Screening for antimicrobial and anti-inflammatory activities and, formulation studies on the extracts of selected medicinal plants topically applied in Ethiopia

A thesis submitted to the School of Graduate Studies of the Addis Ababa University in partial fulfillments for the requirements of the Degree of Master in Pharmaceutics

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February 2004

ACKNOWLEDGEMENTS

Thanks be to God for His unrelenting support in all my life endeavor. Next to this, my deepest gratitude and appreciation are to my advisors, Prof. Tsige Gebre-Mariam and Dr. Kaleab Asres for their constant and constructive advises and follow-up throughout my study.

Last but not least is my appreciation to Addis Ababa University and Ethiopian Health and Nutrition Research Institute (EHNRI) for sponsoring my MSc study and allowing me to use their laboratory facilities, respectively.

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ACRONYMS

AACHRO	African Advisory for Health Research and Development
ACCT	*Agence de Co-operation Culturelle et Technique*
AIDS	Acquired Immuno Deficiency Syndrome
AP-PCR	Arbitrary Primed Polymerase Chain Reaction
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
AUC	Area Under the Curve
COX-2	Cycloxygenase–2
DNA	Deoxyriboneucleic acid
EC	European Commission
GC	Gas Chromatography
GLP	Good Clinical Practice
GMP	Good Manufacturing Practice
HIV	Human Immunodeficiency Virus
HPLC	High Pressure Liquid Chromatography
HPTLC	High Performance Thin layer Chromatography
IFPMA	International Federation of Pharmaceutical Manufacturers Association
LC/MS	Liquid Chromatography/Mass Spectroscopy
LC/NMR	Liquid Chromatography/Nuclear Magnetic Resonance
MDR	Multi-drug Resistance
MIC	Minimum Inhibitory Concentration
NAPRECA	Natural Products for Eastern and Central Africa

OAU/STRC	Organization for African Unity/Scientific and Technical Research Commission
PEG	Polyethlene glycol
R&D	Research and Development
RAPO	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SEM	Standard Error of the Mean
TCM	Traditional Chinese Medicine
TLC	Thin Layer Chromatography
UNIDO	United Nations International Development Agency
USP	United States Pharmacopoeia
UV	Ultraviolet
WHA	World Health Assembly
WHO	World Health Organization
5-HT	5-hydroxytryptamine

Abstract

In an attempt to integrate traditionally used herbal products into modern topical formulation, extracts of the leaves of *Maesa lanceolata* (Myrsinaceae), *Osyris quadripartita* Decn. (Santalaceae), *Steganotaenia araliacea* Hochst ExA. Rich (Apiaceae), *Cadaba farinosa* (Capparidaceae) and; the aerial parts of P*lantago lanceolata* (Plantagonaceae) and *Alachemilla pedata* A. Rich (Rasaceae) have been screened for their antibacterial and antifungal activities. All hydroalcoholic extracts, except *C. farinosa* and *S. araliacea* were active against *E. coli*. And, all extracts had activities against *S. aureus* and *P. aeroginosa*. Similarly, screeneing of the total extracts against *Candida albicans* and *Trichophyton mentagrophytes* indicated that all extracts, except *P. lanceolata* on both strains and *C. farinosa* on *T. mentagrophytes,* were active. But none of the extracts tested displayed activity against *Aspergillus niger*.

The antimicrobial activities of *A. pedata* and *M. lanceolata* were higher than those plants tested and hence, further works have been undertaken on these plants. In an attempt to localize the active ingredients, successive fractionation with petroleum ether, chloroform, acetone and methanol have been carried out. The antimicrobial activity study of the various fractions revealed that the antimicrobial effect of *A. pedata* was because of the non-polar components (petroleum ether fraction) and that of *M. lanceolata*, the activities were distributed among the various fractions. The action against *T. mentagrophytes* of the latter was entirely because of the polar compounds present in the methanol extract.

To assess the clinical utility of these plants, MICs were determined. Accordingly, the results for *A. pedata* were 5 mg/ml against *S. aureus* and *E. coli* and, 10 mg/ml for *C. albicans* using agar-well diffusion technique. The MIC of this plant extract using agar dilution technique on the same

bacteria was 0.125 mg/ml. Similarly; the MIC of *M. lanceolata* was 1.25 mg/ml against the abovementioned bacteria and, 0.625 mg/ml against fungi using agar-well diffusion technique. And, using dilution technique, the result was 1.25 mg/ml for bacteria and 0.0625 mg/ml for fungi.

Anti-inflammatory activities and semi-quantitative standardization works were also conducted and it was found that *M. lanceolata* has significant anti-inflammatory activity at 100 mg/kg and 750mg/kg with better activity at 100 mg/kg. Similarly, *A. pedata* has also displayed anti-inflammatory activity, even though the effect was less than *M. lanceolata.* The water extractive values were 4.66 and 5.24% (W/W) for A. *pedata* and *M. lanceolata*, respectively. Successive extractive values using petroleum ether, chloroform, acetone, methanol and water were (3.20, 4.56), (3.38, 4.36), 1.28, 0.56), (5.38, 6.52), (4.60, 6.64) for *A. pedata* and *M. lanceolata*, respectively. Ash value determination and TLC-fingerprinting were also conducted as part of the standardization work.

Ointments and creams were formulated using the hadroalcoholic extracts of *A. pedata* and *M. lanceolata.* Study on the *in vitro* performances of the proposed formulations indicated that releases from hydrophilic bases were better and polyethylene glycol-based preparations were superior in activity than formulations prepared with hydrophobic bases. Furthermore, the *in vitro* performances of the formulated topical dosage forms were comparable to the activity of locally available marketed antimicrobial products.

1 INTRODUCTION

1.1 Biological scope of the resource for bimolecular discovery

The types of biologicals that are commonly used as sources of biochemical or test resources for biodiscovery programs are microbials, marine organisms and plants. Insects and animals also have limited applications.

Microbials: These are by far the most widely used natural resource in industrial screening programs. The reason is two-fold: economics and reproducibility. The use of microorganisms, over the past few years in antimicrobial research and development has established a sound technological basis for the culture, preservation and manipulation of bacteria and fungi. Microbials are accountable for most of the antibiotics in the therapeutic use today and, in addition, serve as biochemical factories for chemical modifications that cannot be reasonably achieved, otherwise. Antibiotics are also unique chemical substances for further manipulation in laboratory. The shortcomings of microbials may be the limited biochemical scope compared to other natural resources, and the critical need for de-replication at an early stage in screening [1, 2].

Marine organisms: The biodiversity of the marine ecosystem has barely been scratched. In fact, if one looks at the fundamental phyla of life, there are 34; out of which 17 occur on land and 32 in the sea (with some overlap) [3]. These offer a remarkable variety of chemical variation. Over the past thirty years, not less than 3,000 new compounds have been isolated from marine organisms [4]. Many such natural products are unavailable from other natural sources and certainly not through laboratory synthesis. Access to these resources is very expensive, limited to initial structural leads and rarely amenable to commercial development. Even recollection for research purposes is limited by cost and environmental concerns. Another peculiar aspect of

marine organisms is the high incidence of symbiosis and uncertainty as to whether the active compound identified is the product of the bulk organism (e.g., sponge) or a resident invertebrate or microorganisms [2]. Nonetheless, these resources are certainly worthy of access if only for the structural leads and stimulus they provides.

Plants: Paradoxically, the plant genome is much larger than other natural sources (Bacteria: 1000 genes, Fungi: 10,000 genes, Plants: greater than 100,000 genes) and, therein offers a broader biochemical network for the generation of unique and novel chemical structures. A prime example of this scenario is paclitaxel. Paclitaxel is produced by *Taxus brevifolia* and is a cytotoxic agent that is most likely produced to prevent organisms from feeding on the plant. It is a very complicated molecule that contains four fused rings. Due to its complex structure it is highly unlikely that it would have ever been produced synthetically prior to its discovery [5]. For this and various other reasons, drug discovery from plant and hence research in the area has got better appreciation and concern, nowadays.

1.2 Current interest on the use of herbs and their economic importance

Herbs that were once had been the only choice for drug treatment became marginalized because of the advances in synthetic chemistry, especially in the Western World. But, in due course of time, the picture has changed and consistently being changing particularly because of the emergence of chronic and incurable diseases. Hence, throughout the world, especially in Europe and Asia, a tremendous renaissance in the use and appreciation of herbal medicine has taken place. For example, in 1985, the World Health Organization (WHO) estimated that perhaps 80% of the world population relied on herbs for primary health care needs [6]. In France and Germany also, 30 to 40 percent of all medical doctors rely on herbal preparations as their primary

medicines [7]. Obviously, this much increase in the interest on utilization has brought a concomitant economic importance of the area especially to developing countries.

In Germany, estimates showed that over $4 billion dollars are spent on herbal products each year. In Japan, the figure is thought to be even higher. Herbal products represent a major economic force in the United States as well, with an estimated annual sales figure of $3.3 billion dollars for 1998 and rising since then [8]. It has very recently exceeded $11 billion dollars for the purchase of plant-based medicines each year in the United States alone, and $43 billion dollars worldwide [9]. Between 1990 and 1997, this herbal supplements market has had a higher annual average growth rate of 25%. Rising global interest in medicinal plants has also created a sustained and largely underground trade in plant materials, many of which are collected from least developed countries. This is great market opportunity for developing countries; of course, it will be true if these countries can undertake the stringiest tests applied by developed pharmaceutical manufacturers before mass production [10]. Therefore, this is a challenging assignment for developing countries to modernize their system until it satisfies the current market trend and interest.

1.3 R&D trends of herbal-based medicine: the case of Africa and China

1.3.1 Africa

The practice and research in the area of traditional medicine in Africa, where herbal-based treatment is one aspect, is yet to be explored. In 1964, the Organization for African Unity (OAU) set up the Scientific and Technical Research Commission (OAU/STRC) and this in turn organized the Inter-African Symposium on the Development of African Medicinal Plants in Dakar in 1968. The Symposium passed the decision that the efficacy of herbs used by traditional

health practitioners should be tested as much as possible. Hence, since then, institutions like WHO, United Nations International Development Agency (UNIDO) and others have undertaken many initiatives to assist works in this area [11].

For example, under the umbrella of the *Agence de Co-operation Culturelle et Technique (ACCT),* Paris*,* ethno-botanical surveys have been carried out jointly with international teams in Benin, Central African Republic, Comoros, Congo, Gabon, Madagascar, Mali, Mauritius, Niger, Seychelles, Tunisia, Togo, and Rwanda. Moreover, the OAU/STRC has carried out similar surveys in Cameron, Ghana, Uganda, Swaziland, and Western Nigeria. The outcomes of these ethno-botanical surveys have been published and can be consulted in data bases such as those developed by the OAU/STRC in Lagos, Nigeria, by the Natural Products for Eastern and Central Africa (NAPRECA) network and the NARISTAN database on medicinal plants developed by Hoechst/Naristan and used by the University of Cape Town [11].

In continuation of the assistance, the 19th session of the African Advisory for Health Research and Development (AACHRD) in 2000 recommended that the Regional Offices should revitalize research on traditional medicine, particularly for common problems such as HIV/AIDS, tuberculosis, malaria, and related diseases. Then, in 2001, the OAU Heads of State declared at the Summit Meeting in Abuja that research on traditional medicine should be a matter of priority. Later in the same year, the OAU Summit held in Lusaka, declared the period 2001-2010 as the decade for African Traditional Medicine [11]. The OAU/STRC has thus funded 17 research centers all over Africa in order to stimulate research in this virgin area of proof of efficacy of medicinal plants in the region. These initiatives have greatly enhanced the development of medicinal plant research, the drawing up of an African Pharmacopoeia, the conduct of

phytochemical and biological screening of medicinal plants, ethno-botanical surveys and the development of some phytomedicines.

Even though the effort put so far on herbal drugs is appreciable as a good start, R&D in Africa is yet to be encouraged and systematically organized to get meaningful benefit from the area. Unlike of Africa, R&D on herbal drugs has got better sophistication in many parts of the world; a case in point is China's. Comparatively speaking, R&D in China has stepped further a head than that of Africa. Highlight of this case is given below; otherwise the detail is discussed elsewhere [12].

1.3.2 China

Currently, Traditional Chinese medicine (TCM) production in China is being undertaken by not les than 30,000 TCM related enterprises with 12,807 TCM varieties (11,146 plant, 1581 animal, 80 mineral sources). Pertaining to drug research, two approaches are equally valid in China: discovery and development of western medicine from natural sources and research and modernization of herbal medicine, including multi-component prescriptions. In the quest for TCM modernization in the 21st century, the f(w/w) ollowing national goals have been set for China for the period of 1996-2005 [12]:

- Develop 30 highly effective TCM formulas with 10 to enter the international market.
- Establish and improve standardized research and development systems with the following complaint standards: Good manufacturing practice (GMP) standards for TCM cultivation and quality control, Good Laboratory Practice (GLP) standards for TCM pharmacological

experiments and assay and safety evaluation centers, Good Clinical Practices (GCP) for clinical trials; establish 10 standardized TCM raw material production bases and 5 modern high-tech production bases, foster 5 internally recognized TCM based industrial groups.

Furthermore, a legislative decree governing the protection of TCM has also been issued in China to raise the quality of all TCM varieties, protect legal rights and interests of TCM production enterprises, and promote development of R&D activities. Thus, two grades of protection area are given [12].

Hence, Africa should draw lessons from China's experience and gear the direction of R&D work in such a way that it fulfils the current market need of the world. Most researches so far in Africa are no more than observational studies. Screening should not be the sole interest of researchers working in the area. There has to be a means of bringing those herbs proven for efficacy to a state of dosage form to be accessed at least by the local market.

1.4 Approaches for developing formulations from botanicals

The "first generation" of plant drugs was usually simple botanicals employed in more or less their crude form. Several effective medicines used in their natural state such as cinchona, opium, belladonna and aloe were selected as therapeutic agents based on empirical evidence of their clinical application by traditional societies from different parts of the world. Following the industrial revolution, a "second generation" of plant-based drugs emerged based on scientific processing of the plant extract to isolate their "active constituents". The "second-generation" phytopharmaceuticals were pure molecules and some of the compounds were even more

pharmacologically active than their synthetic counterparts. Notable examples were quinine from *Cinchona*, reserpine from *Rauvolfia*, and more recently taxol from *Taxus* species [13].

The sequence for development of pharmaceuticals, in such a case, usually begins with the identification of active lead molecules, then detailed biological assays and formulation of dosage forms in that order. This is followed by several phases of clinical studies designed to establish safety, efficacy and pharmacokinetic profile of the new drug. Possible interaction with food and other medications may be discerned from the clinical trials [13].

In the development of "third generation" phytotherapeutic agents, a top-to-bottom approach is usually adopted. This consists of first conducting a clinical evaluation of the treatment modalities and therapy as administered by traditional doctors or as used by the traditional community as folk medicine. This evaluation is then followed by acute and chronic toxicity studies in animals. Studies should, when applicable, include cytotoxicity studies. It is only if the substance has an acceptable safety index would it be necessary to conduct detailed pharmacological/biochemical studies. Formulation and trial production of the dosage forms are structured to mimic the traditional use of the herb. This is a unique blend of the empiricism of the earlier first generation botanicals with the experimental research used to prove the efficacy and safety of second generation isolated pure compounds [13].

Nowadays, the latter two approaches seem to have got popularity by different manufacturing companies. Even though the second approach will incur high cost, which may frustrate many manufacturers, it is quite rewarding if it is successfully undertaken.

1.5 Production of herbal-based products

1.5.1 Processes involved in the production [14, 15]

Collecting/harvesting: When plants are collected from their natural habitat they are said to be "wild-crafted". In contrast, when they are grown utilizing commercial farming techniques, they are said to be "cultivated". Collection of plants from cultivated sources ensures that the plant collected is the same one desired.

Drying: After harvesting, most herbs have moisture content of 60 to 80 % and cannot be stored without drying. This is necessary so that important compounds can be protected from breakdown by microbial and other contaminating factors. The majority of herbs in widespread use today require relatively mild conditions for drying.

Garbling: Garbling refers to the separation of the portion of the plant to be used from other parts of the plant, dirt and other extraneous matter. This step is often done during collection.

Grinding: This involves mechanically breaking down either leaves, roots, seeds, or other parts of a plant into a uniform size shape and consistency; suitable for encapsulation or extraction. A number of machines can be used to grind herbs, but the most widely used is the hammer mill.

Extraction: Most extracts produced by small manufacturers involve the use of maceration procedures. Larger manufacturers utilize more elaborate techniques to ensure that an herb is fully extracted and that the solvent is reused. For example, high-pressure extraction is used for the separation of both solid and liquid raw substances, the former being fed batch-wise to an extraction vessel, while the latter is extracted continuously in counter-current columns. Liquid

CO_2 flows from collecting vessels to a transfer pump, which compresses it to the required extraction pressure where the gas is heated to the extraction temperature and transferred to the extraction vessel or column. The substances to be extracted are dissolved in the heated CO_2 as they pass through the vessel. This CO_2 mixture, laden with the dissolved substance, is then fed to a separator. By adjusting the pressure and/or temperature, the dissolving power of the CO_2 in the separator is reduced, thus causing the extracts to be precipitated [15, 16].

Concentration: After extraction of the herb, the resulting solutions can be concentrated into fluidextracts or solid extracts. In large manufacturing operations, the techniques and machines used ensure that the extracted plant components are not damaged.

Excipient selection and formulation: Formulations can be prepared in either of the different dosage forms applicable to western drugs. A given dosage form is formulated using the respective component: the drug and the different excipients. The same excipients used in the manufacture of western drugs as well as vitamin and mineral supplements are often used in the production of dosage forms containing herbs or herbal extracts. Many manufacturers provide a list of excipients contained in their products.

1.5.2 Standardization for formulation and its difficulties

Among the reasons why herbal products are notoriously variable is the very fact that they are botanical substances present in complex mixtures of plant constituents. The content of active ingredients in such materials has been shown over and over again to fluctuate with the genetic heterogeneity of a plant species, differences in soil conditions, variation in the seasonal cycle, climatic influences, age of the plant, alteration in weather, sun and shade fluctuations and the

like. Hence, standardized extracts arose out of the need to create a uniform product for clinical trials and quality control tool for reproducible drug treatment [16-18].

The term standardization has been a debatable issue when it is viewed from different perspective, such as consumers, industry and government. In the recent guidance published by the American Herbal Products Association, a much broader definition of standardization is delineated. "Standardization refers to the body of information and controls necessary to produce material of reasonable consistency. This is achieved through minimizing the inherent variation of natural product composition through quality assurance practices applied to agricultural and manufacturing practices" [19]. Hence, standardization can serve at least the following purposes: batch-to-batch consistency, confirmation of the correct amount of extracts per dosage units, and positive control to indicate possible loss or degradation during manufacturing.

Broadly speaking, there are two types of standardization approaches. One approach is based either on identifying and concentrating one or more active constituents, making it closer to the level of isolate or characterize all the possible active and inactive components of the plant material to set standard to each of these components. But with the current technology, it is not possible to quantify the hundreds of chemical constituents present in herbal material in a timely and cost efficient manner. The compromise solution to this dilemma is to select a marker compound(s) and then ensure that every batch of product contains the same amounts of the marker compounds.

This approach is based upon the assumption that the content of other constituents will vary in proportion to the marker compound; that if each batch contains the same standardized amount of marker, the content of other constituent will also be relatively consistent [19, 20].

Marker compounds are one or more constituents that occur naturally in the botanical material and that are selected for special attention. They are often chosen arbitrarily, but the following points may be taken as a good guide for selection of markers. Stability of the constituents, technical ease of analysis, amount of time and cost of analysis, utilities in confirming identification of the herbal botanical, potential relevance to therapeutic effect(s), indicator of product quality and stability, etc.

In the light of the WHO/EC definition that the active ingredient is the whole herb or herbal preparation in its entirety, European experts have adopted a new set of terminology that describe and differentiates the various roles of marker compounds very clearly [19]. These terms are:

Active principles: Compounds with known pharmacological activity that are chemically well defined and generally accepted as the major contributors to the therapeutic effect. Only a very few herbs fall into this category where the potency of the product may be assumed to highly correlate with the content of active principles, thereby justifying adjustments in their content.

Active markers: These are pharmacologically relevant, chemically defined constituents that contribute to efficacy, but for which proof that they are alone responsible for clinical efficacy is still lacking.

Analytical markers: In this case, plants do have neither active principles nor active markers identified. Therefore, characteristic compounds or major compounds may be used as analytical markers for which content ranges may be specified.

Negative markers: Are those undesirable constituents such as allergens, toxins, or compounds that interfere with bioavaiolability. Negative markers are used to screen for the presence of toxic botanicals or undesirable botanical varieties or chemical races, as well as unwanted constituents.

Even though there are exemplary standardization attempt on plant extracts, the task, in general, is not as such easy and liable for long debate. By comparison, precise standardization of synthetic drugs is almost child's play. But, there is a general agreement in that if all the necessary requirements are fulfilled starting from the raw material and all the steps in the production of botanicals is standardized, there could be a chance of getting relatively reproducible results. The requirements may include documentation and quality controlling of the botanicals including all the information that may affect the quality and quantity of the active principles in the final formulation. Some of the areas pertaining to quality control of botanicals and considerations with regard to good manufacturing practices are given in subsequent sections.

1.5.3 Quality control of botanicals

Consistent quality for products of herbal origin can only be assured if the starting materials are defined in a rigorous and detailed manner, besides the control tests carried out at an intermediate stage and finished product in the manufacturing process. The World Health Assembly - in resolutions WHA 31.33 (1978), WHA 40.33 (1987) and WHA 42.43 (1989) has emphasized the need to ensure the quality of medicinal plant products by using modern control techniques and

applying suitable standards. WHO has designed a series of tests for assessing the quality of medicinal plant materials primarily for use in developing countries. These quality control parameters and the detailed procedures are discussed in the document [21, 22].

1.5.4 GMP considerations during production of herbal-based products [22]

Unlike conventional pharmaceutical products, which are usually prepared from synthetic materials using reproducible manufacturing techniques and procedures, the production of herbal medicinal products utilizes material of plant origin which may be prone to contamination, deterioration and variation in quality. Furthermore, the manufacture and quality control of herbal medicinal products often utilize procedures and techniques which are substantially different from those used for conventional pharmaceutical products. Therefore, it is worth noting the various aspects to be considered in manufacturing of these products to get reproducible herbal products.

A. Premises

Storage areas: Medicinal plant materials should be stored in separate areas. The storage area should be well ventilated and equipped in such a way as to give protection against the entry of insects or other animals, especially rodents. Effective measure should be taken to limit the spread of animals and microorganisms introduced with the plant material and to prevent cross-contamination.

Containers should be located in such a way as to allow free air circulation. Special attention should be paid to the cleanliness and good maintenance of the storage areas particularly when dust is generated. Storage of plants, extracts, tinctures and other preparation may require special

conditions of humidity, temperature or light protection; these conditions should be provided and monitored.

Production area: To facilitate cleaning and to avoid cross contamination whenever dust is generated, specific provisions should be taken during sampling, weighing, mixing and processing operations of medicinal plants; for example, dust extraction and dedicated premises.

B. Documentation

In addition to the data described in the "Good Manufacturing Practices for Medicinal Products", the specifications for medicinal plant materials should, as far as possible, include: botanical name with reference to the authors, details of the source of the plant (country of origin, and where applicable, cultivation condition, time of harvesting, collection procedures, possible pesticides used etc.), whether the whole plant or only a part is used, description of plant material, macro- and/or microscopical inspection results, suitable identification tests including identification tests for known active ingredients or markers, methods suitable to determine possible pesticides; contaminants and limits accepted, tests for toxic metals and for likely contaminants, foreign materials and adulterants, test for radioactivity, aflatoxins, microbial contamination etc.

Quantitative and qualitative requirements: Either the quantity of plant material should be stated or the quantity of plant material may be given as a range corresponding to a defined quantity of constituents with known therapeutic activity. The composition of any solvent or solvent mixture and the physical state of the extract must be indicated. If any other substance is added during the manufacture of the plant preparation to adjust the preparation to a certain level of constituents

with known therapeutic activity, or for any other purpose, the added substance must be mentioned as "other ingredients" and the genuine extract as the "active ingredient".

Processing instructions: The processing instruction should give the different operation to be performed with the plant material, such as drying, crushing and sifting, and also the temperatures for the drying process, and the methods to be used to control fragments or the particle size. For the production of plant preparations, the instructions should include details containing any vehicle or solvent, time and temperatures to be observed during extraction, details of any concentration methods used.

C. Quality control

Personnel of quality control unit should have particular expertise in herbal medicinal products in order to carryout identification tests, recognition of adulteration, presence of fungal growth, infestations, non-uniformity within a consignment of medicinal plant material etc. Reference samples of plant materials must be available for use in comparative tests.

D. Sampling

Sampling has to be carried out with special care by personnel with particular expertise since medicinal plant materials are composed of individual plants or plants thereof and presents some heterogeneity.

E. **Stability tests**

It will not be sufficient to determine only the stability of the constituents with known therapeutic activity, since plant materials or plant preparations in their crude form are regarded as the active

ingredient. It must also be shown, as far as possible, for example, by chromatography in the finger print region that other substances present are stable and this proportional content remains constant.

1.6 Skin disorders and herbal treatments

As the body's first line of defense, the skin is continuously subjected to potentially harmful environmental agents including solid matters, liquids, gasses, sunlight and microorganisms. Although the skin may become bruised, lacerated, burned or infected, it has remarkable property that allows for a continuous cycle of healing, shedding and cell regeneration. Importantly, skin may demonstrate outwardly what occurs in the body systemically. A number of systemic diseases are manifested by skin disorders, for example, systemic lupus erythematous, several forms of cancer and Kaposi's sarcoma associated with AIDS. Nowadays, skin disease is among the leading causes of hospital visits, and hence a major concern especially in tropical countries including Ethiopia [23-25].

In general, skin disorders could be resulted from mechanical process, infectious process, inflammatory conditions, allergic reactions, parasitic infestations, overexposure or hypersensitivity to sunlight and neoplasm's. Such disorders and their management are topics of review to many literatures [26-33]. Therefore, only a brief discussion of some of skin disorders by these causative agents is given below.

1.6.1 Mechanical processes

Areas of skin that are rubbed repeatedly may result in necrosis of *stratum spinosum*. This can cause blisters, calluses or corns. A blister is a vesicle or fluid-filled papule originated form the repeated friction caused by repeated rubbing on a single area of the skin. Prolonged and repeated rubbing can produce calluses or area of increased skin production (hyperkeratosis). Similarly, corns are small and well-circumscribed conical keratinous skin usually appear on the toes from rubbing or ill-fitting shoes [34]. These kinds of skin disorders may not be regarded as severe conditions of the skin that threaten normal activity of the person except for some aggravated cases.

1.6.2 Infectious process

Worldwide, infectious diseases are number one cause of death accounting for approximately one-half of all deaths in tropical countries**.** Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is the infectious diseases' mortality rates are actually increasing in developed countries, such as United States. Death from infectious diseases, ranked 5th in 1981, became the third leading cause of death in 1996, with an increase of 58% [35]. The trend is even increasing since that time at alarming rate even though once it was believed that it would be possible to eliminate infectious diseases by the end of the millennium. The increase was attributed to the aggravation in respiratory tract infections and HIV/AIDS. Skin is amongst the parts of the body threatened significantly especially because of the HIV/AIDS pandemic [36, 37].

In general, the skin is subject to a very wide range of lesions; some may be characteristic of specific systemic diseases and fade as the disease regress and others are caused by specific local

infection. As it is the first line of defense, skin is exposed to attack by a number of microorganisms and depending on the virulence of the itching agent and competence of the host's resistance, infection may result. The causative agents could be fungus, bacteria or virus or a combination thereof and the type of infection is named after the causative agent. These infections could either be superficial or deep types. The superficial fungal infections are also termed dermatophytes; they are commonly known as tinea or ringworm. The most common fungal skin infections are the dermatophytoses, pytriasis, versicolor and candidiasis and other less common may include blastomycosis, chromoblastomycosis, mycetoma, mucoromycosis, protothecosis, etc. Fungal infections may sometimes affect the mucous membrane, nails or subcutaneous tissues and can spread to dipper tissues and disseminate especially in immunocompromised patients [34].

Viruses can also invade the skin and cause infection. Basically, viruses are intracellular pathogens with no organized cell structure but consist of either a DNA or RNA core surrounded by a protein coat. They rely completely on live cells for reproduction. By and large, the viruses seen in the skin lesion disorders tend to be DNA-containing viruses. The rapid increase in viral skin disease has been attributed to the use of birth control medication and corticosteroid drugs, which have immunosuppressive properties, and the use of antibiotics, which alter the bacterial flora of the skin [34]. Common viral infection of the skin includes veruceae, herpes simplex and herpes zoster.

1.6.3 Inflammatory processes

Most inflammatory skin diseases are of unknown origin or etiology. They are usually localized to the skin and are rarely associated with specific internal diseases. They produce marked variations in normal skin, usually papulosquamous in nature. Inflammation and erythema are common early manifestations of HIV infection [38]. Such disorders as acne, lichen planus, psoriasis and pityriasis rosea are among the most common one.

1.6.4 Other Skin disorders

These may include allergic skin responses [39], drug-induced skin eruptions [40, 41], insect bites, vectors, ticks and parasites and photosensitivity and sunburn [42].

1.6.5 Herbal treatment of skin disorders

Herbal treatment of infectious skin disease is quite common in different areas of the world. Many literatures reviewed the commonly applied herbs for treating various skin diseases [43-45]. Some of the secondary metabolites from such plants are discussed in section 1.7

1.7 Plant-derived antimicrobial compounds

Substances involved in essential metabolic processes within an organism are identified as “primary metabolites”. For example lipids, porphyrins, amino acids, polyacids (e.g., citric, tartaric, and the like) and many others are among this class. The term “secondary metabolite” is frequently found in literature that addresses the chemistry of plant-derived natural products. The term has no real scientific basis except that over the past half century it has come to represent those products of plant metabolism that are associated with some readily detectable properties

(e.g., taste, color, odor), a biological activity (e.g., toxicity, medicinal or agrochemical use) or simply novel chemical structure [46].

The secondary metabolites are unique and specific variations of the primary products' metabolism. They are derivatives produced by various enzymatic processes of limited and often specific distribution that generate chemical compounds distinct from the primary metabolic products. Discovery scientists in the chemical, pharmaceutical or agrochemical industries have little interest in primary metabolites. In fact, commercial applications, except the obvious uses in nutrition and dietary supplements, are few [46]. Hence, interest is, by and large, given to secondary metabolites such as alkaloids, flavonoids, coumarins, etc. Most secondary metabolites possess antimicrobial effect and some of these are successful in getting access into the arsenal of antibiotic therapy. Representative examples of groups of compounds from plant having antimicrobial activity are given below.

1.7.1 Alkaloids

Alkaloids constitute a major class of chemical group present in plant drugs. Originally, it means "alkali like" which was applied indiscriminately to all the organic bases. Alkaloids may be described as naturally occurring organic substances, having a cyclic nitrogenous nucleus exhibiting basic properties and having a pronounced physiological action [47]. In the plant kingdom, the alkaloids appear to have a restricted distribution in certain families and genera. Among the angiosperms, the *Apocynaceae, Papaveraceae, Ranunclaceae, Rubiaceae, Solanaceae* and *Berberidaceae* are outstanding as alkaloid yielding families [48]. Alkaloids are found to have a range of pharmacological activities such as antimalarial, anti-tumor, relaxing heart and respiratory muscle, anesthetic, analgesic etc.

There are significantly many works done on alkaloids as to their antimicrobial activity. Beriberine, a constituent of many plants is a prototype in the group and has been tested against many strains of microorganisms. This alkaloid has a long history of medicinal use in both Ayurvedic and Chinese medicine. It is present in *Hydrastis canadensis* (goldenseal), *Captis chinensis* (Coptis or golden thread), *Berberis aquifolium*, (Oregon grape), *Berberis vulgaris* (Berbery) and *Berberis aristata* (Tree turmeric) [49]. Currently, the predominant clinical uses of beriberine include bacterial diarrhea, intestinal parasite infections and ocular trachoma infections [49, 50].

Plant antimicrobials are not used as systemic antibiotics at present. The reported MICs are often in the range of 100-1000μg/ml and this is higher than those of common broad-spectrum antibiotics from bacteria or fungi. Gram-negative bacteria have an effective permeability barrier; one of the reasons could be the presence of multi-drug resistance (MDR) pumps that extrude toxins across this barrier. In a study to see the permeability barrier of the cell wall, beriberine was taken as a model compound together with other plant antimicrobials against representative human pathogens: *Pseudomonas aerogenosa, Escherichia coli,* and *Salmonella enterica serovar Typhimurium* [51].

MDR pumps readily extrude beriberine alkaloids, which are cationic antimicrobials. To inhibit the pumping out of this agent the naturally occurring pump inhibitor synthesized by many plants, 5'-methoxyhydrocarpin, is used. Therefore, direct measurement of the uptake of the model drug confirmed that, disabling of the MDRs strongly increases the level of penetration of beriberine into the cells of gram-negative bacteria. In another study, the activity of rhein, the principal antimicrobial from rhubarb, was potentiated 100-2000 fold (depending on the bacterial species).

Other compounds that showed comparable potentiation include plumbagin, resveratol, gossypol, and coumestrol. The finding has implication in that plant antimicrobials might be developed into effective and broad-spectrum antibiotics in combination with inhibitors of MDRs [51, 52].

Beriberine was also derivatized to enhance its antimicrobial activity. A series of compounds bearing 9-O-acyl- and 9-O-alkyl- substituents were synthesized and tested against gram-negative, gram-positive bacteria and fungi. Octanoyl, decanoyl, lauroyl derivatives among the acyl analogs and hexyl, heptyl, octyl, nonyl, decyl, undecyl derivatives among the alkyl analogs showed strong antimicrobial activity against Gram-positive bacteria and fungi [53].

An attempt to enhance lipophilicity, consequently antimicrobial activity, was done by increasing the amphipatic chain length at C-13 side-chain and different modifications of the aromatic rings. 13-Hexylberiberine and 13-Hexylpalmitine exhibited the greatest antimicrobial activity [54-56]. Screening of plant alkaloids for their antimicrobial effect is quite common in the literature but representative samples of alkaloids that are found to have activity are listed in Table 1.1.

Table1.1: List of exemplary plant alkaloids found to have antimicrobial effect

Plant source	Parts	Antimicrobial alkaloid	Test organisms	Ref.
Polyalthia longifolia	Root	Pendulamin A and B	__	57
Erythrina latissima	Seed pods	Erysotrine (+)-10,11-dioxoerysotrine	G^+ & G^- bacteria, yeasst, spores	58
Guatheria multivenia	Root	Liriodenine	*C. albicans, C. neoformans*, *S. aureus*	59
Schizozygia coffaeoides	__	Isoschizagaline	__	60
Zanthoxylum tetraspermum	Stem bark	Acetonyldihydronitidine 8-acetonyldihydroavicine	Antibacterial	61
Pergularia pallida	__	Pergularinine Tylophorinidine	__	62
Murraya koenigii	Fresh leaves	Mahinimbine Murrayanol Mahinine	__	63
Bocconia alborea	__	Dihydrocholerythrine Dihydrosanguinarine	G^+ & G^- bacteria and *C. albicans*	64
Alangium lamarckii	__	*Deoxytubulosine*	__	65
Mitracarpus scaber	Arial part	Benz-[g]-isoquinoline-5,10-dione	AIDS related pathogens	66
Cryptolepis sanguinolenta	__	*Cryptolepine*	*Saccharomyces cerevisiae* *E. coli , C. albicans*	67
Clausena heptaphylla	Leaves	Clausenal	G^+ & G^- bacteria	68

1.7.2 Flavonoids

Flavonoids are hydroxylated phenolic substances occur as a C_6-C_3 unit linked to an aromatic ring [69]. Flavones are also phenolic structures containing one carbonyl group on the aromatic ring and the addition of a 3-hydroxyl group to it yields a flavonol. Many of compounds in these groups have low toxicity in mammals and some of them are widely employed in medicine for various activities such as ateriosclerosis [70, 71], antiulcerogenic activity and hepatoprotective [72-75]. Flavonoids were also demonstrated to have antioxidant activity and because of which they exhibit several beneficial effects, such as anti-inflammatory, antiallergic, antiviral as well as anticancer activities [76-80].

Some flavonoids are known to be synthesized by plants in response to microbial infection. Therefore, it should not be surprising that they have effective antimicrobial activity against a wide array of microorganisms [81]. This activity is probably due to their ability to complex with intracellular soluble proteins and also with bacterial cellwall. Pertaining to the degree of hydroxylation, flavonoids lacking hydroxyl groups on their β-rings are more active against microorganisms than are those with the -OH groups; hence lipophilic compounds would be more disruptive [82]. However, several authors have also found the opposite effect; i.e. the more hydroxylation, the greater the antimicrobial activity [83]. Therefore, it is safe to say that there is no clear predictability for the degree of hydroxylation and toxicity to microorganisms.

Flavonoids also displayed antiviral activity, including anti-HIV and *Herpes simplex* virus type-1 [84]. Recently, a natural plant flavonoid polymer with molecular weight 2100 Da was found to have antiviral activity against two strains of type-1 and type–2 *H. simplex* viruses [85]. In another

experiment, out of twenty-eight flavonoids tested, flavan-3-ol was more effective than flavones and flavonones in selective inhibition of HIV-1, HIV-2 and similar immunodeficiency viral infections [86]. Some of the flavonoids that are active against different organisms are given in Table 1.2.

Table 1.2: Antibacterial, antifungal and antiviral activity of various flavonoids [87].

Flavonoids	Organisms tested
Quercetin	*Staphylococcus aureus, Escherichia coli, Candida tropicalis, Rabies virus, Herpes simplex virus type 1, Respiratory syncytial virus infection*
Naringin	*Staphylococcus aureus, Shigella boydii, Pseudomonas aeroginosa, Respiratory syncytial virus infection,*
Apigenin	*Streptococcus pyogens, Streptococcus viridans, Alternacia tennisima, Immune deficiency viral infection*
Rutin	*Staphylococcus aureus, Shigella boydii, Pseudomonas aeroginosa, Bacilus anthracis, Rabies virus, Parainfluenza virus*
Hespertin	*Staphylococcus aureus, Shigella boydii,*
Baicalin	*Staphylococcus aureus, Pseudomonas aeroginosa*
Chrysin	*Streptococcus jaccalis, Streptococcus baris, Streptococcus pneumoniae,*
Fisetin	*Staphylococcus aureus, Staphylococcus albus,*
Quercetogetin	*Staphylococcus aureus,*
Hydroxyethylrutinose	*Pseudomonas aeroginosa, Clostridium purfringens*
Chloroflavonin	*Candida albicans,*
Datiseten	*Proteus vulgaris*
Echinaelin	*Alternacia tennisima*
Quercetrin	*Rabies virus*

1.7.3 Coumarins

Coumarins are another class of phenolic compounds that owe their class name from “coumarou”, the vernacular name of the tonka bean (*Dipteryx odorata Wild*, Fabaceae), from which coumarine itself was isolated in 1820 [88]. Coumarins belong to a group of compounds known as benzopyrones. This group of compounds arises from the metabolism of phenylalanine via a cinnamic acid or *p*-coumaric acid synthesis and the primary site of synthesis is suggested to be the young actively growing leaves with stems and roots playing a comparatively minor role [89].

The distribution of biologically active coumarins in a wide range of plants seems to correlate with their ability to act as phytoalexins. They accumulate on the surface of the plant parts, in response to traumatic injury during the wilting process, and inhibit the growth and sporulation of fungal plant pathogens [90]. The substituents on the ester or carboxylic acid functional group on the coumarine ring give it a potent inhibitory activity against both Gram-positive and Gram-negative bacteria [91].

There are reports on the efficacies of pure coumarins and their analogs against Gram-positive, Gram-negative bacteria and fungi. Scopletin, for instance, is obtained from *Agyreia aspeciosa* roots, was found to be highly potent against *Alternaria alternate* [92]. Aflatoxin products and fungal growth were also found to be inhibited by *o*-coumaric acid [93]. Interestingly, coumarins such as calanolide A, isolated from C*alophyllum langirum var. austrocoriaceum* and a related coumarin, costatolide from the latex of *C. teysmanii var. inophillide* have also inhibitory effect on DNA-gyrase, which may be linked to the anti-HIV activity [90, 94]. Table 1.3 lists some of the coumarins that are tested for antimicrobial activity.

Table 1.3: Examples of coumaric compounds reported to have antimicrobial properties

Coumarin	Microorganisms	Ref
Psoralen	*Staphylococcus aureaus, Candida* species	95
6,8-dihydroxy-5,7-dimethoxy coumarine	*Streptococcus pneumoniae, Staphylococcus aureaus*	96
Herniarin	*Acaligenes faccalis, Aspergillus* species, *Candida* species	97, 98
Scopletin	*Staphylococcus aureaus, Klebsella. Pneumoniae, Proteus mirabilis, Pseudomonas aeroginosa, Alternaria alternate, Penicillium chrysogenum*	90, 94, 99
Umckalin	*Staphylococcus aureaus, Streptococcus pneumoniae*	96
5,6,7-trimethoxy Coumarine	*Streptococcus pneumoniae, Hemophylus enfloenza*	96
Agelicin	*Aspergillus* species, Candida species, *Cryptococcus pneoformans, Saccharomyces* species	98, 99
5,8-di(2,3-dihydroxy-3-methyl butoxy)-psoralen	*Aspergillus* species	98
Byankangelicin	*Cladosporium* species	98
Oxypeucedanin	*Cladosporium* species	100

1.7.4 Tannins

Tannins, commonly referred to as tannic acid are water-soluble polyphenols that are widely distributed in plants and occur in cell sap often in the distinct vacuoles. Tannins have received a great attention in recent years, since they are shown to occur in many plants that are food to humans, which have got many effects on human health. They have been reported to be responsible for decrease in feed intake, growth rate, feed efficiency, net metabolizable energy, and protein digestibility in experimental animals. Therefore foods rich in tannins are considered to be of low nutritional value [102].

The antimicrobial activity of tannins is reported here and there in the literature for a number of plants. For example, comparison in the antimicrobial activity of plant extracts from *Syzygium jambor* (L.) Alston, *Hamamalis virginiana*, *Krametia triandra, Alchemilla vulgaris and Rubus frutilosus* have correlated with their tannin content. Elimination of tannins in these plants totally suppressed the antimicrobial activities [103]. These compounds were found to form complex with proteins through the so-called non-specific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. Thus, the mode of antimicrobial action of many types of tannin may be related to their ability to inactivate microbial adhesions, enzymes, and cell envelope transport proteins.

1.7.5 Terpenoids

Terpenoids are widely distributed in nature and are found in abundance in higher plants, fungi, marine organisms and insect pheromones as well as in insect defense secretions. The antimicrobial activity of terpenoids is reported for many plant products. A comparable activity to that of cephatoxim against Gram-positive bacteria was observed from diterpenes obtained from

oleoresin of *Copaifera paupera,* (MIC <10 µg/ml) [104]. Similar activities have been found with terpenoids of different plant sources [105-113].

The mechanism of action of terpenoids is not fully understood but speculated to involve membrane disruption by the lipophillic compounds. Accordingly, increasing the hydrophillicity of kaurene diterpenoids by addition of methyl group drastically reduced the antimicrobial activity [114].

The volatile oils (essential oils) are very complex mixture of compounds whose constituents of the oils are mainly monoterpenes and sesquiterpenes. Other compounds include phenylpropenes and specific compounds containing sulfur and nitrogen. Basil (*Osimum basilicum* L.) is a popular culinary herb and its essential oils have been used extensively for many years in food products, perfumery, dental and oral products. Its essential oils and principal constituents were found to exhibit antimicrobial activity against a wide range of Gram-negative and Gram-positive bacteria, yeast and mold. Exhaustive review with regards to its chemical composition, its effect on microorganisms, and possible future use in food preservation or as slow release component of an active package is available elsewhere [115]. Likewise, the antimicrobial activities of many essential oil components of different plant species have been documented [116-118].

Generally, the action of essential oils is the result of the combined effect of both their active and inactive compounds. These inactive compounds might influence resorption, rate of reactions and bioavailability of the active compounds. To add to the complexity of volatile oils, there is evidence that the time of harvest influences the oil composition and consequently the potency of their biological effect including its antimicrobial activity [119-123].

1.7.6 Peptides

Antimicrobial peptides are found in all kingdoms of life, ranging from plants through insects to animals [124-126]. These peptides are termed antimicrobial because they display an unusually broad activity spectrum. The activities may include an ability to kill or neutralize not only Gram-negative and Gram-positive bacteria but also fungi, parasites, cancer cells, and even enveloped viruses like HIV and herpes simplex virus. Moreover, most of the antimicrobial peptides we know today are quite selective for microbes over eukaryotic cells [126].

The endogenous antimicrobial peptides of plants and animals are typically cationic amphipathic molecules consisting from 12 to well over 100 residues. This amphipathic structural arrangement is believed to play a key role in the antimicrobial mechanism of action. The hydrophilic and strongly positively charged domain is proposed to initiate peptide interaction with the negatively charged bacterial surface. Many cationic antimicrobial peptides have been described as membrane active agents [126]. However, the question is still open whether membrane disintegration is the only mechanism by which these peptides kill bacteria or the peptides have various intracellular targets, or both mechanisms play roles in the killing process.

One of such antimicrobial from plants is Zeamatin. It is a large peptide of 22 KDa produced by *Zea maize* and is active against *C. albicans* with a minimum inhibitory concentration of 0.5 μg/ml. This peptide permeabilizes fungal memebrane to cause death [127]. Others could be amphibine H, frangufoline, nummularine, and rugosanine, which are active at the level as low as 5 μg/ml [128, 129].

The abovementioned phytochemicals are not the complete list of all the secondary metabolites found to have activity on microorganisms. It is just a review of the most frequently reported plant-derived compounds.

1.8 Common herbal-based dermatological dosage forms

Dermatological formulations applicable for topical treatment are quite various in kind. Most of those dosage forms prepared from herbal drugs are common with that of Western medicine. Hence, only a quick mention of some of these dosage forms is attempted but the detail could be referred somewhere else [130, 131].

Liquid preparations: liquid preparations for external application to the skin include: simple soaks or baths, applications, liniments, lotions, paints, tinctures, etc.

Semisolid preparations: these are the most common type of dermatological formulations applicable for many herbal products. The most commonly used ones are jells, ointments, creams and pastes

Solid preparations: preparations such as powders and aerosols are also common in topical dosage forms**.** Dusting powders are formulated for application to skin folds by mixing together several finely divided insoluble powders.

1.9 Experimental approach to study the *in vitro* antimicrobial and anti-inflammatory activities

1.9.1 *In vitro* antimicrobial susceptibility testing (AST)

AST was initiated in many countries throughout the world soon after the introduction of antimicrobials for treatment of bacterial diseases. Rapid bacterial identification systems and subsequent improvements in AST were primarily driven by the need to identify the appropriate antimicrobials for successful clinical use. Additionally, the need for laboratory reproducibility of AST methods arose to ensure that data generated was technically accurate and consistent. There are several variations in methodologies, techniques, and interpretive criteria currently being used. The selection of an AST methodology may be based on numerous factors such as ease of performance, flexibility, adaptability to automation or semi-automated systems, cost, reproducibility, reliability, accuracy, and national preference. The most common among the various methods are the agar diffusion assay (e.g. disc diffusion and agar well diffusion) and dilution assay (e.g. broth dilution and agar dilution) [132].

1.9.2 *In vivo* anti-inflammatory screening methods

The complexity of the inflammatory process and the diversity of the drugs that have been found effective in modifying this process have resulted in development of numerous methods of assay for detecting anti-inflammatory substances. The *in vivo* assay techniques include: UV-erythema in guinea pigs [133,134], vascular permeability [134], oxazolone-induced ear edema in mice [135], croton-oil ear edema in rats and mice [136,137], paw edema in rats [138-140], pleurisy tests [141,142], and graneuloma pouch techniques [142]. A few of these methods have been

systematically evaluated for their potential usefulness in screening programs. The most common model among the list has been the prevention of carrageenan-induced edema in rats.

Carrageenan-induced rat paw edema: Carrageenan-induced edema is a model for acute inflammation testing and is believed to have biphasic nature. The first phase is due to release of histamine and serotonin; the second phase is caused by the release of bradykinin, protease, prostaglandin and lysosome. The experiment involves preparing 1% solution of carrageenan from which 0.1ml of the solution is injected into the right hind paw of the rats. The test plant extract at different concentrations and control vehicle are injected either intraperitonealy or administered orally, commonly 30min before the injection of carrageenan solution. The paw volume is measured by volume displacement method just before administration and at different intervals after injection. The resulting data would then be interpreted in different ways [138-140].

1.9.3 Testing the *in vitro* performance of topical antimicrobial formulations

Laboratory tests are available for selecting useful antibiotics for combating systemic infections. The FDA recommended the Kirby-Bauer technique as the standardized disk sensitivity procedure for the determination of antimicrobial susceptibility. In general, there is also, nowadays, an FDA recommended *in vitro* testing methods for dermatological preparation to characterize the performance of a finished dosage forms. The method employs a static diffusion cell system, commonly referred to as the Franz cell, commercially available polyselfone synthetic membrane and an aqueous receptor phase [143].

To determine the *in vitro* drug release, an infinite amount of drug is applied on the donor chamber. Aliquots of the receptor media are removed at 30, 60, 120, 240, and 360 minutes

interval, analyzed and the cumulative amount of drug released is plotted against square root of time. Hence, straight-line relation will be obtained as long as the release is less than 30% of the drug applied. The release rate and slope are obtained by regression analysis. However, standard sensitivity tests are not available for topical antimicrobial formulations. The criteria for drug release from a topical antimicrobial formulation differ from those for other topical products in that penetration through the *stratum corneum* is not necessarily the major consideration. Hence, as they are only for local action, measurement of drug release into an appropriate aqueous medium at physiological pH may be a more realistic *in vitro* model than release and penetration through a membrane system representing the *stratum corneum* [143, 144].

Different methods are proposed as a model for comparing the *in vitro* efficacy of topical products. Some of these methods are discussed here [144-146].

(I) Agar-well diffusion tests

This is essentially a modification of agar cup diffusion tests, which involves formation of evenly distributed holes by removing the plugs with a sterile cork borer. By means of a tarred syringe, a weighed amount of a designated topical agent is added to each well so that the product does not have any contact with the agar. Using a portion of melted media that was maintained at 50^0c over a water bath, a known number of microorganisms is inoculated to form suspension. The suspension is mixed using mechanical agitator and poured on to the previously prepared test plate containing the antimicrobial agents. After solidification, the plate is inverted and incubated and the zone of growth inhibition is recorded [143-146]. General acceptance of this method has been limited by its complexity. Performance of this technique is a time-consuming procedure, about 25

minutes, even for experienced microbiologic technicians. This has led people work in the area to have a modification of this method.

(II) Disk diffusion test

This test procedure has been patterned after the Kirby-Bauer tests. It undergoes similar procedure except that topical antimicrobial agent is loaded on the disk after positioning the filter paper on the swabbed media [144].

(III) Needle extrusion test

This technique is also similar to disk diffusion technique in that it involves similar procedure in agar preparation and bacterial inoculation. But the needle extrusion test differs in the method of application of the antimicrobial cream to the inoculated agar. The topical agent is applied directly to the agar surface by extrusion from a syringe in a straight cylinder measuring 2-3cm in length. The plate is inoculated for certain period of time without inversion and zones of inhibition are recorded [144].

1.10 Literature review on plants selected for this study

1.10.1 *Plantago lanceolata* (Plantagonaceae)

This is an adaptable perennial weed, indigenous to Britain but now naturalized all over the world. It is one of the seven sacred herbs of the druids, sometimes called White Man's Footsteps because it has followed the early settlers wherever they went. This plant is locally termed as "gurteb" and has got application for many skin disorders in Ethiopian traditional medicine. *P. lanceolata* was found to have 2-6% mucilage composed of polysaccharides; 6.5% tannins;

irridoid glycosides, including 0.3-2.5% aucubin and 0.3-1.1% catalpol; over 1% salicylic acid; phenolic carboxylic acids (protocatechuic acid); flavonoids (apigenin, luteolin); enzymes (rennin, invertin, emulsin); ascorbic acid; zinc; silica; citric and oxalic acids; allantoin etc. as constituents [147, 148].

The tannins are astringent, antiseptic and mucolytic and they tonify mucous membranes of the digestion, lung and kidney. *In vitro* bacteriostatic and bactericidal activity have been shown for the cold aqueous extract and attributed to the aglycone, aucubigenin. Experimental research using isolates of *P. lanceolata* has shown an inhibitory effect on mouse ear edema. Furthermore**,** the mucilage has soothing, expectorant, cooling and demulcent, and diuretic effects. Allantoin is a cell proliferant and speeds up tissue healing besides the effects of mucilage, vitamin C and zinc. In a laboratory tests, extracts of this plant reduced plasma lipid, cholesterol, β-lipoprotein, and triglyceride concentration in rabbits with arteriosclerosis. The iridoid glycoside, aucubin has stimulant action in mice, and has also demonstrated protective effects on liver cells [148].

It is used in different forms for different activities. As tincture or tea it is used for sinus congestion, allergies, lung congestion, colitis, excess of production of mucus, diarrhea and dysentery, cystitis, nephritis and other infections. Topical application include as poultice, compress, wash and soak for wounds skin infections, spots, bites and stings [148].

1.10.2 *Maesa lanceolata* Forssk (Myrsinaceae)

M. lanceolata is a tree or shrub 2-20 m tall that grows around margin of evergreen forest, along riverbanks and streams, in open woodland and valleys with altitude ranging from 1350 – 3000 m

throughout tropical Africa extending to South Africa, Madagascar and Arabia. The seeds are used as vermifuge and oil extracted from the seeds is used for greasing the baking plate of Injera and Bread.

Phytochemical screening of various species of the genus resulted in the isolation of different terpenoid saponions. *M. balansae*, for example, was found to have six related triterpenoid saponins which were active against leishmaniasis [149]. Maetenosides, [150], maelexins [151], and maejaposides [152], are new triterpenoid saponins obtained from *M. tenra, M. laxiflora,*and *M. japonica*, respectively. Ten new acylated triterpenoid saponins were also isolated from the leaves of *M. lanceolata*. All saponins identified contained the same tetraglycosidic side-chain but the triterpenoid moiety showed a variable esterification pattern [153,154]. Benzoquinone derivatives such as 2,5-dihydroxy-3- (nonade- 14- enyl)- benzoquinone and lanciaquinone and novel phenolic compounds such as maesol were also isolated from the genus *Maesa* [155,156].

There are many reports in the literature pertaining to the biological activities of *M. lanceolata*. The natural and semisynthetic analogs of substituted 1,4-benzoquinones were evaluated for *in vivo* cytotoxic and antioxidant activities. Maesanin, dihydromaesanin, maesanin di-ethyl ether and isomeric mixtures of benzoquinones exhibited cytotoxic activities against HL-60 cell line while they were inactive against reactive oxygen species generation. In contrast, the isomeric acylated benzoquinones with shorter alkyl substituents showed most prominent antioxidant and antiproliferative effect on the same cells [157].

The methanolic extract of the Rwandan *M. lanceolata* has shown virucidal activity against herpes simplex type-1 and type-2, and vesicular stomatitis viruses. But it was not active against Gram-

negative bacteria and *C. albicans.* From the bioassay-guided fractionation procedure using herpes simplex type-1 virus as a target model, maesasaponin mixture A was isolated from the extract as an active principle [158]. Similarly, from a bioassay-guided fractionation procedure in another experiment using the same virus as a target model, a virucidal saponin mixture, maesasaponin mixture B was isolated from the methanolic extract of the plant. The mixture consisted of six homologous oleanane-type triterpenoid saponins. Besides the virucidal activity, it has also shown molluscicidal, hemolytic, fungistatic and antimutagenic properties [159-163]. Maesanin isolated from *M. lanceolata* was also found to have inhibitory effect on arachidonic acid metabolizing enzyme, 5-lipoxygenase, hence, it could also have an anti-inflammatory effect [164]. The glycosids of *M. chisia* have been demonstrated to possess comparable anti-inflammatory, analgesic and antipyretic activities to that of NSAIDs [165].

1.10.3 *Alchemilla pedata* A. Rich (Rosaceae.)

This is somniferous herb that belongs to the family Rosaceae. It is commonly named by its vernacular name "enchetkitab" and locally used for the treatment of various skin diseases. It grows on moist ground in grazed montane grasslands at altitude ranging from 1800-4000m. Phytochemical screening on the genus *Alchemilla* revealed that there are flavonoids (e.g. quercetin-3-arabinopyranoside) [166-168], tannins [167, 169], polyphenols [170], coumarins [171] etc. Biological activities of some species of the same genus have been determined. For example, *A. vulgaris* is traditionally used for its anxiolytic activity and this effect has been demonstrated by bioassay technique in a controlled experiment [172]. The antibacterial activity of this plant was related with its tannin content [169]. *A. speciosa* was shown to contain a flavonoid quercetin, but its mutagenic property was not comparable to its flavonoid content. The

reduced mutagenic activity was ascribed to the effect of coumarine, called esculetin in the plant [171].

1.10.4 *Cadaba farinosa* (Capparidaceae)

It is a shrub 1-2 m high with many branches bearing numerous small grayish green leaves. Fruits are small, elliptically shaped (spear-shaped at both ends, base and apex, with widest part in the middle) and orange in color. It grows in semiarid and arid areas in the desert grass-bush zone. In Konso, south-west Ethiopia, farmers call it with a nickname “Luqata Sigmama” ('herd's boy fruit'), because herd boys are enjoying the fruits in the bush while they are herding animals. Cattle and goats eat the leaves and young branches throughout the year, although not excessively. During the dry season it is often the only source of green palatable roughage. The infusions of roots have medicinal value against animal diseases (e.g. trypanosomiasis) when mixed with other plants such as *Moringa oleifera*. Phytochemical screening of the stem bark of this plant has resulted in the isolation of constituents such as cadabicilone, cadabicine and cadabicine diacetate as well as other alkaloids and flavonoids [173].

1.10.5 *Osyris quadripartita* Decn. (Santalaceae)

A dioecious tree or shrub, 1-7 m tall with many branches. It grows on rocky slopes and degraded woodland and scrub with altitude 1600 – 2900 m. It grows throughout Africa, southern Asia to china. The bark of this plant has been used for tanning.

1.10.6 *Steganotaenia araliacea* Hochst. ExA. Rich (Apiaceae)

S. araliacea is a small glabrous, deciduous tree, 3-5 m tall with gray bark and ascending branches. It grows on an open woodland and river banks of tropical Africa such as Cameroon, east of Somalia and south west of Africa with altitude 1300 – 2100 m. There are no activity and phytochemical screening report found on *O. quadripartita* and *S. araliacea*.

The above plants were selected on the bases of the information obtained from healers. In Ethiopian traditional medicine, they are widely employed for topical treatment of skin diseases. Therefore, the present works mainly focuses on evaluation of the antimicrobial and anti-inflammatory activities of these plants and prepare reasonable antimicrobial formulations that may be utilized upon further improvement.

Two reasons could be forwarded pertaining to the current interest in the screening of antimicrobial plant extracts for topical application. First, it is very likely that these phytochemicals will find their way into the arsenal of prescribed drugs. This is because of the realization that the effective life span of any antibiotic is becoming more limited. Second, the public is becoming increasingly aware and interested in having more autonomy over their medical care, especially topically seated diseases. A multitude of plant compounds (often unrealistic purity) is readily available over-the-counter from herbal suppliers and natural food-stores, and self-medication with these substances is commonplace. Furthermore, the rapid rate of plant species extinction has made different centers to give attention to utilize the impressive array of knowledge assembled by indigenous people about the plant products to maintain health. Therefore this by itself urges to rationalize the claimed activities and hence, this work is designed to at least pave a way for further work on those plants.

1.11 Objectives of the work

General objective:

The objective of this work is to screen the antimicrobial and anti-inflammatory activities, and undertaking standardization and formulation work on selected traditionally used plants in Ethiopia for various skin disorders.

Specific objectives are:

- To prepare total extracts of the selected traditionally used medicinal plants;
- To screen the antibacterial, antifungal and anti-inflammatory activities of total extracts of the plants;
- To perform fractionation of the constituents by successive extraction with the aim of localizing activities;
- To attempt a preliminary phytochemical screening on the total extracts of selected plants;
- To determine the MICs of the extracts;
- To conduct preliminary semi-quantitative standardization work and
- To formulate the total extracts in a desirable dosage forms and testing for efficiency of the formulations.

2 EXPERIMENTAL

2.1 Materials and test organisms

Chemicals and solvents

The following chemicals and solvents were purchased and used as received. Methanol (LOBA CHMIE Pvt. Ltd., India); Ethanol absolute (Avondale Laboratories Limited Banbury, England); Acetone (Fisher Scientific International Company, United Kingdom); Chloroform (E. Merk, Stockholm); Ethyl acetate, Petroleum ether (80 ^{0}C to 100^{0}C), Sulphuric acid, Glyceryl monostearate, Cetostearyl alcohol and Polyethylene glycol 4000 (BDH Chemicals Ltd., England); Sodium lauryl sulphate (AVONCHEM Ltd., UK); Liquid Paraffin BP (Germany); Cetomacrogol 1000 BPC (The British Drug House, Ltd., London); Petrolatum white, USP (Ethiopian Pharmaceutical Manufacturing, Addis Ababa, Batch No. 101087-1); Polyethylene glycol 400 and Polyethylene glycol 600 (Sigma-Aldrich Chemie GMbH, Germany); Vanillin, Carrageenan Lambda (SIGMA CHEMICAL CO., USA), Ferric chloride (Fischer Scientific Co., Fairview, New Jersey, USA), Hydrochloric acid (Farmitalia Carlo Erba, Milan, Italy), Potassium ferrocyanide (Matheson Coleman & Bell Division, Norwood, Ohio, USA), Lead acetate (Riedel-De Haen AG, Sec Lze, Hannover, Germany) and Ferric sulphate (Prolabo, Rhone-Poulene).

Commercial antimicrobial formulations

The following topical formulations were purchased from drug retail outlets in Addis Ababa and used as positive controls during antimicrobial testing.

Antibacterials

Foban ointment (2% Sodium fusidate, HOE Pharmaceuticals, Lot No. 01021015, Malaysia), Bactroban ointment (2%mupirocin, SmithKline Beecham Pharmaceuticals, Batch No 2352/040818, England), Tetracycline hydrochloride ointment, USP (3% tetracycline, Ambalai

Sarabhai Enterprise Ltd., Batch No 02JUL1, India), Foban cream (2% fusidic acid, HOE Pharmaceuticals, Lot No 649A0005, Malaysia), Sagestan topical cream (0.1% gentamycin PT SANBE FARMA, Batch No BA355, Indonesia).

Antifungals

Cansten® cream (1%bis-phenyl-(2-chlorophenyl)-1-imidazolyl-methane) [Bayer, Germany], Kenazol cream (2% ketoconazol) [Domina pharmaceyticals, Syria], Ketoral® cream (2% ketoconazol) [Beker Pharmaceuticals & Medical Supplies, Turkey, Lot No. 0202003], Denzor cream (2% Ketoconazol and 0.1% Chlorocresol) [HOE Pharmaceuticals Sdn. Bhd., Malayasia, Lot No. 51282015], Nizoral® cream (2% Ketoconazol) [Janssen Pharmaceuticals (Pty) Ltd., South Africa, Lot No. 430BA], Sha Hsien Thin cream (1% Clotrimazol) [Shanghai Medicines and Health Products Import and Export Corporation, China, Batch No. JW02C01], Clotri-denk® cream (1% Clotrimazol and 1% Benzyl alcohol) [Denk Pharma, Germany, Batch No. 11880], Fungoral® cream (2% Ketoconazol) [Ilsan ilac Fabrikasi, Turkey, Batch No. 9011610].

Test specimens and animal models

Microorganisms namely; *Staphylococcus aureus* (ATCC 6538), *Escherchia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (clinical isolate), *Aspergillus niger* (ATCC 10535), *Trichophyton mentagrophytes* (ATCC 18748) were used as a test organisms in the antimicrobial screening. Albino rats (generously supplied from faculty of medicine) were also utilized in the anti-inflammatory testing.

Media and standard drugs for the antimicrobial screening

Nutrient agar (DEFCO Laboratories, USA), Peptone bacteriological (BDH Chemicals Ltd., England), Sabauraud dextrose agar and Tryptone Soya Broth (Oxoid Ltd., England), yeast Extract (UNIPATH Ltd., England), Sodium chloride (East Anglia Chemicals, United Kingdom), Ketoconazole (Medreich/India, working standard No: SKETO 021) and Gentamycin sulphate (Working standard kindly supplied by Drug Adminstration and Control Authority, DACA) were employed in the antimicrobial screening tests. All media and the standard drugs were used as received.

2.2 Methods

2.2.1 Collection of plant materials

The leaves of *Plantago lanceolata* (Faculty compound of Addis Ababa University, Main Campus), *Osyris quadripartita* Decn., *Maesa lanceolata* Forsk and *Alchemilla pedata* A Rich were collected from "Weibla Mariam", one of the outskirts of Addis Ababa, during September and October 2002. The leaves of *Cadaba farinosa* Forsk and *Steganotaenia araliacea* ExA. Rich were collected from Debre-zeit, 45 km south of Addis Ababa, during the same season. The collected plants were identified and samples were deposited in the Natural Herbarium, Department of Biology, Faculty of Science, Addis Ababa University. The collected plants were air-dried and grounded in dark and prepared for extraction.

2.2.2 Preparation of crude extracts

Each of the seven powdered plant materials was extracted using 80% (V/V) methanol. 100g of each of the plant material were taken in three 2.5-liter Erlenmeyer flasks and the methanol was added until a layer of methanol was formed. The maceration was carried out for 48hrs with intermittent agitation. The extracted portion was then filtered with the aid of filter paper (Whatman No 3, Whatman Ltd., England) and the marc was remacerated successively five times. Finally, all the filtrates were collected and concentrated using Rota-vapor (BÜCHI Rota-vapor R-205, Switzerland) at 40^0C. The aqueous residues were dried in an oven at 40^0C and the dried plant extracts were powdered and kept in desiccators for future use.

2.2.3 Antibacterial and antifungal sensitivity testing

These tests have been undertaken according to the methods described elsewhere [132,174, 175]. The procedure was as summarized below.

Preparation of inoculum

Each bacterial culture used for test has been streaked on to a non-inhibitory agar medium to obtain isolated colonies. After an overnight incubation at 37^0C in an Incubator (Heraeus GmbH, D-6450, Germany), well-isolated colonies were selected with a sterile loop and transferred to a tube of non-selective broth and vortexed thoroughly. This in turn is incubated for about 30 min and then compared to 0.5 McFarland standard against a white sheet of paper just before use. The fungal culture was prepared by suspending a stock in a sabauraud molten agar and compared with 0.5 McFarland standard against a white sheet of paper just before use after incubation at 25^0C.

Inoculation procedure

The turbidity adjusted inoculum suspension was dipped with a sterile cotton swab. Pressing firmly against the inside wall of the tube just above the fluid level, the swab was rotated so as to remove excess of the liquid. The swab was then streaked over the entire surface of the neutrient agar medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum. Finally swabbed all-round the edge of the agar surface. This same procedure was followed for the antifungal testing using solidified sabaraud agar medium.

Application of the total extract

After swabbing, a sterile cork borer was used to form a well in such a way that each hole is separated from the other by at least 20mm. 100 µl of each of the extracts at three different concentrations (100 mg/ml, 50 mg/ml, 25 mg/ml) was applied in triplicate into the well using a micropipette (Gilson, France). All these activities were undertaken in an aseptic environment. The dishes were then kept for 2 hrs at environmental condition until the extracts were diffused into the medium and then incubated for 18-24 hrs at 37 ± 1^0C for antibacterial testing, 2days for *C. albicans* and *A. niger* and 7 days for *T. mentagrophytes* at 25^0c. The Petri dishes were then taken out and the inhibition zones were measured. 80% (V/V) methanol and aqueous solution of gentamicin sulphate (0.1mg/ml) were taken as negative and positive controls, respectively for antibacterial activity testing and, 80% (V/V) methanol and ketoconazol (0.3 mg/ml) as negative and positive controls, respectively, in the case of antifungal activity testing.

2.2.4 Preliminary phytochemical screening of *A. pedata* and *M. lanceolata*

Identifications were made by chemical tests for alkaloids, phytosteroids, polyphenols, flavonoids, saponins and phenolic glycosides, according to the already established methods described elsewhere [18]. Summary of the procedure is given below.

Test for alkaloids: Powdered plant material was extracted with 1 % HCl solution in a water bath. The filtrate was then tested for the presence of alkaloids using Dragendorff's and Mayer's reagent. The formation of yellowish orange precipitate for Dragendorff's and whitish opalescence for Mayer's indicates the presence of alkaloids.

Test for phytosteroids: 3-5 drops of concentrated H_2SO_4 was added to the chloroform solution of the petroleum ether extract. The production of red or reddish brown or violet color was taken as indicative of the presence of steroidal compounds.

Test for polyphenols: Three drops of mixture of 1ml 1% $FeCl_3$ and 1ml $K_3Fe(CN)_6$ (prepared separately) was added to the aqueous solution of the plant extract. The formation of a green blue color was taken as an indicator for the presence of polyphenols.

Test for flavonoids: Four drops of 2 % lead acetate solution was added to the alcoholic extract. The development of yellow or orange color indicates the presence of flavonoids.

Test for saponnins: extraction with water was made in a water bath for 5 minutes. 10 ml of the filtered extract was shaken in a large test tube; the formation of honeycomb froth that persisted for half an hour indicated the presence of saponnins.

Test for phenolic glycosides: a crystal of $Fe_2(SO_4)_3$ was added to the aqueous solution of the alcoholic extract of the plant. Formation of dark or violet precipitate was taken as an indicator for the test.

2.2.5 Solvent fractionation of *A. pedata* and *M. lanceolata*

100 g of dried, powdered and ground plant materials were taken and packed in a thimble. Successive and exhaustive extractions were undertaken using petroleum ether ($80\text{-}100^0$c), chloroform, acetone and methanol, respectively by continuous soxhlet apparatus. Each fraction was collected separately and concentrated at reduced pressure using Rota-vapor at 40^0C. The semisolid mass was then dried in an oven at 40^0C. The dried mass was powdered and kept in a desiccator for future use.

2.2.6 Minimum inhibitory concentration (MIC) determination

The MICs were determined according to the method reported [174,175]. Summary of the procedure is given below.

Agar-well diffusion test method

Serial dilutions of *A. pedata* and *M. lanceolata* were prepared by successively diluting the same concentrations of the respective extracts. In the first tube, 100 mg of the plant extract was dissolved in 10 ml of 80% methanol and, in separate nine numbered test tubes; 5 ml of 80%

methanol was taken. The first 5 ml of the extract solution from the 10 ml stock was transferred into the second test tube containing 5 ml of pure solvent making the strength in the second test tube half of the first test tube. 5 ml of the extract solution from the second test tube was transferred to the third. This procedure was repeated until 5 ml was discarded from the 10^{th} test tube. Meanwhile, 24 hr cultures of the test organisms were swabbed onto the solidified nutrient agar media and 100 µl of the solution was then put into the wells using micropipette similar to the sensitivity testing. After incubation, the zone of inhibition was recorded and the concentration below which no activity could be observed was determined as the MIC of the total extract.

Agar dilution test method

A stock solution of each extract was serially diluted twofold in 80% methanol and 2 ml of each solution was incorporated in 20 ml of the appropriate melted agar and sabaurod medium for bacteria and fungus, respectively, under aspetic condition. The mixture was mixed thoroughly with vortex mixer and poured onto Petridishes. The final concentration of the extracts in the solution was ranging from 1 mg/ml to 0.0015 mg/ml. When the media was solidified, overnight culture dilutions were streaked on the surface of the plates and incubated, accordingly. MICs were determined as it is the lowest concentration of the extract inhibiting the visible growth of each microorganism. Dishes treated likewise with methanol (80%) were used in each experiment as negative controls.

2.2.7 Anti-inflammatory activity test

Anti-inflammatory activity was evaluated on the basis of the inhibition of the carrageenan-induced hind paw edema [136,137]. The tests for the two plants were undertaken separately. Hence, two separate sets of experiments with two separate controls were utilized in the

experimentation; otherwise the procedures were essentially similar in both cases. The extracts and water were given orally to the experimental animal and control groups, respectively, 30 min before administration of the edema inducing agent (0.1 ml of a 1% carrageenan suspension in 0.95% NaCl), which was injected subcutaneously into the plantar surface of the left hind paw of male and female albino rats with weights falling in the range of 90-120 g for *M. lanceolata* and 140-190 g for *A. pedata*. The volumes of the right paws were measured just before administering the edema inducing agent and 60, 120, 180 and 240 min after induction of inflammation by water displacement method measured with the help of plethysmometer (Ugo Basile Biological Research Apparatus, 7140, Italy).

Edema was expressed as the increase in paw volume due to carrageenan injection as compared to the paw volume just before administration. The significance of differences between means (against control) was assessed by two-sample t-test, with a significance level of $p < 0.05$. The percent inhibition of edema volume was obtained from the ratio of the mean of edema volume of the test group to that of the control at a specific period of time. Indometacin at 10 mg/kg level was suspended in distilled water and given orally as a positive control.

2.2.8 Formulation of topical antimicrobial dosage forms of *A. pedata* and *M. lanceolata*

Topical formulations were prepared at 10% (W/W) level using bases with different polarities. The composition of the respective bases that may indicate the degree of hydrophilicity is indicated in Table 2.1. First, the bases were prepared for incorporation of the plant extracts by fusion method. Then the preparation of ointments and creams were conducted on an ointment tile

by levigation with a spatula while the bases were still soft to facilitate the uniform incorporation of powdered and sieved plant extracts.

2.2.9 Antimicrobial activity studies of the formulations

Sensitivity test was carried out using the modified agar well diffusion technique as described previously [143-146]. The media for both bacteria and fungi were prepared in the same way to that of the sensitivity testing. 40 ml of these media were dispensed in 14 mm sterile Petridishes and allowed to solidify. 9 holes were made by removing plugs with sterile cork borer in such a way that the wells are separated by not less than 20 mm from each other and 10 mm from the edge of the plate. For antifungal testing, the distance was kept at no less than 30 mm between wells. By means of tarred 5 ml syringe, about 0.1 g of the formulated topical agents was added to each well.

15 ml of molten media, which were maintained at 50^0C over a water bath, were taken in sterile test tubes and inoculated with 0.5 ml of suspension of the respective microorganisms. The agar suspension of microorganisms was mixed on a mechanical agitator and poured onto the previously prepared test plate containing the antimicrobial agents. After the agar solidified, the plates were inverted and incubated at 37^0C. The magnitude of the zones of bacterial growth inhibition around each test agent was then recorded at a specified period.

Table 2.1: List of bases utilized in the formulation of topical dosage forms using the plant extracts.

No.	Name of base	Ingredients	Proportion (%, W/W)	Property of the base
1	Polyethylene glycol	PEG 400	50	Hydrophilic
		PEG 2000	50	
2	Polyethylene Glycol	PEG 400	25	Hydrophilic
		PEG 2000	75	
3	Macrogol blend ointment base	Macrogol 4000	20	Hydrophilic
		Macrogol 600	80	
4	Sodium laurate monostearin cream base	Glyceryl monostearate	5	Oil-in-Water emulsion
		Sodium lauryl sulphate	3	
		Cetosteryl alcohol	2	
		Liquid paraffin	25	
		water to	100	
5	Macrogol cream base	Cetomacrogol emulsifying wax BP	9	Oil-in-Water emulsion
		Liquid paraffin	6	
		White petrolatum	15	
		Water to	100	
6	Simple ointment (BPC, 1979)	Cetosteryl alcohol	5	Emulsified base
		Hard paraffin	5	
		Wool fat	5	
		White petrolatum	85	
7	White petrolatum BP	White petrolatum	100	Non-emulsified hydrophobic base

2.2.10 Preliminary semi-quantitative standardization of the plant extracts [21, 49]

Standardization using extractable matter

Determination of water-soluble and alcohol extractive values: 5 g of air-dried, coarsely powdered plant materials were macerated with 100 ml of water in a closed flask for 24 hrs, shaking frequently during the first 6 hrs and allowed to stand for 18 hrs. The filtrate was collected and 25 ml of it was evaporated to dryness in a tarred evaporating dish at 105^0C to constant weight. The percentage of water-soluble extractive with reference to the air-dried drug was calculated. The same procedure was followed for determining alcohol soluble extractive value, except that absolute ethyl alcohol was employed as a solvent. Each extraction was done three times.

Successive extractive values: 5 g of air-dried, coarsely powdered plant materials were macerated with 100 ml of petroleum ether in a closed flask for 24 hrs, shaking frequently during the first 6 hrs and allowed to stand for 18 hrs. The filtrate was collected and the marc was dried and remacerated using 100 ml of chloroform. Likewise, the filtrate was collected and successive extraction was accomplished using acetone and methanol as solvents. 25 ml of each of the filtrate was evaporated to dryness in a tarred evaporating dish at 105^0C to constant weight. The percentage of each extractive with reference to the air-dried drug was calculated. This was done three times for each solvent.

Standardization using determination of ash values

Total ash: 3 g of the ground air-dried plant materials in a previously ignited and tarred crucible were taken. They were spread evenly and ignited in a Furnace, (Nuber Industrieofunbau, D-2804 Lilienthal/ Bermen, West Germany), by gradually increasing the heat to 550^0C until it was white, indicating the absence of carbon. This was then cooled in a dessicator and weighed.

Acid insoluble ash: To the crucible containing the total ash, 25 ml of HCl (70 g/l) was added and covered with a watch glass. This was boiled for 5 minutes; the watch glass was rinsed with 5 ml of hot water and added to the crucible. The insoluble matter was collected using ashless filter paper by washing with hot water until the filtrate became neutral. The filter paper containing the insoluble matter was transferred to the original crucible, dried and ignited to constant weight. The residue was cooled in a dessicator for 30 min and weighed.

Water soluble ash: To the crucible containing the total ash, 25 ml of water was added and boiled for 5 min. The insoluble matter was collected in an ashless filter paper. After being washed, it was ignited in a crucible for 15 min at a temperature not more than 450^0C. The weight of this residue was subtracted from the weight of the total ash.

Standardization using thin layer chromatography

The successive solvent fractions of both plants including the total extracts were prepared at a concentration of 1 mg/ml in acetone. An aliquot (6 µl) of each solutions was spotted on the Pre-coated TLC plates, (Silicagel, 60 F_{254}, Merk, Germany), 1.5 cm far from one tip of the plate as bands of approximately 1cm length using calibrated micro-capillaries. After complete solvent removal by drying, the plates were immersed into a chamber saturated with the mobile phases.

The chromatograms were developed and analyses were conducted after the mobile phases evaporated. Bands were analyzed and R_f values were calculated under different conditions, which include UV irradiation (both 254 and 366 nm) in a UV-viewing cabinet, (UV-cabinet II, CAMAG), vanillin in sulfuric acid (1% W/V) treatment and simple visual inspection.

Table 2.2: Mobile phases utilized for developing chromatograms of the successive fractions and total extracts of *A. pedata* and *M. lanceolata*

Fraction	Mobile phase	*A. pedata*	*M. lanceolata*
Pet. Ether	Chloroform: ethylacetate: methanol	8:1.5:0.5	8:1.5:0.5
Chloroform	Chloroform: ethylacetate: methanol	8:1.5:0.5	8:1.5:0.5
Acetone	Chloroform: ethylacetate: methanol: formic acid	3:3:3:1	3:3:3:1
Methanol	Chloroform: ethylacetate: methanol: formic acid	3:3:3:1	3:2:4:1
Total extract	Chloroform: ethylacetate: methanol: formic acid	3:3:3:1	3:2:4:1

3 RESULTS AND DISCUSSION

3.1 Extraction and antimicrobial screening of the selected plant materials

Traditionally, a given dried plant can be taken as teas or tinctures or inhaled via steam from boiling suspension of the plant parts. For external purpose, it could either be added to oils or butter and then applied. But integration of such plant-based products into the modern system requires scientific analysis of the components with all the necessary logical pathways. First the plant must be screened for the claimed activity, and the safety and efficacy profiles are assessed including the proposed dosage form of the extract before it is made available for use. There are different research centers mainly focusing on plants as their means of drug innovation endeavor. In these laboratories, activity screening of natural products is one of the earliest steps in the process [176-178]. Screening for antimicrobial action is also one of the commonest exercises.

Initial screening of plants for possible antimicrobial activity typically begins by crude extracts obtained using water or alcohol or a mixture of them as initial solvents, and can be followed by various organic extraction methods. In this work, 80% (V/V) methanol in water was employed and the yields obtained are summarized in Table 3.1.

Table 3.1: Percent yield obtained from 100 g of each of the plant materials after extraction using 80% methanol

Plant name	Parts used	Percent yield
P. lanceolata	Aerial part	21.86
A. pedata A. Rich.	Aerial part	22.25
O. quadripartita Decn.	Leaf	40.11
C. farinosa Forssk.	Leaf	21.96
M. lanceolata Forssk.	Leaf	28.57
S. araliacea Hochst ExA. Rich.	Leaf	25.53

Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction. Actually solvents used in the extraction process should be selected judiciously. In a recent study, ranking of a variety of extractants for their ability to solubilize antimicrobials from plants as well as other factors such as their relative safety and ease of removal from the fraction have been carried out. The focus of the study was to provide a more standardized extraction method for the wide variety of researchers working in diverse settings. Although it was not one of the more frequently used one in studies published to date, acetone received the highest rating followed by methylene dichloride, methanol, ethanol and water, respectively [179].

For the purpose of extracting components, which are soluble in water such as polysaccharides, polypeptides, tannins, mixtures of solvents are quite common; the most common one being alcohol and water. These mixtures of solvents not only extract the abovementioned constituents but also most of the polar and non-polar components of the plant. Therefore, in this work, 80 % (V/V) methanol was chosen to serve as solvent mixture.

As shown in the Table, the extracted amount ranges from 21 to over 40% on dried mass basis. Such a high yield is an indication of the extracting ability of the solvent used with respect to both polar and non-polar components. The maximum yield was obtained from *O. quadripartita* (40.11%) and the minimum yield from *P. lanceolata* (21.86%). These high yields, if the plants are found to be active and promising for further development into formulation, can add advantage to the economic manufacturability of these plant materials. Because formulation of

phytopharmaceuticals is commonly based on the total extracts which do not incur cost of fractionation and other processing.

In many industrial-screening programs, very large number of extracts, perhaps in hundreds of thousands of samples per year are evaluated in a substantial number of assays covering a range of therapeutic areas [176, 177]. But the problem is that the vast majority of samples will probably show no activity in any of the assays. Different approaches have been proposed to increase this very small "hit rates". One of such a scheme was following ethnobotanical data or taking the ground of traditional use [176, 180]. In this study, six commonly used and highly claimed plants for dermatological conditions were selected based on the information collected from traditional healers. It is interesting to note that almost all extracts have shown activity against both bacteria and fungi, Table 3.2 and Table 3.3.

As can be seen from the Table, all the extracts were active against *S. aureus* and *P. aeroginosa*. Similarly, all extracts were active against the Gram-negative bacteria, *E. coli* except *Cadaba farinosa* and *S. araliacea*, and the maximum activity was obtained from *A. pedata.* The activity of *P. lanceolata* against *E. coli* was observed only at higher concentrations. Probably this observation is attributed to the fact that most Gram-negative bacteria show pronounced growth after a short period inhibition upon exposure to sub-therapeutic dose of antimicrobials [181].

Table 3.2: Antibacterial activities of the 80% methanol extracts of the selected plants

Name	Conc. (mg/ml)	E^c	S^a	P^a
P. lanceolata	100	19.0 (0.29)	25.0 (0.58)	21.5 (0.29)
	50	17.7 (0.17)	22.7 (0.88)	20.3 (0.33)
	25	___	24.7 (0.67)	17.7 (0.33)
A. pedata	100	23.2 (0.17)	23.0 (0.58)	22.0 (1.15)
	50	21.0 (0.29)	20.0 (0.58)	19.8 (0.17)
	25	17.3 (0.33)	17.0 (0.58)	19.7 (0.33)
O. quadripartita	100	19.2 (0.44)	15.5 (0.17)	20.3 (0.17)
	50	16.5 (0.50)	17.0 (0.17)	17.5 (0.29)
	25	13.8 (0.17)	19.0 (0.33)	15.2 (0.33)
C. farinosa	100	___	24.3 (0.17)	16.3 (0.44)
	50	___	24.7 (0.88)	15.5 (0.50)
	25	___	26.0 (0.58)	14.3 (0.17)
M. lanceolata	100	14.7 (0.44)	23.4 (0.29)	20.5 (0.50)
	50	13.7 (0.67)	21.3 (0.88)	19.2 (0.44)
	25	12.5 (0.28)	19.0 (0.00)	18.3 (0.33)
S. araliacea	100	___	27.3 (1.5)	18.5 (0.00)
	50	___	25.7 (0.17)	17.5 (0.00)
	25	___	23.0 (0.57)	15.5 (0.29)
Gentamicin	0.1	21.9 (0.64)	26.0 (0)	21.3 (0.60)

Results are given as mean (SEM) of zone of inhibitions in mm (n=3), E^c *= E. coli,* P^a *= P. aerugenosa,* S^a *=S. aureus*

The extracts from *S. araliacea* and *P. lanceolata* exhibited the highest anti-staphylococcal activity and lesser activity was observed from the extract of *O. quadripartita.* The small inhibition zones of the extract of *O. quadripartita* in almost all the strains tested could be associated with diffusion problem of the active constituent(s). If the active constituents were

macromolecules, there could be diffusion problem on the agar media as these molecules move slowly on such a matrix system. Even though it was prepared at the same concentration as those of other extracts, it has thicker consistency which might be responsible for the reduced-size of the colored zone appeared on the dish. Moreover, the plant materials were extracted without prior defating and hence, the high amount of fatty material and pigment might also inhibit the process of diffusion of the active principle(s).

Similar to the activity against *S. aureus*, all the extracts have also showed activity against *P. aeroginosa* and relatively higher activity was observed from *A. pedata*. The activity was even comparable to the positive control, gentamicin sulphate, at the tested level. Minimum activities were recorded for *C. farinosa* and *S. araliacea*.

It is common observation that Gram-negative bacteria are more resistant to many compounds than Gram-positive ones. This is generally ascribed to the morphological differences between these microorganisms. Gram-negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cellwall impermeable to lipophillic solutes with an exclusion limit of 600 Da [182]. Particularly *E. coli* and *P. aeroginosa* are incriminated in several infections for their insensitivity to antibiotics [183].

The mechanisms by which microorganisms generally survive the action of antimicrobial agents are poorly understood and remain debatable. But one of the proposed mechanisms to the resistance is capsule related problem for *P. aeroginosa* and genetic factor for *E. coli* [184]. It was also observed that, after exposure to a given antibiotic agent, *E. coli* was found to decline in number for the first two hour then rapidly increase almost at the same rate as the control. And the

same was true with *P. aeroginosa* after 8 hr [184]. Hence, this is in congruency with the observation in this experiment that almost all of the extracts have shown lesser activity against *P. aeroginosa* and *E. coli* than that of *S. aureus*.

The Gram-positive bacteria should be more susceptible having an outer peptidoglycan layer which is not an effective permeability barrier. In spite of the permeability differences, some of the tested extracts demonstrated reasonable activities against Gram-negative bacteria; especially *A. pedata* which showed better activity on both strains than the other plant extracts.

The activity of *P. lanceolata* collected from Ethiopia is similar to the result obtained elsewhere [148]. In the literature there are quite a number of reports on the traditional use, efficacy, contemporary formulas, and contraindications of this plant. Due to its antimicrobial and anti-inflammatory activities as well as the wound healing effect, this plant finds wider application for many conditions including skin diseases. There are also numerous reports pertaining to its untoward effect, particularly its allergenic problem. The pollens of this plant are claimed to be allergenic [148].

A. pedata has also exhibited good antibacterial activity. Species in the genus *Alchemilla* have shown similar effect and this was attributed to their tannin content. In a preliminary screening of *A. pedata,* it was observed that phenolic glycosides, polyphenols, saponins and phytosteroids existed as components of the total extract. Hence, the activity of this plant might be because of these constituents. *A. speciosa* has also demonstrated activities such as antimutagenic and anti-inflammatory [171]. Even though the antimutagenic activity was not demonstrated in the present

study, *A. pedata* has shown anti-inflammatory activity, justifying its traditionally claimed wound healing activity.

M. lanceolata was active especially against *S. aureus* but less active against the Gram-negative bacteria. In the literature, the genus *Maesa* has many species, which demonstrated very good antimicrobial properties. Saponins are almost always found in every species of plants in this genus. In the preliminary phytochemical screening of *M. lanceolata*, the extract was found positive for the presence of flavonoids, saponins, polyphenols and phytosteroids but negative for phenolic glycosides and alkaloids. These constituents might be responsible for the antibacterial activity of this plant. As it has been shown in many reports, *M. lanceolata* has showed other activities apart from its antimicrobial action. Such activities include: antioxidant, anti-inflammatory, antiviral and antifungal. Therefore, these activities suggest the rational behind the use of this plant in traditional treatment.

In addition to the antibacterial activities, the antifungal effects of the abovementioned plants have also been screened and the results are summarized in Table 3.3.

As shown in Table 3.3 all the tested plants, except *P. lanceolata,* gave comparable activity against standard strains of *C. albicans*. However; their actions against *T. mentagrophytes* were different. Even though all the observed activities against *T. mentagrophyte* were good, strong activity was obtained from the extract of *M. lanceolata*. But the extracts from *C. farinosa* and *P. lanceolata* did not show any activity.

Table 3.3: Antifungal activities of the 80% (V/V) methanol extracts of the selected plants

Name of the plant	Conc. tested (mg/ml)	*C. albicans*	*T. mentagrophytes*
P. lanceolata	100	—	—
	50	—	—
	25	—	—
A. pedata	100	21.8 (0.17)	34.0 (0.57)
	50	20.7 (0.33)	31.0 (0.17)
	25	18.5 (0.29)	23.5 (0.29)
O. quadripartita	100	21.7 (0.33)	33.2 (1.2)
	50	20.5 (0.29)	29.5 (0.17)
	25	19.7 (0.33)	24.0 (0.17)
C. farinosa	100	19.2 (0.17)	—
	50	16.8 (0.17)	—
	25	14.8 (0.17)	—
M. lanceolata	100	21.7 (0.33)	50.0 (1.02)
	50	18.5 (0.5)	47.0 (0.73)
	25	17.7 (0.33)	38.5 (0.87)
S. araliacea	100	18.7 (0.88)	34.5 (0.17)
	50	18.0 (0.57)	34.0 (0.57)
	25	15.0 (0.57)	23.5 (0.33)
Ketokonazol	0.3	37.0	35 .0

Results are given as mean (SEM) of zone of inhibitions in mm (n=3) C^a =C. albicans T^m =T. mentagrophyte

The solvent used for extraction was also employed for dissolving the plant extracts during testing. But it showed no antibacterial or antifungal actions. The positive antifungal control used was ketoconazol at a concentration of 0.3 mg/ml.

Since the antimicrobial activites of *A. pedata* and *M. lanceolata* were superior, they were chosen for further works and the subsequent sections discuss the various tests conducted for these two plants.

3.2 Fractionation, phytochemical screening and MIC determination of *A. pedata* and *M. lanceolata*

3.2.1 Fractionation and antimicrobial activity testing

Locating the active principle from crude plant extract needs extracting this component from the complex matrix of the crude extract to get the pure form. One such attempt is solvent fractionation that involves extracting components depending on their polarities. Nowadays, activity-guided fractionation or target-directed isolation of antibacterial and antifungal drugs can be done with TLC. The technique is also called TLC-bioautography [175, 185].

In the fractionation of the plant materials, which utilizes solvent or solvent mixtures, all fractions are tested and those which exhibit activity are carried through further isolation and purification until the active ingredient(s) are obtained. In this work, preliminary fractionation using petroleum ether, chloroform, acetone, and methanol as solvents was undertaken in a successive manner. After the plant materials were successively and exhaustively extracted with the aforementioned solvents, each fraction of the extracts was then dried and screened for antimicrobial activities. This screening was done in an attempt to localize the fraction in which the active compound was residing. The antimicrobial activity of each fraction is shown in Table 3.4 and 3.5.

As shown in Table 3.4, the antimicrobial activity of *A. pedata* was distributed among the various fractions. This may be expected, as there is no ideal solvent that selectively and exhaustively extracts a given constituent. Therefore a given active component may exist in the various fractions in different proportions. The activity of the fractions could also be due to the presence of more than one active ingredient distributed in the fractions.

Table 3.4: The antibacterial and antifungal activities of the successive fractions and the total extract of *A. pedata*

Fractions	Conc.	E^c	S^a	P^a	C^a	T^m
Pet ether	25	32.0 (0.57)	35.0 (0.60)	25.0 (1.36)	38.0 (1.20)	38.0 (1.92)
	5	---	25.0 (1.7)	16.0 (1.17)	24.0 (2.24)	15.0 (0.33)
Chloroform	25	---	19.0 (1.0)	---	21.5 (1.76)	---
	5	---	15.5 (0.76)	---	18.0 (0.60)	---
Acetone	25	15 (1.00)	21.0 (0.67)	20.0 (0.87)	25.0 (1.35)	22.0 (0.17)
	5	14 (0.67)	17.0 (0.33)	18.0 (0.58)	18.0 (0.44)	16.5 (0.76)
Methanol	25	---	19.0 (0.73)	16.5 (0.44)	20.0 (1.44)	14.5 (0.76)
	5	---	14.5 (0.58)	13.5 (0.50)	---	---
Total	25	15 (0.17)	22.0 (1.45)	17.5 (0.50)	20.0 (0.93)	15.0 (0.32)
	5	14 (0.17)	18.0 (0.29)	14.0 (0.17)	15.5 (0.67)	13.5 (0.74)

Results are given as mean (SEM) of zones of inhibition in mm (n=3); Concentrations are expressed in (mg/ml); E^c = *E. coli;* S^a = *S. aureu;* P^a = *P. aeroginosa;* C^a = *C. albicans;* T^m = *T. mentagrophyte*

The activity of the petroleum ether fraction against *E. coli* was higher as compared to the acetone fraction and the total extract, tested at the same concentration. Where as the chloroform and methanol extracts at the concentrations tested were inactive. From this observation it could be

said that the active principle(s) against *E. coli* in the petroleum ether and the acetone fractions were different. This is because successive fractions of the same plant may leave portion of agiven active ingredient in the second next solvent of higher polarity. On the other hand, the activity of *A. pedata* against *S. aureus* was distributed comparably among the successive fractions except for the petroleum ether extract, which displayed maximum activity. Similar results were obtained with tests against *C. albicans* and *T. mentagrophytes*.

The antimicrobial effect was noticeably higher from the petroleum extract than the activity in the total extract, tested at the same concentration. This difference might be due to a) the active principle(s) are concentrated by using petroleum ether as a solvent; b) the concentration of inhibiting components are decreased by selecting the solvent. Hence, the active principle is most likely non-polar and further activity-guided fractionation work on the non-polar fraction seems to be beneficial.

The antimicrobial effects of the different fractions of *M. lanceolata* are also given in Table 3.5. The chloroform, acetone and the total extracts of *M. lanceolata* showed similar activity against *E. coli,* but the petroleum ether and the methanol fractions are inactive. Moreover, all fractions of *M. lanceolata* inhibited the growth of *S. aureus* and relatively similar activity of the various fractions was obtained against *P. aeruginosa.*

Table 3.5: The antibacterial and antifungal activities of the petroleum ether, chloroform, acetone and methanol fractions, and the total extract of *M. lanceolata*

Fractions	Conc.	E^c	S^a	P^a	C^a	T^m
Pet. ether	25	—	18.8 (0.17)	14.5 (0.29)	17.5 (0.87)	—
	5	—	23.3 (0.17)	13.5 (0.29)	16.5 (0.29)	—
Chloroform	25	13.7 (0.33)	21.0 (0.87)	16.7 (0.44)	—	—
	5	13.0 (0.28)	17.7 (1.67)	14.0 (0.50)	—	—
Acetone	25	15.7 (0.33)	22.2 (0.17)	15.8 (0.44)	20.7 (0.33)	—
	5	13.7 (0.44)	16.7 (0.33)	14.0 (0.29)	16.0 (0.29)	—
Methanol	25	—	17.0 (0.29)	15.5 (0.74)	20.7 (1.30)	42.0 (0.50)
	5	—	13.8 (0.17)	14.2 (0.33)	16.7 (0.44)	25.3 (0.17)
Total	25	14.7 (0.33)	22.0 (1.00)	17.8 (0.17)	19.5 (0.29)	39.0 (0.76)
	5	13.2 (0.17)	18.0 (0.50)	15.5 (0.29)	15.5 (0.87)	27.5 (0.29)

Results are given as mean (SEM) of zones of inhibition in mm (n=3); Concentrations are expressed in (mg/ml); E^c = *E. coli;* S^a = *S. aureu;* P^a = *P. aeroginosa;* C^a = *C. albicans;* T^m = *T. mentagrophyte*

The antifungal effect was similarly distributed into different fractions other than chloroform in the case of *C. albicans*. The activity of the extract against *T. mentagrophytes; however,* was only concentrated in the methanolic fraction and is comparable to the one obtained from the total extract. This result revealed that the active principles against *T. mentagrophytes* are polar compounds. If the water fraction was tested for activity, even stronger result might be obtained.

The antifungal activities of the variuos fractions of *A. pedata* and *M. lanceolata* against clinical isolates of *C. albicans* are given in Table 3.6. All of the fractions of *A. pedata* did not show any

activity against the clinical isolates, although anticandidacl action was demonstrated against the standard strains. This phenomenon might be ascribed to the resistance developed by the clinical isolates.

All the fractions were dissolved in acetone for testing because this solvent did not show any antimicrobial activity. All the fractions of *M. lanceolata* showed antifungal effect against clinical isolate of *C. albicans*. The maximum activity was observed from the methanol fraction and the total extract of this plant.

Table 3.6: Activities of the successive fractions and total extracts of *A. pedata* and *M. lanceolata* against clinical isolate of *C. albicans.*

Fraction tested	Conc. (mg/ml)	*A. pedata*	*M. lanceolata*
Petroleum ether	10	__	16.5
	1	__	19.0
Chloroform	10	__	17.0
	1	__	13.5
Acetone	10	__	__
	1	__	15.5
Methanol	10	__	27.5
	1	__	19.5
Total	10	__	25.5
	1	__	18.17
Acetone		__	__

Results are given as mean (SEM) of the zone of inhibitions in mm (n =3)

3.2.2 **Phytochemical screening**

Isolation of pure, pharmacologically active constituents from plants remains to be a long and tedious process. Several methods could be used for the identification, characterization and screening of the active components of plant extracts. One such technique is chemical screening that will allow localization and targeted isolation of constituents with potential activities. This procedure enables recognition of known metabolites in extracts at the earliest stages of separation and is thus economically very important [1]. For efficient separation and detection of metabolites, good selectivity and sensitivity of detection methods together with the capability of providing on-line structural information is important. This could best be supplied by hyphenated high performance liquid chromatographic, (HPLC), techniques such as LC/MS, LC/UV, LC/NMR [186,187]. Nonetheless, the chemical screening can also give a clue about the type of chemical compounds present.

The phytochemical screening results of total extracts of *A. pedata* and *M. lanceolata* are given in Table 3.7. As shown in the Table, both plants have got different kinds of phenolic compounds and were devoid of alkaloids. The strong antimicrobial activities of these plants might, thus, be related to these compounds.

Table 3.7: Phytochemical screening of the total extracts of *A. pedata* and *M. lanceolata*

Plant	Components screened					
	Alkaloid	Flavonoid	Phenolic glycoside	saponins	polyphenol	phytosteroid
A. pedata	-	+	+	+	+	+
M. lanceolata	-	+	-	+	+	+

3.2.3 MIC determination

Determination of antimicrobial break points in making decision about plant product and any antimicrobial agent, in general, is a bit difficult. But, most commonly, minimum inhibitory concentrations (MICs) for commonly occurring microorganisms, if competently determined by the standard methods described in the literature, are often reproducible. Hence, MIC has been taken as one indicator of the efficacy profile of a given agent [174, 175, 188, 189].

In this work, MICs of the total extracts of *A. pedata* and *M. lanceolata* were determined using the agar well-diffusion and the agar dilution assay methods. The results obtained using the agar-well diffusion method is summarized in Table 3.8. Accordingly, the MICs were in the ranges of 5 to 1.25 mg/ml for the bacteria and between 10 and 0.625 mg/ml for the tested fungi. The MICs for *M. lanceolata* against *E. coli and that of A. pedata* against *T. mentagrophytes* were not within the concentration range tested. The MICs using agar dilution assay were 0.25 mg/ml and 0.0625 mg/ml for *M. lanceolata* against the clinical isolate, *C. albicans,* and *T. mentagrophyte*, respectively. Likewise, the MIC for both *M. lanceolata* and *A. pedata* against *S. aureus* and *E. coli* was 0.125 mg/ml in the latter method.

As was also obtained in this work, the results with agar dilution assay were consistent in giving better MIC values as compared to the well-diffusion assay. This could probably be due to retarded diffusion of plant constituents, especially the insoluble ones in the case of well diffusion technique. Relatively, the components can easily mix with the media in the case of agar dilution method and hence better antimicrobial sensitivity. Many studies use solubilizing agents, such as Tween 80, to overcome the problem of solubility. But, the presence of Tween 80 may cause

various problems such as increasing the susceptibility of some organisms and sometimes even decreasing the susceptibility [190].

Table 3.8: Minimum inhibitory concentrations of the total extracts of *A. pedata* and *M. lanceolata* against the tested bacteria and fungi using agar well diffusion method

	Conc.	E^c	S^a	P^a	C^a	T^m
Alchemilla pedata	10	15.27 (0.17)	16.17 (0.16)	20.16 (1.4)	12.5 (0.29)	___
	5	14.3 (0.17)	14.83 (0.17)	17.5 (0.29)	___	___
	2.5	___	___	___	___	___
	1.25	___	___	___	___	___
Maesa lanceolata	10	___	14.5 (0.29)	18 (1.15)	24.5 (0.29)	35.1 (0.67)
	5	___	12.8 (0.44)	16 (0.29)	23.8 (0.83)	25 (0.87)
	2.5	___	___	15.2 (0.44)	19.2 (0.17)	20.3 (1.9)
	1.25	___	___	14.2 (0.44)	14.2 (0.17)	14.2 (0.17)
	0.625	___	___	___	12.2 (0.17)	___
	0.313	___	___	___	___	___

Results are given as mean (SEM) of zones of inhibition in mm (n=3); Concentrations are expressed in (mg/ml); E^c = *E. coli;* S^a = *S. aureu;* P^a = *P. aeroginosa;* C^a = *C. albicans;* T^m = *T. mentagrophyte*

To make MIC a useful tool in the determination of break points, it must be determined on at least 500 isolates (and at least 300 anaerobes in addition, if relevant, each species in sufficient numbers to establish norms with a note on relative prevalence among clinical isolates), which must include representatives of clinically relevant species appropriate to the class of compound and its proposed clinical use. They must include isolates showing important resistance mechanisms, for example, methicillin resistant *S. aureus* [187].

MIC is also a valuable tool in that it is applicable in making analysis of the relationships such as the time of which the serum or plasma levels exceeds the MIC of relevant organisms, peak serum or plasma level to MIC ratio, and AUC to MIC ratio which are very important in making comparative study of antibiotics and also important in giving a clue about the therapeutic utility of newly obtained antimicrobials. Data for this analysis might be derived from the experimental models of infection, from humans given proper dosage schedules or from clinical studies [187].

From the ongoing discussion, it is evidently shown that the MIC of *M. lanceolata* against the tested fungi is very good. Hence, it seems this plant extract will have therapeutic application upon further fractionation and development.

3.3 Anti-inflammatory activities of *A. pedata* and *M. lanceolata*

The antiedematogenic activities of *A. pedata* and *M. lanceolata* were evaluated at doses of 750 mg/kg and 100 mg/kg taking indomethacin at 10 mg/kg level and only rats given normal saline solution as positive and negative controls, respectively. As reported in the literature, the carrageennan-induced rat paw edema was used as an *in vivo* model for inflammation [191-194]. This is a screening procedure in which the involvement of the cycloxygenase products of arachidonic metabolism and the products of reactive oxygen species are well established [195]. The results of the present study are presented in Table 3.9 and 3.10.

Table 3.9: Anti-inflammatory activity of total extract of *M. lanceolata* on carrageenan-induced rat hind-paw edema.

Treatment	ΔV				% Reduction			
	1 hr.	2hrs.	3hrs.	4hrs.	1hr.	2hrs.	3hrs.	4hrs.
100mg/kg	0.18 (0.031)	0.45 (0.09)	0.65 (0.11)	0.83 (0.05)	62.2%	43%	14.5%	9.2%
750mg/kg	0.21 (0.038)	0.58 (0.03)	0.72 (0.05)	0.75 (0.05)	53.3%	26.6%	5.3%	0%
Negative Control	0.45 (0.025)	0.79 (0.11)	0.76 (0.08)	0.76 (0.07)				

Value represents: mean (SEM) (n=5), ΔV = edema volume

% Reduction = (1- [edema]$_{test}$ / [edema]$_{ontrol}$)x 100%

Table 3.10: Anti-inflammatory activities of the total extract of *A. pedata* and indomethacin *in* carrageenan-induced rat hind-paw edema.

Treatments	ΔV				% Reduction			
	1hr	2hr	3hr	4hr	1hr	2hr	3hr	4hr
(100mg/kg)	0.14 (0.03)	0.21 (0.03)	0.26 (0.04)	0.48 (0.03)	33.3	19.2	21.2	0
750mg/kg	0.20 (0.03)	0.16 (0.05)	0.32 (0.04)	0.51 (0.07)	5	38.5	3.1	0
Positive control (Indomethacin)	0.11 (0.01)	0.17 (0.03)	0.19 (0.05)	0.22 (0.05)	47.6	34.6	42.4	45
Negative control (Normal saline)	0.214 (0.04)	0.264 (0.05)	0.334 (0.06)	0.398 (0.13)				

Value represents: mean (SEM) (n=5), ΔV = edema volume

% Reduction = (1- [edema]$_{test}$ / [edema]$_{ontrol}$) x 100%

Both plant extracts and the positive control, indometacin, have shown antiphlogestic activity. Better activity; however, was observed from the methanolic extract of *M. lanceolata* than that of *A. pedata*. *M. lanceolata* at both dose levels has shown a significant inhibition of edema during the first one hour ($p < 0.001$). *A. pedata*, *M. lanceolata* as well as the positive control showed maximum activity in the first one-hour. However; for *A. pedata* at a dose of 750 mg/kg, the maximum activity was observed during the second hour. In all the tests there were reduction in activity immediately after the maximum result was seen, except for the positive control. This might have something to do with the mechanism of action of the extracts.

It was reported that the carrageenan-induced edema shows three distinct phases, namely; an initial release of histamine and 5-hydroxytryptamine (5-HT), a second phase mediated by kinins and a third phase (about 5 hour of edema) in which the mediators are suspected to be prostaglandins [191,192]. This last phase has been reported to be sensitive to both clinically useful steroidal and non-steroidal anti-inflammatory agents and they are related to cycloxygenase inhibition, especially COX-2 [196]. Some authors classified the phases as two, namely; early phases and the delayed phase where the second phase is because of the secretion of bradykinin and serotonin [197]. Hence, it could be proposed that the extracts might have reduced the secretion of 5-HT and histamine. But this was not the case with *A. pedata* at a dose of 750 mg/kg, which showed maximum activity at the second hour.

It is worth noting that the anti-inflammatory activities of both plant extracts are greater at 100 mg/kg than at 750 mg/kg. This could possibly be as a result of the interference from other components in the crude extracts, which may act negatively to that of the anti-inflammatory

agent. The edema suppressing activity of the indomethacin was comparable to that of the result obtained elsewhere [176].

As inflammation plays a major role in wound healing processes, the anti-inflammatory activity of these plants could be a contributing factor for their traditional use for wound treatment.

3.4 Formulation and dosage form activity testing of the plant extracts

Preliminary formulations were prepared using different bases, which were commonly employed in the preparation of many dermatologicals. During base selection, polarity was considered as the major factor, and hence, bases that might represent relatively different polarity were included. The results of the antimicrobial activity of the formulations are given in Table 3.11.

During the formulation process, it was observed that incorporation of *A. pedata* extract into the macrogol cream base resulted in the liquefaction of the base to the extent of separation of the water phase, indicating incompatibility of the base with the extract components. To asses whether the interaction had effect on the antimicrobial activity of the extract, the formulation was tested for its antimicrobial activity. It was found that the formulation was inactive against *E. coli*, as opposed to the total extract which showed activity against the same organism. However, it was active against *S. aureus and T. mentagrophytes* with good clear zone of inhibition. But it is difficult to conclude about the effect of the interaction based on the results shown here. Therefore, further investigative work is suggested to get meaningful conclusion

Table 3.11: Antibacterial activity of the formulations of *A. pedata and M. lanceolata* in different bases (each formulation contained 10 % W/W of the respective plant extracts)

	Formulation number	*E. coli*	*S. aureus*	*P. aeruginosa*
A. pedata	Form. 1	15.3 (0.25)	22.5	19.3 (0.17)
	Form. 2	—	22 (0.29)	14.0(0.13)
	Form. 3	14.3 (0.44)	17.5 (1.32)	14.7 (0.67)
	Form. 4	—	—	—
	Form. 5	—	—	—
	Form. 6	16.2 (0.72)	20.0 (0.58)	20.8 (1.1)
	Form. 7	15.8 (0.60)	21.3 (0.17)	23.5 (0.76)
M. lanceolata	Form. 1	13.8 (0.44)	20.7 (0.17)	15.7 (0.33)
	Form. 2	—	—	—
	Form. 3	17.0 (1.04)	17.2 (0.44)	16.5 (0.76)
	Form. 4	—	—	—
	Form. 5	—	—	—
	Form. 6	14.8 (0.88)	18.0 (0.58)	15.3 (0.16)
	Form. 7	15.8 (0.44)	14.7 (0.33)	15.5 (1.04)
Control (drugs)	Foban ointment	—	44.7 (1.45)	—
	Bactroban	29.5 (0.5)	57.3 (1.76)	15.8 (0.75)
	Tetracycline ointment	32.7 (0.33)	42.7 (1.20)	19.4 (0.63)
	Foban cream	—	37.0 (1.00)	—
	Gentamycin cream	23.0 (0.87)	21.0 (0.58)	23.7 (0.25)

Formulation 1; extract in Sodium laurylmonosterin creabm base; form 2, in Macrogol cream base; form 3, in Macrogol blend Ointment base; form 4, in Simple ointment base; form 5, White petrolatum BP; form 6, in Polyethylene glycol bases (PEG 400: PEG 2000, 1:1); form 7; in Polyethylene glycol bases (PEG 400: PEG 2000, 1:3).

Evidently, the releases of the active principle(s) from PEG bases were better than most of the bases used in this study. This could be ascribed to the properties of PEGs, of which their solubility in water is the most important one. This makes them suitable for use in different applications. Liquid PEGs with molecular weight of less than 600 are miscible with water in any ratio. But even solid PEGs have excellent solubility in water. For example, up to 50% of PEG 35, 000 can be dissolved at room temperature in water. The solubility and the viscosity are not affected by the presence of electrolyte.

Added advantage of PEGs as a base is the solvent power for numerous substances that are sparingly soluble in water. This action can be ascribed to the formation of a sort of complexes between PEG and the active substances. Most of those complexes are loose and reversible. There are only some examples where the complexes tend to inactivate the active substances, a well known are penicillin G and W, bacitracin and acetylsalicylic acid. Furthermore, some substances react with PEGs by forming precipitates. This includes: phenol, cresol, resorcinol, salicylic acid tannin, potassium iodide, tetraiodo-bismutic acid [196].

The antifungal activities of the formulations were also determined and the results are summarized in Table 3.12. The release studies for antifungal activity showed that PEGs were, once again, good bases that have demonstrated better *in vitro* performance. But, this doesn't necessarily mean that they also have superior performance *in vivo*.

Table 3.12: Antifungal activity of the formulations of *A. pedata* and *M. lanceolata* in different base, (each formulation contained 10 %, w/w, of the respective plant extracts)

	Formulation number	*C. albicans*	*T. mentagrophyte*
A. pedata	Form. 1	__	32.2 (2.04)
	Form. 2	__	23.5 (0.98)
	Form. 3	__	32.3 (2,74)
	Form. 4	__	19.0 (0.28)
	Form. 5	__	__
	Form. 6	__	28.0 (0.43)
	Form. 7	__	29.3 (0.88)
M lanceolata	Form. 1	27.8 (0.75)	43.8 (0.83)
	Form. 2	31.0 (0)	46.2 (0.44)
	Form. 3	31.7 (0.88)	56.1 (1.52)
	Form. 4	19.2 (0.88)	28.5 (0.50)
	Form. 5	20.3 (0.83)	36.3 (3.13)
	Form. 6	32.8 (0.25)	65.5 (3.88)
	Form. 7	31.7 (0.33)	60.7 (2.20)
Positive controls	*Dezor cream*	50.3 (6.84)	27.3 (0.73)
	Ketoral cream	47.5 (2.75)	31.3 (0.88)
	Kenazol® cream	44.7 (1.20)	29.5 (0.28)
	Fungoral® cream	42.5 (2.50)	29.83 (1.17)
	Clotridenk cream	44.7 (0.88)	31.0 (0.73)
	Sha Hsien Thin cream	44.7 (1.45)	47.3 (1.45)
	Nizoral® cream	44.0 (2.30)	38.7 (1.45)
	Cansten® cream	42.0 (3.46)	53.0 (2.00)
Bases	1	__	29.3 (1.45)
	3	__	16.8 (1.75)

Formulation 1; extract in Sodium laurylmonosterin creabm base; form 2, in Macrogol cream base; form 3, in Macrogol blend Ointment base; form 4, in Simple ointment base; form 5, White petrolatum BP; form 6, in Polyethylene glycol bases (PEG 400: PEG 2000, 1:1); form 7; in Polyethylene glycol bases (PEG 400: PEG 2000, 1:3).

From the study on the fractions, it was observed that the activity was more from the non-polar components, making the release from Simple Ointment and White Petrolatum bases nil. This may be because of the fact that the agar medium is hydrophilic and hence, hydrophobic ingredients stay rather longer time in the base than diffusing into the hydrophilic surrounding environment. Even though there seem to have also hydrophilic components with antibacterial activities, the amount released from those bases might have been too small to elicit inhibitory action.

The antifungal activities of the formulations from the extracts, especially from *M. lanceolta*, were comparable with that of the marketed products, at the tested level. Hence, this could be taken as a stimulus to work further on these plants and develop refined dosage forms which may find their way into herbal therapy of skin diseases.

As can be seen from the *in vitro* tests of the marketed products, the various formulations of ketoconazol showed similar activity against fungi. But there were slight differences in activity between different formulations of clotrimazol against the effect on *T. mentagrophyte*; the least activity was obtained from Sha Hsien Thin crem. This was, in a way, a comparative study of the different brands of locally available marketed products. Similarly, the comparative antibacterial activities of marketed formulations are summarized in Table 3.11.

Application of *in vitro* release testing in drug development and its value in topical drug products quality assurance has been discussed. Consensus reached at this workshop was that *in vitro* release methodology is based on sound scientific principles and is of value in assessing product quality. However, it should not be used to compare fundamentally different formulations such as creams, ointments and jells. Furthermore, it was noted that release rate by itself should not be

used as the only measure of bioavailability/ bioequivalence. Nonetheless, *in vitro* release tests can serve as a valuable tool for initial screening of experimental formulations during product development and signals possible bioequivalence or bioinequivalence. *In vitro* release testing may be useful as a quality control tool to assure batch-to-batch uniformity, just as the dissolution test is used to assure quality and performance of solid oral dosage forms [143]. Therefore, the current study was designed to assess the *in vitro* activity of the formulated plant materials as compared to different formulations available on the local market.

3.5 Preliminary semi-quantitative standardization of the extracts of *A. pedata* and *M. lanceolata*

In some countries, safety and efficacy proven standardized herbal drugs can be registered as conventional drugs, i.e., as synthetic or pure drugs. Germany, for instance, has provisions in the drug law to consider herbal drugs as conventional ones. As a result, over the last 50 years Germany has been successful in integrating traditional medicine into modern medicine. Medical practitioners prescribe herbal drugs intensively and there is an increase in interest of patients in herbal drugs. But, the criteria for the quality proof of herbal drugs differ from country to country. Hence, steps have been taken towards a global harmonization of the guidelines such as the International Federation of Pharmaceutical Manufacturers Association (IFPMA), which represents the global research based pharmaceutical industry. The association aims to ensure the same standards of safety, quality, and efficacy for new medicines and more efficient registration for worldwide use. These endeavors are handicapped when the active principles are only partly known or when a mixed preparation contains several raw drugs or extracts. In such cases several approaches could be used [199].

The process of standardization, as it has been described, ranges from quality authentication to quantification of active principle. For quality authentication purpose, genetic make-up inspection is used; as the genetic make-up of herbal species does not vary with their physical form, physiological and external conditions. With the advances in molecular biotechnology in the past decades, different DNA manipulation techniques have been developed and can potentially be used in authentication. The methods developed include: low-Cot DNA fingerprinting, randomly amplified polymorphic DNA (RAPD) or arbitrary primed polymerase chain reaction (AP-PCR) and PCR restriction fragment length polymorphism (PCR-RFLP) [200-202].

The most common and appropriate way to document the quality as a standard is fingerprinting chromatogram of the extract or preparation obtained by HPLC, GC, and TLC. This fingerprinting should demonstrate identity and purity of a drug and should guarantee the therapeutic equivalence of extracts from the same herbal drug. There are also other indirect methods of standardizing quality and quantity of herbal extracts. These may include: solvent extractive value, ash values, water and volatile matter determination, determination of volatile oil content, bitterness value, hemolytic activity, tannins content, swelling index, foaming index, pesticide residues, and radioactive contamination. The evaluation of these parameters gives additional idea about the specific characteristics of crude drug under examination and hence, is used as a means of semi-quantitative preliminary standardization modalities [21, 49]. In this study, some of these quality-controlling exercises have been undertaken as a means of standardization work.

3.5.1 Determinations of solvent extractive and ash values

3.5.1.1 **Solvent extractive values**

Determination of solvent extractive values involves ascertaining the amount of active constituents in a given amount of medicinal plant material when extracted with that solvent. It is commonly employed for materials for which no chemical or biological assay method exists. The extraction of any crude drug with a particular solvent yields a solution containing different phytoconstituents. The composition of these constituents in that particular solvent depends on the nature of the drug and the solvent used. In this study, water and absolute ethanol were used as extracting solvents and the result of the extraction of *A. pedata* and *M. lanceolata* are given in Table 3.13.

As shown in the Table, in both cases, the yields of ethanol were higher than that of the water, showing that penetration power of organic solvents is higher.

Table 3.13: Water and ethanol extractive values of *A. pedata* and *M. lanceolata*

		Yield (of 5g)	Percent yield
Water extractive value	*A. pedata*	0.233	4.66
	M. lanceolata	0.262	5.24
Ethanol extractive value	*A. pedata*	0.401	8.02
	M. lanceolata	0.433	8.66

Successive extraction is also another parameter, which may give better clue about the characteristics of the crude plant material. Since the extraction is successively made by solvents

of various polarities, phytoconstituents are preferentially concentrated in either of the solvent systems. In this work; petroleum ether, chloroform, acetone, methanol and water were used as extractants and the results of this test is given in Table 3.14. Maximum yield was obtained from methanol fraction of *A. pedata* and from water fraction of *M. lanceolata,* where as the minimum yield was obtained from acetone fraction of both plants.

Table 3.14: Yields obtained upon the successive extraction of *A. pedata* and *M. lanceolata*

Fraction	Yield (of 5g)		Percent yield	
	A. pedata	*M. lanceolata*	*A. pedata*	*M. lanceolata*
Petroleum ether	0.160	0.228	3.20	4.56
Chloroform	0.169	0.218	3.38	4.36
Acetone	0.064	0.028	1.28	0.56
Methanol	0.269	0.326	5.38	6.52
Water	0.230	0.232	4.60	6.64

3.5.1.2 Ash values determination

Ash value determination is another method of getting information on standardization of plant materials. The ash of any organic material is composed of their non-volatile inorganic components. Controlled incineration of crude drugs results in an ash residue consisting of an inorganic material. In certain medicinal plants, the percentage variation of the ash values from sample to sample of the same plant is very small and any marked difference indicates a change in quality of that medicinal substance. Different forms of ash could be determined based on the

treatment in which the plant material has undergone. In this work, total ash, acid insoluble ash and water-soluble ash were determined and the results are shown in Table 3.15.

Total ash indicates the total amount of material produced after complete incineration that usually consists of carbonates, phosphates, silicates and silica. As indicated in the Table, total ash value of *M. lanceolata* is 9.32% and that of *A. pedata* is 9.98%. Ashing involves an oxidation of the components of the products and a higher than normal value is an indication of contamination, substitution, or adulteration of herbal drug products.

Table 3.15: Ash values of *A. pedata* and *M. lanceolata*

Ash type	*M. lanceolata*			*A. pedata*		
	Sample(g)	Ash (g)	% ash	Sample (g)	Ash (g)	% ash
Total	3.0028	0.27975	9.320	3.0807	0.3171	9.98
Acid insoluble	3.0028	0.01960	0.652	3.0807	0.0741	2.41
Water soluble	3.0028	0.10910	3.640	3.0807	0.0671	2.18

When total ash is treated with HCl, portion of it is dissolved and the insoluble part is left undissolved. This acid insoluble ash is frequently necessary for evaluation of crude drugs that may indicate contamination with siliceous materials such as sand. Commonly, values of acid insoluble ash for medicinal plants range from 0.5% to 2%, but there are cases where such values could be as much as 12%. The acid insoluble matter of *M. lanceolata* was found to be 0.65% and that of *A. pedata* was 2.41%, the latter being slightly greater than the upper limit in the common

range. Likewise, water-soluble ashes are determined that may indicate whether previous extraction of the water-soluble salts in the plant materials have been undertaken or not.

3.5.2 TLC analysis of *A. pedata* and *M. lanceolata* (TLC fingerprinting)

Traditionally, TLC has been widely used for the analysis of medicinal plants and it is included as a method for identification in monographs of herbal drugs in most Pharmacopoeias throughout the world. For example, in the European Pharmacopoeia, TLC is mentioned as a primary tool for identification as part of monographs on all medicinal plants, most extracts and synthetic drugs. But the general method descriptions for TLC are unusually unspecific and leave much room for individual decisions. Hence, the results obtained with such methods may vary considerably, making it less reproducible. Hence, there is a general trend of considering other reliable methods such as HPLC. Yet, TLC offers a number of advantages that may range from identification of herbal drugs to stability study of the extracts and finished products. In addition, TLC also provides chromatographic drug-fingerprints. It is therefore suitable for monitoring the identity and purity of drugs. The time required for the demonstration of most of the characteristic constituents of a drug by TLC is very short, which is another justification for its wide applicability.

Evaluation of TLC could be qualitative, in which case the aim is simply to identify the individual group of substances. The R_f values indicate the position at which a substance is located in the chromatogram. But reliable identification is only possible by using spectroscopic evaluation alongside TLC. The semi-quantitative evaluation involves several concentration of the substance of interest in the TLC, and evaluation could either be conducted visually or by measurement of the spot diameters or areas. But in quantitative evaluation, the substance after developing the

chromatogram will either be dissolved out of the layer and subjected to further study or evaluated directly on the layer by scanning track-by-track densitometrically. Peak heights and areas are taken as comparison modalities. For better resolution, less band broadening and reproducibility across the plate, HPTLC is employed. Quite many drugs are evaluated by this method [203-206].

The results of chromatographs of *A. pedata* and *M. lanceolata* are given in Table 3.16 and 3.17. Data are displayed as chromatograms with respect to spots observed visually under UV at both 254 and 366 nm. The spots observed after spraying with vanillin in sulfuric acid (1% W/V), were also recorded. The photographs of the chromatograms of the various successive fractions of these two plants are displayed in Fig 3.1- 3.3.

The petroleum ether extract of *M. lanceolata* has resulted into a total of ten bands following various treatments having an R_f values ranging from 0.2 to 0.93. The chloroform, acetone, and methanol fractions of the same plant resulted in a total of 7, 8 and 6 bands with an R_f values ranging from 0.04–0.83, 0.21–0.73, 0.33–0.65 respectively. The 80% methanol extract has also resulted with 14 bands with R_f values ranging from 0.07 t0 0.94. Similarly, the petroleum ether, chloroform, acetone, methanol fractions and total extract of *A. pedata* have resulted in a total of 4, 9, 14, 12 and 7 bands, respectively. The R_f ranges were 0.2 –0.93, 0.04 – 0.83, 0.21 – 0.73, 0.33 – 0.65, and 0.07 – 0.94, respectively. These spots could be either made of pure compound or mixtures of similar compounds. But extracts from natural products are commonly mixtures of large number of components. Therefore, the bands were most likely from mixtures of relatively similar components. These bands together with the photographs of the successive fractions are assumed to characterize the plant material better and hence, can serve as a starting point for complete quantitative standardization.

Table 3.16: TLC analysis by visual inspection, irradiation under UV and vanillin-sulphuric acid spray reagent of the different fractions of *M. lanceolata*

No.	Inspection	R_f values of the fraction				
		Pet. Ether (S_f = 13.5)	Chlor. (sf = 13.5)	Acet. (S_f = 13.1)	MeOH (S_f = 13.8)	T. extract (S_f = 13.8)
1	Visible	0.69 (P)	0.27 (LG)	0.23 (LY)	0.57 (LY)	0.42 (LY)
	254nm	0.31	0.27	0.21 (LV)	0.54 (LV)	0.42
	366nm	0.8 (R)	0.27 (R)	0.41 (LV)	0.47 (LV)	0.47 (LV)
	After spraying	0.2 (LV)	0.04 (LV)	0.21 (LY)	0.33 (FB)	0.07 (P)
2	Visible	0.81 (G)	0.76 (VLG)	0.38 (LY)	__	0.58 (LY)
	254nm	0.69	0.77	0.37 (LV)	0.65 (LV)	0.57
	366nm	0.84 (R)	0.70 (R)	0.76 (LV)	0.57 (LV)	??
	After spraying	0.52 (L.V)	0.07 (LV)	0.37 (LY)	0.37(FB)	0.27 (FB)
3	Visible	0.83 (G)	0.83 (DG)	0.52 (LY)	__	0.67 (LY)
	254nm	0.77	0.83	0.49(LV)	__	0.64
	366nm	0.87 (R)	0.76 (R)	__	__	0.58 (LV)
	After spraying	0.61 (FB)	0.2 (LV)	0.49 (LY)	0.53 (LY)	0.37 (FB)
4	Visible	0.85 (LG)	__	__	__	0.94 (G)
	254nm	0.84	__	0.53 (LV)	__	0.68
	366nm	0.93 (R)	0.81 (R)	__	__	0.80 (LV)
	After spraying	0.84 (G)	0.27 (G)	0.62 (LP)	__	0.54 (LP)
5	Visible	__	__	__	__	__
	254nm	0.88	__	0.61 (LV)	__	0.73
	366nm	__	__	__	__	0.94 (R)
	After spraying	0.84 (IV)	0.83 (G)	0.73 (P)	__	0.75 (P)
6	Visible	__	__	__	__	__
	254nm	__	__	0.73 (LV)	__	__
	366nm	__	__	__	__	__
	After spraying	__	__	__	__	0.89 (DG)

Sf, solvent front; P, pink; R, red; LG, light green; LV, light violet; G, green; FB, fed black; IV, intense violet; VLC, very light green; DG, dark green; LY, light yellowish; LP, light pink; DB, dark black,T, total

Table 3.17: TLC analysis by visual inspection, irradiation under UV and vanillin-sulphuric acid spray reagent of the different fractions of *A. pedata*

No.	Inspection	R_f values of fractions				
		Pet. Ether (S_f = 13.5)	Chloroform (S_f = 13.5)	Acetone (S_f = 13.1)	Methanol (S_f = 13.1)	T. extract (S_f = 13.1)
1	Visible	__	0.27 (VLG)	__	__	0.97 (G)
	254nm	0.66 (G)	0.26 (LG)	0.41 (L V)	0.14(LV)	0.39 (LV)
	366nm	__	0.70 (R)	0.50(L.V.)	0.47(LV)	0.47(LV)
	After spraying	0.62 (FB)	0.04 (LV)	0.24 (DB)	0.16 (DB)	0.15 (FB)
2	Visible	__	0.79 (LG)	__	__	__
	254nm	0.76 (G)	0.78(LG)	0.53 (LV)	0.21 (LV)	0.54 (LV)
	366nm	__	0.79 (R)	0.64 (LV)	0.58 (LV)	-
	After spraying	0.77 (FB)	0.23 (LV)	0.56 (LV)	0.24 (DB)	0.25 (FB)
3	Visible	__	0.83 (G)	__	__	__
	254nm	__	0.83 (G)	0.60 (LV)	0.39 (LV)	__
	366nm	__	0.83 (R)	0.69 (LV)	0.66 (LV)	__
	After spraying	0.84	0.27 (LV)	0.60 (LY)	0.68 (LY)	0.84 (V)
4	Visible	__	__	__	__	__
	254nm	__	__	0.65 (LV)	0.54 (LV)	__
	366nm	__	__	0.77 (LV)	0.76 (LV)	__
	After spraying	__	0.31 (LV)	0.69 (V)	__	__
5	Visible	__	__	__	__	__
	254nm	__	__	0.73 (LV)	0.60 (LV)	__
	366nm	__	__	__	__	__
	After spraying	__	0.55 (LV)	0.73 (LP)	__	__
6	Visible	__	__	__	__	__
	254nm	__	__	0.82	__	__
	366nm	__	__	__	__	__
	After spraying	__	0.83 (G)	0.84 (V)	__	__

Sf, solvent front; P, pink; R, red; LG, light green; LV, light violet; G, green; FB, fed black; IV, intense violet; VLC, very light green; DG, dark green; LY, light yellowish; LP, light pink; DB, dark black; T, total

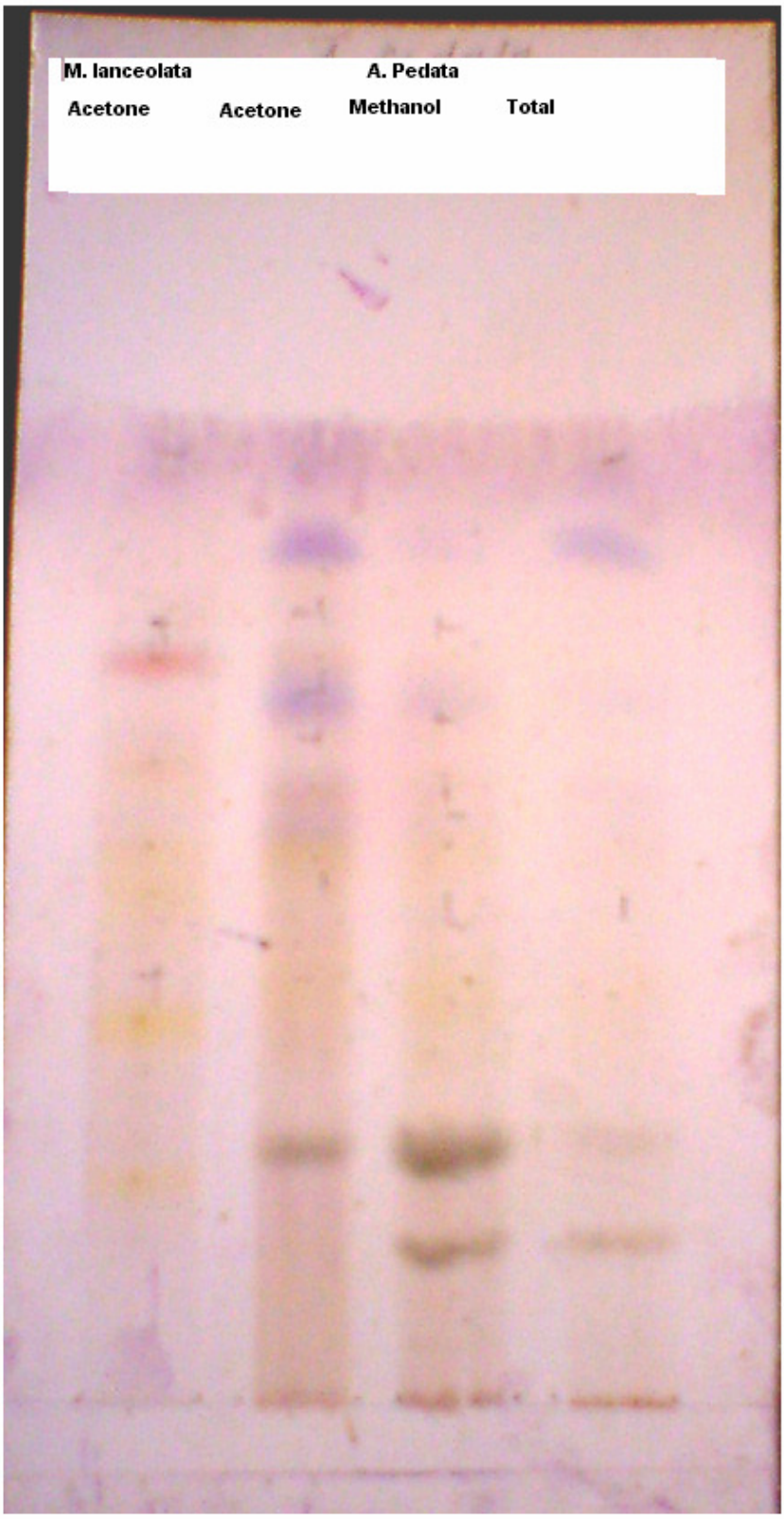

Figure 3.1: TLC chromatogram of the acetone, methanol and total extracts of *A. pedata* and the acetone extract of *M. lanceolata* after treatment with vanillin-sulphuric acid spray reagent

Figure 3.2: Thin Layer Chromatogram of the petroleum ether and chloroform extracts of *A. pedata* and that of *M. lanceolata* after treatment with vanillin-sulphuric acid spray reagent

Figure 3.3: Thin Layer Chromatogram of the methanol and total extracts of *M. lanceolata* after treatment with vanillin-sulphuric acid spray reagent.

4 Conclusions

Correlation of the *in vivo* performances of chemical entities with the *in vitro* activity is dependent on how much the *in vivo* environment is effectively simulated into *in vitro* testing conditions. But commonly, the *in vitro* experiments are short of many aspects of the *in vivo* conditions. For example, the precise nature of Candia's pathogenicity is uncertain; it is probably a combination of factors including adherence, proteinase activity and hyphal production [207, 208]. In addition, the characteristics of candida's pathogenicity are notoriously unpredictable due to genetic instability, its dimorphism and differences between strains [209]. Thus, it is a bit difficult to extrapolate from laboratory experiments to clinical situations. It is quite conceivable that a remedy showing no inhibition *in vitro* could have a significant effect *in vivo* by, for example, modifying the adherence characteristics of the fungus to the host epithelium. This would obviously not be detected in the laboratory by simple inhibition tests. Conversely, however, it is difficult to envisage how an extract showing significant inhibition in simple *in vitro* tests might not have a broadly similar therapeutic effect *in vivo*. Therefore, taking this into consideration the following can be drawn as conclusion of this work.

Almost all the screened plants exhibited both antibacterial and antifungal activities, showing that research undertakings on natural products result into useful outcomes if they start from the already existing indigenous knowledge, either from the community or healers as opposed to simple random screening programs. The activities demonstrated in this work provide the scientific justification for the traditional claims of the plants for various skin conditions.

From the six screened plant materials, the antibacterial activity of *A. pedata* and the antifungal activity of *M. lanceolta* were very good and seem to give reasonable stimulus to further develop them into better phytopharmaceuticals, which can be utilized clinicaly.

In general, the antimicrobial activities of *A. pedata* and *M. lanceolata* could be because of their components, which may include flavonoids, phenolic glycosides, polyphenols and phytosteroids for *A. pedata* and, flavonoids, saponins, polyphenols and phytosteroids for *M. lanceolata.* Much of the antibacterial and antifungal activities of *A. pedata* are because of the non-polar fractions than the polar one. Hence, phytosteroids seem to have contributed a lot to the activity of this plant. Where as, the antibacterial activity of *M. lanceolata* is distributed among the various fractions. This indicates that there could be mixtures of compounds with different polarities that have similar activity. But the antifungal activity of *M. lanceolata* was exclusively because of the polar components.

Besides their antimicrobial activities, *A.pedata* and *M. lanceolata* also have very good anti-inflammatory activities, the latter being superior in this respect. This is an added advantage for their wound healing properties.

Formulation and activity testing of the dosage forms revealed that especially the antifungal activities of the extracts were comparable to that of the marketed products, at the tested level, indicating that these plant extracts will have clinical application upon further development. Furthermore, cream bases were efficient in terms of releasing the active drug from the formulated dosage forms and hence, they can be the very likely vehicle for further development, particularly the polyethylene glycols.

5. Suggestions for further works

Based on the present work the following are forwarded as suggestions for further work:

1. Further investigation should be made to get better activity profile of the tested plant extracts besides the antimicrobial activities. Such tests may include antioxidant and antiviral activities.
2. The antimicrobial screening work should also be conducted on as much strain as possible; especially on clinically important strains to decide the clinical utility of these plant extracts.
3. Further antibacterial and antifungal activities guided fractionation and isolation work should be done on the non-polar fraction of *A. pedata* and the polar components of *M. lanceolata* to get better results.
4. Upon reduction of the dose, both the extracts of *A. pedata* and *M. lanceolata* showed better anti-inflammatory activities. Therefore, testing this activity at small doses may be necessary to characterize the activity profile and dose dependency of the anti-inflammatory properties of these extracts.
5. With regard to the standardization work, it is necessary to perform the experiment with at least two or three batches of the extracts from different time periods to confirm reproducible processing and to guarantee the same efficacy of the preparation. Moreover, further quantification tasks should be conducted which utilizes various approaches such as standardizing the plant extracts with marker compounds or using isolated active substance.
6. The *in vitro* performances of some of the formulated dosage forms were good. But, further works should be undertaken to formulate the extracts at different concentrations and try the *in vivo* performances on animal models.
7. It is worth attempting further tasks like skin sensitivity testing, other toxicity studies and compatibility studies of bases employed for the preparation of the various dosage forms.

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