Biochemical parameters

7.3.1.5.2.1. Total protein and albumin levels

The results of the total protein level in serum in cotton pellet induced granuloma rats are shown in Table 7.10. PAM-200 and PAM-400 groups showed increase in protein levels by 4.32% and 5.76% respectively, while indomethacin group showed decrease in protein level by 3.68% as compared with the control group. In present study, increase in protein level was dose dependent.

The results of the albumin level in serum in cotton pellet induced granuloma rats are shown in Table 7.10. PAM-200 and PAM-400 groups showed increase in albumin level by 4.03% and 18.65% respectively, while indomethacin group showed an increase of 2.87% as compared with the control group. In present study, increase in albumin level was statistically significant in PAM-400 group ($P \leq 0.01$).

Table 7.10 Effect of methanol extract of *Pluchea arguta* on protein and albumin level in cotton pellet induced granuloma rats

Groups	Total protein	% Change	Albumin	% Change
Control	6.25 ± 0.09	-	3.11 ± 0.09	-
PAM-200	6.52 ± 0.07*	4.32↑	3.23 ± 0.05	4.03↑
PAM-400	6.61 ± 0.06*	5.76↑	3.69 ± 0.04***	18.65↑
Indo-2.5	6.02 ± 0.19	3.68↓	3.2 ± 0.16	2.87↑

Data is expressed as mean ± SEM, P≤ (0.05*, 0.001***), PAM- methanol extract of *Pluchea arguta*, ↑ Increase, ↓ decrease

7.3.1.5.2.2. Serum aspartate transaminase (AST) and serum alanine transaminase (ALT) activities

AST and ALT are considered as markers for liver function (Hilaly et al. 2004). The results of the serum AST and ALT activity in cotton pellet induced granuloma rats are shown in Table 7.11. PAM-200 group showed a decrease in AST level by 11.44%, PAM-400 group showed significant increase in AST level by 54.71% (P≤ 0.05) and indomethacin group showed an increase of 90.41% which was highly significant (P≤ 0.01) as compared with the control group. All the three groups showed significant increase in ALT level as compared with the control group. PAM-200 group showed 10.56% (P≤ 0.05) increase in ALT level, PAM-400 group showed 15.85% (P≤ 0.05) increase in ALT level and indomethacin group showed 15.53% (P≤ 0.01) increase in ALT level. Thus, in the present study, there was significant increase in AST and ALT levels in PAM-400 and indomethacin group. However, the ALT values were still within the normal range in all the groups. ALT is located primarily in cytosol and hepatocytes, and this enzyme is considered a more sensitive marker of hepatocellular damage than AST. AST is an enzyme found in the cytoplasm and mitochondria in different tissues, chiefly in the heart and skeletal muscles, liver, kidneys, pancreas and erythrocytes (Chaves and Silva 1998; Aniagu et al. 2004). The increase in AST level in the treated groups may be due to the release of enzymes from the cells of the damaged organ, or to a change in the membrane permeability of the cells (Obici et al. 2008).

Table 7.11 Effect of methanol extract of *Pluchea arguta* on serum aspartate transaminase (AST) and serum alanine transaminase (ALT) activity in cotton pellet induced granuloma rats

Groups	AST	% Change	ALT	% Change
Control	80.88 ± 2.91	-	40.25 ± 073	-
PAM-200	71.63 ± 3.18	11.44↓	44.5 ± 1.32*	10.56↑
PAM-400	125.13 ± 9.82*	54.71↑	46.63 ± 2.4*	15.85↑
Indo-2.5	154 ± 12.32**	90.41↑	46.5 ± 2.54*	15.53↑

Data is expressed as mean ± SEM, P≤ (0.05*, 0.01**), PAM- methanol extract of *Pluchea arguta*, ↑ Increase, ↓ decrease

7.3.1.5.2.3. Alkaline phosphatase (ALP) and acid phosphatase (ACP) activities

The results of alkaline phosphatase and acid phosphatase activity in cotton pellet induced granuloma rats are shown in Table 7.12. PAM-200 and PAM-400 groups showed 15.92% and 11.9% decrease in alkaline phosphatase level respectively as compared with the control group. Indomethacin group showed 30.71% increase in alkaline phosphatase level as compared with the control group. PAM-200 and PAM-400 groups showed 13.15% and 7.78% decrease in acid phosphatase level respectively as compared with the control group. Indomethacin group showed 1.49% increase in acid phosphatase level as compared with the control group. Significant difference was not seen by any group.

Table 7.12 Effect of methanol extract of *Pluchea arguta* on alkaline phosphatase and acid phosphatase activity in cotton pellet induced granuloma rats

Groups	ALP	% Change	ACP	% Change
Control	23.87 ± 4.44	-	14.14 ± 1.57	-
PAM-200	20.07 ± 2.99	15.92↓	12.28 ± 1.55	13.15↓
PAM-400	21.03 ± 2.98	11.90↓	13.04 ± 1.05	7.78↓
Indo-2.5	31.20 ± 5.45	30.71↑	14.35 ± 1.12	1.49↑

Data is expressed as mean ± SEM, PAM- methanol extract of *Pluchea arguta*, ↑ Increase, ↓ decrease

7.3.1.5.2.4. Ascorbic acid content in adrenal gland

The results of ascorbic acid content in adrenal gland in cotton pellet induced granuloma rats are shown in Table 7.13. The ascorbic acid content increased in PAM-400 group (13.38%), while it decreased in PAM-200 group (5.72%) and indomethacin group (1.34%).

Table 7.13 Effect of methanol extract of *Pluchea arguta* on ascorbic acid content in adrenal gland in cotton pellet induced granuloma rats

Groups	Ascorbic acid (µg/mg adrenal)	% Change
Control	8.22 ± 1.08	-
PAM-200	7.75 ± 1.27	5.72↓
PAM-400	9.32 ± 0.78	13.38↑
indo-2.5	8.11 ± 0.33	1.34↓

Data is expressed as mean ± SEM, PAM- methanol extract of *Pluchea arguta*, ↑ Increase, ↓ decrease

7.3.1.5.3. Histopathological studies

The histopathological studies of four tissues (Adrenal, lymph node, spleen and thymus) in cotton pellet induced granuloma rats are shown in Plate 1 and

Plate 2. All the four groups did not produce any changes on cytoarchitecture of adrenal, lymph node, spleen and thymus.

7.3.2. Analgesic activities

7.3.2.1. Acetic acid writhing test

Methanol extract of *Pluchea arguta* was used to distinguish between the central and peripheral analgesic action by acetic acid induced writhing response in mice. This method is not only simple and reliable but also affords rapid evaluation of peripheral type of analgesic action (Gene et al. 1998). In this test the animals react with characteristic stretching behavior, which is called writhing (Ganesh et al. 2008). The writhing response of the mouse to an intraperitoneal injection of noxious chemical is used to screen for both peripherally and centrally acting analgesic activities (Trongsakul et al. 2003).

Intraperitoneal injection of diluted solutions of acetic acid is a well established animal model for tonic visceral pain in rodents. Intraperitoneal injection of acetic acid induces a stereotypic response pattern in the form of abdominal contractions (lengthwise stretches of the torso with a concomitant concave arching of the back) that may persist beyond 6 h after the administration, although most contractions occur within 30 min of the application of the irritant (Koster et al. 1959; Mogil et al. 1996).

The results of analgesic activity of methanol extract of *Pluchea arguta* in acetic acid induced writhing test is shown in Table 7.14. PAM-200 and PAM-400 groups showed 31% and 35% inhibition significantly in abdominal writhes produced by acetic acid respectively as compared with the control group. Thus, PAM-200 and PAM-400 groups produced a significant ($P \leq 0.05$) and dose dependant inhibition of analgesic effect produced by acetic acid. Indomethacin group showed significant inhibition with 46% ($P \leq 0.01$) as compared with the control group. This probably means that the extract is able to reduce the receptor sensitivity to the acetic acid induced pain in a dose dependent manner. The abdominal constriction is related to the sensitization

of nociceptive receptors to prostaglandins. This therefore shows anti-nociceptive activity (Nwinyi et al. 2006).

Table 7.14 Analgesic activity of methanol extract of *Pluchea arguta* in acetic acid writhing test in Swiss albino mice

Groups	Onset time (Sec.)	% Change	Frequency	% Change
Control (D/W)	29 ± 5	-	84 ± 7	-
PAM-200	226 ± 49**	675↑	58 ± 5*	31↓
PAM-400	246 ± 52**	743↑	54 ± 5*	35↓
Indo-2.5	495 ± 51***	1594↑	45 ± 8**	46↓

Data is expressed as mean ± SEM, P≤ (0.05*, 0.01**, 0.001***), PAM- methanol extract of *Pluchea arguta*, ↑ Increase, ↓ decrease

7.3.2.2. Formalin induced paw licking test

The formalin test is a valid and reliable model of nociception, and it is sensitive to various classes of analgesic drugs (Karabay-Yavasoglu et al. 2007). The formalin test may be more useful as model of pain in which the first phase seems to be due to direct chemical activation on nociceptive afferent fibers, whereas the second phase is dependent of peripheral inflammation and changes in central processing (Tjolsen et al. 1992; Sayyah et al. 2004).

The results of analgesic activity of methanol extract of *Pluchea arguta* in formalin induced paw licking are shown in Table 7.15. The onset time of paw licking was measured after formalin injection. PAM-200 and PAM-400 showed 7.29% and 21.53% increase in onset time respectively, while indomethacin group showed 25% increase in onset time as compared with the control group. Onset time of paw licking response of PAM-400 group was almost the same as that of the indomethacin group. PAM-400 and indomethacin groups showed statistically significant increase in paw licking response (P≤ 0.05).

After formalin injection, the frequency of paw licking was measured between 0-5 min, 6-10 min, 11-15 min, 16-20 min and 21-30 min. As compared with the control group, PAM- 200 group showed 14.04% decrease in frequency between 0-5 min, 38.34% between 6-10 min, 62.24% between 11-15 min, 2.74% between 16-20 min and 13.04% between 21-30 min. PAM-400 group showed 38% decrease in frequency between 0-5 min, 80.83% between 6-10 min, 54.13% between 11-15 min, 14.24% between 16-20 min and 13.93% between 21-30 min. Indomethacin group showed 28.01% decrease in frequency between 0-5 min, 72.98% between 6-10 min, 48.62% between 11-15 min, 5.66% between 16-20 min and 11.32% between 21-30 min. Statistically significant decrease in frequency between 0-5 min was seen in PAM-400 group ($P \leq 0.05$) and indomethacin group ($P \leq 0.05$). Statistically significant decrease in frequency between 6-10 min was seen in PAM-400 group ($P \leq 0.01$) and indomethacin group ($P \leq 0.05$). Higher level of frequency in paw licking was observed between 0-5 min and 21-30 min.

Table 7.15 Analgesic activity of methanol extract of *Pluchea arguta* in formalin induced paw licking test in rats

Groups	Onset time (Sec)	% Change	0-5min		6-10min		11-15min		16-20min		21-30min	
			Frequency	% Change	Frequency	% Change	Frequency	% Change	Frequency	% Change	Frequency	% Change
Control	48 ± 3.13	-	16.67 ± 1.05	-	4.33 ± 0.56	-	6.17 ± 1.58	-	5.83 ± 0.91	-	19.17 ± 2.52	-
PAM-200	51.50 ± 4.19	7.29↑	14.33 ± 1.96	14.04↓	2.67 ± 0.61	38.34↓	2.33 ± 0.95	62.24↓	5.67 ± 1.43	2.74↓	16.67 ± 2.03	13.04↓
PAM-400	58.33 ± 1.56*	21.53↑	10.33 ± 2.23*	38↓	0.83 ± 0.40**	80.83↓	2.83 ± 1.14	54.13↓	5 ± 0.68	14.24↓	16.50 ± 1.09	13.93↓
Indo-2.5	60 ± 2.14*	25↑	12 ± 1.34*	28.01↓	1.17 ± 0.98*	72.98↓	3.17 ± 0.91	48.62↓	5.50 ± 1.45	5.66↓	17 ± 1.86	11.32↓

Data is expressed as mean ± SEM, P≤ (0.05*, 0.01**), PAM- methanol extract of *Pluchea arguta*, ↑ Increase, ↓ decrease

7.4. CONCLUSION

The action of methanol extract of *Pluchea arguta* observed in the first phase of carrageenan induced inflammation may be due to the inhibition of early mediators such as histamine and serotonin. The action in the second phase may be due to the inhibition of bradykinin and prostaglandins. The methanol extract of *Pluchea arguta* was active on dextran-induced rat paw edema; it seems that its activity is related to histamine and serotonine mediators from the mast cells (Nishida et al. 1979; Nishida and Tomizawa 1980). Histamine, one of the important inflammation mediators, is a potent vasodilator substance and increases the vascular permeability (Cuman et al. 2001; Linardi et al. 2002). This study showed that all doses of PAM significantly suppressed the edema produced by histamine at 3 h, so it may be suggested that its anti-inflammatory activity is possibly backed by its antihistaminic activity. From the results, it showed that antiedematogenic effects of the PAM on carrageenan, dextran and histamine induced oedema may be related to inhibition of inflammation mediator formation.

It is well known that inhibition of formalin induced edema in rats is one of the most suitable test procedures to screen anti-arthritic and anti-inflammatory agents as it closely resembles human arthritis (Greenwald 1991). Thus formalin-induced arthritis is a model used for the evaluation of an agent with anti-inflammatory activity (Banerjee et al. 2000). Injection of formalin subcutaneously into hind paw of rats produces localized inflammation (Vasudevan et al. 2006). PME and indomethacin administered continuously for 7 days successfully inhibited edema induced by formalin.

As a model of chronic inflammation, cotton pellet induced granuloma in rats was utilized in the present study. During the repair process of inflammation, there is proliferation of macrophages, neutrophils, fibroblasts and multiplication of small blood vessels, which are the basic sources of forming a highly vascularised reddish mass, termed granulation tissue (Swingle 1974; Bhattacharya et al. 1992; Gepdiremen et al. 2004). The results of the present

study showed that PAM reduced the formation of granuloma tissue in a dose-dependent manner, which represented an ability of PAM to inhibit the proliferation phase of inflammatory process.

Acetic acid induced writhing test was non selective anti nociceptive model, since indirectly it induced the release of endogenous mediators stimulated the nociceptive neurons that were sensitive to non steroidal anti-inflammatory drugs (NSAIDs) (Sanchez-Mateo et al. 2006). The results of the present study indicated that methanol extract of *Pluchea arguta* at both the doses could reduce the number of writhing, thus showed powerful anti nociceptive effects.

The most predictive of the models of acute pain is undoubtedly the formalin test (Dubuisson and Dennis 1977). In the formalin test, pretreatment of rats with the PAM at the doses of 200 and 400 mg/kg showed decrease in the paw licking at the first phase of the test (0–5 min) and the second phase (15– 30 min). The ability of PAM to have an effect on both phases shows that the PAM contains active analgesic principle acting both centrally and peripherally.

In conclusion, the results of the present study demonstrated that methanol extract of *Pluchea arguta* produced dose related acute anti-inflammatory activity (Carrageenan, dextran, histamine and formalin), chronic anti-inflammatory activity (Cotton pellet) and analgesic activity (Acetic acid and formalin).

These studies have shown that the methanol extract of *Pluchea arguta* contains some active ingredients with the potential of being good anti-inflammatory and analgesic agents. There is a need for detailed investigation of the mechanism of action of methanol extract of *Pluchea arguta* based on which a possible therapy can be visualized.

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Summary

SUMMARY

An estimate of the World Health Organization (WHO) states that around 80% of the world's population consumes traditional herbal medicines. Use of herbal remedies is on the rise in developing and developed countries. Many plant extracts have been used as a source of medicinal agents to cure urinary tract infections, vaginitis, gastrointestinal disorders, respiratory diseases, cutaneous affections, helmintic infections, parasitic protozoan diseases, inflammatory processes, liver diseases etc. Herbal products have served as a major source of drugs for centuries, and about half of the pharmaceuticals in use today are derived from herbal products. The efficacy and safety of herbal medicines have turned the major pharmaceutical population towards medicinal plants research.

With the availability of primary information, further studies can be carried out like phytopharmacology of different extracts, standardization of the extracts, identification and isolation of active principles and pharmacological studies of isolated compounds. This may be followed by development of lead molecules as well as it may serve for the purpose of use of specific extracts in specific herbal formulation.

In recent years, pathogenic microorganisms have developed resistance to antibiotics in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This situation and the undesirable side effects of certain antibiotics, and the emergence of previously uncommon infections, has forced scientists to look for new antimicrobial substances from various sources, such as medicinal plants.

In the present work, 53 plants were screened for their antimicrobial activity against 15 standard microbial strains (5 Gram positive bacteria, 7 Gram negative bacteria and 3 fungal strains). 53 plants were defatted with petroleum ether and then extracted in methanol and acetone by cold percolation method.

Antimicrobial screening of acetone and methanol extracts of 53 plants was performed by agar disc diffusion method at a concentration of 600 µg/disc. Out of 53 plant extracts, methanol extracts showed 34.72% antibacterial activity against Gram positive bacteria, 19.95% antibacterial activity against Gram negative bacteria and 25.79% against fungal strains studied. Acetone extracts showed 24.53% antibacterial activity against Gram positive bacteria, 19.41% against Gram negative bacteria and 10.69% against fungal strains. Methanol extracts showed more antibacterial activity than acetone extracts. Methanol extracts of *Aristolochia indica* (AIM)*, Argemone mexicana* (AMM)*, Mangifera indica* (MIM) and *Woodfordia fruticosa* (WFM) showed higher antimicrobial activity than other extracts. Therefore, these four plant extracts were selected for further antimicrobial activity against 74 clinically isolated microorganisms and 24 standard microorganisms at two different concentrations (600 and 1200 µg/disc). Methanol extract of *Mangifera indica* showed 100% antibacterial activity against 41 clinically isolated bacterial strains at both the concentrations followed by methanol extract of *Aristolochia indica*. Methanol extracts *of Mangifera indica, Woodfordia fruticosa* and *Aristolochia indica* showed more antibacterial activity against 20 standard bacterial strains. The four extracts did not show any antifungal activity. Eleven antibiotics were studied for antibiotic susceptibility test against 41 clinically isolated bacterial strains and 20 standard bacterial strains. Four antibiotics were studied against 33 clinically isolated *Candida* species and 4 standard fungal strains. Imipenem was the most effective antibiotic against bacterial strains and Nystatin was the most effective antibiotic against fungal strains. From the results it can be concluded that Gram-positive microorganisms were more sensitive to the plant extracts than the Gram-negative microorganisms. This *in vitro* study demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms.

A growing body of evidence indicates that secondary plant metabolites play critical roles in human health and may be nutritionally important. Phytochemical screening of plants has revealed the presence of numerous chemicals including

alkaloids, tannins, flavonoids, steroids, glycosides, saponins etc. The results of preliminary qualitative phytochemical study of the crude powder of 53 plants showed presence of alkaloids, tannins, cardiac glycosides, steroids, flavonoids and saponins. Tannins were present in more number of plants followed by cardiac glycosides and steroids. The results of total phenolic and flavonoid content showed that the methanol extract of *Mangifera indica* showed highest total phenolic content, while acetone extract of *Aristolochia bracteolate* showed highest flavonoid content than other plant extracts.

Inflammatory diseases are very common throughout the world and non-steroidal anti-inflammatory drugs are the most prescribed drugs for the treatment of inflammatory diseases. The greatest disadvantage in presently available potent synthetic drugs is their toxicity and reappearance of symptoms after discontinuation. Therefore, screening and development of drugs for anti-inflammatory activity is still in progress and there is much hope for finding anti-inflammatory drugs from indigenous medicinal plants. Carrageenan induced rat paw edema is a suitable *in vivo* model to predict the value of anti-inflammatory agents, which act by inhibiting the mediators of acute inflammation. Methanol extracts of *Aristolochia indica, Argemone mexicana, Alpinia speciosa, Causarina equissetifolia, Gymnema sylvestre* and *Pluchea arguta* were screened for acute anti-inflammatory study by carrageenan induced rat paw edema model. Out of six plant extracts, the methanol extract of *Pluchea arguta* showed a potent anti-inflammatory activity followed by *Causarina equissetifolia* and *Alpinia speciosa.* Thus, on the bases of availability of plant material and literature search, *Pluchea arguta* (Leaf + stem) was selected for further pharmacognostic, toxicological and pharmacological studies.

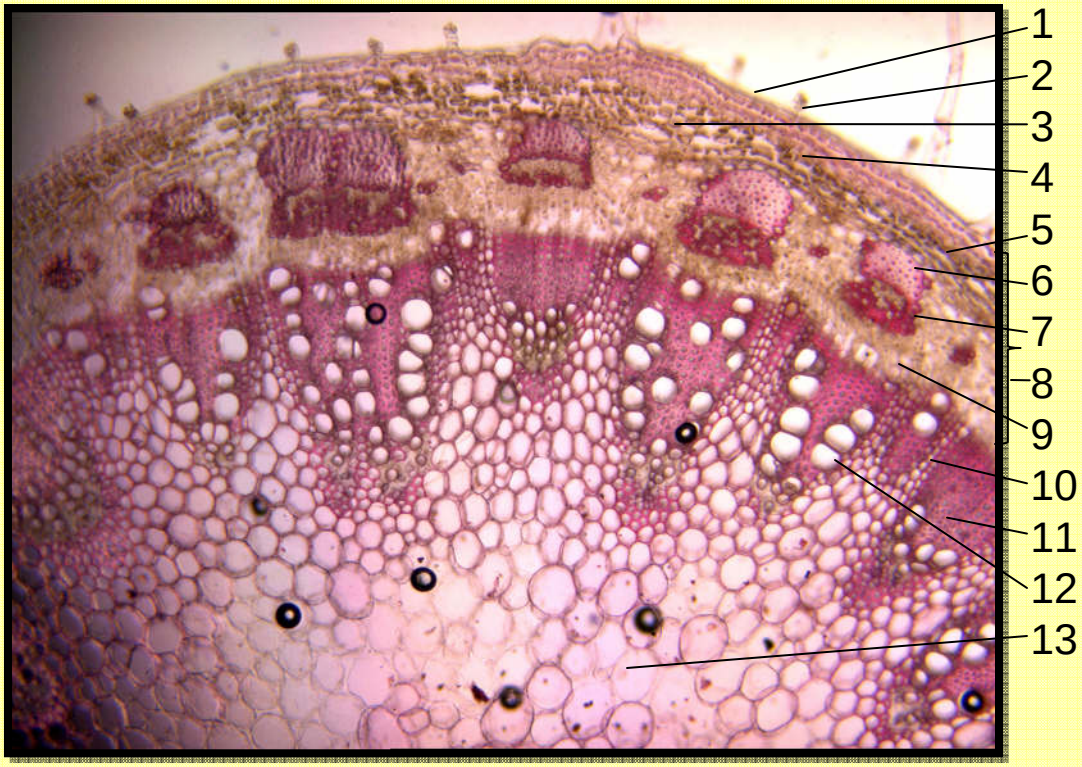
Pluchea arguta Boiss is a member of family Asteraceae and about 1000 genera of this family are well known in the world. Different species of *Pluchea* are used in Ayurvedic system of medicine in various diseases. Various pharmacognostic standards like botanical description, microscopy, ash values, extractive values,

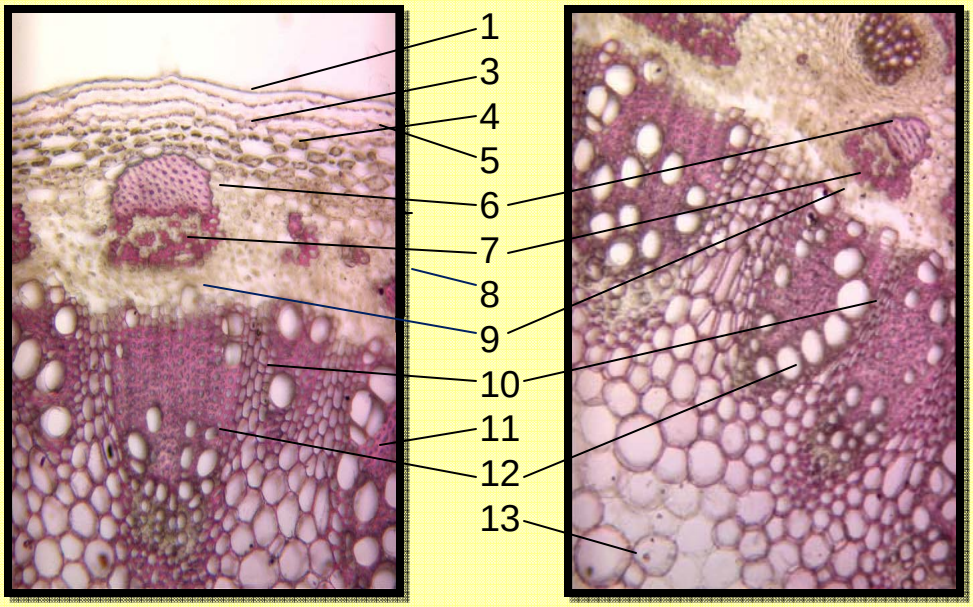
microscopical characteristics of powder, heavy metals, pH, solubility, HPTLC and preliminary phytochemical analysis of *Pluchea arguta* (Leaf + stem) was studied for proper identification of the plant. Standardization procedures and pharmacognostical studies of medicinal plants would reduce drastically much of the accidents in wrong prescriptions of traditional herbal medicines.

Toxicity testing in animals is carried out on new drugs to identify potential hazards before administrating them to humans. Acute toxicity study was undertaken to contribute data on the safety of the methanol extract of *Pluchea arguta.* Methanol extract *Pluchea arguta* extract was found to be nontoxic when oral acute toxicity study in mice was performed at single dose level.

Inflammation is a pathophysiological response of mammalian tissues to a variety of hostile agents including infectious organisms, toxic chemical substances, physical injury or tumor growth leading to local accumulation of plasmic fluid and blood cells. Acute and chronic inflammatory diseases are still one of the most important health problems in the world. Methanol extract of *Pluchea arguta* was studied for anti-inflammatory and analgesic activity by using different models. Methanol extract of *Pluchea arguta* produced dose related acute anti-inflammatory activity (Carrageenan, dextran, histamine and formalin), chronic anti-inflammatory activity (Cotton pellet) and analgesic activity (Acetic acid and formalin). Over all it can be concluded that *Mangifera indica* possessed good antibacterial property and *Pluchea arguta* possessed good anti-inflammatory and analgesic property. Both these plants warrant further investigation to elucidate their mechanism of action.

Fig. 5.1 Macroscopic characteristics of *Pluchea arguta* Boiss.

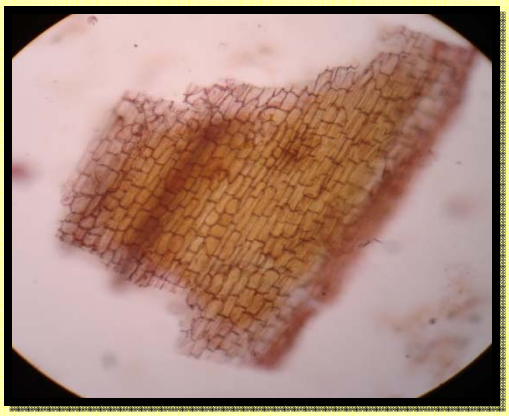




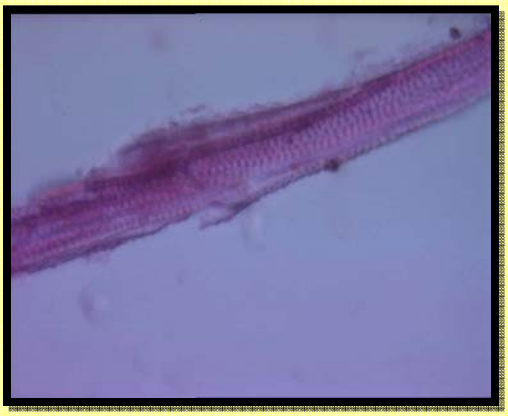
1- Epidermis; 2- Glandular trichome; 3- Endodermis; 4- Collenchyma; 5- Cork layer; 6- Pericycle; 7- Group of sclaride; 8- Cortex; 9- Phloem; 10- Medullary rays; 11- Scleranchymatous cell; 12- Xylem 13- Pith

Fig. 5.2 Microscopic characteristic s of *Pluchea arguta* Boiss. stem

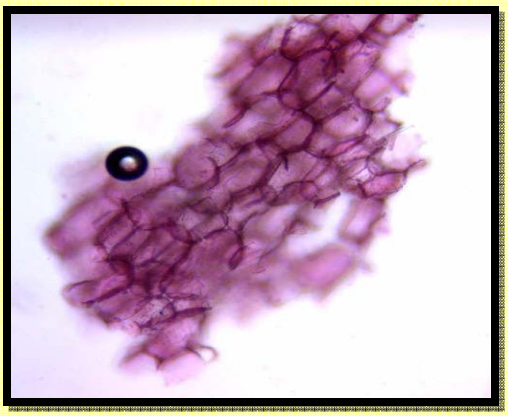
Starch grains



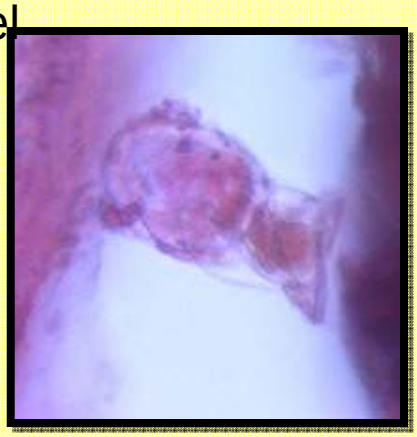
Cork cells



Annular spiral vessel

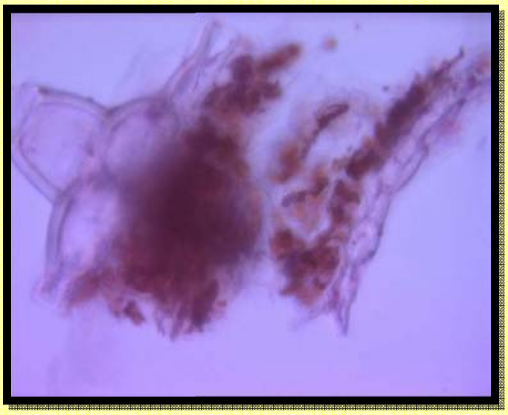


Sclerenchymatous cells

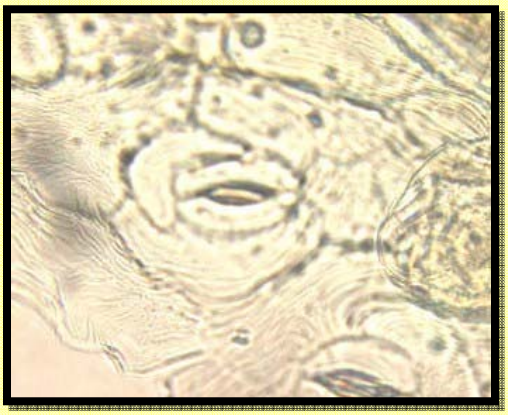


Glandular trichome

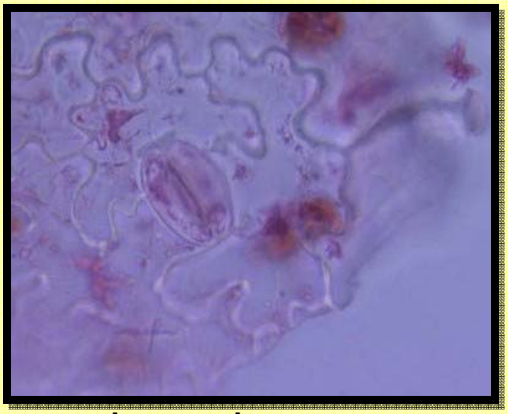
Fig. 5.4 Powdered characteristics of *Pluchea arguta* Boiss. stem



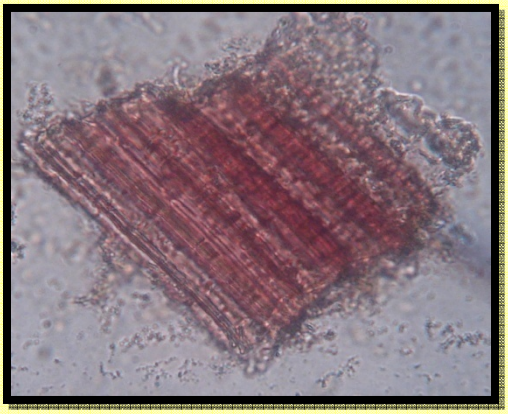
Mesophyll cell



Stomata



Anisocytic stomata with wavy surface

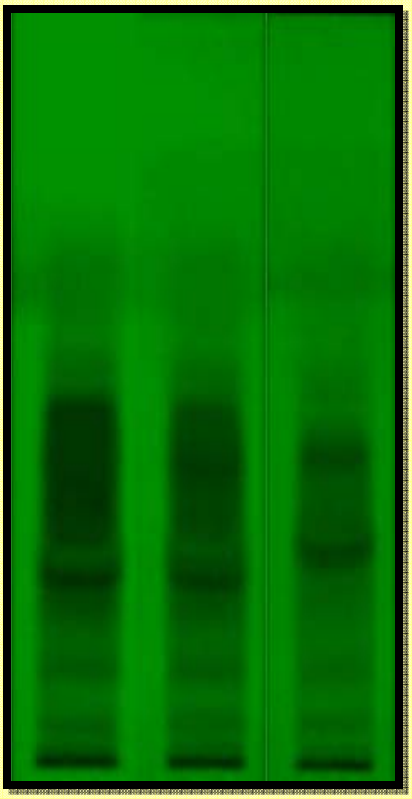


Phloem fiber

Multicellular trichome

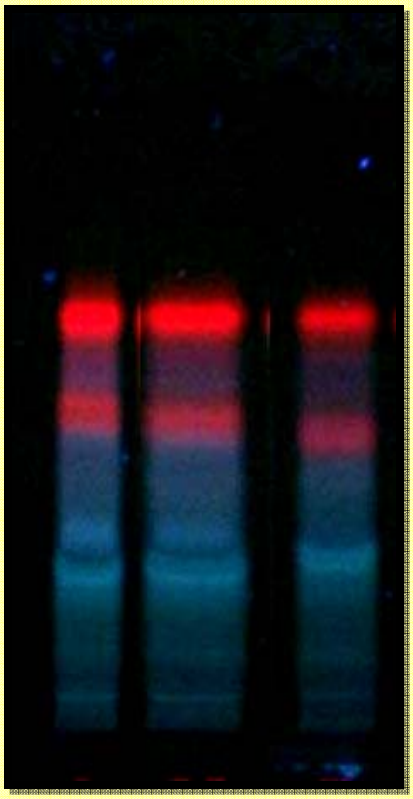
Spiral / annular xylem vessel

Fig. 5.5 Powdered characteristics of *Pluchea arguta* Boiss. leaf



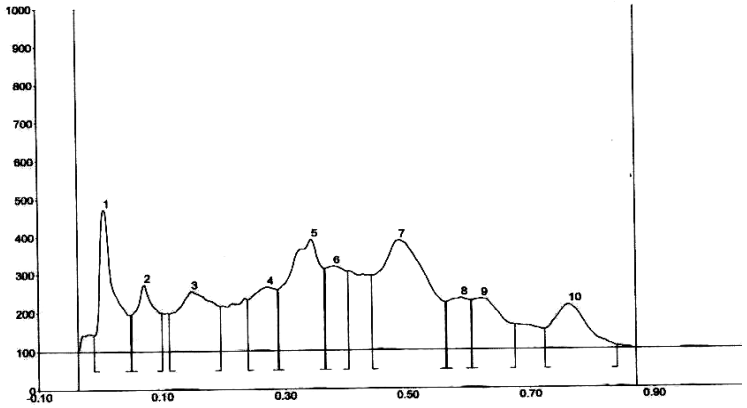
20 µl 15µl
10µl **A**

20 µl 15µl
10µl **B**

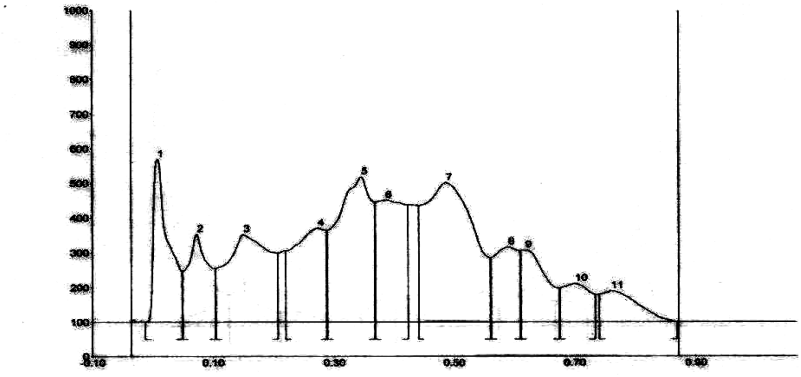


20 µl 15µl
10µl **C**

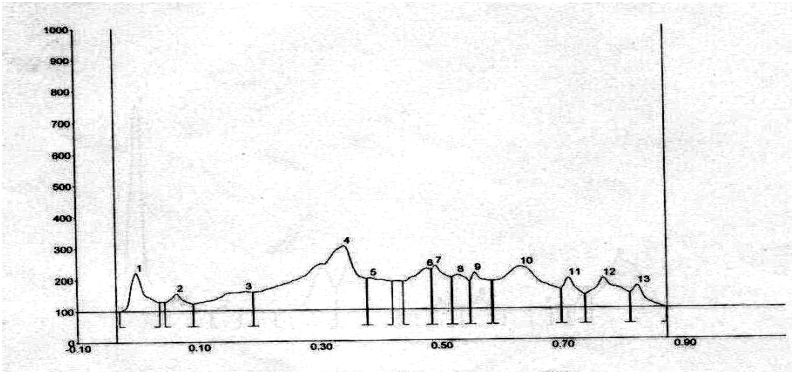
Fig. 5.7 HPTLC fingerprinting of methanol extract of *P. arguta* at (A) 254 nm (B) Visible and (C) 366 nm



A

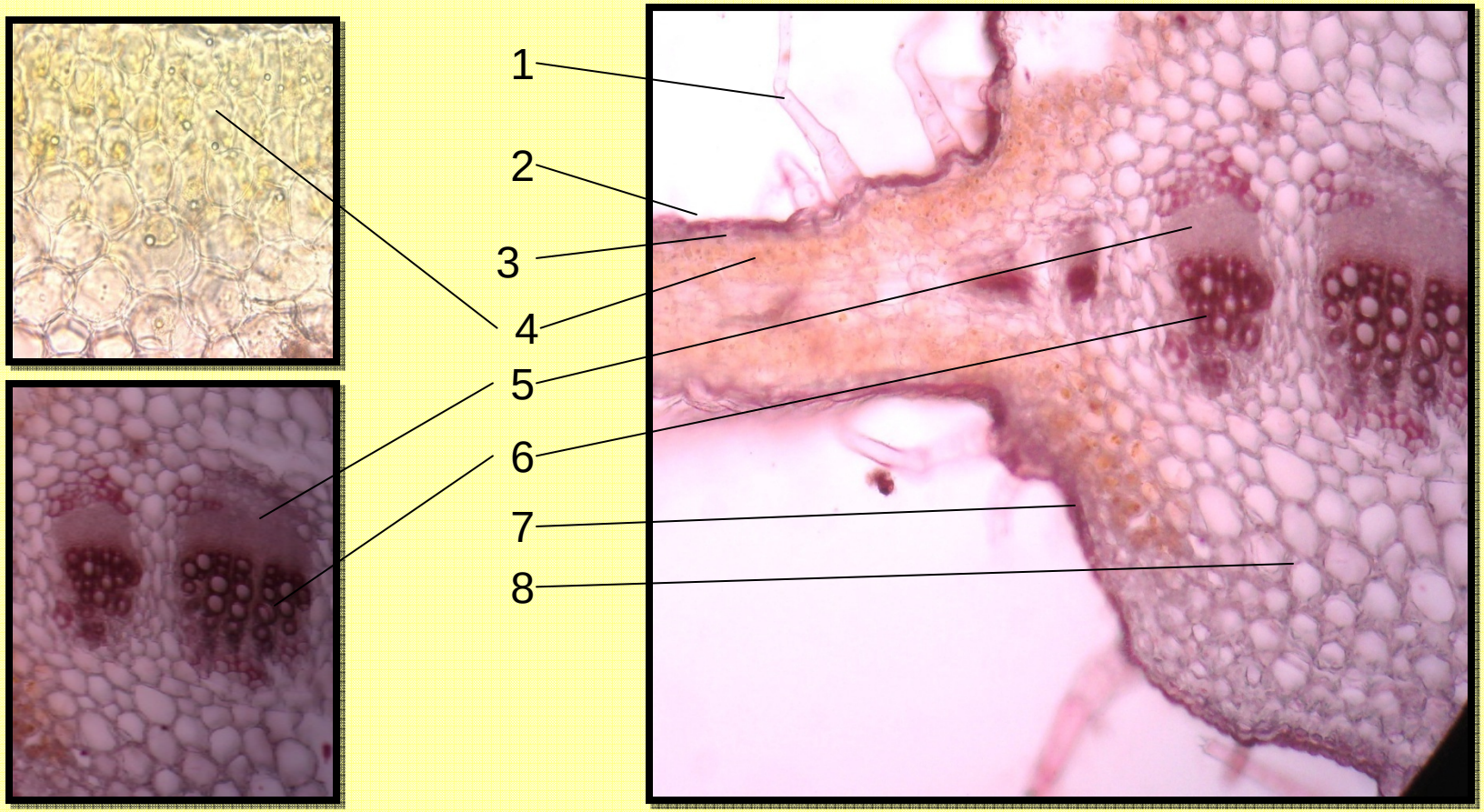


B



C

Fig. 5.6 HPTLC spectra of methanol extract of *P. arguta* at (A) 230 nm (B) 290 nm and (C) 580 nm



1- Multicellular trichome; 2- Cuticle; 3- Upper epidermis; 4- Palisade layer with oil glands; 5- Phloem; 6- Xylem; 7- Lower epidermis; 8- Parenchymatous cells

Fig. 5.3 Microscopic characteristics of *Pluchea arguta* leaf

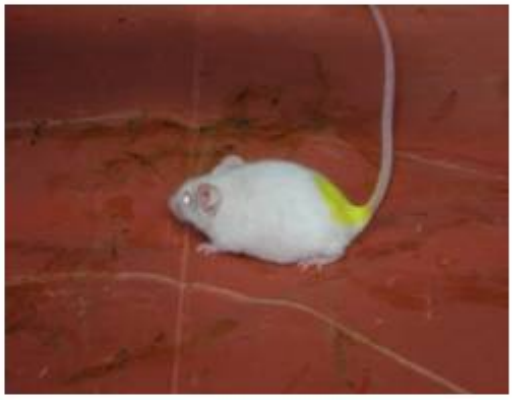
Hypoactivity

Passivity

Relaxation

Analgesia

Tremors

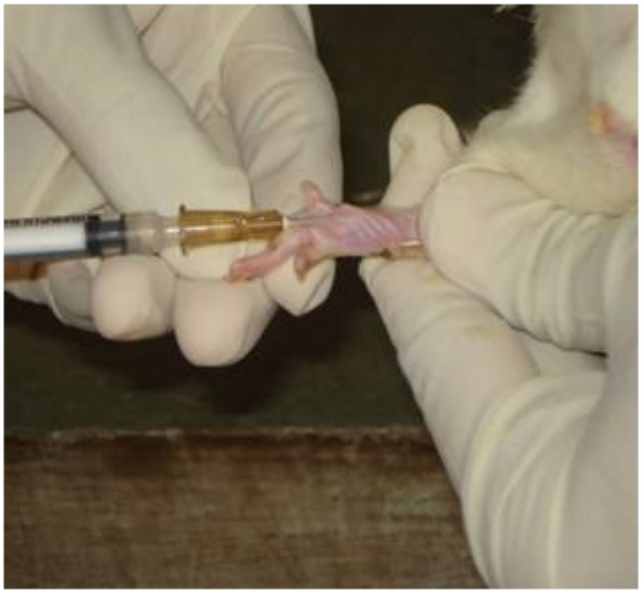


Straub tail

Fig. 6.1 Photographs of different behaviour in toxicity study

DRUG ADMINISTRATION (P.O.)

MEASUREMENT OF PAW VOLUME



SUBPLANTAR INJECTION

Fig. 7.1 Photographs of acute anti-inflammatory study

Plate-1 :Photomicrographs of Adrenal & Lymph node

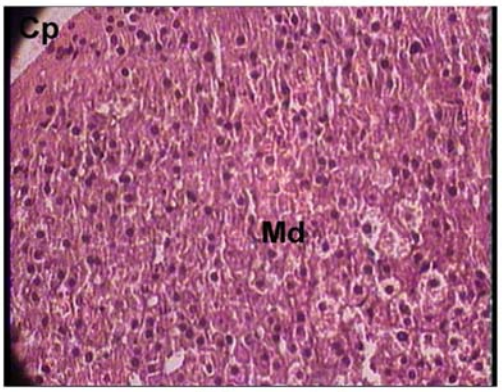


Fig - 1A

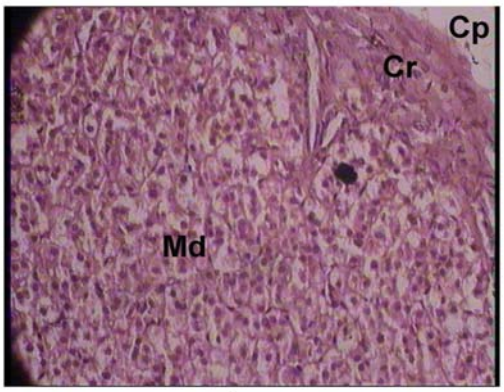


Fig - 1B

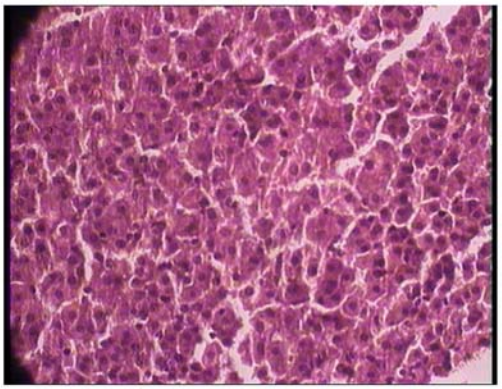


Fig - 1C

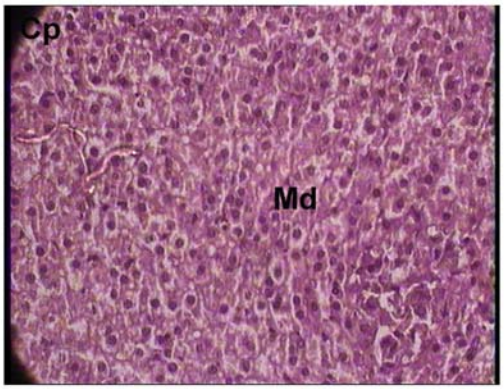


Fig - 1D

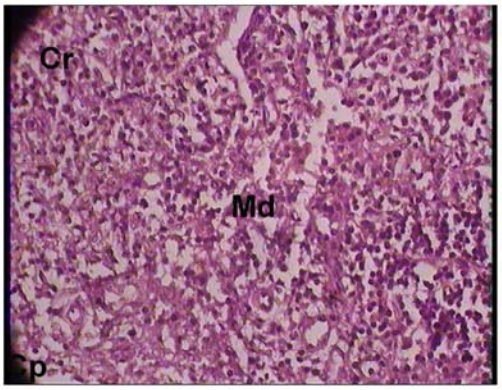


Fig - 1E

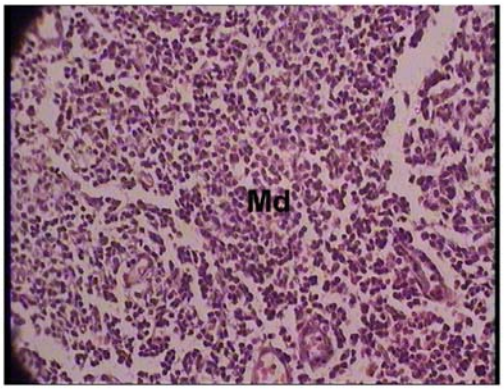


Fig - 1F

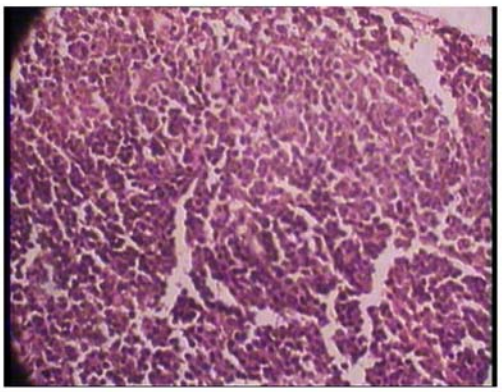


Fig - 1G

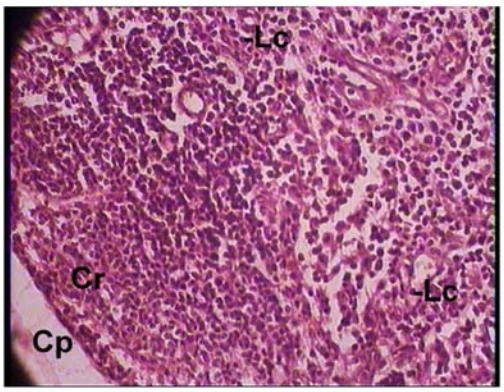


Fig - 1H

Plate-2 :Photomicrographs of Spleen & Thymus

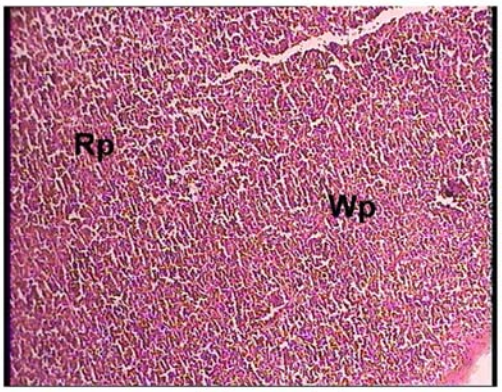


Fig - 2A

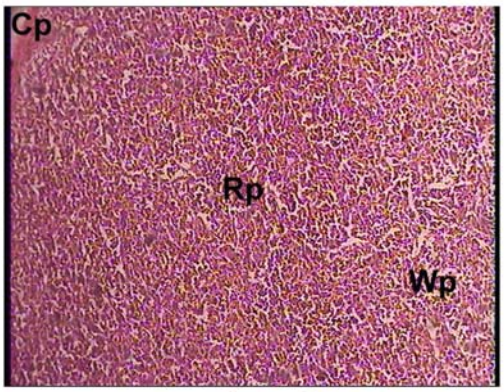


Fig - 2B

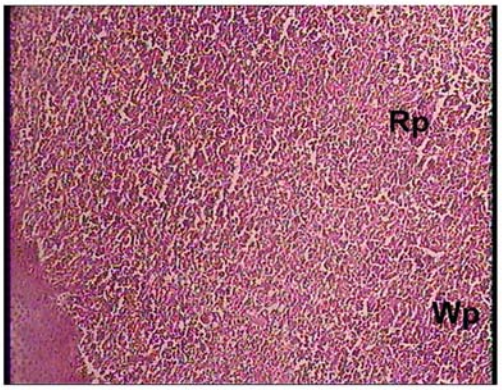


Fig - 2C

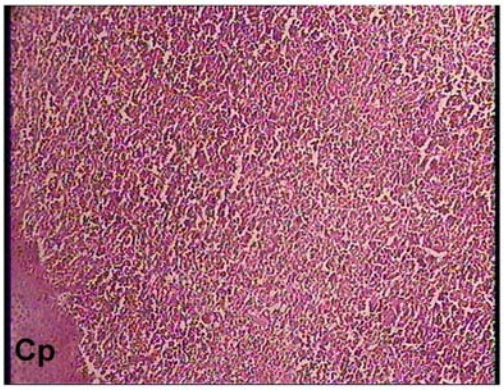


Fig - 2D

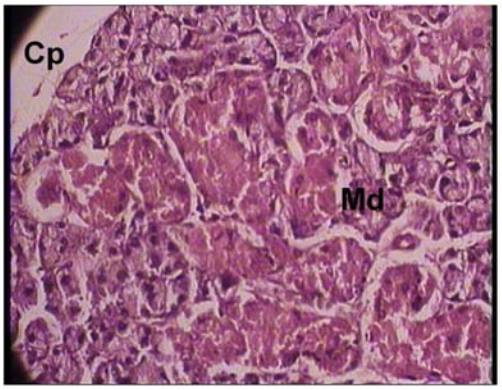


Fig - 2E

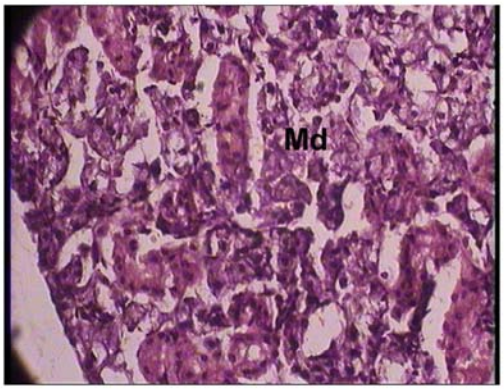


Fig - 2F

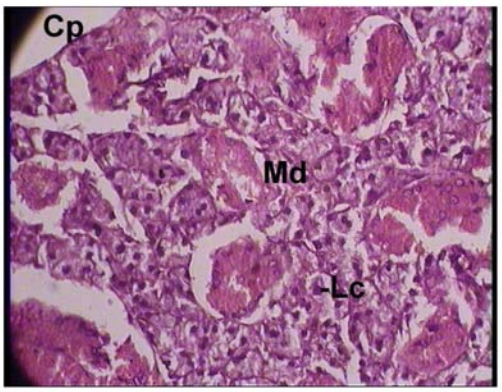


Fig - 2G

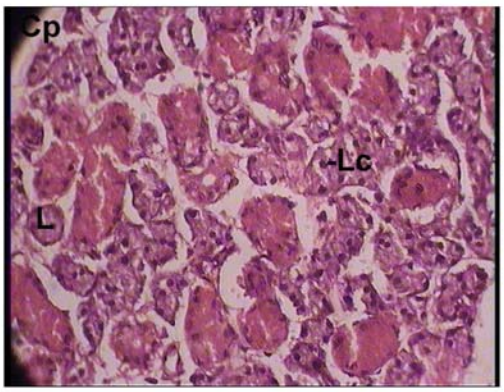


Fig - 2H

Plate 1

Fig. 1A: Photomicrograph of respective section of adrenal gland of albino rats from control group (1 X 400 magnification)
Md- Medulla; Cr- Cortex; Cp- Capsule
Note: Normal cytoarchitecture

Fig. 1B: Photomicrograph of respective section of adrenal gland of albino rats from PAM-200 group (1 X 400 magnification)
Note: Normal cytoarchitecture

Fig. 1C: Photomicrograph of respective section of adrenal gland of albino rats from PAM-400 group (1 X 400 magnification)
Note: Normal cytoarchitecture

Fig.1D: Photomicrograph of respective section of adrenal gland of albino rats from standard group (Indomethacin-2.5) (1 X 400 magnification)
Note: Normal cytoarchitecture

Fig. 1E: Photomicrograph of respective section of lymph node of albino rats from control group (1 X 400 magnification)
Note: Normal cytoarchitecture

Fig. 1F: Photomicrograph of respective section of lymph node of albino rats from PAM-200 group (1 X 400 magnification)
Note: Normal cytoarchitecture

Fig.1G: Photomicrograph of respective section of lymph node of albino rats from PAM-400 group (1 X 400 magnification)
Note: Normal cytoarchitecture

Fig. 1H: Photomicrograph of respective section of lymph node of albino rats from standard group (Indomethacin-2.5) (1 X 400 magnification)
Note: Normal cytoarchitecture

Plate 2

Fig. 2A: Photomicrograph of respective section of spleen of albino rats from control group (1 X 100 magnification)
Rp- Red pulp; Wp- White pulp; Cp- Capsule
Note: Normal cytoarchitecture

Fig. 2B): Photomicrograph of respective section of spleen of albino rats from PAM-200 group (1 X 100 magnification)
Note: Normal cytoarchitecture

Fig. 2C: Photomicrograph of respective section of spleen of albino rats from PAM-400 group (1 X 100 magnification)
Note: Normal cytoarchitecture

Fig. 2D: Photomicrograph of respective section of spleen of albino rats from standard group (Indomethacin-2.5) (1 X 100 magnification)
Note: Normal cytoarchitecture

Fig. 2E: Photomicrograph of respective section of thymus of albino rats from control group (1 X 400 magnification)
Md- Medulla; Lc- Lymphocytes; L- Lobule
Note: Normal cytoarchitecture

Fig. 2F: Photomicrograph of respective section of thymus of albino rats from PAM-200 group (1 X 400 magnification)
Note: Normal cytoarchitecture

Fig. 2G: Photomicrograph of respective section of thymus of albino rats from PAM-400 group (1 X 400 magnification)
Note: Normal cytoarchitecture

Fig. 2H: Photomicrograph of respective section of thymus of albino rats from standard group (Indomethacin-2.5) (1 X 400 magnification)
Note: Normal cytoarchitecture