POTENCY OF SOME MEDICINAL FLORA: PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION

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SECTION I - REVIEW OF LITERATURE

SECTION II SCREENING OF MEDICINAL FLORA

SECTION III ASSESSMENT OF QUALITY, SAFETY AND EFFICACY OF *MANILKARA HEXANDRA* (ROXB.) DUBARD LEAF

PART- II

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Jigna Parekh

Dedicated to my Beloved Mother

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CERTIFICATE

"This is to certify that the Ph.D. thesis entitled 'Potency of Some Medicinal Flora: Phytochemical and Pharmacological Evaluation' embodies the original results of bonafide experimental work carried out by Ms. Jigna Parekh under my guidance and supervision at the Department of Biosciences, Saurashtra University, Rajkot."

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

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PUBLICATIONS AND PRESENTATIONS

RESEARCH PAPERS PUBLISHED

1. **Parekh J,** Inamdar P, Nair R, Baluja S, Chanda S (2005). Synthesis and antibacterial activity of some Schiff bases derived from 4-amino benzoic acid. **J. Serb. Chem. Soc.,** 70: 1155-1161. **(Yugoslavia)**

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Section I

Review of Literature

CHAPTER ONE 1

EMERGING TRENDS IN PHYTOMEDICINE: REVIEW OF LITERATURE

Biodiversity of natural resources like plants, animals, microbes, minerals and marine sources has served not only the primary human needs but also health care since time immemorial (Patwardhan et al., 2004). In every culture, in every age, there are different theories of disease and different systems employed in medicine, but botanical remedies are universal. The healing power of medicinal plants has a long established history. For thousands of years all the cultures have utilized herbs for health care. The widespread use of herbal remedies and healthcare preparations, as those described in ancient texts such as the Veda and the Bible, has been traced to the occurrence of natural products with medicinal properties. Civilized societies have bequeathed myths and compendiums of healing herbs and the herbal remedies from people of preliterate societies continue to surprise us with their extensive green pharmacy (Balick and Cox, 1996). The herbal healing lore was passed from generation to generation by word of mouth.

1.1 HISTORY OF HERBAL MEDICINE

Herbal medicine is the oldest form of healthcare known to mankind. It was an integral part of the development of modern civilization. Fossil records date human use of plants as medicines at least to the Middle Paleolithic age some 60,000 years ago which was evidenced by a burial site of a Neanderthal man uncovered in 1960 (Solecki, 1975). Primitive man observed and appreciated the great diversity of plants available to him. Gradually, each tribe added the medicinal power of herbs in their area to its knowledgebase. All cultures have long folk medicine histories that include the use of numerous plants. Even in ancient cultures, people methodically and scientifically collected information on herbs and developed well-defined herbal pharmacopoeias. Perhaps one of the earliest pharmacopoeias is the De Materia Medica (ca. 79 A.D.) by the Greco-Roman

military physician Dioscorides in the 1st century A.D (Ackerknecht, 1973). Later, in the second century of the present era, Galen (131201 A.D.) shaped pharmaceutical practice for centuries to come (Christopher, 2002). Indeed, well into the 20th century much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native people.

As mute witness to this fact, marshmallow root, hyacinth, and yarrow have been found carefully tucked around the bones of a Stone age man in Iraq. These three medicinal herbs continue to be used today. Marshmallow root is a demulcent herb, soothing to inflamed or irritated mucous membranes, such as sore throat or irritated digestive tract. Hyacinth is a diuretic that encourages tissues to give up excess water. Yarrow is a time-honored cold and fever remedy that may once have been used much as aspirin is used today (Zand et al., 1994).

The entire Middle East has a rich history of herbal healing. There are texts surviving from the ancient cultures of Mesopotamia, Egypt, and India that describe and illustrate the use of many medicinal plant products. The Ebers Papyrus, the most important of the preserved Egyptian manuscripts, was written around 1500 B.C. and includes much earlier information. It contains 876 prescriptions made up of more than 500 different substances, including many herbs (Ackerknecht, 1973).

In 2735 B.C., the Chinese emperor Shen Nong wrote an authoritative treatise on herbs that is still in use today. Traditional Chinese medicine was brought to Japan via Korea, and Chinese-influenced Korean medicine was adapted by the Japanese during the reign of Emperor Ingyo (411-453 A.D.).

The records of King Hammurabi of Babylon (1800 B.C.) include instructions for using medicinal plants and prescribed the use of mint for digestive disorders. Modern research has confirmed that peppermint does indeed relieve nausea and vomiting by mildly anesthetizing the lining of the stomach.

The principal storehouse of the Muslim materia medica is the text of Jami of Ibn

Baiar (1248 A.D.), which lists more than 2,000 substances; including many plant products (Ackerknecht, 1973). The principal Ayurvedic book on internal medicine, the Chark Samhita, describes 582 herbs (Majno, 1975). The main book on surgery, the Sushruta Samhita, lists some 600 herbal remedies. Most experts agree that these books are at least 2,000 years old.

By the seventeenth century, the knowledge of herbal medicine was widely disseminated throughout Europe. The first U.S. Pharmacopoeia was published in 1820. This volume includes an authoritative listing of herbal drugs, with descriptions of their properties, uses, dosages, and tests of purity. It was periodically revised and became the legal standard for medical compounds in 1906.

But as Western medicine evolved from an art to a science in the nineteenth century, information that had at one time been widely available became the domain of comparatively few. The use of herbs, which had been mainstream medical practice, began to be considered unscientific or at least unconventional, and began to fall into relative obscurity.

1.2 MEDICINAL PLANTS: INDIAN WEALTH AND HERITAGE

India is a varietal emporium of medicinal plants and is one of the richest countries in the world as regards to genetic resources of medicinal plants. All known types of agroclimatic, ecologic and edaphic conditions are met within India. The biogeographic position of India is unique which makes India rich in all the three levels of biodiversitysuch as species diversity, genetic diversity and habitat diversity (Krishnaraju et al., 2005). A survey conducted by the All India Coordinated Research Project on Ethnobiology (AICRPE) during the last decade recorded over 8000 species of wild plants used by the tribals and other traditional communities in India for treating various health problems (Laloo et al., 2006).

The Indian subcontinent, with the history of one of the oldest civilization, harbors many traditional health care systems. One of the ancient classics, “Charak Samhita” (Chandra and Sharma, 1986) is the oldest text available on the

complete treatment of diseases which specifies the use of hundreds of herbs in the complete treatment of diseases. The Ayurveda, whose history goes back to 500 B.C., is one of the ancient health care systems, which is a potential source of indigenous drugs. A large number of such herbs are mentioned in “Bhavprakash” (Vaishya, 1835) as well as "Aryavaidhya Kalanidhi” (Kavade Krishnamurthi, 1986). “Indian Materia Medica” (Nandkarni, 1976) also gives a large number of medicinal plants for the treatment of various diseases.

In rural areas, 75 percent of the population is dependent on herbal medicines for healthcare. In the last few decades, herbal medicine has been found to have some impressive credentials. In India, over 2600 plant species have been considered useful in the traditional system of medicine like Ayurveda, Unani, Siddha and Home remedies (Khandelwal, 1999). Number of herbal drugs and their compositions are recommended for combating human ailments in the ancient texts as well as in modern medicine (Sastri, 1962).

1.3 SYNTHETIC DOMINANCE: GROWING THREAT OF ANTIMICROBIAL RESISTANCE

Mankind's discovery of antibiotics ushered in a new age of medicine during the 19th century, an age wherein many predicted an end to diseases that had plagued the mankind for centuries with the appearance of penicillin during World War II as the first miracle drug (Wainwright, 1990). From 1940s to almost 1980s many classes of antibiotics discovered have helped tame many of the terrors of human health. The use of these "wonder drugs", combined with improvements in sanitation, housing, nutrition, and the advent of widespread immunization programmes, led to a dramatic drop in deaths from diseases that were previously widespread, untreatable, and frequently fatal. Over the years, antimicrobials have saved the lives and eased the suffering of millions of people.

Advances in synthetic chemistry for identification of many key chemical molecules offered more opportunities to develop novel compounds. Numerous drugs like sulphonamides, isoniazid, anti-psychotics, anti-histamines and penicillin were

developed from thousands of chemicals (Projan and Shlaes, 2004). Emergence of modern pharmaceutical industry is an outcome of all these different activities that developed potent single molecules with highly selective activity for a wide variety of ailments. These successes resulted in reduced interest in natural products drug discovery. Thus, herbal medicines became the domain of 'old wives tales'.

It was not until the 1970s that antibiotic resistance was considered to be a real threat. In the past, medicine and science were able to stay ahead of this natural phenomenon through the discovery of potent new classes of antimicrobials, a process that flourished from 1930-1970 and has since slowed to a virtual standstill, partly because of misplaced confidence that infectious diseases had been conquered, at least in the industrialized world. In just the past few decades, the development of resistant microbes has been greatly accelerated by several concurrent trends like urbanization, pollution, AIDS epidemic, etc (Levy, 1992). These have worked to increase the number of infections and thus expand both the need for antimicrobials and the opportunities for their misuse.

Recently, infections have become the leading cause of death world-wide which has led to an increase in antibacterial resistance, making it a global growing-problem (Westh et al., 2004). More and more bacteria are developing a resistance to antibiotics (Zajicek, 1996) conferred by randomly mutated genes. Each year infectious diseases cause 14 million deaths worldwide, with mortality increasing even in the United States at an annual rate of 4.8 percent. In 2000, the World Health Organization (WHO) estimated that pneumonia, diarrhoeal disease, and tuberculosis accounted for more than half the deaths due to infectious disease worldwide. The problem is worsened by antibiotic resistance, as well as the emergence of new pathogens with the potential for rapid global spread (Davis, 1994; Service, 1995). In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immume-supression and allergic reactions (Ahmad et al., 1998).

The alarming incidence of antibiotic resistance forced scientists for developing

new and effective therapeutic agents with new principles from botanical medicine with novel modes of action that render them impervious to existing resistance mechanisms (Bhavnani and Ballow, 2000; Essawi and Srour, 2000; Shahidi and Karimi, 2004, Parekh et al., 2005; Nair et al., 2007). Natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanisms of action (Hamil et al., 2003; Machado et al., 2003; Motsei et al., 2003; Barbour et al., 2004). Contrary to the synthetic drugs, antimicrobials of plant origin are affordable and are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases (Iwu *et al*., 1999).

The study of natural products has advantages over synthetic drug design as it leads optimally to materials having new structural features with novel biological activity. Not only do higher plants continue to serve as important source of new drugs, but phytochemicals derived from them are also extremely useful as lead structures for synthetic modicfication and optimization of bioactivity. The starting materials for about one-half of the medicines used today come from natural sources. Virtually every pharmacological class of drugs includes a natural product prototype. Undisputedly, the history of herbal medicine is inextricably intertwined with that of modern medicine. Many drugs listed as conventional medications were originally derived from plants. Salicin, a precursor of aspirin, was originally derived from *Salix alba* (white willow bark) and flowers of *Filipendula ulmaria* (meadowsweet plant) are used as analgesic. Digoxin derived from *Digitalis lantana* (foxglove) is included in a drug class cardiotonic. *Cinchona ledgeriana* bark is the source of malaria-fighting quinine. Vincristine, used to treat certain types of cancer, comes from *Catharantus roseus*. Ephedrine, a bronchodilator used to decrease respiratory congestion, comes from *Ephedra sinica*. The *Papaver somniferum* (opium poppy) yields morphine, codeine, and paregoric, a treatment for diarrhoea. Laudanum, a tincture of the opium poppy, was the favored tranquilizer in Victorian times (Farnsworth and Morris, 1976). The future of higher plants is very promising as they can serve as best sources of medicinal agents in the prevention and treatment of diseases.

1.4 REVIVAL OF TRADITIONAL MEDICINE

The history of interest in phytochemicals reveals that crude drugs were the dominant therapy until the time of World War II. During the late 19th century, Western medicine began to supersede the folk and learned medicine that had been gathered and traded between cultures since the time of the Ancient Egyptians (Root-Bernstein, 1998) and moved away from any interest in folk knowledge of medicinal plants. Today, pendulum is swinging back to an interest in the value of traditional medicine. There is currently a rising recognition of the value of experience and historical knowledge gathered by indigenous cultures with medicinal plants.

Medicinal plants can save lives, livelihood and cultures. Herbal medicines are an important part of the culture and traditions worldwide. It is therefore no surprise that medicinal plants have raised their importance all over the globe. Recently, the renewed interest in medicinal plants as a re-emergent health aid has been fuelled by the extensive antimicrobial resistance along with rising costs of prescription drugs in the maintenance of personal health and well-being and the bioprospecting of new plant-derived drugs (Hoareau and DaSilva, 1999).

The revival of interest in herbal medicines is firstly due to increased awareness of the limited horizon of synthetic pharmaceutical products to control major diseases and secondly due to the current widespread belief that 'green medicine' is safe and more accessible and affordable (Mander, 1998) than the costly synthetic drug many of which have adverse side effects. The past decade has witnessed a tremendous resurgence in the interest and use of medicinal plant products especially from developed countries. According to a WHO estimate, about 80% of the world population relies on traditional systems of medicines for primary health care, where plants form the dominant component over other natural resources (Duraipandiyan et al., 2006).

Today, the renewed interest in traditional pharmacopoeias reveals that researchers are concerned not only with determining the scientific rationale for

the plant's usage, but also with the discovery of novel compounds of pharmaceutical value. Instead of relying on trial and error, as in random screening procedures, traditional knowledge helps scientists to target plants that may be medicinally useful (Cox and Balick, 1994). The burgeoning interest in medicinal plants reflect recognition of the validity of many of the traditional claims for the value of natural products in health care. Many medicinal plants exert specific medicinal actions and may be used in response to specific health problems over short or long term intervals. It is estimated that total of 122 drugs from 94 plant species have been discovered through ethnobotanical leads (Fabricant and Farnsworth, 2001). Now-a-days, many scientists have isolated the medicinal properties of a large number of botanicals, and their healing components have been extracted and analyzed (Nair and Chanda, 2005; Elizabeth, 2005; Aliero and Afolayan, 2005; Rajeshwar et al., 2005; Parekh et al., 2006; Tadhani and Subhash, 2006; Kuzmaa et al., 2007; Parekh and Chanda, 2007). Many plant components are now synthesized in laboratories for use in pharmaceutical preparations.

1.5 HERBAL DRUG RESEARCH TODAY

The goal of herbal drug research and development program is to discover single entity and multicomponent bioactive natural products that may serve as leads for the development of new pharmaceuticals which address unmet therapeutic needs.

Traditional knowledge-driven drug discovery will serve as a powerful search engine and most importantly, will greatly facilitate the focused and safe natural products research to rediscover the drug discovery process. There are over 750,000 plants on earth. Relatively speaking, only a very few of the healing herbs have been studied scientifically. Of these, only about 6% have been screened for biologic activity, and a reported 15% have been evaluated phytochemically (Verpoorte, 2000).

Recently, all over the world, there is an upsurge and interest among scientific institutions, biological research institutions in the use of medicinal plants, crude

extracts or active ingredients to treat various ailments. Almost all of the current research validating herbal medicine has been done in Germany, Japan, China, India, Taiwan, and Russia (Hoareau and DaSilva, 1999). Most of the research that is done on plants continues to focus on identifying and isolating active ingredients, rather than studying the medicinal properties of whole plants. Herbalists, however, consider that the power of a plant lies in the interaction of all its ingredients. Plants used as medicines offer synergistic interactions between ingredients both known and unknown.

Today, rather than using a whole plant, pharmacologists identify, extract, isolate and synthesize individual components to capture the active properties. In addition to active ingredients, plants contain minerals, vitamins, volatile oils, glycosides, alkaloids, bioflavonoids, and other substances that are important in supporting a particular herb's medicinal properties. These elements also provide an important natural safeguard.

Furthermore, an increasing reliance on the use of herbal products in the industrialized societies has led to the extraction and development of several drugs and chemotherapeutics from plants (UNESCO, 1998). Some of the wild plants, traditionally used as herbal remedies by ayurvedic practitioners, secured an important place in modern medicine. Nevertheless, many plants, which have curative properties, are yet to be seriously screened for their medicinal value. In an extensive screening programme of plants used in traditional medicine, researchers provide scientific evidence for their rational use in treating infections and diseases.

1.6 A GLOBAL TREND IN HERBAL DRUG MARKET

Many drugs commonly used today are of herbal origin. Indeed, about 25% of the prescription drugs available in markets contain at least one active ingredient derived from plant material. The World Health Organization (WHO) estimates that 4 billion people, 80% of the world population, presently use herbal medicine for some aspect of primary health care. WHO also notes that of 122 plant-derived

pharmaceutical medicines, about 80% are used in modern medicine that correlated directly with their traditional uses as plant medicines by native cultures.

The herbal drug industry is considered to be a high growth industry of the late 90s and seeing the growing demand, it is all set to flourish in the next century. The trend for the increasing popularity of medicinal herbs in developing countries is well supported by statistical data. The world market for plant-derived chemicals alone exceeds several billion dollars per year. Trade in medicinal plants is growing in volume and in exports. It is estimated that the global trade in medicinal plants is US$ 800 million per year (Wakdikar, 2004). The botanical market, inclusive of herbs and medicinal plants, in the USA, is estimated, at approximately US$ 1.6 billion p.a. China with exports of over 120,000 tonnes p.a., and India with some 32,000 tonnes p.a. dominate the international markets (Dhar et al., 2002). The annual export of medicinal plants from India is valued at Rs. 1200 million (Ramakrishnappa, 2002). It is estimated that Europe, annually, imports about 400,000 tonnes of medicinal plants with an average market value of US$ 1 billion from Africa and Asia (Wakdikar, 2004). A growing awareness of this new contributor to the foreign-exchange reserves of several national treasuries is beginning to emerge. To satisfy growing market demands, surveys are being conducted to unearth new plant sources of herbal remedies and medicines. Based on current research and financial investments into medicinal plants, it seems that they will continue to play important roles in human health (Hoareau and DaSilva, 1999).

1.7 APPROACHES TO HERBAL DRUG DISCOVERY USING HIGHER PLANTS

The plant kingdom harbors an inexhaustible source of active ingredients invaluable in the management of many intractable diseases. Folk medicine is one of the sources to discover new antimicrobial compounds and systematic screening of them may result in the discovery of novel effective compounds (Janovska et al., 2003). The quest for plants with medicinal properties continues

to receive attention as scientists survey plants for a complete range of biological activities, which range from antibiotics to antitumor.

In the light of evidence for the rapid global spread of resistant clinical isolates and the appearance of drug resistant strains among community acquired infections as well as appearance of undesirable side effects of certain antibiotics (WHO, 2002), the need for discovery or development of new antimicrobial agents is of paramount importance (Bradford, 2001). For this reason the search for new antimicrobial drugs became an important alternative. Higher plants have been proved to be a potential source for new antimicrobial agents (Mitscher et al., 1987). Plant based natural constituents 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 may be of value for the synthesis of complex chemical substances.

There are many approaches to the search for new biologically active principles in higher plants (Farnsworth and Loub, 1983). Several reviews pertaining to approaches for selecting plants as candidates for drug discovery programs have been published (Farnsworth, 1966; Farnsworth and Bingel, 1977; Farnsworth, 1984; Phillipson and Anderson, 1989; Kinghorn, 1994; Clark, 1996; Harvey, 2000; Newman et al., 2000). Different approaches include (i) Random selection followed by chemical screening, (ii) Random selection followed by one or more biologic assays, (iii) Follow-up of biologic activity reports, (iv) Follow-up of ethnomedical uses of plants, (v) Plants used in organized traditional medical systems and (vi) Use of databases (Fabricant and Farnsworth, 2001).

Scientific analysis of plant components follows a logical pathway. Plants are collected either randomly or by following leads supplied by local healers. Random collection is to collect readily available plant, prepare extracts, and test each extract for one or more types of pharmacological activity. This, broad screening method is a reasonable approach that eventually should produce useful drugs.

Initial screening of plants for possible antimicrobial activities typically begins by using crude aqueous or alcohol extraction method and can be followed by various organic extraction methods. Since nearly all of the identified components from plants which are active against microorganisms, are aromatic or saturated organic compounds, they are often obtained through initial ethanol or methanol extraction (Vileges et al., 1997). The research on the medicinal plants should be extended with the identification of the active principles in the plants. Scientific examination of the remedies could lead to standardization and quality control of the products to ensure their safety. It is after such evaluations that they can be approved for use in the primary health care. Such research activities could also lead to the development of new drugs.

With the development of various analytical methods of high precision, and advances in molecular biology and genetic engineering, it is now possible to isolate compounds in extremely small quantities, study their chemical structure and therapeutic potentialities and then to alter the molecule to be suitable for production of novel and more selective new therapeutic agents. A number of active constituents have been isolated from plants like *Azardirachta indica* (Randhawa and Parmar, 1993), *Senna alata* (Adedayo, 2001), *Terminalia bellerica* (Elizabeth, 2005).

Major pharmaceutical companies are currently conducting extensive research on plant materials and encourage large scale pharmacological screening of herbs. Natural products to be used in pharmaceutical preparations are either pure compounds or extracts. The screening of plant extracts has been of great interest to scientist for the discovery of new drugs effective in the treatment of several diseases (Dimayuga and Garcia, 1991). Extraction of bioactive compounds from medicinal plants permits the demonstration of their physiological activity and facilitates pharmacology studies leading to synthesis of a more potent drug with reduced toxicity (Pamplona-Roger, 1999; Manna and Abalaka, 2000). Many reports concerning the antibacterial screening of plant extracts of medicinal plants have appeared in the literature (Vlietinck et al., 1995; Lin et al., 1999; Janovska et al., 2003; Geyid and Andualem, 2005; Parekh et al.,

2005; Parekh and Chanda, 2006).

1.8 PHYTOMEDICINAL ACTIONS

The beneficial medicinal effects of phytomedicines typically result from synergistic actions of secondary products present in the plants (Wink, 1999). Plant secondary products have a defensive role against pathogen attack, an attractant role towards pollinators, protective actions to abiotic stresses such as temperature, water status, light levels, UV exposure and mineral nutrients and its role at cellular level as plant growth regulators, modulators of gene expression, and in signal transduction have also been shown (Kaufman et al., 1999; Wink, 1999). In contrast to synthetic pharmaceuticals based upon single chemicals, many phytomedicines exert their beneficial effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites assiociated with a physiological process. This synergistic or additive pharmacological effect can be beneficial by eliminating the problematic side effects associated with the predominance of a single xenobiotic compound in the body (Tyler, 1999). In the role of secondary products as defence chemicals, a mixture of chemicals having additive or synergistic effects at multiple target sites would not only ensure effectiveness against a wide range of herbivores or pathogens but would also decrease the chances of these organisms developing resistance or adaptive responses (Kaufman et al., 1999; Wink, 1999).

1.9 PHYTOMEDICINE: A STRATEGY FOR FUTURE

For future drug development in the 21st century, research should focus not only on bioactive principles (lead compounds or leads), but also on active fractions and active formulations from medicinal herbs.

In vitro screening programmes, using Ethnobotanical approach, are important in validating the traditional use of herbal remedies and for providing leads for newer drugs. The activity identified by an *in vitro* test provides a basic understanding of a plant's efficacy but it does not necessarily confirm that a plant extract is an effective medicine or a suitable candidate for drug development. Certainly this is a

primary concern of ethnopharmacological research in developing new lead compounds. Scientific validation of use of herbal medicine lends support to the continued practice of traditional medicine. Eventually this may lead to more widespread use of traditional medicine in health care system, as in India and China provided thorough toxicological investigations are carried out (Myers, 1984).

The purpose of the present research work is to screen the extracts of some randomly selected plant species from the Saurashtra region of western India that could be useful for the development of new tools for the control of infections and diseases. While pursuing this goal, a systematic evaluation of aqueous and alcoholic extracts of 108 randomly selected plant extracts against a diverse range of bacteria, fungi and yeast was done. Further, the most active plant extract was selected for investigation of pharmacognostical analysis, phytochemical analysis, toxicological studies and pharmacological studies.

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Section II

Screening of Medicinal Flora

CHAPTER TWO 2

ANTI-INFECTIVE POTENTIAL OF MEDICINAL FLORA: AN *IN VITRO* SCREENING FOR ANTIBACTERIAL AND ANTIFUNGAL PROPERTIES

2.1 INTRODUCTION

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress in human medicine. Their impact is particularly large in developing countries due to the relative unavailability of medicines and the emergence of widespread multiple drug resistance due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases (Okeke et al., 2005). Moreover, antimicrobial drugs are sometimes associated with adverse effects on the host including hypersensitivity, immune-supression and allergic reactions (Ahmad et al., 1998). Research on new antimicrobial substances must therefore be continued and all possible strategies should be explored. Besides small molecules from medicinal chemistry, natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases (Clardy and Walsh, 2004). The success story of chemotherapy therefore lies in the continuous search for new drugs to counter the challenge posed by resistant strains of microorganisms. Current research on natural molecules and products primarily focuses on plants since they can be sourced more easily and be selected on the basis of their ethno-medicinal use (Verpoorte et al., 2005).

Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subject of very intense pharmacological studies (Zakaria, 1991; Nascimento, 2000; Bonjar SGH, 2004; Kuzmaa, 2007; Parekh and Chanda, 2007a). In this connection, higher plants continue to be a rich source of therapeutic agents since they produce hundreds to thousands of diverse chemical compounds as secondary metabolites with different biological activities (Hamburger and Hostettmann, 1991). The compounds produced by plants are

active against plant and human pathogenic microorganisms (Mitscher et al., 1987). The remarkable contribution of plants to the drug industry was possible, because of the large number of phytochemical and biological studies carried out all over the world.

Herbal remedies used in the folk medicine provide an interesting and still largely unexplored source for the creation and development of potentially new drugs for chemotherapy which might help overcome the growing problem of resistance and also the toxicity of the currently available commercial antibiotics. From an estimated 250,000 higher plants in the world (Wilson, 1988), only 5-15 % have been studied for a potential therapeutic value (Balandrin et al., 1985; Kinghorn, 1992). A large number of plant species remain yet to be investigated. Therefore it is of great interest to carry out screening of the unexplored plants in order to validate their use in folk medicine and to reveal the active principle by isolation and characterization of their constituents (Kianbakht and Jajaniani, 2003).

Initial screening of plants for possible antimicrobial activities typically begins by using crude aqueous or alcohol extraction and can be followed by various organic extraction methods. Since nearly all of the identified components from plants, active against microorganism are aromatic or saturated organic compounds, they are often obtained through initial ethanol or methanol extraction (Vilegs et al., 1997, Parekh et al., 2005). The screening of plant crude extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes (Maurer-Grimes et al., 1996; Rabe and van Staden, 2000; Srinivasan et al., 2001; Zgoda-Pols et al., 2002; Afolayan, 2003; Nair et al., 2004; Elizabeth, 2005; Parekh et al., 2005; Prashanth Kumar, 2006; Parekh and Chanda, 2006a; Parekh and Chanda, 2007b). Validation and selection of primary screening assays are pivotal to guarantee sound selection of extracts or molecules with relevant pharmacological action and worthy follow-up.

In the light of recent problems of multiple drug resistance and intractable microbial diseases, the need to find new entities with antimicrobial properties becomes pertinent. The present work encompasses the screening of aqueous and

methanol/ethanol extracts of 108 plant species randomly colleted, for their antibacterial property against a wide array of microorganisms. Further, the most active plant extracts showing best antibacterial activity were screened for antifungal activity. The most promising plant extract was selected for further phytochemical and pharmacological activities. This is in pursuance of the efforts to search for drugs from plants and the verification of the scientific basis of some known practices in traditional medicine.

2.2 MATERIALS AND METHODS

2.2.1 ANTIBACTERIAL SCREENING

2.2.1.1 Plant collection

Fresh plants/plant parts were collected randomly from the semi-arid region of western Saurashtra, Gujarat, India. The taxonomic identities of these plants were confirmed by Dr. P. S. Nagar and Dr. N. K. Thakrar, Department of Biosciences, Saurashtra University, Rajkot. The ethnobotanical information on all the plants/plant parts collected for screening along with the voucher specimen numbers and their therapeutic uses are reported in Table 2.1. Fresh plant materials were washed under running tap water, air dried and homogenized to fine powder and stored in air-tight bottles.

2.2.1.2 Preparation of crude plant extract

2.2.1.2.1 Aqueous extraction

Ten grams of dried plant material was extracted in distilled water for 6 h at slow heat. After every two hours it was filtered through eight layers of muslin cloth and centrifuged at 5000 rpm for 15 min. The supernatant was collected. This procedure was repeated twice and after 6 h, the supernatant was concentrated to make the final volume one-fifth of the original volume. The extract was then autoclaved at 121°C and 15 lbs pressure and stored at 4°C (Parekh et al., 2005).

2.2.1.2.2 Solvent extraction

Ten grams of dried plant material was extracted with 100 ml of ethanol/ methanol kept on a rotary shaker for 24 h at room temperature. Thereafter, it was filtered and centrifuged at 5000 rpm for 15 min. The supernatant was collected and the solvent was evaporated to make the final volume one-fifth of the original volume (Parekh et al., 2005). It was stored at 4°C in airtight bottles for further studies.

2.2.1.3 Bacterial strains and growth conditions

The investigated microbial strains are identified strains and were obtained from the National Chemical Laboratory (NCL), Pune, India. The test microorganisms include 5 Gram-positive bacteria *Bacillus cereus* ATCC11778, *Bacillus megaterium* ATCC9885, *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC12228 and *Staphylococcus subfava* NCIM2178; 9 Gram-negative bacteria *Alcaligenes fecalis* ATCC8750, *Enterobacter aerogenes* ATCC13048, *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* NCIM2719, *Proteus mirabilis* NCIM224, *Proteus vulgaris* NCTC8313, *Pseudomonas aeruginosa* ATCC27853, *Pseudomonas pseudoalcaligenes* ATCC17440 and *Salmonella typhimurium* ATCC23564 and 1 yeast *Candida tropicalis* ATCC4563. Bacterial cultures were grown on nutrient broth (Hi-Media) at 37°C for 24 h and yeast was grown on sabouraud dextrose broth (Hi-Media) at 28°C for 48 h. All the microbial cultures were maintained at 4°C on nutrient agar slants (for bacteria) and MGYP slants (for yeast).

2.2.1.4 Assay for antibacterial activity

2.2.1.4.1 Preparation of inoculum

The test bacterial strains were inoculated into nutrient broth and were incubated at 37°C on shaker while yeast culture was inoculated into sabouraud dextrose broth and incubated at 28°C on shaker. The inoculum size was maintained as per 0.5 McFarland standard ($1x10^8$ cfu/ml). The activated inoculum was used for antibacterial assay.

2.2.1.4.2 Antibacterial susceptibility testing

2.2.1.4.2.1 Agar disc diffusion method

The screening of aqueous extracts of different plant species for antibacterial activity was determined by agar disc diffusion method (Bauer et al., 1966; Parekh and Chanda, 2006b). The molten Mueller Hinton Agar No. 2 media (Hi-Media) was inoculated with 200 µl of the inoculum ($1x10^8$ cfu/ml) when the temperature of media reached 40-42°C and then poured into the Petri plate (Hi-Media). Sterile disc (7 mm) (Hi-Media) was saturated with 100 µl of the extract and allowed to dry. The disc was then introduced on the upper layer of the seeded agar plate. For each bacterial strain controls were maintained where pure solvents were used instead of the extract. The plates were incubated at 37°C for 24 h. The result of antibacterial activity was obtained by measuring the diameter of the zone of inhibition. The experiment was performed under strict aseptic conditions for three times to minimize error and the mean values are presented in Table 2.2.

2.2.1.4.2.2 Agar well diffusion method

The screening of alcoholic extracts of different plant species for antibacterial activity was determined by agar well diffusion method (Perez et al., 1990; Parekh et al., 2005). The molten Mueller Hinton Agar No. 2 media (Hi-Media) was inoculated with 200 µl of the inoculum ($1x10^8$ cfu/ml) when the temperature of media reached 40-42°C and then poured into the Petri plate (Hi-Media). After the media was solidified, a well was prepared in the plates with the help of a cup-borer (8.5 mm). The well was filled with 100 µl of the extract. For each bacterial strain controls were maintained where pure solvents were used instead of the extract. The plates were incubated at 37°C for 24 h. The result of antibacterial activity was obtained by measuring the diameter of the zone of inhibition. The experiment was performed under strict aseptic conditions for three times to minimize error and the mean values are presented in Table 2.2.

2.2.2 ANTIFUNGAL SCREENING

2.2.2.1 Plant selection

The primary screening of 108 plant species for antibacterial property yielded many active plant extracts. Amongst them, 20 most active plant extracts were selected for further screening for antifungal property. The selected plants are listed in Table 2.3.

2.2.2.2 Preparation of crude plant extract

Ten grams of dried plant material was extracted with 100 ml of methanol and kept on a rotary shaker for 24 h at the room temperature. Thereafter it was filtered and centrifuged at 5000 rpm for 15 min. The supernatant was collected and evaporated to dryness to give the crude dried extract (Parekh and Chanda, 2006b). The extractive yield (%) of all the plant extracts is shown in Table 2.3.

2.2.2.3 Fungal strains used

The investigated fungal strains are identified strains and were obtained from the National Chemical Laboratory (NCL), Pune, India. The test fungal strains include 7 yeasts viz. *Candida albicans* (1) ATCC2091, *Candida albicans* (2) ATCC18804, *Candida glabrata* NCIM3448, *Candida tropicalis* ATCC4563, *Cryptococcus luteotus* ATCC32044, *Cryptococcus neoformans* ATCC34664, *Trichosporon beigelli* NCIM3404, and 4 moulds viz. *Aspergillus candidus* NCIM883, *Aspergillus flavus* NCIM538, *Aspergillus niger* ATCC6275 and *Mucor heimalis* NCIM873. The fungal strains were grown on sabouraud broth and maintained on MGYP slants (yeast) and potato dextrose agar slants (mould) at 4°C.

2.2.2.4 Assay for antifungal activity

2.2.2.4.1 Preparation of inoculum

The test fungal strains were inoculated into sabouraud dextrose broth and incubated at 28°C on a rotary shaker. The inoculum size was maintained as per

the 0.5 McFarland standard ($1x10^8$ cfu/ml). The activated inoculum was used for antifungal assay.

2.2.2.4.2 Preparation of test compound

The methanol extracts of selected plant species were diluted in 100 % dimethylsulphoxide (DMSO) and the stocks were prepared at the concentration of 25mg/ml, 12.5 mg/ml and 6.75 mg/ml. The antifungal activity was evaluated at three different concentrations viz. 500 µg/disc, 250 µg/disc and 125 µg/disc.

2.2.2.4.3 Antifungal susceptibility testing

The screening of methanol extracts of selected plant species for antifungal activity was determined by agar disc diffusion method (Bauer et al., 1966; Parekh and Chanda, 2006b). The molten sabouraud dextrose agar media (Hi-Media) was inoculated with 200 µl of the inoculum ($1x10^8$ cfu/ml) when the temperature of media reached 40-42°C and then poured into the Petri plate (Hi-Media). Sterile disc (7 mm) (Hi-Media) was saturated with 20 µl of the extract with the concentration of 500 µg/disc, 250 µg/disc and 125 µg/disc and allowed to dry. The disc was then introduced on the upper layer of the seeded agar plate. For each fungal strain, controls were maintained where pure solvents were used instead of the extract. The plates were incubated at 28°C for 48 h. The result of antifungal activity was obtained by measuring the diameter of the zone of inhibition. The experiment was performed under strict aseptic conditions for three times to minimize error and the mean values are presented in Tables 2.4a and 2.4b.

2.3 RESULTS AND DISCUSSION

2.3.1 Antibacterial screening

Although India possesses a rich tradition in the use of medicinal plants and an outstanding flora diversity of vascular plants, little research has been done in the context of phytochemical leads for therapeutic use. The present study

demonstrated that the medicinal knowledge held by the Indian native people is relatively measurable in laboratory-based assays. Moreover, to address the present situation of microbial resistance to antimicrobial agents, evaluation of 108 different plant species from western region of Saurashtra (India) was carried out for potential anti-infective property.

The ethno-botanical details of the plants screened in this work are shown in Table 2.1. As indicated, in some cases leaves, stem, root, bark, rhizomes or whole plant was extracted as it is already known that any part of the plant may contain active constituents (Gordon and David, 2001). In all a total of 216 extracts (aqueous and methanol/ethanol) of 108 different plant species belonging to 54 different families were screened for potential antimicrobial activity. Out of 216 extracts, 143 plant extracts (35 aqueous and 108 methanol/ethanol) showed activity against at least one of the tested microorganism. Although plants differed significantly in their activities against microorganisms tested, more of the extracts showed antibacterial activity against Gram-positive bacteria than Gram-negative bacteria (Rabe and van Staden, 1997; Parekh et al., 2005). Amongst aqueous and methanol/ethanol extracts of the studied plant species, methanol/ethanol extracts were found to be more active against the test microbial strains than the aqueous extracts (Table 2.2).

Some commonly encountered pathogens have been associated with some of the human diseases. The Gram-positive bacterium *Bacillus subtilis* occasionally produce diseases such as meningitis, endocarditis, endophthalmitis, conjunctivitis or acute gastroenteritis in immunocompromised patients. *Bacillus cereus* is a spore forming pathogen associated with various opportunistic clinical infections (Boyd, 1995). *Staphylococcus aureus* is a facultatively anaerobic, Gram-positive bacteria which causes food poisoning and usually grow on the nasal membranes and skin. It also causes boils, abscesses, wound infection, pneumonia, endocarditis, osteomyelitis, toxic shock syndrome and other diseases (Cheesbrough, 2000). The Gram-negative bacterium *Escherichia coli* is present in human intestine and causes lower urinary tract infection, coleocystis or

Table 2.1 Ethnobotanical information of some plant species screened

Botanical name (Family, Genus, Species)	Vernacular Name	Habit	Voucher number	Part(s) extracted	Action/Therapeutic use
ACANTHACEAE					
Andrographis paniculata (Burm.f.) Wall.	Lilu Karyatu	Herb	PSN574	Leaf	anth, antpr, apt, feb, sti, stm, ton, dia, dyspep, fev, fla, rheu
Eranthemum spp. L.	-	Herb	-	Leaf	abs, bo
AMARANTHACEAE					
Achyranthas aspera L.	Agehdi	Herb	PSN635	Whole	antpr, apt, ast, car, col, diu, em, exp, lax, pur, stm, bl dis, bron, co, con, drop, dyspep, dys, gon, itc, lep, pil, pneu, rheu, sca, ul, vom
Celosia argentea L.	Lambadi	Herb	PSN645	Whole	anpy, aphro, bl dis, dia, gon, infl, sor
ANACARDIACEAE					
Mangifera indica L.	Ambo	Tree	-	Leaf	anth, aphro, ast, em, lax, ton, ano, con, dia, diph, dys, infl, leuch, pneu, rheu, syp, ul, wo, vom
APOCYNACEAE					
Carissa congesta Wt.	Karamda	Tree	PSN440	Leaf	dig, stm, bil
Ervatima coronaria Stapf.	Tagar	Shrub	PSN446	Leaf	pur, ton, bil, can, infl, wo
Holarrhena antidysenterica (Heyne. ex Roth.) A. DC.	Kada chaal	Tree	PSN443	Bark	anth, aphro, apt, ast, car, col, feb, ath, bil, bo, bron, dia, diab, drop, dys, fev, hac, infl, lep, leucd, pil, sk, swe, ul, wo
Rauvolfia serpentina (L.) Benth ex Kurz.	Sarpgandha	Shrub	-	Leaf	anth, anthy, diu, lax, sed, dyspep, epi, fev, ins, wo
Thevetia peruviana (Pers.) Merr.	Pili-Karen	Shrub	PSN447	Leaf	abor, ast, cat, em, feb, pur, bron, drop, fev, itc, leucd, pil, rheu, sk,

Botanical name (Family, Genus, Species)	Vernacular Name	Habit	Voucher number	Part(s) extracted	Action/Therapeutic use
ACORACEAE					
Acorus calamus L.	Vaj	Herb	-	Rhizome	verm, dia, fla
ARISTOLOCHIACEAE					
Aristolochia bracteolata Lam.	Kidamari	Herb	PSN662	Seed	anth, antpr, cat, emmen, pur, verm, ecz, fev, gon, sk, syp, ul
ASCLEPIADACEAE					
Calotropis gigantea (L.) R. Br.	Moto Ankado	Shrub	PSN449	Leaf	alex, anal, ast, dig, dip, exp, pur, stm, ton, ath, den fev, drop, infl, lep, leucd, pil, rheu, rw, sca, sk, syp, swe, ul, wo
Tylophora indica (Burm.f.) Merr.	Damnivel	Climber	PSN462	Leaf	car, dip, em, expec, pur, stm, ath, bron, dia, dys, dyspep, fla, gou, ul, wo
ASTERACEAE					
Launaea procumbens (Roxb.) Ram. & Raj.	Moti Bhonpatri	Herb	PSN397	Whole	col, diu, aly, rheu
Saussurea lappa Costus.	Kuth	Herb	-	Root	ath, bron, fla, lep
Tridax procumbens L.	Pardeshi Bhangro	Herb	PSN414	Whole	bron, dia, dys, wo
Vernonia anthelmintica (L.) Willd.	Kalijiri	Herb	PSN415	Whole	fev, ath, co, ul, sk, leucd, lep, dyspep, infl, ast, anth, exp, dmu, diu, stm, feb, gal, ton, pur
Xanthium strumarium L.	Gadariyun	Herb	PSN419	Whole	dip, diu, sed, hp, leuch, mal, sk
BALANITACEAE					
Balanites aegyptiaca (L.) Del.	Engoria	Shrub	PSN112	Whole	alex, anal, anth, pur, verm, bo, bu, co, fra, leucd, sb, sk, sls
BIGNONACEAE					
Spathodea campanulata Beauv.	Kesudo	Tree	PSN563	Aerial parts	Pur, sk
Tecomella undulata (Sm.) Seem.	Ragat-rohido	Tree	PSN566	Leaf, stem	ath, fev, itc, leucd, leuch, rheu, syp

Botanical name (Family, Genus, Species)	Vernacular Name	Habit	Voucher number	Part(s) extracted	Action/Therapeutic use
CAESALPINIACEAE					
Bauhinia variegata L.	Kanchnar	Tree	-	Bark	antd, anth, ast, car, lax, abs, bo, con, dia, diab, infl, mal, ul
Caesalpinia crista L.	Kankach	Shrub	PSN244	Aerial parts	anth, antpr, antpy, antsp, aphro, ast, emmen, feb, ton, ath, elph, fev, leuch, pil, smp, ul, wo
Caesalpinia pulcherrima (L.) Swartz.	Sandhesharo	Shrub	PSN246	Aerial parts	abor, antpr, ast, cat, emmen, pur, sti, ton, ath, bron, chl, mal, tum, ul
Cassia angustifolia Vahl.	Mindhi aval	Shrub	PSN248	Leaf	anth, lax, pur, bil, bron, con, dyspep, fev, gou, infl, jaun, lep, pil, rheu, sk, tum, typh
Cassia fistula L.	Garmalo	Tree	PSN250	Leaf	cat, em, feb, lax, pur, bil, bron, fev, rheu, rw
Cassia tora L.	Kuvadio	Herb	PSN258	Whole	lax, pur, gou, lep, psor, scia, sk
Delonix regia (Boj.) Raf.	Gul-mohor	Tree	PSN260	Pod	feb
CAPPARIDACEAE					
Crataeva religiosa Forst.f.	Varun, Vayvar	Tree	PSN22	Bark skin	anth, ast, lax, stm, verm, anm, calc, fla, gou, wo
CASUARINACEAE					
Casuarina equisetifolia L.	Saru	Tree	PSN713	Leaf, stem	ast, dia, dys, hac
CHENOPODIACEAE					
Basella rubra L.	Poi ni vel	Climber	-	Leaf	aphro, diu, lax, abs, bil, bo, bu, con, gon, hac, ul, vom
Beta vulgaris L.	Beet	Herb	PSN654	Leaf	aphro, car, diu, emmen, exp, pur, ton, con, eac, hac, infl, itc, para, sor, ul
Spinacia oleracea L.	Palak ni Bhaji	Herb	-	Leaf	cat, feb, stm, infl

Botanical name (Family, Genus, Species)	Vernacular Name	Habit	Voucher number	Part(s) extracted	Action/Therapeutic use
COMBRETACEAE					
Quisqualis indica L.	Jhumja vel	Shrub	PSN289	Aerial parts	anth
Terminalia chebula Retz.	Hardae	Tree	PSN292	Seed	anth, aphro, ast, car, dig, diu, feb, fla, lax, pur, stm, ton, ano, co, infl, jaun, ul, wo
COMMELINACEAE					
Commelina benghalensis L.	Motishumliyu	Herb	PSN731	Whole	diu, sti, dia, fev, lep
CONNARACEAE					
Rourea santaloides (Vahl.) Wight &. Arnott	Vardharo	Herb	-	Root	ton, diab, rheu, sk
CONVOLVULACEAE					
Argyreia nervosa (Burm. f.) Boj.	Samudrasosha	Climber	PSN492	Leaf	aphro, diu, pur, sti, rheu, swe
Cressa cretica L.	Paliyo	Herb	PSN496	Whole	anth, aphro, stm, ath, con, ton
Ipomoea pentaphylla Jacq.	Gariya vel	Climber	PSN522	Leaf	rheu, swe
CRUCIFERAE					
Lepidium sativum L.	Ashal/Aserio	Herb	PSN13	Seed	antc
CUCURBITACEAE					
Lagenaria vulgaris Seringe	Tumbada	Climber	PSN328	Fruit	ton, pur
Momordica charantia L.	Karela	Climber	PSN333	Fruit	anth, lax, sed, bron, co, elph, pil, ul
Mukia maderaspatana (L.) M.Roem.	Chadakachima	Climber	PSN335	Aerial parts	exp, sti
CUPRESSACEAE					
Thuja orientalis L.	Arbor-Vitae	Shrub	-	Leaf	arth, ath, bron, co, con, dys, hae, ins, mum, rheu sk
CYPERACEAE					
Cyperus rotundus L.	Moth, Shaiyo	Herb	PSN764	Whole	anth, aro, ast, dip, diu, emmen, stm, dia, dys, infl
Cyperus scarious R.Br.	Nagarmoth	Herb	PSN765	Seed	aro, ast, dip, stm, dia

Botanical name (Family, Genus, Species)	Vernacular Name	Habit	Voucher number	Part(s) extracted	Action/Therapeutic use
EHRETIACEAE					
Cordia dichotoma Forst.	Gunda	Tree	PSN472	Leaf	anth, ast, diu, dmu, exp, pur, ton, co, dyspep, fev, hac, jp, rw, sb, ul
EUPHORBIACEAE					
Croton bonplandianum Baill.	-	Herb	PSN673	Whole	-
Euphorbia hirta L.	Rati dudheli	Herb	PSN683	Whole	ast, stm, co, rw
Euphorbia tirucalli L.	Dandilyo thor	Shrub	PSN690	Stem	car, pur, stm, ath, drop, dyspep, gon, lep, neu, syp
Ricinus communis L.	Erado	Shrub	PSN699	Leaf	anth, aphro, car, cat, diu, gal, pur, ath, bron, co, con, drop, dyspep, fev, hac, infla, lep, lum, para, rheu, rw, sk
FABACEAE					
Abrus precatorius L.	Chanothi	Climber	PSN138	Leaf, stem	aphro, diu, em, pur, ton, dia, dys, para, rheu, scia, swe, ul
Arachis hypogaea L.	Magfali	Herb	PSN152	Leaf	ast, adp, bron, con, fla
Canavalia gladiata DC.	Talvardi	Climber	PSN157	Leaf	can
Cicer arietinum L.	Chana	Herb	PSN159	Whole	abor, anth, aphro, lax, pur, stm, ton, bron, con, dys, dyspep, fla, lep, sk, sb, vom,
Dalbergia sissoo Roxb.	Moto Shisham	Tree	PSN179	Leaf	anth, antpy, aphro, apt, ast, dig, diu, emmen, sti, bron, bu, co, dia, dys, dyspep, fev, gon, gou, haem, infl, lep, leucd, sca, scia, sk, syp, ul, vom
Glycyrrhiza glabra L.	Jethimadha	Shrub	-	Root	anal, aphro, dmu, emmen, exp, lax, pec, adp, anm, bron, co, epi, fev, lep, sk, sor, ul, vom, wo
Mucuna pruriens Bak.	Kauncha	Climber	PSN209	Leaf	sti, dys

Botanical name (Family, Genus, Species)	Vernacular Name	Habit	Voucher number	Part(s) extracted	Action/Therapeutic use
Psoralea corylifolia L.	Babchi	Herb			anth, aphro, dip, diu, lax, rub, sti, stm, der, lep, leucd, sca, ul
Tephrosia purpurea Pers.	Sarpankho	Herb	PSN223	Whole	anth, antpy, dig, diu, lax, pur, anm, ath, bo, bron, dysame, dyspep, elph, fev, fla, gin, gon, haem, infl, pim, sk, syp
Vigna radiata L.	Mag	Herb	PSN235	Whole	aphro, dig, feb, gal, ton, co, con, dia, dyspep, fev, fla, hae, infl, lep, pyr, sk
FUMARIACEAE					
Fumaria indica (Haussk.) Pugsley.	Pitpopdo	Herb	-	Seed	dip, diu
GENTIANACEAE					
Enicostema hyssopifolium (Willd.) I.C. Verd.	Mamejavo	Herb	PSN470	Whole	anth, antpr, ast, car, dig, lax, stm, con, diab, drop, dyspep, fev, fla, her, lep, sb, sk, swe, ul
GUTTIFERAE					
Mesua ferra Linn.	Nagkesar	Tree	-	Seed	aro, ast, col
LABIATAE					
Coleus aromaticus Benth.	Ajma pan	Herb	-	Leaf	apt, dig, ath, co, bron, dia, dyspep, gon, hac, pil
Ocimum americanum L.	Ramtulsi	Herb	PSN625	Whole	aro, dip, sti, ton, bron, car, cat, co, col, diu, dys, toac
Ocimum basilicum L.	Takmaria	Herb	PSN626	Whole	alexi, anpy, anth, car, col, dip, diu, dmu, exp, sti, stm, ath, bron, bu, co, con, dia, dys, haem, infl, mal, rheu, rw, sk
Ocimum kilimanjaricum L.	Kapurtulsi	Herb	-	Whole	col, diu
LAURACEAE					
Cinnamomum tamala Nees & Ebern.	Tamal patra	Tree	-	Leaf	car, diu, dip, gal, sti, co, dyspep, fev, fla

Botanical name (Family, Genus, Species)	Vernacular Name	Habit	Voucher number	Part(s) extracted	Action/Therapeutic use
LILIACEAE					
Aloe barbadensis Mill.	Kuamwar Pant	Shrub	PSN723	Leaf	anth, aphro, car, cat, col, dig, emmen, pur, stm, ton, ath, bil, bron, bu, fev, infl, jaun, lep, lum, pil, sk, ul
Allium sativum L.	Lasan	Herb	-	Whole	alex, anth, antsm, antsp, aphro, car, dig, diu, sti, ath, bron, co, diph,
LYTHRACEAE					
Ammannia baccifera L.	Jal aagiyo	Herb	PSN298	Whole	rheu
Woodfordia fruticosa Kurz.	Dhawadi phool	Shrub	PSN303	Flower	anth, ast, em, feb, sed, sti, bil, bu, diab, hae, lep, sk
MALVACEAE					
Abutilon indicum (L.) Sweet.	Kansaki	Shrub	PSN41	Leaf	anth, aphro, ast, feb, diu, dmu, lax, sed, ton, bo, co, fev, gon, hae, infl, lep, pil, toac, ul, vom
Thespesia populnea (L.) Sol ex Correa.	Paras piplo	Tree	PSN71	Leaf	ast, col, ath, chl, co, dia, diab, dys, gon, haem, her, infl, lep, psor, rw, sca, ul, wo
MORACEAE					
Artocarpus hetrophyllus Lam.	Fanas	Tree	-	Whole	abor, aphro, car, ton, bil, bo, dia, lep, sb, sk, ul, wo
Ficus benghalensis L.	Vad	Tree	PSN703	Aerial roots	aphro, ast, col, dip, em, ton, bil, dia, diab, dys, fev, gon, infla, leuch, lum, pil, rheu, sk, syp, toache, ul, vom
Ficus elastica Roxb.	Rubber plant	Tree	PSN705	Leaf	-

Botanical name (Family, Genus, Species)	Vernacular Name	Habit	Voucher number	Part(s) extracted	Action/Therapeutic use
MUSACEAE					
Musa paradisiaca L.	Kela	Herb	PSN719	Leaf	anth, aphro, apt, ast, col, ton, bil, bron, dyspep, infl, lep, sca
MYRSINACEAE					
Embelica ribes Burm. f	Vavding	Shrub	-	Seed	anth, ast, car, dig, feb, lax, stm, ton, ath, co, con, dyspep, fev, fla, lep, rw, sk, tum
MYRTACEAE					
Eucalyptus citriodora Hook	Nilgiri	Tree	-	Leaf	bron, ath
OLEACEAE					
Jasminum officinale L.	Jui	Shrub	PSN432	Aerial parts	anth, diu, emmen
PEDALIACEAE					
Sesamum indicum L.	Tal	Herb	PSN569	Whole	aphro, ast,col, sti, ton, bu, chl, dys, gon, haem, leucd, mig, ob, pil, ul
PIPERACEAE					
Piper longum L.	Piplimul	Climber	-	Root	anth, aphro, apt car, col, lax, sti, adp, ath, bil, bron, co, fev, gou, ins, infl, jaun, lep, leucd, lum, pil, tum
Piper nigrum L.	Mari	Climber	-	Root	anth, antpr, aphro, car, dig, diu, emmen, rub, sti, stm, ath, bron, co, der, dys, dyspep, fev, fla
PLUMBAGINACEAE					
Plumbago zeylanica L.	Chitrak	Shrub	PSN424	Root	abor, alex, anth, apt, ast, car, exp, lax, stm, ton, bron, dia, dys, dyspep, infl, itc, lep, leucd, pil, rheu, rw, sca, sk

Botanical name (Family, Genus, Species)	Vernacular Name	Habit	Voucher number	Part(s) extracted	Action/Therapeutic use
POACEAE					
Bambusa arundinaceae (Retz.) Roxb.	Vans, bamboo	Tree	PSN793	Leaf	aphro, ast, col, diu, emmen, feb, lax, sti, ton, bil, bron, bu, co, dia, eac, fev, gon, jp, lep, lum, pil, rw
Cymbopogon citratus (DC.) Stapf.	Leelicha	Herb	PSN808	Leaf	alex, anth, aphro, apt, car, dip, lax, rub, sti, stm, ton, bron, bu, dia, epi,fev, fla, lep, neu, rheu
Cynodon dactylon (L.) Pers.	Dhrokhad	Herb	PSN811	Whole	ast, diu, drop, urd, wo
RUBIACEAE					
Gardenia resinifera Roth.	Dikamari	Tree	PSN351	Gum exudate	car, fla, indi, sk
SANTALACEAE					
Santalum album L.	Chandan	Tree	PSN664	Leaf	alex, antpy, aphro, c. ton, dip, diu, dmu, exp, amen, bo, psy, co, dys, jaun, lep
SAPINDACEAE					
Cardiospermum halicacabum L.	Karodiyo	Herb	PSN129	Leaf	ast, dip, diu, dmu, emmen, lax, ton, dysame, eac, fev, hac, lum, pil, rheu, sb, sor, swe, tum
SAPOTACEAE					
Manilkara hexandra (Roxb.) Dubard.	Rayan	Tree	PSN428	Leaf	aphro, col, ton, bil, bron, lep, ul, urd
SCROPHULARIACEAE					
Picrrohiza Kurroa L.	Kadu	Herb	-	Rhizome	verm, ath, con, fev
SOLANACEAE					
Solanum khasianum C. B. Clarke.	Ubhi ringani	Herb	-	Whole	arth, infl
Solanum surattense Burm.f.	Bethi bhony ringani	Herb	PSN543	Whole	apt, car, diu, bron, cd, chp, co, fev, jaun, leud, rheu, swe, toac

Botanical name (Family, Genus, Species)	Vernacular Name	Habit	Voucher number	Part(s) extracted	Action/Therapeutic use
TRAPACEAE					
Trapa natans L.	Singara	Herb	-	Rind	antpy, ,aphro, apt, ast, col, diu, ton, bron, bu, dia, dys, dyspep, fat, fev, hae, infl, lep, lum, phg
UMBELLIFERAE					
Centella asiatica (L.) Urb.	Brahmi	Herb	PSN340	Leaves	apt, b.ton, c.ton, car, diu, stm, ath, bron, hac, lep, sk, syp
Daucus carota L.	Gajar	Herb	PSN343	Aerial parts	anth, aphro, apt, aro, c.ton, car, dig, diu, exp, sti, stm, verm, ano, ath, bron, co, con, dia, diab, dyspep, fla, haem, , jaun, lep, tum
VERBENACEAE					
Gmelina arborea Roxb.	Shivan	Tree	PSN604	Leaf	anth, aphro, ast, col, diu, gal, lax, stm, ton, anm, co, con, diab, dyspep, ecz, fev, gon, gou, haem, lep, leuch, sk, ul
Lantana camera L.	Abhagani	Shrub	PSN605	Whole	antsp, car, cat, dip, ton, ecz, epi, fev, mal, rheu, sk, tum
Vitex negundo L.	Nagod	Tree	PSN611	Leaf	antpy, ast, car, dig, diu, emmen, exp, feb, stm, ton, arth, chl, co, der, dys, dysame, dyspep, fev, fla, gou, hac, haem, infl, lep, rheu, scia, ul, verm, wo
VITACEAE					
Cissus quadrangularis L.	Hadsankar	Climber	PSN127	Stem	anal, fra, mup, pil, tum, ul, wo
Vitis vinifera L.	Darakh	Climber	PSN128	Leaf	aphro, c.ton, dig, diu, emmen, exp, feb, lax, stm, ame, anm, ath, bron,

Botanical name (Family, Genus, Species)	Vernacular Name	Habit	Voucher number	Part(s) extracted	Action/Therapeutic use
ZINGIBERACEAE					
Hedychium spicatum L.	Kapur kachri	Herb	-	Rhizome	ath, bron, infl, nau
ZYGOPHYLLACEAE					
Fagonia cretica L..	Dhamaso	Shrub	PSN98	Whole	alex, ast, emmen, feb, lax, sti, stm, ton, abs, ath, bil, bo, deli, drop, dys, fev, leud, rheu, sb, sca, tac, tum, urd, vom

Key to abbreviations in Table 1

DISEASES

A	**C**	dys - dysentry	**H**	**L**	pil - piles	swe - swellings
abs - abscesses	calc - calculi	dysame -	hac - headache	leucd - leucoderma	pim - pimples	syp - syphilis
adp - abdominal	can - cancer	dysamenorrhoea	hae - haemmorrhage	leuch - leucorrhoea	pneu - pneumonia	**T**
pain	cd - cold	dyspep - dyspepsia	haem - haemorrhoids	lep leprosy	psor - psoriasis	toac - tooth ache
aly - allergy	chl- cholera	**E**	her - hernia	lum - lumbago	psy - psycopathy	tum - tumors
ame - amentia	chp - chest pain	eac-earache	hp - hydrophobia	**M**	pyr - pyrexia	typh typhoid
amen -	co - cough	ecz - eczema	hys - hysteria	mal - malaria	**R**	**U**
amenorrhoea	con - constipation	elph - elephantiasis		mig - migraine	rheu - rheumatism	ul - ulcers
anm - anaemia	**D**	epi - epilpsy	**I**	mum - mumps	rw - ringworm	urd - urinary
ano - anorexia	deli -delirium	**F**	indi - indigestion	mup - muscular	**S**	disorders
arth - arthritis	den fev - dengue	fat - fatigue	infl - inflammations	pain	sb - snake bite	**V**
ath - asthma	fever	fev - fever	ins - insomnia	**N**	sca - scabies	vom - vomiting
B	der - dermatitis	fla - flatulence	itc - itch	neu - neuralgia	scia - sciatica	**W**
bil - biliousness	dia - diarrohea	fra - fracture	**J**	**O**	sk - skin disease	wo - wounds
bl dis - blood diseases	diab - diabetes	**G**	jaun - jaundice	ob - obesity	sls - sleeping	wor - worms
bo - boils	diph - diphtheria	gin - gingivitis	jp - joint pain	**P**	sickness	
bron - bronchitis	drop - dropsy	gon - gonorrhoea		para - paralysis	smp - small pox	
bu - burns		gou - gout		phg - pharyngitis	sor - sores	

MEDICINAL PROPERTIES

A	antpy - antipyretic	**B**	**D**	emmen - emmenagogue	**P**	sed - sedative
abor - abortifacient	antsp - antiseptic	b.ton - brain tonic	dig - digestive	exp - expectorant	pec - pectoral	sti - stimulant
alex - alexipharmic	antsp - antispasmodic	**C**	dip - diphoretic	**F**	pur - purgative	**T**
anal - analgesic	aphro - aphrodisiac	c.ton - cardiotonic	diu - diuretic	feb - febrifuge	**R**	ton - tonic
antd - antidote	apt - apetiser	car - carminative	dmu - demulcent	**G**	rub - rubefacient	
anth - anthelmentic	aro - aromatic	cat - cathartic	**E**	gal - galactagogue	**S**	**V**
anthy - antihypertensive	ast - astringent	col - coolant	em - emetic	**L**	stm - stomachic	verm - vermifuge
antpr - antiperiodic				lax - laxative		

septicemia (Levine, 1987; Singh et al., 2000). *Proteus mirabilis* which is also Gram-negative motile rod, causes urinary tract infections in the elderly and young males often following catheterization or cystoscopy, wound infection also often as a secondary invader of ulcer, pressure sores, burn and damaged tissue and septicemia and occasionally meningitis and chest infections (Cheesbrough, 2000). *Salmonella typhi* is also a pathogenic bacteria causing enteric fevers such as typhoid and paratyphoid, gastroenteritis and septicemia (Cheesbrough, 2000). Infections caused by *Pseudomonas aeruginosa* are among the most difficult to treat with conventional antibiotics (Levison and Jawetz, 1992). *P. aeruginosa* is the most prevalent pathogen capable of causing life-threatening illnesses (Lory, 1990). This bacterium can cause clinically significant infections such as wounds and burn infections, when it is introduced by lumbar punctures and urinary tract infections when introduced by catheters and instruments or irrigating solutions (Murray et al., 1990). In infants or debilitated persons the bacterium may invade the blood stream and result in fatal condition (Lory, 1990). Some strains causing septicemia and pneumonia in cystic fibrosis and immunocompromised patients are becoming difficult to treat with currently available antimicrobial agents (Senda et al., 1996). This organism is one of the most common pathogens associated with bacterial corneal ulcers. Keratitis due to this pathogen also has been observed in those who wear extended-wear contact lenses (Lory, 1990). The yeast *Candida albicans* is common commensals of the gastrointestinal and urogenital tracts of human (Black, 1996; Cheesbrough, 2000) and is the cause of candidiasis in woman (Demarch et al., 1995).

The present investigation revealed that all the plant species from 54 families showed varied levels of antimicrobial activity against tested microorganisms (Table 2.2). Acanthaceae (2 plants) inhibited 28.33 % of the tested microorganisms; Amaranthaceae (2 plants) inhibited 25 %; Anacardiaceae (1 plant) inhibited 70 %; Apocynaceae (5 plants) inhibited 31.33 %; Araceae (1 plant) inhibited 33.33 %; Aristolochiaceae (1 plant) inhibited 20 %; Asclepiadaceae (2 plants) inhibited 18.33 %; Asteraceae (5 plants) inhibited 26.66 %; Balanitaceae (1 plant) inhibited 33.33 %; Bignonaceae (2 plants)

inhibited 23.33 %; Caesalpiniaceae (7 plants) inhibited 39.52 %; Capparidaceae (1 plant) inhibited 20 %; Casuarinaceae (1 plant) inhibited 73.33 %; Chenopodiaceae (3 plants) inhibited 13.33 %; Combretaceae (2 plants) inhibited 68.33 %; Commelinaceae (1 plant) inhibited 10 %; Connaraceae (1 plant) inhibited 26.67 %; Convolvulaceae (3 plants) inhibited 16.67 %; Cruciferae (1 plant) inhibited 8.33 %; Cucurbitaceae (3 plants) inhibited 22.22 %; Cyperaceae (2 plants) inhibited 35 %; Ehretiaceae (1 plant) inhibited 33.33 %; Euphorbiaceae (4 plants) inhibited 50 %; Fabaceae (10 plants) inhibited 22.33 %; Fumariaceae (1 plant) inhibited 30 %; Gentianaceae (1 plant) inhibited 33.33 %; Guttiferae (1 plant) inhibited 33.33 %; Labiatae (4 plants) inhibited 25 %; Lauraceae (1 plant) inhibited 36.67 %; Liliaceae (2 plants) inhibited 23.33 %; Lythraceae (2 plants) inhibited 60 %; Malvaceae (2 plants inhibited 28.33 %; Moraceae (3 plants) inhibited 21.12 %; Musaceae (1 plant) inhibited 36.67 %; Casuarinaceae (1 plant) inhibited 73.33 %; Myrsinaceae (1 plant) inhibited 40 %; Myrtaceae (1 plant) inhibited 66.67 %; Oleaceae (1 plant) inhibited 23.33 %; Pedaliaceae (1 plant) inhibited 30 %; Piperaceae (2 plants) inhibited 30 %; Plumbaginaceae (1 plant) inhibited 40 %; Poaceae (3 plants) inhibited 22.22 %; Rubiaceae (1 plant) inhibited 26.67 %; Santalaceae (1 plant) inhibited 26.67 %; Sapindaceae (1 plant) inhibited 10 %; Sapotaceae (1 plant) inhibited 60 %; Scitaminaceae (1 plant) inhibited 33.33 %; Scrophulariaceae (1 plant) inhibited 30 %; Solanaceae (2 plants) inhibited 15 %; Thujaceae (1 plant) inhibited 30 %; Trapaceae (1 plant) inhibited 73.33 %; Umbelliferae (2 plants) inhibited 25 %; Verbenaceae (3 plants) inhibited 23.33 %; Vitaceae (2 plants) inhibited 50 % and Zygophyllaceae (1 plant) inhibited 33.33 % of the tested microorganisms.

Amongst the 15 microbial strains tested, the most susceptible bacteria was *B. cereus* which is a Gram-positive bacteria and the most resistant bacteria was *E. coli* which is a Gram-negative bacteria. The results (Table 2.2) obtained from screening showed that 47.03 % Gram-positive bacteria while 23.62 % Gram-negative bacteria were inhibited by the aqueous as well as methanol/ethanol extracts. The aqueous extracts were active against 26.11% Gram-positive bacteria and 11.12 % Gram-negative bacteria whereas the methanol/ethanol

extracts were active against 67.96 % Gram- positive bacteria and 36.12 % Gram-negative bacteria. Amongst 108 plant species, the most active plant was *Terminalia chebula* Retz. while the most inactive plant was *Solanum surattense* Burm.f.

For Gram-positive bacteria, the results (Table 2.2) showed that 58.33 % extracts (29.63 % of aqueous extract and 87.04 % of methanol/ethanol) were active against *B. cereus;* 49.07 % extracts (17.6 % of aqueous extract and 80.55 % of methanol/ethanol) were active against *B. subtilis;* 40.74 % extracts (37.96 % of aqueous extract and 43.52 % of methanol/ethanol) were active against *S. aureus,* 35.18 % extracts (29.63 % of aqueous extract and 40.74 % of methanol/ethanol) were active against *S. epidermidis* and 51.84 % extracts (15.74 % of aqueous extract and 87.96 % of methanol/ethanol) were active against *S. subfava*. For Gram-negative bacteria, the results showed that 25 % extracts (12.96 % of aqueous extract and 37.03 % of methanol/ethanol) were active against *A. fecalis;* 15.74 % extracts (2.77 % of aqueous extract and 28.70 % of methanol/ethanol) were active against *E. aerogenes;* 7.40 % extracts (1.85 % of aqueous extract and 12.96 % of methanol/ethanol) were active against *E. coli;* 54.16 % extracts (22.22 % of aqueous extract and 86.12 % of methanol/ethanol) were active against *K. pneumoniae;* 53.70 % extracts (25 % of aqueous extract and 82.40 % of methanol/ethanol) were active against *P. mirabilis;* 16.67 % extracts (4.63 % of aqueous extract and 28.70 % of methanol/ethanol) were active against *P. vulgaris;* 12.96 % extracts (4.63 % of aqueous extract and 21.30 % of methanol/ethanol) were active against *P. aeruginosa;* 17.12 % extracts (16.67 % of aqueous extract and 17.60 % of methanol/ethanol) were active against *P. pseudoalcaligenes* and 9.72 % extracts (9.25 % of aqueous extract and 10.18 % of methanol/ethanol) were active against *S. typhimurium.* For an yeast strain, 18.98 % extracts (0.93 % of aqueous extract and 37.03 % of methanol/ethanol) were active against *C. tropicalis*.

Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Traditional

healers use primarily water as solvent but in the present study it was observed that the plant extracts extracted in organic solvent (methanol or ethanol) showed profoundly distinct antibacterial activity than aqueous extract. This shows that the active components are better soluble in organic solvent as also suggested by de Boer et al., (2005). These observations can be rationalized in terms of the polarity of the compound being extracted by each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the media used in the assay. The growth media also seem to play an important role in the determination of the antibacterial activity. Lin et al., (1999) reported that Mueller-Hinton agar appears to be the best medium to explicate the antibacterial activity and the same was used in the present study.

In general, the plant antibiotic substances appear to be more inhibitory to Gram-positive organisms that to the Gram-negative type since Gram-negative bacteria can be accounted for the presence of thick murein layer, which prevents the entry of inhibitors (Martin, 1995). In present findings also Gram-positive bacteria were found to be more susceptible than Gram-negative bacteria. This could be due to the fact that the cell wall of Gram-positive bacteria is less complex and lack the natural sieve effect against large molecules due to the small pores in their cell envelope (Hawkey, 1998; Gould and Booker, 2000; Parekh et al., 2005; Parekh and Chanda, 2006c; Parekh and Chanda, 2007a). The fact that extracts are more active against Gram-positive bacteria can be attributed to the fact that the cell wall of the Gram-positive bacteria is easier to penetrate than that of Gram-negative bacteria (Rang et al., 1987). Also the organic solvents extracts of all the plant species were found to have good activity against bacteria than yeast. These differences in the activity may be due to the difference in the cell wall pattern (Ozcelik , 1998; Yao and Moellering, 1995).

The negative results obtained against Gram-negative bacteria were not unexpected since this class of bacteria is usually more resistant than Gram-positive bacteria (Tomas-Barberan et al., 1988). Antimicrobial extracts from plants can be assumed to be useful in combating infectious diseases. Therefore,

Table 2.2 Screening of some plant species for potential antimicrobial activity

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
ACANTHACEAE																
Andrographis paniculata (Burm.f.) Wall.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	3	1	-	4	2	-	-	-	-	2	-	-	-	-	2
Eranthemum spp. L.	H_20	4	-	2	11	-	-	-	-	-	2	-	-	-	-	-
	EtOH	1	1	-	-	1	2	-	-	2	1	-	1	-	-	-
AMARANTHACEAE																
Achyranthas aspera L.	H_20	4	-	2	15	-	-	-	-	-	4	-	-	-	-	-
	EtOH	2	1	-	-	-	2	-	-	7	2	-	-	-	-	-
Celosia argentea L.	H_20	-	-	3	7	-	-	-	-	3	-	-	-	-	-	-
	EtOH	2	-	-	-	1	-	-	-	1	-	-	-	-	-	-
ANACARDIACEAE																
Mangifera indica L.	H_20	6	-	2	3	3	-	-	-	4	5	-	3	6	-	-
	EtOH	14	5	12	9	9	11	8	15	22	8	3	7	11	-	-
APOCYNACEAE																
Carissa congesta Wt.	H_20	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	10	2	4	4	2	-	-	-	6	4	-	1	-	-	3
Ervatima coronaria Stapf.	H_20	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	7	-	-	-	1	-	-	-	-	5	-	-	-	-	-
Holarrhena antidysenterica (Heyne. ex Roth.) A. DC.	H_20	-	-	9	-	-	-	-	-	-	-	-	-	9	-	-
	MeOH	6	3	3	3	3	2	2	-	12	2	2	1	13	-	3
Rauvolfia serpentina (L.) Benth ex Kurz.	H_20	-	-	-	4	-	-	-	-	2	3	-	-	-	-	-
	EtOH	3	1	-	2	2	2	-	-	6	3	-	1	-	-	2
Thevetia peruviana (Pers.) Merr.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	12	1	-	1	2	-	-	-	-	1	1	-	-	-	-

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
ARACEAE																
Acorus calamus L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	5	5	-	6	4	2	2	-	9	1	2	-	-	-	6
ARISTOLOCHIACEAE																
Aristolochia bracteolata Lam.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	15	8	20	-	10	-	-	-	15	8	-	-	-	-	-
ASCLEPIADACEAE																
Calotropis gigantea (L.) R. Br.	H_20	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-
	EtOH	2	1	-	-	2	2	-	-	6	1	-	1	-	-	2
Tylophora indica (Burm.f.) Merr.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	2	-	-	-	-	-	-	-	3	-	-	-	-	-	-
ASTERACEAE																
Launaea procumbens (Roxb.) Ram. & Raj.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	15	7	20	12	9	-	-	-	2	8	-	-	-	-	-
Saussurea lappa Costus.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	8	4	5	2	7	3	-	-	12	3	2	-	-	-	2
Tridax procumbens L.	H_20	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-
	EtOH	-	1	-	-	1	-	-	-	4	-	-	1	-	-	1
Vernonia anthelmintica (L.) Willd.	H_20	3	-	2	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	1	1	-	-	2	1	-	-	4	-	-	-	-	-	3
Xanthium strumarium L.	H_20	-	-	3	2	-	-	-	-	-	-	-	-	-	-	-
	EtOH	7	3	4	-	7	-	4	-	9	5	-	-	-	-	-
BALANITACEAE																
Balanites aegyptiaca (L.) Del.	H_20	5	-	4	11	-	-	-	-	-	-	-	-	-	4	-
	EtOH	3	1	-	-	3	1	-	-	3	-	-	-	-	-	2

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
BIGNONACEAE																
Spathodea campanulata Beauv.	H_20	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	-	-	-	-	2	-	3	-	3	-	-	-	-	-	-
Tecomella undulata (Sm.) Seem.	H_20	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	7	3	2	2	3	2	-	-	6	2	-	-	-	-	1
CAESALPINIACEAE																
Bauhinia variegata L.	H_20	-	-	2	-	-	-	-	-	2	-	-	-	-	-	-
	MeOH	8	4	9	7	5	3	-	2	10	3	3	-	8	-	-
Caesalpinia crista L.	H_20	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-
	EtOH	4	1	-	-	1	-	-	-	5	2	-	-	-	-	2
Caesalpinia pulcherrima (L.) Swartz.	H_20	5	2	2	6	3	-	-	-	6	5	2	-	4	3	-
	MeOH	11	3	9	13	7	6	6	5	10	6	5	5	13	8	4
Cassia angustifolia Vahl.	H_20	-	5	6	3	5	-	-	-	3	3	-	-	2	-	-
	EtOH	6	3	-	5	3	-	-	-	-	3	-	-	-	-	-
Cassia fistula L.	H_20	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	8	2	3	-	1	1	-	-	3	2	-	-	-	-	-
Cassia tora L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	7	2	-	1	2	-	-	-	4	1	-	-	-	-	-
Delonix regia (Boj.) Raf.	H_20	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	8	2	6	2	2	3	-	-	6	4	1	-	1	-	4
CAPPARIDACEAE																
Crataeva religiosa Forst.f..	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	-	1	-	3	2	-	-	-	5	1	1	-	-	-	-

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
CASUARINACEAE																
Casuarina equisetifolia L.	H_20	6	1	4	4	3	-	-	-	3	3	-	-	4	7	-
	MeOH	10	3	8	6	5	3	3	3	8	7	8	-	6	9	-
CHENOPODIACEAE																
Basella rubra L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	-	-	-	-	-	-	-	-	5	2	-	-	-	-	-
Beta vulgaris L.	H_20	-	-	2	4	-	-	-	-	1	-	-	-	-	-	-
	EtOH	3	-	-	-	1	-	-	-	1	-	-	-	-	-	-
Spinacia oleracea L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	3	2	-	-	-	-	-	-	1	2	-	-	-	-	-
COMBRETACEAE																
Quisqualis indica L.	H_20	8	-	2	-	5	-	-	-	4	-	-	-	-	-	-
	EtOH	4	2	-	1	2	-	-	-	-	1	-	-	6	-	1
Terminalia chebula Retz.	H_20	11	7	15	8	7	5	5	3	9	8	8	5	12	12	4
	MeOH	27	12	23	21	15	17	7	7	20	16	15	7	22	4	4
COMMELINACEAE																
Commelina benghalensis L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	1	-	-	-	-	-	-	-	2	-	-	-	-	-	2
CONNARACEAE																
Rourea santaloides (Vahl.) Wight &. Arnott	H_20	-	-	3	9	2	-	-	-	-	-	-	-	-	-	-
	EtOH	3	-	-	-	4	-	3	-	6	2	-	-	-	-	-
CONVOLVULACEAE																
Argyreia nervosa (Burm. f.) Boj.	H_20	3	-	5	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	-	1	-	-	2	1	-	-	6	1	-	-	-	-	3

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
Cressa cretica L.	H_20	-	-	3	6	-	-	-	-	-	-	-	-	-	-	-
	EtOH	9	-	-	-	-	-	-	-	4	-	-	-	-	-	-
Ipomoea pentaphylla Jacq.	H_20	6	6	-	5	2	-	-	-	-	4	-	-	-	5	-
	EtOH	4	-	-	-	2	-	-	-	2	2	-	1	-	-	1
CRUCIFERAE																
Lepidium sativum L.	H_20	-	-	12	2	2	-	-	-	-	-	-	-	-	3	-
	MeOH	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-
CUCURBITACEAE																
Lagenaria vulgaris Seringe	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	6	4	1	-	3	-	-	-	7	3	-	-	-	-	-
Momordica charantia L.	H_20	2	-	-	2	-	-	-	-	-	-	-	-	-	-	-
	MeOH	10	7	7	2	4	1	2	-	11	2	-	-	-	-	-
Mukia maderaspatana (L.) M.Roem.	H_20	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	4	-	-	-	-	-	-	-	4	-	-	-	-	-	-
CYPERACEAE																
Cyperus rotundus L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	10	3	4	5	4	3	2	-	5	5	2	-	1	-	-
Cyperus scarious R.Br.	H_20	2	-	-	-	-	-	-	-	-	1	-	-	-	-	-
	MeOH	8	3	3	-	3	-	1	-	4	5	-	-	-	-	3
EHRETIACEAE																
Cordia dichotoma Forst.	H_20	-	3	5	1	-	-	-	-	-	1	-	-	3	-	-
	EtOH	3	1	-	-	2	-	-	-	3	1	-	-	-	-	-

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
EUPHORBIACEAE																
Croton bonplandianum Baill.	H_20	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	4	3	-	2	2	-	-	-	5	2	-	1	-	-	2
Euphorbia hirta L.	H_20	6	2	2	2	2	1	-	-	4	-	1	1	6	4	-
	MeOH	15	3	7	7	4	7	-	-	12	8	15	6	15	15	-
Euphorbia tirucalli L.	H_20	2	-	-	-	-	-	-	-	-	-	-	-	6	-	-
	MeOH	10	2	2	2	4	2	9	2	12	4	8	3	11	-	-
Ricinus communis L.	H_20	2	-	-	-	-	1	-	-	2	2	-	-	3	-	-
	MeOH	9	1	8	-	2	2	-	-	4	1	-	-	-	-	1
FABACEAE																
Abrus precatorius L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	2	1	-	-	3	-	1	-	-	3	-	-	-	-	-
Arachis hypogaea L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	2	1	3	7	3	1	-	-	3	-	-	-	-	-	2
Canavalia gladiata DC.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1
Cicer arietinum L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-
	MeOH	7	3	1	-	3	2	2	-	7	4	2	-	-	-	-
Dalbergia sissoo Roxb.	H_20	-	-	4	1	-	-	-	-	-	1	-	-	-	-	-
	EtOH	2	2	-	2	3	-	-	-	10	2	-	-	-	-	-
Glycyrrhiza glabra L.	H_20	-	-	6	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	12	7	7	-	8	-	3	-	12	7	-	-	-	-	-
Mucuna pruriens Bak.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	-	-	-	-	-	-	-	-	4	2	-	-	-	-	-

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
Psoralea corylifolia L.	H_20	2	-	-	-	-	-	-	-	2	-	-	-	-	-	-
	MeOH	7	8	6	-	5	-	-	-	15	1	-	-	-	-	3
Tephrosia purpurea Pers.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	3	2	-	3	2	-	-	-	-	3	2	-	-	-	2
Vigna radiata L.	H_20	-	-	3	1	-	-	-	-	-	1	-	-	-	-	-
	EtOH	3	1	-	-	2	-	-	-	3	1	-	-	-	-	-
FUMARIACEAE																
Fumaria indica (Haussk.) Pugsley.	H_20	-	-	5	1	-	-	-	-	1	1	-	-	-	-	-
	EtOH	2	2	-	1	1	-	-	-	2	-	-	-	-	-	-
GENTIANACEAE																
Enicostema hyssopifolium (Willd.) I.C. Verd.	H_20	-	-	-	-	-	2	-	-	3	-	-	-	-	-	-
	EtOH	3	2	-	-	1	2	-	-	2	4	-	1	-	-	2
GUTTIFERAE																
Mesua ferra Linn.	H_20	4	5	2	-	4	-	-	-	5	4	-	-	-	-	-
	MeOH	12	16	13	-	16	4	1	-	20	23	3	-	-	-	-
LABIATAE																
Coleus aromaticus Benth.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	3	2	-	-	2	-	-	-	6	2	-	-	-	-	-
Ocimum americanum L.	H_20	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-
	EtOH	6	2	1	-	1	-	-	-	9	9	-	-	-	1	2
Ocimum basilicum L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	10	3	-	2	4	-	-	-	-	5	-	-	-	-	-
Ocimum kilimanjaricum L.	H_20	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	4	4	2	-	4	3	2	3	5	5	4	-	2	-	-

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
LAURACEAE																
Cinnamomum tamala Nees & Ebern.	H_20	-	-	3	1	-	-	-	-	-	1	-	-	-	-	-
	EtOH	6	4	-	3	4	2	-	-	7	4	-	-	-	-	4
LILIACEAE																
Aloe barbadensis Mill.	H_20	-	-	4	-	-	-	-	-	-	1	-	-	-	-	-
	EtOH	1	1	-	2	2	-	-	-	2	1	-	-	-	-	-
Allium sativum L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	4	2	-	-	2	-	3	-	13	3	-	-	-	-	-
LYTHRACEAE																
Ammannia baccifera L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	6	3	3	-	3	-	-	6	3	2	2	-	16	-	-
Woodfordia fruticosa Kurz.	H_20	12	2	9	-	3	7	10	10	10	6	-	5	11	9	-
	MeOH	17	6	15	9	10	14	8	14	19	10	8	9	21	8	-
MALVACEAE																
Abutilon indicum (L.) Sweet.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	-	1	-	-	2	-	-	-	11	-	1	-	-	-	1
Thespesia populnea (L.) Sol ex Correa.	H_20	3	-	3	-	-	-	-	-	2	1	-	-	-	-	-
	EtOH	5	4	4	-	2	4	-	-	7	-	-	3	2	-	-
MORACEAE																
Artocarpus hetrophyllus Lam.	H_20	-	-	3	9	-	-	-	-	-	-	-	-	-	-	-
	EtOH	7	2	4	-	6	-	3	-	-	4	-	-	-	-	-
Ficus benghalensis L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	7	2	3	2	2	1	-	-	6	4	-	-	-	-	1
Ficus elastica Roxb.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	-	-	-	-	-	-	-	-	6	3	-	-	-	-	-

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
MUSACEAE																
Musa paradisiaca L.	H_20	-	8	-	8	2	-	-	-	-	5	-	-	-	3	-
	EtOH	1	-	-	-	1	-	-	-	-	1	1	1	-	-	1
MYRSINACEAE																
Embelica ribes Burm. f	H_20	2	-	3	3	2	-	-	-	2	2	-	-	5	-	-
	EtOH	4	1	-	3	2	-	-	-	-	2	-	-	-	-	-
MYRTACEAE																
Eucalyptus citriodora Hook	H_20	4	-	-	7	-	-	-	-	2	12	-	-	3	-	-
	EtOH	12	7	10	15	9	12	-	6	17	11	5	9	15	14	3
OLEACEAE																
Jasminum officinale L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	3	1	-	1	2	-	10	-	1	2	-	-	-	-	-
PEDALIACEAE																
Sesamum indicum L.	H_20	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-
	EtOH	5	1	1	-	2	-	-	-	2	4	-	-	-	1	1
PIPERACEAE																
Piper longum L.	H_20	-	2	-	3	-	-	-	-	-	-	-	-	-	-	-
	EtOH	7	2	-	-	6	-	3	-	8	4	-	-	-	6	-
Piper nigrum L.	H_20	3	1	3	-	-	-	-	-	2	-	-	-	4	-	-
	MeOH	-	2	-	-	2	-	-	-	-	5	1	-	-	-	-
PLUMBAGINACEAE																
Plumbago zeylanica L.	H_20	3	1	1	-	-	-	-	-	-	2	-	-	-	-	-
	MeOH	7	4	6	2	4	-	-	-	11	2	2	-	-	-	-

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
POACEAE																
Bambusa arundinaceae (Retz.) Roxb.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	-	-	-	-	-	-	-	-	3	-	-	-	-	-	2
Cymbopogon citratus (DC.) Stapf.	H_20	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
	EtOH	7	3	1	-	2	-	-	-	3	3	-	-	-	2	3
Cynodon dactylon (L.) Pers.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	2	2	1	-	1	1	2	-	3	1	-	-	-	-	1
RUBIACEAE																
Gardenia resinifera Roth.	H_20	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	11	7	3	-	7	-	-	-	13	6	-	-	-	-	-
SANTALACEAE																
Santalum album L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	2	4	9	-	2	2	2	-	5	2	-	-	-	-	-
SAPINDACEAE																
Cardiospermum halicacabum L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	-	-	2	-	2	-	-	-	3	-	-	-	-	-	-
SAPOTACEAE																
Manilkara hexandra (Roxb.) Dubard.	H_20	-	-	2	4	2	3	-	-	2	-	2	-	-	-	-
	MeOH	11	4	7	10	4	7	2	2	10	7	5	-	7	-	-
SCITAMINACEAE																
Hedychium spicatum L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	4	2	-	4	4	2	2	-	6	2	2	-	-	-	3
SCROPHULARIACEAE																
Picrrohiza Kurroa L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-
	MeOH	10	3	2	-	4	3	-	-	5	3	2	-	-	-	-

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
SOLANACEAE																
Solanum khasianum C. B. Clarke.	H_20	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-
	EtOH	3	2	-	-	2	-	-	-	3	2	-	1	-	-	1
Solanum surattense Burm.f.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-
THUJACEAE																
Thuja orientalis L.	H_20	4	-	-	-	-	-	-	-	3	-	-	-	-	-	-
	EtOH	2	1	-	1	2	-	-	-	3	1	2	-	-	-	-
TRAPACEAE																
Trapa natans L.	H_20	5	-	7	-	2	-	-	-	2	3	2	-	10	-	-
	MeOH	14	7	11	11	9	8	4	5	14	12	10	15	16	11	10
UMBELLIFERAE																
Centella asiatica (L.) Urb.	H_20	-	2	2	7	-	-	-	-	-	-	-	-	-	-	-
	EtOH	5	-	4	-	6	-	3	-	6	2	-	-	-	-	-
Daucus carota L.	H_20	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	4	1	-	-	-	-	4	-	6	2	-	-	-	-	-
VERBENACEAE																
Gmelina arborea Roxb.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	3	1	-	2	2	-	-	-	5	4	-	-	-	-	-
Lantana camera L.	H_20	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-
	EtOH	5	3	-	6	4	-	-	-	-	3	2	-	-	-	-
Vitex negundo L.	H_20	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-
	EtOH	12	4	1	-	11	-	-	-	4	2	-	-	-	-	3

Botanical name (family, genus, species)	Extract	Inhibition Zone (mm)#														
		Bc*	Bs*	Sa*	Se*	Ss*	Af**	Ea**	Ec**	Kp**	Pm**	Pv**	Pa**	Pp**	St**	Ct***
VITACEAE																
Cissus quadrangularis L.	H_20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MeOH	3	-	-	-	1	1	-	-	-	2	-	4	-	-	-
Vitis vinifera L.	H_20	6	3	3	4	3	3	5	-	7	3	-	2	10	2	-
	MeOH	12	3	6	3	5	1	5	6	6	7	2	2	3	-	-
ZYGOPHYLLACEAE																
Fagonia cretica L.	H_20	-	-	-	4	-	-	-	-	-	4	-	-	-	-	-
	EtOH	3	1	-	-	2	7	-	1	7	7	-	1	-	-	-

H_20: Aqueous extract, EtOH: Ethanol extract, MeOH: Methanol extract;

#Values are the mean of inhibition zone diameter and subtracted from the control;

*Gram positive bacteria - Bc: *Bacillus cereus,* Bs: *Bacillus subtilis,* Sa: *Staphylococcus aureus,* Se: *Staphylococcus epidermidis,* Ss: *Staphylococcus subfava;*

**Gram negative bacteria - Af: *Alcaligenes fecalis,* Ea: *Enterobacter aerogenes,* Ec: *Escherichia coli,* Kp: *Klebsiella pneumoniae,* Pm: *Proteus mirabilis,* Pv: *Proteus vulgaris,* Pa: *Pseudomonas aeruginosa,* Pp: *Pseudomonas pseudoalcaligenes,* St: *Salmonella typhimurium;*

***Yeast - Ct: *Candida tropicalis*

there is a compelling reason to suppose that anti-infective agents could be active against human pathogens as was suggested by folkloric and historical accounts (Kirtikar and Basu, 1968; Nandkarni, 1976).

The present investigation justified the folklore use of some plant species. The potential for developing antimicrobial drug through screening of higher plants is rewarding as it will lead to the development of a phytomedicine to act against microbes (Hostettmann and Terreaux, 2000). Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose without any side effects that are often associated with synthetic compounds (Hamburger and Hostettmann, 1991). The most active plant extracts was further subjected for the evaluation of antifungal potency.

2.3.2 Antifungal screening

Fungal infections remain a significant cause of morbidity and mortality despite advances in medicine and the emergence of new antifungal agents (McNeil et al., 2001). Immunocompromised patients are particularly at risk of developing these infections with *Candida and Aspergillus spp.* as mycoses being the most commonly identified (Florea et al., 2002). Patients who develop candidemia have a greater chance of prolonged hospitalization and have a mortality rate as high as 60%. Also the prevalence of *Candida spp.* that are resistant to triazole antifungal agents is increasing, making treatment options a concern. Aspergillosis carries a 100% mortality rate if left untreated (Denning, 2000). In addition to this, there is a high incidence of cryptococcal infections which can be due to the explosion of acquired immune deficiency syndrome (AIDS) epidemic around the world and the use of more potent immunosuppressive agents by increasing numbers of organ transplant recipients (Mitchell and Perfect, 1995; Dromer et al., 1996). Cryptococcal meningitis, the most common infection of cryptococcosis is usually chronic and uniformly fatal if untreated (Collazos, 2003).

Some antifungal drugs, such as polyene macrolides (amphotericin-B) and azoles (itraconazole and fluconazole) are currently used in antifungal therapies with

certain limitations due to side effects as toxicity and emergence of resistant strains (Terrel, 1999; Saag et al., 2000). Although there are numerous treatment options, no broad-spectrum antifungal agents with an acceptable safety profile and with both intravenous and oral formulations are available at this time. These factors prompt the need for development of new antifungal agents in order to widen the spectrum of activities against pathogenic fungal species and combat strains expressing resistance to the available antifungals (Selitrennikoff, 2001).

The methanol extracts of twenty selected medicinal plants were screened for antifungal activity against 11 fungal strains. The extractive yields of the selected plants are summarized in Table 2.3. The results of antifungal activity of screened plant species against some strains of yeast is shown in Table 2.4a and that against moulds are shown in Table 2.4b. The antifungal activity was evaluated at three different concentrations (500 µg/disc, 250 µg/disc and 125 µg/disc). The moulds were more susceptible than yeast. All the concentrations of the extracts investigated, inhibited the fungal species with varying degree of sensitivity.

Table 2.3 Extractive yields (%) of the plant species selected for determining antifungal activity

Plant species	Extractive yield (%)
Ammannia baccifera L.	08.13
Bauhinia variegata L.	05.46
Caesalpinia pulcherrima (L.) Swartz.	10.27
Casuarina equissetifolia Forest.	13.17
Cyperus rotundus L.	07.35
Euphorbia hirta L.	09.70
Euphorbia tirucalli L.	08.10
Eucalyptus citriodora Hook.	21.33
Glycyrrhiza glabra L.	11.90
Holarrhena antidysenterica (Heyne. ex Roth.) A. DC.	15.60
Launaea procumbens Ram. & Raj.	09.43
Manilkara hexandra (Roxb.) Dubard	19.98
Mangifera indica L.	10.58
Mesua ferra L.	07.29
Saussurea lappa Costus.	11.56
Terminalia chebula Retz.	36.92
Trapa natans L.	02.49
Vitex negundo L.	13.48
Vitis vinifera L.	07.00
Woodfordia fruticosa kurz.	20.93

Ahmad et al., (1998) and Sengul et al., (2005) reported that methanol was a better solvent for the consistent extraction of antimicrobial substances from medicinal plants compared to other solvents such as water, ethanol, and hexane. Our work also supported this conclusion (Parekh and Chanda, 2006c). Therefore methanol was used for plant extraction in this study and antimicrobial activities were quantitatively assessed by the presence or absence of inhibition zone and zone diameters.

Amongst *Candida spps., Candida glabrata* was the most resistant strain followed by *Cryptococcus neoformans* and *Candida tropicalis.* The methanol extracts of *Glycyrrhiza glabra* showed moderate activity against *Candida glabrata,* that of *Eucalyptus citriodora, Holarrhena antidysentrica, Trapa natans* and *Woodfordia fruticosa* showed some activity against *Candida tropicalis* at the lowest concentration (125 µg/disc). The higher concentrations (500 and 250 µg/disc) of all the screened plants did not show any activity against *Candida albicans* (1) and *Candida albicans* (2) except *Glycyrrhiza glabra* extract which was active against both these strains while *Bauhinia variegata* extract was active against *Candida albicans* (1) (Table 2.4a).

Similarly the higher concentrations (500 and 250 µg/disc) of all the screened plants did not show any activity against *Cryptococcus luteolus* and *Cryptococcus neoformans.* These were resistant to most of the screened plant extracts except *Trapa natans* and *Woodfordia fruticosa* which showed slight activity at two lower concentrations (250 and 125µg/disc) (Table 2.4a).

The most susceptible yeast was *Trichosporon begelli.* All the 3 concentrations of all the plant extracts showed some activity against this fungal strain but to a varied degree. *Euphorbia hirta, Trapa natans, Vitis vinifera* extracts were active only at the lowest concentration. The methanol extract of *Holarrhena antidysentrica, Bauhinia variegata* and *Glycyrrhiza glabra,* showed best antifungal activity against *Trichosporon begelli. Bauhinia variegata, Caesalpinia pulcherrima, Casuarina equissetifolia, Meusa ferra, Mangifera indica, Woodfordia fruticosa, Saussurea lappa, Terminalia chebula* and *Glycyrrhiza glabra* showed antifungal

activity in all the three concentrations against *Trichosporon begelli.* Poor activity was shown by *Cyperus rotundus* and *Trapa natans* (Table 2.4a). These two plants showed good antibacterial activity (Parekh and Chanda, 2006b; Parekh and Chanda, 2006c) but they turned out to be poor antifungal agents.

The inability of the extracts to inhibit the growth of *Candida spps.* in the experiment showed that the plant drug cannot be used in treatment of fungal infections, like eczema, candidiasis which is caused by *Candida albicans.*

The methanolic extracts of all the screened plants showed good antifungal activity against the strains of moulds screened (Table 2.4b). The three Aspergillus spps. were more susceptible than *Mucor heimalis.* The lowest concentration of all the plants almost did not show any activity against *Aspergillus candidus* and *Mucor heimalis,* while the other two higher concentrations showed good antifungal activity. The lowest concentration of all the plants showed good antifungal activity against *Aspergillus flavus* while the highest concentration showed inhibitory effect against *Aspergillus flavus* and *Aspergillus niger.*

The effect of plant extracts was different with different fungal strains. The methanolic extracts of *Caesalpinia pulcherrima, Cyperus rotundus* and *Woodfordia fruticosa* showed best antifungal activity against *Aspergillus candidus. Manilkara hexandra* showed the best antifungal activity against *Aspergillus flavus* followed by *Eucalyptus citriodora, Ammania baccifera, Glycyrrhiza glabra* and *Trapa natans.* The methanolic extract of *Bauhinia variegata* followed by *Holarrhena antidysentrica, Eucalyptus citriodora* and *Euphorbia tirucalli* showed best antifungal activity against *Aspergillus niger* and *Glycyrrhiza glabra* was the best plant active against *Mucor heimalis.* The most resistant mould was *Mucor heimalis* where only highest concentration i.e. 500 µg/disc showed activity in almost all the plant extracts. Amongst all the plant extracts screened, *Glycyrrhiza glabra* and *Bauhinia variegata* extracts showed remarkable antifungal activity against all the tested moulds in all the three concentrations. The minimum antifungal activity was shown by methanol extract of *Ammania baccifera* (Table 2.4b).

Table 2.4a Screening of some Indian medicinal plants for antifungal activity against some strains of yeast.

Plant species	Inhibition Zone (mm)*																				
	Candida albicans (1) ATCC2091			*Candida albicans* (2) ATCC18804			*Candida glabrata* NCIM3448			*Candida tropicalis* ATCC4563			*Cryptococcus luteotus* ATCC32044			*Cryptococcus neoformans* ATCC34664			*Trichosporon beigelli* NCIM3404		
	Concentration of plant extract (µg /disc)																				
	500	250	125	500	250	125	500	250	125	500	250	125	500	250	125	500	250	125	500	250	125
Ammannia baccifera L.	-	-	-	-	11	-	-	-	-	-	-	-	-	-	12	-	-	-	-	-	9
Bauhinia variegata L.	11	9	9	-	11	-	-	-	-	-	-	-	-	9	-	-	-	-	11	10	9
Caesalpinia pulcherrima (L.) Swartz.	-	-	10	-	-	9	-	-	-	-	-	-	-	-	-	-	-	-	11	10	11
Casuarina equissetifolia Forest.	-	-	9	-	-	-	-	-	-	-	-	-	-	9	-	-	-	-	10	10	10
Cyperus rotundus L.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-	-
Euphorbia hirta L.	-	-	-	-	-	9	-	-	-	-	-	-	-	-	10	-	-	-	-	-	9
Euphorbia tirucalli L.	-	-	9	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-	9	10	-
Eucalyptus citriodora Hook.	-	-	10	-	-	9	-	-	-	-	-	10	-	-	-	-	-	-	9		10
Glycyrrhiza glabra L.	10	9	9	11	9	9	10	-	-	-	-	-	-	-	-	-	-	11	10	12	13
Holarrhena antidysenterica (Heyne. ex Roth.) A. DC.	-	-	-	-	-	-	-	-	-	-	-	10	-	-	-	-	-	-	10	12	11
Launaea procumbens Ram. & Raj.	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9	9
Manilkara hexandra (Roxb.) Dubard	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-	-	-	9	-	9
Mangifera indica L.	-	-	10	-	-	9	-	-	-	-	-	-	-	-	-	-	-	-	12	9	-
Mesua ferra L.	-	-	9	-	-	11	-	-	-	-	-	-	-	-	-	-	-	-	9	11	10
Saussurea lappa Costus.	-	-	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	9	10	12
Terminalia chebula Retz.																			10	10	9
Trapa natans L.	-	-	-	-	-	-	-	-	-	-	-	11	-	11	9	-	-	-	-	-	11
Vitex negundo L.	-	9	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	9
Vitis vinifera L.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10
Woodfordia fruticosa kurz.	-	-	10	-	-	-	-	-	-	-	-	10	-	10	9	-	-	-	13	12	11

*values are mean of three replicates and include disc diameter (7 mm)

Table 2.4b Screening of some Indian medicinal plants for antifungal activity against some strains of moulds.

Plant species	Inhibition Zone (mm)*											
	Aspergillus candidus NCIM883			*Aspergillus flavus* NCIM538			*Aspergillus niger* ATCC6275			*Mucor heimalis* NCIM873		
	Concentration of plant extract (µg /disc)											
	500	250	125	500	250	125	500	250	125	500	250	125
Ammannia baccifera L.	-	12	-	16	11	-	-	14	11	9		
Bauhinia variegata L..	11	12	10	10	20	18	18	15	13	11	12	12
Caesalpinia pulcherrima (L.) Swartz.	13	11	10	10	13	15	-	12	10	10	12	-
Casuarina equissetifolia Forest.	11	-	-	-	15	16	-	13	10	12	10	13
Cyperus rotundus L.	13	12	-	-	-	12	-	14	11	-	-	12
Euphorbia hirta L.	9		11	-	13	14	13	11	10	9	-	-
Euphorbia tirucalli L.	-	-	-	12	10	9	15	10	10	-	-	10
Eucalyptus citriodora Hook.	11	-	-	17	19	21	15	15	12	9	-	-
Glycyrrhiza glabra L.	16	12	11	15	19	20	13	16	14	15	14	12
Holarrhena antidysenterica (Heyne. ex Roth.) A. DC.	11	10	10	-	10	11	15	14	16	10	10	-
Launaea procumbens Ram. & Raj.	10	11	-	-	16	14	11	11	12	11	10	-
Manilkara hexandra (Roxb.) Dubard	12	-	12	20	12	17	13	15	11	-	14	10
Mangifera indica L.	12	12	-	-	-	15	12	15	13	12	-	-
Mesua ferra L.	10	-	-	-	12	12	-	12	13	11	10	-
Saussurea lappa Costus.	10	11	-	11	10	20	13	12	11	10	10	-
Terminalia chebula Retz.	10	13	10	-	15	15	14	17	16	13	-	-
Trapa natans L.	9	-	-	15	12	10	12	-	12	-	-	-
Vitex negundo L.	10	11	-	-	10	11	10	10	15	10	-	-
Vitis vinifera L.	10	10	-	12	10	10	-	10	16	10	-	-
Woodfordia fruticosa kurz.	13	11	13	12	12	17	-	15	12	15	11	-

*values are mean of three replicates and include disc diameter (7 mm)

The overall results suggest that *Aspergillus flavus* was the most susceptible fungal strain and the most resistant was *Candida glabrata.* The results of antifungal activity of the screened plants did not show any concentration effect. Certainly indigenous plants are reservoirs of novel antimicrobials; they would play important roles in providing us with newer bioactive leads in future. Plants with high anti-mould activity presented here or in similar studies would act as bedrocks for expanding our knowledge to attain novel antimicrobial agents.

2.4 CONCLUSIONS

From our investigation for screening of different plant species for antibacterial and antifungal potency, the results obtained confirm the therapeutic potency of some plants used in traditional medicine. The results obtained revealed that plant extracts in organic solvent proved to be more active than those in water. The plants species determind better antibacterial activity than antifungal activity. The most susceptible bacteria was *B. cereus* which is a Gram-positive bacteria and the most resistant bacteria was *E. coli* which is a Gram-negative bacteria. Some of the plant species namely *Woodfordia fruticosa* kurz., *Trapa natans* L., *Terminalia chebula* Retz., *Mesua ferra* L., *Manilkara hexandra* (Roxb.) Dubard, *Mangifera indica* L., *Eucalyptus citriodora* Hook., *Bauhinia variegata* L., *Caesalpinia pulcherrima* (L.) *Swartz., Vitis vinifera* L. *and Euphorbia hirta* L. showed the best antimicrobial activity amongst all the screened plant species. Therefore, these results form a good basis for selection of candidate plant species for further phytochemical and pharmacological investigation.

On the basis of the results evaluated for *in vitro* antibacterial and antifungal potencies of the selected plants, availability and literautre search, *Manilkara hexandra* (Roxb.) Dubard leaf (Sapotaceae) was selected for further pharmacognostic, phytochemical, toxicological and pharmacological evaluations.

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Section III

Assesment of Quality, Safety & Efficacy of *Manikara hexandra*

CHAPTER THREE 3

ASSESSMENT OF QUALITY OF *MANILKARA HEXANDRA* (ROXB.) DUBARD LEAF (SAPOTACEAE): PHARMACOGNOSTICAL AND PHYTOCHEMICAL PROFILE

This chapter is divided into two parts. Part I describes the pharmacognostical studies and Part II describes the phytochemical studies.

PART- I

3.1 INTRODUCTION

Knowledge of plants and healing has been closely linked from the time of man's earliest social and culture grouping. Besides medicine, mankind is almost completely dependent on plants for requirements such as food, shelter and clothing. Therefore, one of the biggest myth attending the use of herbs today is the idea that because something is "natural", it is completely safe. This is dangerous notion because some of the drugs that are available from plants are very toxic in natural state. So, the use of herbal medicine however, can be made relevant and popular after evaluating them for their quality, safety and efficacy (WHO, 1991).

The therapeutic activity of herbs is because of various constituents present in them. The therapeutic effects of herbal products is inconsistent and varies because the chemical constituents vary; they depend on various factors and one of them is the source. In some plants toxic constituents are also present therefore it is essential to evaluate their quality, safety and efficacy. Correct Identification and quality assurance of the starting material is, therefore an essential prerequisite to ensure reproducible quality of herbal medicine, which contributes to its safety and efficacy (Joshi et al., 2004). The present work is aimed at the pharmacognostical and phytochemical analysis of *Manilkara hexandra* (Roxb.) Dubard leaf.

3.2 MATERIALS AND METHODS

3.2.1 Identification and collection of the plant material

Fresh leaves of *Manilkara hexandra* (Roxb.) Dubard were collected in the month of August 2005, from Anand Agricultural University, Anand. The authenticity of the plant was confirmed by comparing their morphological characters with the description in books (Anjaria et al., 2002). Besides, the identity of the plant was also confirmed by Dr. Sriram, Research Scientist, Anand Agricultural University, Anand and Dr. P. S. Nagar, former taxonomist, Department of Biosciences, Saurashtra University, Rajkot. The voucher specimen (PSN 428) was deposited at Department of Biosciences, Rajkot. For further confirmation, the microscopic characteristics of this plant was studied and compared with available literature. The fresh plant material collected was thoroughly cleaned by washing under running tap water and air-dried in shade for seven days. It was then homogenized to fine powder and stored in air-tight bottles for further studies.

3.2.2 Successive extraction

Ten grams of dried leaf powder of *Manilkara hexandra* (Roxb.) Dubard was successively extracted by Soxhlet extraction method using three solvents with increasing polarity viz. petroleum ether, acetone and methanol. The solvent was evaporated under reduced pressure and the extract thus obtained was stored in air-tight bottles at 4ºC for further experiments. The respective yields (%) are shown in Table 3.1.

3.2.3 Pharmacognostical studies

The quality of *Manilkara hexandra* (Roxb.) Dubard leaf was assessed as per WHO guideline (WHO, 2000)

3.2.3.1 Determination of macroscopic characteristics

Macroscopic observations of *M. hexandra* (Roxb.) Dubard leaf was done. It comprised of shape, size, surface characteristics, texture, color, consistency,

odour, taste, etc (Khandelwala, 1988).

3.2.3.2 Determination of microscopic characteristics

Microscopic evaluation of *M. hexandra* (Roxb.) Dubard leaf was done as a whole (leaf) as well as in the powder form (Khandelwala, 1988).

3.2.3.2.1 Paraffin method for microtomy

Transverse sections of the leaves of *M. hexandra* (Roxb.) Dubard were taken by using a microtome. Permanent mount of leaf was prepared using paraffin method (Johansen, 1940). It involved the following steps:-

1) Collection of material and fixing:

The leaves of *M. hexandra* (Roxb.) Dubard were collected in a single collection since only one kind or type of material in a collection gives uniform fixation, dehydration and infiltration. FAA (Formaldehyde: Acetic acid: Absolute alcohol: Distilled water, 10: 5: 70: 15) (Berlyn and Miksche, 1976) was used as a fixing fluid which gives perfect fixation. The fixing fluid (FAA) was placed in a vial which was atleast ten times the volume of plant material. The plant material was kept in the fixing fluid (FAA) for atleast 24 h.

2) Washing:

The plant tissue was washed with 70 % alcohol since It is essential to wash out the fixing fluid (FAA) thoroughly before proceeding with dehydration and infiltration.

3) Dehydration:

The plant tissue was subjected to dehydration for complete removal of water. For the process of dehydration, the plant tissue was passed through TBA series (Tertiary butyl alcohol series). The following table shows the solutions taken in the TBA series for dehydration.

TBA series for dehydration

% TBA	Volume of solution (ml)		
	n-Butanol	Alcohol	D/W
10 %	10	50	40
30 %	30	50	20
50 %	50	50	-
70 %	70	30	-
90 %	90	10	-
100 %	100	-	-
100 %	100	-	-

The plant tissue was kept for 24 h in each series. The plant tissue was then ready for infiltration. Now equal parts of paraffin wax was transferred to the glass vials containing plant tissue till the solution was saturated with wax. During this stage paraffin diffused gradually to all the plant tissues with tert-butyl alcohol. Thereafter, the vials containing tissue and those containing only paraffin wax were placed in oven at 56°C overnight. This allowed tert-butyl alcohol to evaporate. Next day the tissues were transferred in the vial containing only pure paraffin wax and kept in oven at 56°C for complete evaporation of tert-butyl alcohol. This process was repeated twice during the next 6 h.

1) Embedding:

In the process of embedding the melted wax was poured into the Petri-dishes and then the plant tissues were properly arranged onto the base. Thereafter, the Petri-dish was fully covered with rest of the melted paraffin wax. The entire mass within the dish was cooled. The blocks were prepared to be attached to wooden blocks.

2) Microtoming and Mounting:

The section of the plant tissue was obtained by a rotary microtome. The micron scale was set for the desired thickness of 10-15 µm. The sections were placed on

the slides and fixed by egg albumin.

3) Staining and Mounting:

Before staining, paraffin wax was totally removed from the slides by passing them through xylene and alcohol series. The sections were stained by Safranin and Fast green (Berlyn and Miksche, 1976). After staining, the slides were mounted with DPX.

The slides were passed through the following series:-

100 % Xylene (5 min) → 100 % Xylene (5 min) → Alcohol : Xylene (1:3) (3 min) → Alcohol : Xylene (1:1) (3 min) → Alcohol : Xylene (3:1) (3 min) → 100 % Alcohol (3 min) → 90 % Alcohol (3 min) → 70 % Alcohol (3 min) → 50 % Alcohol (3 min) → 30 % Alcohol (3 min) → 20 % Alcohol (3 min) → 10 % Alcohol (3 min) → D/W (3 min) → SAFRANIN (overnight) → D/W (1 h) → 10 % Alcohol (3 min) → 20 % Alcohol (3 min) → 30 % Alcohol (3 min) → 50 % Alcohol (3 min) → 70 % Alcohol (3 min) → 90 % Alcohol (3 min) → FAST GREEN (2-3 dips) → 100 % Alcohol (3 min) → 100 % Alcohol (3 min) → Xylene : Alcohol (1:3) (5 min) → Xylene : Alcohol (1:1) (5 min) → Xylene : Alcohol (3:1) (5 min) → Xylene (5 min) → Xylene (5 min) → DPX → cover slip

3.2.3.2.2 Method for powder study

Manilkara hexandra (Roxb.) Dubard leaf powder was cleared with chloral hydrate solution, stained with phloroglucinol and HCl staining reagent and mounted with glycerin. The different characteristics of powder are shown in the Figure 3.3.

3.2.3.3 Determination of proximate parameters

Proximate analysis of crude powder and different extracts of *Manilkara hexandra* (Roxb.) Dubard leaf (MCR, MPE, MAC and MME) was done. Following determinations were made:-

- Loss on drying
- Total Ash
- Acid insoluble ash
- Petroleum ether soluble extractive
- Alcohol soluble extractive
- Water soluble extractive
- Solubility
- Melting point
- pH
- Heavy metal analysis

3.2.3.3.1 Determination of loss on drying

The loss on drying was determined by weighing 2 g of crude powder of *M. hexandra* (Roxb.) Dubard leaf (MCR) 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.

3.2.3.3.2 Determination of total ash

The total ash value of crude powder of *M. hexandra* (Roxb.) Dubard leaf (MCR) was determined by incinerating 1 g of accurately weighed crude powder in a tarred silica crucible. It was incinerated in a muffle furnace at a temperature not exceeding 450ºC until free from carbon, then cooled and weighed.

3.2.3.3.3 Determination of acid insoluble ash

The total ash obtained as described in the section 3.2.3.3.2 was boiled for 5 min with 25 ml dilute HCl. The insoluble matter was collected on the filter paper placed in a Gooch crucible, washed with water and heated till the constant weight was obtained. The percentage of acid insoluble ash was calculated with reference to the sample taken initially.

3.2.3.3.4 Determination of petroleum ether soluble extractive

Five grams of crude powder of *M. hexandra* (Roxb.) Dubard leaf (MCR) 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. From the weight of the dried residue, the percentage of extractable matter was calculated with reference to the sample taken initially.

3.2.3.3.5 Determination of alcohol soluble extractive

Five grams of crude powder of *M. hexandra* (Roxb.) Dubard leaf (MCR) 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. From the weight of the dried residue, the percentage of extractable matter was calculated with reference to the sample taken initially.

3.2.3.3.6 Determination of water soluble extractive

Five grams of crude powder of *M. hexandra* (Roxb.) Dubard leaf (MCR) 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. From the weight of the dried residue, the percentage of extractable matter was calculated with reference to the sample taken initially.

3.2.3.3.7 Determination of solubility

The qualitative solubility test of the different extracts of *M. hexandra* (Roxb.) Dubard leaf (MPE, MAC, and MME) was determined for different solvents with different polarities.

3.2.3.3.8 Determination of melting point

The melting point of the crude powder and different extracts of *M. hexandra* (Roxb.) Dubard leaf (MCR, MPE, MAC, and MME) were done at Department of Chemistry, Saurashtra Unviersity, Rajkot, by open capillary method (Apparao et al., 1971; Sukhwal et al., 1995).

3.2.3.3.9 Determination of pH

The crude powder of *M. hexandra* (Roxb.) Dubard leaf and its different extracts (MCR, MPE, MAC, and MME) were dissolved in distilled water and were kept in water bath for 20 min. It was then filtered and the pH of the filtrate was noted down with the help of a Systronic pH meter (pH system 361).

3.2.3.3.10 Determination of heavy metals

Contamination of medicinal plant materials with heavy metals can be attributed to many cases including environmental pollution and traces of pesticides. Therefore, detection of heavy metals is important for herbal drugs. The analysis for heavy metals like arsenic, chromium, cobalt, lead, mercury and nickel for crude powder and different extracts of *M. hexandra* (Roxb.) Dubard leaf (MCR, MPE, MAC, and MME) were done at Choksi Laboratries Limited, Vadodara.

3.2.3.4 Determination of microorganisms

Medicinal plant materials normally carry a great number of bacteria and moulds, often originating in soil. Current practices of harvesting, handling and production may cause additional contamination and microbial growth. The determination of bacteria and moulds may indicate the quality of the plant material. Therefore, total bacterial and total fungal counts as well as specific count for *Escherichia coli, Salmonella typhi* and *Pseudomonas aruginosa* for crude powder of *M. hexandra* (Roxb.) Dubard leaf was carried out using reported methods (Pharmacopoeia of India, 1985).

3.3 RESULTS AND DISCUSSION

The extractive yield of MPE, MAC and MME is shown in Table 3.1. Maximum extractive yield was obtained by MME which was 23 % while minimum extractive yield was of MAC which was 4.8 %.

Table 3.1 The extractive yields of different extracts of *Manilkara hexandra* (Roxb.) Dubard leaf

Plant extract	Extractive yield (%)
MPE	5.5
MAC	4.8
MME	23

MPE: Petroleum ether extract, MAC: Acetone extract, MME: Methanol extract

3.3.1 Pharmacognostical studies

3.3.1.1 Macroscopic characteristics (Figure 3.1)

Kingdom	Plant
Division	Angiospermae
Class	Dicotyledons
Subclass	Gamopetalae
Series	Heteromerae
Order	Ebenales
Family	Sapotaceae
Genus	*Manilkara*
Species	*hexandra*

Plant: *Manilkara hexandra* (Roxb.) Dubard; Syn. *Mimusops hexandra* Roxb.

Family: Sapotaceae

Vernacular name: Bakula (Kannad), Khiri, Khirakuli (Oriya); Khirkhejur (Bengali); Khirni (Hindi); Ranjana, Rayan, Raini (Marathi); Rayana, khirni (Gujarati); Mangi-pala, Pala (Telugu); Palla, Palai (Tamil).

Habit: It is an evergreen, glabrous tree with shady head. It measures about 10-15 m.

MACROSCOPY OF MANILKARA HEXANDRA (ROXB.) DUBARD LEAF

FIGURE 3.1 Macroscopic characteristics of *Manilkara hexandra* (Roxb.) Dubard leaf

Leaf: The leaves are alternate, coriaceous, elliptic, oblong or obovate, oblong, rounded or emarginated at apex, glabrous, dark green and polished above, paler beneath. It measures about 2.5-11 x 1-6 cm. Its taste is slightly bitter.

Flower: Inflorescence is axillary, solitary or in fascicles of 2-6 flowers. Flowers are creamy-white and small. Calyx consists of 6 sepals in 2 whorls of 3 each with rusty-tomentose outside. Corolla consists of 18 petals, gamopetalous, white, brownish red when dry. Androecium consist of 16 stamens in 2 series of 6 each, alternating with staminodes, inner whorl stamens 6, epipetalous; outer whorl reduced to 6 staminodes, alternating with the stamens, bifid or denticulate at the apex, glabrous, toothed or laciniate but not petaloid. Gynoecium consist of 12 carpels, ovary syncarpous, 12-celled with one ovule in each cell.

Fruit: Berries, oblong, ovoid or ellipsoid, smooth, with plenty of latex, slightly curved, 1/2 seeded, reddish yellow when ripe.

Seeds: Ovoid, smooth, reddish-brown to black in color, shining surface.

3.3.1.2 Microscopic characteristics

3.3.1.2.1 T.S. of leaf (Figure 3.2)

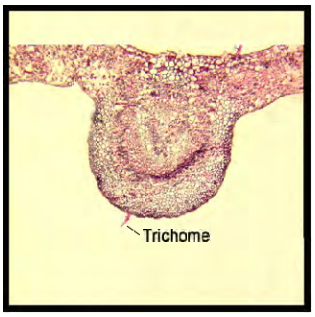
The transverse section (T.S.) of *M. hexandra* (Roxb.) Dubard leaf was examined for microscopic characteristics. The T.S. of leaf showed following tissue system: (1) Epidermis, (2) Mesophyll and (3) Midrib.

(1) Epidermis:

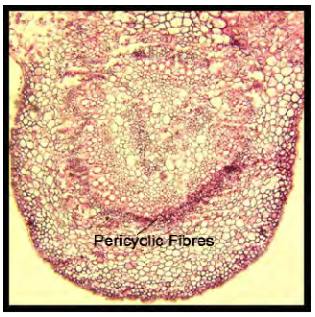
Upper Epidermis: The single layered epidermal cells were straight wall, rectangular in shape and covered with thin cuticle. The simple, unicellular, conical and thick trichomes with single covering were present.

Lower Epidermis: Similar to upper epidermis. The lower epidermis had more number of trichomes than the upper epidermis. The anomocytic stomata were present in lower epidermis. Prisms of calcium oxalate were present in the lower

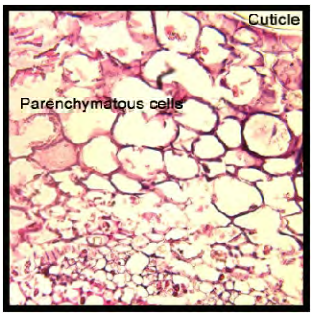
MICROSOCPY OF *MANILKARA HEXANDRA* (ROXB.) DUBARD LEAF



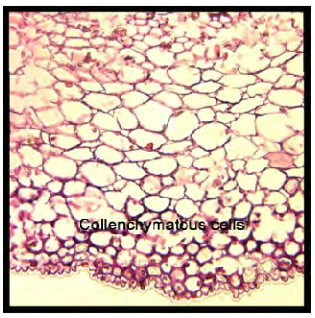
T. S. OF LEAF



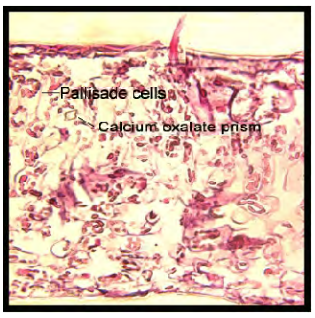
MIDRIB



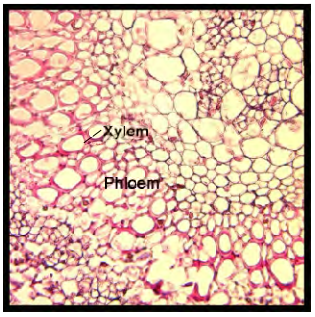
UPPER EPIDERMIS



LOWER EPIDERMIS



LAMINAE WITH CALCIUM OXALATE PRISM



VASCULAR BUNDLES

FIGURE 3.2 Photomicrographs of the microscopic characteristics of *Manilkara hexandra* (Roxb.) Dubard leaf.

epidermal cells.

(2) Mesophyll:

Leaf was dorsiventral. Therefore, mesophyll was divided in to single layered elongated palisade cells under upper epidermis and a loose tissue of irregular spongy parenchymatous cells having large intercellular spaces above lower epidermis.

(3) Midrib:

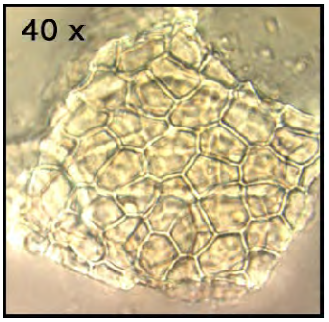
The T. S. of midrib showed meristele in the form of an arc which contained xylem towards the dorsal side and phloem towards the ventral side. Epidermal layers were continuous. Below upper epidermis and above lower epidermis, the thick walled cellulosic collenchyma cells were present. The vascular bundle was collateral i.e. xylem and phloem were present on the same radius side by side. The xylem was lignified while phloem was non-lignified. Thick walled pericyclic fibers containing sclerenchyma cells were present between the phloem and cortical region and surrounded the vascular bundle.

3.3.1.2.2 Powder characteristics (Figure 3.3)

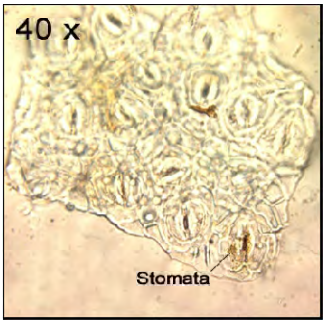
The crude powder of *M. hexandra* (Roxb.) Dubard leaf was dark green in color with characteristic odour and slightly bitter taste. The specific characteristics determined from the powder study were:-

- Upper epidermis in surface view showed straight wall cells
- Lower epidermis in surface view showed some what wavy walled cell with anomocytic stomata
- Simple covering unicellular trichome
- Group of pericyclic fibers
- Prisms of calcium oxalate crystals were found
- Xylem vessels in longitudinal sectional view showed spiral thickening.

POWER STUDY OF *MANILKARA HEXANDRA* (ROXB.) DUBARD LEAF

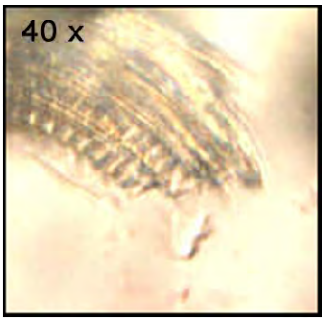


UPPER EPIDERMIS

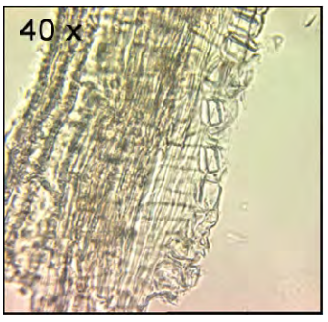


LOWER EPIDERMIS

TRICHOME



SPIRAL XYLEM VESSELS



CALCIUM OXALATE PRISM

PITTED PERICYCLIC FIBRES

FIGURE 3.3 Photomicrographs of the specific characteristics determined from the powder study of *Manilkara hexandra* (Roxb.) Dubard leaf.

3.3.1.3 Proximate analysis

The results obtained from various determinations are complied in Table 3.2- 3.4.

Table 3.2 Determination of proximate parameters of crude powder of *Manilkara hexandra* (Roxb.) Dubard leaf

Proximate parameters	Average value % w/w
Loss on drying	4
Total ash	6
Acid insoluble ash	1
Petroleum ether soluble extractive	3.74
Alcohol soluble extractive	10.6
Water soluble extractive	12.3

The result of proximate analysis of crude powder of *M. hexandra* (Roxb.) Dubard leaf (MCR) is shown in Table 3.2. The average values are expressed as percentage of air-dried material. MCR showed 4 % of loss on drying. It contained 6 % of total ash and about 1 % of acid insoluble ash. The percent extractive yield of crude powder extracted in petroleum ether was 3.74 %; that extracted in alcohol was 10.60 % and extracted in water was 12.30 %.

Table 3.3 Determination of solubility of different extracts of *Manilkara hexandra* (Roxb.) Dubard leaf

Solvents	Solubility		
	MPE	MAC	MME
Hexane	++	+	+
Heptane	++	+	+
Benzene	+++	++	++
Diethyl ether	++	+++	++
Petroleum ether	++	+	+
1-4 dioxan	++	+++	++
Tetrahydrofuran	-	++	+++
Ethyl acetate	++	++	++
Chloroform	+++	++	++
Acetone	++	++	++
Dimethylformamide	+	+++	+++
Dimethylsulphoxide	++	+++	+++

Solvents	Solubility		
	MPE	MAC	MME
Dimethylsulphoxide	++	+++	+++
1-Butanol	++	++	++
1-Propanol	++	+++	+++
Acetic acid	++	++	++
Ethanol	++	+++	+++
Methanol	+	+++	+++
2-methoxy ethanol	++	+++	+++
Triacetin	+	+	+
Toluene	-	++	+++
Distilled water	-	+	+
Tap water	-	++	++
2-methyl Propanol	++	++	+
Dichloromethane	+++	++	++
Amyl alcohol	++	++	+
Benzyl alcohol	++	+++	++
Benzaldehyde	+++	+++	++
Orthophosphoric acid	+	++	+++
Formic acid	++	+++	+++

(-): Not Soluble, (+): Sparingly soluble, (++): Soluble, (+++): Highly soluble, MPE: Petroleum ether extract, MAC: Acetone extract, MME: Methanol extract

The three extracts MPE, MAC and MME of *M. hexandra* (Roxb.) Dubard were evaluated for qualitative solubility test for about 29 solvents with varied polarities (Table 3.3). MME and MAC were almost soluble in all the solvents while MPE showed comparatively less solubility due to its hydrophobic nature.

Table 3.4 Determination of melting point and pH of the crude powder and different extracts of *Manilkara hexandra* (Roxb.) Dubard leaf

Plant extract	Melting Point (°C)	pH
MCR	< 300	5.51
MPE	206-210	ND
MAC	< 300	5.01
MME	< 300	4.82

MCR: Crude powder, MPE: Petroleum ether extract, MAC: Acetone extract, MME: Methanol extract, ND: Not done

The results of melting point and pH of the crude powder and different extracts of *M. hexandra* (Roxb.) Dubard are shown in Table 3.4. The melting point of crude powder (MCR), acetone extract (MAC) and methanol extract (MME) was < 300°C and that of petroleum ether extract (MPE) was 206-210°C. All the samples were acidic in nature. The methanol extract (MME) was the most acidic in nature.

Table 3.5 Determination of heavy metals in crude powder and different extracts of *Manilkara hexandra* (Roxb.) Dubard leaf

Plant extract	Heavy Metals (ppm)					
	Mercury (Hg)	Lead (Pb)	Chromium (Cr)	Cobalt (Co)	Arsenic (As)	Nickel (Ni)
MCR	0.071	NDT	NDT	2.07	NDT	NDT
MPE	0.09	NDT	NDT	2.36	0.045	NDT
MAC	0.046	NDT	NDT	2.12	0.027	NDT
MME	0.094	NDT	NDT	2.02	NDT	NDT

MCR: Crude powder, MPE: Petroleum ether extract, MAC: Acetone extract, MME: Methanol extract, NDT: Not detected

The presence of heavy metals in the medicinal plants exceeding certain limit tends to cause a health hazard. Thus, the crude leaf powder of *M. hexandra* (Roxb.) Dubard and its extracts (MPE, MAC and MME) were analysed for the presence of heavy metals. The results (Table 3.5) showed that lead, chromium and nickel were not present in any of the sample drugs; mercury and cobalt was present in all the four sample drugs with varying values. The presence of arsenic was present only in two sample drugs (MPE and MAC). The crude drug contained 0.071 ppm mercury and 2.07 ppm cobalt; MPE had 0.09 ppm mercury, 2.36 ppm cobalt and 0.045 ppm arsenic; MAC had 0.046 ppm mercury, 2.12 ppm cobalt and 0.027 ppm arsenic and MME had 0.094 ppm mercury and 2.02 ppm cobalt. Although, there was minor presence of some heavy metals but the extracts did not exceed the limit given according to the WHO guidelines (1991). Therefore, the sample drugs investigated were free from heavy metal contamination.

3.3.1.4 Determination of microorganisms

The crude powder of *Manilkara hexandra* (Roxb.) Dubard leaf was evaluated for microbial contamination. They were free from moulds; however 10^2 colonies of yeast were observed per gram. The samples possessed around 10^2 colonies of Enterobacteriaceae but were free from *P.aeruginosa* and *S. typhimurium.*

PART- II

3.4 INTRODUCTION

Plant cells are highly sophisticated chemical factories where a large variety of chemical compounds are manufactured with great precision and ease from simple raw materials at normal temperature and pressure. The knowledge of chemical constitutions of plants is desirable for the discovery of therapeutic agents. In addition, the knowledge of the chemical constituents of plants would further be valuable in discovering the actual value of folkloric remedies (Farnsworth, 1966).

Medicinal plants are of great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are saponins, alkaloids, tannins, flavonoids, steroids and phenolic compounds (Hill, 1952).

3.5 MATERIALS AND METHODS

3.5.1 Phytochemical studies

The qualitative phytochemical analysis and HPTLC fingerprinting were done to evaluate the phytochemical constituents from the crude powder and different extracts of *Manilkara hexandra* (Roxb.) Dubard leaf (MCR, MPE, MAC, and MME).

3.5.1.1 Qualitative Phytochemical Screening

Preliminary chemical tests were carried out for MCR, MPE, MAC, and MME to identify different phyto-constituents (Harborne, 1973; Trease and Evans, 1989).

3.5.1.1.1 Alkaloids

The methanolic extract of the sample drug was evaporated to dryness on 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 few drops of Mayer's reagent; one portion was treated with equal amount of Dragondroff 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. A (+) score was recorded if the reagent produced only a slight opaqueness; a (++) score was recorded if a definite turbidity but no flocculation was observed and a (+++) score was recorded if heavy precipitate or flocculation was observed (Salehi-Surmaghi et al., 1992).

3.5.1.1.2 Flavonoids

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

3.5.1.1.3 Saponins

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

3.5.1.1.4 Tannins

The water extract of the sample drug was treated with alcoholic $FeCl_3$ reagent. Blue color indicated the presence of tannins (Segelman et al., 1969).

3.4.1.1.5 Steroids

Liebermann-Burchard reaction was performed for the presence of steroids. A chloroformic solution of the sample drug was treated with acetic anhydride and few drops of concentrated H_2SO_4 were added down the sides of test tube. A blue green ring indicated the presence of terpenoids.

3.5.1.1.6 Cardiac glycosides

Keller-kiliani test was performed for the presence of cardiac glycosides. The sample drug was treated with 1ml mixture of 1 volume of 5% $FeCl_3$ solution and 99 volume of glacial acetic acid. To this solution few drops of concentrated H_2SO_4 was added. Appearance of greenish blue color within few minutes indicated the presence of cardiac glycosides (Ajaiyeobu, 2002).

3.5.1.2 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 HPTLC fingerprinting of the different extracts of *M. hexandra* (Roxb.) Dubard leaf (MPE, MAC and MME) was carried out at Anchrom Enterprises (I) Pvt. Ltd., Mulund, Mumbai.

3.5.1.2.1 Sample preparation

100 mg of different extracts of *M. hexandra* (Roxb.) Dubard leaf (MPE, MAC, and MME) were dissolved in 10 ml methanol and centrifuged for 15 min. The supernatant was then loaded on the plates. The amount of each sample loaded was 100 µg.

3.5.1.2.2 Development of HPTLC plates

The HPTLC was carried out using a Hemilton 100 µl HPTLC syringe, Camag Linomat IV automatic spoting device, Camag twin trough chamber, Camag TLC scanner-3, win CATS integration software, aluminium sheet precoated with silica gel G 60F_{254} (Merck, India), 200 µm thickness.

Prior to use, these plates were washed with methanol in a pre-saturated twin trough chamber. 10 µl of test sample was spotted in the form of band using Linomat IV automatic spotter (Camag, Switzerland). The plates were developed using the solvent system Toluene: Ethyl acetate: Formic acid (7: 2.5: 0.5), Rectangular twin trough chambers were used for development. Chamber was saturated with solvent system for about 10 min before inserting the plates. The plates were developed to a height of 5-6 cm, then the plates were taken out and air dried.

3.5.1.2.3 Detection and scanning of HPTLC

Using UV light at 254 nm, substances which had fluorescence in this region appeared as dark zones against yellow-green fluroscent background. One of the TLC plates were derivatized by spraying with anisaldehyde-sulphuric acid reagent till the band developed. The plates were scanned at 366 nm using Camag scanner-3. Number of peaks and peaks height of the resolved bands were recorded.

3.6 RESULTS AND DISCUSSION

3.6.1 Preliminary phytochemical analysis

The results of preliminary phytochemical analysis of the crude powder and the different extracts of *M. hexandra* (Roxb.) Dubard leaf (MCR, MPE, MAC and MME) is shown in Table 3.6. All the four samples contained maximum amount of cardiac glycosides and steroids. Tannins and saponins were present in all the extracts except MPE. Alkaloids were present in MCR, MPE, MAC and MME with

Wagner's test. The alkaloids were present only in MCR and MPE with Dragendroff's test and in MCR and MME with Mayer's test. Flavonoids were absent in all the four drug samples.

Table 3.6 Preliminary qualitative phytochemical analysis of crude powder and different extracts of *Manilkara hexandra* (Roxb.) Dubard leaf

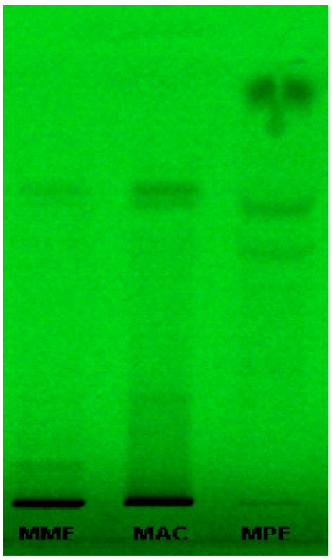
Phytochemical tests	*Manilkara hexandra* leaf extracts			
	MCR	MPE	MAC	MME
Alkaloids				
Dragondroffs test	+	+	-	-
Mayers test	++	-	-	+
Wagners test	+++	+	+ + +	+++
Flavonoids				
Shinoda test	-	-	-	-
Saponins				
Frothing test	+	-	+++	++
Tannins				
$FeCl_3$ test	+++	-	+++	+ ++
Steroids				
Liebermann-Burchard reaction	+++	+++	+++	+++
Cardiac glycosides				
Keller-kilianni test	+++	+++	+++	+++

(-): No presence, (+): Less presence, (++): Moderate presence, (+++): High presence, MCR: Crude powder, MPE: Petroleum ether extract, MAC: Acetone extract, MME: Methanol extract,

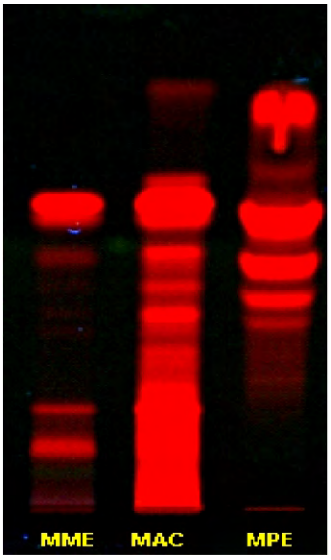
3.6.1 HPTLC fingerprinting

The three extracts of *M. hexandra* (Roxb.) Dubard leaf (MPE, MAC and MME) were subjected to HPTLC fingerprinting. The results showing the Rf values and the percentage of the constituents in each extract are shown in Table 3.7. The HPTLC plates showing different bands for all the extracts are shown in Figure 3.4. The MPE showed 7 peaks, MAC showed 14 peaks and MME showed 13 peaks (Figure 3.5). There were four constituents common in MAC and MME with Rf values of 0.06, 0.21, 0.50 and 0.55 and there were two constituents common in MPE and MAC with Rf values of 0.23 and 0.67. None of the constituents were

THIN LAYER CHROMATOGRAPHY



TLC AT 254nm



TLC AT 366nm

FIGURE 3.4 Thin layer chromatography (TLC) of *Manilkara hexandra* (Roxb.) Dubard leaf; MPE: Petroleum ether extract, MAC: Acetone extract, MME: Methanol extract

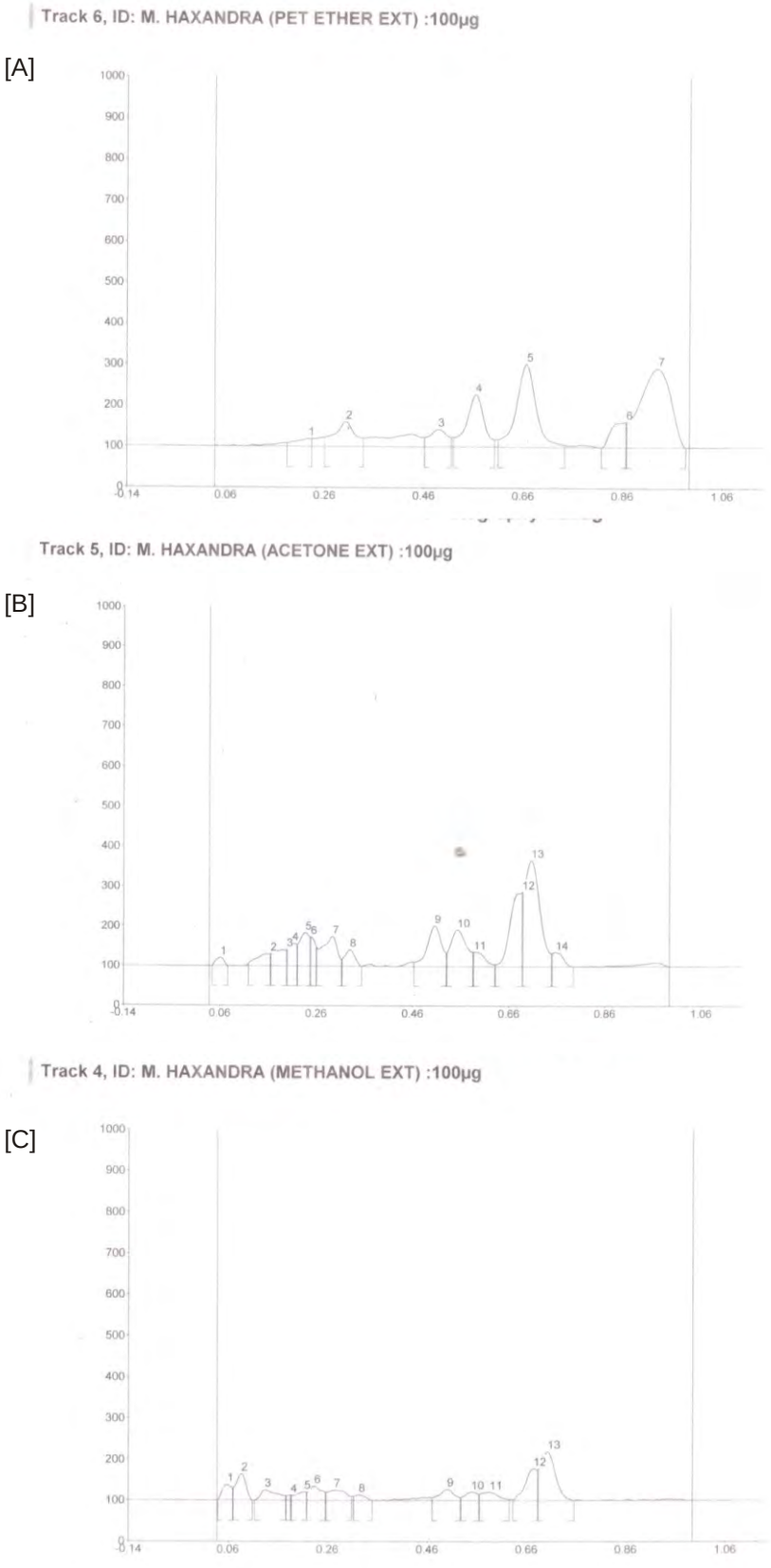


Figure 3.5 HPTLC spectra of (A) Petroleum ether extract (B) Acetone extract (C) Methanol extract of *Manilkara hexandra* (Roxb.) Dubard leaf.

common in MPE and MME. The constituents can be further isolated and purified to find its potency for biological activities.

Table 3.7 HPTLC (High Performance Thin Layer Chromatography) fingerprinting of different extracts of *Manilkara hexandra* (Roxb.) Dubard leaf

Petroleum ether (MPE)			Acetone Extract (MAC)			Methanol Extract (MME)		
Peak	Max Rf	Area (%)	Peak	Max Rf	Area (%)	Peak	Max Rf	Area (%)
1	0.23	2.08	1	0.06	1.19	1	0.06	5.58
2	0.3	8	2	0.16	2.99	2	0.08	9.95
3	0.49	5.06	3	0.2	3.68	3	0.13	6.61
4	0.56	14.49	4	0.21	3.31	4	0.18	0.81
5	0.67	26.17	5	0.24	5.79	5	0.21	3.47
6	0.87	5.95	6	0.25	2.41	6	0.23	6.88
7	0.93	38.25	7	0.29	7.79	7	0.27	7.17
			8	0.33	2.88	8	0.32	2.27
			9	0.5	10.17	9	0.5	6.26
			10	0.55	10.13	10	0.55	4.04
			11	0.59	2.9	11	0.58	5.43
			12	0.69	15.6	12	0.67	14.3
			13	0.71	28.47	13	0.7	27.23
			14	0.76	2.69			

MPE: Petroleum ether extract, MAC: Acetone extract, MME: Methanol extract

3.7 CONCLUSIONS

From the present study, it can be concluded that crude powder (MCR) of *Manilkara hexandra* (Roxb.) Dubard leaf was dark green in color with characteristic odour and bitter taste. The extractive yield of MPE, MAC and MME was 5.5 %, 4.8 % and 23 % respectively. The crude powder and different extracts of *M. hexandra* (Roxb.) Dubard leaf (MCR, MPE, MAC and MME) were free from heavy metal and microbial contamination. All the four sample drugs were acidic in nature. MPE, MAC and MME were maximally soluble in polar solvents. All the sample drugs (MCR, MPE, MAC and MME) possessed maximum amount of

cardiac glycosides and steroids while they were totally devoid of flavonoids. Except MPE, all the other sample drugs contained tannins and saponins. Alkaloids were present in all sample drugs with varying degree. The HPTLC fingerprinting showed maximum 14 peaks for MAC, followed by 13 peaks for MME and 7 peaks for MPE. Hence, the determination of pharmacognostical and phytochemical profile of *Manilkara hexandra* (Roxb.) Dubard leaf authenticates the further usage of this plant material for evaluating safety and efficacy.

3.8 REFERENCES

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Trease GE, Evans WC (1997). *Trease and Evan's* Pharmacognosy, Vol -35, pp. 371.

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CHAPTER FOUR 4

ASSESSMENT OF *IN VITRO* ANTIMICRIOBIAL ACTIVITY OF *MANILKARA HEXANDRA* (ROXB.) DUBARD LEAF AGAINST SOME HUMAN PATHOGENS

4.1 INTRODUCTION

Nature has been a source of medicinal agents for thousands of years since it comprises of compounds that are highly diverse and often provide highly specific biological activities. This follows from the proposition that essentially all natural products have some receptor binding capacity (Verdine, 1996). Herbal medicines have been the basis of treatment for various diseases and physiological conditions in traditional methods practiced such as Ayurveda, Unani and Siddha (Kumar et al., 2006). To promote the proper use of herbal medicine and to determine their potential as sources for new drugs, it is essential to study medicinal plants, which have folklore reputation in a more intensified way (Awadh Ali et al., 2001). Over the past 20 years, there has been a lot of interest in the investigation of natural materials as sources of new antimicrobial agents (Recio, 1989). Some natural products have been approved as new antimicrobial drugs, but there is a continuous and urgent need to screen more and more plant species and discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action to combat new and re-emerging infectious diseases of today's era (Rojas et al., 2003; Bonjar, 2004; Prashanth Kumar, 2006).

Bacteria and fungi cause some important human diseases, especially in immunocompromised or immunodeficient patients. Despite the existence of potent antibacterial and antifungal agents, the past three decades have seen a dramatic increase in microbial resistance to antimicrobial agents (Baquero, 1997) that lead to repeated use of antibiotics and insufficient control of diseases (NCID, 2002), imposing the need for a permanent search and development of new drugs (Benkeblia, 2004). Many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Even today, plant materials continue

to play a major role in primary health care as therapeutic remedies in many developing countries (Zakaria, 1991). In an effort to discover new lead compounds, many researchers are increasingly turning their attention to folk medicine looking for new leads to develop better drugs against microbial infections (Benkeblia, 2004; Parekh et al., 2005).

Plant based antimicrobials represent a vast untapped source of medicines and there is a need for further exploration of plant antimicrobials. It is estimated that several plant-derived drugs prescribed in the industrialized world were discovered by studying folk knowledge. However, less than one-half of 1 percent of all plant species in the world has been studied for potential pharmacological activity (Balick and Cox, 1996). Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc (Gordon and David, 2001; Parekh et al., 2006) i.e. any part of the plant may contain active components. Scientific analysis of plant components may give a new source of antimicrobial agents with possibly novel mechanisms of action (Motsei et al., 2003; Barbour et al., 2004). World Health Organization has also approved the study of medicinal plants for the development of new drug lead (WHO, 2000). In recent years, many researchers all over the world have screened plant extracts to detect and utilize secondary metabolites with relevant biological activities as medicinal agents (Balandrin et al., 1985; Aliero and Afolayan, 2005; Parekh and Chanda, 2006a; Parekh and Chanda, 2007).

The primary screening of several plant extracts resulted in the selection of *Manilkara Hexandra* (Roxb.) Dubard (Sapotraceae) as the promising plant to be investigated for further experiments. This work is aimed at the investigation of antibacterial as well as antifungal property of different extracts of *Manilkara hexandra* (Roxb.) Dubard leaf against an array of human pathogens including Gram-positive and Gram-negative bacteria as well as some fungi like yeast and moulds. The impelling motive behind the present work was to emphasize the possibilities and scientific importance for the use of *M. hexandra* (Roxb.) Dubard leaf extracts in treating common ailments due to microbial infections encountered worldwide.

4.2 MATERIALS AND METHODS

4.2.1 Plant collection

As per section 3.2.1 from chapter 3

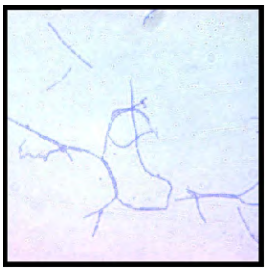
4.2.2 Successive Extraction

As per section 3.2.2 from chapter 3

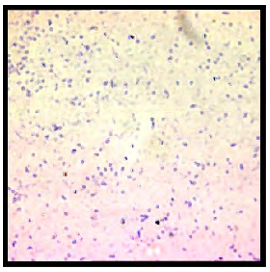
4.2.3 Microorganisms and growth conditions

The investigated microbial strains were obtained from the National Chemical Laboratory (NCL), Pune, India. The microbial strains investigated include 9 Gram-positive bacteria: *Bacillus cereus* ATCC11778, *Bacillus megaterium* ATCC9885, *Bacillus subtilis* ATCC6633, *Corynebacterium rubrum* ATCC14898, *Micrococcus flavus* ATCC10240 *Staphylococcus aureus* (1) ATCC25923, *Staphylococcus aureus* (2) ATCC29737, *Staphylococcus epidermidis* ATCC12228, *Staphylococcus subfava* NCIM2178; 14 Gram-negative bacteria: *Alcaligenes fecalis* ATCC8750, *Citrobacter freundii* ATCC10787, *Enterobacter aerogenes* ATCC13048, *Escherichia coli* ATCC25922, *Klebsiella aerogenes* NCTC418, *Klebsiella pneumoniae* NCIM2719, *Proteus mirabilis* NCIM2241, *Proteus morganii* NCIM2040, *Proteus vulgaris* NCTC8313, *Pseudomonas aeruginosa* ATCC27853, *Pseudomonas pseudoalcaligenes* ATCC17440, *Pseudomonas putida* ATCC12842, *Pseudomonas testosteroni* NCIM5098, *Salmonella typhimurium* ATCC23564; 7 yeast: *Candida albicans* (1) ATCC2091, *Candida albicans* (2) ATCC18804, *Candida glabrata* NCIM3448, *Candida tropicalis* ATCC4563, *Cryptococcus luteolus* ATCC32044, *Cryptococcus neoformans* ATCC34664, *Trichosporon beigelii* NCIM3404 and 4 moulds: *Aspergillus candidus* NCIM883, *Aspergillus flavus* NCIM538, *Aspergillus niger* ATCC6275, *Mucor hiemalis wehmer* NCIM873. Bacterial cultures were grown on nutrient broth (Hi-Media) at 37°C for 24 h and the fungal cultures were grown on sabouraud dextrose broth (Hi-Media) at 28°C for 48 h. All the microbial cultures of bacteria, yeast and mould were maintained on nutrient agar slants, MGYP slants, PDA (potato dextrose agar) slants respectively at 4° C.

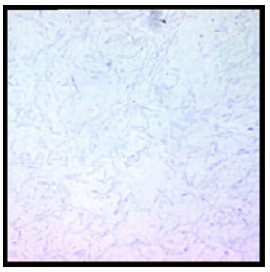
PHOTOMICROGRAPH OF GRAM-POSITIVE BACTERIA



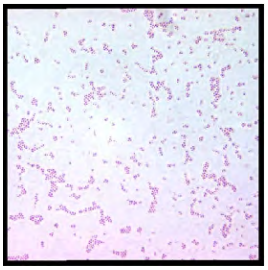
Bacillus cereus
ATCC11778



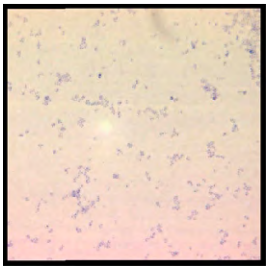
Bacillus megaterium
ATCC9885



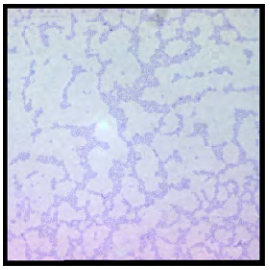
Bacillus subtilis
ATCC6633



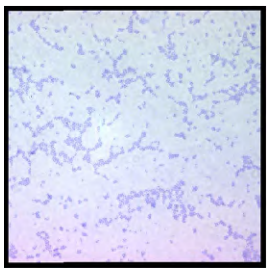
Corynebacterium rubrum
ATCC14898



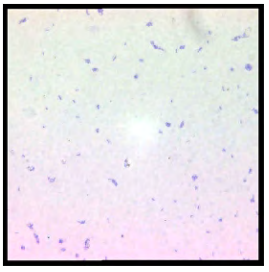
Micrococcus flavus
ATCC10240



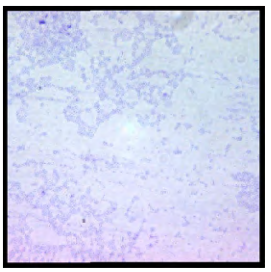
Staphylococcus aureus (1)
ATCC25923



Staphylococcus aureus (2)
ATCC29737



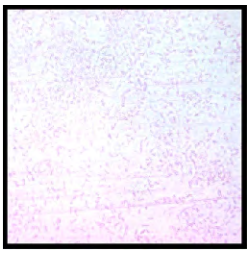
Staphylococcus epidermidis
ATCC12228



Staphylococcus subflava
NCIM2178

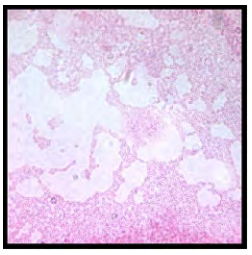
PHOTOMICROGRAPH OF GRAM-NEGATIVE BACTERIA

Alcaligenes fecalis
ATCC8750

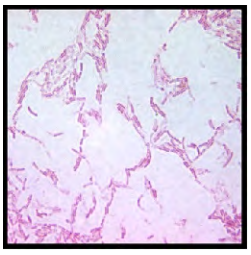


Citrobacter freundii
ATCC10787

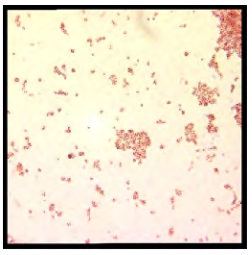
Enterobacter aerogenes
ATCC13048



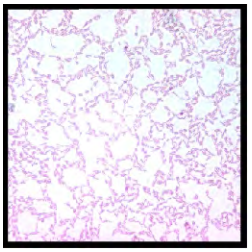
Escherichia coli
ATCC25922



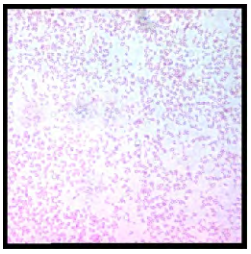
Klebsiella aerogenes
NCTC418



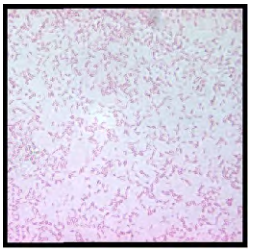
Klebsiella pneumoniae
NCIM2719



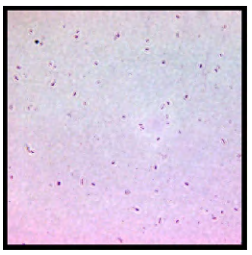
Proteus mirabilis
NCIM2241



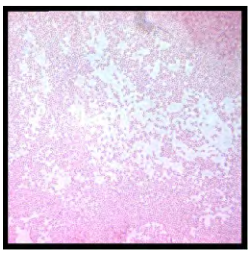
Proteus morganii
NCIM2040



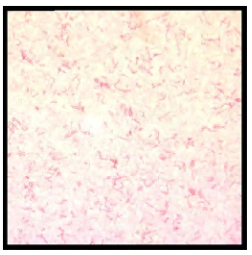
Proteus vulgaris
NCTC8313



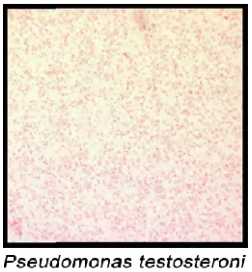
Pseudomonas aeruginosa
ATCC27853



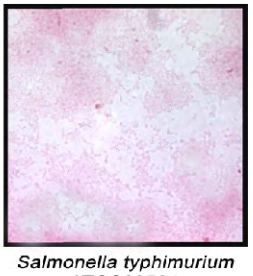
Pseudomonas putida
ATCC12842



Pseudomonas pseudoalcaligenes
ATCC17440

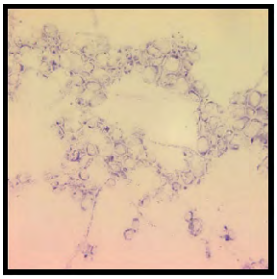


Pseudomonas testosteroni
NCIM5098

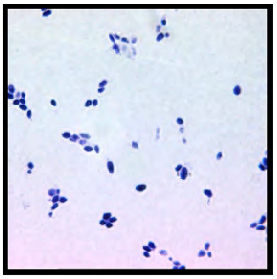


Salmonella typhimurium
ATCC23564

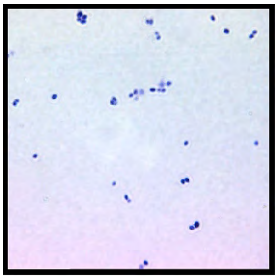
PHOTOMICROGRAPH OF FUNGI



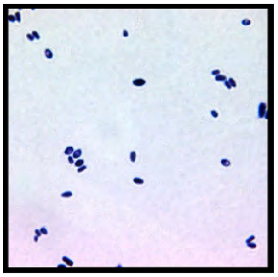
Candida albicans (1)
ATCC2091



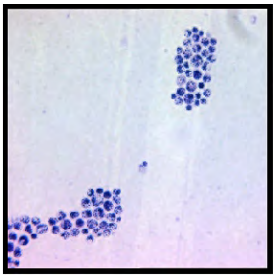
Candida albicans (2)
ATCC18804



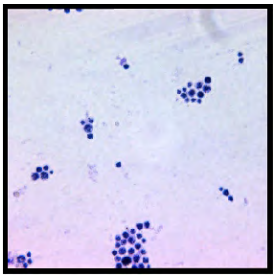
Candida glabrata
NCIM3448



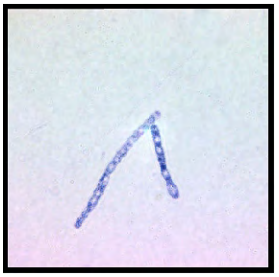
Candida tropicalis
ATCC4563



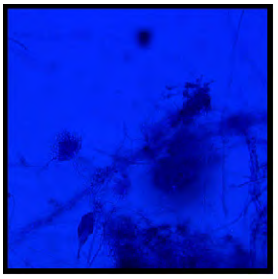
Cryptococcus luteolus
ATCC32044



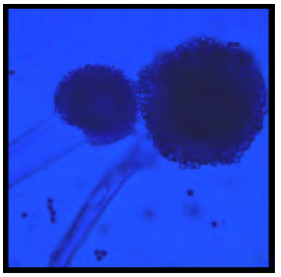
Cryptococcus neoformans
ATCC34664



Trichosporon beigelii
NCIM3404

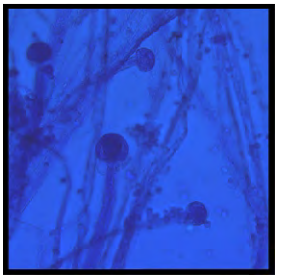


Aspergillus candidus
NCIM883



Aspergillus flavus
NCIM538

Aspergillus niger
ATCC6275



Mucor hiemalis wehmer
NCIM873

4.2.4 Standard antimicrobics Used

The investigated microorganisms were tested against standard antimicrobics. The standard antimicrobics used for bacterial strains were Piperacillin (100 µg /disc) and Gentamicin (10 µg/disc) and that for fungal strains were Amphotericin B (100 units/disc) and Fluconazole (10 µg/disc).

4.2.5 Antimicrobial assay

4.2.5.1 Preparation of inoculum

The test bacterial strains were inoculated into nutrient broth and were incubated at 37ºC on a rotary shaker whereas test fungal strains were inoculated into sabouraud dextrose broth and incubated at 28ºC on a rotary shaker. The inoculum size was maintained as per the 0.5 McFarland standard ($1x10^8$ cfu/ml). The activated inoculum was used for antimicrobial assay.

4.2.5.2 Preparation of test compound

The petroleum ether, acetone and methanol extracts of *M. hexandra* (Roxb.) Dubard leaf were diluted in 100 % dimethylsulphoxide (DMSO) and the stocks were prepared at a concentration of 25mg/ml and 12.5 mg/ml. The antimicrobial activity was evaluated at two different concentrations viz. 500 µg/disc and 250 µg/disc.

4.2.5.3 Antimicrobial susceptibility testing

The antimicrobial activity of petroleum ether, acetone and methanol extracts of *M. hexandra* (Roxb.) Dubard leaf was determined by agar disc diffusion method (Bauer et al., 1966; Parekh and Chanda, 2006b). The molten Mueller Hinton Agar No. 2 media (Hi-Media) for bacteria and Sabouraud dextrose agar media (Hi-Media) for fungi was inoculated with 200 µl of the inoculum ($1x10^8$ cfu/ml) when the temperature of media reached 40-42°C and then poured into the Petri plate (Hi-Media). Sterile disc (7 mm) (Hi-Media) was saturated with 20 µl of the test

compound (MPE, MAC and MME) with the concentration of 500 µg/disc and 250 g/disc and allowed to dry. The disc was then introduced on the upper layer of the seeded agar plate. For each microbial strain negative controls were maintained where pure solvent (DMSO) were used instead of the extract since it does not possess any antimicrobial effect (Pelczar et al., 1993) and for positive control the standard antimicrobics with known concentration were used. The bacterial plates were incubated at 37°C for 24 h and the fungal plates at 28°C for 48 h. The result of antimicrobial activity was obtained by measuring the diameter of the zone of inhibition. The values in the result are expressed as mean ± SEM. The values were compared with the standard antimicrobics. The experiment was performed under strict aseptic conditions for three times to minimize error. The results are presented in Table 4.1.

4.2.6 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration was determined by disc diffusion method (NCCLS, 1993 protocol, Okigbo et al., 2005). The Mueller Hinton agar plates for bacteria and sabouraud dextrose agar plates for fungi, containing an inoculum size of $1x10^8$ cfu/ml, were used. Two-fold serial dilutions of petroleum ether, acetone and methanol extracts of *M. hexandra* leaf, ranging from 250-32,000 µg/ml, were prepared in 100 % pure DMSO (dimethylsulphoxide). The prepared dilutions were impregnated on sterile discs and were placed aseptically on seeded agar plates. Appropriate controls were maintained. All the plates were incubated at 37°C for bacteria and at 28°C for fungi. The lowest concentration within the plate, which did not show any clear zone around the disc, was considered as MIC. The results of MIC are presented in Table 4.2.

4.3 RESULTS AND DISCUSSION

4.3.1 Antimicrobial activity

The presence of antibacterial and antifungal substances in higher plants is well established (Dhar et al., 1968; Bhakuni et al., 1974). Plants have provided sources for novel drug compounds as plants derived medicines have made

significant contribution towards human health. Phytomedicine can be used for the treatment of diseases as is done in case of Unani and Ayurvedic system of medicines or it can be the base for the development of medicine, a natural blueprint for the development of new drugs (Javed and Ali, 2002). Much of the exploration and utilization of natural products as antimicrobial agents arise. Though soil microorganisms or fungi produce most of the clinically used antibiotics, higher plants can be very good source of antibiotics.

Present study was conducted to investigate the antimicrobial potential of the petroleum ether, acetone and methanol extracts of *M. hexandra* (Roxb.) Dubard leaf at two different concentrations. The microorganisms used for the antimicrobial studies were Gram-positive and Gram-negative bacteria, yeast and moulds. The results of antimicrobial activity of *M. hexandra* (Roxb.) Dubard leaf extracts are summarized in Table 4.1.

The results showed that methanol extracts (MME) were better than the other two extracts (MPE and MAC) which implied that antimicrobial activity is better with the polar solvents. Also, the antimicrobial activity observed was concentration dependent for each extract. The mean zones of inhibition produced against the test microorganisms ranged between 8-22 mm. The highest zone of inhibition was obtained at the higher concentration with Enterobacter aerogenes. Amongst all the microbial strains investigated, a Gram-negative bacteria- *Citrobacter freundii,* yeasts- *Candida albicans* (2), *Candida glabrata, Cryptococcus luteolus, Cryptococcus lneoformans* and amongst moulds- *Aspergillus candidus, Aspergilllus niger* were most resistant strains which did not show any activity at all whereas a Gram-positive bacteria - *Bacillus cereus* and Gram-negative bacteria *Enterobacter aerogenes* and *Pseudomonas putida* were most susceptible bacteria (Table 4.1). However, all the three extracts were more active against bacterial strains than the fungal strains.

The methanol extract inhibited 76.47 % of microbial strains a higher concentration and 64.70 % at lower concentration. The methanol extract was totally inactive at the lower concentration (250 µg/disc) against *Staphylococcus epidermidis,*

Table 4.1 *In vitro* antimicrobial activity of *Manilkara hexandra* (Roxb.) Dubard against some medically important microorganisms

Microorganisms	Inhibition Zone (mm) [a]									
	Manilkara hexandra (Roxb.) Dubard leaf extracts [b] [Extractive yield in % [c]]						Antimicrobics [d]			
	MPE [5.5]		MAC [4.8]		MME [23]					
	Concentration of plant extract (µg /disk)						Pc	G	Ap	Fu
	500	250	500	250	500	250	100 µg /disc	10 µg/disc	100 units/disc	10 µg/disc
Gram-positve bacteria										
Bacillus cereus ATCC11778	10 ± 0	9 ± 0	13.5 ± 0.31	12.5 ± 0.31	14.5 ± 0.31	12 ± 0	16 ± 2	12 ± 1	-	-
Bacillus megaterium ATCC9885	-	-	9.5 ± 0.31	-	12 ± 0	10.5 ± 0.31	11.5 ± 0.5	20 ± 1	-	-
Bacillus subtilis ATCC6633	-	8.75 ± 0.15	-	-	10 ± 0	9 ± 0	17.5 ± 2.5	14 ± 1	-	-
Corynebacterium rubrum ATCC14898	-	-	9.75 ± 0.15	-	11 ± 0	9 ± 0	23.5 ± 0.5	20.5 ± 0.5	-	-
Micrococcus flavus ATCC10240	-	8 ± 0	11.5 ± 0.31	9 ± 0	11.75 ± 0.47	10.25 ± 0.15	32.5 ± 2.5	36.5 ± 0.5	-	-
Staphylococcus aureus (1) ATCC25923	-	-	9.5 ± 0	-	10 ± 0	8 ± 0	25 ± 3	17.5 ± 0.5	-	-
Staphylococcus aureus (1) ATCC29737	-	-	10.5 ± 0.47	-	11 ± 0	9 ± 0	24 ± 3	14 ± 1	-	-
Staphylococcus epidermidis ATCC12228	-	-	10.5 ± 0.31	-	9.25 ± 0.15	-	10 ± 1	20 ± 2	-	-
Staphylococcus subflava NCIM2178	-	-	-	-	10.25 ± 0.15	8.5 ± 0	21.5 ± 0.5	13.5 ± 0.5	-	-
Gram-negative bacteria										
Alcaligenes fecalis ATCC8750	-	-	-	-	9 ± 0	-	-	16 ± 2	-	-
Citrobacter freundii ATCC10787	-	-	-	-	-	-	19 ± 1	10 ± 0	-	-
Enterobacter aerogenes ATCC13048	11 ± 0.63	9.5 ± 0.31	20.5 ± 0.31	17 ± 0	22 ± 0	17 ± 0.63	10 ± 1	15 ± 1	-	-
Escherichia coli ATCC25922	-	-	-	-	9 ± 0	8 ± 0	14 ± 1	21 ± 1	-	-
Klebsiella aerogenes NCTC418	9 ± 0	-	13.75 ± 0.15	12 ± 0	14.75 ± 0	12 ± 0	17 ± 0	11.5 ± 0.5	-	-
Klebsiella pneumoniae NCIM2719	9 ± 0	-	11 ± 0	9 ± 0	11.75 ± 0.15	11 ± 0	25 ± 0	21 ± 1	-	-
Proteus mirabilis NCIM2241	-	8 ± 0	9 ± 0	8 ± 0	10 ± 0	9 ± 0	22.5 ± 2.5	24 ± 1	-	-
Proteus morganii NCIM2040	-	-	11.25 ± 0.15	10.5 ± 0.31	13.75 ± 0.15	12	18.5 ± 0.5	28 ± 0	-	-
Proteus vulgaris NCTC8313	-	-	-	-	10 ± 0	8 ± 0	12.5 ± 2.5	21.5 ± 1.5	-	-
Pseudomonas aeruginosa ATCC27853	-	-	9.5 ± 0.31	9 ± 0	10.5 ± 0.31	9 ± 0	21.5 ± 1.5	19 ± 1	-	-
Pseudomonas putida ATCC12842	9 ± 0	9 ± 0	12 ± 0	9 ± 0	13 ± 0	11.25 ± 0.15	35 ± 0	30.5 ± 0.5	-	-
Pseudomonas pseudoalcaligenes ATCC17440	-	9 ± 0	12.75 ± 0.15	11 ± 0.15	12.5 ± 0.31	12 ± 0	23.5 ± 1.5	25.5 ± 0.5	-	-
Pseudomonas testosteroni NCIM5098	-	-	10 ± 0	-	11 ± 0	9 ± 0	-	14.5 ± 0.5	-	-

Salmonella typhimurium ATCC23564	-	-	-	-	10 ± 0	9±0	20 ± 1	21 ± 1	-	-
Yeast										
Candida albicans (1) ATCC2091	-	-	9 ± 0.31	8.5 ± 0	9 ± 0	-	-	-	12.5 ± 0.5	-
Candida albicans (2) ATCC18804	-	-	-	-	-	-	-	-	17± 0	22 ± 1
Candida glabrata NCIM3448	-	-	-	-	-	-	-	-	18.5 ± 0.5	28 ± 1
Candida tropicalis ATCC4563	9 ± 0	10 ± 0	-	-	-	-	-	-	12 ± 1	-
Cryptococcus luteolus ATCC32044	-	-	-	-	-	-	-	-	15.5 ± 0.5	18.5 ± 0.5
Cryptococcus neoformans ATCC34664	-	-	-	-	-	-	-	-	14 ± 1	20 ± 0
Trichosporon beigelii NCIM3404	9.5 ± 0.31	10.75 ± 0.15	8 ± 0	-	8 ± 0	-	-	-	16.5 ± 0.5	24.5 ± 0.5
Mould										
Aspergillus candidus NCIM883	-	-	-	-			-	-	20 ± 0	17.5 ± 0.5
Aspergillus flavus NCIM538	-	-	15.5 ± 0.31	11.5 ± 0.31	16.5 ± 0.31	13 ± 0.63	-	-	18 ± 0	21 ± 1
Aspergillus niger ATCC6275	-	-	-	-			-	-	19 ± 1	14 ± 0
Mucor hiemalis wehmer NCIM873	-	-	9 ± 0	-	12.25 ± 0.15	10.5 ± 0.31	-	-	16.5 ± 0.5	-

-: no activity, Negative controls did not show any activity.

[a.] values are mean ± SEM; mean values include the diameter of the paper disc (7 mm)

[b.] MPE-Petroleum ether extract, MAC-Acetone extract, MME-Methanol extract

[c.] Percentage extract yield (w/w) was estimated as dry extract weight/dry material weight×100.

[d.] Pc-Piperacillin, G-Gentamicin, Ap- Amphotericin-B, Fu-Fluconazole

Alcaligenes fecalis, Citrobacter freundii and all the seven yeast spps. tested. In case of acetone extract, 58.82 % of microorganisms were inhibited at higher concentration while 35.30 % were inhibited at lower concentration. On the other hand the petroleum ether extract did not show any appreciable activity against all the microorganisms used in present investigation; it could inhibit only 20.58 % of microorganisms at higher concentration while 26.47 % at the lower concentration (Table 4.1).

The extracts were compared to the standard antibiotics. The standard antibacterial agents used were Piperacillin (100 µg/disc) and Gentamicin (10 µg/disc) while the standard antifungal agents used were Amphotericin-B (100 units/disc) and Fluconazole (10 µg/disc) (Table 4.1).

4.3.2 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations of the extracts of *M. hexandra* (Roxb.) Dubard are reported in Table 2. The most active extracts were subjected to the evaluation of MIC. The MIC of *M. hexandra* was evaluated within a range of concentration from 250-32,000 µg/ml.

The results of petroleum ether extract (MPE) showed MIC ranging from 2000-16000 µg/ml. The MIC of petroleum ether extract for *Bacillus cereus* and *Micrococcus flavus* was 2000 µg/ml; for *Proteus mirabilis* and *Pseudomonas putida* it was 4000 µg/ml; for *Bacillus subtilis, Enterobacter aerogenes, Escherichia coli, Pseudomonas pseudoalcaligenes, Aspergillus flavus* and *Mucor hiemalis* was observed as 8000 µg/ml and MIC for *Klebsiella pneumoniae* was observed as 16,000 µg/ml (Table 4.2).

The results of acetone extract (MAC) showed MIC ranging from 250-16,000 µg/ml. The MIC of acetone extract for *Pseudomonas testosteroni* was 250 µg/ml while for *Klebsiella pneumoniae* it was <250 µg/ml; for *Bacillus cereus, Micrococcus flavus, Klebsiella aerogenes, Proteus morganii, Pseudomonas aeruginosa* and *Pseudomonas pseudoalcaligenes* MIC was 2000 µg/ml; for

Bacillus megaterium, Corynebacterium rubrum, Staphylococcus aureus (2), *Pseudomonas putida* and *Trichosporon beigelii* it was 4000 µg/ml; for *Staphylococcus aureus* (1), *Staphylococcus epidermidis, Enterobacter aerogenes, Proteus mirabilis,* and *Mucor hiemalis* was 8000 µg/ml and the MIC values for *Candida albicans* (1) was 16,000 µg/ml.

The results of methanol extract (MME) showed MIC ranging from 250-8000 µg/ml. The MIC of methanol extract for *Klebsiella pneumoniae* was < 250 µg/ml; for *Bacillus cereus, Micrococcus flavus, Klebsiella aerogenes, Proteus mirabils* and *Proteus morganii* was 1000 µg/ml; for *Alcaligenes fecalis, Pseudomonas pseudoalcaligenes, Pseudomonas testosteroni, Salmonella typhimurium, Trichosporon beigelii* and *Aspergillus flavus* it was 2000 µg/ml; the MIC value for *Bacillus megaterium, Corynebacterium rubrum, Staphylococcus aureus* (1), *Staphylococcus aureus* (2), *Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas putida* and *Mucor hiemalis* was 4000 µg/ml and that for *Bacillus subtilis, Staphylococcus epidermidis, Staphylococcus subfava, Enterobacter aerogenes, Escherichia coli,* and *Candida albicans* (1) it was 8000 µg/ml.

The results showed that methanol extract of *M. hexandra* (Roxb.) Dubard leaf possessed measurable in vitro antimicrobial activity against many of the microorganisms implicated in the pathogenesis of the human infections. The broad range of inhibition found implied that the extract had a comparable antimicrobial activity. This may be because of presence of tannins which were detected to an appreciable amount in phytochemical analysis. Tannins, (non-crystalizable substances), have astringent actions, which form the basis for their therapeutic applications (Edward et al., 1970). It has been found that plants which contain tannins possess antimicrobial activity (Trease and Evans, 1989). Hence the significant antibacterial activity of the extract could be ascribed to the tannins constituent. However, further work is needed to isolate the active principle from the active plant extracts and to carry our pharmaceutical studies.

Table 4.2 Minimum Inhibitory Concentration (MIC) of methanol extract of *Manilkara hexandra* (Roxb.) Dubard.

Microorganisms	MIC of *Manilkara hexandra* leaf extracts (µg /ml)		
	MPE	MAC	MME
Gram-positve bacteria			
Bacillus cereus ATCC 11778	2000	2000	1000
Bacillus megaterium ATCC 9885	NA	4000	4000
Bacillus subtilis ATCC 6633	8000	NA	8000
Corynebacterium rubrum ATCC 14898	NA	4000	4000
Micrococcus flavus ATCC 10240	2000	2000	1000
Staphylococcus aureus (1) ATCC 25923	NA	8000	4000
Staphylococcus aureus (2) ATCC 29737	NA	4000	4000
Staphylococcus epidermidis ATCC 12228	NA	8000	8000
Staphylococcus subflava NCIM 2178	NA	NA	8000
Gram-negative bacteria			
Alcaligenes fecalis ATCC 8750	NA	NA	2000
Enterobacter aerogenes ATCC 13048	8000	8000	8000
Escherichia coli ATCC 25922	8000	NA	8000
Klebsiella aerogenes NCTC 418	NA	2000	1000
Klebsiella pneumoniae NCIM 2719	16000	<250	<250
Proteus mirabilis NCIM 2241	4000	8000	1000
Proteus morganii NCIM 2040	NA	2000	1000
Proteus vulgaris NCTC 8313	NA	NA	4000
Pseudomonas aeruginosa ATCC 27853	NA	2000	4000
Pseudomonas putida ATCC 12842	4000	4000	4000
Pseudomonas pseudoalcaligenes ATCC 17440	8000	2000	2000
Pseudomonas testosteroni NCIM 5098	NA	250	2000
Salmonella typhimurium ATCC 23564	NA	NA	2000
Yeast			
Candida albicans (1) ATCC 2091	NA	16000	8000
Trichosporon beigelii NCIM 3404	NA	4000	2000
Mould			
Aspergillus flavus NCIM 538	8000	4000	2000
Mucor hiemalis wehmer NCIM 873	8000	8000	4000

NA: No activity
MPE: Petroleum ether extract of *M. hexandra,*
MAC: Acetone extract of *M. hexandra,*
MME: Methanol extract of *M. hexandra.*

The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against microbes. Plant based antimicrobials represents 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. Therefore, continuous further exploration of plant derived antimicrobials is needed today.

4.4 CONCLUSIONS

The present study for assessing the potent extracts of *Manilkara hexandra* (Roxb.) Dubard leaf, reveals that methanol extract was the most active amongst the three extracts. Methanol extract was thus selected for further studies on toxicity and pharmacological effect on diabetes.

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CHAPTER FIVE 5 ASSESSMENT OF SAFETY OF *MANILKARA HEXANDRA* LEAF: ACUTE TOXICITY STUDIES

5.1 INTRODUCTION

Toxicology basically is defined traditionally as "the science of poisons." The word toxicology is derived from toxicon - a poisonous substance into which arrow heads were dipped and toxikos - a bow (Robyn, 1996). A poison is any substance has a harmful effect on a living system (EHSC, 2004). A more descriptive definition of toxicology is "the study of the adverse effects of chemicals or physical agents on biological material with special emphasis on the harmful effects." After gaining relevant information on the harmful effects of a compound the levels for its safe usage or the degree of its safeness is established, this is known as its 'Biosafety level'.

Toxicology is a relatively young biological science that involves a complex interrelationship among dose, absorption, distribution, metabolism and elimination (Robyn, 1996). A toxic substance is a material which has toxic properties. Knowledge of how toxic agents damage the body has progressed along with medical knowledge. Toxicity depends not only on the dose of the substance but also on the toxic properties of the substance. The relationship between these two factors is important in the assessment of therapeutic dosage in pharmacology and herbalism (Klaassen et al., 1995; Hayes, 2001).

5.1.1 History

The historical development of toxicology began with early cave dwellers who recognized poisonous plants and animals and used their extracts for hunting or in warfare. By 1500 BC, written records indicated that hemlock, opium, arrow poisons, and certain metals were used to poison enemies or for state executions. Gradually the concept of toxicology was developed to determine the

effectiveness of a particular compound.

By the time of the Renaissance certain concepts fundamental to toxicology began to take shape. Orfila, a Spanish physician, is often referred to as the founder of toxicology.

Orfila was the first one who prepared a systematic correlation between the chemical and biological properties of poisons. He demonstrated effects of poisons on specific organs by analyzing autopsy materials for poisons and their associated tissue damage. Paracelsus (1493-1541) was one of the first to distinguish between the therapeutic and toxic properties of substances. Paracelsus is often quoted for his statement: "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy." He determined that specific chemicals were actually responsible for the toxicity of a plant or animal poison. He further documented that the body's response to those specific chemicals depended on the dose received. His studies revealed that small doses of a substance might be harmless or beneficial whereas larger doses could be toxic. This is now known as the dose-response relationship, a major concept of toxicology (EHSC, 2004).

The 20th century is marked by an advanced level of understanding of toxicology. The knowledge of toxic effects on organs and cells is now being revealed at the molecular level. It is recognized that virtually all toxic effects are caused by changes in specific cellular molecules and biochemicals.

5.1.2 Toxicity effects

In traditional toxicology the organism tends to be regarded as the primary unit for the expression of toxic effects. The adverse effects caused by a toxic substance may occur in many forms, ranging from immediate death to subtle changes not realized until months or years later. Consequently, the onset and duration of effects, as well as the effects themselves (e.g., changes in behavior and alterations in blood flow, renal functions, and metabolic parameters), become

signs of impaired homeostasis and are inevitably bound to the intact organism.

All chemicals are toxic under some condition of exposure. Therefore, it is necessary to define these conditions as well as the quantity involved in the exposure in order to compare the toxicity characteristics of chemicals.

Toxic effects are classified as either acute, sub chronic or chronic. The dose-response concept is the basis for all toxicity assessments. It is used differently to evaluate acute effects, sub chronic effects and chronic effects.

5.1.3 Acute Toxicity

Acute toxicity studies are the most common of the toxicity or safety evaluations. Acute toxicity is usually defined as the adverse change(s) occurring immediately or after short time following a single dose or short period of exposure to a substance or substances within 24 h. An adverse effect is any effect that results in functional impairment and/or biochemical lesions that may affect the performance of the whole organism or that reduce the organ's ability to respond to an additional challenge. Consequently, a chemical that enters the organism via the oral route during a restricted time and produces any adverse effect with little delay is acutely toxic. However, the term acute oral toxicity is most often used in connection to lethality and LD_{50} determinations.

5.1.3.1 Objective

In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step. Acute studies are generally performed first to generate data to improve the design and conduct of longer-term studies including sub chronic and chronic toxicity tests and others devoted to reproductivity, carcinogenicity and neurotoxicity testing. If designed appropriately, these studies may also provide initial information on the biological activity, toxicokinetics of a chemical and useful to gain insight into its mechanism of action (Fan and Chang, 1996; Walum, 1998).

The short-term hazard from a chemical is determined by identifying the acute toxicity by the most likely routes of exposure. Acute toxicity effects are also investigated when exposed to a chemical taken orally, absorbed through the skin or by inhalation. A study of acute toxicity determines the dose-dependent adverse effect and from the comprehensive acute toxicity profile of a substance various appropriate data may be collected. This may include the incidence of lethality. If the dose-dependent lethality incidence is determined in a precise manner, it is usually expressed as an LD_{50} (Gad and Chengelis, 1988).

5.1.3.2 Median lethal dose test (LD50)

The term LD_{50} (lethal dose 50 or median lethal dose) is defined as statistically derived dose of a substance that, when administered in an acute toxicity test, is expected to cause death in 50 % of the treated animals exposed for a specified time (Oliver, 1986). For a classical LD_{50} study, laboratory mice and rats are species typically selected. Materials can be classified as hazardous waste on the basis of LD_{50} of the material. A number of protocols exist for conducting LD_{50} studies (Litchfield and Wilcoxon, 1949; Bruce, 1985).

Determination of lethality (LD_{50}) is one of the many measures of toxicity evaluated in acute toxicity studies. The focus of acute toxicity is to identify lethal effects and target organs, with emphasis on behavioral, gross anatomical, hematological biochemical and histopathological changes. Dose selection in acute toxicity tests should aim to produce a dose-response curve that will enable an acceptable estimation of the median lethal dose (LD_{50}) which usually occurs within 24 h after initiation of exposure (Fan and Chang, 1996).

The LD_{50} value (precise or approximate) is currently the basis for toxicologic classification of chemicals. Thus, LD_{50} values based on animal experiments are used to estimate the lethal dose of substances for humans (Martin et al., 1989).

5.1.3.3 Application

Acute toxicity studies in animals are usually necessary for any pharmaceutical intended for human use. The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity. Acute toxicity studies may also aid in the selection of starting doses for Phase 1 human studies, and provide information relevant to acute overdosing in humans (Guidance for Industry, 1996).

5.1.4 Botanicals: Toxicological evaluation

Plants are an important part of culture and traditions all over the globe. Most of the people today are reliant on herbal medicines for their health care needs. Herbal prescriptions and natural remedies are commonly employed in developing countries for the treatment of various diseases, which is an alternative way to compensate for some deficiencies in orthodox pharmacotherapy (Sofowora, 1989; Zhu et al., 2002). The fact that herbal medicines contain ingredients to maintain health and to cure ailments is well known. However, the fact that they may contain toxic substances which are harmful or even dangerous to health is least known. Moreover there is limited scientific evidence regarding safety and efficacy to back up the continued therapeutic application of these remedies.

Plants commonly used in traditional medicine are assumed to be safe. This safety is based on their long usage in the treatment of diseases according to knowledge accumulated over centuries. Virtually all our knowledge is derived from human exposures to herbs leading to acute toxicities and little information is available from prior animal experimentation (Sheehan, 1998).

It has been reported by researchers that many plants used as food or in traditional medicine are potentially toxic, mutagenic and carcinogenic (Schimmer et al., 1988, 1994; Higashimoto et al., 1993; Kassie et al., 1996; De Sa Ferrira and Ferrao Vargas, 1999). Herbs may be contaminated with undeclared toxic botanicals or heavy metals (De Smet, 1992). Several plants used in traditional

medicine can cause damage to the genetic material and therefore have the potential to cause long-term damage in patients when administered as medical preparations. Thus, the rationale for their utilization has rested largely on the treatment of various ailments with caution and rigorous long-term toxicological and clinical studies (Zhu et al., 2002; Santamaria et al., 2007). Now, with the upsurge in the use of herbal medicines, a thorough scientific investigation of these plants will go a long way in validating their folkloric usage (Sofowora, 1989).

It is essential to evaluate the quality and safety of herbs before being prescribed for any medicinal use. Considering the aforesaid, crude powder (MCR) and methanol extract (MME) of *Manilkara hexandra* (Roxb.) Dubard leaf was evaluated for the acute toxicity studies.

5.2 MATERIAL AND METHODS

5.2.1 Experimental animals

Swiss albino mice of either sex were used for acute toxicity studies. The animals were obtained from Sarabhai Research Center (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. The animals were kept in a group of 6-8 per cage and facilitated with standard environmental condition of photoperiod (12:12 h dark:light cycle) and temperature (27 ± 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 ethical committee.

5.2.2 Drug preparation

The crude powder (MCR) and methanol extract (MME) of *Manilkara hexandra* (Roxb.) Dubard leaf was suspended in water (5% gum acacia drops as surfactant) and four doses of 390, 780, 1560 and 3120 mg kg^{-1} body weight were prepared.

5.2.3 Experimental groups

Nine groups of mice, each consisting of three mice were kept for determination of acute toxicity of MCR and MME at four doses as mentioned in section 5.2.2. Amongst nine groups, one group was the control group. The bioassays were conducted according to the World Health Organization guideline for the evaluation of the safety and efficiency of herbal medicines (WHO, 1992).

5.2.4 Drug administration

The test drug, MCR and MME as mentioned in section 5.2.2, was administered orally (p.o.) to all the groups of mice. The control group received water. The test drug (MCR and MME) was administered one hour prior to the experiment.

5.2.5 Behavioural assessment

The acute toxicity effect of MCR and MME of *Manilkara hexandra* (Roxb.) Dubard leaf was assessed by gross behaviour model (Morpugo, 1971). 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. The animals were observed for different parameters of bahavioural changes. After drug administration, the behaviour modifications were observed every hour till 5 h and then at 24 h, 48 h and 72 h. The mortality was observed for 10 days after treatment. The observed result was recorded as the score of 0-3 point scale relative to the average intensity of the phenomena observed. Various parameters of gross behaviour studied are (Figure 5.1):

TOXICOLOGICAL STUDY

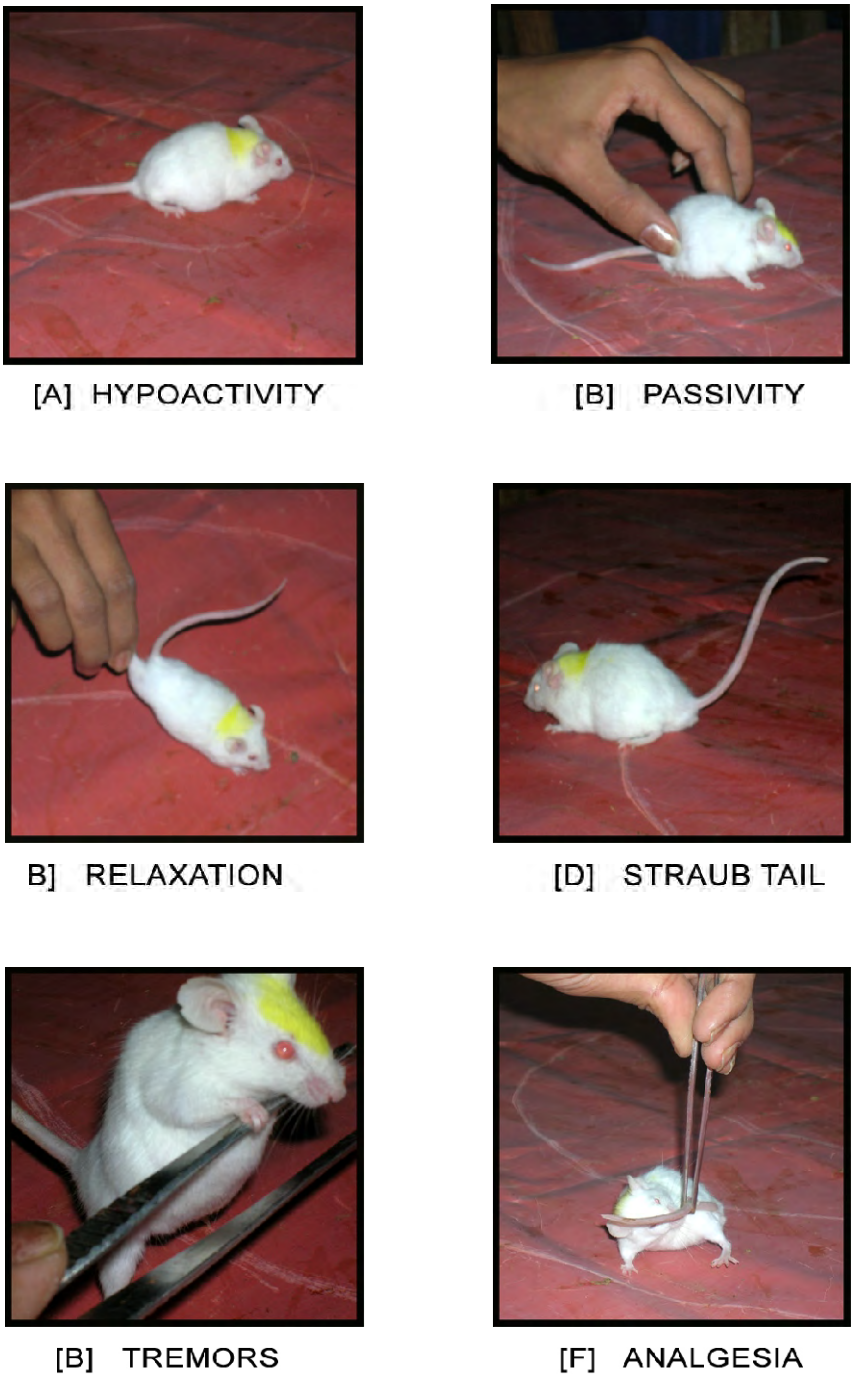
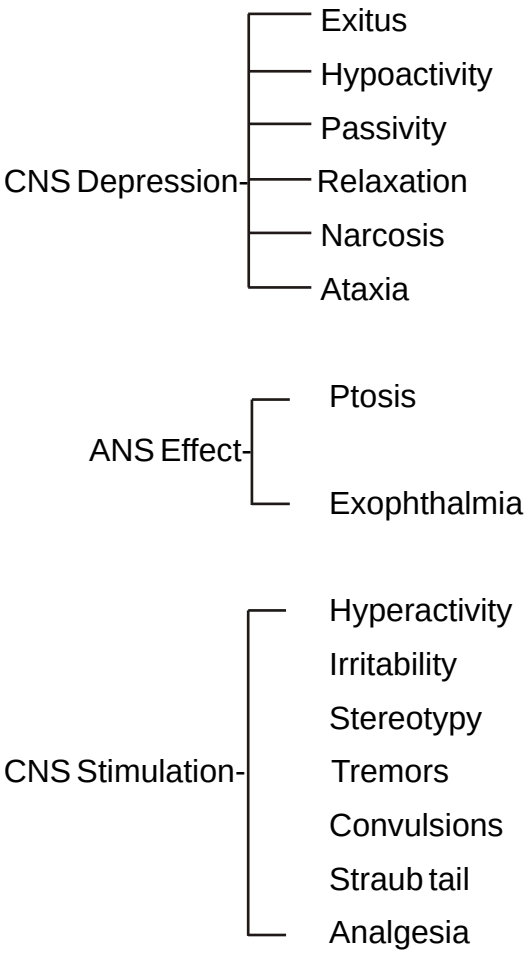


FIGURE 5.1 Photomicrographs demonstrating different behaviour in toxicity studies



5.2.6 Determination of LD_{50}

The experimental observations of acute toxicity study showed mortality with methanol extract of *Manilkara hexandra* (Roxb.) Dubard leaf (MME). Therefore, MME was evaluated for determining LD_{50} value. The doses from 2000 to 9000 mg kg^{-1} were taken for determining LD_{50}. Each group consisted of six mice. Mortality was observed within each group for 10 days after drug administration.

5.3 RESULTS AND DISCUSSION

The results of acute toxicity studies of crude powder (MCR) and methanol extract (MME) of *M. hexandra* (Roxb.) Dubard leaf is shown in Table 5.1 and Table 5.2 respectively. The methanol extract exhibited pronounced effect on gross behaviour of mice as compared to the crude powder. The crude powder as well as methanol extract showed dose dependent effect on gross behaviour with the increasing dose levels i.e. 390, 780, 1560 and 3120 mg kg^{-1} showing strong CNS depression and weak CNS stimulation.

Mild to moderate hypoactivity or reduced locomotion was noted at all dose levels for crude powder (MCR) of *M. hexandra* (Roxb.) Dubard leaf. MCR showed no effect or mild effect at lower doses while moderate effect at the highest dose level (3120 mg kg^{-1}) for passivity and relaxation. Mild effect for irritability was observed at 2 h for 3120 mg kg^{-1}. Mild effect of stereotypy was observed at 1 h, 24 h and 48 h at 390 mg kg^{-1} and 780 mg kg^{-1} dose level while analgesic effect was observed at 1 h at 780 and 3120 mg kg^{-1} dose level. There was no mortality observed at any dose levels for crude powder (MCR).

The methanol extract (MME) of *M. hexandra* (Roxb.) Dubard leaf showed marked effect for hypoactivity at the highest dose level (3120 mg kg^{-1}) while moderate effect at 390 and 780 mg kg^{-1} dose levels. The hypoactive effect increased with the increasing time period. Moderate effect for passivity and relaxation was observed at the highest dose level (3120 mg kg^{-1}) while there was either no effect or very mild effect at other dose levels. MME showed weak CNS stimulation. Irritability was not observed. Mild effect for stereotypy was observed at all dose levels except the highest dose level (3120 mg kg^{-1}). Mild effect of analgesia was observed at 1 h for 390 mg kg^{-1} dose level while at 1 h and 2 h for 3120 mg kg^{-1} dose level. Mortality was observed at the highest dose level i.e. 3120 mg kg^{-1} with MME. Animal death was preceded by symptoms, such as hypoactivity and lethargy. The further evaluation of LD_{50} at the dose levels 2000-9000 mg Kg^{-1} revealed that methanol extract (MME) was devoid of acute toxicity at the studied dose level.

Table 5.1 Effect of crude powder of Manilkara hexandra (Roxb.) Dubard leaf on gross behaviour of mice

Treatment mg kg-1	Parameters of Gross Behaviour																				
	Morta-lity	CNS Depression																			
		Hypoactivity							Passivity							Relaxation						
		1h	2h	3h	4h	5h	24h	48h	1h	2h	3h	4h	5h	24h	48h	1h	2h	3h	4h	5h	24h	48h
Control	-	1	-	1	1	1	-	1	1	-	-	-	-	-	-	1	-	-	-	-	-	-
MCR-390	-	1	2	2	2	2	2	1	-	-	-	1	-	1	-	-	-	-	1	-	1	-
MCR-780	-	1	-	1	1	1	1	-	1	1	-	1	-	-	-	-	1	-	1	-	-	-
MCR-1560	-	1	1	2	2	2	1	2	-	1	1	1	1	-	1	-	1	-	1	-	1	1
MCR-3120	-	2	1	2	1	1	-	1	2	2	2	2	1	-	1	1	1	2	2	1	-	1

Treatment mg kg-1	CNS Stimulation																				
	Irritability							Stereotypy							Analgesia						
	1h	2h	3h	4h	5h	24h	48h	1h	2h	3h	4h	5h	24h	48h	1h	2h	3h	4h	5h	24h	48h
MCR-390	-	-	-	-	-	-	-	1	-	-	-	-	1	1	-	-	-	-	-	-	-
MCR-780	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-
MCR-1560	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MCR-3120	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-

MCR: Crude powder; -: No effect; 1: Mild effect; 2: Moderate effect; 3: Marked effect.

Table 5.2 Effect of methanol extract of Manilkara hexandra (Roxb.) Dubard leaf on gross behaviour of mice

Treatment mg kg-1	Parameters of Gross Behaviour																				
	Morta-lity	CNS Depression																			
		Hypoactivity							Passivity							Relaxation						
		1h	2h	3h	4h	5h	24h	48h	1h	2h	3h	4h	5h	24h	48h	1h	2h	3h	4h	5h	24h	48h
Control	-	1	-	1	1	1	-	1	1	-	-	-	-	-	-	1	-	-	-	-	-	-
MME-390	-	1	2	2	2	3	2	2	-	1	-	-	1	-	-	-	-	-	-	1	-	-
MME-780	-	-	1	2	1	2	2	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-
MME-1560	-	1	-	1	2	1	1	2	-	-	-	1	1	-	1	-	-	-	-	1	-	-
MME-3120	2	3	3	3	3	3	-	-	-	2	2	2	2	-	-	2	1	2	1	2	-	-

Treatment mg kg-1	CNS Stimulation																				
	Irritability							Stereotypy							Analgesia						
	1h	2h	3h	4h	5h	24h	48h	1h	2h	3h	4h	5h	24h	48h	1h	2h	3h	4h	5h	24h	48h
Control	-	-	-	-	-	-	-	1	-	-	-	-	-	-	1	-	-	-	-	-	-
MME-390	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
MME-780	-	-	-	-	-	-	-	-	1	1	-	1	-	-	-	-	-	-	-	-	-
MME-1560	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	-
MME-3120	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-

MME: Methanol extract; -: No effect; 1: Mild effect; 2: Moderate effect; 3: Marked effect.

5.4 CONCLUSIONS

The acute toxicity studies of crude powder (MCR) and methanol extract (MME) of *M. hexandra* (Roxb.) Dubard leaf showed that they did not possess any toxic effect at the studied dose levels and are safe till the dose level of 9000 mg kg^{-1}.

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CHAPTER SIX 6

ASSESSMENT OF ANTI-DIABETIC ACTIVITY OF *MANILKARA HEXANDRA* LEAF IN ALBINO RATS

6.1 INTRODUCTION

Diabetes is one of the most common noncommunicable diseases and a serious life-long condition appearing worldwide. The etiology of diabetes is a complex interaction of genetic and environmental factors. It is a heterogeneous group of metabolic disorders characterized physiologically by dysfunction of pancreatic cells and deficiency in insulin secretion or insulin activity and clinically by hyperglycemia or impaired glucose tolerance and other manifestable disorders (Nyholm et al., 2000). It is an endocrinological syndrome abnormally having high levels of sugar in the blood. This may be either due to insulin not being produced at all, is not made at sufficient levels, or is not as effective as it should be.

Diabetes is still a serious health problem all over the world since it is associated with increased morbidity and mortality rate. When compared with the general population, mortality and morbidity increase in diabetes is mainly due to the associated chronic complications both specific (microvascular) and nonspecific (macrovascular). Since the disease prevails in both genders and in all age groups, the general public has a concern about its control and treatment (Safdar et al., 2004).

6.1.1 Classification

Diabetes is classified by underlying cause. The most common forms of diabetes are categorized as Type 1, or insulin-dependent diabetes mellitus (IDDM) - an autoimmune disease in which the body's own immune system attacks the pancreatic cells, rendering it unable to produce insulin and Type 2, or non-insulin-dependent diabetes mellitus (NIDDM) - in which there is resistance to the effects of insulin or a defect in insulin secretion (DeFronzo, 1997, Xie et al., 2002).

Type 2 diabetes commonly occurs in adults associated with obesity. There are many underlying factors that contribute to the high blood glucose levels in these individuals. An important factor is the resistance to insulin in the body essentially ignoring its insulin secretions. A second factor is the decreased production of insulin by the cells of the pancreas. Therefore, an individual with Type 2 diabetes may have a combination of deficient secretion and deficient action of insulin.

In contrast to Type 2 diabetes, Type 1 diabetes most commonly occurs in children and is a result of the body's immune system attacking and destroying the cells. The trigger for this autoimmune attack is not clear, but the result is the end of insulin production (ECDCD, 2003).

6.1.2 History

The term “Diabetes” was first used around 250 B.C. It is a Greek word meaning “to syphon”, reflecting how diabetes seemed to rapidly drain fluid from the affected individual. The Greek physician Aretaeus noted that affected individuals passed increasing amounts of urine as if there was “liquefaction of flesh and bones into urine”. The complete term “diabetes mellitus” was coined in 1674 by Thomas Willis. Mellitus is Latin for honey, which is how Willis described the urine of diabetics.

Historical accounts reveal that as early as 700-200 BC, diabetes mellitus was a well recognized disease in India and was even distinguished as two types, a genetically based disorder and other one resulting from dietary indiscretion (Oubre et al., 1997). Ancient Hindu writings document how black ants and flies were attracted to the urine of diabetics. The Indian physician Sushruta in 400 B.C. described the sweet taste of urine from affected individuals, and for many centuries to come, the sweet taste of urine was a key to the diagnosis.

Physicians have observed the effects of diabetes for thousands of years. One of the effects of diabetes is the presence of glucose in the urine (glucosuria). For much of the time, little was known about this fatal disease that caused weight loss

of body, extreme thirst, and frequent urination. It was in 1922 that the first patient was successfully treated with insulin. Till the mid-1800s, the treatments offered for diabetes varied tremendously. A breakthrough in the puzzle of diabetes came in 1889. German physicians Joseph von Mering and Oskar Minkowski surgically removed the pancreas from dogs. The dogs immediately developed diabetes. Now that a link was established between the pancreas and diabetes, research focused on isolating the pancreatic extract that could treat diabetes. Dr. Frederick Banting succeeded in his experiments of isolating a pancreatic extract. The diabetic dog was kept alive for eight days by regular injections until supplies of the extract, at that time called "isletin", was exhausted. Experiments on dogs showed that extracts from the pancreas caused a drop in blood sugar, caused glucose in the urine to disappear, and produced a marked improvement in clinical condition. A young boy, Leonard Thompson, was the first patient to receive insulin treatment in the year 1922 and lived for thirteen years. Over the next 70 years, insulin was further refined and purified. A revolution came with the production of recombinant human DNA insulin in 1978. Instead of collecting insulin from animals, new human insulin could be synthesized. In 1923, Banting and Macloed were awarded the Nobel Prize for the discovery of insulin. In his Nobel Lecture, Banting concluded the following about their discovery: “Insulin is not a cure for diabetes; it is a treatment.”

6.1.3 Epidemiology

Present status projects that incidence of diabetes is on rise. Present number of diabetics worldwide is 150 million and according to new estimates from researchers at the World Health Organization (WHO), there will be an increase of about 300 million or more by the year 2030 (Warner, 2004). Only in year 2001, about 441,004 deaths were registered and 49,855 of them provoked by diabetes, representing 11.2% of the total population (INEGI/Health Ministry, 2002). In United States, diabetes is the sixth leading cause of death (NIDDK, 1995). The prevalence of diabetes mellitus is rapidly increasing worldwide and India is estimated to have 31 million diabetics from the total population of the world

(Warner, 2004). Diabetes is predicted to become one of the most common diseases in the world within a couple of decades, affecting at least half a billion people.

The driving force behind the high prevalence of diabetes is the rise of obesity, sedentary lifestyle, consumption of energy rich diet, etc. (Yagnik, 2001). The diabetes epidemic is accelerating in the developing world, with an increasing proportion of affected people in younger age groups.

The prevalence of Type 2 diabetes is now at epidemic proportions. Type 2 diabetes has a significant impact on the health, quality of life, and life expectancy of patients, as well as on the health care system. Type 2 diabetes accounts for about 90-95 % of population while Type 1 diabetes accounts for about 5 -10% of the total population. In the past, Type 2 was rarely seen in the young, but recent reports describe Type 2 diabetes being diagnosed even in children and adolescents (WHO, 2003).

6.1.4 Sugar Regulation: Carbohydrate, protein and lipid metabolism

Glucose is an essential fuel for the body and is the main source of energy for the tissue cell. The amount of glucose in the blood is controlled mainly by the hormones insulin and glucagon. The rise in blood glucose following a meal is detected by the pancreatic cells, which respond by releasing insulin. Glucose is transported into the cell by Type 2 glucose transporters (GLUT2). As glucose metabolism proceeds, ATP is produced which closes ATP-gated potassium channels in the cell membrane. Positively charged potassium ions (K^+) are now prevented from leaving the cell. The rise in positive charge inside the cell causes depolarization thereby opening the voltage-gated calcium channels and allowing calcium ions (Ca^{2+}) to flood into the cell. The increase in intracellular calcium concentration triggers the secretion of insulin via exocytosis.

Insulin increases the uptake and use of glucose by tissues such as skeletal muscle and fat cells. Once inside the cell, some of the glucose is used immediately

via glycolysis. Any glucose that is not used immediately is taken up by the liver and muscle where it can be converted into glycogen (glycogenesis). When glycogen stores are fully replenished, excess glucose is converted into fat in a process called lipogenesis by increasing the number of glucose transporters (GLUT4) expressed on the surface of the fat cell, causing a rapid uptake of glucose. Glucose is converted into fatty acids that are stored as triglycerides for storage.

The rise in glucose also inhibits the release of glucagon, inhibiting the production of glucose from other sources, e.g., glycogen break down. Glucose may also indirectly contribute to protein synthesis by synthesis of amino acids. Glucagon is the main hormone opposing the action of insulin and is released when food is scarce. Glucagon also helps the body to switch to using resources other than glucose, such as fat and protein during starvation.

6.1.5 Pathophysiology and complications

Diabetes mellitus is characterized by chronic hyperglycemia together with biochemical alterations of carbohydrate, lipid and protein metabolism associated with absolute or relative deficiencies in insulin secretion and/or insulin action (Arky, 1982; Andreoli et al., 1990).

Type 2 diabetes is known to have a strong genetic component along with some environmental determinants. Although the disease is genetically heterogeneous, there appears to be a fairly consistent phenotype once the disease is fully manifested. Whatever the pathogenic causes, the early stage of Type 2 diabetes is characterized by insulin resistance in insulin-targeting tissues, mainly liver, skeletal muscle and adiposities. Insulin resistance develops from obesity and physical inactivity (Gerick, 1998; Chisholm et al., 1997). Insulin resistance in these tissues is associated with excessive glucose production by the liver and impaired glucose utilization by peripheral tissues, especially muscles. These events undermine metabolic homeostasis, but may not directly lead to overt

diabetes in early stage. The chronic over-stimulation of insulin secretion gradually diminishes and eventually exhausts the islet cell reserve. A state of absolute insulin deficiency renders the fully blown clinical diabetes (DeFronzo, 1988; Olefsky, 1999). The transition of impaired glucose tolerance to Type 2 diabetes can also be influenced by ethnicity, degree of obesity, distribution of body fat, sedentary lifestyle, aging and other concomitant medical conditions (Clark, 1998).

In diabetic patients the most common acute complications are metabolic problems and infection. The long-term complications are macrovascular complications (hypertension, dyslipidemia, myocardial infraction, stroke), microvascular complications (retinopathy, nephropathy, diabetic neuropathy, diarrhea, neurogenic bladder, impaired cardiovascular reflexes, sexual dysfunction), and diabetic foot disorders (Davidson, 1991).

Hyperglycemia is caused by the increased production of glucose by the liver (driven by glucagon) and the decreased use of glucose of insulin by peripheral tissues (because of the lack of insulin). If there is too much insulin in the body compared to the amount of blood sugar, and the blood sugar falls below normal levels, a condition known as hypoglycemia occurs.

Acute metabolic complications like diabetic ketoacidosis (DKA) is a metabolic state emerging in some diabetics (Motala et al., 2000). As in starvation, diabetics use non-glucose sources of energy, such as fatty acids and ketone bodies, in their peripheral tissues. Because the ketones are weak acids, they acidify the blood. The result is the metabolic state of diabetic ketoacidosis. Hypertriglyceridemia is also seen in DKA. The liver combines triglycerol with protein to form very low density lipoprotein (VLDL). It then releases VLDL into the blood. In diabetics, the enzyme that normally degrades lipoproteins (lipoprotein lipase) is inhibited by the low level of insulin and the high level of glucagon. As a result, the levels of VLDL are high in DKA. Hyperlipidaemia is known to accelerate renal injury by activation of cytokine dependent pathways and stimulation of macrophage proliferation leading to progression of incipient/overt nephropathy (Park et al., 1998).

Oxidation of circulating low-density lipoprotein (LDL) has been linked to the initiation and progression of atherosclerosis and ultimately to the pathogenesis of cardiovascular disease (Steinberg et al., 1989).

Oxidative stress is also suggested as one of the mechanism underlying diabetes mellitus, which affects carbohydrate, lipid and protein metabolism with an increased production of damaging free radicals that may be due to auto-oxidation of glucose and glycosylated proteins (Jones et al., 1985; Lyons, 1991; Giugliano et al., 1995). Glycation (Vlassara et al., 1994) and hyperglycemic pseudohypoxia (Williamson and Lilo, 1993) generates a redox imbalance of reactive oxygen species (ROS) inside the cells, especially in the liver (Gallou et al., 1993). Reactive oxygen species are thought to be implicated in the pathogenesis of various human diseases. They are generated endogenously under physiological and pathological conditions (Halliwell, 1984).

Defence against the reactive oxidants produced during aerobic metabolism is a complex process and is provided by a system of antioxidant enzymes (superoxide dismutase [SOD], glutathione peroxidase [Gpx], and catalase [CAT]) and low-molecular-weight antioxidant compounds such as vitamins E and C and carotenoids (Halliwell, 1997; Halliwell and Gutteridge, 1989; Sies, 1993). Since expression of antioxidant enzymes may be induced at the transcriptional level by oxidative stress (Kullik and Storz, 1994), it is possible that the metabolic changes accompanying diabetes may induce these enzymes. These enzymes are capable of preventing excess radical production, neutralizing free radicals and repairing the damage caused by them. Damage caused by free radicals is possibly involved in cell destruction and in the pathogenesis of diabetes mellitus (Oberley, 1988). In times of increased free radical production, individuals may become deficient in these antioxidants. This may ultimately lead to cell death with widespread pathological consequences (Baynes, 1991). Impaired antioxidant defense is implicated in the development of cardiovascular complications in non-insulin-dependent diabetes (NIDDM). There is increasing evidence from epidemiological studies, animal experiments, and *in vitro* investigations that an

increased intake of antioxidants is associated with a diminished risk for several diseases (Stahl and Sies, 1997).

Aldose reductase inhibitors (ARIs) is a key enzyme in the polyol pathway which inhibit glucose flux through the polyol pathway, reduce sorbitol and fructose buildup and ameliorate neurovascular abnormalities in experimental diabetes (Tomlinson et al., 1982; Greene et al., 1992; Cameron et al., 1996). Sorbitol does not readily diffuse across cell membranes, and the intracellular accumulation of sorbitol has been implicated in the chronic complications of diabetes such as peripheral neuropathy, retinopathy, and cataracts. The polyol pathway becomes more active under hyperglycemic conditions and usually results in the accumulation of both sorbitol and fructose. Heightened activity of the polyol pathway could lead to the depletion of NADPH, which has been reported to be a cofactor for fatty acid desaturases (Brenner, 1977). The ability of sorbitol dehydrogenase inhibitors to relieve abnormalities in diabetes has also been examined, but inconsistent results have been obtained (Tilton et al., 1995; Cameron et al., 1997).

6.1.6 Biochemical changes in diabetes mellitus

Diabetes mellitus is a progressive disorder characterized by hyperglycemia associated with malfunctioning of the vital organs of the body due to biochemical alterations of glucose, lipid and protein metabolism (Joslin, 1994; Lebovitz, 1994).

Liver and kidney participates in the uptake, oxidation and metabolic conversion of free fatty acids, synthesis of cholesterol, phospholipids and triglycerides. Liver is an insulin dependent tissue, which plays a pivotal role in glucose and lipid homeostasis and is severely affected during diabetes (Seifter and England, 1982). Decreased glycolysis, impeded glycogenesis and increased gluconeogenesis are some of the changes of glucose metabolism in the diabetic liver (Baquer, 1998). Kidney plays an important role in protein and lipid metabolism and exhibits a characteristic pattern of changes during diabetes (Aragno et al., 1999). Due to hyperglycemia the delicate filtering system in the

kidney becomes destroyed which leads to the leakage of large blood proteins such as albumin.

Estimation of total protein is one way to assess liver function. When disease affects the liver cells, the cells lose their ability to make albumin. Therefore, the measure of total protein is a rather indirect and inadequate indication of liver function. Albumin is the major protein of the blood and increased presence in serum indicates malfunctioning of the liver.

Amino transferases like alanine transferase (ALT) and aspartate transferase (AST) measure the concentration of intracellular hepatic enzymes that have leaked into the circulation and serve as a marker of liver injury. The enzyme SGPT (ALT) speeds up chemical organic reactions that occur within cells. The enzyme SGOT (AST) is found in very high concentration in the heart and in the liver and in moderately large amounts in the skeletal muscle, kidneys, and pancreas. A characteristic rise in transaminases level is found in Type 2 diabetics (Harris, 2005). The elevation of the enzyme acid phosphatase level in serum is also involved with the malfunctioning of liver or kidney during diabetes (Bottini et al., 2004).

Creatinine is used for skeletal muscle contraction. The serum creatinine level provides a more sensitive test of kidney function because kidney impairment is the most common cause of elevated creatinine. The elevated levels of the urea also indicate the problem of kidney (Almdal and Vilstrup, 1988).

During diabetes a profound alteration in the concentration and composition of lipid occurs (Sochor et al., 1985). Triglycerides is a true fat and an important lipid component which takes the available cholesterol and makes it "stick" to the artery walls causing fat deposit build up. The cholesterol is not a true fat and is carried around and attaches to the major fat particles that are called lipoprotein. An elevated level of triglyceride and cholesterol level is associated with diabetes and increases the risk of coronary diseases (Al-Shamaony, 1994). HDL (Good cholesterol) is a lipoprotein that accepts free cholesterol from the tissue for

transport to the liver. HDL helps remove lipids (fats) from artery tissues and also protects plasma LDL from damaging the artery, thus limiting plaque buildup. LDL (Bad cholesterol) typically contain 60-70% of the total serum cholesterol and therefore its concentration closely correlates with the concentration of total cholesterol. The common alterations in lipid and lipoprotein profile in type 2 diabetes also involves an elevation in serum VLDL concentrations, a dense LDL phenotype, and low levels of HDL cholesterol and are strongly associated with heart disease (De Man et al., 1996).

6.1.7 Alloxan induced diabetes mellitus

Much of the evidence for the induction of diabetes mellitus comes from the studies of alloxan which produces diabetes in experimental animals (Wolff 1993). Alloxan appears to selectively destroy the islets of Langerhans by oxidant production. Current evidence suggests that the selective cytotoxicity of alloxan is due to the function of 3 factors: efficient uptake, oxidant production by redox coupling of the drug with intracellular reductant (ascorbate and thiols) coupled with low levels of glutathione peroxidase in islets (Malaisse, 1982). Several studies on enzymes involved in hepatic glucose metabolism in rats with alloxan induced diabetes have shown well defined changes, which consist primarily of a decrease in the activity of glucokinase, hexokinase (Sheela and Augusti, 1992), and an increase in the activity of gluconeogenic enzymes including aminotransferases (AST and ALT). (Tanaka et al., 1988; Rawi et al., 1998) and glucose-6-phosphatase (Sheela and Augusti, 1992; Shibib et al., 1993).

6.1.8 Treatment: An approach to botanicals

Pharmacological treatment of diabetes mellitus is based on oral hypoglycemic agents and insulin (Committee Report, 1997). Different types of oral hypoglycemic agents such as biguanides, sulphonylurea and thiazolididinones are available along with insulin for the treatment of diabetes mellitus (Holman and Turner, 1991). These drug therapies (i.e., oral glucose-lowering agents and insulin injection) along with having adverse effects (Sameul and Julio, 1997) are

not always satisfactory in maintaining glycemia and avoid late stage diabetic complications (Kameshwara et al., 1997; Valiathan, 1998). Hence, their use is restricted by their limited action, pharmacokinetic properties, secondary failure rates and accompanying side effects (Bailey et al., 1989). Moreover, these therapies only partially compensate for metabolic derangements seen in diabetics and do not necessarily correct the fundamental biochemical lesion (Taylor and Agius, 1988).

The complete cure of the disease has been eluding physicians for centuries and the quest for the development of more effective antidiabetic agents is pursued relentlessly. Currently available treatment is far from satisfactory and is expensive. Treatment and care of diabetes represents a substantial portion of the national health care expenditure, over $105 billion annually. This represents a substantial portion of health care expenditure more than one of four Medicare dollars (DRWG, 1999).

Inspite of large number of hypoglycemic drugs available in the market, more and more people are approaching for an alternative treatment for diabetes mellitus in the form of herbal medicine (Bruce and David, 1999; Chang et al., 2006). Nowadays there is a growing interest in herbal remedies because of their effectiveness, minimal side effects in clinical experience and relatively low costs as compared to the synthetic therapeutic agents (Venkatesh et al., 2003). In India, indigenous remedies have been used in the treatment of diabetes mellitus since the time of Charaka and Sushruta (6th Century BC) (Grover et al., 2001).

Plants have been an exemplary source of medicine and many of the currently available drugs have been derived directly or indirectly from them. India has about 45,000 plant species and the research conducted in last few decades on traditional plants reports the ethno-botanical information of about 800 plants that may possess anti-diabetic potential (Swanston et al., 1990; Alarcon-Aguilara et al., 1998). Very few of the traditional plant treatments for diabetes have received scientific scrutiny to assess their efficacy despite the fact that World Health Organisation has recommended that pharmacological and a scientific

examination of such plants warrants attention (WHO, 1980). Herbal drugs or their extracts are prescribed widely, even when their biological active compounds are unknown. Therefore studies with plant extracts are useful to know their efficacy and mechanism of action and safety. Medicinal plants useful in diabetes were reviewed recently (Shukla et al., 2000; Grover et al., 2002). The hypoglycemic effect of some herbal extracts has been confirmed in animal and human models of Type 2 diabetes (Dey et al., 2002). There are reports of using herbal extracts for the treatment of diabetes mellitus in humans (ICMR, 1998). Adverse effects are indeed a cause of concern (Gupta and Raina, 1998), however, available evidence suggests that herbal medicines are relatively safe (Bailey and Day, 1989).

The potential role of the medicinal plants as hypoglycemic agents has been reviewed by several authors (Wang and Ng, 1999; Jouad et al., 2001; Grover et al., 2002; Yeh et al., 2003; Biesalski, 2004; Gupta et al., 2005; Antia et al., 2005; Djomeni Dzeufiet et al., 2006; Reyes et al., 2006). Many Indian medicinal plants are reported to be useful in diabetes (Nandkarni and Nandkarni, 1976; Kirithikar and Basu, 1995). Several of the most studied and commonly used medicinal herbs for diabetes include *Ginseng species, Momordica charantia* (BitterMelon), *Trigonella foenum graecum* (Fenugreek), *Gymnema sylvestre* (Gurmar), *Allium cepa* (Onion) and *Allium sativum* (Garlic), *Petrocarpus marsupium, Vaccinium myrtillus* (Bilberry), *Atriplex halimus* (Salt Bush), *Aloe vera* (Dey et al., 2002). A wide array of plant derived active principles representing numerous chemical compounds like alkaloids, glycosides, polysaccharides, guanidine, steroids, carbohydrates, glycopeptides, terpenoids etc has demonstrated activity consistent with their possible use in the treatment of NIDDM (Marles and Farnsworth, 1995).

Medicinal plants have been used for diabetes safely and with reasonable success (Marles and Farnsworth, 1994; Duke et al., 1998). Despite the great strides that have been made in understanding and management of diabetes mellitus, serious complications continue to confront patients and physicians. The graph of diabetes related mortality is raising unabated (Olefsky, 2000). Therefore, search

for new anti-diabetic drugs continue.

6.1.9 Diabetes: Hope for the future

Although there are no definitive preventive measures that can be taken against diabetes at this time, except for identifying persons at high risk and encouraging appropriate management. Research into the causes and control of this disease continues to provide the possibility of new cures. The therapy of diabetes will surely be altered dramatically over the next few decades (Clinton, 1998).

Research continues on the islet cell transplantation techniques, including research that uses stem cells derived from pancreatic ducts. There has been laboratory success with transplantation of islet cells derived from stem cells in mice, but not yet in humans (ADA, 2003).

Looking further into the future, researchers are studying the use of gene therapies to correct the genetic defects that are the original cause of diabetes. As successful gene therapy will affect a true cure of the disease, it seems likely that researchers will continue to pursue its development, despite many hurdles. At this point, however, gene therapy for treatment of diabetes appears far away in the future (ADA, 2003).

Considering the aforesaid, the study is aimed to demonstrate the hypoglycaemic and antihyperglycemic activity of the crude powder (MCR) and methanol extract (MME) of *Manikara hexandra* (Roxb.) Dubard leaf in diabetic animals with a view to explore its use for the treatment of diabetes mellitus in humans. Pharmacological action of *M. hexandra* (Roxb.) Dubard for anti-ulcer activity is reported by Shah et al., (2004).

6.2 MATERIALS AND METHODS

6.2.1 Drug dose and preparation

The dried leaf powder of *Manilkara hexandra* (Roxb.) Dubard and its methanol extract extracted using Soxhlet extractor were used as the drug. The dose

considered for the experiment on rats was obtained on conversion of the human dose of *M. hexandra* (Roxb.) Dubard which is 3 g kg^{-1}. The conversion factor of human dose to rat dose is 0.018 (Paget and Barnes, 1964). Hence the drug dose of animal with body weight of 200 g was 60 mg. The required dose of *M. hexandra* (Roxb.) Dubard crude powder (MCR) and methanol extract (MME) was prepared in distilled water. The standard drug glipizide was given at the dose of 0.5 mg kg^{-1} body weight.

6.2.2 Experimental animals

Wistar albino rats of either sex (200-300 g) were used for the antidiabetic 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 as bedding material at the animal house of Department of Biosciences, Saurashtra University, Rajkot. The animals were kept in a group of 6-8 per cage and facilitated with standard environmental condition of photoperiod (12:12 h dark: light cycle) and temperature (27 ± 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 ethical committee.

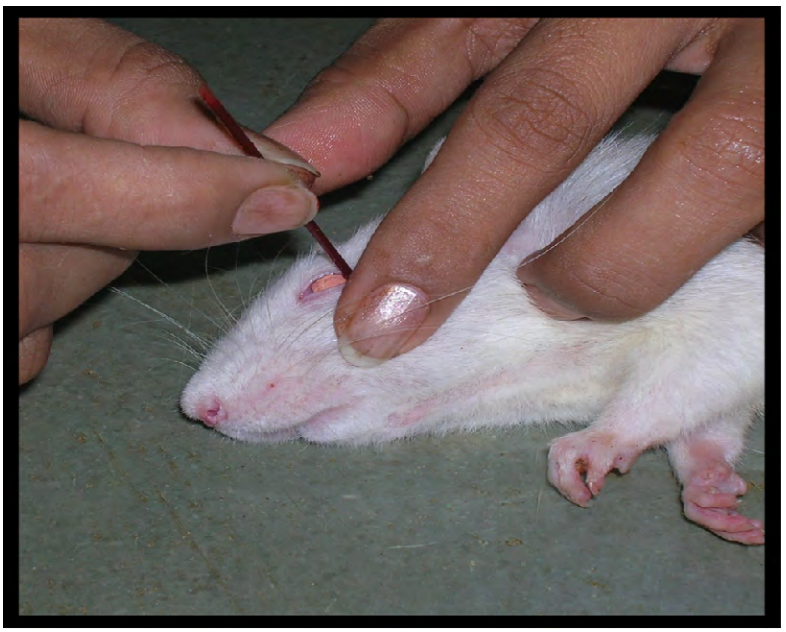
6.2.3 Drug administration

The test drugs, cude powder drug (MCR), methanol extract (MME) and glipizide (G) was administered orally (p.o.) according to the body weight of all the groups of rats with the help of a gastric catheter.

6.2.4 Experimental design

6.2.4.1 Hypoglycemic study of *M. hexandra* (Roxb.) Dubard leaf on normal fasted rats

To investigate hypoglycemic effect of the crude powder and methanol extract of *Manilkara hexandra* (Roxb.) Dubard, the normal rats were fasted overnight. They



[A] DEMONSTRATION OF REMOVAL OF BLOOD FROM RETRO-ORBITAL PLEXUS OF ALBINO RAT

[B] DEMONSTRATION OF READING BLOOD GLUCOSE LEVEL BY ONE TOUCH GLUCOMETER

FIGURE 6.1

were divided into 3 groups of 6 each. Fasting blood glucose (FBG) level was measured with one touch glucometer (Johnson & Johnson). The animals were anesthetized by ether and the blood was collected by puncturing the retro-orbital plexuses with the help of sterile glass capillaries. Thereafter, one group received distilled water which served as the control group. The other two groups received suspension of crude powder and methanol extract with dose of 300 mg kg^{-1} body weight respectively. Blood samples were collected at 1 h, 2 h, 3 h and 4 h after drug administration and the blood glucose levels were measured with glucometer.

6.2.4.2 Sucrose tolerance test (STT) of *M. hexandra* (Roxb.) Dubard leaf on normal rats

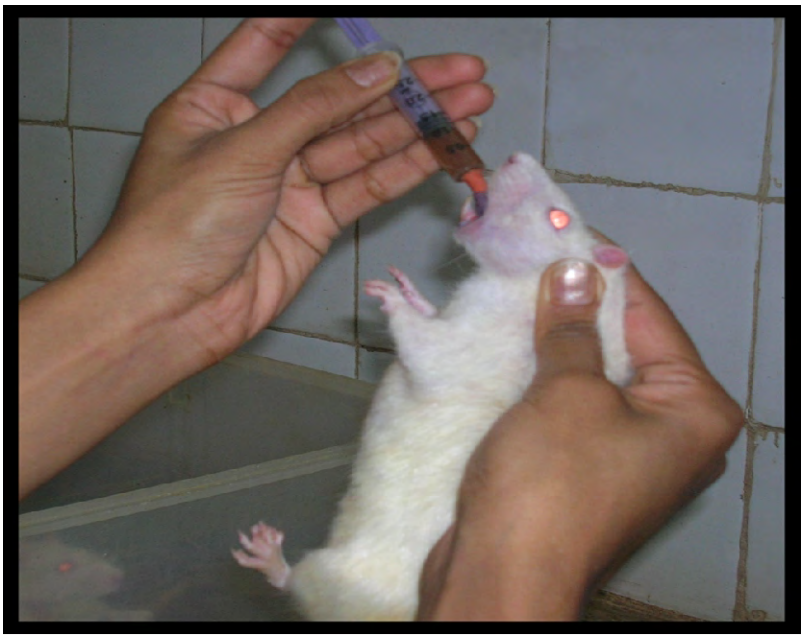
A modified glucose tolerance test (GTT) was carried out on normal rats by sucrose loading. To investigate sucrose tolerance test (STT) in crude powder and methanol extract of *Manilkara hexandra* (Roxb.) Dubard, rats were tagged, weighed and divided into 3 groups of 6 each. The initial blood glucose level of the rats in each group was measured with glucometer. One group received water which served as the control group. The other two groups received suspension of crude and methanol extract with dose of 300 mg kg^{-1} respectively. The rats of all groups were loaded with sucrose at the dose of 40 mg kg^{-1} body weight, orally (p.o.) 1 h after drug administration. The blood glucose levels were measured at 2 h, 4 h, 8 h and 24 h after sucrose loading. The animals were anesthetized by ether and blood was collected by puncturing the retro-orbital plexuses with the help of sterile glass capillaries.

6.2.4.3 Efficacy of the *M. hexandra* (Roxb.) Dubard leaf in alloxan induced diabetic rats

6.2.4.3.1 Induction of experimental diabetes

The rats selected for the study were weighed and their initial blood glucose level was measured with glucometer. A freshly prepared solution of alloxan (Loba

[A] DEMONSTRATION OF INTRAPERITONIAL (I.P.) INDUCTION OF ALLOXAN IN DIABETES



[B] DEMONSTRATION OF DRUG ADMINISTRATION OF DRUG ORALLY (P.O.) BY A CATHETER

FIGURE 6.2

Chemie) (90 mg kg^{-1} body weight) in 0.1 M citrate buffer, pH 4.6 was injected intraperitoneally (i.p.) to rats of either sex for two consecutive days at an interval of 24 h. The total dose of alloxan induced in the rats was 180 mg kg^{-1} body weight. At the third day of first induction, blood glucose levels were measured to confirm the development of diabetes. The diabetic rats exhibiting blood glucose levels in the range of 200- 590 mg dL^{-1} were selected to determine the efficacy of crude powder and methanol extract of *Manilkara hexandra* (Roxb.) Dubard leaf.

6.2.4.3.2 Drug treatment

The alloxan induced diabetic rats of either sex were weighed and divided into 4 groups of 6 each. Group I was given water, p.o. daily and served as control group. Group II and Group III received suspension of the crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf p.o., at the dose of 300 mg kg^{-1} body weight. The standard group received the suspension of glipizide p.o., at a dose of 0.5 mg kg-1 body weight. All the groups were given the drug treatment for a period of 7 days.

6.2.4.3.3 Sacrificing of the animals

The animals were sacrificed by stunning and severing of neck blood vessels on the 8th day of treatment. The body weight of animals was noted prior to sacrificing them. The blood was collected after sacrificing the animals for evaluating some biochemical parameters like total serum protein, serum albumin, total serum cholesterol, HDL-cholesterol, serum triglyceride, serum urea, serum creatinine, aspartate transaminase (AST), alanine transaminase (ALT), serum acid phosphatase and blood glucose. The glycogen content was estimated from the liver sample. All the sacrificed animals were dissected and the important organs like liver, kidney, heart and pancreas were removed carefully. The absolute weight of liver, kidney and heart was noted. All the organs (liver, kidney, heart and pancreas) were kept in 4% formalin solution and were subjected to histopathological studies.

6.2.4.3.4 Biochemical parameters

The absorbance of all the biochemical parameters was measured in a UV-VIS spectrophotometer (Shimadzu).

6.2.4.3.4.1 Serum glucose

The estimation of serum glucose was done by GOD/POD method (Trinder, 1969) using Glucose Tablet format estimation kit (Bayer Diagnostics).

Principle: Glucose is oxidized by glucose oxidase (GOD) into gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of peroxidase (POD) oxidizes the chromogen 4-aminoantipyrene/phenolic compound to a red coloured compound. The intensity of the red coloured compound is proportional to the glucose concentration and is measured at 505 nm.

Reagents: Reagent I: Buffer/ Enzymes/ Chromogen
Reagent II: Standard (Glucose 100 mg dL^{-1})

Assay: 2000 µl of Reagent I was added in clean test tubes. 20 µl of serum was added for test, 20 µl of Reagent II was added for standard and 20 µl of distilled water was added for blank. It was then mixed well and incubated at 37°C for 15 min. The absorbance was read at 505 nm against blank.

Calculation:

Serum Glucose (mg dL^{-1}) = Absorbance of test X Calibration factor (64.1)

6.2.4.3.4.2 Serum albumin

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

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.

Reagents: Reagent I- Albumin reagent

Reagent II- Albumin standard 4.0 g dL^{-1}

Assay: 2 ml Reagent I was added to all the test tubes. Thereafter, 20 µl serum was added for the test and 20 µl Reagent II was added for the standard. It was then mixed well and incubated at room temperature for 1 min. The absorbance was read at 630 nm against reagent blank.

Calculation:

$$\text{Serum albumin (g dL}^{-1}) = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 4.0$$

6.2.4.3.4.3 Serum total protein

The total serum protein was estimated by modified Biuret method (Gornall et al., 1949) using the Total Protein test kit (Span Diagnostics Ltd.).

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 to complex cupric ions and maintains their solubility at alkaline pH. Absorbance data are proportional to protein concentrations.

Reagents: Reagent I- Biuret reagent

Reagent II- Protein standard 6.5 g dL^{-1}

Assay: 3 ml of Reagent I was added to all the test tubes. Thereafter, 30 µl serum was added for the test and 30 µl Reagent II was added for the standard. It was then mixed well and incubated at 37ºC for 5 min. The absorbance was read at 578 nm against reagent blank.

Calculation:

$$\text{Serum total protein concentration (g dL}^{-1}) = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 6.5$$

6.2.4.3.4.4 Glycogen estimation

Estimation of glycogen is carried out by the method of Caroll et al., (1956).

Reagents: Reagent I- KOH solution (30%)
Reagent II- Anthrone reagent
Reagent III- Stock glucose solution, 1gm 100 ml^{-1}
Reagent IV- Working glucose solution, 40 µg ml^{-1}

Assay: 50 mg of liver was digested by boiling it in 2 ml 30% KOH for 20 min. It was then left in KOH till the tissue was completely digested. It was cooled and 2-3 ml of ethanol was added. Again it was boiled for 1-2 min, cooled and centrifuged. The supernatant was discarded and again 2-3 ml of 95% ethanol was added to the pellet. It was again boiled a little and cooled. Thereafter it was kept in refrigerator for 30 min in order to precipitate the glycogen. Thereafter it was centrifuged and the supernatant was discarded. The precipitate was diluted with 10 ml of distilled water and from that 1 ml was added for test while 1 ml distilled water was added for blank. Then 4 ml of anthrone reagent was added to each tube and kept the tubes in ice bath for 5 min. Immediately the tubes were transferred to boiling water bath for 4 min. It was again immediately cooled in ice for 5 min. The absorbance was read at 620 nm.

Calculation:

$$\text{Glycogen content (mg dL}^{-1}) = \frac{\text{O.D. of test X concentration of std. X dilution X 100}}{\text{O.D. of std. X wt. of tissue (mg) X conversion factor (1.11)}}$$

6.2.4.3.4.5 Serum urea

The serum urea was estimated by Enzymatic Urease (Berthelot) method (Fawcett

and Scott, 1960) using Urea Berthelot test kit (Span Diagnostics Ltd.).

Principle: Urea in the presence of water and urease produces ammonia and carbon dioxide. Under alkaline conditions, ammonia so formed reacts with hypochlorite and phenolic chromogen to form a green colored indophenol which is measured at 578 nm. The intensity of the colour is proportional to the concentration of urea in the sample.

Reagents: Reagent I- Urea enzyme reagent
Reagent II- Urea chromogen reagent
Reagent III- Urea standard 50 mg dL^{-1}
Reagent IV- Purified water

Solution I: To Reagent I add 50 ml Reagent IV and mix gently. Enter the date of reconstitution and store at 2-8ºC. It is stable for 45 days.

Solution II: Dilute the contents of Reagent II, with 160 ml reagent 4. Alternatively dilute the contents of Regent II as per the requirement. It is stable for 4 months.

Assay: 1500 µl Solution I was added to clean test tubes. 20 µl serum was added for the test and 20 µl Reagent II was added for the standard. It was then mixed well and incubated at 37ºC for 3 min; then 1500 µl of Solution II was added. It was then mixed well and incubated at 37ºC for 5 min. The absorbance was read at 578 nm against reagent blank.

Calculation:

$$\text{Serum urea (mg dL}^{-1}) = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 50$$

6.2.4.3.4.6 Serum creatinine

The serum Creatinine was estimated by Alkaline Picrate method (Bonses and Taussky, 1945) using Creatinine test kit (Span Diagnostics Ltd.).

Principle: Creatinine in a protein free solution reacts with alkaline picrate and produces a red coloured complex, which is measured colorimetrically.

Reagents: Reagent I- Picric acid

Reagent II- Sodium Hydroxide, 0.75N

Reagent III- Stock Creatinine standard, 150 mg %

Assay:

Step A. Deproteinization of test samples

3000 µl Reagent I was added to clean test tubes. Thereafter 500 µl of serum and 500 µl of purified water were added for the test. It was then mixed well and kept in boiling water bath exactly for 1 min. It was then cooled immediately under running tap water and centrifuged.

Step B. Color Development

2000 µl of the supernatant was added to clean test tubes. 500 µl of working standard prepared from Reagent III and 500 µl of purified water was added to the tubes. Then 1500 µl Reagent I and 500 µl Reagent II was added to the tubes. It was then mixed well and allowed to stand at room temperature exactly for 20 min. The absorbance of blank, standard and test was read at 520 nm.

Calculation:

$$\text{Serum creatinine (mg dL}^{-1}) = \frac{\text{Absorbance of test - Absorbance of blank}}{\text{Absorbance of standard - Absorbance of blank}} \times 3.0$$

6.2.4.3.4.7 Serum aspartate transaminase (AST) (SGOT) *(E.C.2.6.1.1)*

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

Principle: 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.

Reagents: Reagent I- Buffered Aspartate á KG Substrate, pH 7.4
Reagent II- DNPH color reagent
Reagent III- Sodium hydroxide, 4N
Reagent IV- Working Pyruvate standard, 2 mM

Solution 1: Dilute 1 ml of Reagent III to 10 ml with purified water.

Assay: 250 µl Reagent I was added in clean test tubes and Incubated at 37ºC for 5 min. 50 µl of serum was added for the test, 50 µl Reagent IV for standard and 50 µl distilled water was added for the blank. It was mixed well and incubated at 37ºC for 60 min. Thereafter, 250 µl Reagent II was added to all the tubes, mixed well and allowed to stand at room temperature for 20 min. Now 2500 µl Solution 1 was added to all the tubes, mixed well and allowed to stand at room temperature for 10 min. The absorbance of blank, standard and test was read at 505 nm.

Calculation: Mark the absorbance of test on Y-axis of the standard curve and extrapolate it to the corresponding enzyme activity on X-axis.

6.2.4.3.4.8 Serum alanine transaminase (ALT) (SGPT) *(E.C.2.6.1.2)*

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

Principle: 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.

Reagents: Reagent I- Buffered Alanine KG Substrate, pH 7.4
Reagent II- DNPH color reagent
Reagent III- Sodium Hydroxide, 4N
Reagent IV- Working Pyruvate standard, 2 mM

Solution 1: Dilute 1 ml of Reagent III to 10 ml with purified water.

Assay: 250 µl Reagent I was added to clean tubes and was incubated at 37ºC for 5 min. 50 µl of serum was added for the test, 50 µl of Reagent IV was added for the standard and 50 µl distilled water was added for the blank. It was then mixed well and incubated at 37ºC for 30 min. 250 µl of Reagent II was added to the tubes; mixed well and allowed to stand at room temperature for 20 min. Then 2500 µl Solution 1 was added, mixed well and allowed to stand at room temperature for 10 min. The absorbance of blank, standard and test was read at 505 nm.

Calculation: Mark the absorbance of test on Y-axis of the standard curve and extrapolate it to the corresponding enzyme activity on X-axis.

6.2.4.3.4.9 Serum acid phosphatase

The serum acid phosphatase was determined by King's method (King and Jagatheesan, 1959) using Acid Phosphatase test kit (Span Diagnostics Ltd.).

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 presence of oxidizing agent potassium ferricyanide and forms an orange red coloured complex, which is measured colorimetrically. The color intensity is proportional to the enzyme activity. Since tartrate inhibits the prostratic fraction of the enzyme, the difference in acid phosphatase activity without and with tartrate represents the activity of the prostratic fraction.

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- Stock phenol standard, 10 mg %

Preparation of working solutions:

Solution 1: Reconstitute each vial of Reagent I with 2.0 ml of purified water and mix well.

Solution 2: Dissolve Reagent IV in 25 ml of purified water

Solution 3: Dissolve Reagent V in 25 ml of purified water

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

Assay: The clean test tubes were marked as blank, standard, control and test. 500 µl Solution 1 was added in control and test. Then 500 µl of purified water was added in control and test and in blank and standard 1100 µl and 600 µl of purified water was added respectively. It was mixed well and incubated at 37ºC for 3 min. Thereafter, 500 µl of working standard solution was added in standard and 100 µl of serum was added in test. It was mixed well and incubated at 37ºC for 60 min. Now 500 µl Reagent II was added in all blank, standard, control and test; 100 µl of serum was added in control; 500 µl Reagent III , Solution 2 and Solution 3 were added in all blank, standard, control and test. The tubes were mixed well and the absorbance was read at 510 nm.

Calculation:

Serum acid phosphatase (KA units) :

$$\text{Total} = \frac{\text{Absorbance of test} - \text{Absorbance of control}}{\text{Absorbance of standard} - \text{Absorbance of blank}} \times 5.0$$

6.2.4.3.4.10 Serum triglyceride

The serum triglyceride was estimated by Enzymatic GPO method (Cole et al.,

1997; Rifai et al., 1999) using Triglyceride EP* LS test kit (Euro Diagnostic Systems Pvt. Ltd.).

Principle:

$$\text{Triglycerides} \rightarrow \text{Glycerol + Fatty acid}$$

$$\text{Glycerol + ATP} \rightarrow \text{Glycerol- 3-Phosphate + ADP}$$

$$\text{Glycerol- 3-Phosphate + } O_2 \rightarrow \text{Dihydroxyacetone phosphate + } H_2O_2$$

$$2H_2O_2 \text{ + Aminoantipyrine + 4-Chlorophenol} \rightarrow \text{Quinoneimine + HCl + 4 } H_2O$$

Reagents: Reagent I- Monoreagent

Reagent II- Triglyceride standard 200 mg dL^{-1}

Assay: 2000 µl Reagent I was added to clean test tubes. 20 µl of serum was added for the test and 20 µl Reagent II was added for the standard. It was then mixed well and incubated at 37ºC for 10 min. The absorbance was read at 500 nm against reagent blank.

Calculation:

$$\text{Serum triglyceride (mg } dL^{-1}) = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ X Conc. of standard (40)}$$

6.2.4.3.4.11 Serum cholesterol

The serum cholesterol was estimated by CHOD-PAP method (Trinder, 1969) using Cholesterol EP LS test kit (Euro Diagnostic Systems Pvt. Ltd.).

Principle: Cholesterol esters are hydrolysed by Cholesterol Esterase (CE) to give free cholesterol and fatty acids. In subsequent reaction, cholesterol oxidase (CHOD) oxidises the 3-OH group of free cholesterol to liberate Cholest-4-en-3-one and hydrogen peroxide. In the presence of the peroxidase (POD), hydrogen oxide couples with 4-aminoantipyrene (4-AAP) and phenol to produce red quinoneimine dye. Absorbance of colored dye is measured at 505 nm and

proportional to the amount of total cholesterol concentration in the sample.

Reagents: Reagent I- Monoreagent

Reagent II- Cholesterol standard 200 mg dL^{-1}

Assay: 2000 µl Reagent I was added to all the test tubes. Thereafter, 20 µl serum was added for the test, 20 µl Reagent II for standard and 20 µl distilled water in blank. It was then mixed well and incubated at 37ºC for 10 min. The absorbance was read at 505 nm against reagent blank.

Calculation:

$$\text{Serum cholesterol (mg } dL^{-1}) = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc. of standard (40)}$$

6.2.4.3.4.12 HDL-cholesterol

The HDL-cholesterol was estimated by PTA method (Friedwaid et al., 1972) using HDL-cholesterol test kit (Euro Diagnostic Systems Pvt. Ltd.).

Principle: The chylomicrons, VLDL and LDL are precipitated from serum by the addition of phosphotungstic acid and magnesium chloride. After centrifugation, high density lipoprotein fractions recovered as clear supernatant and the cholesterol content is estimated by enzymatic method.

Reagents: Reagent I- Precipitating reagent

Reagent II- Cholesterol reagent

Reagent III- Standard 50 mg dL^{-1}

Assay: 200 µl of serum sample and 200 µl of Reagent I was added to all the test tubes. It was mixed well and allowed to stand for 10 min at room temperature. The test tubes were centrifuged for 10 min at 3000 rpm. The clear supernatant was separated and used for HDL-cholesterol estimation. 1000 µl Reagent II was added in clean test tubes. 50 µl separated supernatant for the test and 50 µl Reagent 3 for standard was added. It was then mixed well and incubated at 37ºC

for 10 min. The absorbance was read at 505 nm against reagent blank.

Calculation:

$$\text{HDL-cholesterol (mg dL}^{-1}) = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc. of standard (25)} \times 2$$

6.2.4.3.4.13 LDL-cholesterol

The LDL-cholesterol value was calculated from the values of serum cholesterol, serum triglyceride and HDL-cholesterol estimated (Friedewald et al., 1972). The Frieldewald's formula for the calculation of LDL-cholesterol is expressed as follows:

$$\text{LDL-cholesterol} = \text{Cholesterol} - \left[\frac{\text{Triglyceride}}{5} + \text{HDL-cholesterol}\right]$$

6.2.4.3.4.14 VLDL-cholesterol

The VLDL-cholesterol value was calculated from the values of serum cholesterol and serum triglyceride estimated (Friedewald et al., 1972). The Frieldewald's formula for the calculation of VLDL- cholesterol is expressed as follows:

$$\text{VLDL-cholesterol} = \left[\frac{\text{Triglyceride}}{5}\right]$$

6.2.4.3.5 Changes in the body weight and their organs

The changes in the body weight during 7 days treatment was determined. The changes in absolute and relative weight of organs like liver, kidney and heart of all the experimental groups was evaluated after seven days treatment.

6.2.4.3.6 Histopathological study

Histopathological study of liver, kidney, heart and pancreas was performed.

6.2.4.3.6.1 Histology of studied organs

6.2.4.3.6.1.1 Heart

The heart is centrally located in the thoracic cavity. It contains four chambers. The two dark colored chambers at the top are the atria, and the bottom chambers are the ventricles. The heart is covered by a thin membrane called the pericardium. Valves guard the exits of the chambers, preventing backflow of blood. The wall of heart includes the cardiac muscle, a fibrous skeleton for attachment of the valves, and a specialized internal conducting system involved in the regulation of heart rate. The fibrous skeleton, comprised of dense connective tissue, is situated around the openings of the two arteries leaving the heart and around the openings between the atria and the ventricles. The wall of the heart contains three layers: the epicardium, myocardium and the endocardium. The layers are essentially the same in the atria and the ventricles. The epicardium consists of a lining of mesothelial cells and an underlying layer of connective tissue. Blood vessels and nerves supplying the heart lie within the epicardium, often surrounded by adipose tissue. The myocardium consists of cardiac muscle. The myocardium can be divided into three layers: an inner layer of endothelium and subendothelial connective tissue, a middle layer comprised of connective tissue and smooth muscle cells, and an outer layer, also called the subendocardial layer, which is continuous with the connective tissue of the myocardium. The interventricular septum serves as the wall between the right and left ventricles. The interatrial septum is much thinner than the interventricular septum. Fibrous thread like cords, called the chordae tendinae, extend from the free edge of the atrioventricular valves to muscular projections from the wall of the ventricles called papillary muscles.

6.2.4.3.6.1.2 Liver

Liver is an organ suspended just under the diaphragm. There are four parts of the liver- median or cystic lobe which is located at top, there is a central cleft left lateral lobe - large and is covered by the stomach, right lateral lobe- partially divided into

an anterior and posterior lobule, hidden from view by the median lobe caudate lobe- small and folds around the esophagus and the stomach, seen most easily when liver is raised.

The rat liver stained with hematoxylin and eosin shows a classic lobule and a liver acinus located. The arrangement of the portal triad, hepatocytes, sinusoids, and central veins within the lobule and acinus can be seen. At higher power the portal triad with hepatic artery, portal vein, bile duct and connective tissue can be located. The portal vein is the largest vessel to have a thin wall. The hepatic artery is small, with 1-3 muscle layers in the tunica media. The bile duct is lined with simple cuboidal epithelium. The hepatic sinusoids are seen and the wall of the sinusoid is lined with endothelial cells and Kupffer cells. Kupffer cells have nuclei, which tend to be larger and more ovoid than the flattened endothelial cells. The space of Disse is visible between the sinusoidal endothelial cell and the hepatocyte. The hepatocytes are mono- and binucleate cells, and the presence of small and large nuclei. The ergastoplasm of the hepatocytes appears as darkly stained, irregularly shaped areas in the. The central vein is lined by endothelial cells with minimal underlying connective tissue. There is a continuation of a sinusoid with the central vein and the serosa of this organ can be seen.

6.2.4.3.6.1.3 Kidney

The two bean-shaped kidneys lie up against the dorsal body wall. The kidney is divided into two regions, an outer cortex and an inner medulla. The nephron (the functional unit of the kidney) is organized so that Bowman's Capsule and the proximal and distal tubules are located in the cortex and the loop of Henle and the collecting tubules are located primarily in the medulla. In the cortical region Bowman's capsules are relatively abundant and appear as spherical structures with a coiled mass of capillaries, the glomerulus in the center. Surrounding the Bowman's capsule are the elements of proximal and distal convoluted tubules cut in various plains of section. The cells making up the walls of the tubules are cuboid in shape and contain a prominent nucleus. In the medulla there are

elements from the loop of Henle and the collecting tubules which cut mostly in longitudinal section.

6.2.4.3.6.1.4 Pancreas

The rat pancreas is a filamentous organ divided into 3 anatomic regions- gastroplenic, duodenal and biliary. There are exocrine and endocrine glands in the pancreas.

The exocrine pancreas consists of several thousand loosely connected lobes or tubuloacinar glands. The lobes are separated by their connective tissue septa and each lobe contains a group of branched ducts with their acini. Acini may have a multitude of shapes and sizes. They are long, club-shaped acini with humped surfaces as well as single round acini. 3-5 acini assemble to a cluster. Their individual ducts combine to a common duct. A single layer of pyramidal shaped cells forms the secretory acini. The supranuclear and cytoplasmic spaces are densely packed with secretory granules. is filled with secretory vesicles containing the precursors of digestive enzymes. The first portion of the duct system extends into the centre of the acini, which is lined by small centroacinar cells. These are numerous capillaries in the interstitial connective tissue. They form the first part of intercalated ducts. Intercalated ducts are lined by low columnar or cuboidal epithelium. The columnar cells of the secretory cells of the acini conain basal nuclei. They empty into interlobular ducts, which are lined by a columnar epithelium. Interlobular ducts in turn empty into the main pancreatic duct, which is lined by a tall columnar epithelium.

The endocrine glands are the islets of Langerhans. Islets are comprised of a heterogenous population of the endrocrine cells, including insulin-producing -cells, glucagon-secreting -cells, somatostatin-secreting -cells and polypeptide (PP) secreting cells.

6.2.4.3.6.2 Methods for histopathological studies

The organs were transferred to 10% formalin (37-40%) solution for fixation and

thereafter processed for histopathological studies following the procedure of 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. Any changes in the cytoarchitechture of the tissue were determeined.

6.2.4.3.6.2.1 Fixation

Fixation is the process of preserving, hardening and preventing postmortem changes of the tissues. The tissues were excised out immediately after sacrificing, cleaned off extraneous tissues, cut in to pieces of appropriate thickness so that the fixative readily penetrated throughout the tissue to be fixed. Tissue was then transferred to 10% formaldehyde solution (37-40% formaldehyde) and allowed to remain until they were processed.

6.2.4.3.6.2.2 Tissue processing

The processing of tissue 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. Tissues were thoroughly washed by placing them under running tap water and then passed through a series of 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°C) (3 changes)	-	30 minutes each

Thereafter, the tissues were embedded in paraffin wax to prepare tissue blocks, which are oriented so that sections are cut in desired plane of the tissue. After trimming them to suitable size the tissues were fixed to metal object holder.

6.2.4.3.6.2.3 Section Cutting

A smear of 5% Mayer's egg albumin was prepared and smeared on to 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 the section floated in water on slide between 55-60°C, water was drained off and slide was dried on hot plate at about 50°C for 30 minutes.

6.2.4.3.6.2.4 Staining Procedure

Reagent: 1). Mayer's heamotoxyline stain

2). Eosin stain, 2% w/v in alcohol

After fixing the section on slide, the sections 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

When the slides were passed through all the above reagents, the slides were mounted with D.P.X. (Diphenyl Phthalate Xylene) and cover slip was placed. During mounting the slide care should be taken for avoiding the air bubbles. 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.

6.2.5 Statistical analysis

Data is expressed as mean ± S.E.M. for all the experiments. The biochemical parameters were analyzed statistically using analysis of variance (ANOVA) (significance level was fixed at $F<0.05$) followed by Dunnett's multiple comparison test (significance level was fixed at $^{d}P<0.05$). Statistical difference amongst the data was analyzed by Students “t” test. The minimum level of significance was fixed at $P<0.05$.

6.3 RESULTS AND DISCUSSION

Diabetes is possibly the world's fastest growing metabolic disease, so there arises need for more appropriate therapies. Management of diabetes without any side effects is still a challenge to the medical community. Despite insufficient evidences to support its therapeutic efficacy, the use of herbal medicine has increased considerably.

6.3.1 Hypoglycemic study of *Manilkara hexandra* (Roxb.) Dubard leaf on normal fasted rats

The hypoglycemic effect of *M. hexandra* (Roxb.) Dubard leaf is shown in Table 6.1. The blood glucose level of the control group at 1st h was 69.6 ± 5.74 mg/dL, that of crude drug (MCR) treated group was 56.6 ± 2.32 which was 18.67 % less that the control group and the blood glucose level of the methanol extract (MME) treated group at 1st h was 63.8 ± 3.19 mg/dL which was 8.33 % less than the control group. The blood glucose levels at the 2nd h for control group was 60.2 ± 2.07, that of crude drug administered group was 60.8 ± 1.40 mg/dL which was 0.99 % higher than the control group and that of the methanol treated group was 69.8 ± 3.45 mg/dL which was 15.94 % higher as compared to the control group. The blood glucose level at the 3rd h for the control group was 70.0 ± 3.52 mg/dL, that of crude drug treated group was 61.2 ± 2.51 mg/dL which was 12.57 % less

than the control group and that of the methanol treated group was 64.8 ± 5.56 mg/dL which was 7.42 % less than the control group. The blood glucose level at the 4th h of the control group was 70.8 ± 1.20 mg/dL, of the crude drug treated group was 63.8 ± 3.24 mg/dL which was 9.88 % less than the control group and that of the methanol group was 72.2 ± 3.63 mg/dL which was 2.82 % higher than the control group.

6.3.2 Sucrose tolerance test (STT) of *Manilkara hexandra* (Roxb.) Dubard leaf on normal rats

The data related to the evaluation of antihyperglycemic activity of *M. hexandra* (Roxb.) Dubard leaf on the sucrose fed normal rats is shown in the Table 6.2.

The percent elevation of the blood glucose level of the control group as compared to the initial level was 21.61 ± 5.39 mg dL^{-1} at 2nd h, 20.29 ± 6.91 mg dL^{-1} at 4th h, 27.76 ± 10.64 mg dL^{-1} at 8th h and 15.98 ± 10.77 mg dL^{-1} at 24th h.

The percent change as compared to the initial blood glucose level for the crude drug group was 38.34 ± 5.33 mg dL^{-1} at 2nd h which was 77.41 % ($P< 0.05$, $^{d}P< 0.05$) higher than the control group; at 4th h the percent rise of the blood glucose level as compared to the initial level was 47.10 ± 4.67 mg dL^{-1} which was 132.13 % ($P< 0.05$) more than the control group; at 8th h the percent change in blood glucose level was 31.26 ± 6.97 mg dL^{-1} which was 12.60 % higher than the control group and at 24th h the glucose level was 20.13 ± 4.10 mg dL^{-1} which was 25.96 % more than the control group.

The percent change in the blood glucose level of the methanol drug treated group as compared to the initial level was 36.56 ± 3.77 mg dL^{-1} at 1st h which was 69.18 % higher than the control group; at 4th h the glucose level was 36.05 ± 2.38 mg dL^{-1} which was 77.67 % higher as compared to the control group; the elevation of blood glucose at 8th h was 62.03 ± 9.90 mg dL^{-1} which was 123.45 % more than control group and at 24th h the rise in glucose level was 32.59 ± 5.67 mg dL^{-1} which was 103.94 % more than the control group.

Table 6.1 Hypoglycemic effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on normal fasted albino rats

Treated Group (mg kg^{-1})	1 h		2 h		3 h		4 h	
	Glucose level (mg dL^{-1})	% Change	Glucose level (mg dL^{-1})	% Change	Glucose level (mg/dL^{-1})	% Change	Glucose level (mg/dL^{-1})	% Change
Control	69.6 ± 05.74	-	60.2 ± 02.07	-	70.0 ± 03.52	-	70.8 ± 01.20	-
MCR-300	56.6 ± 02.32	18.67*↓	60.8 ± 01.40	0.99↑	61.2 ± 02.51	12.57↓	63.8 ± 03.24	9.88↓
MME-300	63.8 ± 03.19	8.33↓	69.8 ± 03.45	15.94↑	64.8 ± 05.56	7.42 ↓	72.2 ± 03.63	2.82 ↑

Values are Mean ± SEM, n = 5, Decrease-↓, Increase-↑, MCR: Crude powder, MME: Methanol extract, *: P< (0.05)

Table 6.2 Antihyperglycemic activity of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on oral sucrose loaded albino rats

Treated Group (mg kg^{-1})	1 h		2 h		3 h		4 h	
	Glucose level (mg dL^{-1})	% Change	Glucose level (mg dL^{-1})	% Change	Glucose level (mg dL^{-1})	% Change	Glucose level (mg dL^{-1})	% Change
Control	21.61 ± 05.39	-	20.29 ± 06.91	-	27.76 ± 10.64	-	15.98 ± 10.77	-
MCR-300	38.34 ± 05.33	77.41*@↑	47.10 ± 04.67	132.13*↑	31.26 ± 06.97	12.60↑	20.13 ± 04.10	25.96↑
MME-300	36.56 ± 03.77	69.18↑	36.05 ± 02.38	77.67↑	62.03 ± 09.90	123.45↑	32.59 ± 05.67	103.94↑

Values are Mean ± SEM, n = 5, Increase- ↑, MCR: Crude powder, MME: Methanol extract, *: P< (0.05), @,d: P< (0.05)

6.3.3 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf on blood glucose level in alloxan induced diabetic rats

Alloxan is widely used as an agent for the development of experimental diabetes to induce selective dysfunctioning of pancreatic -cells (Rerup, 1970; Lenzen and Panten, 1988) and the destruction is almost complete (Khan and Shechter, 1991). Alloxan causes massive reduction in insulin release, through the destruction of -cells of the islets of Langerhans (Oberley, 1988). *In vitro* studies have shown that alloxan is selectively toxic to pancreatic cells, leading to the induction of cell necrosis (Jorns et al., 1997). The cytotoxic action of alloxan is mediated by reactive oxygen species, with a simultaneous massive increase in cytosolic calcium concentration, leading to a rapid destruction of cells (Szkudelski, 2001).

Diabetes mellitus is characterised by hyperglycaemia together with biochemical alterations of glucose and lipid metabolism (Arky, 1982). Experimental diabetes in animals has provided considerable insight into physiologic and biochemical derangement of the diabetic state. Insulin deficiency leads to various metabolic aberrations in the animals, viz. increased blood glucose, decreased protein and albumin content, increased cholesterol and triglycerides levels, increase in alkaline phosphatase, acid phosphatase and transaminases (alanine transaminase and aspartate transaminase) activities (Shanmugasundaram et al., 1983). Fat tissue acts as an endocrine organ, promoting lipolysis via release of mediator substances. This leads to a rise in the concentration of free fatty acids in the blood. This, in turn, not only exacerbates hyperinsulinemia and insulin resistance, but has other undesirable effects such as stimulation of the synthesis of triglyceride-rich very-low-density lipoproteins (VLDL). Cholesterol is likewise esterified by these free fatty acids and bound to high-density lipoprotein (HDL) to form HDL-cholesterol or to low-density lipoprotein (LDL) to form LDL-cholesterol. Reduced levels of HDL-cholesterol and increased levels of LDL-cholesterol particles have been found in Type 2 diabetes. Taking these factors into consideration the effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and

its extract on a number of biochemical parameters in alloxan induced diabetic rats was studied.

6.3.3.1 Biochemical parameters

6.3.3.1.1 Serum glucose level

Glucose measurement is useful in the diagnosis and treatment of pancreatic islet cell carcinoma and of carbohydrate metabolism disorders, including diabetes mellitus, neonatal hypoglycemia, and idiopathic hypoglycemia. The results of the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum glucose level in alloxan diabetic rats is shown in Table 6.3.

Table 6.3 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on alloxan induced hyperglycemia in rats

Treated Group (mg kg^{-1})	Glucose Level (mg dL^{-1})	% Decrease
Control	298.19 ± 70.71	-
MCR-300	261.39 ± 33.47	12.34 ↓
MME-300	271.46 ± 25.58	8.96 ↓
G-0.5	245.43 ± 38.80	17.69 ↓

Values are Mean ± SEM, n = 5, Decrease ↓, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The crude drug (MCR) and methanol drug (MME) showed decrease in serum glucose level as compared to the control group. The serum glucose of the control group was 298.19 ± 70.71 mg dL^{-1}. The serum glucose of the crude drug at the dose of 300 mg kg^{-1} was 261.39 ± 33.47 mg dL^{-1} which was 12.34 % less than the control group while the serum glucose level of the methanol drug at the same dose level was 271.46 ± 25.58 mg dL^{-1} which was 8.96 % less than the control group. The serum glucose level of the standard drug glipizide (G) at the dose of 0.5 mg kg^{-1} was 245.43 ± 38.80 mg dL^{-1} which was 17.69 % less as compared to the control group. However, the observed decrease in blood glucose level in test drug administered groups was found to be statistically non-significant in comparison to control diabetic rats.

6.3.3.1.2 Serum albumin level

Albumin measurement is used in the diagnosis and treatment of numerous diseases primarily involving the liver or kidneys. The results of the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum albumin level is shown in Table 6.4.

Table 6.4 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum albumin level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum Albumin Level (g dL^{-1})	% Decrease
Control	3.80 ± 0.11	-
MCR-300	3.62 ± 0.14	4.73 ↓
MME-300	3.45 ± 0.11	9.21 ↓
G-0.5	3.66 ± 0.12	3.68 ↓

Values are Mean ± SEM, n = 5, Decrease ↓, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The serum albumin level of the diabetic control group was 3.80 ± 0.11 g dL^{-1}. The serum albumin levels of the crude drug (MCR) was 3.62 ± 0.14 g dL^{-1} which was 4.73 % less than the control group while that of methanol group (MME) was 3.45 ± 0.11 g dL^{-1} which was 9.21 less than the control group. The standard drug glipizide (G) showed 3.66 ± 0.12 g dL^{-1} of serum albumin level with the percent decrease of 3.68 as compared to the control group. The changes were not statistically significant in comparison to diabetic control group.

The decrease in albumin level has been attributed to several causes like massive necrosis of liver, deterioration of liver function, hepatic resistance to insulin and glycogen impairment of oxidative phosphorylation (Ezzat et al., 1989; Guler et al., 1994; Rao, 1995). Nevertheless, hypoalbuminemia is a common problem in diabetic animals and is generally attributed to the presence of diabetic nephropathy (Porte and Halter, 1981). In the present study the test drugs did not modulate albumin level to significant extent indicating lack of effect on albumin

turnover.

6.3.3.1.3 Serum total protein level

Total protein measurement is used in the diagnosis and treatment of a variety of diseases involving the liver or kidney as well as other metabolic disorders. The data related to the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum total protein level is shown in Table 6.5.

Table 6.5 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum protein level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum Protein Level (g dL^{-1})	% Change
Control	7.08 ± 0.18	-
MCR-300	6.84 ± 0.06	3.38 ↓
MME-300	7.18 ± 0.14	1.41 ↑
G-0.5	6.99 ± 0.15	1.27 ↓

Values are Mean ± SEM, n = 5, Decrease ↓, Increase ↑, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The serum protein level of the control group was 7.08 ± 0.18 g dL^{-1}. The serum protein level of the crude drug (MCR) administered group was 6.84 ± 0.06 g dL^{-1} with the percent decrease of 3.38 as compared to control group while that of methanol extract (MME) group was 7.18 ± 0.14 g dL^{-1} with slight increase of 1.41 % than the control group. The serum protein level of the standard drug glipizide (G) administered group was 6.99 ± 0.15 g dL^{-1} which was 1.27 % less than the control group. However, none of the observed changes in the test drug treated group were found to be statistically significant.

6.3.3.1.4 Glycogen level

The data on the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the glycogen level in the liver is shown in Table 6.6.

Table 6.6 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on glycogen level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Glycogen Level (mg/100mg tissue)	% Increase
Control	0.41 ± 0.09	-
MCR-300	0.71 ± 0.04	$73.17^{*@\#}\uparrow$
MME-300	0.47 ± 0.06	$14.63^{\#}\uparrow$
G-0.5	0.48 ± 0.03	$17.07^{\#}\uparrow$

Values are Mean ± SEM, n = 5, Increase ↑, *: P< (0.05), @: ^{d}P< (0.05), #: F< (0.05), MCR: Crude powder, MME: Methanol extract, G: Glipizide

The glycogen content in the control group was 0.41 ± 0.09 mg/100 mg tissue. The glycogen level of the crude drug (MCR) treated group increased to 0.71 ± 0.04 mg/100 mg tissue which was 73.17 % (P< 0.05, ^{d}P< 0.05, F< 0.05) higher than the control group and also for the methanol extract (MME) administered group the glycogen levels increased to 0.47 ± 0.06 mg/100 mg tissue which was 14.63 % (F< 0.05) higher as compared to the control group. The glycogen content of the standard drug glipizide (G) treated group was 0.48 ± 0.03 mg/100 mg tissue which was 17.07 % (F< 0.05), higher than the control group.

The hyperglycemia and decrease in liver glycogen content observed in diabetic group are due to lack of insulin, increased gluconeogenesis and/or glycogenolysis (Kobayashi and Olefsky, 1979; Defronzo and Simonson, 1992). Impairment of liver glycogen synthesis in diabetic rats has been reported by Whitton and Hems, (1975) and by Huang et al., (2000). Anti-diabetic drugs are expected to reverse this decrease. In the present study the crude drug (MCR) showed marked and significant elevation of glycogen as compared to the methanol extract (MME) and the standard drug glipizide treated groups. This indicated that at the dose level studied MCR possessed weak anti-diabetic activity.

6.3.3.1.5 Serum urea level

In diabetes there is a relationship between glucose homeostasis and renal damage (Rasch, 1979a, b). The diabetic hyperglycemia induces elevation of the plasma levels of urea which is considered as significant marker of renal dysfunction (Almdal and Vilstrup, 1988). The data on the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum urea level is shown in Table 6.7.

Table 6.7 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum urea level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum Urea Level (mg dL^{-1})	% Change
Control	31.13 ± 6.51	-
MCR-300	24.80 ± 5.52	20.33↓
MME-300	32.77 ± 5.10	5.26 ↑
G-0.5	25.62 ± 3.62	17.69↓

Values are Mean ± SEM, n = 5, Decrease-↓, Increase-↑, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The serum urea level in the control group was 31.13 ± 6.51 mg dL^{-1}. The serum urea level in the crude drug (MCR) administered group was 24.80 ± 5.52 mg dL^{-1} which was 20.33 % less than the control group while that of the methanol extract (MME) administered group was 32.77 ± 5.10 mg dL^{-1} which was 5.26 % higher than the control group. The serum urea level of the glipizide (G) administered group was 25.62 ± 3.62 mg dL^{-1} which was 17.69 % less than the control group.

The highly significant increase in serum urea concentration of diabetic rats reported earlier (Ganong, 1995) is attributed to depletion of serum protein, increase in the rate of circulating amino acids and deamination that consequently leads to the formation of large amount of ammonia which is eventually converted to urea. The breakdown of amino acids during gluconeogenesis in the liver results in increased production of urea, fostering negative nitrogen balance (Ganong, 1995). However, in the present study the serum urea level in diabetic control rats

was within the normal range. The decrease observed in crude drug and glipizide may be indicator of their effect on gluconeogenesis.

6.3.3.1.6 Serum creatinine level

Creatinine measurement serves as a test for normal glomerular filtration. Elevated levels are associated with acute and chronic renal insufficiency and are indicated as significant marker of renal dysfunction (Almdal and Vilstrup, 1988). The data on the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum creatinine level is shown in Table 6.8.

Table 6.8 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum creatinine level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum Creatinine Level (mg dL^{-1})	% Decrease
Control	2.94 ± 0.50	-
MCR-300	2.73 ± 0.51	7.14↓
MME-300	2.48 ± 0.26	15.64↓
G-0.5	2.51 ± 0.15	14.62↓

Values are Mean ± SEM, n = 5, Decrease-↓, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The serum creatinine level of the control group was 2.94 ± 0.50 mg dL^{-1}. The serum creatinine levels of crude drug (MCR) administered group was 2.73 ± 0.51 mg/dL which was 7.14 % less as compared to the control group while that of the methanol extract (MME) administered group was 2.48 ± 0.26 mg dL^{-1} which was 15.64 % less than the control group. The serum creatinine level of the standard drug glipizide (G) administered group was 2.51 ± 0.15 mg dL^{-1} which was 14.62 % less than the control group.

The creatinine level observed in diabetic control rats is comparatively higher. This indicates possible renal dysfunction. The moderate decrease observed in test drug administered groups is indicative of reversal of this dysfunction.

6.3.3.1.7 Serum aspartate transaminase level (AST)

Aspartate transaminase (AST) measurements are used in the diagnosis and treatment of certain types of liver and heart disease. Elevated levels of the transaminases can signal myocardial infarction, hepatic disease, muscular dystrophy, or organ damage. The data on the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum aspartate transaminase level is shown in Table 6.9.

Table 6.9 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum aspartate transaminase level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum Aspartate Transaminase Level (IU/L)	% Increase
Control	221.09 ± 22.26	-
MCR-300	236.03 ± 22.07	6.75 ↑
MME-300	240.98 ± 26.42	8.99 ↑
G-0.5	237.11 ± 23.76	7.24 ↑

Values are Mean ± SEM, n = 5, Increase-↑, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The control group showed 221.09 ± 22.26 IU/L of the serum aspartate transaminases (AST) level. The serum AST level increased in both the test drugs (MCR and MME) and standard drug glipizide (G). For the crude drug treated group the serum AST level was 236.03 ± 22.07 IU/L which was 6.75 % more than the control group while that of the methanol extract group was 240.98 ± 26.42 IU/L which was 8.99 % more than the control group. The AST level of the glipizide administered group was 237.11 ± 23.76 IU/L which was 7.24 % more than the control group.

6.3.3.1.8 Serum alanine transaminase level (ALT)

Alanine aminotransferase (ALT) measurements are used in the diagnosis and treatment of certain liver diseases and heart diseases. Elevated levels of the transaminases can indicate myocardial infarction, hepatic disease, muscular

dystrophy, or organ damage. Alanine transaminase is a more liver-specific enzyme than asparate aminotransferase. The data on the effect of crude powder and methanol extract of M. hexandra (Roxb.) Dubard leaf on the serum alanine transaminase level is shown in Table 6.10.

Table 6.10 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum alanine transaminase level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum Alanine Transaminase Level (IU/L)	% Increase
Control	099.79 ± 10.81	-
MCR-300	107.71 ± 12.63	7.93 ↑
MME-300	126.75 ± 20.18	27.01↑
G-0.5	127.09 ± 23.87	27.35 ↑

Values are Mean ± SEM, n = 5, Increase-↑, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The control group showed 99.79 ± 10.81 IU/L of serum alanine transaminases (ALT) level. The serum ALT level for the crude drug (MCR) treated group was 107.71 ± 12.63 IU/L which was 7.93 % more than the control group while that of the methanol extract (MME) treated group was 126.75 ± 20.18 IU/L which was 27.01 more than the control group. The serum ALT level of the standard drug glipizide (G) administered group was 27.35 % more than the control group.

The activities of serum AST, ALT which are known as the marker enzymes for liver damage, showed weak to moderate elevation in all the experimental groups investigated here. Several studies on rats with alloxan diabetes have shown an increase in the activity of gluconeogenic enzymes including aminotransferases (AST and ALT). (Tanaka et al., 1988; Rawi et al., 1998). Although the activities of aspartate and alanine transaminase in the serum have been found to be a useful indicator of liver damage in the diagnosis and study of acute hepatic disease, these enzymes are located not only in the liver, but also in the extrahepatic tissues (He and Aoyama, 2003). The rise in the activity of alanine transaminase is due to hepatocellular damage and is usually accompanied by a rise in aspartate

transaminase (Mohan Rao et al., 1989). Since only moderate and statistically non-significant elevation was observed in transaminase activities, it can be suggested that the test drug has no modulatory effect on diabetes induced increase in the ALT and AST levels.

6.3.3.1.9 Serum acid phosphatase level (ACP)

The data on the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum acid phosphatase level is shown in Table 6.11.

Table 6.11 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum acid phosphatase level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum Acid Phosphatase Level (KA units)	% Change
Control	2.08 ± 0.40	-
MCR-300	2.15 ± 0.11	03.36↑
MME-300	2.52 ± 0.36	21.15↑
G-0.5	1.96 ± 0.20	05.76↓

Values are Mean ± SEM, n = 5, Decrease-↓, Increase-↑, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The serum acid phosphatase (ACP) level of the control group was 2.08 ± 0.40 KA units. The serum ACP level of the crude drug (MCR) treated group was 2.25 ± 0.11 KA units which was 3.36 % more than the control group; for the methanol extract (MME) administered group, the ACP level was 2.52 ± 0.36 KA units which was 21.15 % more than the control group and for the standard drug glipizide (G) treated group, it was 5.76 % less than the control group.

The elevation of the ACP level in serum is involved with the malfunctioning of liver or kidney during diabetes (Bottini et al., 2004). It has been shown that the alloxan injection causes a significant increase in the activity of acid phosphatase (Witek et al., 2001). The observed effect indicates lessening of the diabetic induced changes; however, there was no exact correlation.

Lipid profile

Apart from the regulation of carbohydrate metabolism, insulin also plays an important role in the metabolism of lipids. Insulin is potent inhibitor of lipolysis. The abnormal high concentration of serum lipids in diabetic subject is mainly due to increased mobilization of free fatty acids from the peripheral fat depots, insulin inhibits the hormone sensitive lipase (Al-Shamaony et al., 1994). The marked hyperlipidemia that characterizes the diabetic state may therefore be regarded as a consequence of the uninhibited actions of lipolytic hormones on the fat depots (Garber, 1998). The increase in concentration of free fatty acids in liver and kidney may be due to lipid breakdown.

In the alloxan-induced diabetes increased levels of total cholesterol, LDL-cholesterol, VLDL-cholesterol and triglyceride and decreased level of HDL cholesterol were found, all of which contribute to the coronary artery disease (Palumbo, 1998; Arvind et al., 2002). Lowering of serum lipid levels through dietary or drug therapy is associated with a decrease in the risk of vascular disease (Rhoads et al., 1976).

6.3.3.1.10 Serum triglyceride level

Triglyceride measurement is used in the diagnosis of diabetes mellitus, nephrosis, liver obstruction, and other diseases involving lipid metabolism and various endocrine disorders and in the treatment of patients with these diseases. Accumulation of triglycerides is one of the risk factors in coronary heart disease. The data on the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum triglyceride level is shown in Table 6.12.

Table 6.12 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum triglyceride level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum Triglyceride Level (mg dL^{-1})	% Change
Control	135.98 ± 02.40	-
MCR-300	130.56 ± 05.78	3.98 ↓
MME-300	154.13 ± 23.68	13.34 ↑
G-0.5	136.43 ± 04.87	0.33 ↑

Values are Mean ± SEM, n = 5, Decrease-↓, Increase-↑, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The serum triglyceride level of the control group was 135.98 ± 2.40 mg dL^{-1}. The serum triglyceride level of the crude drug (MCR) treated group was 130.56 ± 5.78 mg dL^{-1} which was 3.98 % less than the control group while that of methanol extract (MME) administered group was 154.13 ± 23.68 mg dL^{-1} which was 13.34 % more than the control group and that of the glipizide (G) treated group was 136.43 ± 4.87 mg dL^{-1} which was 0.33 % more than the control group. There was negligible decrease in crude drug treated group however this change was not statistically significant.

6.3.3.1.11 Serum cholesterol level

An elevated cholesterol level is associated with diabetes, nephrosis, hypothyroidism, biliary obstruction, and those rare cases of idiopathic hypercholesterolemia and hyperlipidemia; low levels are associated with hyperthyroidism, hepatitis, and sometimes severe anemia or infection. The results of the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum cholesterol level is shown in Table 6.13.

Table 6.13 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum cholesterol level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum Cholesterol Level (mg dL^{-1})	% Change
Control	78.34 ± 05.42	-
MCR-300	67.72 ± 04.41	13.55 ↓
MME-300	83.65 ± 13.98	6.77 ↑
G-0.5	79.52 ± 08.29	1.50 ↑

Values are Mean ± SEM, n = 5, Decrease-↓, Increase-↑, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The serum cholesterol level of the control group was 78.34 ± 5.42 mg dL^{-1}. The serum cholesterol level of the crude drug (MCR) treated group was 67.72 ± 4.41 mg dL^{-1} which was 13.55 % less than the control group while that of the methanol extract (MME) group was 83.65 ± 13.98 mg dL^{-1} which was 6.77 % more than the control group and that of the standard drug glipizide (G) treated group was 79.52 ± 8.39 mg dL^{-1} which was 1.50 % more than the control group. The crude drug showed little decrease in the cholesterol level which indicated that the drug might possess components aiding in reduction of cholesterol levels in diabetic conditions.

6.3.3.1.12 Serum HDL-cholesterol level

The main anti-atherogenic lipoprotein (HDL) is involved in the transport of cholesterol from peripheral tissues into liver (Segal et al., 1984) and thereby it acts as a protective factor against coronary heart disease (Gordon et al., 1977). The results of the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum HDL-cholesterol level is shown in Table 6.14.

Table 6.14 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum HDL-cholesterol level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum HDL-Cholesterol Level (mg dL^{-1})	% Decrease
Control	27.65 ± 2.54	-
MCR-300	22.58 ± 1.58	18.33 ↓
MME-300	23.13 ± 3.06	16.34 ↓
G-0.5	25.44± 4.26	7.99 ↓

Values are Mean ± SEM, n = 5, Decrease-↓, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The serum HDL-cholesterol level of the control group was 27.65 ± 2.54 mg dL^{-1}. The serum HDL-cholesterol level showed moderate decrease in both the test drugs (MCR and MME) and the standard drug glipizide (G) administered groups. The level of HDL-cholesterol of the crude drug treated group was 22.58 ± 1.58 mg/dL which was 18.33 % less than the control drug while that of the methanol extract group was 23.13 ± 3.06 mg dL^{-1} which was 16.34 % less than the control group and that of the standard drug was 25.44 ± 4.26 mg dL^{-1} which was 7.99 % less than the control group.

6.3.3.1.13 Serum LDL-cholesterol level

LDL is known as bad cholesterol as it transfers cholesterol from the liver to circulation. LDL-cholesterol particles play an important role in the formation of atherosclerotic plaques on the internal lining of blood vessels. If these plaques become dislodged and carried away in the blood, they can obstruct small blood vessels and thus cause myocardial infarction and strokes. The data on the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum LDL-cholesterol level is shown in Table 6.15.

Table 6.15 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum LDL-cholesterol level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum LDL-Cholesterol Level (mg dL^{-1})	% Change
Control	23.50 ± 4.53	-
MCR-300	19.03 ± 3.73	19.02↓
MME-300	29.69 ± 7.75	26.34↑
G-0.5	26.79 ± 6.50	14.0 ↑

Values are Mean ± SEM, n = 5, Decrease-↓, Increase-↑, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The serum LDL-cholesterol level of the control group was 23.50 ± 4.53. mg dL^{-1}. The serum LDL-cholesterol level of the crude drug (MCR) treated group was 19.03 ± 3.73 mg dL^{-1} which was 19.02 % less than the control group while that of the methanol extract (MME) group group was 29.69 ± 7.75 mg dL^{-1} which was 26.34 % more than the control group and that of the standard drug glipizide (G) administered group was 26.79 ± 6.50 mg dL^{-1} which was 14 % more than the control group. There was little decrease in LDL-cholesterol level in crude drug treated group while standard drug glipizide could not reduce the LDL-cholesterol level which indicated that crude drug has better potential in lowering LDL-cholesterol levels in diabetes. However, none of the changes were statistically significant.

6.3.3.1.14 Serum VLDL-cholesterol level

The level of glycemic control is the major determinant of total and very low density lipoprotein (VLDL) concentrations (Laakso, 1995). The data on the effect of crude powder and methanol extract of *M. hexandra* (Roxb.) Dubard leaf on the serum VLDL-cholesterol level is shown in Table 6.16.

Table 6.16 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its methanol extract on serum VLDL-cholesterol level in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Serum VLDL-Cholesterol Level (mg dL^{-1})	% Change
Control	27.19 ± 0.48	-
MCR-300	26.11 ± 1.15	3.97↓
MME-300	30.82 ± 4.73	13.35 ↑
G-0.5	27.28 ± 0.97	0.33↑

Values are Mean ± SEM, n = 5, Decrease-↓, Increase-↑, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The serum VLDL-cholesterol level of the control group was 27.19 ± 0.48 mg dL^{-1}. The serum VLDL-cholesterol level of the crude drug (MCR) group was 26.11 ± 1.15 mg dL^{-1} which was 3.97 % less than the control group while that of the methanol extract (MME) group was 30.82 ± 4.73 mg dL^{-1} which was 13.35 % more than the control group and that of the standard drug glipizide (G) administered group was 27.28 ± 0.97 mg dL^{-1} which was 0.33 % more than the control group. The crude drug showed negligible decrease in VLDL-choloesterol level while standard did not show any reduction in the increased VLDL-choloesterol level induced by diabetes.

6.3.3.2 Change in the weight of the organs and body weight of the experimental animals

6.3.3.2.1 Weight of liver

The absolute and relative weights of the rat liver of all the experimental groups are shown in the Table 6.17.

Table 6.17 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its extracts on absolute and relative weight of liver in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Absolute Weight of Liver		Relative Weight of Liver (g/100 g body weight)	
	Weight (g)	% Increase	Weight (g)	% Increase
Control	7.14 ± 0.63	-	3.06 ± 0.13	-
MCR-300	7.49 ± 0.50	4.90↑	3.10 ± 0.11	1.30↑
MME-300	7.64 ± 0.58	7.00↑	3.37 ± 0.11	10.13↑
G-0.5	7.21 ± 0.55	0.98↑	3.28 ± 0.15	7.18↑

Values are Mean ± SEM, n = 5, Increase-↑, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The absolute weight of the liver for the control group was 7.14 ± 0.63 g while the relative weight was 3.06 ± 0.13 g. The absolute weight of the liver for crude drug (MCR) group was 7.49 ± 0.50 g which was 4.90 % more than the control group whereas the relative weight was 3.10 ± 0.11 g which was 1.30 % more than the control group. The absolute weight of the liver for the methanol extract (MME) treated group was 7.64 ± 0.58 g which was 7 % more than the control group while the relative weight was 3.37 ± 0.11 g which was 10.13 % more than the control group. The absolute weight of the liver for the glipizide (G) administered group was 7.21 ± 0.55 g which was 0.98 % more than the control group while the relative weight was 3.28 ± 0.15 g which was 7.18 % more than the control group. The observed changes in the test drug administered were found to be statistically non-significant in comparison to diabetic control group

6.3.3.2.2 Weight of kidney

The absolute and relative weights of the rat kidney of all the experimental groups are shown in the Table 6.18.

Table 6.18 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its extracts on absolute and relative weight of kidney in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Absolute Weight of Kidney		Relative Weight of Kidney (g/100 g body weight)	
	Weight (g)	% Decrease	Weight (g)	% Decrease
Control	1.28 ± 0.06	-	0.56 ± 0.06	-
MCR-300	1.17 ± 0.07	08.59 ↓	0.48 ± 0.01	14.28 ↓
MME-300	1.04 ± 0.05	18.75*@↓	0.46 ± 0.02	17.85 ↓
G-0.5	1.06 ± 0.08	17.18 ↓	0.48 ± 0.02	14.28 ↓

Values are Mean ± SEM, n = 5, Decrease-↓, MCR: Crude powder, MME: Methanol extract, G: Glipizide, *: P< (0.05), @: ^{d}P< (0.05)

The absolute weight of the kidney for the control group was 1.28 ± 0.06 g whereas the relative weight was 0.56 ± 0.06 g. The absolute weight of the kidney for crude drug (MCR) group was 1.17 ± 0.07 g which was 8.59 % less than the control group while the relative weight was 0.48 ± 0.01 g which was 14.28 % less than the control group. The absolute weight of the kidney for methanol extract (MME) treated group was 1.04 ± 0.05 g which was 18.75 % (P< 0.05, ^{d}P< 0.05) less than the control group while the relative weight was 0.46 ± 0.02 g which was 17.85 % less than the control group. The absolute weight of the kidney for the glipizide (G) administered group was 1.06 ± 0.08 g which was 17.18 % less than the control group while the relative weight was 0.48 ± 0.02 g which was 14.28 % less than the control group. However, none of the observed changes in the test drug treated group were found to be statistically significant.

6.3.3.2.3 Weight of heart

The absolute and relative weights of the rat liver heart of all the experimental groups are shown in the Table 6.19.

Table 6.19 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its extracts on absolute and relative weight of heart in alloxan induced hyperglycemic rats

Treated Group (mg kg^{-1})	Absolute Weight of Heart		Relative Weight of Heart (g/100 g body weight)	
	Weight (g)	% Increase	Weight (g)	% Increase
Control	0.75 ± 0.03	-	0.32 ± 0.01	-
MCR-300	0.80 ± 0.04	6.66↑	0.33 ± 0.01	3.12↑
MME-300	0.81 ± 0.04	8.00↑	0.36 ± 0.02	12.5↑
G-0.5	0.79 ± 0.07	5.33↑	0.36 ± 0.03	12.5↑

Values are Mean ± SEM, n = 5, Increase-↑, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The absolute weight of the heart for the control group was 0.75 ± 0.03 g while the relative weight was 0.32 ± 0.01 g. The absolute weight of the heart for the crude drug (MCR) treated group was 0.80 ± 0.04 g which was 6.66 % higher than the control group while the relative weight was 0.33 ± 0.01 g which was 3.12 % higher than the control group. The absolute weight of the heart for the methanol extract (MME) treated group was 0.81 ± 0.04 g which was 8 % higher than the control group while the relative weight was 0.36 ± 0.02 g which was 12.5 % higher than the control group. The absolute weight of heart for the glipizide (G) administered group was 0.79 ± 0.07 g which was 5.33 % higher than the control group whereas the relative weight was 0.36 ± 0.03 g which was 12.5 % higher than the control group. The observed changes in the test drug administered were found to be statistically non-significant in comparison to diabetic control group

6.3.3.2.4 Change in the body weight

The change in the body weight during the treatment of 7 seven days is shown in Table 6.20.

Table 6.20 Effect of *Manilkara hexandra* (Roxb.) Dubard leaf powder and its extracts on body weight of albino rats

Treated Group (mg kg^{-1})	Change in Body weight (g)			
	4^{th} Day		7^{th} Day	
	Body wt. (g)	% Decrease	Body wt. (g)	% Decrease
Control	8.58 ± 2.43	-	11.86 ± 4.61	-
MCR-300	1.39 ± 2.33	83.79↓	5.18 ± 3.30	56.32↓
MME-300	0.61 ± 1.90	92.89↓	1.82 ± 3.28	87.65↓
G-0.5	3.70 ± 3.68	56.87↓	3.94 ± 3.16	66.77↓

Values are Mean ± SEM, n = 5, Decrease-↓, MCR: Crude powder, MME: Methanol extract, G: Glipizide

The body weight of the control group as compared to the initial body weight on the on the 4^{th} day was 8.58 ± 2.43 g and on the 7^{th} day it increased more to 11.86 ± 4.61 g. The body weight for the crude drug treated group on the on 4^{th} day was found to be 1.39 ± 2.33 g which was 83.79 % less than the control group and on the 7^{th} day it was 5.18 ± 3.30 g which was 56.32 % less than the control group. The body weight for the methanol extract treated drug on the 4^{th} day was 0.61 ± 1.90 g which was 92.89 % less than the control group while on the 7^{th} day it was 1.82 ± 3.28 g which was 87.65 % less than the control group. The body weight for the standard drug on the 4^{th} day was 3.70 ± 3.68 g which was 56.87 % less than the control group while on the 7^{th} day it was 3.94 ± 3.16 g which was 66.77 % less than the control group.

HISTOPATHOLOGY OF HEART

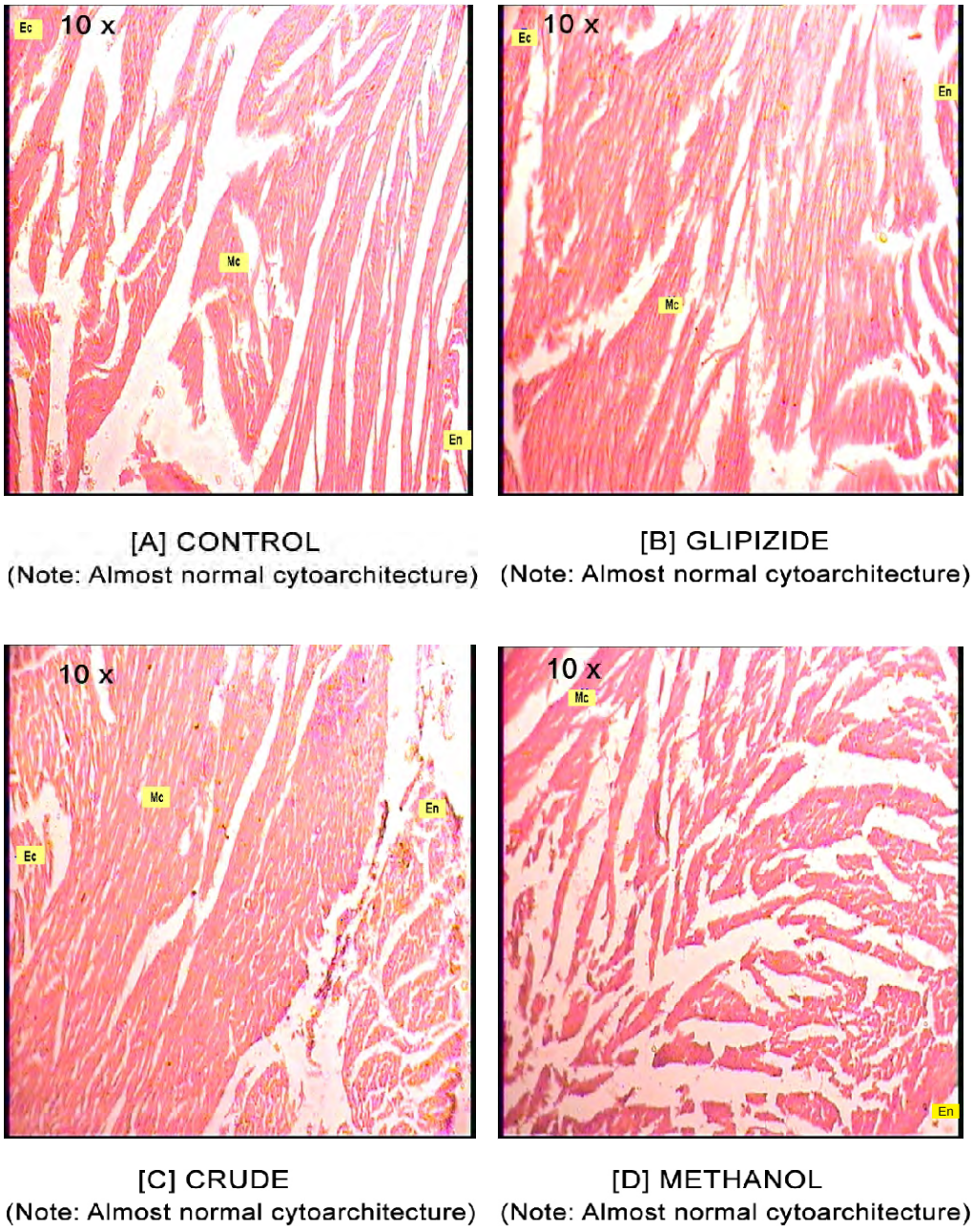
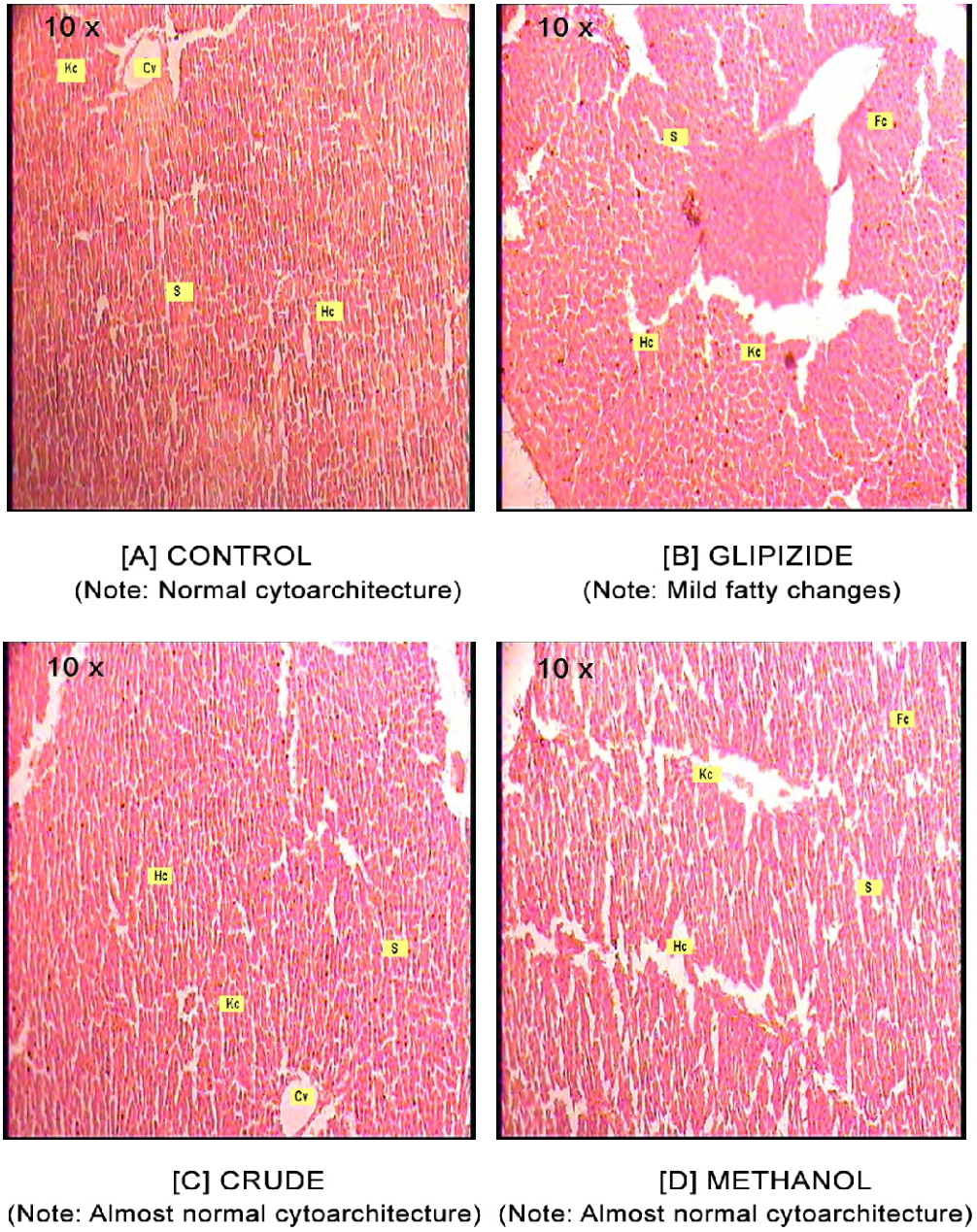


FIGURE 6.3 Photomicrographs of T.S. of heart from diabetic control [A], glipizide [B], crude [C] and methanol [D] administered groups
Ec- Epicardium, Mc- Myocardium, En- Endocardium

HISTOPATHOLOGY OF LIVER



[A] CONTROL
(Note: Normal cytoarchitecture)

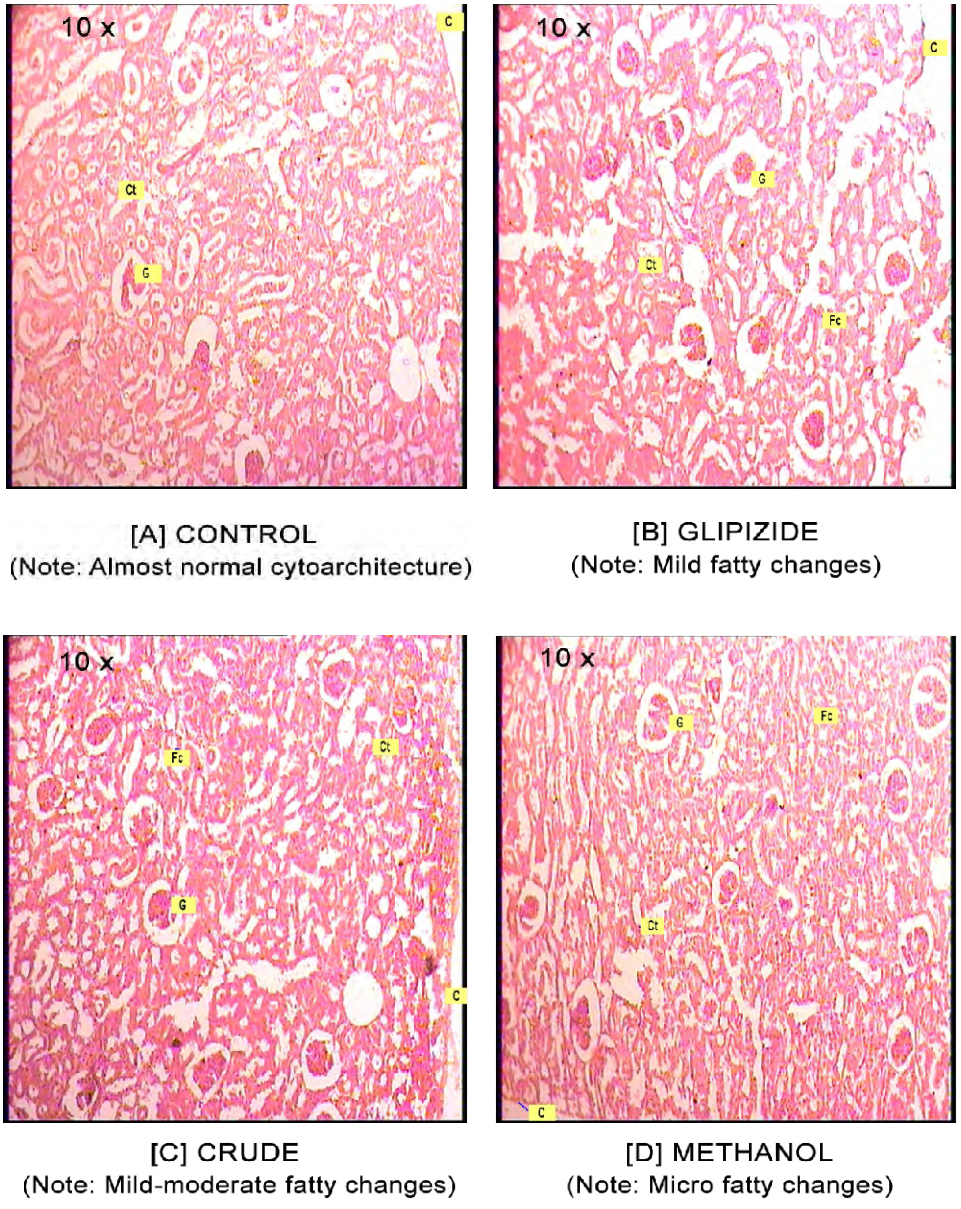
[B] GLIPIZIDE
(Note: Mild fatty changes)

[C] CRUDE
(Note: Almost normal cytoarchitecture)

[D] METHANOL
(Note: Almost normal cytoarchitecture)

FIGURE 6.4 Photomicrographs of T.S. of liver from diabetic control [A], glipizide [B], crude [C] and methanol [D] administered groups, Cv-Central vein, Hc- Hepatic cell, Kc- Kupffer cells, S- Sinusoid, Fc- Fatty changes

HISTOPATHOLOGY OF KIDNEY



[A] CONTROL
(Note: Almost normal cytoarchitecture)

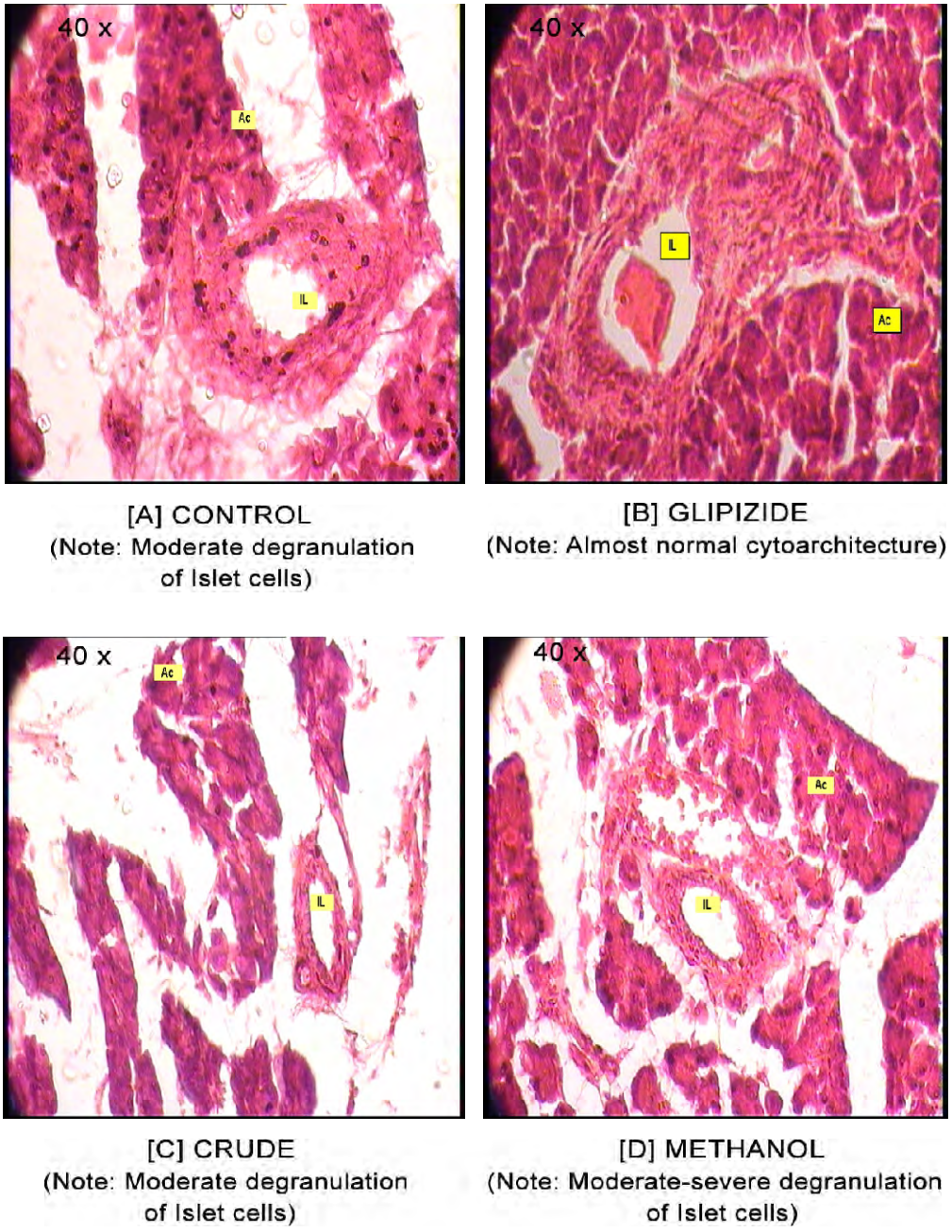
[B] GLIPIZIDE
(Note: Mild fatty changes)

[C] CRUDE
(Note: Mild-moderate fatty changes)

[D] METHANOL
(Note: Micro fatty changes)

FIGURE 6.5 Photomicrographs of T.S. of kidney from diabetic control [A], glipizide [B], crude [C] and methanol [D] administered groups, G- Glomerulus; Ct- Convolulated tubule; C- Capsule; Fc- Fatty changes

HISTOPATHOLOGY OF PANCREAS



[A] CONTROL
(Note: Moderate degranulation of Islet cells)

[B] GLIPIZIDE
(Note: Almost normal cytoarchitecture)

[C] CRUDE
(Note: Moderate degranulation of Islet cells)

[D] METHANOL
(Note: Moderate-severe degranulation of Islet cells)

FIGURE 6.6 Photomicrographs of T.S. of pancreas from diabetic control [A], glipizide [B], crude [C] and methanol [D] administered groups, IL- Islet of Langerhans; Ac- Acini cells.

6.3.3.3 Histopathological study

The histopathological study of four tissues was performed. Heart, liver, kidney and pancreas were selected to observe effect of drug treatment on cytoarctitecture of tissue in alloxan induced diabetic animals (Figures 6.3-6.6).

The crude extract (MCR), methanol extract (MME) and glipizide (G) treatment did not produce any gross changes in cell density and pattern in heart (Figure 6.3) and liver (Figure 6.4) section with control group. In heart no significant pathological changes were observed as a result of diabetes.

Mild fatty degenerative changes were observed in the kidney section of all the treated groups (MCR, MME and G) including control group. This indicated that the drug treatment had no significant effect on cytoarchitecture of kidney (Figure 6.5).

During histological examination of pancreas moderate degenerative changes were observed in the cells of islets of Langerhans as a result of injection of alloxan toxicant. Crude drug (MCR) and methanol extract (MME) were not able to prevent the observed degenerative changes but in comparision to control group less degeneration was observed in glipizide (G) treated group ((Figure 6.6). This indicated moderate protection of cell.

6.4 CONCLUSIONS

The crude powder (MCR) and methanol extract (MME) of *Manilkara hexandra* (Roxb.) Dubard leaf produced lowering of blood glucose level at 1st h. The treatment of MCR on normal fasted rats produced significant hypoglycemic effect as compared to MME at the dose level 300 mg kg^{-1}. Both the drugs MCR and MME showed increase in blood glucose level after sucrose loading on normal rats till 24th h. Hence, crude powder and methanol extract of *M. hexandra* leaf does not show sucrose tolerance at the studied dose level.

The present study showed that the crude powder (MCR) and methanol extract (MME) of *M. hexandra* leaf showed decreased level of glucose in alloxan diabetic

rats. However, there was a significant elevation in the glycogen level in the crude drug treated group as compared to methanol extract and and glipizide (G) treated groups. The crude drug of *M. hexandra* (Roxb.) Dubard leaf showed reduction in the triglyceride level, total cholesterol level, LDL level and VLDL level as compared to the diabetic control group. Similarly the effect of aqueous extract of *Scoparia dulcis* on lipid profile was reported by Latha and Pari (2005) and extract of Azadirachta indica leaf extract on lipid profile was reported by Chattopadhyay and Bandyopadhyay (2005). Hence, the results of the present study indicated that crude drug of *M. hexandra* (Roxb.) Dubard leaf may possess antihyperlipidemic activity if studied at higher dose levels. The methanol extract and glipizide treated group did not show any significant changes in biochemical parameters studied.

The histopatholgical studies showed that the tissues possessed almost normal cytoarchitecture. Therefore, the results show that the crude drug (MCR) of *Manilkara hexandra* (Roxb.) Dubard leaf possesses weak antidiabetic activity at the dose level of 300 mg kg^{-1}. Further studies of *Manilkara hexandra* (Roxb.) Dubard leaf at the higher dose level may show better antidiabetic activity.

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SUMMARY

Medicinal plants, since time immemorial, have been used in virtually all cultures as a source of medicine. Encompassing concepts and methods for protection and restoration of health, traditional medicine has served as a fount of alternative medicine, new pharmaceuticals and health care-products. Recently, public, scientific and medical interest in this key topic has soared as the importance of the indigenous collective wisdom in the use of herbal preparations, has been recognized.

Worldwide, at least 122 chemical substances of known structure are extracted and purified for medicinal purposes. There are hundreds of species recognized to possess therapeutic value. Although these are not purified into separate compounds, many are believed to exert therapeutic effects good enough to be proven effective by modern analysis.

Recently the search for components with novel biological properties has gained increasing importance due to growing world wide concern about the alarming increase in the rate of deadly diseases like infections by antibiotic resistant microorganisms, AIDS, cancer, diabetes, tuberculosis etc. This situation has forced scientists for searching newer drugs from various sources like medicinal plants which are the good source of novel chemotherapeutic agents. Considering the bioprospectus of medicinal flora, the present study was conducted to investigate antimicrobial properties of some plants of Saurashtra region against a wide array of microorganisms. Amongst these, the most promising plant was selected for further evaluation of pharmacognostical studies, phytochemical studies, toxicological studies and pharmacological activity.

In the present work, 108 different plant species belonging to 54 different families were screened for their potential antimicrobial property against 5 Gram-positive bacteria, 9 Gram-negative bacteria and 1 yeast by agar well and agar disc diffusion method. Out of 216 extracts, 143 plant extracts (35 aqueous and 108

methanol/ethanol) showed activity against at least one of the tested microorganism. Although plants differed significantly in their activities against microorganisms tested, more of the extracts showed antibacterial activity against Gram-positive bacteria than Gram-negative bacteria. Amongst the 15 microbial strains tested, the most susceptible bacteria was *B. cereus* which is a Gram-positive bacteria and the most resistant bacteria was *E. coli* which is a Gram-negative bacteria.

Amongst aqueous and methanol/ethanol extracts of the studied plant species, methanol/ethanol extracts were found to be more active against the test microbial strains than the aqueous extracts. Amongst 54 families, 8 families viz. Anacardiaceae, Casuarinaceae, Combretaceae, Lythraceae, Myrtaceae, Sapotaceae, Trapaceae, and Vitaceae inhibited more than 50 % bacterial strains. Amongst 108 plant species, the most active plant was *Terminalia chebula* Retz. while the most inactive plant was *Solanum surattense* Burm.f.

The methanol extracts of 20 selected medicinal plants were screened for antifungal activity against 11 fungal strains. The antifungal activity was evaluated by agar disc diffusion method at three different concentrations (500 µg/disc, 250 µg/disc and 125 µg/disc). The moulds were more susceptible than yeast. All the concentrations of the extracts investigated, inhibited the fungal species with varying degree of sensitivity. *Aspergillus flavus* was the most susceptible and *Candida glabrata* was the most resistant fungal strain. The antifungal activity of the screened plants did not show any concentration effect.

On the basis of the results evaluated for *in vitro* antibacterial and antifungal potencies of the plants, *Woodfordia fruticosa* kurz., *Trapa natans* L., *Terminalia chebula* Retz., *Mesua ferra* L., *Manilkara hexandra* (Roxb.) Dubard, *Mangifera indica* L., *Eucalyptus citriodora* Hook., *Bauhinia variegata* L., *Caesalpinia pulcherrima* (L.) Swartz., *Vitis vinifera* L. and *Euphorbia hirta* L. showed the best antimicrobial activity. *Manilkara hexandra* (Roxb.) Dubard (Sapotaceae) leaf was selected for further pharmacognostic, phytochemical, toxicological and pharmacological evaluations on the basis of availability and literature search.

Manilkara hexandra (Roxb.) Dubard is an evergreen tree with 3-12 m tallness. The leaves of *M. hexandra* (Roxb.) Dubard are dark green in color and bitter in taste. The crude powder was successively extracted with petroleum ether, acetone and methanol. The methanol extract (MME) had the maximum yield of 23 % while acetone extract (MAC) had minimum extractive yield of 4.8 %.

The microscopic study of *Manilkara hexandra* (Roxb.) Dubard leaf was done by evaluating different characteristics from the T.S. and crude powder of leaf. T.S of leaf showed upper epidermis with epidermal cells which were single layered, straight walled, rectangular in shape and covered with thin cuticle. The simple, unicellular, conical and thick trichomes with single covering were present. The lower epidermis had more number of trichomes than the upper epidermis. The anomocytic stomata were present in lower epidermis. Prisms of calcium oxalate were present in the lower epidermal cells. The vascular bundle was collateral. Leaf was dorsiventral. The specific characteristics observed in powder studies were anomocytic stomata, simple covering unicellular trichome, group of pericyclic fibers, prisms of calcium oxalate crystals and xylem vessels in longitudinal sectional view showed spiral thickening.

The result of proximate analysis of crude powder (MCR) of *M. hexandra* (Roxb.) Dubard leaf showed 4 % of loss on drying, It contained 6 % of total ash and 1 % of acid insoluble ash. The percent extractive yield of crude powder extracted in petroleum ether was 3.74 %; that extracted in alcohol was 10.60 % and extracted in water was 12.30 %. MME and MAC were soluble in almost all the solvents while MPE showed comparatively less solubility due to its hydrophobic nature. All the samples were acidic in nature. All the sample drugs (MCR, MPE, MAC and MME) showed minor presence of some heavy metals but the extracts did not exceed the limit reported by WHO guidelines. The sample drugs investigated were free from heavy metal and microbial contamination.

The results of preliminary phytochemical analysis of crude powder and different extracts of *M. hexandra* (Roxb.) (MCR, MPE, MAC and MME) Dubard showed maximum presence of cardiac glycosides and steroids in all the four sample

drugs. Tannins and saponins were present in all the extracts except MPE. Alkaloids were present in MCR, MPE, MAC and MME with Wagner's test. The alkaloids were present only in MCR and MPE with Dragendroff's test and in MCR and MME with Mayer's test. Flavonoids were absent in all the four drug samples. The HPTLC fingerprinting showed maximum 14 peaks for MAC, followed by 13 peaks for MME and 7 peaks for MPE. There were four constituents common in MAC and MME with Rf values of 0.06, 0.21, 0.50 and 0.55 and there were two constituents common in MPE and MAC with Rf values of 0.23 and 0.67. None of the constituents were common in MPE and MME. The constituents can be further isolated and purified to find its potency for biological activities.

Bacteria and fungi cause some important human diseases. Despite the existence of potent antibacterial and antifungal agents, the past three decades have seen a dramatic increase in microbial resistance to antimicrobial agents, imposing the need for a permanent search and development of new drugs. Plant based antimicrobials represent a vast untapped source of medicines and there is a need for further exploration of plant antimicrobials. In the present study, the antimicrobial potential of the petroleum ether, acetone and methanol extracts of *M. hexandra* (Roxb.) Dubard leaf were evaluated at two different concentrations against 9 Gram-positive, 14 Gram-negative, 7 yeast and 4 moulds by agar disc diffusion method. The results showed that methanol extract (MME) was better than the other two extracts (MPE and MAC) which implied that antimicrobial activity is better with the polar solvents. Also, the antimicrobial activity observed was concentration dependent for each extract.

Amongst all the microbial strains investigated, a Gram-negative bacteria- *Citrobacter freundii,* yeasts- *Candida albicans* (2), *Candida glabrata, Cryptococcus luteolus, Cryptococcus neoformans* and mould- *Aspergillus candidus,* were most resistant strains whereas a Gram-positive bacteria - *Bacillus cereus* and Gram-negative bacteria *Enterobacter aerogenes* and *Pseudomonas putida,* yeast - *Trichosporon begelli* and mould- *Aspergillus flavus* were most susceptible strains.

The results showed that methanol extract (MME) of *M. hexandra* (Roxb.) Dubard leaf possessed measurable in vitro antimicrobial activity against many of the microorganisms implicated in the pathogenesis of the human infections. The broad range of inhibition implied that the extract had a comparable antimicrobial activity. The MIC of *M. hexandra* was evaluated within a range of concentration from 250-32,000 µg/ml.

Toxicology is a relatively young biological science that involves a complex interrelationship among dose, absorption, distribution, metabolism and elimination. A toxic substance is a material which has toxic properties. Knowledge of how toxic agents damage the body has progressed along with medical knowledge. Toxicity depends not only on the dose of the substance but also on the toxic properties of the substance. The relationship between these two factors is important in the assessment of therapeutic dosage in pharmacology and herbalism. Considering the aforesaid, crude powder (MCR) and methanol extract (MME) of *Manilkara hexandra* (Roxb.) Dubard leaf was evaluated for the acute toxicity studies.

The results of acute toxicity studies showed that the crude powder as well as methanol extract showed dose dependent effect on gross behaviour. The methanol extract exhibited pronounced effect on gross behaviour of mice as compared to the crude powder. Mild to moderate hypoactivity or reduced locomotion was noted at all dose levels for crude powder (MCR) of *M. hexandra* (Roxb.) Dubard leaf. Mortality was not observed at any dose levels for crude powder (MCR). The methanol extract (MME) of *M. hexandra* (Roxb.) Dubard leaf showed marked effect for hypoactivity at the highest dose level. Mortality for methanol extract (MME) was observed at the highest dose level i.e. 3120 mg kg^{-1}. Animal death was preceded by symptoms, such as hypoactivity and lethargy. The further evaluation of LD_{50} at the dose levels 2000-9000 mg Kg^{-1} revealed that methanol extract (MME) was devoid of acute toxicity at the studied dose level therefore, crude drug (MCR) and methanol (MME) extract are safe till the dose level of 9000 mg kg^{-1}.

Diabetes is a growing public health concern worldwide causing severe and costly complications including blindness, cardiac and kidney diseases. The diabetic epidemic is accelerating in the developing world, with an increasing proportion of affected people in younger age groups. Worldwide, approximately 120 million people have diabetes and that could double in 10 years. In addition to different synthetic drugs, plant remedies play an effective role in diminishing the suffering. In the present work, *M. hexandra* (Roxb.) Dubard leaf was evaluated for antidiabetic activity.

The hypoglycemic study of the crude powder (MCR) and methanol extract (MME) of *Manilkara hexandra* (Roxb.) Dubard leaf produced lowering of blood glucose level at 1st h. The treatment of MCR on normal fasted rats produced significant hypoglycemic effect as compared to MME at the dose level 300 mg kg^{-1}. In the sucrose tolerance test, both the drugs MCR and MME showed increase in blood glucose level after sucrose loading on normal rats till 24th h. Hence, crude powder and methanol extract of *M. hexandra* leaf does not show sucrose tolerance at the studied dose level.

The present study showed that the crude powder (MCR) and methanol extract (MME) of *M. hexandra* leaf showed decreased level of glucose in alloxan induced diabetic rats. However, there was a significant elevation in the glycogen level in the crude drug treated group as compared to methanol extract and glipizide (G) treated groups. The crude drug of *M. hexandra* (Roxb.) Dubard leaf showed reduction in the triglyceride level, total cholesterol level, LDL level and VLDL level as compared to the diabetic control group. Hence, the results of the present study indicated that crude drug of *M. hexandra* (Roxb.) Dubard leaf may possess good antihyperlipidemic activity if studied at higher dose levels. The methanol extract and glipizide treated group did not show any significant changes in biochemical parameters studied.

The histopatholgical studies showed that the tissues (heart, liver, kidney and pancreas) possessed almost normal cytoarchitecture. Therefore, the results show that the crude drug (MCR) of *Manilkara hexandra* (Roxb.) Dubard leaf

possesses weak antidiabetic activity at the dose level of 300 mg kg^{-1}. Further studies of *Manilkara hexandra* (Roxb.) Dubard leaf at the higher dose level may show better antidiabetic activity.

Overall, it can be concluded that *M. hexandra* (Roxb.) Dubard leaf possessed moderate antimicrobial property. Also, it possesses weak antidiabetic activity however, the results were not significant. It showed lipid lowering property. This study therefore warrants investigation to isolate and identify the antidiabetic and antihyperlipidemic principles to elucidate their exact mechanism of action.