

**STUDIES ON EXTRACTS OF SOME MEDICINAL PLANTS
TRADITIONALLY USED FOR DERMATOLOGICAL
DISORDERS IN ETHIOPIA**

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LIST OF ACRONYMS

ATCC	American Type Culture Collection
ACV	acyclovir
AIDS	acquired immunodeficiency syndrome
CAM	complementary/alternative medicine
DACA	Drug Administration and Control Authority
EHNRI	Ethiopian Health and Nutrition Research Institute
HIV	human immunodeficiency virus
HSV	Herpes simplex virus
ICME	undefatted, 80 % methanol extract of <i>I. confertiflora</i>
MEST	the mouse ear swelling test
MIC	minimum inhibitory concentration
MRSA	methicillin resistant <i>S. aureus</i>
PEE	petroleum ether extract
SL	sesquiterpene lactones
TM	traditional medicine
TLC	thin layer chromatography
UV	ultra violet
VZV	varicella zoster virus
WHO	World Health Organization

ABSTRACT

Key words: traditional medicine, medicinal plants, antimicrobial activities, anti-inflammatory test, skin sensitization test, topical formulations.

The majority of the populations in the developing world rely on traditional medicine for their primary healthcare needs. Herbal therapy predominates in traditional medical practices as well as in complimentary/alternative medicine practiced in the developed world. Among the indications where traditional herbal medicines are used, skin and skin related disorders, which also happen to be common diseases in the communities, rank among the top. This study had the objective of evaluating the extracts of four medicinal plants traditionally used for skin diseases, namely *Inula confertiflora*, *Clematis simensis*, *Zehneria scabra* and *Pycnostachys abyssinica*, for some of their claimed activities by both *in vitro* and *in vivo* methods.

The 80 % methanol extract of the dried, ground plant materials was prepared. The plant extracts were then tested for antimicrobial activity against common bacterial and fungal pathogens by the agar well diffusion method. Furthermore, the 80% methanol extract of *I. confertiflora* was subjected to minimum inhibitory concentration (MIC) determination, *in vivo* studies such as anti-inflammatory and skin sensitization tests as well as *in vitro* tests such as preliminary screening for the presence of some plant constituents, TLC analysis, and evaluation of topical antimicrobial formulations of the plant extracts.

The results of the study indicated all of the plant extracts to exhibit antimicrobial activities against one of the most common bacterial pathogens, namely *Staphylococcus aureus* (ATCC).

Although these activities were not impressive especially as compared to the positive control used, they lend some credibility to the traditional uses of the plants. Good antifungal activity was demonstrated by one of the plant extracts (*I. confertiflora*) against *Trichophyton mentagrophytes*, which was further corroborated by the agar dilution method. *I. confertiflora* (80% methanol) extract proved to exert a good anti-inflammatory activity at a dose of 1000 mg/kg but not at a lower dose (500 mg/ml) in the carrageenan-induced paw edema test. These activities support the traditional use of this plant. Furthermore, the 80% methanol extract of *I. confertiflora*, was not found to be a skin sensitizer in the mouse ear swelling test as opposed to its petroleum ether counterpart, which demonstrated a strong sensitizing property. Some secondary metabolites such as sesquiterpene lactones and flavonoids were detected, which may be responsible for some of the demonstrated pharmacological activities of this plant. Evaluation of topical formulations of the 80% methanol extract of *I. confertiflora* demonstrated that the hydrophilic formulations exhibited higher antimicrobial activities compared to the lipophilic formulations. The activity of the hydrophilic formulations against *T. mentagrophytes* was comparable to the commercially available antifungal products tested. These bases could thus be used as a starting point for further formulation studies.

1. INTRODUCTION

1.1 Overview on traditional herbal medicine

The term “traditional medicine” refers to ways of protecting and restoring health that existed before the arrival of modern medicine. Thus, in all countries of the world there exists traditional knowledge related to the health of humans and animals. The importance of traditional medicine (TM) as a source of primary healthcare was first officially recognized by the World Health Organization (WHO) in the Primary Health Care Declaration of Alma Ata (1978) and has been globally addressed since 1976 by Traditional Medicine Program of the WHO. That program defined TM as: “the sum total of all the knowledge, skills and practices, whether explicable or not, based on the theories, beliefs and experiences indigenous to different cultures, and used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical, mental or social imbalance. The terms complementary/alternative/non-conventional medicine are used interchangeably with TM in some countries, although these latter terms more appropriately refer to a broad set of health care practices that are not part of the country’s own tradition and are not integrated into the dominant health care system [1,2].

The WHO estimates that about 80% of the population living in the developing countries relies almost exclusively on TM for their primary health care needs. The wide spread use of TM among both rural and urban population could be attributed to cultural acceptability, physical accessibility and economic affordability, as well as efficacy against certain types of diseases, as compared to modern medicine [3,4]. In practice, TM may include the following components: acupuncture, traditional birth attendants, mental healers and herbal medicine. Surgery, bleeding, bone setting,

dentistry, as well as use of thermal waters are also among those mentioned in African literatures [5-8].

On the other hand, complementary/alternative medicine (CAM) is being increasingly used in the developed countries with prevalence as high as 65%. The most popular therapies used include herbal therapy, chiropractic, relaxation techniques, vitamin therapy as well as massage therapy. People seemed to be motivated to use CAM largely because they had misgivings about pharmaco-therapy in particular and the conventional system in general. Other considerations include minimization of the risks of adverse effects of synthetic drugs and also on account of the increasing cost of personal health maintenance [9-11]. WHO estimates state that 50% of Canadians and 75% of people in France have tried CAM, which often includes herbal remedies. In Japan, 85% of doctors prescribe not only modern medicine but also the traditional herbal medicine (called Kampo), which is covered by health insurance [12].

In fact, herbal medicine has been found to be among the most popular therapy in complementary/alternative medicine, representing a market value of about US\$ 43 billion a year. In the United States alone, over 1500 herbal medicines are sold annually for a total of nearly US\$ 5 billion and now constitute the fastest growing sector of the US pharmaceutical market. It is estimated that Europe, annually imports about 400,000 tons of medicinal plants with an average market value of US\$ 1 billion from Africa and Asia. China with exports of over 120,000 tones p.a. and India with some 32,000 tones p.a. dominate the international market [10-12].

Medicinal plants also play a major role and constitute the backbone of TM practices. Indian Materia Medica includes about 2000 drugs of natural origin almost all of which are derived from

different traditional system and folklore practices. Out of these drugs derived from traditional system, 400 are of mineral and animal origin while the rest are of vegetable origin [2, 3]. The materia medica of the ancient African healers, on the other hand consisted of mixtures of various herbs, animal parts and clays. The Ebers Papyrus, one of the oldest in medical literature, listed several recipes used by ancient African healers and the list was dominated by numerous food plants, in keeping with the belief at that time that “every disease to which men are liable is occasioned by the substances whereon they feed” [6]. Studies done locally have also demonstrated herbal medicine therapy to predominate over other forms of TM [4, 13-16].

The growing popularity of herbal remedies is fuelling and is to some extent fuelled by increasing scientific interest in herbal medicine. WHO estimates that of the 35, 000 – 70,000 species of plants that are used for medicinal purposes around the world, some 5000 have been submitted to biomedical scrutiny. Furthermore, scientific evidence of efficacy is beginning to emerge from randomized, controlled trials in which herbs compare favorably with placebo. Examples include Saint John’s wort for mild depression, ginkgo biloba for some forms of dementia, saw palmetto for benign prostatic hyperplasia, horse chestnut seeds for chronic venous insufficiency, Echinacea preparations for colds, and garlic for cholesterol lowering, to mention a few [12, 17, 18].

Of the many indications where traditional herbal medicines have been used, skin and skin related disorders rank among the top where up to one-third of these TM compared to 1-3% of modern drugs are used for treatment of wounds or skin disorders [13, 19]. Indeed, skin disorders are among the most prevalent in the world. Prevalence studies done locally have also observed similar trends where skin diseases have been identified among the leading causes of hospital visits with even more unreported cases [20, 21].

1.2 Prevalence of cutaneous diseases in developing nations

Skin diseases are amongst the most common causes of morbidity in rural and urban areas of developing countries accounting for a high proportion of visits to healthcare centers. For instance, a study done to determine the prevalence of skin diseases in a rural community in the southwestern part of Ethiopia found more than half of all the households and 80% of the selected children to manifest one or more skin diseases [21, 22]. This high prevalence is similar to the results obtained in a rural Tanzanian community and that done on primary school children in Turkey [23, 24].

Prominent skin conditions identified in cities and rural areas of Ethiopia, which also happen to show similar trends to a study in Tanzania include, parasitic infestations (e.g., scabies, pediculosis), bacterial (e.g., impetigo), fungal (e.g., dermatophytoses, candidiasis) and viral (e.g., herpes, warts) infections as well as inflammatory diseases (e.g., atopic dermatitis, contact dermatitis, seborrhoeic dermatitis) [20-23, 25-29]. Some of the prevalent skin diseases along with the conventional treatments are briefly reviewed.

1.3 Common cutaneous diseases

1.3.1 Cutaneous infections

The normal skin of healthy subjects is very resistant to invasion by most microorganisms. Infection hence develops when the right combination of causative factors exists and a particular microorganism usually represents only one of the etiologic agents. There are almost always a number of interacting causes for infection of any body tissue, some direct, some indirect, which create circumstances leading to infection and aid in its persistence [30].

Bacterial skin infection

Cutaneous bacterial infections may be divided into primary and secondary types. Primary infections tend to have a characteristic morphology and course, are incited initially by a single organism, and arise in normal skin. Secondary infections, on the other hand originate in diseased skin as a superimposed condition, and this results in an acute or chronic intermingling of the underlying disease; the infection may not follow a characteristic course, and the role that bacteria are playing may be difficult to assess [30]. Primary infections are most frequently incited by *Staphylococci*, especially *Staphylococcus aureus*, as well as *Streptococci*, mainly group A *Streptococci* [31-35]. These are also the most common invaders in secondary infections, but gram-negative organisms also often colonize dermatitic skin, though they do not frequently produce true secondary infection except in special locations such as the external ear, or in certain types of chronic lesions. Some of the most common bacteria isolated include *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis* and *Bacteroides fragilis* [36-42].

Management

Topical antibacterial agents offer a useful alternative to systemic agents in certain circumstances. Uses include prophylaxis of infection for burns, traumatic wounds, and intravascular catheters, as well as eradication of *S. aureus* nasal carriage and treatment of primary and secondary infections [43-46]. Commonly used agents include mupirocin, tetracycline, gentamicin and fusidic acid. Choice of antimicrobial is made according to the situation at hand. For example, topical antimicrobials of choice in case of burn wounds include bacitracin, neomycin, silver sulfadiazine and mafenide. On the other hand, foul smelling anaerobically infected pressure sores and leg ulcers have been successfully treated by topical metronidazole preparations [47, 48].

Nevertheless, topical antibiotics should only be used short term because of the risks of bacterial resistance and contact allergy [46, 49]. Systemic toxicity is also a possibility; for example the topical use of neomycin in patients with extensive damage may result in deafness. Cutaneous infections that are widespread and that are accompanied by systemic signs (such as fever, malaise) are among those conditions where a systemic therapy is indicated [30, 47, 50].

An antistaphylococcal penicillin such as flucloxacillin is usually indicated where the pathogen is *S. aureus*, but erythromycin is a suitable alternative in penicillin-allergic patients [47]. Successful treatment in perioperative antibiotic prophylaxis as well as in the management of infections in surgical and chronic wounds, which are characterized by a polymicrobial aerobic-anaerobic micro flora, is by the use of broad-spectrum systemic antimicrobial agents [51]. There are increasing reports, however, of high resistance rates to commonly used antimicrobials among the frequent bacterial pathogens [52].

Fungal skin infections

Superficial fungal infections can and do occur in both healthy and compromised individuals. The most vulnerable hosts belong to several high-risk groups characterized by specific life-styles or intercurrent diseases. Cutaneous mycoses are the result of one or more pathogenic or opportunistic organisms that belong to the groups of dermatophytes, yeasts and non-dermatophytic moulds, the most common fungal skin infections being the dermatophytoses, pityriasis versicolor and candidiasis [47, 53-56].

Dermatophytoses (ring worm, tinea) are infections by dermatophytes, a group of fungi, which include soil-dwelling organisms, and human and animal pathogens from 3 genera - *Microsporum*, *Trichophyton* and *Epidermophyton*. These genera consist of many species showing different propensities to affect various body sites. Dermatophytic infections are generally restricted to the non-living cornified layers of the skin, hair and nail and are traditionally named according to the anatomic location of the infected body surface. The major types are tinea barbae, facie, capitis, corporis, cruris, pedis, manuum and unguium. The most frequently isolated causative dermatophytes are *T. rubrum*, *T. mentagrophytes* and *E. floccosum* in case of tinea pedis while *M. canis*, *T. mentagrophytes*, *T. rubrum* as well as *T. violaceum* are common causative agents in case of tinea capitis [27, 53, 57-60].

Candidiasis, the general term for pathogenic infection with *Candida*, can be divided into superficial mucocutaneous infection, deep local infection, or dissemination. *Candida* infections of the skin and nails are one of the most common infections worldwide. *Candida* species are commonly found in the human gastro-intestinal tract, mouth and vagina. Predisposition factors for pathogenic *Candida* infection include prolonged antibiotic therapy, steroid therapy, diabetes mellitus, skin trauma and immunodeficiency, with the most severe infections now occurring in patients with AIDS. *C. albicans* has been identified as the most common etiologic agent although other species such as *C. parapsilosis* and *C. tropicalis* are also common [47, 61-63].

Ptyriasis versicolor is a superficial infection caused by the commensal yeast *Malassezia furfur*. It is more common in tropical than in temperate latitudes and sun exposure may trigger the infection. Immunocompromised patients, including those receiving corticosteroids, those patients

on antibiotic therapy as well as diabetic patients are predisposed to this infection. Common sites of infection are the back, arms, face and trunk [64-67].

Onychomycosis is a common fungal infection of the nails. Infections of the nail vary greatly in their manifestation, from changes that are hardly detectable to the full-blown infection in which the nail plate is almost completely disintegrated [30, 55]. Causative organisms belong to the groups of dermatophytes, yeasts and nondermatophytic moulds although the role of nondermatophytes is controversial. The majority of toenail infections are caused by dermatophytes; *T. rubrum* and *T. mentagrophytes* being isolated with the greatest frequency [68, 69]. In infections of fingernails, *Candida* species (*C. albicans* being predominant) can be isolated as frequently as the dermatophytes [47, 70-73].

Management

The management of mild, localized, superficial mycotic infection begins with topical therapy. Nonspecific agents that have a long history of topical use include benzoic acid, crystal violet, selenium sulphide, tolnaftate and salicylic acid. A major breakthrough in efficacy came with the introduction of synthetic azole derivatives including ketoconazole, miconazole, econazole and clotrimazole. Other unrelated products such as ciclopirox, amorolfine, terbinafine and haloprogin also show good antimycotic activity [47].

Oral antifungals are indicated or required to treat hyperkeratotic areas such as nails, palms, and soles, patients with disabling or extensive disease, patients intolerant to or who have failed topical therapy, those with chronic infection, those with granulomatous lesions and patients immuno-suppressed by disease or by therapy. These oral drugs active against common fungal

skin infections belong to the imidazoles (ketoconazole), triazoles (fluconazole, itraconazole) and allylamines (terbinafine). However, even with the new systemic antifungals (itraconazole, terbinafine) significant proportion of patients are not cured and those who have been successfully treated experienced relapses [46, 47, 74, 75, 76].

Viral skin infections

Herpes simplex infections

Herpes simplex virus (HSV), which is distributed worldwide, is most often classified into serotypes HSV-1 and HSV-2. Transmission is by direct contact with infected secretions, HSV-1 being primarily associated with oral transmission and HSV-2 with genital transmission. Primary infections are usually in the perioral, ocular, or genital areas, but any skin site may be involved if the skin is damaged or in immunocompromised patients. Most primary HSV-1 infections are asymptomatic but may occasionally present as acute gingivostomatitis and pharyngitis. HSV then becomes latent within sensory nerve ganglia from where it can be reactivated by various triggers such as stress, bacterial infection, fever, irradiation (including sunlight), or menstruation. Reactivation leads to a prodromal period before the lesions emerge. Infections commonly recur as herpes labialis, also known as fever blisters or cold sores. Ocular herpes is also generally caused by HSV-1. Genital herpes is usually caused by HSV-2 and tend to be a more severe condition than other herpes simplex infections, especially in women [47, 77, 78].

Varicella zoster infections

Primary varicella-zoster virus (VZV) infection produces varicella (chickenpox), a mild contagious disease, which presents with a characteristic generalized vesicular eruption, fever, and malaise. In temperate countries, it is a typical childhood disease with only 2% of varicella

occurring after the age of 20. On the other hand, a higher incidence of varicella is seen in adults in tropical climates when it can be severe and is potentially fatal in immunocompromised patients. Patients who recover from primary infections with varicella zoster have lifelong immunity against chickenpox. However, a permanent latent infection of sensory nerve ganglia is established, and reactivation produces herpes zoster, which is characterized by painful vesicular eruptions localized to a single dermatome of skin, and sometimes preceded by a prodromal phase with fever, malaise, and headache. Involvement of the trigeminal nerve can lead to sight-threatening ophthalmic herpes zoster. As with chickenpox, herpes zoster is more serious in immunocompromised patients and may be more severe, prolonged, or disseminated. Chronic pain that presents after the rash has healed is termed postherpetic neuralgia (PHN) and occurs in about 10% of patients who have had herpes zoster [47, 79-81].

Management

Currently the most effective and widely used antiviral agent for HSV and VZV infections is aciclovir (ACV). Antiviral therapy for HSV infections, while relieving symptoms and reducing the duration of viral shedding does not prevent recurrences. On the other hand, management of chickenpox in otherwise healthy patients is usually symptomatic and the use of antivirals is not recommended for the treatment of uncomplicated cases. The place of antiviral agents in the treatment of herpes zoster is however well established. Antiviral treatment can reduce pain and potentially decrease the incidence of PHN, minimize complications and propagation of the rash, and reduce viral shedding. In any case, ACV may be given orally, topically or intravenously depending on the severity and nature of the infection. Topical treatment is generally less effective than systemic therapy and is of little use if systemic symptoms predominate [47, 77-79]. Cases of contact dermatitis have also been reported with the topical use of ACV [82]. Resistance to ACV

has been reported but is generally confined to immunocompromised patients, although recurrences may occur during suppressive therapy [83].

1.3.2 Dermatitis/Eczema

These are one of the most common inflammatory skin disorders. The terms “eczema” and “dermatitis” may be used interchangeably, and describe the same clinical and histological entity. Both words are derived from Greek, “eczema” meaning “to boil”: describing the characteristic tiny blisters of the condition and “dermatitis” meaning inflammation of the skin. There are several patterns of the condition, and a common convention is to describe as “eczema” those that are endogenous or constitutional, and as ‘dermatitis’ those that are exogenous or due to contact. Some of the most common types are reviewed [30, 84].

Contact dermatitis (that includes those inflammations due to external agents) is classically broken down into two categories; irritant contact dermatitis (which includes all non-immunologic mechanisms) and allergic contact dermatitis (due to an acquired immunologic response) [30, 84].

A very large number of compounds found in daily life, either at home or at work, may be responsible for allergic contact dermatitis. Common culprits are metals such as nickel, topical medicaments such as betamethasone valerate (a steroidal anti-inflammatory), ointment bases and preservatives, balsams and fragrances, dyes, plants and rubber compounds [30, 84-88]. Irritant contact dermatitis is the commonest cause of hand eczema, and is seen particularly in housework, as well as with oils and greases in industry. Contact with anything that dehydrates the skin, particularly water, detergents and soaps, and degreasers, removes the natural protective oils of the skin, allowing evaporation of water and penetration of irritants. The longer the skin is exposed to such treatment, the more likely is the development of dermatitis [84, 89-91].

Atopic dermatitis is a genetically acquired, chronic relapsing skin condition with its expression principally determined by environmental allergens, infections, as well as skin barrier defects. It is characterized by severe itching, eczematous skin lesions with often distinctive distribution and dryness of the skin [92, 93]. In addition, colonization and secondary infections with *Staphylococci* and *Streptococci* have been observed to exacerbate atopic dermatitis [94, 95]. It affects about 5-20% of children worldwide with prevalence rates in the western world increasing in the last few decades. Symptoms can also persist or begin in adulthood [96, 97].

Seborrheic dermatitis is an erythematous-squamous condition of unknown etiology with a prevalence of approximately 2.5%. It involves the areas of the body with a high density of sebaceous glands, i.e., the face, scalp and upper trunk, and occurs after puberty, when these glands become active. One of the most commonly named pathogenic factor of seborrheic dermatitis is the overgrowth of *Pityrosporum ovale*, yeast that are normal commensal on the skin [84, 98, 99].

Management

Therapy should include avoidance of the allergen and also of agents that dehydrate the skin. The same basic principles of treatment apply regardless of the type of eczema. The mainstay of dermatitis treatment is the use of liberal quantities of moisturizers, as in all types the basic problem is the loss or deficiency of the skin's lipid layer. Adequate use of emollients will reduce the requirement for topical steroid, both in quantity and in potency. As eczema is an inflammatory condition, specific treatment must be anti-inflammatory, which in practice means corticosteroids, normally used topically. As pruritus is often the most distressing feature of

eczema, the patient may benefit from the short-term use of a sedative antihistamine [47, 84, 100-102].

Chronic low-grade infection is common in eczema and dermatitis, and using a steroid alone may exacerbate this. Significant infection is best managed with oral antibiotics, and topical steroid/antibiotic combinations avoided, as the topical antibiotic can prove to be potent sensitizer. Chronic infection can be controlled using a topical steroid/clioquinol combination of appropriate potency, which is antiseptic rather than an antibiotic. In the case of seborrhoeic dermatitis, topical antifungal preparations of imidazoles (such as ketoconazole) are utilized to reduce the population of *P. ovale* on the skin. The disease runs a chronic, relapsing course, and hence regular or intermittent use is usually necessary [84, 94, 103, 104].

1.3.3 Dermatologic manifestations in HIV/AIDS

AIDS is a condition characterized by the development of life-threatening opportunistic infection or malignancies in a patient with severe depression of the T-cell mediated immune system caused by infection with human immune deficiency virus (HIV). There are currently over 34 million people worldwide infected with human immune deficiency virus (HIV) with 15,000 new patients infected each day. The acquired immunodeficiency syndrome (AIDS) pandemic has particularly affected the third world and currently over 70% of those infected reside in sub-Saharan Africa [84, 105, 106].

Infection with HIV may be associated with a large number of clinical manifestations and cutaneous diseases have been observed to be among the most prevalent. In fact, mucocutaneous lesions directly related to human immuno-deficiency virus (HIV) infection usually present as

initial manifestations of immune deficiency. The most common mucocutaneous lesions are Kaposi's sarcoma, histoplasmosis, oro-esophageal candidiasis, oral hairy leukoplakia, and, in Asia, *Penicillium marneffe* infection. In addition, skin lesions such as psoriasis, seborrhoeic dermatitis, and nodular prurigo, may be among the initial presentations in HIV infected patients [107-112]. While some cutaneous findings are nearly exclusive to HIV-seropositive individuals, many are found in the general population. However, HIV-infected individuals often have an increased prevalence or severity, atypical presentations, or difficulty with treatment of the disease [113-115].

1.4 Phytotherapy and skin conditions

The traditional practice of topically treating dermatologic conditions with plant-derived medicines predates the cultures of ancient Egypt and remains vital today in both traditional medical practices as well as complementary/alternative medicine. The use of such medicinal plant extracts for the treatment of skin disorders arguably has been based largely on historical/anecdotal evidence, since there has been limited data available in the scientific literature, particularly with regard to safety/efficacy of plant extracts in controlled trials [19, 116].

Some of the limited works done on the adverse and beneficial effects of medicinal plants on skin and skin disorders are reported in the literature. Beneficial aspects of medicinal plants on skin include: healing of wounds and burn injuries; antifungal, antiviral, antibacterial and acaricidal activity against skin infections such as acne, herpes and scabies; activity against inflammatory/immune disorders affecting skin (e.g. psoriasis) and antitumour promoting activity against skin cancer. Adverse effects of plants on skin include: irritant contact dermatitis caused mechanically (spines, irritant hairs) or by irritant chemicals in plant sap; phytophotodermatitis resulting from

skin contamination by plants containing furanocoumarins, and subsequent exposure to UV light; and immediate or delayed hypersensitivity contact reactions mediated by the immune system in individuals sensitized to plants or plant products. Some of the commonly used medicinal plants will be reviewed in light of the points mentioned above [19, 117].

1.5 Common phytotherapeutics used for skin disorders

1.5.1 Tea Tree Oil

Perhaps one of the most commonly mentioned (and utilized) natural products for topical use is tea tree oil – the essential oil derived from the Australian native plant, *Melaleuca alternifolia*. Tea tree oil is a complex mixture of approximately 100 terpenes and hydrocarbons including 1, 8-cineol, terpinen-4-ol, ρ -cymene, linalool, α -terpinene, γ -terpinene, α -terpineol and terpinolene, the main component being terpinen-4-ol comprising at least 30% of the oil. Tea tree oil exhibits a broad range of pharmacological activities that have been attested by various *in vitro* and *in vivo* studies [118].

Antibacterial activity

One of those activities that has been extensively studied is its promising antibacterial efficacy against the commonly occurring skin and soft tissue pathogens, such as *S. aureus* (including methicillin resistant *S. aureus* or MRSA), β -hemolytic *Streptococci*, *Enterococcus durans*, *E. coli*, *P. mirabilis*, *Enterobacter aerogenes* and *Propionibacterium acne* but not *P. aeruginosa* among the gram-negative bacteria. Studies done to determine the susceptibility of the microorganisms to components of the essential oil have found out the strong activities of terpinen-4-ol followed by those of linalool and α -terpineol [118-125]. Reports of clinical trials,

albeit very few exist that have demonstrated the antibacterial efficacy of tea tree oil preparations [118, 126-128].

Antifungal activity

Tea tree oil is also known to inhibit a wide range of fungi *in vitro* including *Cryptococcus neoformans*, *A. niger*, *A. flavus*, *E. floccosum*, *M. audonii*, *M. canis*, *T. mentagrophytes* and *T. rubrum*. Also the yeasts *M. furfur* as well as *C. albicans* (along with other *Candida* species) have been well inhibited *in vitro* [118, 129-134]. Clinical studies done to examine the therapeutic efficacy of tea tree oil in cutaneous fungal infection have demonstrated the therapeutic efficacies of tea tree oil preparations in the treatment of common fungal infections including tinea pedis, onychomycosis and dandruff [135-139]

Antiviral activity

Among the few reported studies on the antiviral activity of tea tree oil, its activity against HSV is notable. In an *in vitro* study, noncytotoxic concentrations of tea tree oil demonstrated high levels of virucidal activity against HSV-1 and HSV-2 [140]. Anti-HSV activity was evaluated clinically in a controlled trial with the participation of 20 patients affected by recurrent herpes labialis (or cold sores). The study reported some benefit from tea tree oil treatment that was comparable in some aspects to commercially available topical therapies [141].

Miscellaneous activities

Besides anecdotal evidence for its anti-inflammatory properties, tea tree oil and its components have demonstrated anti-inflammatory activity *in vivo* in experimental animals as well as in clinical trials involving humans [142-144]. Beneficial effects of tea tree oil have also been

observed in wound healing (animal study) as well as in an *in vitro* activity against *S. scabiei* that was comparable to commercially available acaricides [145, 146].

Adverse effects

Reports of beneficial effects as well as the overall strong demand for natural remedies and aromatic substances have contributed to the considerable increase in the commercial production of tea tree oil. However, the numbers of case reports that describe cutaneous as well as systemic toxicities are on the rise. Allergic contact dermatitis to tea tree oil is the most often reported adverse reaction according to studies done in experimental animals and in humans with cineole identified as one of the allergens [118, 135, 147-152]. In addition, immediate type hypersensitivity reactions as well as other systemic toxicity manifestations in both human and veterinary use have been associated with topical use while ataxia and drowsiness that require hospitalization were among those symptoms reported after ingestion of tea tree oil preparations by children [153-157].

1.5.2 Garlic

Garlic (*Allium sativum*) has been used as a medicine and condiment since time immemorial and still enjoys popularity as a “cure for all” remedy. The oldest recorded literature from the Sumerians is dated at 2600-2100 BC. Although garlic was exalted throughout history, the quest to understand its action began only recently. Botanically, *A. sativum* is a member of the Liliaceae family, along with onions, chives and shallots. Studies done on the chemistry of garlic have identified a number of components such as diallyl trisulfide, diallyl disulfide, allicin and ajoene that have demonstrated wide spectrum of pharmacological activities [158, 159]. Those activities that may be of relevance in topical application will be briefly reviewed.

Antifungal activity

Various studies have been done that have demonstrated the antifungal activity of garlic extracts. Those fungi that are among the common pathogens of superficial infections and have proven *in vitro* susceptibility to garlic extracts include *Candida* (*C. albicans*, *C. tropicalis*), *Trichophyton* (*T. rubrum*, *T. mentagrophytes*, *T. violaceum*), *Microsporum* (*M. canis*, *M. audoninii*), *Epidermophyton floccosum* as well as *Aspergillus* (*A. niger*, *A. flavus*, *A. fumigatus*) [160-164]. Excellent results are reported from controlled clinical trials in the treatment of dermatophytic infections including tinea pedis, tinea corporis and tinea cruris by garlic extracts (especially one of its component, ajoene), even in comparison to standard drugs [165-167].

Antibacterial and antiviral activities

The antibacterial activity of garlic extracts has been demonstrated by various *in vitro* studies. Common skin and soft tissue pathogens that are well inhibited by garlic include *S. aureus* including MRSA, *E. coli* and *P. aeruginosa* [158, 160, 162, 168, 169]. On the other hand, very little work has been done to investigate the antiviral properties of garlic compared to its other antimicrobial activities. The few studies have reported garlic extract to exhibit *in vitro* antiviral activity against HSV types 1 and 2 as well as against human cytomegalovirus (HCMC) [158, 170, 171]. In a study involving 5 patients (all children of 5 years of age), a clinician reported of the successful clearance of hand warts by use of raw garlic cloves, with only one patient complaining of itching [172].

Adverse effects

Despite these and many other therapeutic benefits of garlic, adverse effects have been reported especially after topical application. For example, garlic in its fresh form has been found to be a

potent irritant especially under occlusive conditions and also in a crushed form [173, 174]. Allergic contact dermatitis to garlic has also been an area of concern. Studies done have found diallyl disulfide, allylpropyl and allicin as the principal allergens [152, 175-177]. Reports, albeit rare, of immediate allergic reactions such as localized urticaria, also exist after contact and ingestion of raw garlic [178, 179]. In addition, use of garlic topically has been implicated as the cause of chemical burns to the skin. In this case, infants have been found to be especially susceptible because their skin is delicate and does not have enough keratinous material in the outer layer of the epidermis to protect against this agent [180, 181]. Finally, intake of garlic has been associated with the induction of pemphigus (an inflammatory skin condition) in human and provoking acantholysis *in vitro* [182-184].

1.5.3 Aloe Gel

One of the most widely used herbal preparations for the treatment of skin conditions is aloe gel. Often referred to as aloe vera gel, this is the mucilaginous gel obtained from the cells making up the inner portion of the leaf *Aloe barbadensis* Mill (family Liliaceae), sometimes referred to as *A. vera* (L.) Webb & Berth. It has been claimed that aloe has several important therapeutic properties and it is nowadays used in a variety of commercial products including sun creams, cosmetics and lotions. One of the most popular indications for aloe vera (AV) preparations is in the treatment of wounds [159].

Wound healing activity

Various studies, mostly *in vivo*, have been conducted that have explored the therapeutic activity of AV and its components on wound healing. These studies have examined the wound healing activities of AV in experimentally induced wounds of laboratory animals, and found out AV

treatments of wound enhance the process of wound healing by influencing phases such as inflammation, fibroplasia, collagen synthesis and maturation and wound contraction [185-190]. In addition, wounds treated by both topical application and oral administrations of AV were found to result in therapeutic results [191, 192]. The wound healing activities of AV preparations have been tried and found to be promising in controlled clinical trials in the treatment of conditions such as partial thickness burn wound and full-face dermabrasion [193-195].

Nevertheless, reports of works that question the claimed effectiveness of AV on wound healing exist. One example is that done on an *in vivo* burn wound model (using guinea pigs) where AV gel hindered the healing process compared to the standard used [196]. Among the clinical studies that challenge the claims are those dealing with the cases where AV preparations were not superior and were even cause of delayed healing in the treatment of wounds of pressure ulcers and after gynecologic surgery [197, 198].

Anti-inflammatory activity

AV has shown a broad spectrum of anti-inflammatory activity in various models of inflammation including via the topical and systemic routes of administration in the *in vivo* models. The major sugar in aloe gel, mannose-6-phosphate and a C-glucosyl chromone are constituents that have been identified to possess anti-inflammatory activity [186, 199-205]. AV preparations have been evaluated for anti-inflammatory activity in controlled clinical trials whereby encouraging results have been obtained in the alleviation of irritant contact dermatitis [206] and in the efficacy and tolerability when used against psoriasis [207].

Miscellaneous activities

Furthermore, AV extracts have been found to possess cytotoxic activity *in vitro* against carcinoma cell lines, peroxidase activity that may be useful in skin protective activity, as well as antimicrobial activity against bacteria and herpes virus. Beneficial activities have also been reported in its prevention of UV-B induced immune suppression in skin as well as in the treatment of lichen planus [197, 208-216].

Adverse effects

Despite its many reported merits, the use of AV has been associated with a number of adverse effects. One of those reported adverse effects is related to the increased sensitivity of skin to UV light after topical application of aloe emodin (an aloe vera component), which may subsequently lead to photocytotoxicity as demonstrated on human skin fibroblasts *in vitro* [217, 218]. The cytotoxicity potential of a low molecular weight fraction obtained from AV has also been demonstrated even in the absence of UV light and was found to be of similar potency to sodium dodecyl sulfate (a well known toxic substance) [219]. Apart from the above-mentioned, carcinogenic effects, severe dermatitis as well as allergic reactions on skin were among those reported with the use of AV [220-222].

1.5.4 Arnica

Arnica, also known as leopard's bane or mountain tobacco, is an extract derived from *Arnica montana* in case of the European drug, while other species such as *A. fulgens*, *A. cordifolia* and *A. sororia* (Family Compositae) are also included in the American product. Many homeopathic formulations of arnica including tinctures, ointments, creams, gels, and tablets are on the market. Arnica has been used as a homeopathic remedy for hundreds of years and has been advocated by

manufactures for the treatment of pain, stiffness, and swelling associated with trauma as well as for the prevention and quickened resolution of bruises [223, 224].

Anti-inflammatory activity

The anti-inflammatory activity of arnica is one of the most studied and has been demonstrated in various *in vitro* studies as well as *in vivo* studies. It has been found that the active ingredients mediating this pharmacological effect are mainly sesquiterpene lactones (SLs) such as helenalin and 11 α , 13 - dihydrohelenalin [224-228]. Despite its wide popularity, especially in homeopathic medicine, reports of clinical trials that support the acclaimed therapeutic activities of arnica are very few. Among these studies, beneficial effects of arnica preparations have been reported in mild to moderate osteoarthritis of the knee, reduction of pain in patients recovering from hand surgery and alleviating the feeling of stiffness during hard physical exertion [229-231]. Most of the clinical studies carried out however do not support the claim that homeopathic arnica is efficacious beyond a placebo effect in conditions associated with tissue trauma [224, 232-238].

Miscellaneous activities

Arnica has also been shown to exhibit promising activities including inhibitory activity against periodontopathic bacteria, cytotoxic activity against cancer cell lines, wound healing activities as well as immunostimulant activity. Studies done in mice have in addition shown its usefulness in ameliorating cytogenetic damage induced by single and multiple exposures to ultrasonication [239-245].

Adverse effects

The widespread use of arnica has however been accompanied by increasing reports of adverse effects that were mainly related to its allergenic potential. Studies done have identified SLs such as helenalin (which also possess therapeutic activity) to be the main sensitizers. In one prospective study done in order to determine the prevalence of delayed-type hypersensitivity to arnica, 5 patients out of 443 individuals (~1.13%) referred for chronic hand or face dermatitis showed positive reactions to arnica after patch testing [245-251].

1.5.5 Chamomile

The herb, commonly known as German or Hungarian chamomile, and which consists of the flower heads of *Matricaria recutita* L., also referred to as *Chamomilla recutita* or *Matricaria chamomilla* Lpp. has been used as a medicinal herb worldwide for thousands of years and has become increasingly popular in recent decades. Chamomile has been widely used as an anti-inflammatory for various afflictions of the skin and mucous membranes and as an antiinfective for many minor illnesses. Extracts of the plant or its volatile oil are used in the form of ointments, lotions, vapor baths, and the like, all intended for local application. Internally the drug is taken as a strong tea. The herb yields about 0.5% of a volatile oil (-)- α -bisabolol and matricin being among the principal constituents and flavonoids such as apigenin and luteolin [223, 252].

Anti-inflammatory activity

The anti-inflammatory activity of chamomile extract has been the subject of many researches. Studies carried out include both *in vitro* and *in vivo* experiments with the latter being predominant. Chamazulene (a transformation product of matricin) has been cited as one of the components contributing to the anti-inflammatory activity [253, 254]. Promising anti-

inflammatory activities have been exhibited by chamomile containing preparations in controlled clinical trials as compared to standard steroidal and nonsteroidal anti-inflammatory agents [255-259].

Miscellaneous activities

Chamomile extracts have exhibited chemopreventive activity, as well as antibacterial, antifungal and antiviral activity against common pathogens [260-266]. The therapeutic efficacy of chamomile extract in wound healing was also attested in a controlled clinical trial [267].

Adverse effects

Although German chamomile is one of the oldest and most valued of medicinal plants and is widely available in various formulations, patients with Compositae contact sensitivity are routinely warned of applying such products. This warning largely stems from reports of contact dermatitis from chamomile containing preparation as well as from positive reactions to patch testing with chamomile extracts. In fact a number of reports exist that detail both immediate and delayed type allergic reactions to chamomile containing preparations in sensitive patients. These studies have identified the SL, anthecotulide, as the main allergen [245, 268-274].

1.6 Review on medicinal plants selected for this study

1.6.1 *Inula confertiflora* A. Rich (family Compositae)

I. confertiflora is a shrub that is highly branched above, the branches being striated, tomentellous and leafy at extremities. The leaves are elongate-lanceolate, acute, narrowed at the base and shortly petioled with alternate arrangement. In addition, the leaves are tomentellous beneath and

nearly glabrous above with a denticulate margin. The capitula are yellow in color, sub-hemispherical in shape and many flowered [275].

I. confertiflora (vernacular name 'tikur weynagift' in Amharic) is a plant that is widely used locally for skin and eye diseases. According to a traditional report of use, the plant has successfully been used for skin conditions such as fungal infections (tinea capitis and t. corporis), wound infections, herpes infections as well as for eczematous lesions. Topical preparations of the plant for application to the eye and skin are reported including ointments made by triturating the powdered plant material (leaves and flowers) with butter or honey [12]. Nevertheless, report of scientific work on any pharmacological activity or on its phytoconstituents is unavailable. A number of studies are however reported on the pharmacological activity as well as on the phytoconstituents of the genus *Inula*, some of which are reviewed.

Antifungal activity

The antifungal activity of *Inula* spp. is among the most widely studied. Extracts of *I. viscosa* have shown significant antifungal activity against *M. canis*, *M. gypseum*, *T. mentagrophytes*, *T. rubrum*, *T. violaceum* and also against the yeast, *C. albicans*. Isolates of *I. viscosa* that have demonstrated antifungal activities include a SL (tomentosin) as well as flavonoids [276-281]. Another species, *I. racemosa* has demonstrated antifungal activity against *A. flavus*, *A. niger*, *C. albicans* and *C. tropicalis*. The active constituent in this case was the SL, alantolactone [282, 283].

Antibacterial and antiviral activity

Reports, although very few exist of the antibacterial and antiviral activities of *Inula* species. In one of these studies, *I. viscosa* was able to demonstrate *in vitro* activity against both gram-positive bacteria such as *S. aureus* and *P. vulgaris* and gram-negative bacteria such as *E. coli* and *P. aeruginosa* [284, 285]. Good *in vitro* activity against HSV 2 was also demonstrated by another species, *I. japonica* [286].

Anti-inflammatory activity

A number of studies also exist on the anti-inflammatory activities of *Inula* species. In one study constituents from *I. viscosa* including flavonoids (rhamnocitrin, 7-O-methylaromadendrin, and 3-O-acetylpadmatin), a SL (inuvicolide), a sesquiterpene acid (ilicic acid) as well as a digalactosyldiacylglycerol (inugalactollipid), have all demonstrated topical anti-inflammatory activity against 12-O-tetradecanoylphorbol-13-acetate-induced ear edema in mice. In addition, inuvicolide via systemic administration was able to reduce the phospholipase-induced paw edema [287-290]. *I. viscosa* extracts have also demonstrated antioxidant activity in an *in vitro* study [291].

Ergolide, a SL isolated from *I. britannica*, was able to induce significant suppression of the expression of inducible nitric oxide synthase and cyclo-oxygenase-2, both of which play important roles in the inflammatory signaling pathway. Oral administration of *I. britannica* in addition demonstrated significant suppression of antibodies production against ovalbumin-induced immunization. Strong *in vitro* antioxidant activities have also been shown against glutamate induced oxidative stress by flavonoids of *I. britannica* such as patuletin, nepetin, and axillarin [292-294]. On the other hand, the alcoholic extract of the root of *I. racemosa* has

exhibited potent antiallergic effect in experimental model of type I hypersensitivity – an egg albumin induced passive cutaneous anaphylaxis and mast cell degranulation in albino rats - that was comparable to disodium cromoglylate. In addition, alantolactone isolated from the petroleum ether extract of the root of *I. racemosa*, demonstrated significant anti-inflammatory activity against carrageenan-induced paw edema [295, 296].

Anticancer activity

Different *Inula* species have also demonstrated potent antiproliferative and cytotoxic activities. These activities were demonstrated in various *in vitro* studies against human tumor cell lines. Cytotoxic-guided fractionation has led to the identification of a number of SLs such as alantolactone, isoalantolactone, ergolide and bigevolin as active constituents [297-300].

Adverse effects

The main adverse effect associated with the use of *Inula* species is allergic contact dermatitis, the main allergens being SLs. Evidence of allergic contact dermatitis has been provided by *in vitro* tests such as the lymphocyte proliferation test as well as *in vivo* tests in mice. In one such study done on the constituents of *I. helenium*, the SL alantolactone was found out to be a better sensitizer when compared with another allergen, isoalantolactone [301]. The sensitization potential of *I. helenium* has also been demonstrated in humans by patch testing using SLs mix (composed of costunolide, dehydrocostus lactone and alantolactone) in Compositae allergic patients. Another report dealing on a clinical study reports of an individual who developed anal itching and a vesicular dermatitis of the hands and forearms when she treated her hemorrhoids with an infusion of *I. viscosa*. She was strongly patch test positive to two fractions of an ether

extract of the plant (possibly representing the SLs 2-desacetoxyxanthinin and inuviscolide) as well as to alantolactone and isoalantolactone [245, 302].

1.6.2 *Clematis simensis* Fresen. (Family Ranunculaceae)

C. simensis is a woody climber that can go up to 10m or more, sometimes with long branches lying on the ground. The stem is pubescent, longitudinally ribbed and furrowed. The leaves on the other hand are pinnate while the leaflets are ovate to ovate lanceolate, margin at the base and apex entire, serrate with widely spaced mucronate teeth in the middle. The upper surface of the leaves has scattered hairs while the lower one is tomentose. The inflorescence is many flowered, the flowers being pale yellow to white in color [303].

C. simensis (vernacular name- 'azo hareg' in Amharic) is one of the common medicinal plants in use by the Ethiopian society. The dried powder and fresh pulverized leaves have been used to dress wounds, and also for the treatment of eczema, tinea capitis and tropical ulcers. The seeds are reportedly used against rheumatic pain while the sap is used as a febrifuge and against bloat in animals. There are also reports of the plant being eaten as a cooked vegetable. Other uses include administration of the juice of the fresh leaves topically through the nasal route for the treatment of headache and cold. A recent study has however reported a burning sensation as a side effect to the topical application of the leaves of *C. simensis* used in combination with another plant from the same family, *Ranunculus multifidus* [13-16, 304-306].

There are some reports on the traditional use of other *Clematis* species. The infusion from the leaves of *C. hirsuta* is drunk for the treatment of gonorrhoea, syphilis and sore throat. The leaves have also been used for the treatment of leprosy, fever and various skin diseases. To clear a stuffy

nose the flowers are sniffed, which causes sneezing. It is also used for headache and cold in the head. The leaves of another species, *C. grandiflora* have also been used for headaches and skin diseases [6, 306].

Antibacterial and antifungal activities

Various scientific works have been done worldwide on the medicinal activities of the genus *Clematis*. However, the antimicrobial screening on various fractions of *C. simensis* done locally is the only work that is available in the literature on this species. In this work, the polar (aqueous and methanol) fractions of the dried, powdered leaf extracts of *C. simensis* have been shown to exhibit promising activity against certain bacteria (such as *S. aureus* and *P. aeruginosa*) and fungi (*C. albicans*) [304]. In a study done in Rwanda, the leaf extracts of *C. hirsuta*, a medicinal plant used traditionally have shown pronounced antifungal activity against *C. albicans* and the dermatophytes (*T. rubrum*, *E. floccosum* and *M. canis*) while in another study *C. cirrhosa* showed activity against *T. violaceum* at an MIC less than 40 µg/ml [279, 307]. A wide spectrum of antibacterial activity was also demonstrated by the methanolic extracts of *C. papuasica* leaves and stem bark, which was increased on fractionation [308].

Anti-inflammatory activity

Studies done have documented anti-inflammatory activities of *Clematis* species. In one study, *C. flammna* flowering herbs exhibited inhibitory effects on *in vitro* inflammatory models. Another study done on *C. chinensis*, a traditional Chinese medicine that has been used as anti-inflammatory and antitumor found saponins to be the bioactive compounds [309, 310].

1.6.3 *Zehneria scabra* (Linn. F.) Sond. (Family Cucurbitaceae)

Z. scabra is a climbing or trailing herb that can go up to 10 m. Old stems become woody with corky-ridged bark. The leaves are ovate, broadly ovate or pentagonal, more or less scabrid-punctate above, sparsely setulose to densely tomentose beneath while the flowers are white coloured, becoming cream then yellow with age [311].

Z. scabra (vernacular name - 'hareg ressa') is one of the commonly used medicinal plants in Ethiopian traditional medical practices and has reportedly been used topically for the treatment of alopecia, wound and eczema (as part of a poly-herbal preparation) [12, 311]. The ash from the burnt plant has been used as a burn remedy while the wash prepared from pounded leaves has been used for the treatment of skin rashes and also to treat calves for fleas in the East African region [306].

Antibacterial and antifungal activities

Scientific works to testify to any of the claims are almost nonexistent albeit for one work that conducted antimicrobial activity test for the various extracts of the dried, powdered leaves of *Z. scabra*. The results were promising with the aqueous and methanolic extracts exhibiting interesting inhibitory activity against *S. aureus*, *P. aeruginosa* and *C. albicans* [304].

1.6.4 *Pycnostachys abyssinica* Fresen. (Family name Labiatae)

P. abyssinica is a shrub with stout woody main branches, the branchlets being finely pubescent. The leaves are ovate, acute, crenate, pubescent on both sides, and are oppositely arranged. Whorls are condensed into a dense terminal spike. Furthermore, the corolla-tube is longer than

the calyx-tube that is campanulate in shape. The corolla is violet in color and is glabrous outside [312].

P. abyssinica (vernacular name - 'ye'eroo') is a not so common medicinal plant that is nevertheless used by some sects of the society. It has been reported by some individuals to be a useful remedy for tinea capitis and other skin diseases although no such report exists in the literature. An ethnobotanical survey conducted locally reported on the medicinal activities of *Pycnostachys* spp. (vernacular name - 'famfa') whereby the decoction of the leaves were used orally for the treatment of 'mitch' (a local inflammatory condition) and topically for the treatment of eye infection [15]. A study done in the East African region reported the use of the leaves of *P. umbrosa* as an eyewash [307].

1.7 Study on the *in vitro* efficacy of topical antimicrobial formulations

Clinical indications for topically applied antimicrobials include infections of the skin surface, damaged skin, open wounds and burns, and the anterior nares. Consequently, the major consideration of drug release from a topical antimicrobial is surface bioavailability but not necessarily penetration through the stratum corneum. This is so because surface microorganisms are the primary targets of such formulations. Thus, an important criteria for developmental studies is confirming that the formulation releases and does not bind the medicament. The key parameter for any product however remains its efficacy as demonstrated in controlled clinical trials. The time and expense associated with such trials make them unsuitable as routine testing methods, even more so with preliminary studies. Therefore, *in vitro* surrogate tests are often used as evaluative tools [313-317].

In these *in vitro* studies, measurement of drug release into appropriate media such as aqueous medium has been found out to be a more realistic *in vitro* model than release and penetration through a membrane system, representing the stratum corneum. In case of topical antimicrobial formulations, an evaluative tool that in addition confirms the antimicrobial efficacy would be highly beneficial. Among the few methods available for evaluation of an antimicrobial semisolid product, agar well diffusion is the most commonly used. Relying on the zone of inhibited growth around the well, this method gives quantitative results, that may however be limited by the extent of diffusional properties of the active ingredient through the agar [313, 314, 318, 319].

The usefulness of the agar well diffusion method as a model for comparison of clinical efficacy of antimicrobial products has been well demonstrated in various *in vitro* studies as well as *in vivo* settings. In one of these studies, *in vitro* results showed gentamicin in a cream carrier to exhibit a zone of inhibition that was twice that observed with gentamicin ointment. When these agents were evaluated *in vivo* in mice and also on contaminated burns of rats, the gentamicin cream effectively eliminated the bacteria, while the gentamicin ointment offered no therapeutic benefit. The usefulness of agar well diffusion has also been demonstrated in a clinical setting. With the aid of this testing method, topical agents could be selected for use in the burn wound on the basis of their *in vitro* activity against the specific burn pathogens. By selecting the most appropriate topical agent on the basis of this method, the incidence of burn wound cultures exceeding 10^5 organisms has been reduced to only 8 % [318, 320, 321].

1.8 Objectives of the study

1.8.1 General objective

To conduct *in vitro* and *in vivo* studies on extracts of four selected medicinal plants that are traditionally used for dermatological disorders in Ethiopia and prepare dermatological formulations of the promising extract(s).

1.8.2 Specific objectives

- To prepare the crude plant extracts of the selected medicinal plants;
- To study the antibacterial and antifungal activities of the crude extracts;
- To carry out some *in vitro* and *in vivo* activities on one of the plant materials with promising antimicrobial activities including:
 - Determination of some preliminary standardization parameters;
 - Conducting *in vivo* anti-inflammatory and skin sensitization tests;
 - Conducting *in vitro* evaluation on the antimicrobial performance of dermatological formulation prepared from the crude extract.

2. EXPERIMENTAL

2.1. Materials

Reagents and chemicals

Methanol (Loba Chemie, Mumbai, India), petroleum ether, chloroform, dimethyl sulfoxide, ethyl acetate, sulphuric acid, aluminium chloride, glyceryl monostearate, polyethylene glycol 4000, cetostearyl alcohol and silica gel G6 (BDH Chemicals Ltd, Poole, England), acetone and ferric chloride (Fischer Scientific Co., Fairview, New Jersey, USA), ethanol (Avondale Laboratories, Banbury, Oxon, England) formic acid (LabPak Ltd., Mill Lane, Fillongley) diethyl ether (Bio-Lab Laboratories Ltd, Israel), hydrochloric acid and acetic acid (Farmitalia Carlo Erba, Milan, Italy), potassium ferricyanide (Matheson Coleman & Bell Division, Norwood, Ohio, USA), lead acetate (Riedel-De Haen AG, Sec Lze, Hannover, Germany), ferric sulphate (Prolabo, Rhone-Poulenc), bismuth subnitrate (May & Baker Ltd., Dagenham, England), potassium iodide (Evans Medical Ltd., Liverpool, England), silver chloride (Nen Tech Ltd., Brixworth, Northants, UK), sodium lauryl sulfate (Avonchem Ltd., UK), polyethylene glycol 400 (Sigma-Aldrich Chemie GmbH, Riedstr, Steinheim, Germany), white soft paraffin (Ethiopian Pharmaceutical Manufacturing, Addis Ababa, Ethiopia), liquid paraffin BP (New Cross, Germany), cetomacrogol BPC 1000 (The British Drug House Ltd., London, UK), tetracycline hydrochloride (Lot No 114F-0163), vanillin and carrageenan lambda (Sigma Chemical Co., St. Louis, Mo, USA), ketoconazole (BNo KT-4061, Flamingo Pharmaceuticals, India), and indomethacin (Lot No. TOO-0045, Indukern Chemie, Switzerland) were used as received.

Pharmaceuticals

Foban® ointment (2% w/w sodium fusidate, Lot No. 01021015) and Foban® cream (2% w/w fusidic acid, Lot No. 82200005) from Hoe Pharmaceuticals, Bactroban® ointment (2% w/w mupirocin, BN 2352/040818) from Beecham Pharmaceuticals, Tetracycline Hydrochloride USP ointment (3% w/w, BN 02 Jul 1) from Sarabhai Ltd., Nizoral® cream (2% w/w ketoconazole, Lot No. 430BA) from Janssen-Cilag and Canesten® cream (1% w/w clotrimazole, BN BXB5551) from Bayer AG were used as received.

Test organisms

The following bacteria and fungi were used: *S. aureus* (ATCC 259C23), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 956140), *T. mentagrophytes* (ATCC 18748) and *A. niger* (ATCC 10535) from Rockville, MD, USA while *S. aureus* and *C. albicans* are clinical isolates obtained from the Department of Infectious Diseases, EHNRI.

Media

Mueller Hinton agar (Lot No 402928/1 from Fluka Biochemika (Switzerland), Nutrient broth (Lot No 143 36566), Sabouraud Dextrose agar (Lot No 251385) and Sabouraud liquid medium (Lot No 19037092) all from Oxoid Ltd. (England).

Animals

Swiss albino mice (6-8 weeks old, male and female) and Pirbright guinea pigs (200-350g in weight, male and female) used for the skin sensitization and anti-inflammatory tests respectively were obtained from the EHNRI.

2.2 Methodology

2.2.1 Collection of plant materials

The leaves and flowers of *I. confertiflora* were collected from Entoto area in the month of December 2002. The leaves of *Z. scabra*, *P. abyssinica* and *C. simensis* were collected from different places in Addis Ababa in the months of May and June 2002. The plant materials were then dried in open air (under shade) and powdered. The National Herbarium, Department of Biology, Addis Ababa University, to which sample specimens were also deposited (under sample specimen identification numbers BM 01 upto BM 04 for *I. confertiflora*, *C. simensis*, *P. abyssinica* and *Z. scabra* respectively), made taxonomic identifications.

2.2.2 Preparation of crude extracts

100g of previously dried and powdered plant materials of all except *I. confertiflora* were defatted by maceration in petroleum ether for 24 hours with occasional shaking. The petroleum ether extract was set aside for future tests. The marc remaining after defatting was placed in an oven (Gallenkamp, England) for a period of 24 hours, at a temperature not exceeding 40 °C, to allow sufficient drying. Then after, the dried marcs of the three plant materials as well as the plant material of *I. confertiflora* were extracted by maceration (with occasional shaking) using 80% methanol as menstruum. The miscella was changed every 24 hours and this process was repeated five times. The volume of the menstruum used was that volume enabling complete immersion of the plant material and differed among the plants; it ranged from 500-800 ml on the first day to 300-500 ml on days 2-5. The miscella were concentrated by use of a rota vapor (Buchi, model R-144, Switzerland) and then dried in an oven at temperatures not exceeding 40°C. The dried extracts were weighed, transferred into jars and then stored in a dessicator for future use. The

petroleum ether extract of *I. confertiflora* was prepared using similar extraction techniques and stored for further investigation. Extraction of all the plant materials was done in triplicates.

2.2.3 Antimicrobial testing

2.2.3.1 Inoculum

Cultures of the bacteria and fungi were maintained on nutrient and Sabouraud dextrose agar respectively at 4 °C. Prior to the test, bacterial and fungal cultures were prepared as follows. The bacterial cultures were sub-cultured in nutrient broth at 37 °C for 24 hours while the culture of *C. albicans* was sub-cultured in liquid Sabouraud dextrose medium for 48 hours at 25 °C. The turbidity of the broth culture was then equilibrated with 0.5 McFarland standard. Mature cultures of *A. niger* and *T. mentagrophytes* were inoculated into Sabouraud dextrose broth to prepare the test inocula which were similarly equilibrated with 0.5 McFarland standard [322].

2.2.3.2 Antibacterial activity test

The antibacterial activity test of the crude plant extracts against *S. aureus* (ATCC), *S. aureus* (isol.), *E. coli* (ATCC) and *P. aeruginosa* (ATCC) was carried out by the hole-plate (agar-well) diffusion method [302, 323- 325]. The agar plate was prepared for each organism as follows. 0.2 ml of the standardized inoculum was mixed with 20 ml of sterile Mueller Hinton® agar (maintained at 45-50 °C in a molten state) using a mixer (WhirliMixer, Fisherbrand, England), and then poured into sterilized petridishes (90 millimeter in diameter) and set aside. After congealing, the seeded agar was punched out with a sterile hole borer at spaced out positions to make 4 holes (9 millimeter in diameter). Three of the holes were filled with 0.1 ml of the test sample solution/suspension (concentrations of 40, 20, 10, 5, 2.5 and 1.25 mg/ml) of the crude

extracts to give 4, 2, 1, 0.5, 0.25 and 0.125 mg of extract per hole, while the fourth one was filled with 0.1 ml of tetracycline Hcl (0.3 mg/ml) in distilled water to give 0.03 mg per hole. The plates were then left at room temperature for 2 hours (to favor diffusion over microbial growth) and then incubated (Gallenkamp, England) at 37°C for 24 hours. Each test was done in triplicates. Preliminary studies have confirmed the vehicles, 80% methanol (used to dissolve/suspend the 80% methanol extracts) and dimethyl sulfoxide (used to dissolve the petroleum ether extracts and TLC fractions) to be inactive against the tested bacteria. The antibacterial activity was evaluated by measuring the diameter of the zone of inhibition excluding the hole size by use of an antibiotic zone reader (Fisher-Lilly, USA).

2.2.3.3 Antifungal activity test

Antifungal activity test of the crude plant extracts against *T. mentagrophytes* (ATCC), *A. niger* (ATCC) and *C. albicans* (isol.) was similarly conducted using the hole-plate (agar-well) diffusion method [302, 323]. 0.2 ml of the inoculum was mixed with 20 ml of sterile Sabouraud Dextrose® agar (maintained at 45-50°C in molten state), and then poured into sterilized petridishes (90 millimeter in diameter) and set aside. After congealing, the seeded agar was punched out with a sterile hole borer at spaced out positions in order to make 4 holes (9 mm in diameter). Three holes were filled with 0.1 ml of test sample solution/suspension (containing similar concentrations as for the antibacterial test) while the fourth one was filled with ketoconazole (0.3 mg/ml) dissolved in methanol to make 0.03 mg per hole. In case of the activity test of the petroleum ether extract of *I. confertiflora* against *T. mentagrophytes*, single holes were made in 70-millimeter sterilized petridishes to which 15 ml of molten seeded agar was poured, in light of the wide inhibition and zone overlapping observed in preliminary studies. The plates were then

left at room temperature for 2 hours and then incubated (B&T Unitemp, England) at 25 °C for 48 hours in case of *C. albicans* and *A. niger*, and for 7 days in case of *T. mentagrophytes*. Each antifungal activity was done in triplicates. Preliminary tests confirmed the vehicles chloroform (used to dissolve the petroleum ether extracts) and 80% methanol to be inactive against the tested fungi. The antifungal activity was similarly evaluated by measuring the diameter of the zone of inhibition excluding the hole size by use of an antibiotic zone reader in case of the 90 mm petridishes and a caliper (NSK, Nippon, Japan) in case of the 70 mm petridishes.

2.2.3.4 Minimum inhibitory concentration (MIC) determination

The MIC of the undefatted 80% methanolic extract of *I. confertiflora* (ICME) was determined against *S. aureus* (ATCC) and *T. mentagrophytes* (ATCC) by the agar dilution method [280, 322, 323, 326]. Dilutions of the extract were prepared in 80% methanol, which has been confirmed to be devoid of antimicrobial activity against the test organisms in preliminary studies. Slants were prepared in sterilized test tubes by mixing 9 ml of molten agar (Mueller Hinton® and Sabouraud dextrose® for *S. aureus* and *T. mentagrophytes* respectively) with 1 ml of the extract dilution to give final concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.90625 and 1.9531µg/ml in the agar media. After solidification, the surface of each slant was inoculated with liquid microbial culture in case of *S. aureus* and with approximately 23 2-millimeter portions of mature mycelial cultures in case of *T. mentagrophytes*. Incubation period was 24 hours at 37 °C for *S. aureus* and 7 days at 25 °C for *T. mentagrophytes*. Each test was done in triplicates. Inhibition of growth was judged by comparison with growth in control tubes prepared without plant extracts, which were set up at the same time as the test tubes. The MIC was defined as the lowest concentration of the extract preventing growth of the microorganisms.

2.2.4 Anti-inflammatory activity test: Carrageenan-induced paw edema test

The anti-inflammatory activity was evaluated according to the carrageenan-induced paw edema method [327, 328]. Pirbright guinea pigs (in groups of 7) received ICME (1000 or 500 mg/kg), water as a negative control, or indomethacin (10 mg/kg) as a positive control. They were treated per os at a dose volume of 10 ml/kg, 1 hour before the subplantar injection of λ -carrageenan (0.1 ml of 1% suspension in 0.9 % saline) in the right hind paw and 0.9 % saline solution in the left paw. Paw volume was measured with a plethysmometer (Ugo Basile, model 7140, Italy) before (V_i) and 3 hour after injection of λ -carrageenan (V_f).

Inflammation (I) was expressed as the increase in paw volume measured after carrageenan injection and compared with the preinjection value for individual animals. Results reported as anti-inflammatory effect (A), were calculated by comparing the percent inflammation of the test group to the control group receiving the vehicle (water). The significance of the drug induced changes was estimated by the student's t-test making use of a statistical software, GraphPad InStat.

The anti-inflammatory effect was calculated using the following formula:

$$\%A = (\% \bar{I}_c - \% \bar{I}_s) / \% \bar{I}_c \times 100 \quad (1),$$

where $\% \bar{I}_c$ and $\% \bar{I}_s$ correspond to the mean inflammation in controls and sample treated animals respectively.

$$\text{Percent inflammation } (\%I) = [(V_f - V_i) / V_i] \times 100 \quad (2)$$

where V_f and V_i are final and initial paw volumes respectively.

$$\% \bar{I} = \sum \% I / n \pm \text{SEM} \quad (3)$$

where n is the number of animals in each assay and SEM is the standard error of the mean.

2.2.5 Skin sensitization test: the mouse ear swelling test (MEST)

The basic procedure followed is based on a standard method that has been used to evaluate test substances for their potential to cause dermal sensitization in mice [329, 330]. This test evaluates contact sensitization by quantitatively measuring mouse ear thickness. Some modifications have been made based on other findings and also on local availability of materials [331]. The test materials evaluated for their sensitization potential in this case were the petroleum ether extract (PEE) and the ICME dissolved in petroleum ether: acetone (9:1) and in 80% methanol respectively. For each material investigated, a pretest group of 8 mice, a test group of 15 mice, and a control group of 10 mice were utilized. Test and control mice were observed for 1 week before the start of the actual study for any signs of illness. The mice were housed five per cage and allowed water and feed *ad libitum*.

Concentrations utilized for the actual test were the highest which were minimally or mildly irritating to the stomach (for induction) and nonirritating to the ear (for challenge). To establish these concentrations, an irritation probe was conducted for each extract prior to the actual MEST. Two mice were used in each of four groups for each extract. Each group was induced (including shaving, tape stripping, and four induction applications over a 4-day period) and mock “challenged” once on the ears. Concentrations that were applied to the different groups of mice included 10, 20, 40 and 80 mg/ml in case of the PEE and 5, 10, 20 and 40 mg/ml in case of the ICME. Concentrations for the probe were selected based on existing data, solubility in vehicle

and best judgment. Ears were both visually evaluated for erythema and measured for thickness using a micrometer (Vernier, USA) at 24h after application of test substances to detect any irritation swelling. The abdominal skin of all animals was observed for dermal irritation 24h after the last induction application. Based on the results of the pretest (irritation probe) data, a judgment was made as to which concentration would be used for topical induction applications to the belly and for topical challenge application to the ear in the actual test. Accordingly, concentrations of 10 mg/ml and 40 mg/ml were chosen for PEE and ICME respectively for both the induction and challenge applications.

In the actual MEST, similar procedures were used. Induction was carried out by application of 100µl of the extract in solvent or solvent alone (for controls) to the center of the shaved region. Tape stripping and topical application of the appropriate solution to the belly region were repeated for three additional days. Seven days after the final topical application, 20µl of the extract solution was applied to both the ventral and dorsal surfaces of the left ear of each animal (15 test and 5 control group). The right ear was likewise dosed with 20µl of the vehicle. At both 24 and 48 hours after the challenge, animals were anesthetized with ether and the thickness of both ears measured. The extract that was judged to be a nonsensitizer after the first challenge application was subjected to a second and final challenge 7 days after the first challenge. The five remaining 'naïve' control group animals were challenged for comparison to the test group animals. Control mice from the first challenge were not rechallenged because they had been exposed to the test substance and were no longer true negative controls. The procedures used for the first challenge in both challenge application as well as ear thickness measurement (24 and 48h later) were used for the re-challenge.

1. Positive responses were defined on an animal-by-animal basis as cases where the test ear demonstrated at least a 20% greater thickness than the control ear. This effect criterion was selected because it has previously been shown to guarantee a level of false positive of less than 1 in 1000. The percentage of animals in a test group considered “positive” was then calculated and recorded as percent responders.
2. In addition, percent ear swelling was calculated for the test group. The left (A) and right (B) ear thickness measurements were added. Percent ear swelling equaled that sum of A (test ear thickness measurements) divided by the sum of B (control ear thickness measurements) multiplied by 100.

$$\% \text{ Ear swelling} = A/B \times 100 \quad (4)$$

2.2.6 Preliminary screening for the presence of some active plant constituents

Phyto-chemical screening to detect for the presence of some secondary metabolites in *I. confertiflora* was carried out following standard methods.

Test for presence of sesquiterpene lactones

Petroleum ether extract prepared earlier was used to detect the presence of sesquiterpene lactones by a thin layer chromatography (TLC) method. For this 1 mg/ml of the extract was dissolved in chloroform and TLC was run on a 20320-plate (of thickness 0.25 mm) prepared in the laboratory using a mobile phase, petroleum ether-chloroform-acetone in the ratio of 5:3:2. After development of the chromatogram, detection was carried out by use of a spraying reagent, 5% solution of aluminum chloride in ethanol. The formation of visible violet or brown color on the

plate or yellow, brown and green fluorescence under UV light at 366 nm evident after 10-15 minutes after heating at 120°C in an oven was indicative of the presence of sesquiterpene lactones [332].

Test for alkaloids

Two grams of ground plant material was treated in a test tube with 10 ml of 1 % HCl for 30 minutes in a water bath. The suspension was filtered through cotton into a test tube and was divided into two parts. Five drops of each of Dragendorff's reagent (composed of bismuth subnitrate, acetic acid and potassium iodide) and Mayer's reagent (composed of mercuric chloride and potassium iodide) were added to the respective parts of the solution and the formation of yellowish orange precipitate (Dragendorff's reagent) or whitish opalescence (Mayer's reagent) indicated the presence of alkaloids [333].

Test for phytosterols

One gram of ground plant material was macerated with petroleum ether, filtered and concentrated. The concentrated residue was then dissolved in chloroform to which 3 to 5 drops of conc. H₂SO₄ was added carefully. The production of a red or reddish brown or violet color was taken as indicative of the presence of steroidal compounds [332].

Test for polyphenols

To 2 ml of filtered solution of aqueous macerate of a plant material, 3 drops of a mixture of 1 ml 1% FeCl₃ and 1 ml of 1% K₃Fe (CN)₆ (prepared separately) were added. The formation of a green blue color indicated the presence of polyphenols. The test was repeated with 2 ml of the alcoholic macerate of the plant material [333].

Test for flavonoids

To 2 ml of the alcoholic extract, 3 to 5 drops of 2% lead acetate solution was added. The development of yellow or orange color indicated the presence of flavonoids [333].

Test for phenolic glycosides

When a crystal of ferric sulphate was added to 1 ml of aqueous solution of the dried alcoholic extract, the formation of a dark violet color that precipitated was taken as indication of the presence of phenolic glycosides [333].

Test for saponins

Formation of honeycomb froth

Ground plant material (about 3g) was heated for 5 minutes in a test tube with 30ml of distilled water. The solution was filtered through a filter paper at room temperature. About 10 ml of the filtered solution was shaken in a large test tube; the formation of honeycomb froth that persisted for half an hour indicated the presence of saponins [333].

Hemolysis test

Two ml of the filtered solution obtained above was added to 2 ml of 10% v/v whole sheep blood in normal saline and thoroughly mixed. The mixture was then centrifuged and supernatant noted. This was compared with a control tube containing 2 ml of 10% v/v blood in normal saline diluted with 2 ml of normal saline. After mixing and centrifuging the contents of the control tube, a pale yellow supernatant and precipitate of red blood cells was obtained. The presence of saponin in the crude drug was detected if the supernatant was found to be red. This test is based on the

observation that saponin glycosides hemolyse red blood cells, releasing haemoglobin into the solution. Thus the supernatant is yellow in the control tube (normal saline does not disrupt the red blood cells) but red in the test mixture (cell membrane disrupted, so haemoglobin leaks into the serum) [3].

2.2.7 Determination of preliminary standardization parameters

Some preliminary standardization parameter determinations were carried out on the air-dried material of one of the plants, *I. confertiflora*.

2.2.7.1 Determination of solvent extractive values

Solvent extractive values were determined for some common solvents including water, 80% methanol and 70% ethanol using the following procedure [3]. 5 g of the air-dried plant material, coarsely powdered was macerated with 100 ml of menstruum in a closed flask for 24 hours. The flask was shaken periodically during the first 6 hours and allowed to stand for 18 hours. Thereafter, the miscella was filtered and 25 ml of this filtrate was first dried in a tared evaporating dish in a water bath (Gallenkamp, England), and then transferred to an oven for further drying at 105°C and weighed. The percentage of solvent soluble extractive with reference to the air-dried drug was then calculated.

2.2.7.2 Determination of ash values

Total ash

3 g of accurately weighed, ground air-dried plant material was placed in a previously ignited and tared crucible of platinum. The material was spread in an even layer and then ignited using a

furnace (Nuber Industrieofunbau, model D-2804 Lilienthal, West Germany) by gradually increasing the heat to 500-600°C until it was white, indicating the absence of carbon. It was then cooled in a dessicator and weighed. The percentage of the total ash with reference to the air-dried material was then calculated [3].

Acid insoluble ash

25 ml of hydrochloric acid was added to the crucible containing the total ash. The crucible was then covered with a watch glass and boiled for 5 minutes. The watch glass was then rinsed with 5 ml of hot water that was transferred to the crucible. The insoluble matter was collected in an ashless filter paper (Whatman, England), which was then washed with hot water until the filtrate was neutral. The filter paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to constant weight. The residue was allowed to cool in a dessicator for 30 minutes and weighed without delay. The percentage of the acid insoluble ash with reference to the air-dried material was then calculated [3].

Water-soluble ash

25 ml of distilled water was added to the crucible containing the total ash and boiled for 5 minutes. The insoluble matter was collected in an ashless filter paper, washed with hot water and then ignited in a crucible for 15 minutes at a temperature not exceeding 450°C. The weight of the remaining residue in mg was subtracted from the weight of the total ash. The result was then used to calculate the percentage of water-soluble ash with reference to the air-dried material [3].

2.2.7.3 Determination of moisture content

Moisture content was determined by estimating the loss on drying after heating in an oven. For this 5g of the plant material was dried at a temperature of 105°C. The material was weighed periodically until no more than 0.25 percent was lost in one hour's drying. The total weight was expressed as a percentage of the initial weight of the sample [3].

2.2.8 Chromatographic techniques

2.2.8.1 Qualitative thin layer chromatography (TLC) analysis

Analytical TLC procedures were carried out on both the PEE and ICME. Solutions (1 mg/ml) of the petroleum ether and whole 80% methanol extracts were prepared in chloroform and methanol respectively, of which 10µl was applied as short bands. TLC was performed using 5×20 silica gel 60 F₂₅₄ pre-coated plates (0.2mm, E. Merck, Darmstadt). The mobile phase used for development of the chromatograms were petroleum ether – chloroform – acetone (5:3:2) and chloroform – ethyl acetate – methanol – formic acid (3:3:3:1) for the PEE and the ICME respectively. Visualization was done in visible light, in UV – 254 and 366nm (CAMAG UV cabinet), and finally in visible light after spraying with 1% vanillin in concentrated sulfuric acid.

2.2.8.2 Preparative thin layer chromatography (TLC)

Preparation of PEE TLC fractions

Preparative TLC was carried out for PEE. Each plate was arbitrarily divided into four zones based on information obtained from preliminary studies. In the preliminary study, a chromatogram was developed on a 20×20 TLC plate of 0.25 mm thickness by application of 1 mg/ml solution of the PEE as a band using a mobile phase system composed of petroleum ether –

chloroform – acetone (5:3:2). In this preliminary experiment, visible zones, those observed under UV 366 and the visible zones obtained after spraying with vanillin-sulphuric acid reagent were used as a basis to divide the plate into four areas. Accordingly, fraction I was the decided to be between R_f 0.12 and 0.03 inclusive, fraction II between R_f s 0.40 inclusive and 0.12 exclusive, fraction III between R_f s 0.73 exclusive and 0.40 exclusive and fraction 4 above R_f 0.73 inclusive up to solvent front.

Based on the above determination, a 160 mg/ml solution of the extract was prepared in chloroform and approximately 200 μ l of this solution was applied as a band on 20×20 preparative TLC plates of 0.5 mm thickness. The mobile phase used for the development of the PEE was the one mentioned above and which was used in the qualitative TLC analysis. Eventhough the chromatographs gave more or less well-separated zones, tailing was present. Thus the separation plan was not strictly followed and thus visible zones and vizualization under UV 366 were used as guides for separation. In addition, separation was aided by using the spray reagent mentioned above on the edge of the plates. The separated TLC fractions were scraped from the developed chromatogram and washed with chloroform and chloroform-acetone mixture as required. Fractionation by use of the above procedure was carried out on 32 - 20×20 TLC plates to obtain enough fractions for subsequent antimicrobial activity tests. The resulting fractions were dried and stored in a similar manner as the previously prepared extracts.

Antimicrobial activity of PEE TLC fractions

The TLC fractions were screened for antimicrobial activities by using the agar well diffusion method described earlier. 10 mg/ml of each of the fractions was prepared and used for the activity

test against the microorganisms used earlier in the antimicrobial test of the crude extracts. 90 mm petridishes were used for this study. Each test was done in triplicates. Preliminary studies confirmed dimethyl sulphoxide used as a vehicle, to be inactive against the tested organisms. The antimicrobial activities were similarly evaluated by measuring the diameter of inhibition zone excluding the zone size using an antibiotic zone reader.

2.2.9 Evaluation of the antimicrobial activity of suggested topical formulations of *I. confertiflora*

2.2.9.1 Preparation of the suggested topical formulations

The suggested formulation bases used are as follows:

(I) Sodium laurate monostearin cream [345] – (oil-in-water emulsion type)

Glyceryl monostearate	5%
Sodium lauryl sulphate	3%
Cetostearyl alcohol	2%
Liquid paraffin	25%
Water to	100%

(II) Cetomacrogol Cream (BP 1988) – (oil-in-water emulsion type)

Cetomacogol emulsifying ointment	9%
White soft paraffin	15%
Liquid paraffin	6%
Water to	100%

(III) Polyethylene glycol (PEG) ointment (USPXXII) – (water-soluble type)

PEG 400	60%
PEG 4000	40%

(IV) Hydrophilic base – (oil-in-water type)

Cetostearyl alcohol	16%
Sodium lauryl sulphate	1%
White soft paraffin	40%
Water to	100%

(V) White soft paraffin – (hydrocarbon base)

A 10 % w/w dispersion of the ICME was prepared in each of the above bases by incorporation of the powdered extract using the method of trituration. The bases without the extract were used as negative controls while the following commercial products (obtained from local dispensing outlets) were used as comparison:

Antibacterials: Bactroban® ointment, Foban® cream, Foban® ointment, and Tetracycline Hydrochloride ointment.

Antifungals: Canesten® cream and Nizoral® cream.

2.2.9.2 Antimicrobial efficacy tests of the topical formulations

Antimicrobial activity of the five suggested formulations, commercial products and the negative controls were carried out by a modified agar-well diffusion method [320]. In this 15 ml of molten

agar Mueller-Hinton® agar and 10 ml of Sabouraud dextrose® agar (for *S. aureus* and *T. mentagrophytes* respectively) were added to sterile petridishes of 90 mm and 70 mm diameter respectively, and allowed to solidify. Three holes that were evenly distributed for the 90 mm plates and a single hole for the 70 mm plate (9 mm in diameter) were formed in the agar plate by removing plugs made with a sterile cork borer. By means of disposable syringes, ~0.11g of the test preparation was added to each hole. 10 ml of molten agar was then inoculated with 0.2 ml broth culture of *S. aureus* (ATCC.) or *T. mentagrophytes* (ATCC) prepared in a similar manner as for the antimicrobial tests carried out earlier. The agar suspension of organisms was then mixed and poured onto the previously prepared test plates containing the antimicrobial formulations. After the agar solidified, the plates were inverted and incubated. Incubation was 24 hours at 37°C in case of *S. aureus* and 7 days at 25 °C for *T. mentagrophytes*. The diameter of zones of inhibition was measured excluding hole size using a caliper, after the stated incubation period.

3. RESULTS AND DISCUSSION

3.1 Crude extracts yield

Solvent or the extraction agent used in the preparation of phyto-pharmaceuticals must be suitable for dissolving the important therapeutic drug constituents. In addition, solvents used should be easy to remove, inert, nontoxic, and not easily flammable. Accordingly, water-lower alcohol cosolvents have been commonly used especially in preliminary studies such as the present one. It is hypothesized that alcoholic solvents efficiently penetrate cell membranes, permitting the extraction of high amounts of endocellular components in contrast to lower polarity solvents such as chloroform, which are limited to extracting mostly extra-cellular material. In this manner, alcohols dissolve chiefly polar constituents together with medium and low polarity compounds extracted by co-solubilization. Bearing these possible problems in mind, in general hydro-alcoholic cosolvents such as 80 % methanol seem to possess the optimum solubility characteristics for initial extraction. In the present study, 80% methanol was chosen as the menstrum based on the above-mentioned points [3, 334].

The plant parts used for the extraction were the leaves for all the plants except for *I. confertiflora*, where both the leaves and flowers were used. The decision for the selection of the plant parts used was made based on the traditional use of these plants [12]. The yield of the 80% methanol plant extracts in grams and percentage of dried plant material are given in Table 3.1 below. The lowest yield was obtained from *Z. scabra* leaves while the highest yield was that from the *C. simensis* leaves.

Table 3.1: Yield of the plant extracts from 100g of plant material using 80 % methanol as menstruum.

Name of the plant	Family	Part of the plant extracted	Percentage yield
<i>C. simensis</i>	Ranunculaceae	Leaves	27.1±1.2*
<i>P. abyssinca</i>	Labiatae	Leaves	21.9±1.3*
<i>Z. scabra</i>	Cucurbitacea	Leaves	12.7±1.2*
<i>I. confertiflora</i>	Compositae	leaves and flowers	22.2±1.2*

* n=3

3.2 Antimicrobial tests

3.2.1 Antibacterial test

All of the 80% methanol extracts tested have exhibited antibacterial activities as can be observed in Table 3.2, but only against the gram-positive bacteria, *S. aureus* (ATCC); one exception is the 80% methanol extract of *C. simensis*, which in addition exhibited activity albeit weak against *P. aeruginosa*. Of the petroleum extracts, only the extract obtained from *I. confertiflora* demonstrated activity, which was found to be higher than the 80 % methanol extract of the same plant, thus the reason for preparing the 80% methanol extract without de-fatting with petroleum ether. For all of the extracts tested, a concentration dependent increase in the antibacterial activity was observed. The commercially available antibiotic, tetracycline was found to exhibit antibacterial activities against all of the pathogens tested that was higher than that exhibited by the plant extracts. In addition, it was observed that the antimicrobial activity of tetracycline was higher against the gram-positive organism as compared to the gram-negative ones. This phenomenon has been observed elsewhere and one reason for this may be the fact that gram

Name of the plant		Concentration (mg/per hole)	Zone of inhibition (mm diameter)			
			<i>S.a^s</i>	<i>S.aⁱ</i>	<i>E.c</i>	<i>P.a</i>
80% methanol extracts	<i>C. simensis</i>	0.25	-	-	-	-
		0.5	3	-	-	-
		1.0	4	-	-	-
		2.0	5	-	-	-
		4.0	9	-	-	3
	<i>P. abyssinica</i>	0.25	-	-	-	-
		0.5	3	-	-	-
		1.0	6	-	-	-
		2.0	10	-	-	-
		4.0	13	-	-	-
	<i>Z. scabra</i>	0.25	-	-	-	-
		0.5	3	-	-	-
		1.0	4	-	-	-
		2.0	5	-	-	-
		4.0	6	-	-	-
	<i>I. confertiflora</i>	0.25	-	-	-	-
		0.5	2	-	-	-
		1.0	3	-	-	-
		2.0	5	-	-	-
		4.0	8	-	-	-
Petroleum ether extract	<i>I. confertiflora</i>	0.25	-	-	-	-
		0.5	4	-	-	-
		1.0	8	-	-	-
		2.0	9	-	-	-
		4.0	11	-	-	-
Tetracycline HCl		0.03	29	26	24	21

Table 3.2: Diameter of inhibition zones of the crude plant extracts against selected bacteria; concentration of extract is the actual amount filled into each hole ; *S.a^s* = *S. aureus* (ATCC); *S.aⁱ* = *S. aureus* (isol); *Ec* = *E. coli* (ATCC); *Pa* = *P. aeruginosa*(ATCC)

negative bacteria are more resistant to the action of antimicrobials compared to their gram positive counterparts as a result of the more complex cell wall of the former [313].

The results of the antibacterial activity study for the extracts of *C. simensis* and *Z. scabra* slightly differ from that of another study done locally which found both of these extracts to be active against standard strains of *S. aureus*, *E. coli* and *P. aeruginosa* with *C. simensis* demonstrating higher activities [304]. Nevertheless, taking into consideration the fact that *S. aureus* is the most important and common pathogen causing both primary and secondary skin infections, the activities demonstrated by the 80% methanol extracts of the four plants against the above pathogen may be the basis for their traditional ethno-medical use for a variety of skin and wound infections.

3.2.2 Antifungal test

Variable antifungal activity was exhibited by the extracts of *I. confertiflora* (as can be seen in Table 3.3) while neither the 80% methanol nor the petroleum ether extracts of the other three plant extracts were able to exert any inhibition on the tested fungal strains. Remarkable antifungal activity was observed against *T. mentagrophytes* by both the petroleum ether and 80% methanol extracts of *I. confertiflora* while weak activity was exhibited by the petroleum ether extract against *A. niger*.

The activity of the petroleum ether extract of *I. confertiflora* against *T. mentagrophytes* was observed to be higher than that of the 80% methanol extract. This is indicative that the active antifungal constituents are concentrated in the nonpolar fraction, which may contain the SLs that are common constituents in the genus and are widely reported to exhibit interesting antifungal activity especially against the dermatophytes [276]. The activity of a commonly used antifungal agent, ketoconazole against *T. mentagrophytes*, was found to be lower than both the petroleum ether and 80% methanol extracts of *I. confertiflora* at the tested concentrations. On the other

hand, all of the plant extracts tested were devoid of any inhibitory activity against *C. albicans* although a study done locally by a similar method had reported the extracts of *C. simensis* and *Z. scabra* to exhibit some activity against *C. albicans* (ATCC) [304].

	Name of the plant	Concentrations (mg/hole)	Zone of inhibition (mm diameter)		
			<i>Tm</i>	<i>Ca</i>	<i>An</i>
80% methanol extract	<i>C. simensis</i>	4.0	-	-	-
	<i>P. abyssinica</i>	4.0	-	-	-
	<i>Z. scabra</i>	4.0	-	-	-
	<i>I. confertiflora</i>	0.25	-	-	-
		0.5	2	-	-
		1.0	4	-	-
		2.0	8	-	-
4.0	21	-	-		
<i>Petroleum ether extract</i>	<i>I. confertiflora</i>	0.0625	-	-	-
		0.125	2	-	-
		0.25	5	-	-
		0.5	10	-	-
		1.0	14	-	-
		2.0	21	-	-
		4.0	30	-	3
	Ketoconazole	0.03	18	40	16

Table 3.3: Diameter of inhibition zones of the crude plant extracts against selected fungi; concentration of extract is the actual amount filled into each hole; *Tm* = *T. mentagrophytes* (ATCC); *Ca* = *C. albicans* (isol);

The activity of *I. confertiflora* extracts against *T. mentagrophytes*, one of the most commonly isolated pathogens in dermatophytic infections may be the basis of the traditional use of this plant

for conditions such as tinea capitis and tinea corporis. Extracts of *C. simensis* and *P. abyssinica*, which have been used traditionally for tinea capitis, were however unable to inhibit the growth of the *T. mentagrophyte* strain used in this study at the maximum concentration tested.

3.2.3 Minimum inhibitory concentration (MIC) determination

Minimum inhibitory concentrations (MICs) are considered the gold standard for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definite answer when a borderline result is obtained by other methods of testing or when diffusion methods are not appropriate [326]

Among the plants studied, the 80 % methanol extract of *I. confertiflora* has been found to exhibit an overall superiority in its antimicrobial activity compared to the other plant extracts according to the agar well diffusion method. The detectable antimicrobial activities of the *I. confertiflora* extract were against *S. aureus* (ATCC) and *T. mentagrophytes* (ATCC); these susceptible organisms were thus chosen for further investigation on the antimicrobial activity of the 80 % methanol extract by the dilution method.

The MIC values of the 80% methanol extract of *I. confertiflora* against *S. aureus* and *T. mentagrophytes* (ATCC) were found to be 125 µg/ml and 3.9 µg/ml respectively. These values are indicative of a higher antimicrobial activity than would be assumed from the agar well diffusion test carried out. In the agar well diffusion test it is to be recalled that the smallest inhibitory concentration against both microorganisms (*S. aureus* and *T. mentagrophytes*) was 5

mg/ml. The relatively lower antimicrobial activity obtained in the agar diffusion test could be due to poor diffusion of the active constituents through the polar media, agar. This argument seems plausible for the antifungal activity where the discrepancy between the two antimicrobial methods is higher because the active component is concentrated in the nonpolar fraction as observed in the higher activity of the petroleum ether extract. Similar observations in the antimicrobial studies of other antimicrobial compounds have been reported [318, 335-337].

3.3 Evaluation of anti-inflammatory activity

One of the characteristics of living tissue is its ability to react to injury. The reaction of living tissue to injury, which comprises a series of changes of the terminal vascular bed, blood, and connective tissues, that tend to eliminate the injurious agents to repair the damaged tissue may be called as “inflammation”. Repair begins during the active phase of inflammation, but reaches completion usually after the injurious influence has been neutralized. Both inflammation and repair generally serve useful purposes. Without inflammation, bacterial infections would remain unencountered, wounds would never heal, and injured tissues and organs might be permanently defected. However, inflammation may be potentially harmful; inflammatory reactions underline the genesis of diseases such as crippling rheumatoid arthritis and allergic reactions that can be life threatening [3].

There are two types of inflammation, acute and chronic inflammation. The classical signs of acute inflammatory reactions are: warmth, redness, pain, swelling and loss of function. The intensity and the localization of the reaction are determined by both severity of the injurious agent and the reactive capacity of the host. Chronic inflammation is also characterized by pain,

redness and swelling but it does not subside in a period of days, but may instead have a relentless, damaging course of several weeks, months or years [3].

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 carrageenan-induced paw edema test is one of the commonly used experimental animal models of acute inflammation. This method has proved to be a suitable test for evaluating anti-inflammatory drugs and has frequently been used to assess the anti-edematous effect of natural products [3]. The results of the anti-inflammatory test carried out by this method are presented in Table 3.4.

The anti-inflammatory effect of the 80% methanol extract of *I. confertiflora* (ICME) was assessed by the carrageenan induced paw edema method using guinea pigs as animal models. In the groups that received ICME at a dose of 1000 mg/ml, the inflammatory (I) response (17.8 %) was significantly lower as compared to those that received the vehicle (60.7 %). Furthermore, this extract was able to exhibit an anti-inflammatory effect (% A) of 70.7 that was observed to be better as compared to the positive control used (34.6).

Table 3.4: Effect of the 80 % methanol extract of *I. confertiflora* (ICME) on the carrageenan-induced inflammation in guinea pigs.

Sample	Dose (mg/kg)	% I	% A
Water	-	60.7± 4.4	-
ICME	500	57.1± 5.0	5.9
	1000	17.8± 3.5*	70.7
IND	10	39.7± 6.4**	34.8

Values are expressed as % anti-inflammatory effect (% A) compared to the control group (n = 7) receiving the vehicle (water) and mean % inflammation (% I) ± SEM; Significance of drug induced reduction in the inflammation were estimated by the student's t-test, * P < 0.0001, ** P < 0.02.

The %A of ICME is also indicative of a good anti-inflammatory activity as compared to other studies done on similar model of inflammation and dose levels [338, 339]. This activity may be the basis of the traditional claim for this plant for some inflammatory diseases such as eczema. ICME dose level of 500 mg/kg was however not able to achieve a significant reduction in the edematous response.

3.4 Skin sensitivity test

There seems to be an overall misconception in the lay community that herbal agents come from 'natural' plants and are therefore 'naturally safe' or 'intrinsically harmless'. The skin seems to be an important target organ of a variety of adverse clinical effects such as allergic skin reactions for herbal drugs. Allergic contact dermatitis has in fact been repeatedly reported to a number of herbal preparations including those containing *Aloe barbadensis*, *Melaleuca alternifolia*, *Calendula officinalis*, and *Eucalyptus globules*, to mention a few common ones [117, 152, 245].

I. confertiflora is one of the commonly used medicinal plants in Ethiopian traditional medicine mainly applied topically. In addition, this plant comes from one of the largest families of flowering plants, Compositae, which is also currently held as the most allergenic family in Europe [245]. Taking these facts into consideration and also in light of the increasing reports of adverse effects of herbal drugs in dermatology, it was deemed appropriate to conduct a skin sensitization test on the extracts of *I. confertiflora*.

Antigen-specific T-cell mediated hypersensitivity reactions occur from 24 to 48 hour after exposure. The T-lymphocytes that have previously been specifically sensitized to an antigen migrate to the region of exposure “recruiting” macrophages leading to the accumulation of basophiles and accompanied by mediator release. The result is erythema and edema in the region of contact. The erythema is the basis of the traditional guinea pig sensitization tests. The edema portion of the sensitization response can be detected when the site of exposure to an antigen is the skin, but it is not easily detected from the background, and therefore is not used as an endpoint marker. When the ear is used as a “challenge” site, however, a response is very evident and easy to quantitatively assess [329].

Conventional tests for identifying potential dermal delayed contact sensitizers have a number of disadvantages, such as cost, length of test, a large amount of required animal care space, difficulty in assessing pigmented materials as antigens, and having a subjective (rather than objective) endpoint. Several of these disadvantages are reflections of the guinea pig as a model and the methodology of evaluating response in terms of observing and subjectively “grading” skin erythema. The mouse ear swelling test (MEST) and variations on it were developed to

overcome these disadvantages [331, 340,341]. The table below contains the results of the MEST done for both petroleum ether and 80% methanol extract of *I. confertiflora*.

Table 3.5: Results of the skin sensitization test conducted by the mouse ear swelling test (MEST) of the petroleum ether extract and the undefatted, 80 % methanol extract of *I. confertiflora*.

Extract	Challenge	Test parameters	Time after challenge	
			24 h	48h
PEE	Initial	% Responsiveness	82.7	42.9
		% Ear swelling	130	121
ICME	Initial	% Responsiveness	0	0
		% Ear swelling	103	104
	Second	% Responsiveness	0	0
		% Ear swelling	103	102

According to the mouse ear swelling ear skin sensitivity test, the petroleum ether extract (PEE) of *I. confertiflora* at a concentration of 10 mg/ml was found to be a dermal sensitizer with 82.7% responsiveness and 130% ear swelling 24 hours post challenge. In addition, the pretest conducted revealed a strong irritant effect of this extract at concentrations higher than 20 mg/ml. Skin sensitization elicited by this extract might be due to the presence of SLs that may be present in the extract. SLs are wide spread in plants of the Compositae family and have been widely implicated as the most important cause of allergic contact dermatitis in this group of plants [245, 274]. The 80% methanol extract of the same plant at a concentration of 40 mg/ml was however not found to be a dermal sensitizer according to the MEST conducted. Measurements done 24 hours post challenge have resulted in the % responsiveness of 0 and the % ear swelling of 103 in

both the initial and the second (confirmatory) challenge done a week later. Based on the obtained result, it can be deduced that the 80% methanol extract is not a strong sensitizer in contrast to the petroleum ether extract.

The difference observed between the two extracts, namely the petroleum ether and the 80% methanol extracts, may be because of the presence of the sensitizing components in less amount in the 80% methanol extract as compared to the non polar fraction, petroleum ether [245]. Furthermore, the presence of compounds with anti-inflammatory activity observed in the 80% methanol extract might have contributed to the lessening of the sensitizing potential of this extract. No report of this sensitizing property exists in the traditional use of the plant. This may be because the polar (aqueous) is traditionally used and as has been observed in this test the allergenic components seem to reside in the non-polar fraction.

The performance of the MEST has been evaluated across a broad range of materials in various studies. These studies have found the MEST to be comparable to the conventional guinea pig skin sensitization tests in predicting human sensitization responses. Even so the database for the MEST, though not small, is not as extensive as that for some of the guinea pig tests [329, 342]. In addition, especially in the test procedure followed in the present test, probably strong sensitizers were only identified because enhancement techniques used in other tests were not used.

3.5 Preliminary screening for the presence of some active plant constituents

Phytochemical screening is a useful tool that gives an idea on some constituents that may be responsible for the pharmacological activities of the plant and serve as a stepping-stone for further isolation studies. In the present case, preliminary phytochemical screening was carried out

on one of the plant materials, namely *I. confertiflora*, which was chosen mainly because of its interesting antimicrobial and anti-inflammatory activities as well as its allergenic potential observed in the nonpolar fraction.

The results of the preliminary screening (Table 3.6) indicated the presence of some active constituents such as flavonoids, saponins and sesquiterpene lactones. Flavonoids are one of the most widely distributed natural products in plants. Studies done on this group of compounds have reported a variety of biological activities including anti-inflammatory, antiallergic, antibacterial, antiviral, and anticarcinogenic activities [17, 261, 288, 343]. This group of compounds may thus be responsible for some of the observed pharmacological activities of the extracts of *I. confertiflora* such as the antibacterial and anti-inflammatory activities.

Table 3.6: Results of preliminary screening of the *I. confertiflora* plant material for the presence (+) or absence (-) of some plant constituents.

Plant constituent	Presence/absence
Polyphenols	+
Flavonoids	+
Phytosteroids	+
Sesquiterpene lactones	+
Phenolic glycosides	-
Alkaloids	
Dragendroff's	-
Mayer's	-
Saponins	
Froth test	+
Hemolysis test	+

Saponins are another group of plant constituents with a wide distribution in higher plants. Reported pharmacological activities that may be of relevance to dermatology include its anti-inflammatory and antimicrobial efficacy. Drugs containing them are in addition usually sternutatory or otherwise irritating to the mucous membrane, which possibly explains similar observation with the crude plant material [17, 344]. Whenever one deals with a plant from the Compositae family, SLs are expected. This group of compounds are responsible for many of the pharmacological activities of the Compositae plants including antifungal, anti-inflammatory, antiproliferative, cytotoxicity against solid tumours, and last but not least their infamous allergenic potential [245, 274, 276, 287, 297, 298]. In the present case, the sesquiterpene lactones detected in the petroleum ether extract may be responsible for the antifungal and anti-inflammatory activities and the skin sensitization property demonstrated in the present study.

3.6 Determination of preliminary standardization parameters

The increasing demand for herbal medicines, both in the developing and developed countries has inevitably led to maintaining the quality and purity of the herbal raw materials and finished products. The standardization problem relating to herbal drugs mainly arises from the complex composition of drugs that are used in the form of the whole plant, plant parts or extract obtained therefrom. To ensure reproducible quality of a herbal remedy, proper control of the starting material is essential. [3]. Some of these preliminary standardization parameters have been attempted on one of the plants that has exhibited interesting pharmacological activities, namely *I. confertiflora* and the results given in Table 3.7 below.

Table 3.7: Determination of preliminary standardization parameters the dried plant material of *I.confertiflora*

Determination	(%)
Total ash	10.9
Acid insoluble ash	0.7
Water soluble ash	5
Moisture content	8.4
Water soluble extractive	10.6
Ethyl alcohol soluble extractive	5
Methyl alcohol soluble extractive	4.6

3.6.1 Determination of solvent extractive values

This method determines the relative amount of active constituents in a given amount of medicinal plant material when extracted with solvents. It is employed for that material for which no chemical or biological assay methods exist. It is mentioned that the determination of water-soluble and alcohol soluble extractives, is used as a means of evaluating crude drugs which are not readily estimated by other means [3].

The extraction of any crude drug with a particular solvent yields a solution containing different phyto-constituents. The composition of these phytoconstituents in that particular solvent depends upon the nature of the drug and solvent used. The determination of solvent extractive value thus permits the selection of a suitable solvent. The use of a single solvent can also be the means of providing preliminary information on the quality of a particular drug sample; for example, in a drug where the extraction procedure for the constituents commences with water as the solvent, any subsequent aqueous extraction on the re-dried residue will give a very low yield of soluble matter. Low yield of soluble matter obtained can also be indicative of poor quality, adulteration

with any unwanted material or incorrect processing of the crude drug during the process of drying, storage, etc [3].

3.6.2 Determination of ash values

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 (metallic salts and silica). This value varies within fairly wide limits and is therefore an important parameter for the purpose of evaluation of any drug. In case of certain drugs, especially at commercial level of processing, the marked difference indicates a change in quality such as the unwanted parts of drugs or more direct contamination, such as by sand or earth, which is immediately detected by the elevated ash value [3, 333]. The ash value can be determined by three different methods to measure the total ash, the acid insoluble ash and the water-soluble ash.

Ashing involves an oxidation of the components of the product. A high ash value is indicative of contamination, substitution, adulteration or carelessness in preparing the crude drug. Total ash is designed to measure the total amount of material produced after complete incineration of the ground drug at as low temperature as possible (about 450°C) to remove all the carbons. At higher temperatures, the alkali chloride may be volatile and may be lost by the process. The total ash usually consists of carbonates, phosphates and silicates that include both plant-derived ash as well as those residues that may adhere to the plant surface such as soil contaminants [3].

Water-soluble ash is that part of the total ash content which is soluble in water. It is good indicator of either previous extraction of the water-soluble salts in the drug or incorrect preparation. Acid insoluble ash value on the other hand indicates contamination with silicious

material e.g., earth and sand. Comparison of this with the total ash value of the same sample will differentiate between contaminating materials and variations of the natural ash of the drug. The value for this acid insoluble ash varies from 0.5% (agar) to as much as 12% (*Hyoscyamus*). If no figure is stated in the individual monograph, it is assumed that the acid insoluble ash should not exceed 2% [3]. The result obtained in case of the *I. confertiflora* plant material was well below the stated figure and thus complies.

3.6.3 Determination of moisture content

Moisture is an inevitable component of crude drugs, which must be eliminated as far as is practicable. The objectives of drying fresh material are to aid in their preservation, protection from enzymatic or hydrolytic reactions, to facilitate subsequent comminution and reduce their weight and bulk. Since the moisture requirements for enzymatic activity and that which microorganisms demand, vary not only with the species, but also with other environmental factors (e.g., temperature, light etc.), it is difficult to state a precise upper limit of moisture that can be permitted in crude drugs [3]. The moisture content in case of air-dried plant material of *I. confertiflora* was 8.4%.

3.7 Chromatographic techniques

3.7.1 Qualitative TLC analysis

TLC chromatogram of the petroleum ether extract exhibited two visible zones of R_f values 0.61 and 0.73. Observation under UV-366 yielded two fluorescent zones (both of pink red color) with R_f s 0.77 and 0.84, while no zone was observed under UV-254 nm. This latter observation may be indicative of the absence of flavonoids in the petroleum ether extract, as flavonoids are known to exhibit quenching zones under UV-254 [345]. Visualization of this chromatogram after spraying

with vanillin-sulphuric acid reagent yielded a total of ten distinct visible zones with R_f values ranging from 0.12 to 0.99 as demonstrated on Figure 1.

TLC chromatogram of the 80 % methanol extract on the other hand gave a single visible zone of R_f 0.92. When this chromatogram was observed under UV-254, three quenching zones (R_f values 0.65, 0.84 and 0.92) were observed. Observation in UV-366 gave three light blue fluorescent zones (R_f values 0.35, 0.49 and 0.65) and one pink red fluorescent zone (R_f 0.92). Visualization carried out after spraying with vanillin-sulphuric acid reagent gave two visible zones – a blue-violet zone (R_f 0.87) and a pink-red zone (R_f 0.67) as can be seen, albeit vaguely on Figure 2. These two zones obtained may be representative of saponins that have been found to be present in the plant, as it has been reported that these groups of compounds exhibit these colors after spraying with this reagent. The blue fluorescent zones in UV-366 and the quenching zones in UV-254 nm may be indicative of the flavonoids that have been found to be present in the plant [345].

I. confertiflora
Pet. ether



Fig 1: TLC chromatogram of the petroleum ether extract (PEE) of *I. confertiflora* using a mobile phase, petroleum ether – chloroform - acetone after in the ratio of 5:3:2 after spraying with vanillin-sulphuric acid reagent.

I. confertiflora
Whole extract



Fig 2: TLC chromatogram of the undefatted, 80% methanol extract of *I. confertiflora* (ICME) using a mobile phase chloroform – ethylacetate – methanol – formic acid in the ratio of 3:3:3:1 after spraying with vanillin-sulphuric acid reagent.

3.7.2 Preparative TLC

3.7.2.1 Yield of the TLC fractions

TLC is a useful and simple method of separation that has found great use in pharmacy especially in the natural products field. In this study it was used to fractionate the petroleum ether extract of *I. confertiflora*, chosen mainly because the antimicrobial activity (especially the antifungal activity) of the plant is concentrated in it. Approximately, a total of 1024 mg of the extract (as solution in chloroform) was applied on the plates and the result obtained as fractions' yield is given below.

Table 3.8: Fractions obtained after carrying out preparative TLC on the petroleum ether extract (PEE) of *I. confertiflora* using a plate of 0.5 mm thickness.

TLC fractions	R _f range (approximate)	Approximate yield (mg)*
1	0.03 – 0.12 (both sides inclusive)	118
2	0.12 (exclusive) – 0.40 (inclusive)	174
3	0.40 – 0.73 (both sides exclusive)	260
4	0.73 – 1.0 (both sides inclusive)	320

* Yield was calculated from a total of approximately 1024 mg of the extract applied as a band (200µl of a 160 mg/ml extract) to 32 - 20×20 TLC plates.

3.7.2.2 Antimicrobial activity of the TLC fractions

The results of the TLC fractionation indicated that antimicrobial activity of the petroleum ether fractions was concentrated in the components with higher R_f values (higher than 0.40) (Table 3.7). The fact that different antimicrobial activities were exhibited by these fractions is interesting in that, the components having antibacterial and antifungal activities may be different. Similar to

the results obtained for the crude extracts of *I. confertiflora*, inhibitory activity was observed only against *S. aureus* (ATCC) and *T. mentagrophytes* (ATCC), and not against the other test organisms (data not shown).

Table 3.9: Diameter of inhibition zones of the TLC fractions of the petroleum ether extract (PEE) of *I. confertiflora* against *S. aureus* (*Sa^s*) and *T. mentagrophytes* (*Tm*) at a concentration of 10 mg/ml

PEE fractions	<i>Sa^s</i>	<i>Tm</i>
1	-	-
2	-	-
3	2	7
4	7	2

3.8 Evaluation of *in vitro* antimicrobial activity of suggested topical formulations

The 80% methanol extract of *I. confertiflora* has exhibited interesting antimicrobial activity, anti-inflammatory activity and a low sensitizing potential. More importantly, this plant is used traditionally for a variety of skin conditions and trials to formulate the extract of this plant as a phytopharmaceutical topical preparation may increase its antimicrobial and anti-inflammatory efficacies.

The nature of vehicle in which a drug is formulated has considerable effect on its efficacy, which may be more pronounced in case of topical drug delivery because the vehicle remains at the site of administration [346]. This influence of the vehicle has been well observed in the results obtained in the present study, where different antimicrobial activities have been demonstrated by the different topical formulations of *I. confertiflora* (Table 3.8). Higher antimicrobial activities

were demonstrated by the hydrophilic formulations as compared to the hydrophobic formulation. On the other hand, the antifungal activity of the formulations was higher than their antibacterial activities commensurate with the activities of the crude plant extract. The hydrophilic bases could thus be used as a starting point for further formulation studies of this extract.

One of the major factors that affect the efficacy of topical formulations is the affinity of the active ingredients to the vehicle. Thus solutes “held loosely” in the vehicle exhibit high escaping tendencies and high release while those solutes “held firmly” exhibit low escaping tendencies and consequently low release [346]. The antimicrobial activity, especially that against *T. mentagrophytes* has been observed to be noticeably higher by the nonpolar (petroleum ether) extract of *I. confertiflora* as compared to that by the 80% methanol extract. This observation leads to the assumption that at least the antifungal components of the 80 % methanol extract are nonpolar in nature and that relatively polar vehicles would give a more efficacious formulation.

The above phenomenon has in fact been observed in the performance profiles of the *I. confertiflora* formulations where negligible antimicrobial activity was demonstrated by the hydrophobic formulation (in white soft paraffin base) while comparatively higher activities were demonstrated by the hydrophilic formulations. Similar results were obtained in other studies done to compare different topical formulations of nonpolar compounds, namely mupirocin and a volatile oil (*Ocimum*). In these studies, hydrophilic formulations produced higher zones of microbial inhibition as compared to the hydrophobic preparations [314, 315].

Table 3.10: Diameter of inhibition zone of topical *I. confertiflora* formulations and commercial products against *S. aureus* (*S.a^s*) and *T. mentagrophytes* (*Tm*).

Suggested <i>I. confertiflora</i> formulations	Zone of inhibition (mm diameter)	
	<i>S.a^s</i>	<i>Tm</i>
I	8	38
II	1	11
III	1	14
IV	2	29
V	-	1
Base demonstrating activity		
I	4	6
Commercial products		
Bactroban® ointment	31	
Foban® ointment	22	
Foban® cream	22	
Tetracycline HCl ointment	16	
Nizoral®		10
Canesten®		37

The results obtained in this study have indicated that *I. confertiflora* extract in the hydrophilic bases (water soluble ointment and oil-in-water creams) demonstrated a good performance. Of these, the formulation in the sodium laurate cream base exhibited the highest inhibitory activity against both *S. aureus* and *T. mentagrophytes*. The sodium laurate cream base has however exhibited some activity against the test organisms. This antimicrobial activity of the base has been observed in a similar study and the inherent antimicrobial activity was ascribed to the surfactant, sodium lauryl sulphate (SLS), present in the cream base [315]. Incidentally, another

base used in the present study for Formulation IV also has SLS as one of its ingredients, although it did not exhibit detectable antimicrobial activity. This phenomenon may be because of the presence of SLS in lower amount in this base. On the other hand, the water content of the sodium laurate cream base used for Formulation I and hence polarity seem to be higher compared to that used for Formulation IV. This phenomenon may be responsible for the more efficacious formulation obtained with this base by enabling the ready release of the nonpolar, active antimicrobial components.

The hydrophilic formulations of *I. confertiflora* extract compared well to the commercial antifungal products in their activity against *T. mentagrophytes*. The commercial antibacterial products have however shown good antibacterial activities and all were better than the extract formulations in their activity against *S. aureus* as can be seen on Table 3.8. Of these, Bactroban® ointment exhibited the highest activity similar to another study carried out to compare similar products [314].

The agar well diffusion method is widely used for evaluation of topical antimicrobial formulations. However, limitations have been cited especially in the evaluation of formulations containing nonpolar active compounds whereby low or no zones of microbial inhibition were exhibited as compared to some modifications to the method. Some of the modifications proposed include the needle extrusion test (where the topical agent was applied directly to the agar surface), the disk diffusion test (where the topical agent was added to the surface of a disk) as well as a method where the semisolid formulation was placed in a strip of dialysis tubing (clamped off at each end) that was in turn placed on a seeded agar plate. In the latter method, the

contact time needed to kill the microorganism was determined by removing tubed samples at specified times [318, 320].

Taking into consideration the limitations observed with the agar well diffusion method for evaluation of especially nonpolar compounds, it would be worth a try to explore modifications to the presently used method. Nevertheless, the agar well diffusion method used in the present study has achieved its intended purpose of assigning different activities to the different formulations. Based on the nature of the base of the extract formulation, negligible activity was demonstrated by the hydrophobic based formulation while detectable and promising activities were exhibited by the hydrophilic formulations that were in some cases better than the commercially available products. This method went further to enable differentiation of the performance of the hydrophilic formulations in their activity against *T. mentagrophytes*.

4. CONCLUSION

From the foregoing study, it can be concluded that all of the tested 80% methanol extracts demonstrated antibacterial activities against *S. aureus* (ATCC) albeit weak as compared to the positive control. The only plant extract that exhibited activity against other bacteria is *C. simensis*, which was able to show an almost negligible activity against *P. aeruginosa*. Antifungal activity was demonstrated only by the crude extracts of *I. confertiflora* while the other plant extracts were devoid of any activity at the tested concentrations. *I. confertiflora* 80% methanol extract exhibited a good activity against *T. mentagrophytes* with the active components concentrated in the non-polar fraction as observed in the higher activity by the petroleum ether extract. Furthermore, *I. confertiflora* extract exhibited a higher antimicrobial activity when tested by the agar dilution method as compared to the agar well diffusion method.

The 80% methanol extract of *I. confertiflora* exhibited a promising anti-inflammatory activity in the carrageenan-induced paw edema test, which was observed at a higher dose (1000 mg/kg) but not at a lower dose (500 mg/kg). In the skin sensitization test carried out by the mouse ear swelling test, the petroleum ether extract of *I. confertiflora* proved to be a strong skin sensitizer while the 80% methanol extract gave a negative result in the same test model proving that it was at least not a strong sensitizer. Some active plant constituents such as flavonoids and sesquiterpene lactones, which might be responsible for the observed pharmacological activities of the extracts of *I. confertiflora*, were found to be present.

I. confertiflora 80% methanol extract formulations in hydrophilic (emulsifying and water soluble) bases proved to be more efficacious compared to that in the hydrophobic (white soft

paraffin) base. Of the hydrophilic bases the sodium laurate monostearin cream base gave the highest antimicrobial activity.

RECOMMENDATIONS FOR FURTHER WORK

Based on the present study, the following are areas to be considered for further work:

- The antimicrobial activity test was conducted by the agar well diffusion method for all the crude extracts with one exception. It is recommended to carry out antimicrobial tests by other methods such as the dilution method for all the extracts to check if there are differences in activity. In addition, only a few species of microorganisms were used in the study. Antimicrobial tests are recommended to be done on more strains of the same species and more species that are of relevance to skin disorders.
- It is recommended to carry out anti-inflammatory activity test on the other plant extracts and on other models of inflammation.
- Skin sensitization tests should be carried for all the plant extracts that are potentially used for dermatological application. In addition, tests should be designed to enable not only strong but also moderate and weak sensitizers.
- Some preliminary standardization parameters have been determined for one of the plant materials. It would be useful to carry out further standardization works especially on the promising plants.
- In case of *I. confertiflora*, both the leaves and flowers were extracted and used for analysis in light of the traditional use of the plant. To be able to identify which one of the plant parts is responsible for the specific pharmacological activity, it would be desirable to extract the parts individually and test for activity.
- To enable identification of the active constituent(s) for the demonstrated pharmacological activities, whether beneficial or adverse, it is highly recommended to carry out bioassay-guided fractionation.

- For those plant extracts that are promising, further formulation studies should be conducted.

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