CLINICAL EVALUATION AND MONOGRAPH DEVELOPMENT FOR A GHANAIAN POLYHERBAL PRODUCT (*EAF-2011*) USED IN THE MANAGEMENT OF SUPERFICIAL MYCOSES

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DECLARATION

I hereby declare that this submission is my own work towards the award of a Doctor of Philosophy and that to the best of my knowledge, it contains no material previously published by another person for the award of any degree of the University, except where due acknowledgement has been made in text.

The experimental work described in this thesis was carried out at the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST, the Clinical Research, Microbiology and Pharmacology Departments of the Centre for Plant Medicine Research, Mampong-Akuapem.

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ABSTRACT

Herbal medicines are the most accessible form of healthcare product for majority of the world's population and have been used over time to address the health needs of several societies. In the present study, a Ghanaian polyherbal product (ointment) from the Centre for Plant Medicine Research comprising: *Alchornea cordifolia, Eugenia caryophyllata, Psidium guajava, Zanthoxylum zanthoxyloides* and *Tridax procumbens*, coded *EAF-2011* and used in the management of superficial fungal skin diseases was assessed for its quality, safety and effectiveness. Qualitative chemical fingerprinting of the ointment indicated the presence of phytochemicals including alkaloids, phenols, triterpenes and flavonoids. Thin layer chromatograms also produced three marker spots whose properties make them suitable for use as analytical markers. Quantitative chemical assay of three flavonoid compounds in the product *EAF-2011* using High Performance Liquid Chromatography (HPLC) showed the presence of 8.6810% ($^{w}/_{w}$) of rutin (RU), 0.2670% ($^{w}/_{w}$) of quercetin (QE) and 0.0610% ($^{w}/_{w}$) of kaempferol (KA). A twelve (12) month stability study that assessed the product for its pharmaceutical quality using organoleptic and physicochemical tests, Thin Layer Chromatography (TLC), HPLC and an antimicrobial assay during the storage of the product under ambient conditions revealed marginal changes in chemical constituents with one of the spots obtained on thin layer analysis undergoing a colour change to purple compared to the baseline colour of brown. The concentration of quercetin was also undetectable after the 6^{th} month of assay during the HPLC analysis. This change in chemistry was however considered insignificant as the biological activity of the product remained unaffected over the period of study based on the results of the antimicrobial assay. An *in-vivo* chronic toxicity and skin sensitisation test using male Sprague-Dawley rats showed that three concentrations of the herbal extracts [i.e. 2% ($^{w}/_{w}$), 5% ($^{w}/_{w}$) and 10% ($^{w}/_{w}$)] had no adverse effect on the haematological, biochemical and urine analytical

parameters of the animals used. The ointment did not induce any histological changes in skin, liver, kidney and spleen of the animals used. In the clinical study involving 84 participants diagnosed with superficial mycoses, the 10% ($^{w}/_{w}$) concentration of the herbal product was most efficacious with 91.3% of participants randomised to the group achieving the primary outcome of complete cure compared to 30.0% achieved with the standard treatment of Whitfield ointment after 3 months. The efficacy of two (2) other concentrations of the herbal product tested [2% ($^{w}/_{w}$) and 5% ($^{w}/_{w}$)] was also comparable to Whitfield ointment. The products tested were also safe for human use as haematological, biochemical and urine biochemistry parameters were normal at the end of the study for all the treatment groups. Re-evaluation of the component raw materials of the product using a combination and interactive study to establish their contribution to the overall activity of the product showed that *Eugenia caryophyllata*, *Alchornea cordifolia* and *Zanthoxylum zanthoxyloides* had better activity individually than the total crude extract of five plants used in the formulation of the original product. The combination of *Eugenia caryophyllata* 60% ($^{w}/_{w}$) and *Alchornea cordifolia* 40% ($^{w}/_{w}$) was selected after further screening and analysis using the fractional inhibitory concentration (FIC) and an isobolographic analysis. A new product (*RF-2013)* formulated using this combination as the recipe at a concentration of 5% ($^{w}/_{w}$) was clinically evaluated against superficial fungal skin infections in another human trial. This product was subjected to a randomised controlled single blind study in 15 participants with the 10% ($^{w}/_{w}$) *EAF-2011* as the control treatment. Primary outcome was achieved by all participants receiving the control treatment compared to the 60% attained in the 5% ($^{w}/_{w}$) *RF-2013*. Based on the number of participants and their time taken to achieve the primary outcome, the 10% ($^{w}/_{w}$) *EAF-2011* which is the original formulation was proposed as the preferred treatment in the management of superficial fungal skin infections.

DEDICATION

This dissertation is dedicated to Anthony, Florence, Kobina, Nyamenaorye and my dearest Maame.

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Thanks be to God and Father of our Lord Jesus Christ, who has blessed me with every spiritual blessing to accomplish every good work He has placed in me.

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LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
AST	Aspartate Transaminase
BAS	Basophils
CDE	Cutaneous Drug Eruptions
CONSORT	Consolidated Standards for Reporting Trials
CREAT	Creatinine
DMSO	Dimethyl Sulphoxide
EOS	Eosinophils
FIC	Fractional Inhibitory Concentration
GGT	Gamma Glutamyl Transferase
HB	Haemoglobin
HCT	Haematocrit
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HPV	Human Papilloma Virus
HSV	Herpes Simplex Virus
ICH	International Committee on Harmonisation
IUPAC	International Union of Pure and Applied Chemistry
IFN-c	Interferon c
LYM	Lymphocytes
MCHC	Mean Corpsular Haemoglobin Concentration
MCH	Mean Corpsular Haemoglobin
MCV	Mean Corpsular Volume

MPV	Mean Platelet Volume
MON	Monocytes
NEU	Neutrophils
OTC	Over the Counter drugs
PBS	Phosphate Buffer Saline
PLT	Platelets
RBC	Red Blood Cells
RDW	Red Blood Cell Distribution Width
RCT	Randomised Controlled Trial
R_f	Retardation Factor
S.G	Specific Gravity
TC	Total Crude of Herbal Extracts
TLC	Thin Layer Chromatography
TSSS	Total Signs and Symptoms Score
WBC	White Blood Cells
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1. General Introduction

The African continent remains an abundant source of several plant species with medicinal properties that have been employed in the treatment of ailments for centuries. In Ghana, herbal medicine and other traditional therapies remain the most accessible form of primary healthcare for majority of the population. Statistics put the figure at about 80% of the general population in the developing world (WHO, 2002). The prospects of herbal medicines in addressing the healthcare needs of society lie in their widespread use and potential at providing alternative treatment options to the conventional medicines available.

Medicinal plants continue to play a very important role in the treatment of infections with this role increasing in recent years due to the challenge of microbial resistance. Numerous plants species are therefore being screened for their possible antimicrobial properties both as crude preparations and chemical isolates towards the development of new agents (Nichols, 1999; Senekal, 2010). This search has become even more imperative due to the cost, morbidity and mortality rates associated with treatment failures. The challenge of antimicrobial resistance is not only a problem in systemic infections but also in localised infections such as those that affect the skin and its appendages (Ogunbiyi *et al.*, 2005).

1.2. The Burden of Skin Diseases in Humans

The skin is the largest organ of the human body and one of the most complex in structure. It has the basic role of protecting and maintaining the balance between the

internal and external environment of man (McKay and Aldridge, 2006; Grice and Segre, 2011). Its specific roles include controlling the exchange of gas between the human body and the environment and also regulating the temperature of the body. The skin also contains sensory organs that interact with the environment, provides a physical barrier against the penetration of inorganic matter and acts as a biochemical defence against viruses and other pathogens. It also provides a cushion against trauma and is equipped with dendritic cells to trigger immune responses (Giacomoni *et al.*, 2009). Despite its protective structure, the skin's exposure to the outside world makes it prone to the harsh environmental conditions. This barrier may hence be breached and become subject to various diseases; common among these are the fungal, bacterial and viral diseases as well as malignancies.

Skin diseases are among the most common health problems worldwide and are associated with a considerable and multidimensional burden which includes psycho-social and financial consequences on the patients, their families and on society. There are currently more than 3000 conditions that affect the skin (Bickers *et al.*, 2004), from chronic and incurable skin diseases such as psoriasis and eczema; associated with significant morbidity in the form of physical discomfort and impairment of patients' quality of life, to malignant diseases such as melanomas which carry mortality of about 20,000 per year in sub-Saharan Africa (Lundberg *et al.*, 1999; Weinstock and Gardstein, 1999). This burden of mortality has been comparable to those attributed to meningitis, hepatitis B, obstructed labour and rheumatic heart disease conditions that continue to receive great attention (Mathers *et al.*, 2001). However, in assigning health priorities, skin diseases are sometimes thought of in planning terms, as small-time players in the global league of illnesses compared with diseases that cause significant

mortality such as HIV/AIDS, community-acquired pneumonia and tuberculosis. This position has lead to the neglect of superficial skin diseases (Hay *et al.*, 2006).

Superficial mycoses also referred to as dermatophytosis are a group of very common infections. These infections have usually received less attention like most skin diseases because they are considered to carry lesser morbidity and mortality (Mathers *et al.*, 2001). Estimates however put the prevalence of this class of skin infection which is very common in children at about 10% to 20% of the general population (Havlickova *et al.*, 2008). In Africa, there have been similar reports on the prevalence in different countries: Ethiopia (49.2%), Tanzania (34.7%) and Nigeria (40.4%) (Oladele *et al.*, 2010). Ghana's trend of infections are slightly less, with rates between 10.6% and 12.5% reported in the urban areas, while prevalence rates in some rural communities have been recorded as 18.4% (Doe *et al.*, 2001; Boakye, 2008). Again, using a comparative assessment of disability-adjusted life years (DALYs), it was reported that the impact of fungal skin diseases is comparable to conditions like gout, arthritis, endocrine diseases and panic disorders that continue to receive much more attention (Hay *et al.*, 2006).

1.3. Quality and Clinical Assessment of Herbal Medicines

Herbal medicines are a viable treatment option for fungal skin infections with the management of the condition using medicinal plants still very popular and widespread (Abad *et al.*, 2007). Notwithstanding the numerous *in vitro* and *in vivo* evidence in literature, there is still a wide gap in proving the clinical value of these medicinal agents using randomised controlled trials (RCT) (Guo *et al.*, 2007). This deficit in evidence or data has been noted in several reviews as posing a great challenge towards the advancement of herbal medicine practice as evidence based practices and

treatments are accepted as the norm in clinical medicine today (Martin and Ernst, 2004; Simaan, 2009). In this regard, every treatment is expected to be subjected to scientific evaluation to establish its benefit to risk ratio for users especially when there is an existing treatment (Donald and McCullough, 2009). Currently, the World Health Organisation (WHO) advocates the use of well-established, randomised controlled clinical trials (RCT) to provide the highest level of evidence for efficacy and safety of medicinal products. Such studies facilitate the confirmation of medicines in therapy including herbals in different regions of the world (WHO, 2002). Other protocols like the Consolidated Standards for Reporting Trials (CONSORT) have also provided guidelines for such evaluations.

Aside the lack of clinical evidence, the quality of Herbal Medicinal Products (HMP) also remains an outstanding issue that needs to be addressed. The complex nature of medicinal plants and their products unlike orthodox medicines makes their quality control very challenging. A typical issue is the selection of constituents that are ideal for use as marker(s) for the raw materials and their products during quality control (Folashade *et al.*, 2012). In cases where characteristic markers are available, uncertainties about the role of such constituents in the therapeutic activity of products limits the inferences that can be drawn by either their presence or absence in a particular herbal medicinal product. However, as in the case of the clinical evaluation of HMP's, protocols and guidelines for the quality control have been made available by regulators and other authorities such as the International Union Pure and Applied Chemistry (IUPAC) (Moshihuzzaman and Choudary, 2008). These guidelines when properly applied generate adequate data that can be applied as a standard with which subsequent products can be compared to ensure consistency and maximize the therapeutic effect produced by herbal medicinal agents.

1.4. Justification for the Study

The Centre for Plant Medicine Research, Mampong-Akuapem (CPMR) is an agency of the Ministry of Health, Ghana which is mandated to carry out research towards the development of herbal medicines for public use in the country. The institution has been collaborating in this regard with traditional practitioners for over 30 years. These collaborations have resulted in the formulation of the over 20 herbal medicinal products currently in use by the institution for the management of numerous disease conditions (CPMR, 2014).

The product under study coded as *EAF-2011* and comprising five plants is the result of one such collaboration between a traditional practitioner who had been using the formulation for over 20 years. *EAF-2011* which is a topical preparation was adapted by the CPMR for the management of superficial fungal infections but has remained unprescribed formally at the institution due to the lack of documented standards on quality and the absence of clinical evidence. The use of five plants for the formulation of the product has also made it unattractive due to the cost of production and issues about conservation of medicinal plants.

1.5. Aim of the Study

The study aimed to develop a monograph for the polyherbal product *EAF-2011*, using data from preclinical and clinical assessments of the product.

1.5.1. Specific Objectives

i. To evaluate the pharmaceutical stability of *EAF-2011* using a physicochemical assessment, thin layer profiling, high performance liquid chromatography and an antimicrobial assay.

ii. To develop chemical standards for *EAF-2011* using basic phytochemical screening, thin layer chromatography and high performance liquid chromatography.

iii. To evaluate the safety of *EAF-2011* using a preclinical skin sensitisation and chronic toxicity study of the product in Sprague-Dawley rats.

iv. To assess the clinical safety and effectiveness of *EAF-2011* at different concentrations of 2% ($^{w}/_{w}$), 5% ($^{w}/_{w}$) and 10% ($^{w}/_{w}$) of the total crude extract in an emulsifying ointment base with Whitfield's ointment as the standard drug.

v. To re-evaluate the relevance of each component plant material used in the formulation using an interactive combination assay of the product and propose a new formulae for the product.

vi. To compare the clinical effectiveness of the reformulated product (*RF-2013)* with the most effective concentration of the original product.

CHAPTER 2

LITERATURE REVIEW

2.1. Skin Diseases

Skin diseases are grouped under the major categories of microbial, dermatitis and immune related diseases, malignant and benign neoplasm and exogenous skin diseases that occur as a result of injuries. This classification encompasses over 3000 skin diseases some of whose epidemiology and the clinical forms exhibited form a subset of an already prevalent skin condition e.g. *Tinea coporis* and its clinical sub form of *Tinea incognito* (Bickers *et al.*, 2004).

2.1.1. Microbial Skin Diseases

Microbial skin diseases are classified as fungal, bacterial or viral in origin. This group accounts for the largest cause of all skin diseases and are discussed below.

2.1.1.1. Cutaneous Fungal Diseases

Cutaneous fungal diseases are among the most common of all skin diseases affecting an estimated 10-20% of the general population (Havlickova *et al.*, 2008). Dermatophytosis, *Tinea vesicolor* and cutaneous candidiasis are the major components of this group of infections. *Malassezia folliculitis*, *Tinea nigra*, Black and White Piedra form the other subset belonging to this class, but they are rarely diagnosed and also very uncommon (Ameen, 2010).

2.1.1.1.1. Dermatophytosis

Dermatophytosis is a broad term used to describe superficial fungal skin diseases. They usually are infections of keratinized structures, such as the nails, hair shafts and stratum

corneum of the skin. Three genera of fungi: *Trichophyton, Epidermophyton* and *Microsporum,* are classified as the dermatophytes (Ameen, 2010). Infections coming from these organisms are distinguished from the other superficial fungal infections like *Tinea versicolor* whose causative organism is *Malassezia furfur.* Although not part of the normal skin flora, they are well adapted to adhere to it because of their use of keratin as a source of nutrients. These infections are very common and highly variable in their clinical presentations. Microorganisms implicated in dermatophytosis are found in humans (anthropophilic), animals (zoophilic) and in the soil (geophilic). The zoophilic and geophilic forms are now very rarely found in humans (Wagner and Sohnle, 1995).

2.1.1.1.2. Classification of Dermatophytes

Traditionally, dermatophytes are classified based on the anatomical sites they infect on the human body: *Tinea barbae,* infection of the skin of the bearded area and neck; *Tinea capitis,* infection on skin of the scalp and head; *Tinea corporis,* infection of skin of the trunk and extremities; *Tinea cruris,* infection of the skin of the groin, proximal thigh, and buttock; *Tinea faciale,* infection of skin of the face; *Tinea manuum,* infection of skin of the palm, soles, and interdigital webs; *Tinea pedis* (athlete's foot), infection on skin of the foot and *Tinea unguium* (onychomycosis), infection of the nail (Dahl, 1994; Wagner and Sohnle, 1995; Jones, 1998).

2.1.1.1.3. Epidemiology of Dermatophytes

Microorganisms commonly implicated in dermatophytosis are *Trichophyton rubrum, Trichophyton mentagrophytes var. interdigitale, Microsporum canis,* and *Epidermophyton floccosum.* These vary in their occurrence at the various parts of the

human body and are highlighted in Table 2.1 as the dermatophytes implicated in the various classes of infections and their sources.

Apart from the variation in distribution according to the anatomical sites, others have partial geographic restriction, such as *Trichophyton schoenleinii* (Eurasia, Africa), *Trichophyton soudanense* (Africa), *Trichophyton violaceum* (Africa, Asia, and Europe), and *Trichophyton concentricum* (Pacific Islands, Far East, and India) (Ameen, 2010).

Most cases of *Tinea capitis*, *Tinea unguim* and *Tinea coporis* in both developed and underdeveloped countries are caused by the *Trichophyton* and *Microsporum spp*. The zoophilic forms of these microorganisms are increasingly becoming rare due to improvements in the standards of living and rather interestingly an increased incidence in the cases of the anthropophilic forms (Foster *et al.*, 2004). This change has led to the domination of *Trichophyton rubrum* as the major causative organism and a decline in infections with species like *Epidermophyton. floccosum* and *Microsporum. audouinii* (Borman et al., 2007).

2.1.1.1.4. Predisposing Factors

Factors that enhance the thriving of dermatophytes on the skin are known to be local; a moist and warm skin environment is likely to enhance the growth of most dermatophyte after exposure. Several other systemic conditions are also known to increase this risk including individuals with collagen vascular disease, systemic corticosteroid therapy or Cushing's disease, haematologic malignancy, chronic mucocutaneous candidiasis and diabetes mellitus. Advanced age has also been related to an increased incidence of dermatophytosis. Another increasing contributor is

immunosupression majority of which are from HIV and AIDS and abuse of steroids (Drake *et al.*, 1996).

Table 2.1: Summary of the microorganisms implicated in the various classes of dermatophytosis and their sources. (Wagner and Sohnle, 1995)

Type of infection	**Anthropophilic**	**Zoophilic**	**Geophilic**
Tinea pedis	*T. rubrum* *E. floccosum*	*T. mentagrophytes,*	
Tinea cruris	*E. floccosum* *T. rubrum*	*T. mentagrophytes*	
Tinea barbae	*T. rubrum*	*T. mentagrophytes* *T. verrucosum,*	
Tinea unguium (onychomycosis)	*T. rubrum* *E. floccosum*	*T. mentagrophytes*	
Tinea capitis	*T. tonsurans* *T. schoenleini* *T. violaceum*	*M. canis* *T. mentagrophytes*	
Tinea corporis	*T. rubrum,* *T. concentricum* *T. tonsurans* *E. floccosum*	*M. canis*	*M. gypseum*

2.1.1.1.5. *Tinea vesicolor*

This is a superficial fungal infection characterized by skin pigmentary changes due to colonization of the stratum corneum by a dimorphic lipophilic fungus in the normal flora of the skin known as *Malassezia furfur*. *Tinea versicolor* is also known as *Pityriasis versicolor* and less commonly as *Dermatomycosis furfuracea*, *Tinea flava*, or *Achromia parasitica*. It has a worldwide distribution but is more common in the tropics due to the relative high temperature and humidity (Adamski, 1995).

This condition was thought of initially as a post-pubertal disease; however evidence has shown that *Tinea versicolor* is not uncommon in children. This may be caused by

hormonal changes and/or increases in sebum secretion. Studies revealed a prevalence of about 4.9% in children between the ages of 5 months to 13 years and accounts for 3% of dermatological cases recorded. Though widely believed, *Tinea vesicolor* is not a result of poor hygiene and is non-contagious (Bergbrant and Brobeg, 1994; Savin, 1996).

The pathogenesis of *Tinea vesicolor* is similar to that of the dermatophytes. Depressed cellular immunity is believed to play a major role. Individuals who are malnourished, those on immunosuppressive treatments, those with seborrheic dermatitis and Cushing's syndrome are also at high risk. Hereditary factors seem to play a role in the disease; a positive family history accounted for approximately 17% of infections in one study, while conjugal cases were less commonly reported (Savin, 1996).

2.1.1.1.6. Cutaneous Candidiasis

Although very rare, the incidence of cutaneous fungal skin diseases is increasing in number due to the surge in cases of immunosuppression from HIV and AIDS. Most are caused by the yeast *Candida albicans*. Other rare *Candida* species implicated are *Candida parapsilosis* or *Candida tropicalis* which may present in an acute or a chronic form. These *Candida spp* can also infect the nails (paronychia) and feet (athletes foot). Clinical features of cutaneous candida infections include erythema, oedema, pustule formation and a characteristic creamy exudate. (Silva-Lizama, 1995).

2.1.1.2. Bacterial Skin Infections

Bacterial skin infections may range from simple infections like folliculitis to deep seated ones that may extend to the dermis. Humans are natural hosts for many bacterial species that colonize the skin as normal flora. *Staphylococcus aureus* and

Streptococcus pyogenes are infrequent resident flora, but they account for a wide variety of bacterial pyodermas (Trent *et al.*, 2001).

The clinical presentation of bacterial infections include: shallow crusted ulcers, which are due to untreated staphylococcal or streptococcal impetigo that extend deeply penetrating the dermis; folliculitis, a superficial infection of the hair follicles characterized by erythematous, follicular-based papules and pustules; furuncles, deeper infections of the hair follicle characterized by inflammatory nodules with pustular drainage, which can coalesce to form larger draining nodules (carbuncles) commonly referred to as boils and acne and dry scaly erythematous sebaceous follicles all have *S. aureus* as the usual pathogen (Carroll, 1996).

2.1.1.3. Viral Skin Diseases

Viral skin infections include herpes simplex and herpes zoster. These two categories of viruses are responsible for common skin ailments such as cold sores and shingles. The herpes simplex viruses (HSV), referred to as HSV-1 and HSV-2, are responsible for several common and recognizable ailments such as cold sores that appear on the lips and sexually-acquired genital herpes. Among the varied presentations of HSV-1 infection, cold sores on and around the lips and mouth are the most common manifestation of HSV, with approximately 20-40% of adults experiencing these outbreaks in a year (Simmons, 2002).

Herpes zoster (HZ) is caused by the *Varicella-zoster* virus which is also responsible for causing chicken pox. Following chicken pox, the virus becomes dormant in the peripheral nervous system, where it can remain for several years. When reactivated, the virus re-emerges as herpes zoster or “shingles,” producing an itchy or painful rash that

usually follows the pattern of distribution of the affected nerve and a feeling of malaise, all of which may last for two to three weeks (Dwyer and Cunningham, 2002).

Human papilloma virus (HPV) is another virus that infects cells of the epithelium, including the epidermis of the skin and the surface layer of mucous membranes. There are more than eighty (80) different serotypes of HPV (Severson *et al.*, 2001). HPV may remain dormant without visible symptoms in infected individuals, but it also may result in non genital or genital warts, condylomata (wart-like growths), and polyps. While non-genital warts rarely pose a serious health problem, they can cause physical impairment and psychosocial discomfort. HPV induced lesions may become malignant, resulting in cancers with cervical cancer being one of the most common (Bellew *et al.*, 2004).

2.2. The Role of Traditional Medicine in Primary Healthcare Delivery

The profile of traditional therapies has been on the rise since the Alma-Ata declaration of 1978. This declaration continues to influence the attention given to primary healthcare of which traditional medicine plays a major role due to its accessibility and widespread use especially in developing countries (Ameh *et al.*, 2010). In Ghana, the predominant form of traditional medicine practised involves the use of medicinal plants and their products.

2.2.1. Factors Affecting the Rising Profile and Demand for Herbal Medicines

Generally, anxiety about the adverse effects caused by allopathic drugs, improvements in the access to health information, changing values and reduced tolerance of paternalism are factors that have been recorded as influencing the growing demand for natural remedies (WHO, 2002).

In developing countries like Ghana, accessibility to this form of treatment and the cost involved are the major factors at play. Socio-cultural compatibility has also been noted and this has been attributed to the fact that most of these practices have evolved and continue to be shaped by the culture of the society where they emerged from (Chatora, 2003).

In the western and developed nations, the increased usage of herbal remedies has been attributed to the increasing incidence of chronic diseases whose management with orthodox medicines is faced with challenges (Thorne *et al.*, 2002). This factor is also applicable to developing countries. It has also been recorded that most of the patients in the western countries who carry these chronic and terminal diseases are now willing to accept various practices whose foundations are based on a different understanding of diseases and health; another factor driving the demand for herbal medicines.

2.2.2. Challenges Affecting the Practice of Herbal Medicine and the Way Forward

Although there is a growing demand and preference for botanicals, the practice continues to be plagued by several challenges. First is the absence of documented evidence on the efficacy of most products in use leading to most traditional practitioners especially in Africa, relying on folkloric knowledge and personal experience as a basis for the administration of products. This deficit in evidence is also noted to limit the widespread acceptability of herbal medicines into mainstream healthcare (De Smet, 2002; Edzard, 2005).

The quality of products administered is another issue of concern as medicinal plants are known to show variations in the quantity of therapeutic constituents due to factors like climate, temperature and post harvest handling. These variations in constituents have

implications for the efficacy and safety of natural products (Moshihuzzaman and Choudary, 2008).

In addressing the challenge with the quality of herbal medicines, standardisation of products has been recommended. This ensures that a predefined quantity and therapeutic effect of an ingredient in each dose of the medicinal product is maintained to give an assurance of quality, safety and reproducibility of the products (Choudhary and Sekhon, 2011; Kunle *et al.*, 2012). The quantitative assay of active and analytical markers in standardisation of HMP's is thus becoming quite common. In situations where these methods are not available, the basic phytochemical screening is still considered relevant as a means to standardise medicinal products (Mitra and Kannan, 2007).

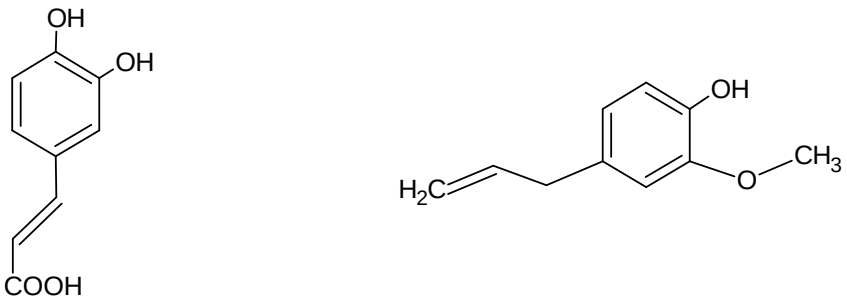
Long term safety studies like the preclinical chronic toxicity for new products, the use of clinical studies and post market surveillance of products with history of long use are very important in assuring users about the safety of herbal medicines (Firenzuoli and Gori, 2007).

In the case of providing clinical evidence on the effectiveness of products, the use of randomised controlled trials is most beneficial and provides the highest level of evidence. However when such methods cannot be used, documentation of individual case studies is also accepted as providing evidence for use although with several limitations (WHO, 2000). Applying these methods to the practise of herbal medicine is essential for the development of the field and will quicken the process of integration of traditional medicines into the conventional system of healthcare.

2.3. Plants as Sources of Antimicrobials

Medicinal plants have been used for the management of infections for centuries. Metabolites synthesised by these plants are primarily used for defence against attacks from microbes, herbivores and protection against adverse environmental conditions. These metabolites are responsible for the therapeutic effect produced by plants when applied in humans as medicinal agents (Cordell, 2000; Hussin *et al.*, 2009).

The role of medicinal plants as antimicrobial agents can be considered to be twofold. First, the application of the crude extracts serve as treatments for infections and secondly, their metabolites can be used as novel compounds for the development of newer synthetic antimicrobials. Examples exist of plants that are known for their antimicrobial activity with some metabolites isolated from them also showing similar activities (Rios and Recio, 2005). *Thymus vulgaris* and *Eugenia caryophyllata* are well known for this property with their activity attributable to the presence of phenolic compounds like caffeic acid and eugenol which have also been reported to have some antimicrobial activity (Figure 2.1a & b) (Suresh *et al.*, 1992; Ali *et al.*, 2005).



(a) Caffeic acid (b) Eugenol

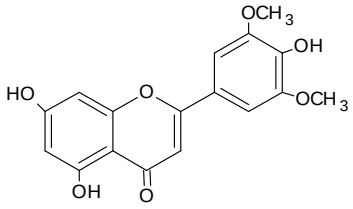
Figure 2.1: Phenolic compounds which have shown antimicrobial activity

The flavonoids, which also carry a phenolic nucleus, have become a subject of interest due to their diverse medicinal properties. Catechin, a flavonoid from green tea (*Camellia sinensis*) showed activity against *Staphylococcus mutans* and *Vibro cholera* (Cushnie and Lamb, 2005). This flavonoid has also been documented for its antiviral activity (Khullar, 2010). Wachtera *et al.*, (1999) identified the isolate 5, 7, 4-trihydroxy-8-methyl-6-(3-methyl-[2-butenyl])-(2*S*)-flavanone from *Eysenhardtia texana* and documents that it possessed activity against the opportunistic pathogen *Candida albicans*, an activity also indicated for the flavonoid 7-hydroxy-3, 4-(methylenedioxy) flavan, isolated from *Terminalia bellerica* fruit rind.

Another flavone isolated from *Artemisia giraldi*, identified as 6,7,4-trihydroxy-3,5-dimethoxyflavone together with 5,7,4-trihydroxy-3,5-dimethoxyflavone (Figure 2.2 a & b) were reported to exhibit activity against *Aspergillus flavus* a species of fungi that causes invasive diseases in immunosuppressed patients (Zheng et al., 1996).

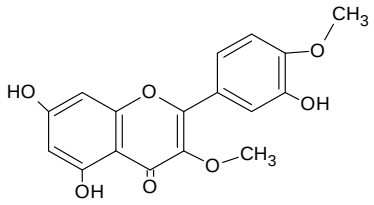
Apart from these, numerous ubiquitous flavonoids like rutin, quercetin, kaempferol, (Figure 2.2 c, d & e); myricetin and isorhamnetin have also been widely reported for their antimicrobial activity. The role of these flavonoids in the antifungal activity of medicinal plants against strains like *Candida albicans, Trichophyton mentagrophytes, Epidermophyton floccosum, Trichoderma spp.* and *Aspergillus spp.* is established (Hussin *et al.*, 2009; Mahule *et al.*, 2012; Dubey *et al.*, 2013).

(a)



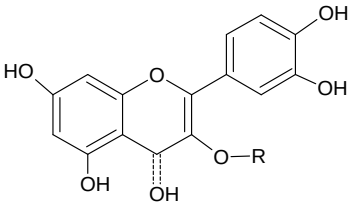
6,7,4-trihydroxy-3,5-dimethoxyflavone

(b)



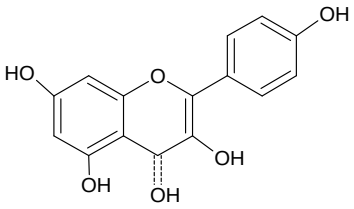
5,7,4-trihydroxy-3,5-dimethoxyflavone

(c)



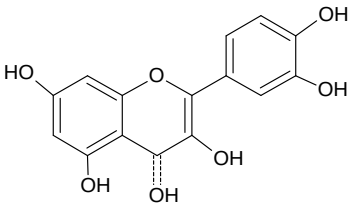
Rutin

(d)



Kaempferol

(e)



Quercetin

Figure 2.2: Some flavonoids that have shown antimicrobial activity

The quinones another derivative of the phenols, are also known for their antimicrobial activity with the anthraquinone from *Cassia italica,* shown to be bacteriostatic for *Bacillus anthracis, Corynebacterium pseudodiphthericum* and *Pseudomonas aeruginosa;* and bactericidal for *Pseudomonas pseudomalliae*. Studies have also shown quinones to have good to moderate antifungal activity against *Colletotrichum spp* and hence their prospects for use in the treatment of fungal infections in humans. Lawsone, (2-hydroxy-1,4-naphthoquinone) another quinone from Lawsonia is also recorded to

have antimicrobial activity against *Mycobacterium tuberculosis* (Cowan, 1999; Meazza *et al.*, 2003).

Apart from the quinones and flavonoids, tannins which are polymeric phenolic compounds widely distributed in botanicals, have over centuries been exploited for their antimicrobial activity (Haslam, 1996; Stern *et al.*, 1996). These metabolites were shown to have significant antimicrobial activity against *Helicobacter pylori* in a screening of 41 tannins. These tannins were from several plant species among which were *Psidium guajava* and *Euphorbia hirta* which form an important part of the list of plants that are used in the treatment of infections (Funatogawa *et al.*, 2004).

Plants such as *Aframomum melegueta*, *Piper guineense*, *Xylopia aethiopica* and *Zingiber officinale*, all known for their high terpenoid contents have been documented for this antimicrobial activity (Konning *et al.*, 2004). Other examples include the compound capsaicin from *Capsicum frutescens* which has activity against *Helicobacter pylori* and *Artemisia annua* which contains artemisinin and is effective against protozoans (Taylor *et al.*, 1996; Xu *et al.*, 1996).

2.4. Review of the Plants used in Study

The product under study *EAF-2011* is formulated from five plants namely: *Alchornea cordifolia, Eugenia caryophyllata, Psidium guajava, Zanthoxylum zanthoxyloides* and *Tridax procumbens.* All the plants have been previously documented as possessing *in-vitro* antimicrobial activity. The biological activity of the plants can be attributed in part to the presence of flavonoids with compounds like myricetin, rutin, quercetin, kaempferol and caryophyllene derivatives present in some of the plants, noted to have

some antimicrobial activity (Cowan, 1999). A review of the medicinal plants in the product is elaborated below.

2.4.1. Alchornea cordifolia (Schum. & Thonn.) Muell.Arg.

Alcornea cordifolia is also known as the Christmas tree and belongs to the family Euphorbiaceae. The plant is known in the Akan language as *Agyamma* or *Ogyamma;* as *Gboo* in Ga and in Ewe as *Avovlo, Ahame* or *Ayarba* (GHP, 2007).

2.4.1.1. Description

Alchornea cordifolia is a shrub that can grow up to 5 m high. The stems are armed with blunt spines; leaves are long-petiolate, broadly ovate and cordate at the base. The apex of the leaves are shortly acuminate, entire or with slightly dentate margin. The leaves can also be identified by their finely stellate-puberulous or slightly glabrescent underside. The glands in axils of the basal nerves are arranged in alternates about 10-28 cm long, 6-16 cm broad; flowers of the plant are greenish-white with lax pendulous spikes or raceme; styles are long and permanent on mature fruits. The fruit of the plant are two-celled, small, stellate and pubescent (GHP, 2007).

2.4.1.2. Chemical Constituents

The secondary metabolite content showed tannin as the highest (9.8%) followed by flavonoids (9.1%) which are well known for their antimicrobial activities. The presence of saponins, anthraquinones, traces of alkaloids and cardiac glycosides have also been detected in the leaves of the plant (Adeshina *et al.*, 2010; George *et al.*, 2010).

Other chemical isolates from the plant are: acetyl aleuritolic acid, diisopentyl-guanidine and *β*-sitosterol see Figure 2.3 (Mavar-Mangaa *et al.*, 2008).

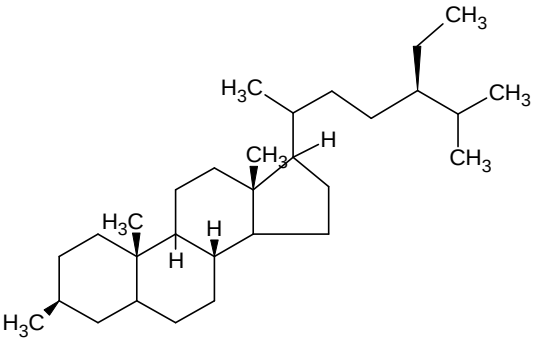
2.4.1.3. Medicinal Uses

Traditionally the plant has been used as an antifungal, antidiarrhoeal, antirheumatic, anti-inflammatory and as an anti-protozoan agent (Ayisi and Nyadedzor, 2003).

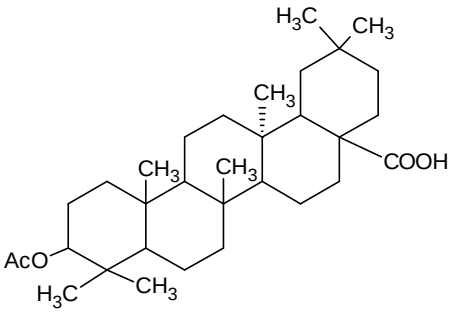
2.4.1.4. Antimicrobial activity of the Plant

Adeyemi *et al.,* (2008) reported that the ethanolic and aqueous extract of the plant had bactericidal effects on several diarrhoeagenic bacteria like *Helicobacter pylori*, *Salmonella typhi*, *Salmonella enteritidis*, *Shigella flexneri* and enterohaemorrhagic *Escherichia coli* (EHEC). This effect also makes the plant useful for the management of gastric ulcers and several other diarrhoeal diseases.

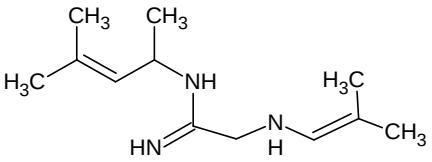
Mavar Manga (2004), Ebi, (2001) and Tona (1999) have also reported the antibacterial activity of the plant against *Pseudomonas aeruginosa*, *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli*. The results emphasised the broad spectrum activity of the plant against both gram-negative and gram-positive bacteria (Pesewu *et al*., 2008).



(a) β-sitosterol



(b) Acetyl aleuritolic acid



(c) Diisopentenyl-guanidine

Figure 2.3: Some chemical isolates from *Alchornea cordifolia*

Okeke *et al*., (1999) reported that a concentration of 40 mg/ml, *Alchornea cordifolia* was active against the fungi species: *Candida pseudotropicalis, Cladosporum cucumerium, Candida albicans, Trichophyton rubrum, Fusarium solanii, Aspergillus avus, Cochlibolus lunatus, Trichoderma spp.* and *Epidemophyton spp.* The butanolic and alcoholic fractions of the plant have also been reported on for their activity against *Candida albicans* and *Trichophyton violaceum* (George *et al.*, 2010).

2.4.2. Eugenia caryophyllata (Thumb)

Eugenia caryophyllata belongs to the family Myrtaceae. The plant is commonly known as clove and referred to as *Pepre* in Twi. This plant is however not indigenous to Ghana.

2.4.2.1. Description

Eugenia caryophyllata is a tree that grows as high as 10-15 m. Its leaves are petiolated, green, shiny, with translucent spots, inflorescence in panicle; floral buds up to form spikes with white flowers (Mshana *et al.*, 2000).

2.4.2.2. Medicinal Uses

Clove has been used for the treatment of abdominal pain, rheumatism and toothaches. It is also well known to be effective against fungal, bacterial and viral infections.

2.4.2.3. Chemical Constituents of the Plant

Chemical constituents isolated include eugenol that forms about 78% of the essential oil constituents and *β*-caryophyllene (1%) (Figure 2.4). Both constituents have been documented for their antifungal, antibacterial and antiviral prospects (Pawar and Thaker, 2006).

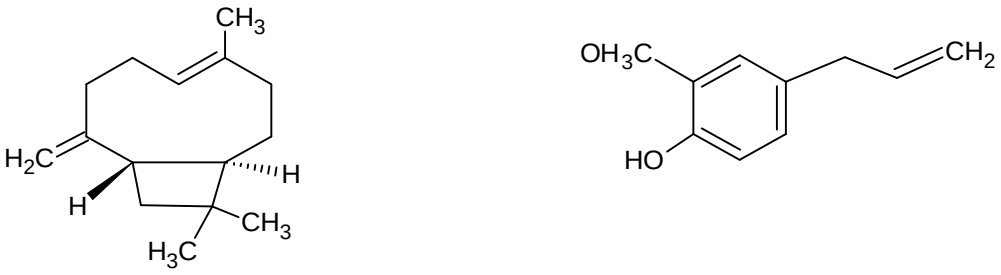
2.4.2.4. Antimicrobial Properties of *Eugenia caryophyllata*

Studies from Chami *et al*., (2005) and Ogata *et al.*, (2000) have confirmed the antifungal, antibacterial and antiviral properties of the essential oil of the plant. The action of *Eugenia caryophyllata* against mites in scabies infection has also been documented (Pasay *et al.*, 2010).

Other studies also report the plant as having activity against *Candida albicans, Candida tropicalis, Candida krusei, Trichophyton. rubrum, Trichophyton mentagrophytes* and *Geotrichum candidum* isolated and identified from clinical samples of patients. In the study that compared the essential oils and eugenol with ketoconazole and itraconazole, the essential oil and eugenol was shown to have comparable efficacy to the other test

agents. However, in that same experiment *Candida krusei* was resistant to the essential oil of the plant (Gayosoa *et al.*, 2005). The positive effect of the plant was confirmed by Ahmad *et al.*, (2005) who noted that the essential oils from the plant were efficacious against opportunistic fungal pathogens such as *Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigatus*. The oil was also found to be extremely successful in the treatment of experimental murine vaginitis in model animals.

The antibacterial spectrum of the plant includes activity against *Campylobacter jejuni*, *Salmonella enteritidis*, *Escherichia coli*, *Staphylococcus aureus, Bacillus cereus and Listeria monocytogenes* (Larhsini *et al.*, 2001; Burt and Reinders, 2003; Pinto *et al.*, 2009).



(a) β -caryophyllene (b) Eugenol

Figure 2.4: The major constituents of *Eugenia caryophyllata*

2.4.3. Zanthoxylum zanthoxyloides (Lam)

Zanthoxylum zanthoxyloides belongs to the family Rutaceae and is commonly referred to as Fagara. It is known to the Akan as *Okanto*, the Ga as *Haatso* and the Ewe as *Xetsi*.

2.4.3.1. Description

Fagara is a tree that grows up to 12 m high, usually branching low, often shrubby with a rough bark that also has fine longitudinal fissures. The leaves are imparipinnate, alternate (5 to 9 pairs of leaflets), rounded, often emarginate and horizontally spread. The trunk, rachis and occasionally the median vein are lined with large thorns. The fruit is a capsule that has two valve-like openings containing a single shiny black seed (Adjanohoun *et al.*, 1980).

2.4.3.2. Chemical Constituents

Chelerythrine, berberine and canthine-6-one (Figure 2.5) are alkaloids isolated from the plant and reported to possess strong antibacterial activity (Odebiyi and Sofowora, 1979; Tsuchiya *et al.*, 1996). Other metabolites like saponins, tannins, aliphatic and aromatic amides, lignans, coumarins, sterols and flavonoids have also been detected (Adesina, 2005).

2.4.3.3. Medicinal Usage

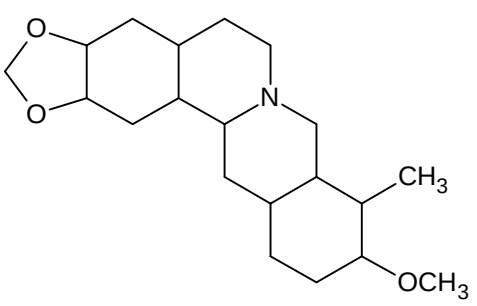
Traditionally, the plant has been used as a chewing stick for dental hygiene and as treatment for toothaches, elephantiasis, sexual impotence, gonorrhoea, malaria, dysmenorrhoea and abdominal pain (Odebiyi and Sofowora, 1979; Rotimi *et al.*, 1988).

2.4.3.4. Antimicrobial Properties of the Plant

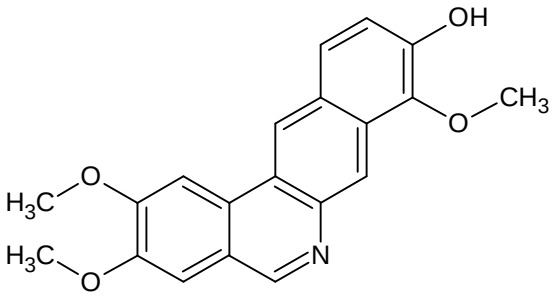
The efficacy of the plant against odontopathogens is widely known and recorded. *Zanthoxylum zanthoxyloides* has appreciable activity against *Staphylococcus aureus* and *Eikenella corrodens* (Muhammad and Shinkafi, 2007). Flavonoid compounds isolated from the plant are also reported to have antibacterial properties as reported by Odebiyi and Sofowora (1979) and Tsuchiya *et al.*, (1996).

Taiwo *et al.*, (1999) reported that extracts from *Zanthoxylum zanthoxyloides* were active against the following oral microorganisms: *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Eikenella corrodens* and *Campylobacter rectus*.

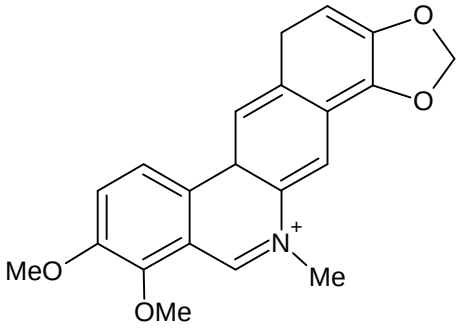
The antifungal potential is also known with extracts of the plant exerting inhibitory effect on *Microsporum canis, Trichophyton rubrum* and *Trichophyton mentagrophytes* (Banso and Ngbede, 2006). The efficacy of the ethanolic and aqueous plant extracts have been established against *Candida albicans, Aspergillus flavus, Microsporium gypseum* and *Trichophyton metagrophytes*. This property was also shown by the root, leaves and stem extracts of the plant against *Candida albicans, Cryptococcus neoformans* and seven other filamentous fungi (Ngane *et al.*, 2000).



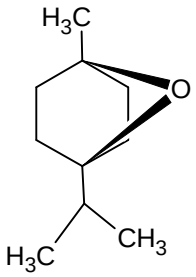
(a) Fagaronine



(b) Berberine



(c) Cherelythrine



(d) Canthine-6-one

Figure 2.5: Some chemical isolates from *Zanthoxylum zanthoxyloides*

2.4.4. Psidium guajava (Linn)

Psidium guajava; family Myrtaceae is commonly known to the Akan as *Oguawa or Eguaba.* The Ga refer to it as *Gowa* and the Ewe as *Goa.*

2.4.4.1. Description

Psidium guajava (Linn) is a shrub that grows to about 5-8 m high; its leaves are simple, opposite, entire and ovate 3-5 cm long and 2.5-4 cm broad; glabrous and 8-15 prominent lateral nerves beneath. It also has white pedunculate solitary flowers, 1.5-2 cm in diameter. The fruits are spherical with persistent sepals on top and a white or pink pulp with numerous seeds (Dutta *et al.*, 2000; Mshana *et al.*, 2000).

2.4.4.2. Chemical Constituents

The plant is rich in several minerals (Conway, 2002; Medina and Pagano, 2003) with constituents like ursolic acid, *β*-sitosterol, caryophyllene oxide and caryophyllene isolated from the plant. These isolates are known for their antimicrobial properties. Quercetin (Fig 2.7a) limonene (Fig 2.6a), α-pinene (Fig 2.6b), and *β*-pinene (Fig 2.6c) have also been identified in the plant (Dweck, 2008).

2.4.4.3. Medicinal Uses

Psidium guajava has been used for the treatment of diarrhoea and the leaves specifically for chronic diarrhoea, cough, urinary tract infections and toothaches. The stem bark is used for treating boils and the fruits for pharyngeal abscess (Heinrich *et al.*, 1998; GHP, 2007).

2.4.4.4. Antimicrobial Properties of *Psidium guajava*

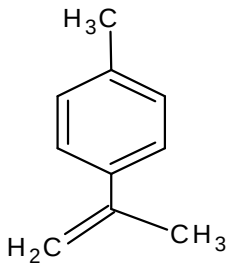
Guava like other plants is traditionally used for the treatment of infections. In the treatment of fungal skin diseases, Dutta *et al.,* (2000) showed that the leaves of the plant were active against the dermatophytes *Trichophtyon tonsurans, Trichophyton rubum,* and *Microsporum fulvum*.

The stem bark and leaf extracts have also been shown to have antifungal and antibacterial properties against some fungi such as *Microsporum gypseum, Trichophtyton mentagrophytes,* and bacteria like *Staphylococcus aureus,* and *Staphylococcus epidermidis* which are commonly implicated in urinary tract infections and therefore validating its traditional use (Yamada, 2004). Tinctures of the stem bark and leaves have exhibited activity against *Trichophyton tonsurans, Trichophyton rubrum, Trichophyton beigelii, Microsporum fulvum, Microsporum gypseum* and

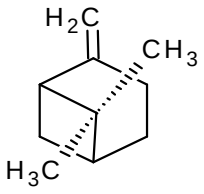
Candida albicans (Dutta *et al.*, 2000). The antifungal activity has also been demonstrated in a methanolic extract (Rabe and van Staden, 1997).

Like *Alchornea cordifolia, Psidium guajava* has been shown to be active against different species of diarrheagenic *Escherichia coli, Salmonella,* and *Shigella*. *Psidium guajava* showed inhibitory effects against two species of *Salmonella, Shigella flexneri, Shigella virchow,* and *Shigella dysenteriae,* and two varieties of enteropathogenic *E. coli* (Lin et al., 2002). Adbelrahima *et al.,* (2002) have also indicated that a methanolic extract had activity against *Proteus mirabilis, Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus* and *Proteus vulgaris*.

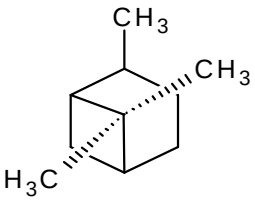
Lutterodt, (1992), Tona *et al.,* (1999) and Goncalves *et al.,* (2005) reported on the antidiarrhoeal activity of the plant with some isolated compounds from the plant: quercetin and quercetin-3-arabinose, showing similar effects (Heinrich, 1996; Zhang *et al.,* 2003).



(a) Limonene



(b) α - pinene



(c) β - pinene

Figure 2.6: Some chemical constituents of *Psidium guajava*

2.4.5. Tridax procumbens (Linn)

Tridax procumbens belongs to the family Asteraceae. The plant is known to the Ewes as *Fomizibge* (Mshana *et al*., 2000).

2.4.5.1. Description

Tridax procumbens (Linn) is annual or sometimes perennial when mowed. It is a hispid herb with trailing stems branching low from a tap root. It has opposite leaves up to 7 cm long and 3 cm broad, ovate-rhomboid; florets on solitary capitula on slender peduncles (Mshana *et al.*, 2000).

2.4.5.2. Chemical Constituents

Tridax procumbens is known to contain *β*-sitosterol (Figure 2.3a), quercetin (Figure 2.7a) and kaempferol (Figure 2.2d) these constituents have been reported as being present in *Eugenia caryophyllata, Psidium guajava* and *Alchornea cordifolia*. Luteolin (Figure 2.7b) is another constituent found in *Tridax procumbens* which has been shown to possess antifungal properties (Ali *et al.*, 2001).

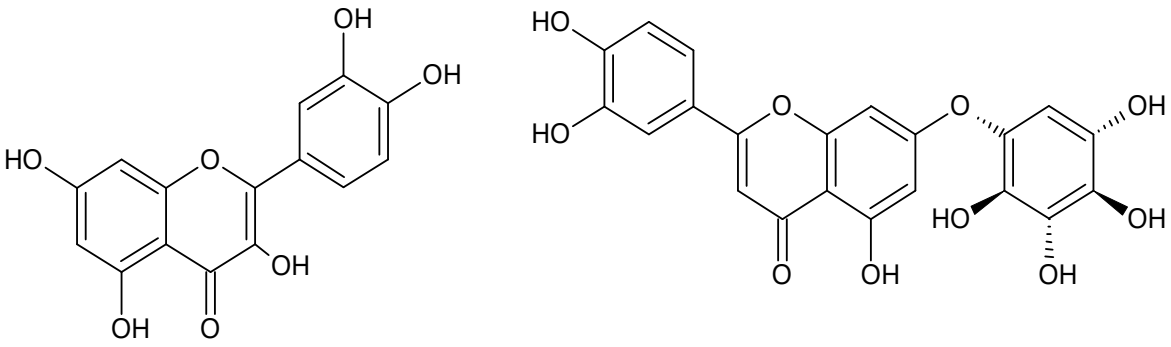
2.4.5.3. Medicinal Uses

The plant has been used to treat jaundice and whitlow. The aerial parts are used for malaria. Other reported uses include the leaf juice as an antiseptic and insecticide. The juice is also used to control bleeding from wounds, dysentery, diarrhoea and for the prevention of premature alopecia (GHP, 2007).

2.4.5.4. Antimicrobial Properties of *Tridax procumbens*

The plant is reported to be active against both gram-positive and gram-negative bacteria as well as stimulating wound healing (Udopa *et al.*, 1991; Taddel and Rosas Romero, 2000).

The antimicrobial activity of the aqueous extracts of the plant investigated against bacterial pathogens: *Staphylococcus aureus, Bacillus subtilus, Escherichia coli, Klebsiella pneumonia* and the fungi *Candida albicans* showed inhibitory activity against all the tested organisms (Sharma and Sharma, 2010).



(a) Quercetin

(b) Luteolin

Figure 2.7: Some constituents of *Tridax procumbens*

CHAPTER 3

ASSESSMENT OF PRODUCT QUALITY TOWARDS THE DEVELOPMENT OF STANDARDS

3.1. Introduction

The quality control of herbal medicines and their products continue to pose a challenge for regulators for want of standards to judge quality by; for users because of potential adulteration with the increased risk for harms. Reports are available on toxic and adverse reactions associated with herbal products that may be the result of poor quality. These untoward effects may be the result of poor agricultural practices, adulteration, misidentification and poor manufacturing procedures (Yi-Zeng *et al.*, 2004; Songlin *et al.*, 2008).

Aside the associated toxicity, poor quality products lead to decreased clinical efficacy and thus increased cost of treatment for patients. Numerous methods have been suggested to address these issues. Each of these methods provide varying levels of guarantee about quality hence the suggestion to apply multiple methods during the quality assessment of herbal medicines; involving both chemical and biological assays (Choudhary and Sekhon, 2011).

The process of development of standards for the herbal product in this study was undertaken to provide some specifications for subsequent manufacturing processes to ensure quality.

3.2. Plant Collection and Extraction

3.2.1. Materials and Methods

3.2.1.1. Plants Used in the Study

The plants used in the product comprised the dried leaf of *Alchornea cordifolia* (Schum. & Thonn.) Muell.Arg. (Euphorbiaceae) and *Psidium guajava* (Linn) (Myrtaceae); the dried whole plant of *Tridax procumbens* (Linn) (Asteraceae), the dried stem bark of *Zanthoxylum zanthoxyloides* (Lam) (Rutaceae) and the dried flower buds of *Eugenia caryophyllata* (Thumb) (Myrtaceae).

3.2.1.2. Plant Collection and Authentication

The plants used were sourced from Mampong-Akuapem and its environs in November and December, 2010 except *Eugenia caryophyllata* which was purchased from some commercial collectors. The plants were authenticated by a botanist at the Plant Development Department, Centre for Plant Medicine Research (CPMR), Mampong-Akuapem. Voucher Specimen Numbers were allocated (Table 3.1) and specimen deposited at the herbarium of the CPMR.

Table 3.1: Voucher specimen numbers of plant materials used

Plant Material	**Voucher Specimen Number**
Alchornea cordifolia	CSRPM 368
Psidium guajava	CSRPM 50
Tridax procumbens	CSRPM 256
Zanthoxylum zanthoxyloides	CSRPM 330
Eugenia caryophyllata	CSRPM 001CM

3.2.1.3. Plant Preparation

The various plant materials were air-dried in a cool, dry place under shade for 2 weeks at an ambient temperature, between 23ºC and 27ºC. The materials were pulverized using a hammer mill and sieved through 2 mm screen to obtain a coarse powdered material.

3.2.1.4. Extraction

Extracts of each powdered plant material was prepared by macerating 1 kg of the powdered plant material in 5 litres of 70% ($^{v}/_{v}$) ethanol for 3 days and then filtering. The ethanol was recovered using the Rotary evaporator (Buchi™ R210) and the fluid extract evaporated on a water bath over 72hrs. The dried extracts were kept in a desiccator prior to formulation of the ointment.

3.2.1.5. Yield of Plant Extracts

The yield per kilogram of the five (5) plants used was between 154.7-231.93 g as shown in Table 3.2. The percentage yield was calculated using the formula:

Weight of Dry Extract (g) / Total Weight of the Powdered Material (kg) x 100

Table 3.2: Yield of the plants extracts using 70% ($^{v}/_{v}$) ethanol

Plant Extract	**Yield ($^{g}/_{kg}$)**	**Percentage Yield ($^{w}/_{w}$)**
Alchornea cordifolia	154.70	15.47
Eugenia caryophyllata	185.54	18.55
Zanthoxylum zanthoxyloides	115.97	11.59
Psidium guajava	231.93	23.19
Tridax procumbens	154.62	15.62

3.2.1.6. Formulation of Extract Concentrations of *EAF-2011*

The herbal product was prepared according to the proprietary formula for the product obtained from the CPMR. The individual extracts were combined in a ratio according to this formula to obtain the total crude extract (TC) which was subsequently incorporated into the ointment base at various concentrations of 2%, 5% and 10% ($^{w}/_{w}$). The individual extracts were combined according to the formula:

Alchornea cordifolia	30% ($^{w}/_{w}$)
Eugenia caryophyllata	25% ($^{w}/_{w}$)
Psidium guajava	20% ($^{w}/_{w}$)
Tridax procumbens	15% ($^{w}/_{w}$)
Zanthoxylum zanthoxyloides	10% ($^{w}/_{w}$)

3.2.1.7. Formulation of the Ointment Base for *EAF-2011*

The plant extracts were incorporated into an emulsifying ointment base (B.P.) prepared by the fusion method (Marriott *et al.*, 2006). The ointment base composition was:

Emulsifying wax (B.P.)	30% ($^{w}/_{w}$)
White soft paraffin	20% ($^{w}/_{w}$)
Liquid Paraffin	50% ($^{w}/_{w}$)

3.3. Development of Standards for the Finished Herbal Product and its Component Raw Materials

Standardisation of the finished herbal product and its raw materials involved the use of basic phytochemical screening, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

3.3.1. Basic Phytochemical Screening

3.3.1.1. Materials and Methods

The individual plant extracts, the total crude extract (TC) and the finished herbal product were each screened for alkaloids, saponins, phenols, flavonoids, sterols and triterpenes, anthracenosides and cyanogenic glycosides as stated below.

3.3.1.2. Pretreatment of the Ointment for Phytochemical Screening

Five (5) grams of the ointment was macerated in 0.5N KOH for 24hrs. This mixture was refluxed for 1 hr to separate the ointment base from the incorporated total crude extract. The mixture was then cooled and filtered using a Whatman No.1 paper. The filtrate was then extracted with ether in a separating funnel. Half of the resultant extract was evaporated over a steam bath to dryness and redissolved in distilled water to form an aqueous fraction. The aqueous (AQF) and ether (ETF) portions were subsequently used for the phytochemical analysis. All the phytochemical analysis was performed according to the methods described by Odebiyi and Sofowora (1978), Sofowora (1993) and Evans (2002).

3.3.1.3. Alkaloid Test

An amount of 5 mls of HCl 1% ($^{v}/_{v}$) was added to 5 mls of the ether extract (ETF). The HCl fraction was divided into two portions with one serving as a control. Few drops of Mayer's reagent was then added to one part and observed for turbidity and formation of a yellowish to brown precipitate which indicates a positive test. A confirmatory test was performed by partitioning the ether extract between 2% ($^{v}/_{v}$) H_2SO_4/$CHCl_3$ (1:1) ($^{v}/_{v}$) in a separating funnel. The chloroform phase was further extracted with H_2SO_4. The aqueous phase was combined and the pH adjusted to 9-10 with dilute ammonia (NH_3). The basified aqueous phase was extracted with chloroform and all the

chloroform phases combined. This chloroform extract was then spotted onto a filter paper, dried and sprayed with freshly prepared Dragendorff's spray reagent. A yellowish to orange stain was recorded as a positive test.

3.3.1.4. Test for Phenolic Compounds

An amount of 5 mls of the aqueous fraction (AQF) was put in a test tube and 1% ($^{v}/_{v}$) alcoholic ferric chloride added. The formation of an intense green, purple, blue or black colour is recorded as a positive test.

3.3.1.4.1. Flavonoid Test

About 10 mls of the ether fraction (ETF) was evaporated to dryness in a test tube. An amount of 2 mls of 50% methanol, two chips of metallic magnesium and few drops of HCl were also added. A positive test is recorded when there was the formation of a red to orange colour.

3.3.1.5. Saponin Test

An amount of 5 mls of the aqueous fractions (AQF) was put in a test tube and vigorously shaken. The development of a persistent froth lasting more than 15 minutes indicated a positive test for saponins.

3.3.1.6. Anthraquinone Test

Approximately 1 ml of dilute ammonia was added to 3 mls of the ether extract (ETF). The formation of a red colour at the bottom of the test tube indicated a positive test.

3.3.1.7. Triterpene and Sterol Test

A quantity of 10 mls of the ether fraction (ETF) was evaporated to dryness and 0.5 mls of acetic anhydride and chloroform were added. A portion was transferred into a dry test tube and 1 ml of concentrated H_2SO_4 added at the bottom of the tube with a pipette. A positive test was recorded when a brownish or violet ring was formed at the interphase of the formation of a greenish/violet supernatant layer.

3.3.1.8. Cyanogenic Glycoside Test

An amount of 5 mls of the ether extract (ETF) was put into a conical flask. Moistened sodium picrate paper was suspended in the mouth of the flask by means of a cork. The flask was then warmed over a water bath for about 2 minutes and observed. A positive test was recorded when the test paper turned from yellow to reddish purple.

3.3.1.9. Results of Basic Phytochemical Screening

The phytochemical constituents of the individual plant extracts compared to the combined crude extract (TC) and the ointment are shown in Table 3.3. The results indicated the presence of phenols and flavonoids in all the test samples. Saponins were detected in *Alchornea cordifolia*, *Eugenia caryophyllata* and *Zanthoxylum zanthoxyloides* but were however absent in the total crude extract (TC) and the ointment. Anthracenosides were also detected in *Eugenia caryophyllata* but were absent in the total crude extract and the ointment. Cyanogenetic glycosides were absent in all the test samples. The phytochemical screening did not indicate any difference between the total crude extract (TC) and the finished product.

Table 3.3: Results of the phytochemical screening of the various samples.

Plant Extract	Alkaloids	Phenols	Sterol/ Triterpenes	Saponins	Flavonoids	Anthracenosides	Cyanogenetic Glycosides
Alchornea cordifolia	-	+	-	+	+	-	-
Eugenia caryophyllata	-	+	+	-	+	+	-
Psidium. guajava	-	+	-	+	+	-	-
Zanthoxylum zanthoxyloides	+	+	+	-	+	-	-
Tridax procumbens	+	+	-	+	+	-	-
Total Crude Extract	+	+	+	-	+	-	-
Ointment (EAF-2011)	+	+	+	-	+	-	-

Key: (-) Absent; (+) Present

3.3.2. Thin layer chromatography

3.3.2.1. Material and Methods

Thin layer chromatography was performed on the ointment, the total crude extract and the raw materials to obtain a fingerprint or a profile that could be compared with subsequent formulations.

3.3.2.2. Pretreatment of the Herbal Product and Raw Materials

One gram of total crude extract (TC) and the ointment were separately extracted in 70% ($^{v}/_{v}$) ethanol for three days. The ethanol was recovered using the Rotary evaporator and the aqueous fraction remaining subsequently extracted with ethyl acetate in a separating funnel (1:2) ($^{v}/_{v}$). The ethyl acetate fraction was spotted on TLC plates and were developed according to guidelines indicated by Nyarko *et al.*, (2005) and Furniss *et al.*, (1989).

3.3.2.3. Development and Detection Methods

Silica gel 60 F_{254} precoated plates (Merck) were used for the chromatography. Detection was done under Ultraviolet Lamp at 365 nm and 254 nm and with anisaldehyde detecting reagent by heating to a temperature of 105ºC.

3.3.2.4. Results of Thin Layer Chromatography

The chromatographic fingerprint for the individual plant extracts, total crude extract and the ointment were obtained by developing the silica gel plates using the solvent system of petroleum ether and ethyl acetate (4:1). Spots were detected by visualisation under ultraviolet light and heating to a temperature of about 105ºC after spraying with anisaldehyde reagent.

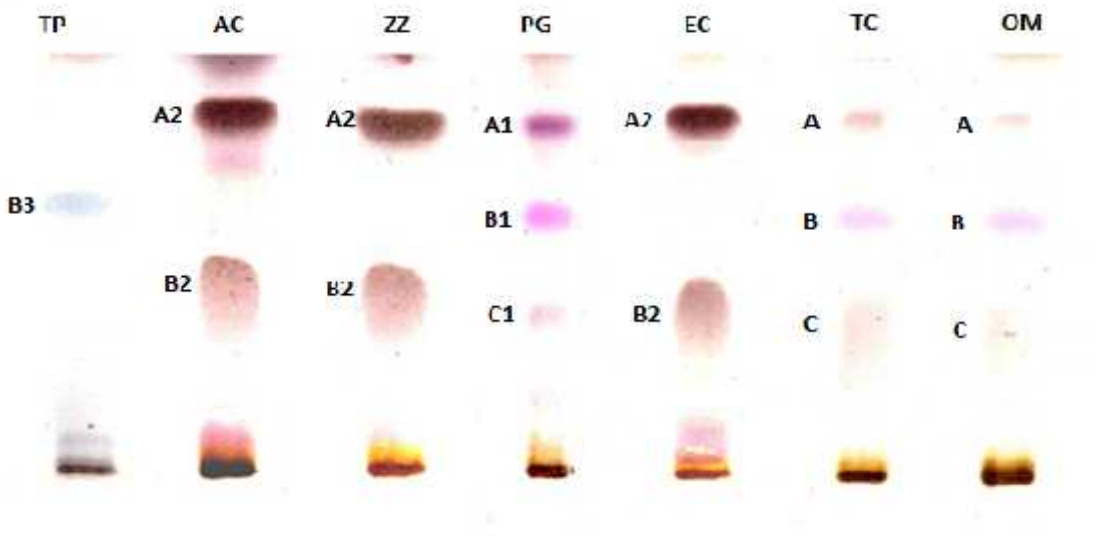


Figure 3.1: Chromatographic profile of the individual plant extracts, total crude extract and the ointment. Solvent system: petroleum ether: ethyl acetate (4:1). Derivatisation: Anisaldehyde-H_2SO_4 detecting regent. R_f values for spots A, A1, A2-0.82; B, B1, B3-0.54; C, C1-0.36.
Key: *Tridax procumbens* (TP), *Alchornea cordifolia* (AC), *Zanthoxylum zanthoxyloides* (ZZ), *Psidium guajava* (PG), *Eugenia caryophyllata* (EC), Total crude extract (TC) and Ointment (OM).

The chromatographic profiles for the plant extracts, total crude extract and the ointment are shown in Figure 3.1. The fingerprint obtained for the total crude extract and the ointment produced similar spots (A, B and C) of nearly the same R_f values, shape and colour. Again, the fingerprints for the extracts of *Alchornea cordifolia, Zanthoxylum zanthoxyloides* and *Eugenia caryophyllata* were comparable: two spots (A2 and B2) of nearly the same R_f values, shape and colour were obtained. *Psidium guajava* also produced three spots (A1, B1 and C1) with spot B1 being detetcted in the final product as spot B. The fingerprint for *Tridax procumbens* had a spot (B3) which also shared a similar R_f value with spot B1 from *Psidium guajava* but of a different colour.

3.3.3. High Performance Liquid Chromatography

3.3.3.1. Materials and Methods

3.3.3.1.1. Solvents and Chemicals

Solvents used: methanol (MEOH), acetonitrile (ACN), acetic acid (CH_3COOH) and terahydrofuran (THF) were of HPLC grade (Sigma Aldrich). Deionised water was prepared by a Milli-Q Water purification system (Millipore, MA, USA). The standard compounds rutin, quercetin and kaempferol were also purchased from Sigma-Aldrich, USA.

3.3.3.1.2. HPLC Instrumentation and Conditions

The chromatographic system comprised an Agilent Chemstation HPLC system consisting of 1260 quarternary HPLC pump, ASL Prep autosampler, degasser and a multiple wavelength detector. The column used was a Superclo C18 reversed phase column (5µm pore size, Ø 4.6 mm × 250 mm), purchased from Sigma Aldrich, USA.

3.3.3.1.3. Development of Chromatographic Conditions

Mobile phase for the detection of the three compounds was developed by varying five (5) solvents: tetrahydrofuran (THF), methanol (MEOH), acetonitrile (ACN), H_2O and acetic acid (CH_3COOH) in different ratios. The solvent system of MEOH, ACN and 1% CH_3COOH (40:15:45) was settled upon based on the separation and retention time (resolution), height of the peak and the area produced. Detection wavelength for the samples was selected after analysing fingerprints produced using a multiple wavelength detector (250-368 nm). The wavelength of 345 nm was selected as the most suitable based on the fingerprints produced. Flow rate and injection volume were set at 1.0 ml/min and 20 µl respectively. Column temperature was also kept at ambient temperature of 26 ^oC.

3.3.3.1.4. Extraction and Preparation of Raw Plant Materials

The herbal materials used were extracted according to the method described in section 3.2.1.4. The resultant extracts were then lyophilised and kept in a desiccator prior to analysis.

3.3.3.1.5. Preparation of Herbal Extracts

The lyophilised plant materials and the herbal product were each reconstituted in methanol to achieve a concentration of 100 mg/ml and then sonicated for 20 minutes. The ointment base for the finished herbal product was separated from the incorporated herbal extract by macerating 5 g of the product in 50 mls of 0.5N KOH for 24hrs refluxing for 1hr and then filtering. The filtrate was extracted with methanol (1:2) ($^{v}/_{v}$) in a separating funnel. All the herbal extracts were filtered through 0.45 µm PTFE membrane syringe filters (Thermo Fischer Scientific, USA) prior to injection; each injection was done in triplicate.

3.3.3.1.6. Preparation of Standard Solutions

Standard stock solutions of the three flavonoid compounds: rutin, quercetin and kaempferol, were prepared by dissolving them in methanol. A twofold serial dilution was prepared from the stock solution (1 mg/ml) of the compounds to obtain ten (10) different concentrations in the range of 1 mg/ml – 3.9×10^{-5} mg/ml. Samples were then injected in triplicates.

3.3.3.2. Results of High Performance Liquid Chromatography

3.3.3.2.1. Validation of Chromatographic Method

The developed chromatographic method was validated for linearity and range, precision, recovery/accuracy, limits of quantitation, limits of detection and system

suitability according to guidelines by International Conference on Harmonisation (ICH) (ICH, 1997).

3.3.3.2.2. Linearity and Range

An analysis of the peak area (y - axis) versus concentration (x - axis) was done (Table 3.4). Correlation coefficient (r^2) for all samples tested were >0.998, indicating a strong linear relationship between the peaks area and concentration. Retention times (R_T) for rutin, quercetin and kaempferol were 3.549 (± 0.030) mins, 4.999 (± 0.004) mins and 6.561 (± 0.030) mins respectively. Chromatographic fingerprints and calibration curve plots obtained for concentrations injected are shown as Appendices VIII to XI.

3.3.3.2.3. System Suitability

System suitability analyses were performed to verify the efficiency and reliability of the HPLC system for the analysis to be performed. Results shown in Table 3.5 indicated the system had a theoretical plate number >2000 and % Relative Standard Deviation (RSD) for area precision and retention time < 1%. % RSD was calculated as [(Standard deviation) / (Average)] x 100.

Table 3.4: Validation data from the calibration curves of the standard flavonoid compounds

Flavonoid Compound	**Regression Equation**	**Correlation Coefficient (r^2)**	**Linearity Range (mg/ml)**
Rutin	y=719.424 x +12.61	0.9995	0.0313-1.0
Quercetin	y= 813.008 x -24.12	0.9996	0.0625-1.0
Kaempferol	y=4241.882 x -31.91	0.9997	0.0156-1.0

Table 3.5: System suitability analysis for the HPLC assay for the three flavonoid compounds

Standard	Theoretical Plates (>2000)	Injection Precision for area (% RSD)	Injection Precision for retention time (% RSD)
Rutin	6180.019	0.18	0.17
Quercetin	8641.921	0.07	0.034
Kaempferol	11774.866	0.04	0.06

3.3.3.2.4. Precision

Inter-day repeatability analysis was performed as a measure of precision of the HPLC method. Testing confirmed that the procedure was robust to some random environmental conditions such as temperature and humidity. Percentage relative standard deviation (% RSD) for all samples analysed was < 2% (Table 3.6).

3.3.3.2.5. Accuracy and Recovery

The accuracy of the method was also demonstrated by the recovery studies after the injection of 1.5 mg/ml of rutin, quercetin and kaempferol individually (Table 3.7). The peak area produced after this indicated percentage recovery for rutin as 99.13%, quercetin (104%) and kaempferol (99.33%) and the RSD for the entire test samples as < 1%.

3.3.3.2.6. Limits of Detection and Quantitation

Limits of detection (LOD) and quantitation (LOQ) for the HPLC method were determined using the signal to noise ratio. LOD was determined as 3.3 times the signal to noise ratio and the LOQ was also determined as 10 times the signal to noise ratio using the calibration curve method. LOD and LOQ for rutin were 0.0048 mg/ml and

0.0148 mg/ml respectively, quercetin (0.00304 mg/ml and 0.00921 mg/ml) and kaempferol (0.000142 mg/ml and 0.00043 mg/ml) respectively.

3.3.3.2.7. Determination of Flavonoid Content in Plant Raw Materials and *EAF-2011*

The validated chromatographic conditions were subsequently used to determine the content of rutin, quercetin and kaempferol in the five plant materials and the finished herbal product (*EAF-2011*) (Table 3.8). Concentrations of rutin when present were found to be higher than other constituents e.g. 8.586 ($^{w}/_{w}$) in *Psidium guajava.* The finished herbal product also had all the assayed flavonoid compounds present in it albeit in quantities that were lower than expected. Chromatograms for the analytes are attached as Appendices XII to XVI.

Table 3.6: Results of inter-day repeatability test presented as %RSD for the HPLC method

Standard	**Precision for R_T**	**Precision for Peak Area**
Rutin		
1 mg/ml	0.12	0.05
0.5 mg/ml	0.06	0.11
Quercetin		
1 mg/ml	0.21	0.01
0.5 mg/ml	0.18	0.02
Kaempferol		
1 mg/ml	0.08	0.01
0.5 mg/ml	0.04	0.04

Table 3.7: The percentage recovery for standard flavonoids using the HPLC method

Flavonoid	Peak Area (n=3)	% RSD	% Recovery
Quercetin (SD)	1240.0 ± 1.617	0.25	104.0
Kaempferol (SD)	6315.0 ± 6.786	0.25	99.13
Rutin (SD)	1085.0 ± 5.131	0.00	99.13

Table 3.8: Flavonoid contents of the plant materials and the product

Plant Materials	Rutin ($^{w}/_{w}$)	Quercetin ($^{w}/_{w}$)	Kaempferol ($^{w}/_{w}$)
Eugenia caryophyllata	0.00	0.408 ± 0.001	0.0771 ± 0.00
Psidium guajava	8.586 ± 0.057	0.543 ± 0.00	0.1003 ± 0.00
Alchornea cordifolia	2.554 ± 0.001	0.0536 ± 0.00	0.00
Zanthoxylum zanthoxyloides	5.806 ± 0.001	0.00	0.0174 ± 0.00
Tridax procumbens	2.439 ± 0.01	0.1044 ± 0.00	0.0505 ± 0.00
Herbal product (*EAF-2011*)	8.681 ± 0.00	0.2665 ± 0.00	0.0610 ± 0.00

Results are Mean ± S.E.M; n= 3

3.4. Stability Studies of *EAF-2011*

The stability of the product, stored under normal shelf conditions was studied by assessing the physical, chemical and biological properties of the product. An organoleptic test, a thin layer and high performance liquid chromatographic profiling, and an antimicrobial assay were performed on the ointment. These studies were conducted at the baseline, month 3, month 6 and month 12 according to requirements of the WHO, (2007).

3.4.1. Materials and Methods

3.4.1.1. Organoleptic tests

The organoleptic features: colour, odour and consistency of the product were observed over the duration of the stability studies. The results represented the impression from two independent assessors.

3.4.1.2. Acidity or Alkalinity (pH)

An amount of 1 g of the ointment was dispersed in 100 mls of distilled water by vigorous shaking and warming over a water bath to melt the ointment. The mixture was allowed to cool at room temperature to separate the insoluble wax from the aqueous phase. The aqueous fraction was then decanted and the pH determined using the digital pH meter (Eutech Inst, USA).

3.4.1.3. Thin Layer Chromatography

The chromatographic conditions developed and described in section 3.3.2 were applied for the stability assessment.

3.4.1.4. High Performance Liquid Chromatography

The developed and validated HPLC method described in section 3.3.3 was used for this assessment.

3.4.1.5. Antimicrobial Assay

The agar well diffusion method was used for the antimicrobial assay as described in the B.P., (1988). An amount of 16 g of the ointment was dissolved in 100 mls of 20% dimethylsulphoxide (DMSO). Sabouraud and Mueller Hinton agar was used as the media for the fungal and bacterial organisms respectively. Petri dishes of 100 mm

diameter in dimension were filled with 25 mls of the respective media to a depth of 4 mm and allowed to solidify. Microorganisms tested included *Microsporum canis* (ATCC 36299), *Trichophyton rubrum* (ATCC 10218), *Candida albicans* (ATCC 10231) and *Staphylococcus aureus* (ATCC 25923). Each plate was then flooded with about 100 µl the pathogenic microorganism and the plates allowed to dry at room temperature for one hour. A sterilised cork borer of internal diameter of 4 mm was used to bore holes in the media. The herbal products were dispensed into the bored holes and the filled Petri dishes were kept in the refrigerator for 6 hours to allow absorption of the extract into the media and then incubated at 27^{o}C for 72hrs for the fungal organisms and 38^{o}C for 24 hrs for the bacterial organisms. DMSO was used as the negative control for all tests, Ketoconazole as the positive control for the fungal organisms and Ciprofloxacin for the bacterial organisms. After the incubation period, the diameter of each zone of inhibition was measured with a caliper. The measure of the zone of inhibition at each period of testing was compared and used as an indicator of the stability of the product.

3.4.2. Results of Stability Study

3.4.2.1. Organoleptics and pH

The study of the organoleptic characters of the ointment at baseline time showed the product to be dark brown in colour, aromatic in odour, smooth with no lumps or grittiness in consistency. Similar observations were subsequently made during the period of observation. The results of the organoleptic characteristcs and pH over the period are reported in Table 3.9. An insignificant change in pH values was recorded at the end of the study.

3.4.2.2. Thin Layer Chromatography

The chromatographic profile obtained for the ointment and the total crude extract was repeated over a one year period. The R_f values obtained for each period of time were not different. The chromatographic profile is presented as Figure 3.2. The baseline time and after one year of study for both test samples showed the ointment and the crude extracts to be stable as the spots obtained at the end of the study were not different from that obtained at the start of the study.

Table 3.9: The organoleptic characters and pH of the ointment during the 12 month stability study.

Time	Colour	Odour	Consistency	pH
0	Dark Brown	Aromatic	No grittiness or lumps observed	5.42 @ 29.2 ^{0}C
3	Dark Brown	Aromatic	No grittiness or lumps observed	5.29 @ 30.2 ^{0}C
6	Dark Brown	Aromatic	No grittiness or lumps observed	5.67 @ 30.4 ^{0}C
12	Dark Brown	Aromatic	No grittiness or lumps observed	5.71 @ 29.4 ^{0}C

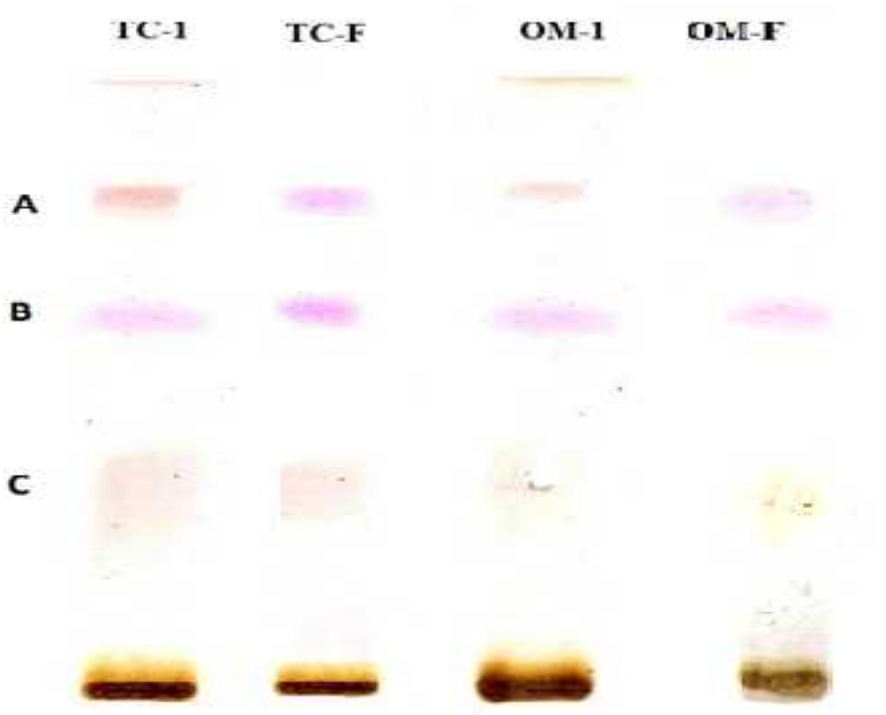


Figure 3.2: Chromatographic profile of the ointment and total crude extract at the baseline and the end of the study for the stability study. Solvent system: petroleum ether: ethyl acetate (4:1). Derivatisation: Anisaldehyde-H_2SO_4 Detecting Regent.

Key: Total crude extract at the start (TC-1) and Total crude extract after a year (TC-F), Ointment at the start (OM-1), Ointment after a year (OM-F)

3.4.2.3. High Performance Liquid Chromatography

Results of the quantitative assay of the flavonoids in the product at the baseline, sixth and at 12 months indicated that rutin and kaempferol were present at all times. The amount of quercetin was undetectable after the baseline assay. The fingerprints obtained are reported as Appendices XVII to XIX.

Table 3.10: Flavonoid content of the herbal product over a period of 12 months.

Time	Rutin ($^{w}/_{w}$)	Quercetin ($^{w}/_{w}$)	Kaempferol ($^{w}/_{w}$)
0	8.6810 ± 0.00	0.2665 ± 0.00	0.0610 ± 0.00
6	8.6550 ± 0.00	0.00	0.0589 ± 0.00
12	8.6550 ± 0.00	0.00	0.0597 ± 0.00

Results are Mean ± S.E.M; n= 3

3.4.2.4. Antimicrobial Assay

The antimicrobial assay of the ointment against three fungal pathogens: *Micosporum canis, Trichophyton rubrum,* and *Candida albicans* and one bacterial pathogen (*Staphylococcus aureus*) produced similar results over the one year-study period (Table 3.11). The differences observed in the readings were not significantly different at the baseline and after one year. The ointment also showed almost similar activity against the bacterial pathogen.

Table 3.11: Zones of inhibition produced by the ointment (*EAF-2011*) over the one year period.

Month	*Microsporum canis*	*Trichophyton rubrum*	*Candida albicans*	*Staphylococcus aureus*
0	12.7 ± 0.22	13.3 ± 0.12	10.3 ± 0.56	20.3 ± 0.01
3	12.3 ± 0.31	13.0 ± 0.32	10.0 ± 0.45	19.7 ± 0.20
6	13.3 ± 0.25	14.0 ± 0.00	10.3 ± 0.34	20.0 ± 0.47
12	13.2 ± 0.88	13.8 ± 0.71	10.2 ± 0.67	19.8 ± 0.84

Results are Mean ± S.E.M; n= 5

3.5. Chronic Toxicity Tests and Skin Sensitisation Testing

Skin sensitisation and chronic toxicity were conducted for the product to identify any possible toxic effect prior to the start of the clinical study. All experiments were performed according to the protocol described by Nyarko *et al.*, (2005) and conducted in accordance with accepted principles for laboratory animal use and care (EU directive of 1986: 86/609/EEC).

3.5.1. Materials and Method

3.5.1.1. Reagents and Chemicals

Test Kits: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Gamma-glutamyl-transferase (γ-GT), Bilirubin (direct and total), Albumin, Creatinine and Urea were purchased from Cypress Diagnostics, Belgium. Urine test strips (UroColor™ 10) were supplied by Standard Diagnostics Inc. (Kyonggi-do, Korea).

3.5.1.2. Animals

Male Sprague-Dawley rats weighing 200-250 g used were obtained from the Animal Unit of the Centre for Plant Medicine Research (CPMR), Mampong-Akuapem, in the Eastern region of Ghana. The animals were allowed feed and water *ad libitum.* The feed was obtained from Ghana Agro Food Company (GAFCO) Tema, Ghana.

3.5.1.3. Skin Sensitisation

The hair on the lateral portion (about 9 cm^3) of albino rats were trimmed and shaved with a razor blade. The rats were divided into 5 groups (n=5). One group was injected intradermally with 0.1 ml of 5% ($^{w}/_{v}$) of the 10% *EAF-2011* dissolved in glycerol. The duration of the resultant swelling was observed, as well as any ulceration that appeared subsequently. The various dosage concentrations of the ointment were also applied

topically twice daily to the three (3) groups of rats and the animals were observed daily for 6 months for any ulceration, irritation and/or inflammation. The last group did not receive any treatment and served as the negative control.

3.5.1.4. Chronic Toxicity

The four groups of five rats were each kept in four separate metal cages. Group 1 was kept as control and they received sterilised water and no treatment for six months. Animals in groups 2, 3 and 4 had the shaved areas of the skin treated twice daily with 2% ($^{w}/_{w}$), 5% ($^{w}/_{w}$) and 10% ($^{w}/_{w}$) of the ointment respectively. Blood and urine samples were taken for analysis at the baseline-time and then repeated at the 1^{st}, 3^{rd} and 6^{th} months after treatment.

3.5.1.5. Urinalysis

Samples for urinalysis obtained from the animals by involuntary discharges were collected on a clean ceramic tile at day 0 (baseline), 1^{st}, 3^{rd} and 6^{th} month. Analysis of urine for glucose, bilirubin, ketones, specific gravity, pH, proteins, urobilinogen, nitrates, blood and leucocytes were done using urine reagent strip UroColor™ 10 from Standard Diagnostics Inc. (Kyonggi-do, Korea).

3.5.1.6. Blood Sampling

Blood samples from the test animals were obtained by tail bleeding into Eppendorf tubes without an anticoagulant and then centrifuging for 5 minutes (Denley BS 400, England), serum was stored at -40^{o}C for prior to biochemical and haematological analysis.

3.5.1.7. Serum Biochemical Analysis

Serum Alanine transaminase (ALT), Aspartate transaminase (AST), Gamma glutamyl transferase (γ-GT), Total and Direct Bilirubin, Albumin, Creatinine and Urea of the samples were determined using protocols from Cypress Diagnostic Kits, Belgium with a semi-automated blood chemistry analyser, photometer 4040.

3.5.1.8. Haematological Analysis

Red blood cells (RBC) count, White blood cells (WBC) count, and Haematocrit (HCT), Haemoglobin (Hb) as well as other haematological parameters such as Mean cell volume (MCV), Mean corpsular haemoglobin concentration (MCHC), Mean platelet volume (MPV) and Platelet (PLT) count were determined with Haema-Screen 18 (Hospitex Diagnostics, Italy) in accordance with established protocol.

3.5.1.9. Histology

Two rats from each group were euthanized after 6 months of treatment and biopsies were taken from the liver, spleen, kidneys and the skin. The skin biopsy was obtained from the area where the ointment was applied. Specimen was preserved in 10% buffered formalin (Formaldehyde solution) and later were embedded in paraffin wax and sectioned to a uniform thickness of 10 µm and then stained with hematoxylin and eosin. Sections from all the groups were evaluated under a microscope for any morphological changes.

3.5.1.10. Statistical Analysis

One-way analysis of variance (ANOVA) and independent sample *t*-Test was conducted between control and test to determine statistical significance in all instances. All

Statistical tests were performed with Graphpad prism software version 5.0 and *p*-value <0.05 was considered statistically significant.

3.5.2. Results of Chronic Toxicity and Skin Sensitivity Testing

3.5.2.1. Skin Sensitivity Testing

The skin sensitivity test using the male Sprague- Dawley rats showed no dermal irritation in the form of ulcerations during the 6 months of treatment. Subcutaneous papules that were formed after intradermal injection of the ointment dissolved in glycerol resolved without any ulcerations after 72hrs of observation.

3.5.2.2. Chronic Toxicity Studies

The results from the chronic toxicity study indicated that the ointment did not cause any significant change in the haematological, biochemical and urine parameters after the six months of administration. The results of the haematological, biochemical and urine parameters are presented in Tables 3.12, 3.13 and 3.14 respectively.

3.5.2.3. Haematological Data

Haematological indices showed no significant difference ($p<0.05$) between the test and control animals for all parameters in all the treatment groups. This implied the ointment had no effect on the blood cells of the animals (Table 3.12).

Table 3.12: Post treatment effect of ointment on haematological parameters of rats

Haematological Parameters	TREATMENT			
	CONTROL	2%	5%	10%
NEU (%)	13.37 ±0.98	14.57 ± 2.41	18.03 ± 0.69	28.53 ± 11.79
LYM (%)	81.93 ±0.81	56.37 ± 20.34	56.27 ± 19.62	66.90 ± 11.36
MON (%)	3.93 ± 0.29	29.67 ± 23.97	27.83 ± 22.54	3.40 ± 1.32
EOS (%)	0.30 ± 0.00	2.13 ± 1.83	3.00 ± 2.65	0.70 ± 0.15
BAS (%)	0.47 ± 0.07	0.47 ± 0.03	0.47 ± 0.07	0.47 ± 0.07
RBC (10^6/mm^3)	8.68 ± 0.16	5.82 ± 2.71	5.86 ± 2.78	8.01 ± 0.64
HB (g/dl)	16.50 ±0.23	13.58 ± 2.62	14.01 ± 2.79	15.10 ±1.30
HCT (%)	48.70 ±0.45	36.90 ± 10.10	37.67 ± 10.94	44.00 ± 4.11
MCV (μm^3)	56.00 ±1.00	53.10 ± 1.99	52.77 ± 3.23	54.67 ± 0.88
MCH (pg)	19.03 ±0.33	32.30 ± 13.35	31.30 ± 11.85	18.87 ± 0.09
MCHC (g/dl)	33.90 ±0.20	29.63 ± 4.82	29.30 ± 5.30	34.43 ± 0.32
RDW (%)	10.83 ±0.58	10.87 ± 0.15	18.47 ± 7.82	10.80 ± 0.46
PLT (10^3/mm^3)	614.0±128.6	587.67 ±219.9	772.00 ± 47.3	696.67 ± 44.6
MPV (μm^3)	5.80 ± 0.12	6.27 ± 0.48	5.87 ± 0.07	5.97 ± 0.26
WBC(10^3/mm^3)	12.0 ± 0.56	11.50 ± 1.93	11.43 ± 0.81	11.80 ± 0.90

Results are Mean ± S.E.M; n=5

Key: HB - Haemoglobin, HCT - Haematocrit, BAS - Basophils, LYM - Lymphocytes,MCHC - Mean Corpsular Haemoglobin Concentration, MCH - Mean Corpsular Haemoglobin, MCV - Mean Corpsular Volume, MPV - Mean Platelet Volume, MON – Monocytes, NEU - Neutrophils, PLT - Platelets, RBC - Red Blood Cells, RDW - Red Blood Cell Distribution Width , WBC - White Blood Cells.

3.5.2.4. Serum Biochemical Data

No significant difference was observed ($p<0.05$) between the test and control animals for all parameters in all the treatment groups. Implying the ointment has no harmful effect on the liver and kidneys of the animals.

Table 3.13: Post treatment effect of the ointment on the liver and kidneys of the rats.

		TREATMENTS		
	CONTROL	2%	5%	10%
Kidney Function				
UREA (mmol/l)	3.43 ± 0.30	3.13 ± 0.30	2.93 ± 0.07	2.90 ± 0.10
CREAT (µmol/l)	36.07 ± 3.33	25.07 ± 2.93	44.75 ± 6.93	28.33 ± 3.31
Liver Function				
ALBUMIN (g/L)	30.85 ± 2.08	32.35 ± 1.01	32.82 ± 1.39	32.70 ± 0.87
ALT (u/L)	104.67±0.91	98.67 ± 3.72	99.67 ±13.17	87.10 ± 8.98
AST (u/L)	139.45 ±8.51	170.14 ± 11.08	132.07 ± 28.09	147.43±25.87
GGT (u/L)	1.20 ± 0.23	1.10 ± 0.15	1.43 ± 0.35	2.60 ± 0.62
ALP (u/L)	4.67 ± 0.88	4.67 ± 0.33	3.33 ± 0.88	4.00 ± 0.58

Results are Mean ± S.E.M; n= 5

Key: ALT – Alanine Transaminase, AST – Aspartate Transaminase, Creat – Creatinine, GGT – Gamma Glutamyl Transferase, ALP – Alkaline Phosphatase,

3.5.2.5. Urinalysis

There was no significant difference between the controls and test implying there was no effect on the kidneys of animals used.

Table 3.14: Post treatment effect of the ointment on urine parameters of rats

Urine Parameter	Treatment			
	Control	10%	2%	5%
Urobilinogen	-	-	-	-
Glucose	-	-	-	-
Ketones	-	-	-	-
Specific Gravity	1.024	1.022	1.023	1.012
Blood	-	-	-	-
pH	7.0	7.2	7.3	7.3
Proteins	++	+	++	+
Nitrites	-	-	-	-

Key: (-): absent; (+): present in moderate quantities; (++) present in large quantities

3.5.2.6. Results of Histological Analysis

Examination of the gross features of the organs isolated after termination detected no abnormalities. No significant differences were observed in the organ weight: body ratio of the control and the test animals receiving the herbal treatments as shown in Table 3.15. Microscopic examination of the tissues did not indicate any histopathological changes to the tissues sampled. The skin epidermis, dermis and adnexal tissues were regular in pattern. The spleen had regular red and white pulps. In the kidneys the

glomeruli, tubules and interstitium were normal and there was no indication of an inflammation or necrosis. The liver's portal tracts, hepatocytes and sinusoids were normal with the absence of inflammation or fibrosis. There was also no hepatocyte degeneration. The features were identified in the 2%, 5% and 10% herbal treatment. Samples micrographs from histopathology are presented as Appendix XXI; Figure 7.19-22.

Table 3.15: Post treatment effect of *EAF-2011* on the organ weights (weight to body ratio) of rats

Organ	**Control**	**2%**	**5%**	**10%**
Kidney	2.29 ± 0.16 (0.0098)	2.24 ± 0.57 (0.0094)	2.16 ± 0.24 (0.0098)	2.21± 0.84 (0.0097)
Spleen	0.71± 0.64 (0.0031)	0.71 ± 0.15 (0.0030)	0.63 ± 0.11 (0.0030)	0.65 ± 0.18 (0.0031)
Liver	12.61 ± 1.71 (0.0523)	12.19 ± 2.90 (0.0491)	10.50 ± 0.85 (0.0560)	11.29 ± 2.82 (0.0532)

Results represent the Mean ± SEM; n=5

3.6. Discussion

The complex nature of herbal medicines and their products makes their quality control very challenging. The first and most important step in this assessment involved the authentication of the plant materials by a botanist and the retention of voucher specimen. The documentation of the yields obtained from the extraction process also formed an essential part of this process as variations can affect the content of chemical constituents in the finished product.

Qualitative chemical fingerprinting using the basic phytochemical screening and the thin layer chromatography indicated the presence of various groups of secondary metabolites that are known to possess some antimicrobial activities. These metabolites included triterpenes; some of which have been reported by Shai *et al.,* (2008) to have antifungal activity against *Candida albicans.* The triterpenes isolated were from *Curtisia dentata* and included ursolic acid which is also found in *Psidium guajava.* The presence of phenolic compounds was also detected; their therapeutic importance as antifungal agents has been reported in *Barringtonia racemosa.* In this report by Hussin *et al.*,(2009), it was established that the ability of *Barringtonia racemosa* to resist pathogenic fungi increased when the phenolic constituents in the plant was higher. Specific mention has been made of the flavonoids also detected in the product and its starting raw materials, with quercetin and kaempferol previously identified in *Eugenia caryophyllata, Psidium guajava* and *Tridax procumbens* documented to have activity against *Candida albicans;* a common fungal pathogen (Jindal and Kumar, 2011). The antifungal properties of the alkaloids present in the product is also worth noting as berberine and a furoquinoline present in *Zanthoxylum zanthoxyloides* have been shown to be active against *Trichophyton, Epidermophyton, Micosporum* and *Candida spp* isolated from some clinical samples (Zhao *et al.,* 1998; Volleková *et al.,* 2003).

The presence of these secondary metabolites was also confirmed by the thin layer chromatography following the detection of spots whose colour after derivatisation indicated the presence of phenolic compounds which are known to give either a violet, dark brown or blue colour on spraying with anisaldehyde detecting reagent (Furniss *et al.*, 1989).

The quantitative HPLC which assayed three flavonoid compounds (rutin, quercetin and kaempferol) further affirmed the results of the basic phytochemical screening and the thin layer chromatography. Rutin was present in all the starting raw materials except *Eugenia caryophyllata*. Quercetin and kaempferol were also undetected in *Zanthoxylum zanthoxyloides* and *Alchornea cordiflolia* respectively. This absence can be ascribed to the limit of detection of the HPLC system employed. The finished herbal product however showed the presence of all the flavonoids tested.

In the stability study for the finished herbal product performed over a one year period, results from the physical assessment using the organoleptic features and pH of the product indicated that the product was stable. This chemical stability was also demonstrated to a large extent using the thin layer chromatography and HPLC. Marginal declines of 2.6% and 0.13% were recorded in the quantity of rutin and kaempferol respectively after a year compared to the baseline. Significantly, quercetin also was undetectable after the first month of assay indicating a possible change in this constituent. The biological assessment of stability using the *in vitro* antimicrobial assay showed that the product did not undergo any significant detoriation in quality. The zones of inhibition produced during the assessment were comparable over the study period.

The final aspect of the quality evaluation was on the safety of the product. The skin sensitivity test ruled out any irritations and immune response that may be elicited on the administration of the polyherbal product. This finding was also confirmed on the histopathological examination of the sampled skin tissue by the absence of immunomodulatory structures like the mast cells whose presence indicates an immune reaction (Bala *et al.*, 2000). Very importantly, the ointment did not induce any significant change in the haematological (Table 3.12), biochemical (Table 3.13) and urine (Table 3.14) parameters after the chronic toxicity study. This result meant that the product was not likely to have any harmful effect on the human subjects; a requirement for the conduct of the clinical study.

3.7. Conclusion

The polyherbal herbal product (*EAF-2011*) has been shown to be safe and stable over a one year period with the results of the qualitative and quantitative chemical fingerprinting separately providing adequate standards by which the product can be assessed. Combination of this data can significantly contribute to the standards for a monograph of the product.

CHAPTER 4

CLINICAL EVALUATION OF *EAF-2011*

4.1. Introduction

The highest form of evidence on safety and efficacy that can be provided for any medicinal agent comes from clinical studies, especially in randomised controlled trials (RCT). Currently, most herbal medicines lack clinical evidence on their use and even when such data is available, queries have always been raised about the quality of methodological procedure used in these evaluations. It is therefore recommended that standard and acceptable methods are used in the clinical evaluation of herbal medicines to provide evidence that will enable acceptance of medicinal plant products into conventional system of care by regulatory bodies (WHO, 2004).

The clinical study of the herbal product (*EAF-2011*) was carried out to evaluate its safety and effectiveness to fulfil the criteria stipulated by the WHO for medicinal agents since such data was absent. The absence of any adverse reactions from the prior skin sensitisation and chronic toxicity study (section 3.5) as well as the history of use for the product all provided a basis for the human trial to be undertaken. The methods used in the study were in line with the recommendations of the Consolidated Standards for Reporting Trials (CONSORT) (Gagniera *et al.*, 2006).

Participants were randomly assigned to the treatment groups that comprised three different concentrations of the herbal product and a control treatment of Whitfield ointment. The trial was also designed to blind both participants and investigators as to which treatment group participants were assigned. Safety analysis was also performed for participants to confirm the results of the chronic toxicity and skin sensitivity testing.

4.2. Methods

4.2.1. Ethical Approval and Conduct of Trial

Ethical approval was obtained from the Ethics Committee for Human Research of the Centre for Plant Medicine Research (see Appendix I). The trial was performed according to guidelines stipulated by the Helsinki declaration for the conduct of Medical Research (WHO, 2001). The activities participants were involved in during the study are as summarised in Figure 4.1.

4.2.2. Trial design

A prospective randomised double-blind parallel controlled method was used for the study.

4.2.3. Randomisation and Blinding

A blocked randomisation was used; participants were assigned to the treatment using a randomisation ratio of 3:3:3:1 i.e. 2%, 5%, 10% of the herbal treatment and the control respectively. Randomisation was achieved by making participants pick numbered papers without replacing from a box containing folded papers with codes for the assignment of groups. Allocation was done to attain the desired mix of the assignment ratio at the end of a 10^{th} recruitment.

A subgroup of 30 participants was also randomly selected to undergo microscopy and mycological culture using the same randomisation ratio. Blinding was achieved by making an independent individual collate information and assign the treatment for each participant. This individual was not involved in any of the trial related procedures including follow up assessments and kept the treatment assignment information until

the completion of the study. The treatment assignment data was only obtained after submission of the data for statistical analysis.

4.2.4. Study Sites

The study was undertaken at the clinic of the Centre for Plant Medicine Research and some selected first cycle institutions in the Akuapem-North District.

4.2.5. Criteria for Participant Selection

A selection and exemption criteria as stated below was set up for eligible participants.

4.2.5.1. Participant Inclusion Criteria

Participants included in the study were male or female between the ages of 6 and 65 years clinically diagnosed with any superficial fungal skin infection. Participants were also expected to be able to complete the informed consent process and also to comply with the protocol or if a minor, has a parent or guardian who is able to complete the informed consent form.

4.2.5.2. Participant Exclusion Criteria

Participants were excluded from the study if they were patients with kidney or liver dysfunction, pregnant women and immunocompromised patients. Individuals who were also on any orthodox medication that could affect the outcome of the trial e.g. corticosteroids and immunomodulating agents were excluded. Acutely ill-individuals were also exempted from the study.

4.2.5.3. Withdrawal from the Study

Withdrawal criteria for participants taking part in the study were listed as individuals who were unable to comply with the protocol and those who developed any hypersensitive reactions whether local or systemic to the medications. Participants who developed any systemic complications deemed detrimental to them and those who recorded an extension of the lesions or an increase in Total Signs and Symptoms Score of more than 2 for each of the selected parameter were also withdrawn from the study.

4.2.6. Sample Size

The study was designed to have 30 participants for each herbal treatment group and 10 participants in the control treatment with an assignment ratio of 3:3:3:1. The risk of making a type II error (statistical power) was set at 0.20 and a difference in treatment (Total signs and symptom score) of 2.00 considered clinically relevant. Type I error (α level) was 0.01 and the population standard deviation assumed to be 2.30. Sample size was calculated using the formula:

$$N = f(\alpha, ß) \times \frac{2\sigma^2}{(\mu_1 - \mu_2)^2}$$

N is the sample size

$f(\alpha, ß)$ is the critical factor

σ is the population standard deviation

$(\mu_1 - \mu_2)$ is the difference between the sample and population mean considered clinically significant

4.2.7. Informed Consent Forms

Participants were asked to complete an informed consent form and children considered too young to complete the form were requested to report with a parent or guardian. The

details of the trial were always explained to subjects in the local dialect or any understood language by the investigator before forms were signed or thumb printed.

4.2.8. Schedule of Evaluation

Participants on recruitment into the trial (Day 0); were followed up for clinical assessment and observation twice during the first month (Day 14, 28); twice during second month (Day 42, 56) and once during the last month (Day 90). Monitoring for relapse was done 30 days after completion (Day 120).

4.2.9. Treatment Dosage

The participants were advised to apply their respective ointments to the affected parts of the body morning and evening daily after bathing.

4.2.10. Assessment of Effectiveness and Classification of Therapeutic Response

On the day of recruitment, skin scrapings were taken from the 30 participants included for the microscopic and mycological analysis. Each participant was then graded using a clinical score as shown in Appendix II. This grading was repeated on subsequent visits. Primary assessment of effectiveness was based on a clinical score, microscopic examination and results of a culture for the fungal infection.

The clinical score used the Total Signs and Symptoms Score (TSSS) (Friedlander *et al.*, 2002). This is a rating using a four point scale where; *0- absent*; *1-mild*; *2-moderate* and *3-severe* for each of the selected signs and symptoms that are characteristic for the condition.

Microscopic examination observed skin scrapings from subjects for characteristic hyphae. Samples were then cultured on the appropriate media for identification of the causative fungal organisms.

4.2.11. Definition of Clinical Effectiveness of the Product and Primary Outcome

Effectiveness or complete cure was defined as clinical cure (Total Clearance) or a TSSS of 0 for all the population randomised; a negative microscopic examination and a negative culture in the population included in the mycological studies.

4.2.12. Secondary Outcomes

Secondary outcome measures were defined as effective treatment when participants had a TSSS of ≤ 3 with individual scores of ≤ 1for at least one of the signs and a negative microscopy and mycology in that population.

4.2.13. Assessment of Safety

On the day participants were recruited for the study (Day 0), blood samples were drawn for analysis from the 24 participants randomised for the safety study. Parameters considered were the Full Blood Count (FBC), Renal Function Test (RFT), Liver Function Test (LFT) and a urinalysis. This was repeated at the end of the study. The list of safety indicators and reference ranges used are included as Appendix V**.**

4.2.14. Adverse Drug Effects

On each visit for monitoring and review, adverse reactions to the product were recorded. This included a review of all the systems to detect any of such reactions. The adverse reporting sheet is attached as Appendix VI.

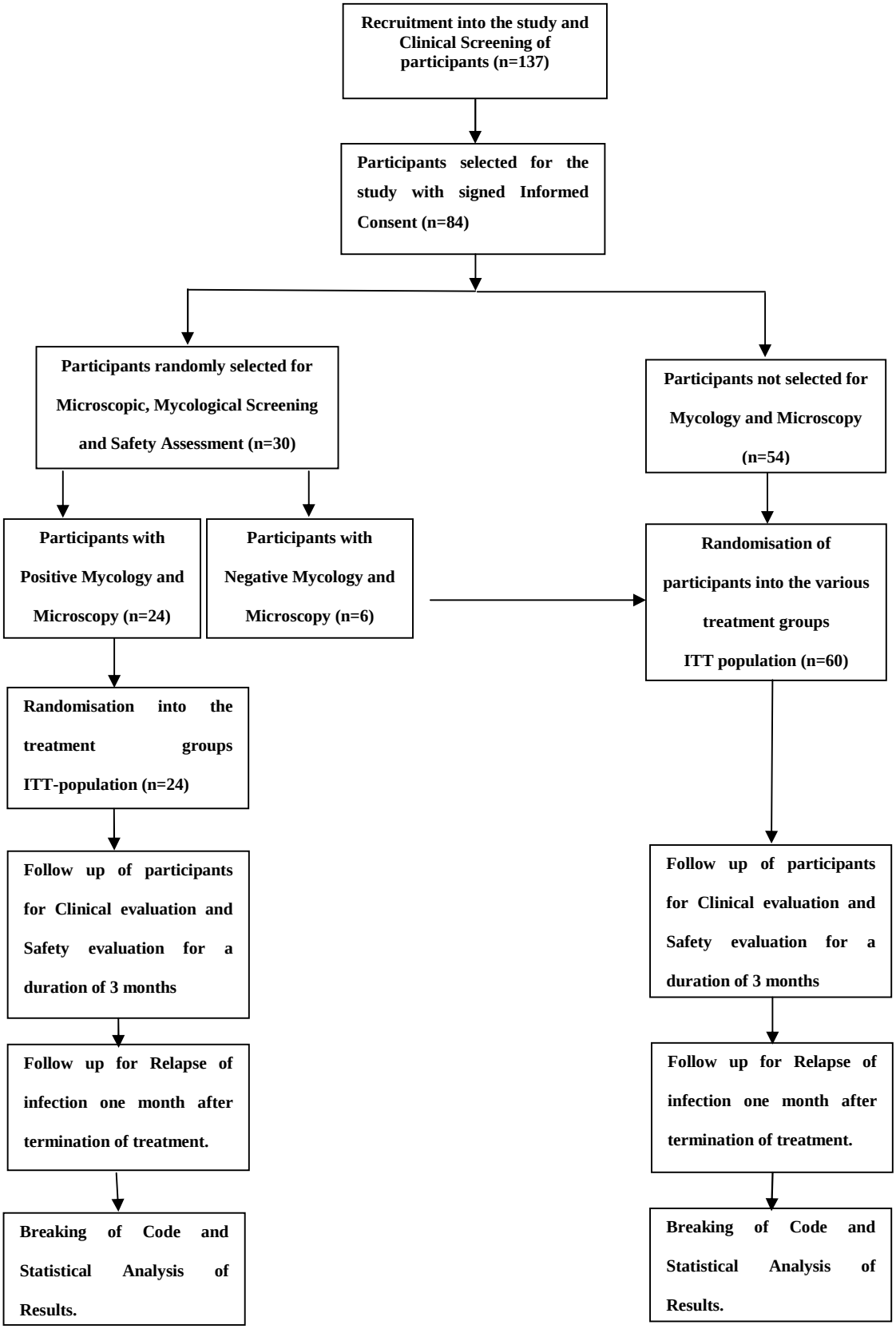


Figure 4.1: Schematic diagram of the randomisation groups and the sequence of activities participants were involved in during the study

4.2.15. Statistical Analysis

The hypothesis of interest for the primary efficacy outcome was that each of the herbal extract had at least comparable activity to Whitfield's ointment. A one way analysis of variance (ANOVA) was also used to compare the differences between the treatment groups. When differences were detected, an independent *t*-test was used in the analysis of the primary efficacy outcome by comparing the three herbal extract concentrations with the Whitfield ointment. Safety analysis was also performed using an independent *t*-test comparing the herbal treatments to the control. An α-level of 1% was set for the detection of statistical significance. All analyses were done using the intention to treat (ITT) population.

4.3. Results of Clinical Studies

4.3.1. Randomisation Groups

There were 4 treatment groups in the study: a positive control group of Whitfield's ointment, 2%, 5% and 10% herbal extract concentrations of *EAF-2011*. A number of 84 participants were randomised into these groups and constituted the intention-to-treat (ITT) population.

According to the protocol schedule, there were two subgroups of the ITT population. The first subgroup comprised 30 participants randomly selected to undergo the microscopy and mycological culture of their skin scrapings. Results were positive for 24 of the participants and they underwent a separate randomisation using the same randomisation ratio as per the trial protocol. The second subgroup also comprised 60 participants; 54 from the population not included in the microscopy and mycological studies and 6 from the population that failed the microscopy and mycological screening

as shown in Figure 4.1. The two subgroups accounted for the total ITT population of 84.

The distribution of participants in the treatment groups were: 3 participants for Whitfield treatment group, 7 participants each for the 2%, 5% and the 10% herbal extract concentration groups in the mycology group. In the non-mycological group, 7 participants were randomised into the Whitfield treatment group. The 2%, 5% and 10% *EAF-2011* treatment groups had 16, 21 and 16 participants respectively being randomised into them. Figure 4.2 also gives a schematic representation of the distribution of participants and a summary of the results. The mycology group also formed the population included in the safety assessment of the treatments. All participants were assessed for adverse events.

4.3.2. Participant Demographics and Disease Characteristics

The mean age across the treatment groups are shown in Table 4.1. The ages across the groups were comparable and the distribution according to sex was also similar. However, of the participants randomised to the 2% *EAF-2011* treatment group, 43.48% were males and 56.52% were females. This result was a deviation from the recorded trend in this study where the male population was higher than the female across the groups.

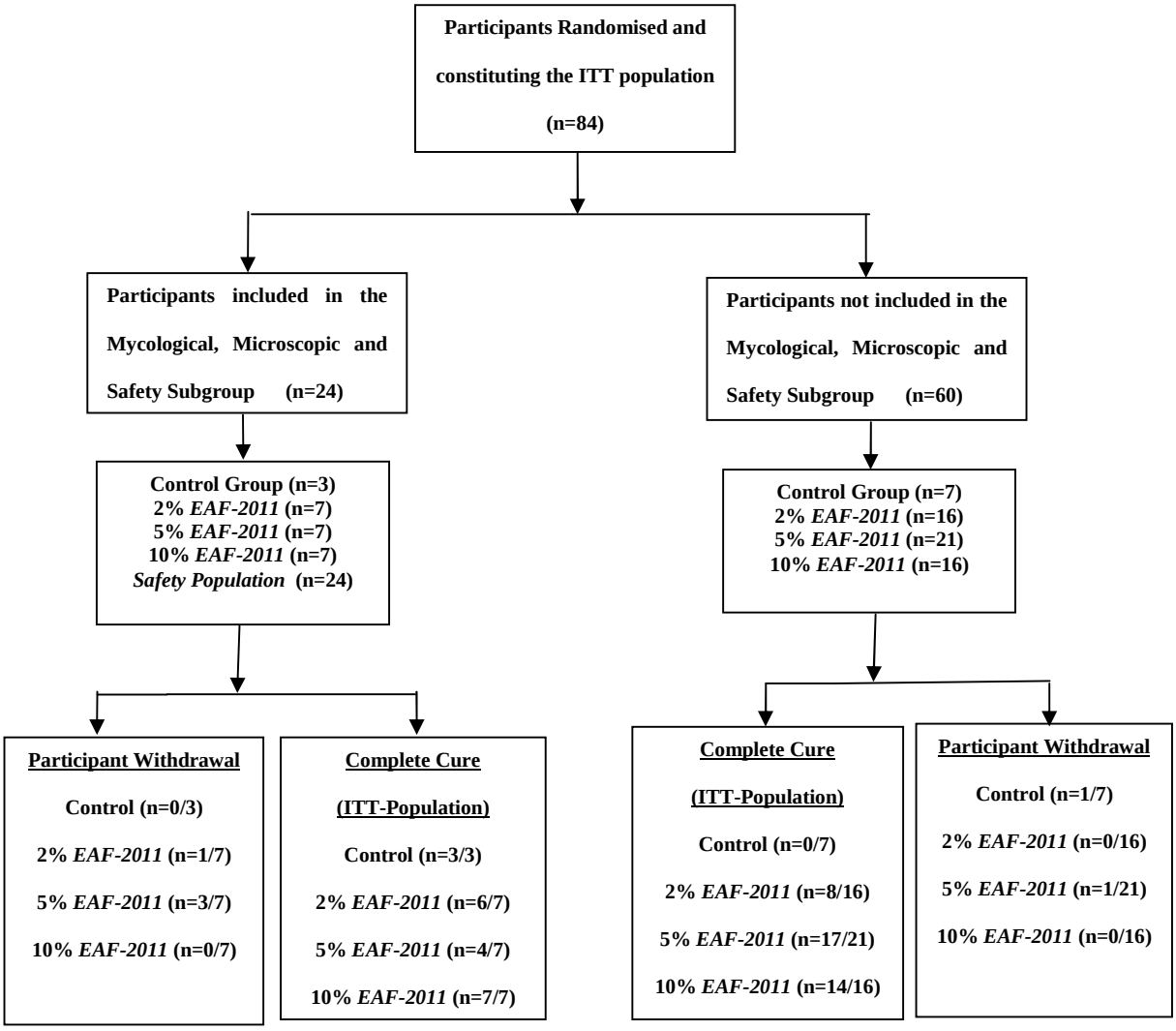


Figure 4.2: Summarised results of the clinical study; randomisation groups, treatment outcome and participant withdrawal.

Table 4.1: Participant Demographics at the baseline of the study (ITT population)

	Control (n=10)	2% (n=23)	5% (n=28)	10% (n=23)
Age (SD)	12.40 (1.96)	13.48 (8.31)	14.57 (8.91)	13.57 (6.56)
Sex:				
Male (%)	6 (60)	10 (43.48)	20 (71.40)	15 (65.21)
Female (%)	4 (40)	13 (56.52)	8 (28.57)	8 (34.78)

Tinea capitis was the most prevalent infection recorded among participants (Table 4.2). *Microsporum spp* were identified in 16 (66.66%) of the 24 participants in the mycology subgroup with 5 cases being confirmed as *Microsporum canis*. The other cultures were identified as yeast like cells; most likely *Malassezia furfur* which are known for their role in *Tinea versicolor.* Participants with previous infections also accounted for 71.42% of the ITT population. The study also recorded 52.38% of participants with an immediate relation living in the same household having a related or similar infection. An analysis of the duration of infections in participants showed 46.42% with infections lasting more than 12 months, 44.05% between 3 to 12 months and 10.71% having the infection for less than 3 months.

4.3.3. Treatment Efficacy

4.3.3.1. Primary Efficacy Outcome

Complete cure was achieved in 34.78% of participants randomised to the 2% *EAF-2011* treatment group. In the 5% and 10% *EAF-2011* treatment groups, 75.0% and 91.3% of participants recorded complete cure respectively. These were higher compared to the Whitfield ointment group which had 30.0% complete cure rate at the end of the study period (Table 4.3).

Table 4.2: Disease characteristics of participants at the baseline (ITT population)

Type of Infection		Control (n=10)	2% (n=23)	5% (n=28)	10% (n=23)
Tinea capitis n (%)		5 (50)	11 (47.82)	15 (53.57)	14 (60.86)
Tinea coporis n (%)		5 (50)	8 (34.78)	8 (28.57)	5 (21.73)
Tinea barbae n (%)		0	1 (4.34)	0	0
Pityriasis vesicolor n (%)		0	3 (13.04)	5 (17.85)	4 (17.39)
Participants with previous infections n (%)		8 (80)	15 (65.21)	19 (67.85)	18 (78.26)
Relations with similar infections n (%)		4 (40)	7 (30.43)	17 (60.71)	16 (69.56)
Duration of current infection n (%)	< 3 months	0	2 (8.69)	4 (14.28)	3 (13.04)
	3-12 months	6 (60)	9 (39.13)	13 (46.42)	9 (39.13)
	>12months	4 (40)	12 (52.17)	12 (42.85)	11 (47.82)

4.3.3.2. Secondary Efficacy Outcomes

Effective treatment was recorded in 82.65%, 85.71% and 95.65% of the participants in the 2%, 5% and 10% herbal extract treated groups respectively. The Whitfield ointment group had 50% of participants also achieving effective treatment. The comparative achievement of the secondary efficacy outcome across the groups is as presented in Table 4.3.

Negative microscopy and mycology were recorded in all the participants who completed the study. This outcome was achieved by day 56 on follow-up. When analysed using the ITT population, the 2% and 5% *EAF-2011* treated group had a clearance rate of 85.71% and 57.14% respectively, while the 10% *EAF-2011* and the Whitfield groups had a 100% clearance rate.

4.3.3.3. Treatment Differences across Groups

The mean TSSS across the Whitfield, 2%, 5% and 10% *EAF-2011* treatment populations at the end of the study were compared to each other. The difference between the groups after a one way analysis of variance (ANOVA) was at the borderline of significance (*p*-value of 0.0145; α-level: 1%). However differences existed in the percentage cure rates at each follow up period with the 10% *EAF-2011* treatment showing the highest cure rate at the end of the study (Figure 4.3).

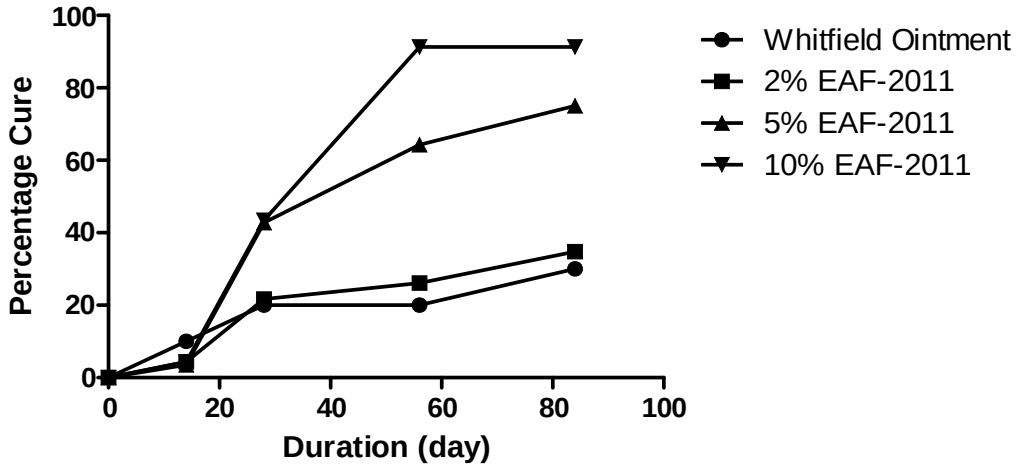


Figure 4.3: The number of participants achieving the primary end point (complete cure). Results are expressed as the percentage of participants achieving primary cure at each follow up period.

Table 4.3: Results of Primary and Secondary Efficacy Outcomes (ITT population)

Efficacy Outcome	**Whitfield Ointment (n=10)**	**2% (n=23)**	**5% (n=28)**	**10% (n=23)**
Primary Efficacy Outcome *(Complete Cure)* n (%)	3 (30)	8 (34.78)	21 (75.0)	21 (91.30)
Secondary Efficacy Outcomes *(Effective Treatment)* n (%)	5 (50)	19 (82.65)	24 (85.71)	22 (95.65)
(Mycological Cure) n (%)	3 (100)	6 (85.71)	4 (57.14)	7 (100)

4.3.3.3.1. Comparison of the Herbal treatments with Whitfield's ointment

The mean Total Signs and Symptoms Score (TSSS) for participants receiving the herbal treatment declined over the treatment duration. This trend was also observed for the control treatment of Whitfield ointment. The decline in TSSS was however not significantly different from the Whitfield group for participants receiving the 2% and 5% *EAF-2011* at the end of the study (Table 4.4). The clinical effect of the 10% *EAF-2011* was however better than Whitfield ointment with participants in this group recording a mean change in TSSS of -8.66 (0.21). The effect of the treatments on the TSSS of participants are summarised as Figure 4.4.

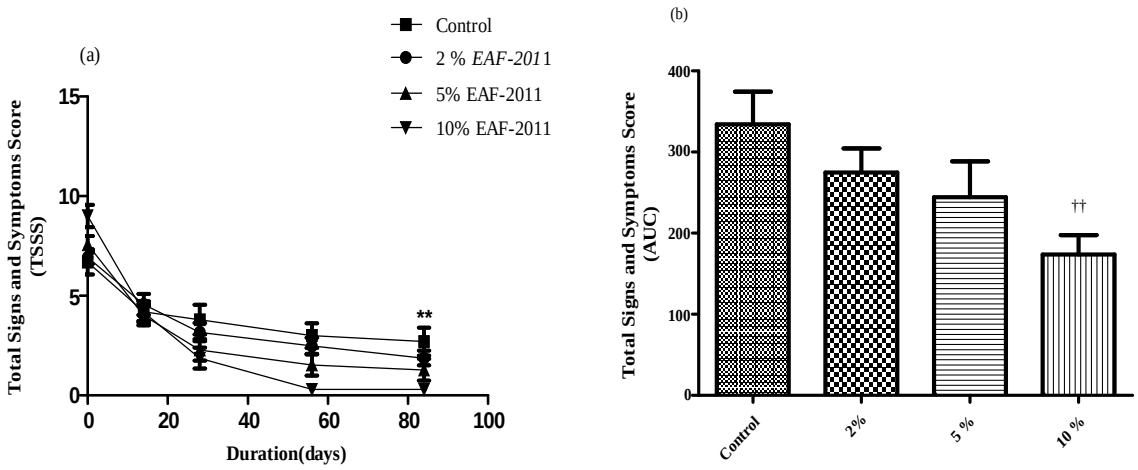


Figure 4.4: Effect of the three (3) concentrations of *EAF-2011* and Whitfield's ointment on the time course curve (a) and the TSSS presented as the area under the curve (AUC) (b). Data is presented as mean ±SD compared with the control (an independent *t*-test at an α-level of 1% showed no significant difference between the treatments).

Table 4.4: Summary of statistical analysis comparing the three (3) concentrations of *EAF-2011* to control treatment of Whitfield Ointment

Herbal Treatment	**Change in TSSS**	***p-value***	**Confidence Interval**
2% *EAF-2011*	-5.04 (0.37)	0.312	(-3.19 to 1.52)
5% *EAF-2011*	-6.29 (0.54)	0.125	(-3.93 to 1.09)
10% *EAF-2011*	-8.66 (0.21)	0.008	(-4.71 to -0.08)

4.3.4. Safety Analysis

The baseline and end-of-study haematological, biochemical and urine analytical parameters are shown in Tables 4.7, 4.8 and 4.9 respectively. The parameters analysed were the white blood cell (WBC) count, haematocrit (HCT), haemoglobin (HB), red blood cells (RBC) and platelets (PLT) for the haematological analysis. alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin (ALB), urea and creatinine were also included for the serum biochemical analysis. The urinalysis comprised evaluation of urobilinogen, glucose, ketones, specific gravity, blood, pH and proteins levels for participants in the safety subgroup.

The mean for all the safety variables were different in terms of the recorded values at the end of the study compared to the baseline. Statistical comparison of the means revealed significant differences for haemoglobin (HB), aspartate aminotransferase (AST) and urea in the 2% *EAF-2011* treatment population. The white blood cell count in the 10% *EAF-2011* treatment population was also significantly different from their

baseline mean. The Whitfield ointment group also had significant differences for haemoglobin, hematocrit and albumin. However, these changes with the exception of the haemoglobin reading from the 2% *EAF-2011* treatment group, were not clinically significant since the absolute values were within the normal reference limits.

Table 4.5: Baseline and end-of-study safety variables for the Control and 2% *EAF-2011* treatment groups; ITT population. Mean (SD)

	CONTROL		**2 % *EAF-2011***	
Parameter	Baseline	End of Study	Baseline	End of Study
WBC ($10^3/mm^3$)	7.80 (1.40)	5.50 (2.16)	9.10 (0.20)	6.10 (3.07)
RBC ($10^6/mm^3$)	4.64 (0.24)	4.08 (0.24)	4.45 (0.32)	3.91 (0.25)
HB (g/dl)	11.27 (0.24)	10.08 (1.29)*	11.0 (1.01)	10.95 (0.79)*
HCT (%)	33.87 (1.44)	30.75 (3.45)*	33.53 (2.91)	30.18 (2.39)*
PLT($10^3/mm^3$)	275.0 (90.31)	203.0 (29.49)	274.3 (140.6)	191.0 (68.64)
AST (u/L)	32.75 (1.34)	32.50 (0.99)	39.35 (6.85)	52.90 (5.37)**
ALT (u/L)	12.75 (8.13)	18.00 (1.41)	8.20 (0.70)	16.55 (9.26)
ALP (u/L)	329.0 (21.92)	330.0 (22.63)	405.0 (116.0)	493.5 (176.1)
GGT (u/L)	31.70 (2.04)	43.92 (9.46)	38.73 (10.70)	38.75 (13.43)
ALB (g/L)	30.60 (12.16)	32.55 (12.52)**	41.25 (40.80)	40.80 (9.19)
UREA (mmol/l)	1.55 (0.92)	1.05 (0.35)	0.35 (0.04)	2.30 (0.14)*
CREAT(µmol/l)	66.85 (16.05)	58.20 (3.96)	60.15 (1.49)	67.70 (14.85)

Key: HB - Haemoglobin, HCT - Haematocrit, PLT - Platelets, RBC - Red Blood Cells, White Blood Cells. ALT – Alanine Transaminase, AST – Aspartate Transaminase, GGT – Gamma Glutamyl Transferase, ALP – Alkaline Phosphatase, CREAT – Creatinine

Table 4.6: Baseline and end-of-study safety variables for the 5% *EAF-2011* and 10% *EAF-2011* treatment groups; ITT population. Mean (SD)

	5 % *EAF-2011*		**10 % *EAF-2011***	
Parameter	Baseline	End of Study	Baseline	End of Study
WBC ($10^3/mm^3$)	5.25 (1.34)	4.65 (1.36)	7.30 (1.01)	4.63 (1.02)*
RBC ($10^6/mm^3$)	4.36 (0.63)	3.91 (0.25)	4.63 (1.02)	4.50 (0.18)
HB (g/dl)	12.0 (2.17)	11.30 (0.76)	11.57 (0.25)	11.53 (1.57)
HCT (%)	35.38 (6.09)	34.30 (2.01)	34.62 (0.81)	34.95 (5.07)
PLT ($10^3/mm^3$)	210.3 (69.98)	138.3 (46.45)	270.3 (113.2)	164.0 (16.27)
AST (u/L)	14.75 (7.49)	16.68 (5.68)	22.60 (17.09)	20.25 (14.44)
ALT (u/L)	12.73 (9.61)	9.28 (0.55)	12.08 (8.52)	15.35 (5.72)
ALP (u/L)	404.5 (33.98)	522 (32.42)	460.5 (217.8)	472.0 (200.6)
GGT (u/L)	31.98 (12.75)	31.20 (6.57)	35.76 (4.50)	36.05 (8.98)
ALB (g/L)	33.98 (6.42)	33.45 (2.18)	43.35 (2.26)	48.10 (10.71)
UREA (mmol/l)	1.25 (0.72)	1.50 (0.53)	1.20 (0.74)	1.28 (0.97)
CREAT(µmol/l)	31.45 (8.24)	36.88 (7.92)*	47.93 (8.14)	44.55 (20.63)

Key: HB - Haemoglobin, HCT - Haematocrit, PLT - Platelets, RBC - Red Blood Cells, White Blood Cells. ALT – Alanine Transaminase, AST – Aspartate Transaminase, GGT – Gamma Glutamyl Transferase, ALP – Alkaline Phosphatase, CREAT – Creatinine

Table 4.7: Results of urinalysis at the end-of-study for all the treatment groups. Mean (SD)

Parameter	Control	2%	5%	10%
Urobilinogen	-	-	-	-
Glucose	-	-	-	-
Ketones	-	-	-	-
Specific Gravity	**1.020**	**1.005**	**1.010**	**1.005**
Blood	-	-	-	-
pH	**7.5**	**6.0**	**7.0**	**6.5**
Proteins	+	-	-	+

Key: (-): absent; (+): present in moderate quantities

4.3.5. Relapse of Infection

Participants who recorded the relapse are shown in Table 4.10. The mean TSSS for these individuals were 2, 4 and 2 for 2%, 5% and 10% herbal extract concentrations respectively. None of the participants in the Whitfield group recorded a relapse.

Table 4.8: Participants recording a relapse 30 days after termination of treatment (ITT Population).

	Control	2%	5%	10%
Number of participants	0/10	4/23	7/28	4/23
Mean TSSS	0	2	4	2

4.3.6. Adverse Reactions

None of the participant reported of any adverse reactions to the product. Routine examination during the follow up period also did not record any adverse findings.

4.4. Discussion

The outcome of the clinical study showed an average age of 13.50 yrs across the groups. The ages and disease characteristics recorded were confirmatory of the population that were prone to fungal skin infections as previously reported by Dagnew and Erwin (1991) and Wu *et al.*,(2000). The most prevalent infection by percentage was *Tinea capitis*, which has been reported among children in endemic regions. Additionally, the *Microsporum spp* which was the most common fungal organisms isolated during the mycological study has also been reported to be one of the most dominant dermatophyte among Africans (Seebacher *et al.*, 2008).

Results of the primary outcome of the study (complete cure) showed all the treatments to be effective in the management of the investigated fungal skin diseases. The differences in the achievement of the outcome was established to be concentration and time dependent with the number of participants achieving complete cure increasing at each follow-up period (Figure 4.3). This was clearly supported by data from the participants randomised to the 2% herbal extract concentration group who recorded an increase in the primary outcome of 21.74%, 26.09% and 34.78% on day 28, 56 and 84 respectively. Similar trends were recorded in the other treatment groups including the Whitfield ointment group. At the end of the study, complete cure was achieved in 30.0% of the Whitfield ointment group, 34.78%, 75.0% and 91.30% of the 2%, 5% and 10% herbal treatment groups respectively.

Although participants in the herbal extract group who achieved the primary outcome were higher than the control, statistical analysis of the results showed the 2% and 5% concentration of the herbal extracts had comparable activity to Whitfield ointment. The 10% herbal group however showed better activity than Whitfield ointment.

The secondary efficacy outcome in this study assessed the time taken for participants to achieve mycological and microscopic cure in that subgroup and graded individuals who did not achieve complete clearance of the disease characteristics. The outcome assumed a maximum TSSS of 3 with individual scores ≤ 1 which meant that participants were expected to have mild symptoms for three of the assessed parameters at the end of the study. Secondary end point of effective treatment was attained by 50%, 82.65%, 85.71% and 95.65% of the Whitfield, 2%, 5% and 10% *EAF-2011* treatments respectively. The results of the secondary outcomes (see Table 4.3) showed a similar trend of increasing percentages of participants achieving the outcome with increasing extract concentration as in the primary outcome.

The other secondary outcome: time taken for mycological clearance, was comparable between the Whitfield ointment and 10% extract concentration with all the participants of these groups achieving total clearance by day 28. The 2% and 5% extract concentrations recorded 57.1% and 71.42% clearance at day 28 respectively. The remaining participants in the 2% and 5% groups had mycological and microscopic clearance on day 56. The results further characterised the therapeutic advantage of the 10% *EAF-2011* over the other herbal extract concentration groups.

Safety evaluation of the product indicated no adverse events after an active surveillance of harms during the follow up periods. Although results of the serum biochemistry was normal, significant differences were noted for some other parameters, the safety of the herbal products at the dosage concentrations can still be confirmed as the changes were within the established reference ranges for all but the mean haemoglobin concentration of the 2% herbal product group. However, this decline in haemoglobin may not be

attributable to treatment as similar changes were absent in groups that received higher concentration of the herbal product.

In monitoring for disease relapse, some individuals in the herbal extract group recorded a recurrence of the condition. This situation was absent for the Whitfield ointment group. Individuals who had the relapse were largely made up of those with family members having similar infections and/or participants who had a previous history of the current infection being present for at least 3 months prior to the study and therefore the situation could have been the result of possible re-infection from other infected relatives.

4.5. Conclusion

The different extract concentrations of the herbal product had comparable activity to the standard treatment of Whitfield ointment. However, the 10% ($^{w}/_{w}$) concentration can be considered as a viable alternative to the conventional treatment of Whitfield ointment as it was shown to be safe and more effective.

CHAPTER 5

RE-EVALUATION OF COMPONENT RAW MATERIALS

5.1. Introduction

It is an obligation that in the formulation and use of herbal medicines, especially when the products are made from two or more plant materials, there is reliance on scientific evidence for these multiple combinations (Parasuraman *et al.*, 2014). Such evidence-based use of herbal medicines has positive implications for both users and society. For society, the sustainable exploitation of these finite resources will ensure the sustenance of the communities which have their livelihood or existence directly linked to the conservation of their flora (Hamilton, 2004). Thus, conservation of medicinal plants has become an issue of international concern as the ever increasing demand for herbal medicines continues to put enormous pressure on the raw materials for herbal medicinal products (HMP) which are mostly sourced from the wild.

The implication for users of such mixed products is seen in at least three ways. First, the risk of adverse reactions and other unwanted side effects may be greatly increased with an increase in the number of plant materials used in a product (Zeng and Jiang, 2010). Therefore a clear demonstration of the benefits of such combinations must be evident for such products, if multiple ingredients are to be used. Secondly, reliance on scientific evidence optimises the therapeutic benefits derived from these products through correct dosing which in turn reduces treatment failures. Finally, the cost of production of these medicines may also be greatly reduced through such data with the economic burden on clients also decreased due to the cost effectiveness of the products.

The component raw materials in the polyherbal product, *EAF-2011* previously listed in section 3.2.1.1 were reviewed to establish the contribution of the individual plants to the therapeutic activity of the product. Plant materials were subjected to an *in vitro* antimicrobial assay against the common dermatophytes and yeast organisms using the microtitre plate dilution method. *Staphylococcus aureus* was also included in the assay because of its role in skin diseases. The Minimum Inhibitory Concentrations (MIC) recorded by each of the plant materials was compared to that of the original formulation and the results of an interactive assay were to inform the selection of the starting material(s) of the new product which should have comparable or better efficacy relative to the original formulation.

5.2. Materials and Methods

5.2.1. Preparation of Plant Materials

The extracts of plant materials used in the formulation of the product prepared according to the method described in section 3.2.1.4 were lyophilised. The total crude extract (TC) used in the formulation of the product was also obtained by combining the lyophilised plant materials according to the recipe listed in section 3.2.1.6.

5.2.2. Preparation of Cultures and Test Organisms

The media preparation and process of culturing of pathogens used in the experiments were performed as detailed in the National Committee for Clinical Laboratory Services (NCCLS) guidelines (2003). The microorganisms chosen for analysis were selected based on their dermatological relevance. Strains of microorganism used were of the American Type Culture Collection (ATCC). Three dermatophytes and one yeast organism with dermatological importance were selected for the assays and included: *Trichophyton rubrum* (ATCC 10218), *Epidermophyton flocossum* (ATCC 9664),

Microsporum canis (ATCC 36299) and the yeast *Candida albicans* (ATCC 10231) were also tested. *Staphylococcus aureus* (ATCC 25923) was selected as the bacterial strain. Bacterial culture was grown in Meuller Hinton Broth (MHB) (Oxoid), for 18-24 hours at 37 °C. Dermatophytes: *Trichophyton rubrum* (ATCC 10218), *Epidermophyton flocossum* (ATCC 9664) and *Microsporum canis* (ATCC 36299) and *Candida albicans* (ATCC 10231) were grown and maintained in Sabouraud's Dextrose Agar (Oxoid) incubated at 25 °C for seven days at 100% relative humidity. Bacterial cultures were streaked on Meuller Hinton Agar (Oxoid), while the dermatophytes were streaked on Sabouraud's Dextrose Agar plates, which were then incubated under suitable conditions to confirm their purity. DMSO and a dilution of the microorganisms alone served as the negative controls

5.2.3. Determination of Minimum Inhibitory Concentration (MIC) of Plant Extracts

A serial micro-dilution assay using the micro-titre plate dilution technique was used to determine the Minimum Inhibitory Concentration (MIC) values for the extracts of the component plant materials and the total crude extract. Using aseptic manipulation, 100µl of Phosphate Buffered Saline (PBS) was placed in each well of a 96 well micro-titre plate. The plant extracts at starting concentrations of 100 mg/ml in 2% Dimethyl Sulfoxide (DMSO) were transferred to the first column of the micro-titre plate. Serial dilutions were performed on each plate, and thereafter the cultures with an approximate innoculum size of 1×10^6 colony forming units/ml (CFU/ml) were introduced. A volume of 100 µl of the culture was added to all the wells. Tests were performed in duplicates. Each plate was subsequently sealed with a sterile adhesive sealing film. All micro-titre plates were incubated under the appropriate conditions. Ketoconazole (Sigma Aldrich, USA) was used as the reference agent for the fungal strains and Ciprofloxacin (Sigma Aldrich, USA) for the bacterial strain.

5.2.4. Detection of Microbial Activity

Testing for bacterial and fungal growth after incubation was done by adding 40 µl (0.04% $^{w}/_{v}$) of *p*-iodonitrotetrazolium chloride (INT) (Sigma Aldrich, USA) to each well. The plates were subsequently incubated again for between 2-4hrs for the bacterial strain and 24-36hrs for the fungal strains. The development of a pink to reddish colour in the well after incubation was recorded as a microbial growth. Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of the plant extract that showed no visible microbial growth.

5.2.5. Selection of Plant Extracts with Significant Antimicrobial Activity

Individual plant extracts with significant antimicrobial activity (MIC < 1.00 mg/ml) against half of the test microorganisms were then selected for the interactive combination study.

5.2.6. Interactive Combination Studies

The potential synergistic, additive, non interactive (indifferent) or antagonistic interaction between the selected plants extracts from section 5.2.5 were investigated using two approaches. First, the component plant extracts at a starting concentration of 100 mg/ml were mixed in ratios of 1:1. The MIC values were determined for each combination to establish the interaction and the sum of the Fractional inhibitory Concentration (∑FIC) was calculated for each combination using the following equation;

FIC (i) = MIC (a) in combination with (b) / MIC (a) independently

FIC (ii) = MIC (b) in combination with (a) / MIC (b) independently

(i) and (ii) in this study represents the different plants in combination. The sum of the FIC, known as the FIC index was thus calculated as ∑FIC = FIC (i) + FIC (ii). This

may be classified as either synergistic (≤ 0.50), additive (0.50-1.00), indifferent (>1.00-4.00) or antagonistic (>4.00) (Van Vuuren and Viljoen, 2008).

The combinations with notable interactions, defined as synergistic or additive activity for more than half of the test organisms, were further investigated at various ratios against the selected pathogens. The MIC assay was conducted on four (4) ratio combinations i.e. 80%: 20%; 60%: 40%; 40%:60% and 20%: 80% for the eventual product. The results were then plotted on an isobologram using Sigma Plot® Software (Version 11.0), allowing for a figurative representation of the interaction. The isobolograms were interpreted by examining the data points of the ratio where the MIC for each concentration is determined in relation to the independent MIC's. Data points falling below or on the 0.50 line on the isobologram were interpreted as synergistic. Points between 0.50 and/or on the 1.00 line were interpreted as additive and points >1.00 - ≤4.00 were defined as either non-interactive or antagonistic for points >4.0 (Van Vuuren and Viljoen, 2011). Positive and negative controls were included in all assays which were also undertaken in duplicate and the mean values noted.

5.3. Results

5.3.1. Minimum Inhibitory Concentrations of Plant Extracts Screened

All of the five (5) plants screened demonstrated some activity against the test fungi and bacteria. The level of antimicrobial activity demonstrated generally varied with the test organisms. MIC's for *Tridax procumbens* were higher than the other plant extracts tested (Table 5.1). The five (5) plant extracts also failed to show any significant activity against *S. aureus.* However, the activity demonstrated by *A. cordifolia* (MIC: 1.563 mg/ml), *Z. zanthoxyloides* (MIC: 1.563 mg/ml) and *E. caryophyllata* (MIC: 1.563

mg/ml) was better than the Total Crude Extract (MIC: 3.125 mg/ml) used in the formulation of the final product.

Table 5.1: Average MIC (mg/ml) for the plant extracts screened using the micro-dilution assay

Plant Extract	*T. rubrum*	*E. flocossum*	*M. canis*	*C. albicans*	*S. aureus*
A. cordifolia	3.125	0.0781*	0.0781*	0.0781*	1.563
T. procumbens	25.00	3.125	3.125	1.563	6.25
Z. zanthoxyloides	6.250	0.0391*	0.0781*	0.0391*	1.563
P. guajava	3.125	0.078*	3.125	1.563	6.25
E. caryophylata	0.0781*	0.0391*	0.0781*	0.0781*	1.563
Total Crude Extact	1.563	3.125	0.0781*	3.125	3.125

* Indicates plant extracts with significant antimicrobial activity

5.3.2. Preliminary Interactive Combination Studies of the Selected Plants

Three (3) plants: *Eugenia caryophyllata, Zanthoxylum zanthoxyloides and Alchornea cordifolia* were selected for the interactive combination studies based on their MIC's reported in Table 5.1. Results for the binary and a triple combination are also reported in Table 5.2 together with their sum of Fractional Inhibitory Concentration (∑FIC) for the binary mixture.

The combination of *E. caryophyllata* and *Z. zanthoxyloides* in a ratio of 1:1 was additive against all the test strains except *S. aureus*. The combination was non-interactive against the latter with ∑FIC of 4.0. *Z. zanthoxyloides* and *A. cordifolia* were antagonistic in effect when tested against *C. albicans* (∑FIC: 59.94) and non-interactive against the four (4) fungi and bacterial strains (Table 5.2). The combination

of *A. cordifolia* and *E. caryophyllata* was synergistic in effect against *S. aureus* and additive against *M. canis, C. albicans and E. flocossum* from the ∑FIC. A non-interactive effect was noted when the combination was tested against *E. flocossum.* A summary of the interactive studies is also illustrated as an isobologram in Figure 5.1.

The activity demonstrated by the MIC of the triple combination of *E. caryophyllata, Z zanthoxyloides* and *A. cordifolia* was also not better than the three (3) binary combinations.

5.3.3. Interactive Combination Studies for Alchornea cordifolia and Eugenia caryophyllata

Results for the interactive study of *Alchornea cordifolia* and *Eugenia caryophyllata* in varying percentages showed the combination of *A. cordifolia* 40% ($^{w}/_{w}$) with *E. caryophyllata* 60% ($^{w}/_{w}$) as most efficacious against all the microbial strains tested (Table 5.3). The combination demonstrated synergistic activity against *S. aureus, C. albicans, M. canis and T. rubrum.* An additive effect was recorded in the test against *E. flocossum* (Figure 5.5). The other combinations were also synergistic or additive in effect when tested against *C. albicans* (Figure 5.3). Other notable combinations are listed: the synergistic effect of the *A. cordifolia* 20% ($^{w}/_{w}$) with *E. caryophyllata* 80% ($^{w}/_{w}$) against *T. rubrum,* Additive effect of *A. cordifolia* 20% ($^{w}/_{w}$) with *E. caryophyllata* 80% ($^{w}/_{w}$) and the 60% ($^{w}/_{w}$) *A. cordifolia* with 40% ($^{w}/_{w}$) *E. caryophyllata* against *E. flocussum* and the additive activity of *A. cordifolia* 60% ($^{w}/_{w}$) with *E. caryophyllata* 40% ($^{w}/_{w}$) against *M. canis.*

Table 5.2: MIC (mg/ml) [∑FIC] for binary combinations of *A. cordifolia, Z. zanthoxyloides* and *E. caryophyllata* at a ratio of 1:1

Plant Extract	*T. rubrum*	*E. flocossum*	*M. canis*	*C. albicans*	*S. aureus*
E. caryophyllata & *Z. zanthoxyloides*	0.0391 [0.506]	0.0391 [1.00]	0.0781 [1.00]	0.0195 [0.748]	3.125 [4.0]
Z. zanthoxyloides & *A. cordifolia*	6.250 [3.00]	0.0391 [1.50]	0.0781 [2.00]	1.563 [59.94]	1.563 [2.0]
A. cordifolia & *E. caryophyllata*	0.0391 [0.511]	0.0195 [0.748]	0.0195 [0.499]	0.0195 [0.499]	0.0781 [0.075]
A.cordifolia + *E. caryophyllata* + *Z. zanthoxyloides*	1.563	1.563	3.125	1.563	0.0781

Table 5.3: ∑FIC for the percentage combinations of *Alchornea cordifolia* (AC) and *Eugenia caryophyllata* (EC) against the test fungi and bacterium

A. cordifolia + *E. caryophyllata*	*T. rubrum*	*E. flocossum*	*M. canis*	*C. albicans*	*S. aureus*
80% A.C + 20% E.C	1.013	1.20	0.999	0.498	2.00
60% A.C + 40% E.C	1.013	0.748	0.748	0.498	0.10
40% A.C + 60% E.C	0.256	0.748	0.251	0.251	0.025
20% A.C + 80% E.C	0.128	0.748	0.999	0.498	0.049

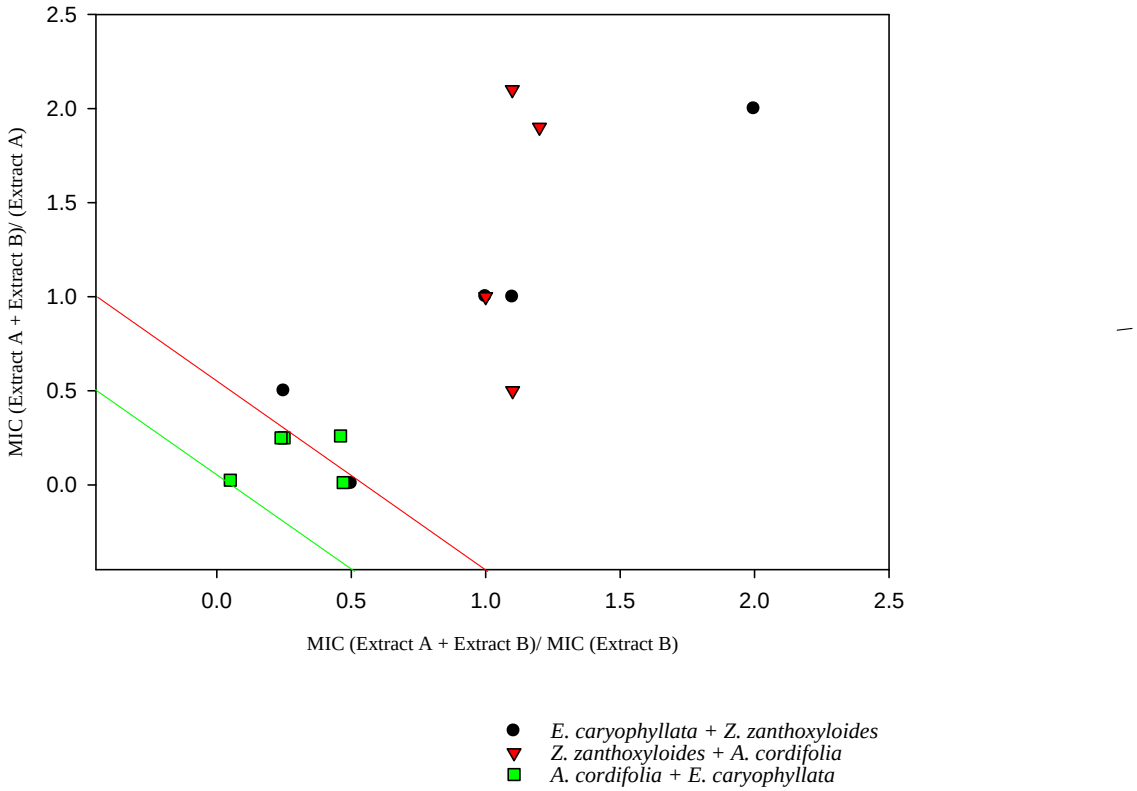


Figure 5.1: Isobolographic representation of the 1:1 combinations of *A. cordifolia + E. caryophyllata; E. caryophyllata + Z. zanthoxyloides* and *Z. zanthoxyloides + A. cordifolia* against the five test strains. Data points falling below or on the 0.50 (Green Line) were interpreted as synergistic. Points between 0.50 and/or on 1.00 (Red Line) were interpreted as additive and points >1.00 were defined as non-interactive.

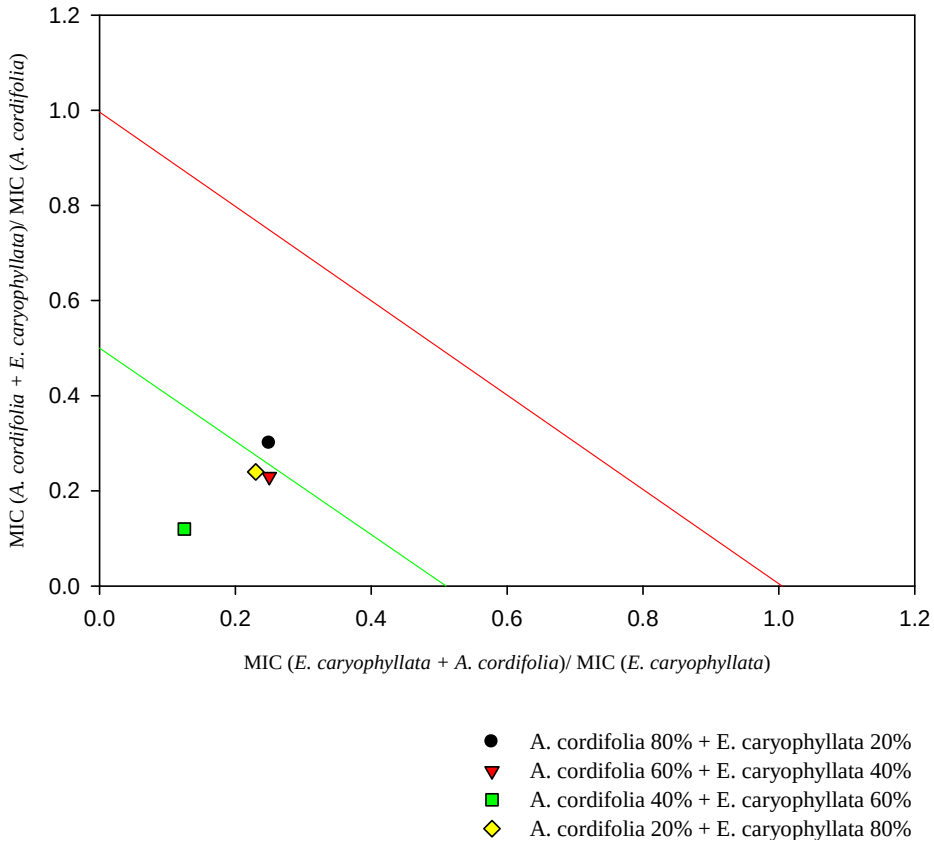


Figure 5.2: Isobologram of *Alchornea cordifolia* and *Eugenia caryophyllata* in varying combinations against *Staphylococcus aureus*. Data points falling below or on the 0.50 (Green Line) were interpreted as synergistic. Points between 0.50 and/or on 1.00 (Red Line) were interpreted as additive and points >1.00 were defined as non-interactive.

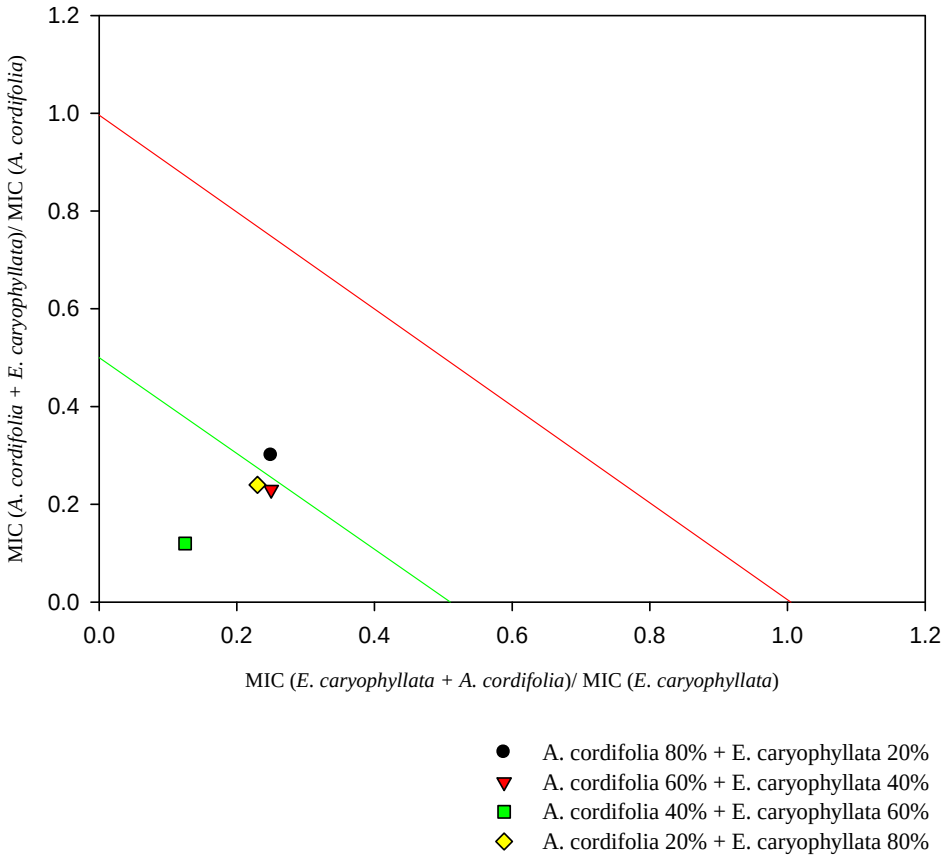


Figure 5.3: Isobologram of *Alchornea cordifolia* and *Eugenia caryophyllata* in varying combinations against *Candida albicans*. Data points falling below or on the 0.50 (Green Line) were interpreted as synergistic. Points between 0.50 and/or on 1.00 (Red Line) were interpreted as additive and points >1.00 were defined as non-interactive.

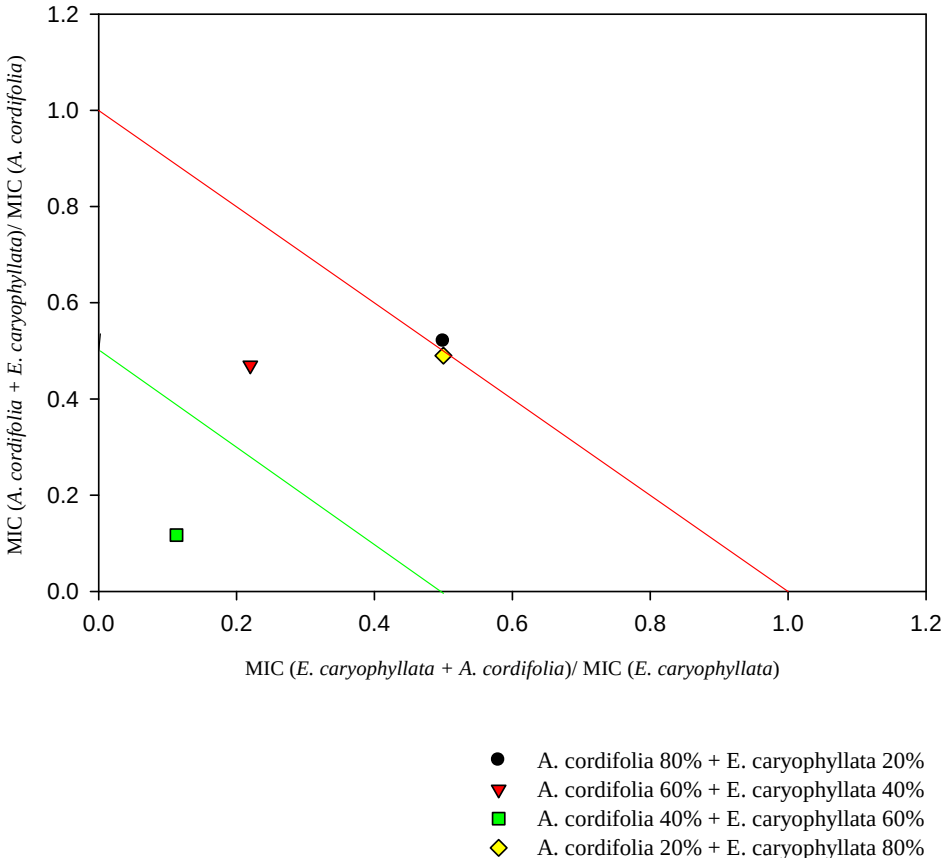


Figure 5.4: Isobologram of *Alchornea cordifolia* and *Eugenia caryophyllata* in varying combinations against *Microsporum canis*. Data points falling below or on the 0.50 (Green Line) were interpreted as synergistic. Points between 0.50 and/or on 1.00 (Red Line) were interpreted as additive and points >1.00 were defined as non-interactive.

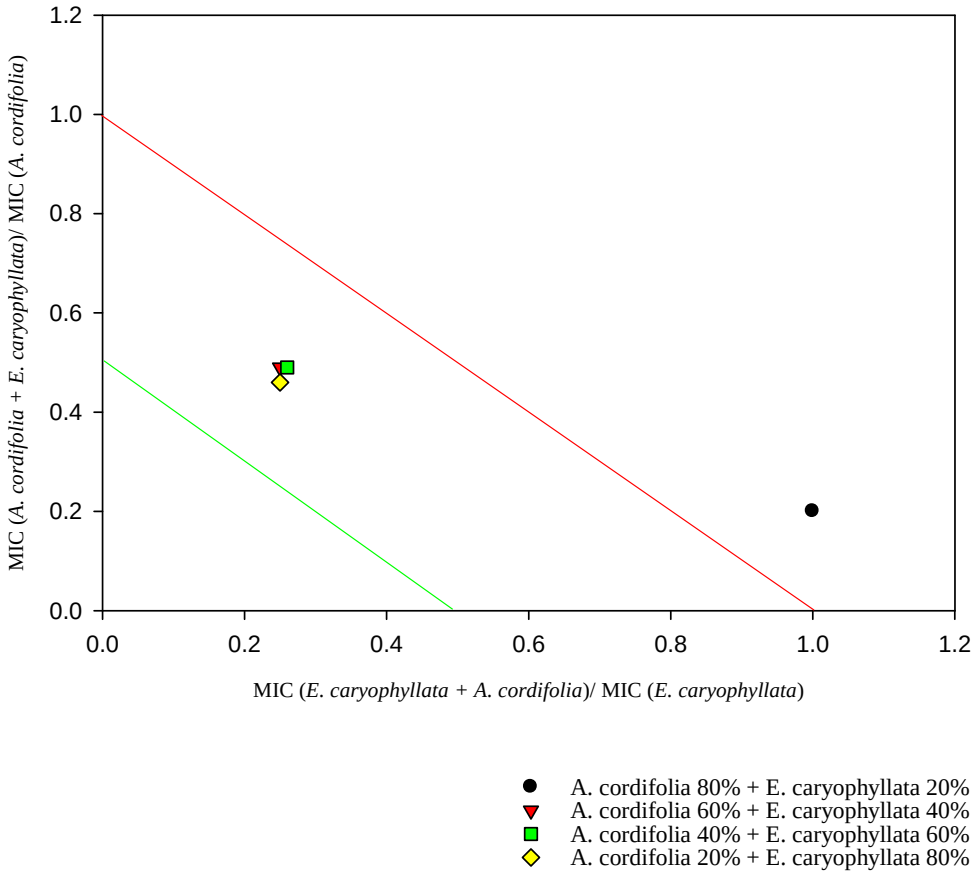


Figure 5.5: Isobologram of *Alchornea cordifolia* and *Eugenia caryophyllata* in varying combinations against *Epidermophyton flocossum*. Data points falling below or on the 0.50 (Green Line) were interpreted as synergistic. Points between 0.50 and/or on 1.00 (Red Line) were interpreted as additive and points >1.00 were defined as non-interactive.

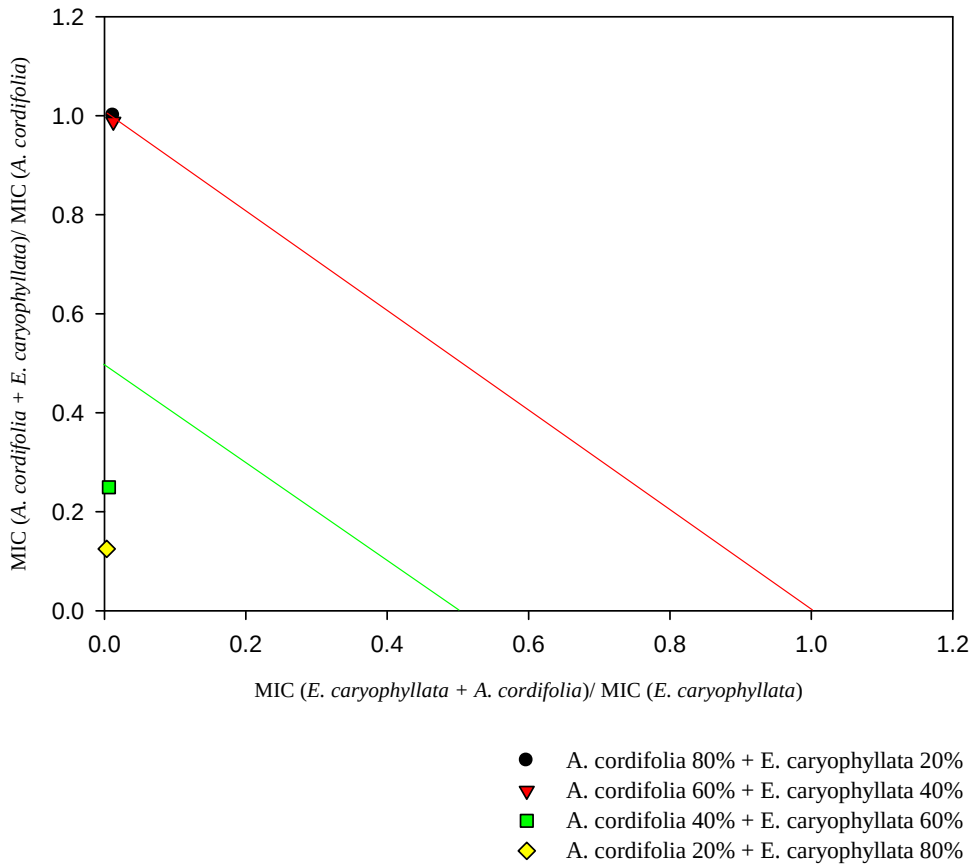


Figure 5.6: Isobologram of *Alchornea cordifolia* and *Eugenia caryophyllata* in varying combinations against *Trichophyton rubrum.* Data points falling below or on the 0.50 (Green Line) were interpreted as synergistic. Points between 0.50 and/or on 1.00 (Red Line) were interpreted as additive and points >1.00 were defined as non-interactive.

5.4. Discussion

The re-evaluation of the component plant materials used in the production of the polyherbal product did indicate the relevance of each starting material. Generally, positive activity was noted for each of the plant materials and the total crude extract tested (Table 5.1). *Eugenia caryophyllata* proved most efficacious with significant activity against all the fungal strains tested. *Zanthoxylum zanthoxyloides* and *Alchornea cordifolia* also showed significant activity.

Although there have been previous reports indicating the antifungal activity of *Psidium guajava* and *Tridax procumbens* against the test strains (Pandey and Shweta, 2011; Manjamalai *et al.*, 2012), in this report, the activity shown cannot be described as significant. The *in vitro* activity of the starting materials when compared with that of the total crude extract indicated a possible advantage of using a single plant formulation. However, the possibility of obtaining a synergistic action when medicinal plants are used in combination informed the interactive combination study (Williamson *et al.*, 1996; Patwardhan and Mashelkar, 2009).

Minimum inhibitory concentrations reported in Table 5.2 also showed the binary combinations as generally having better activity than that of the triple combination of *Eugenia caryophyllata, Zanthoxylum zanthoxyloides* and *Alchornea cordifolia.* Significantly, the combination of *Eugenia caryophyllata* and *Alchornea cordifolia* indicated synergistic and additive activity against all the microorganisms. The combination was therefore considered a better option than the mixtures of *Zanthoxylum zanthoxyloides* with *Alchornea cordifolia* and *Eugenia caryophyllata* in combination with *Zanthoxylum zanthoxyloides.*

This positive interaction was confirmed using the isobologram with data points plotted indicating that an optimum biological effect can be attained by the combination of the *Eugenia caryophyllata* and *Alchornea cordifolia* at the ratio of 60% ($^{w}/_{w}$) and 40% ($^{w}/_{w}$) respectively (Table 5.3). This combination was synergistic against *Staphylococcus aureus* (Figure 5.2), *Candida albicans* (Figure 5.3), *Microsporum canis* (Figure 5.4) and *Trichophyton rubrum* (Figure 5.6) and could be recommended as the suitable formulation despite showing only additive effect against *Epidermophyton flocossum*.

5.5. Conclusion

The results of the interactive study indicated that treatment effect may be better when the combination of *Eugenia caryophyllata* 60% ($^{w}/_{w}$) and *Alchornea cordifolia* 40% ($^{w}/_{w}$) is used as the recipe for the formulation of the product. The relevance of scientific evaluation of component materials in polyherbal formulations has also been highlighted by the study.

CHAPTER 6

EVALUATION OF THE REFORMULATED HERBAL PRODUCT (*RF-2013*)

6.1. Introduction

In the previous chapter the component materials in the product were re-evaluated, the interactive combination study indicated the mixture of *Eugenia caryophyllata* 60% ($^{w}/_{w}$) and *Alchornea cordifolia* 40% ($^{w}/_{w}$) as being most efficacious.

However, the clinical application of the data obtained from the *in vitro* study is limited in the absence of evidence from a well run clinical study. Again the availability of clinical data from the randomised double-blind controlled study conducted for the original formulation, which also indicated the 10% ($^{w}/_{w}$) *EAF-2011* as having better effect than the standard treatment of Whitfield ointment also means that the efficacy of the reformulated product needs to be evaluated in a clinical study to provide a basis for use as an alternative treatment. It was therefore imperative to subject the reformulated product (*RF-2013)* to a randomised controlled trial to allow for a fair comparison of its effectiveness with the 10% ($^{w}/_{w}$) *EAF-2011*.

Prior to the start of this clinical study the newly formulated ointment was subjected to a skin sensitisation and a chronic toxicity study.

6.2. Methods

6.2.1. Reformulation and Standardisation of the Herbal Product

The reformulated herbal product comprising *Eugenia caryophyllata* 60% ($^{w}/_{w}$) and *Alchornea cordifolia* 40% ($^{w}/_{w}$) was incorporated into an emulsifying ointment base (B.P) according to the method described in section 3.2.1.6-7. The ointment was formulated to achieve a concentration of 5% ($^{w}/_{w}$) for the final product. Products were also standardised using the methods in sections 3.3.1.2 and 3.3.3.1

6.2.2. Skin Sensitivity and Chronic Toxicity Study

Skin sensitivity and chronic toxicity testing was carried out according to the methods described in section 3.5 the duration of the study was for 6 months.

6.2.3. Clinical Study

6.2.3.1. Ethical Approval and Conduct of Trial

Approval was obtained from the Ethics Committee for Human Research of the Centre for Plant Medicine Research, Mampong - Akuapem (see Appendix I). The trial was performed according to guidelines stipulated by the Helsinki declaration for the conduct of Medical Research (WHO, 2001).

6.2.3.2. Trial Design and Randomisation

A single blind randomised controlled trial was carried out for the study at a randomisation ratio of 2:1 for the 5% ($^{w}/_{w}$) *RF-2013* and 10% ($^{w}/_{w}$) *EAF-2011* respectively. Randomisation was achieved by making participants pick, without replacing, a folded paper with the names of the test products in a box. Allocation was done to attain this ratio at the end of a 15^{th} recruitment.

6.2.3.3. Study Sites and Treatment Received

The study was undertaken at the clinic of the CPMR. Participants received either the 5% ($^{w}/_{w}$) *RF-2013* or the control treatment of 10% ($^{w}/_{w}$) *EAF-2011*.

6.2.3.4. Criteria for Participant Selection

Selection and exemption criteria for the study used are described in section 4.2.6.1-3.

6.2.3.5. Sample Size

Study was designed to have 30 participants in the reformulated product and 15 participants in the control treatment of 10% ($^{w}/_{w}$) *EAF-2011* based on an assignment ratio of 2:1 respectively. The risk of making a type II error (statistical power) was set at 0.20 and a difference in treatment (Total signs and symptom score) of 2.00 considered clinically relevant. Type I error (α level) was 0.01 and the population standard deviation assumed to be 2.30. Sample size was calculated using the formula in section 4.2.6.

6.2.3.6. Informed Consent Forms

Participants were asked to complete an informed consent form but children considered too young to complete the form were requested to report with a parent or guardian. The details of the trial were always explained in the local dialect or any understood language by the investigator before forms were given out to be signed or thumb printed.

6.2.3.7. Schedule of Evaluation

Participants on recruitment into the trial (Day 0); were followed up for clinical assessment and observation twice during the first month (Day 14, 28); twice during second month (Day 42, 56) and once during the last month (Day 84).

6.2.3.8. Treatment Dosage

The participants were advised to apply daily their respective ointments to the affected parts of the body i.e. morning and evening.

6.2.3.9. Assessment of Effectiveness and Classification of Therapeutic Response

Each participant was graded using a clinical score as shown in Appendix II. This was repeated on subsequent visits. Primary assessment of effectiveness was based on a clinical score.

The clinical score used the Total Sign and Symptoms Score (TSSS) with modifications (Friedlander *et al.*, 2002). This is a rating using a four point scale where; *0- absent*; *1-mild*; *2-moderate*; *3-severe* for each of the selected signs and symptoms that are characteristic for the condition.

6.2.3.10. Definition of Clinical Effectiveness of the Product and Primary Outcome

Effectiveness or Complete cure was defined as clinical cure (Total Clearance) or a TSSS of 0 for all the population randomised.

6.2.3.11. Adverse Drug Effects

On each visit for monitoring and review, adverse effects to the product were recorded. This included a review of all the systems to detect any such reactions. The adverse reporting sheet is attached as Appendix VI.

6.2.3.12. Statistical Analysis

The hypothesis of interest for the primary efficacy outcome was that the reformulated herbal product *(RF-2013)* should have comparable activity to the 10% *EAF-2011*. An independent *t*-test was also used to compare the difference between the two treatment groups. An α-level of 1% was set for the detection of statistical significance. All analyses were done using the intention to treat (ITT) population.

6.3. Results

6.3.1. Standardisation of the Product

Basic phytochemical screening of the product indicated the presence of sterols and triterpenes, flavonoids and phenolic compounds. The product after an HPLC assay was found to contain rutin: 2.280% ($^{w}/_{w}$), quercetin: 0.422% ($^{w}/_{w}$) and kaempferol: 0.078% ($^{w}/_{w}$).

6.3.2. Skin Sensitivity and Chronic Toxicity Testing of the Product

The skin sensitivity test using Sprague- Dawley rats did not indicate dermal irritation in the form of ulcerations during the 6 months of treatment. Subcutaneous papules that were formed after intradermal injection of the ointment dissolved in glycerol resolved without any ulcerations after 72hrs of observation.

The reformulated product did not also have any adverse effect on the kidney, liver and the haematological profile of treated rats. No significant differences were observed between the non treated control and the animals that received the herbal product (Table 6.1-3). This observation was confirmed from the histological assessment which did not indicate any change in the tissue structure of the kidney, liver, spleen and the skin (Table 6.4; Appendix XXI, Figure 7.23)

Table 6.1: Post treatment effect of the ointment on haematological parameters of rats

Haematological Parameters	TREATMENT	
	Control	Reformulated Product
NEU(%)	14.56 ±0.43	14.03 ± 0.19
LYM(%)	89.77 ±0.90	86.71 ± 12.02
MON(%)	4.12 ± 0.11	4.83 ± 0.95
EOS(%)	0.29 ± 0.10	0.20 ± 0.50
BAS(%)	0.31 ± 0.03	0.27 ± 0.07
RBC(10^6/mm^3)	7.33 ± 0.34	6.96 ± 2.08
HB(g/dl)	17.01 ± 0.39	16.71 ± 1.09
HCT(%)	39.70 ± 0.51	41.90 ± 12.40
MCV(μm^3)	55.80 ± 2.70	53.75 ± 1.03
MCH(pg)	17.56 ±1.93	16.40 ± 10.13
MCHC(g/dl)	28.10 ±0.19	30.78 ± 4.97
RDW(%)	13.63 ± 3.67	14.65 ± 4.50
PLT(10^3/mm^3)	530.4 ± 105.1	652.00 ± 35.3
MPV(μm^3)	6.11 ± 2.02	6.37 ± 1.15
WBC(10^3/mm^3)	10.0 ± 1.86	12.43 ± 0.10

Results are Mean ± S.E.M; n=5

Key: HB - Haemoglobin, HCT - Haematocrit, BAS - Basophils, LYM - Lymphocytes,MCHC - Mean Corpsular Haemoglobin Concentration, MCH - Mean Corpsular Haemoglobin, MCV - Mean Corpsular Volume, MPV - Mean Platelet Volume, MON – Monocytes, NEU - Neutrophils, PLT - Platelets, RBC - Red Blood Cells, RDW - Red Blood Cell Distribution Width, WBC - White Blood Cells.

Table 6.2: Post treatment effect of the ointment on the liver and kidneys of the rats.

	TREATMENTS	
	Control	Reformulated Product
Kidney Function		
UREA (mmol/l)	1.09 ± 0.71	2.39 ± 0.40
CREAT (mmol/l)	46.13 ± 2.67	42.67 ± 5.62
Liver Function		
ALBUMIN (g/L)	34.35 ± 3.98	34.57 ± 5.43
ALT (u/L)	98.15±0.80	97.55 ±6.90
AST (u/L)	141.65 ±7.01	126.11 ± 32.07
GGT (u/L)	2.50 ± 0.41	1.55 ± 0.21
ALP (u/L)	3.51 ± 1.70	2.56 ± 0.15

Results are Mean ± S.E.M; n= 5

Key: ALT – Alanine Transaminase, AST – Aspartate Transaminase, Creat – Creatinine, GGT – Gamma Glutamyl Transferase, ALP – Alkaline Phosphatase

Table 6.3: Post treatment effect of the ointment on urine parameters of rats

Urine Parameter	Treatment	
	Control	Reformulated Product
Urobilinogen	-	-
Glucose	-	-
Ketones	-	-
Specific Gravity	1.022	1.022
Blood	-	-
pH	7.0	7.2
Proteins	++	+
Nitrites	-	-

Key: (-): absent; (+): present in moderate quantities; (++) present in large quantities

Table 6.4: Post treatment effect of *EAF-2011* on the organ weights (weight to body ratio) of rats

Organ	**Control**	**Reformulated Product**
Kidney	1.94 ± 0.26	2.20 ± 0.14
	(0.0097)	(0.0085)
Spleen	0.62± 0.04	0.51 ± 0.01
	(0.0028)	(0.0026)
Liver	13.23 ± 4.06	11.67 ± 0.40
	(0.0553)	(0.0561)

Results represent the Mean ± SEM; n=5

6.3.3. Clinical Study

6.3.3.1. Patient Demographics and Disease Characteristics

A total of fifteen (15) participants were involved in the study. The mean age of participants was 14.40 (± 3.96) for participants randomised to the control treatment of 10% *EAF-2011* and 11.50 (± 4.31) for participants in the reformulated product group (Table .6.5).

Table 6.5: Demographical data of participants involved in the study

	Control (10% *EAF-2011*)	Reformulated Product (5% *RF-2013)*
Age (SD)	14.40 (3.96)	11.50 (4.31)
Sex		
Males (%)	4 (80)	8 (80)
Females (%)	1 (20)	2 (20)

Dermatophytic infection noted in the participants was *Tinea capitis, Tinea coporis* and *Pityriasis vesicolor*. Most participants also reported with infections that had been present for between three (3) months to one year. Four (4) participants in the reformulated product group reported they had relations with similar infections (Table 6.6).

Table 6.6: Disease characteristics for participants in the treatment groups

Type of Infection	Control (10% *EAF-2011*)	Reformulated Product (5% *RF-2013)*
Tinea capitis (%)	2 (40)	4 (40)
Tinea coporis (%)	1 (20)	1 (10)
Pityriasis vesicolor (%)	2 (40)	5 (50)
Participants with previous infections (%)	3 (60)	7 (70)
Relations with similar infections (%)	0 (0)	4 (40)

Table 6.7: Mean change in TSSS (SD) for the 10% *EAF-2011* and the 5% *RF-2013*

Time	10% *EAF-2011*	5% *RF-2013*	*p-value*	Confidence Interval
Day 14	- 4.40 (1.75)	- 2.50 (0.88)	0.318	(-1.970 to 1.910)
Day 28	- 2.20 (1.22)	- 1.70 (1.75)	0.196	(-4.550 to -0.680)
Day 56	-2.40 (0.55)	- 1.00 (2.10)	0.013	(-0.123 to 5.723)
Day 84	-	- 1.40 (2.48)	-	-

6.3.3.2. Treatment Efficacy

Baseline TSSS between the 2 groups were comparable: *EAF-2011* group had a TSSS of 9.6 (± 2.3) with the reformulated product recording a mean TSSS of 8.4 (±2.55). The control treatment demonstrated better activity than the reformulated product. The group recorded a 100% cure rate by day 56 compared to a 10% cure rate for the reformulated

product. The percentage cure for the latter group increased to 60.0% on day 84 using the intention to treat (ITT) population and 75.0% without the ITT population. The difference between the two treatments is shown in Table 6.7 and Figure 6.1. The study also had two (2) participants from the reformulated product dropping out due to loss on follow up.

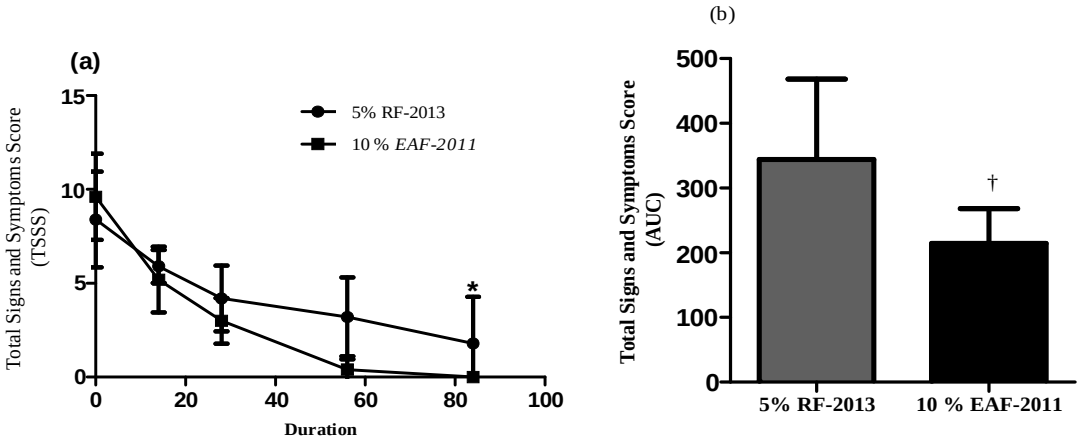


Figure 6.1: Effect of the Reformulated product (*RF-2013*) on the TSSS of participants with time (a), calculated as the area under the curve (AUC) (b). Data is presented as mean (SD) compared with the control (an independent *t*-test at an α-level of 1% showed a significant difference between the treatments; *p*-0.013, CI: -0.123 to 5.723)

6.3.3.3. Safety Analysis

The safety analysis of the reformulated herbal product and the control treatment involved an active surveillance of harms. The surveillance employed the WHO checklist for adverse drug effects. Participants receiving both herbal treatments did not report any adverse effects during the study period of three (3) months.

6.4. Discussion

The need to provide evidence for clinical use of the reformulated herbal product informed the clinical study reported. Baseline demographics for both groups in the study are comparable to the populations that are usually known for these infections (Wu *et al.*, 2000). Again, the most prevalent infection was *Tinea capitis* as in the previous study carried out for the original formulation. The study however failed to recruit the predetermined number of participants due to the unavailability of participants with the relevant infection, the lack of time and financial capacity.

Participants in the 5% *RF-2013* group recorded a decline in their TSSS over the entire study period of three (3) months. When compared to the control treatment of 10% *EAF-2011*, the reformulated product was lower in the achievement of the primary outcome of complete cure. The control group recorded a cure rate of 60% and 100% by day 56 and 84 respectively. The reformulated product *(RF-2013)* however had a 60.0% cure rate by the end of the study. Difference in treatment reported in Table 6.3 indicates a lower TSSS (CI: -0.123 to 5.723) for the 10% *EAF-2011* compared to the TSSS observed for participants using the reformulated product by day 56 of treatment. This difference was statistically significant with *p*-value of 0.013.

The results mean that the reformulated product was lower in efficacy than the control treatment. However with the cure rate of 60.0% obtained for the reformulated product, the product may still have some potential worth exploring. Indeed challenges such as sample size and the loss of participants during follow up could all have affected the weight of the effect recorded in this study. The inferiority of the clinical effect of the reformulation compared to original product due to lower content of constituents is also another possibility.

Quality evaluation of the product also indicated the presence of secondary metabolites well known for their antifungal activity. The product also had the presence of rutin, quercetin and kaempferol from the HPLC assay.

6.5. Conclusion

The original formulation of the product from this study would still be the treatment of choice when the number of participants and the time taken to attain the primary outcome is considered.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1. General Discussion

Herbal medicines have been praised for their potential in meeting the health needs of developing countries. However, this form of medical practice is challenged with numerous issues that have been limiting the general acceptability of their products into the conventional system of healthcare. At the core of these challenges is the lack of documented evidence. This absence of evidence is applicable to all aspects of the field from clinical use through to product quality (Ernst, 2002; Martin and Ernst, 2004). In this report on a Ghanaian polyherbal skin product (*EAF-2011*), steps were taken to address some of these pertinent issues. First, there was a development of chemical standards for the product; the results of which could serve as a potential monograph for the product, a clinical evaluation, followed by an *in vitro* re-evaluation of the component raw materials used in the product for their relevance and finally a clinical study of a proposed formulation after the *in vitro* re-evaluation.

The development of standards started with the authentication of the raw materials used in the product. Voucher specimen were retained for comparison with materials that would be collected in the future. This authentication and retention of specimen is very vital in the manufacturing process as misidentification and adulteration of plant materials have a direct effect on product quality with reports available for situations where such errors have been linked to serious adverse events for users (Ang-Lee *et al.*, 2001).

The other arm of the standards development involved the chemical fingerprinting of the raw materials and the finished product. This evaluation included the use of a basic phytochemical screening, thin layer and high performance liquid chromatography. The basic phytochemical screening indicated the presence of characteristic secondary metabolites in the plant extracts and the product (See Table 3.3). The presence of these markers serves as another means of ensuring the consistency of subsequent products. However, the sole reliance on such qualitative assays for standardisation of products comes with some limitations such as an uncertainty about the specificity and quantity of medically active constituents in the raw materials and its product (EMEA, 2008; Songlin *et al.*, 2008). Despite these limitations, the basic phytochemical screening continues to be relevant especially when used with other methods of standardisation. This relevance was shown in the thin layer chromatograms illustrated in Figure 3.1. The colours obtained for the fingerprints indicated the presence of phenolic compounds or their derivatives in the samples tested; metabolites also detected from the phytochemical screening.

The properties of the markers obtained from the thin layer chromatography such as clear detection and well defined separation from the other spots obtained also made them ideal candidates for use as analytical markers. *Tridax procumbens* had one distinct spot (Figure 3.1; B3) and *Psidium guajava* three spots (Figure 3.1; A1, B1 and C1). Profiles for *Alchornea cordifolia*, *Zanthoxylum zanthoxyloides* and *Eugenia caryophyllata* had the markers (Figure 3.1; A2 and B2) shown to be identical in shape, colour and R_f value. The final product also indicated the presence of all the marker spots found in the starting materials.

This combination of the phytochemical screening and thin layer chromatography in the standardisation process provides a better guarantee about the quality of the product (Goldman, 2001; Mitra and Kannan, 2007). The methods are also still very relevant for lower middle income countries like Ghana where logistics for quantitative assays are either very limited or costly to operate and in most cases completely absent.

In the quantitative assessment using the HPLC, three (3) flavonoid compounds i.e. rutin, quercetin and kaempferol, were assayed. These compounds were selected as potential markers due to documented evidence about their antimicrobial activity and also the availability of some previous reports indicating their presence in the plants (Basile *et al.*, 2000; Santas *et al.*, 2010).

The HPLC method used was validated in conformity with the recommendations of the ICH to ensure reproducibility of the results (ICH, 1997). The method produced correlation coefficients that met the approved standard of 0.998 making the regression equations reported in Table 3.4 applicable for the quantitative assay of the flavonoid contents. Retention times were ideal with the maximum time of ≈ 6.6 mins obtained for kaempferol. The short retention time means that the results can be obtained quickly and will be economically beneficial as the cost of running samples will be minimal. The chromatographic fingerprints (Appendix XI to XVI) provide another qualitative means of ensuring product and raw material consistency in addition to the phytochemical screening and thin layer chromatography. The standards reported: the basic phytochemical, thin layer chromatography and the high performance liquid chromatography provide sufficient data which when applied during the manufacturing process can ensure final products are of the right quality.

Apart from the chemical constituents, the quality of medicinal agents is also directly linked with their stability hence the demonstration of pharmaceutical stability by these products is critical (Thakur *et al.*, 2011). Organoleptic features recorded at the baseline remained unchanged during this period (Table 3.9). The pH values increased from an initial 5.42 to 5.71 but this was not beyond the recommended limit (± 10%) of the declared or the initial value and also within the limits allowed for a topical preparation and may therefore be considered insignificant (Kruse and Sultan, 2009). The thin layer fingerprint also showed a change in the colour of the spot labelled as "A" (Figure 3.2); the result of a possible change in chemical constituents. A similar change in chemical composition was seen during the HPLC analysis with an inability to detect the presence of quercetin during the 6^{th} and 12^{th} month of assay (Table 3.10).

The chemical changes did not however affect the biological activity of the product as the antimicrobial assay (Table 3.11) using the zones of inhibition produced over the one year period as a measure of stability, revealed some consistency in the product. The stability of the ointment (*EAF-2011*) was very important as changes in quality has implications on the clinical safety and effectiveness of the product (Thakur *et al.*, 2011)

Overall, the features exhibited by the markers on the chromatograms; resolution and stability are recommended when analytical markers are used in the standardisation of herbal medicines (EMEA, 2008; Duron *et al.*, 2009). The bioassay can also be employed as a standard in addition to the other methods of standardisation elaborated to address one of the challenges with herbal medicines; the possible variations in active constituents. This method is not new in natural product research and has been employed during the development of standards for botanicals (Barnes, 2003; Valerio and Gonzales, 2005). It is thus certain that in the standardisation of natural products,

the use of a single method may prove limiting but multiple methods will assure the validity of quality and consistency of products.

The final part of the quality assessment involved the skin sensitivity and chronic toxicity testing. Although the product had a history of use in humans, it is difficult identifying drug related toxicity that develops late after exposure to a treatment as well as reactions that lack an acute clinical presentation (Debbiea *et al.*, 2012). The ointment was shown to be safe at the concentrations tested after the chronic toxicity and skin sensitivity evaluation which is required for obtaining an approval for human testing.

In the clinical study, evidence gathered showed *EAF-2011* to be effective and safe for the management of dermatophytosis. The method used in the study; the prospective, randomised, double-blind, parallel controlled approach, is now widely employed in clinical studies as it eliminates bias that is usually encountered during trials (WHO, 2000; Sathian *et al.*, 2009). Key to this approach is the use of a well established comparator treatment in this case Whitfield ointment a known conventional antifungal agent; blinding of both the investigators and participants to treatment received as well as the randomisation of participants to the treatment groups making the evidence gathered for the product very reliable. A notable limitation however was the inability to recruit the required number of 30 participants per each group of the herbal product.

The three (3) concentrations tested were all efficacious when compared to Whitfield ointment. Whitfield ointment is one of the standard treatments for superficial fungal skin infections (GNDP, 2010) and was preferred because the packaging would allow sufficient blinding of both the investigator and the participants. The herbal products were also prepared and stored in a similar package. The 10% ($^{w}/_{w}$) herbal product

proved to be a better treatment and was more effective than the standard therapy. This concentration of the product had 91.30% of participants achieving the primary outcome of complete cure compared to the 30% for the Whitfield ointment group. The possible therapeutic benefit of each herbal product compared to the control calculated as the confidence interval for each group again showed the 10% herbal extract concentration to be the most effective with this group recording a confidence interval of -4.71 to -0.08 and a *p-value* of 0.008 (Table 4.6). The confidence interval represented the range of decline in TSSS for a participant receiving the 10% *EAF-2011* compared to another participant receiving the control treatment of Whitfield ointment**.** The results when extrapolated clinically means that a participant receiving the 10% herbal extract concentration, will at the end of the study be expected to experience a decrease in TSSS between 4.71 and 0.08 more than another participant on the control treatment would record.

The secondary outcomes in this trial was based on the premise that in true clinical practice it is possible that some individuals might not meet the primary end point but there is also the need to grade the relative benefit such patients may derive from the treatments administered. Such benefits would also influence the selection of the best extract concentration of the product or the possible application of the product in other specific complaints such as excoriation of the skin and unexplained itching.

Significantly, none of the participants reported any adverse effects; parameters of safety using the blood and urine indices were also normal during the study. The result also confirms the finding of the skin sensitisation and chronic toxicity test. Again, the use of the active surveillance of harms makes the data obtained reliable contrary to the passive

method which depends on participants reporting an unexpected event they experience (Ioannidis *et al.*, 2004).

Although therapeutic response obtained for the herbal treatments and specifically the 10% ($^{w}/_{w}$) *EAF-2011* group implies the treatment can be considered as an alternative to the conventional treatment of Whitfield ointment, a potential concern that may hinder the widespread acceptability and use of the product will be with the number of plant materials used in the formulation. This situation may arise since conservation of plant materials has become an issue of importance and hence the need to demonstrate the relevance of each plant material in the bioactivity of traditional polyherbal formulations.

The five (5) plants used in the current formulation were in this regard re-evaluated to establish their contribution to the overall efficacy of the product. Comparison was done using the basic combination method where synergy was expected if the MIC of the combination was lower than the plant extract singularly and antagonism if the reverse occurred. The MIC's of the individual plants were generally lower than the original combination (Table 5.1). Selection of the plants for the interactive combination studies was based on a highly significant antimicrobial activity against 50% of the microorganism tested. Significant activity was defined as an MIC of <1mg/ml against the test organism (Rios and Recio, 2005). *Psidium guajava* and *Tridax procumbens* which have been documented for their antifungal activity did not show any significant activity in this study. Possible explanations for this result could be the differences in the methods used and the variations in medicinally active constituents due to solvents extracts tested or the geographical source of plant materials used. The extracts used in some of these previous reports were methanol and acetone and the assay methods

generally involved the disc diffusion (Nair and Chanda, 2007; Dhiman *et al.*, 2011; Manjamala *et al.*, 2012). A limited number of these reports also involved the dermatophytes hence conclusions about the inefficacy of these two plants can be limited to the solvents and the assay methods used in this study: 70% ($^{v}/_{v}$) hydroalcoholic extract and the microtitre dilution respectively. While the hydro-alcoholic extract used in this study allows the use of eventual products in humans, the microtitre dilution method ensures results can be verified and experiments replicated hence they are preferred for antimicrobial assays (Klancnik *et al.*, 2010).

Alchornea cordifolia, *Zanthoxylum zanthoxyloides* and *Eugenia caryophyllata* which satisfied the set criteria after the basic combination study were also evaluated as binary and a triple combination (Table 5.2). The triple combination product proved to be less efficacious against the test strains compared to the binary combinations. An antagonistic effect was also observed when the combination of *Alchornea cordifolia* and *Zanthoxylum zanthoxyloides* was tested against *Candida albicans*. Contrary to traditional perception that combination of several plants will always produce better therapeutic effects, most of the combinations were merely additive and non-interactive. Based on the results of the fractional inhibitory concentration (FIC) and the isobologram shown in Table 5.2 and Figure 5.1 the combination of *Alchornea cordifolia* and *Eugenia caryophyllata* was proposed for the new product.

The therapeutic activity of *Alchornea cordifolia* and *Eugenia caryophyllata* was maximised by performing different ratio combinations of the two plants. The mixture of *Alchornea cordifolia* 40% ($^{w}/_{w}$) and *Eugenia caryophyllata* 60% ($^{w}/_{w}$) produced synergistic activity against all of the test organisms except for *E. flocossum*. Inferences that can be drawn from the ratio combinations include the fact that at certain ratios the

level of antimicrobial activity for particular plants can be enhanced to improve therapeutic response against certain strains of microorganisms. The situation provides an avenue for addressing the current burden of antimicrobial drug resistance. Such combinations will not be unique to herbal medicines as they are being widely used in the orthodox pharmaceuticals (Che *et al.*, 2013).

The mixture of *Alchornea cordifolia* 40% ($^{w}/_{w}$) and *Eugenia caryophyllata* 60% ($^{w}/_{w}$) was proposed as the new recipe for the product based on the synergistic activity demonstrated against most of the microorganisms tested. This newly reformulated product was subjected to a clinical evaluation, after the skin sensitivity and chronic toxicity study indicated that the possibility of any adverse effect occurring on exposure to the treatment was minimal. The study was undertaken using a RCT but single blinded unlike the first study involving the original product which was double blinded. The activity of the reformulated product was lower compared to the control treatment.

Participants attaining the primary outcome of complete cure for the 10% ($^{w}/_{w}$) *EAF-2011* was 100% compared to the 60% for the 5% ($^{w}/_{w}$) *RF-2013*. This cure rate would increase to 75% when analysis is done without the withdrawals. The weight of change as indicated by the confidence interval (Table 6.7), area under the curve (Figure 6.1b) and the time taken to achieve the primary outcome (Figure 6.1a) will however continue to make the 10% ($^{w}/_{w}$) *EAF-2011* the preferable treatment.

Despite the weight of the evidence being in favour of the 10% ($^{w}/_{w}$) *EAF-2011*, the reformulated product may still be therapeutically relevant as the product was tested at a concentration of 5% ($^{w}/_{w}$). As observed in the initial clinical evaluation of the original formulation, the activity of the reformulated product may be concentration dependent

which means that the reported effect may be increased upon increasing the concentration of the content of the ointment.

In summary, the outcomes of the study; the standards developed and the clinical evidence provided, should result in an improved product that would be acceptable to regulator and very beneficial for users.

7.2. Conclusions and Recommendations

This study has produced qualitative and quantitative chemical standards that can be used as a monograph for the product when combined with the results from the stability study. Preclinical analysis of the ointment also established it to be safe in an animal model and also stable over the one year period of evaluation.

The first human trial indicated that the three herbal extract concentrations of *EAF-2011* are effective for the management of superficial fungal infections when compared to Whitfield ointment. The 10% herbal extract concentration was established to be the preferred treatment because a higher number of participants achieved the primary and secondary outcomes when compared to the other concentration used and Whitfield ointment. Participants in this group took the least time in achieving these outcomes and importantly the outcomes were attained without any related harms from the use of this concentration of *EAF-2011*. All the products were established to be safe for use from the results of both the preclinical toxicity studies and the clinical trial as well.

In vitro antimicrobial re-evaluation of the component raw materials used in the product indicated the combination of *Alchornea cordifolia* 40% ($^{w}/_{w}$) and *Eugenia caryophyllata* 60% ($^{w}/_{w}$) as possessing better activity than the mixture of the five (5)

plants. This finding was very important as conservation of botanicals has become an important issue in the manufacture of herbal medicines. This combination thus formed the recipe for a proposed reformulated product.

However, in a second clinical study which compared the activity of the reformulated product to the original formulation, the superiority of the 10% ($^{w}/_{w}$) *EAF-2011* was confirmed as it showed better activity: a higher number of participants reaching the primary outcome and the shorter time taken to achieve this outcome.

The use of the product (*EAF-2011*) is thus recommended with the 10% ($^{w}/_{w}$) concentration suggested as the concentration of choice. However, the reformulated product can be clinically re-evaluated at different concentrations and in a larger number of study participants as the efficacy of the recipe may be dependent on these factors as demonstrated in the first clinically study.

It is recommended that an accelerated stability study should be undertaken for *EAF-2011* to establish the actual shelf-life since the stability study conducted was done with a proposed shelf life of one year for the product. Also, a multicentre clinical study can be carried out for populations from different areas to verify the generalisability of the results gathered from the current study. A phase IV study can be commenced to continue the documentation of adverse reactions that may be associated with the use of the product in the general population.

Finally, from the results of the study, increasing the herbal extract concentration in *EAF-2011* from the current 5% ($^{w}/_{w}$) to the more effective 10% ($^{w}/_{w}$) would be most

beneficial for users as treatment duration would be shorter for effective patient compliance.

REFERENCES

Abad, J. M., Ansuategui, M. and Bermejo, P. (2007). Active antifungal substances from natural sources. *ARKIVOC* **vii**: 116-145.

Abdelrahima, S. I., Almagboulb, A. Z., Omerb, M. E. A. and Elegamib, A. (2002). Antimicrobial activity of *Psidium guajava* L. *Fitoterapia* **73**: 713-715.

Adamski, Z. (1995). Studies of a role played by lipophilic yeasts *Malassezia furfur (Pityrosporum ovale, Pityrosporum orbiculare)* in different dermatoses. *Postepy Dermatol (Poznan)* **12**: 349-454.

Adeshina, G. O., Onaolapo, J. A., Ehinmidu, J. O. and Odama, E. L. (2010). Phytochemical and antimicrobial studies of the ethyl acetate extract of *Alchornea cordifolia* leaf found in Abuja, Nigeria. *J Med Plants Res* **4**(8): 649-658.

Adesina, S. K. (2005). The Nigerian Zanthoxylum; Chemical and Biological values. *Afr. J. Trad.* **2**(3): 282-301.

Adeyemi, I. A., Omonigbehin, A. E., Smith, S., Oluwatosin, O. and Jumoke, S. (2008). Antibacterial activity of extracts of *Alchornea cordifolia* (Schum and Thonn) Mull.Arg., *Boerhavia diffusa* (L) and *Bridellia micrantha* (Hoscht) Baill. used in traditional medicine in Nigeria on *Helicobacter pylori* and four diarrhoeagenic bacterial pathogen. *Afr J Biotech* **7**: 3761-3764.

Adjanohoun, E. J., Ake, A. L., Fionet, J. J., Guinko, S., Koumare, M., Ahyi, A. M. R. and Raynol, J. (1980). Traditional medicine and Pharmacopoeia. Contribution to the Revision of Ethnobotanical and Floristic studies of Mali. Paris, Agency for Cultural and Technical Cooperation**:** 273.

Ahmad, N., Alam, M. K., Shehbaz, A., Khan, A., Mannan, A., Hakim, R. S., Bisht, D. and Owais, M. (2005). Antimicrobial activity of clove oil and its potential in the treatment of *Vaginal candidiasis. J Drug Target* **13**: 555-561.

Ali, M., Ravinder, E. and Ramachandram, R. (2001). A new flavonoid from the aerial parts of *Tridax procumbens*. *Fitoterapia* **72**: 313-315.

Ali, S. M., Khan, A. A., Ahmed, I., Musaddiq, S., Ahmed, S. K., Polasa, H., Rao, V. L., Habibullah, M. C., Sechi, A. L. and Ahmed, N. (2005). Antimicrobial activities of Eugenol and Cinnamaldehyde against the human gastric pathogen *Helicobacter pylori*. *Annals of Clinical Microbiology and Antimicrobials* **4**: 20.

Ameen, M. (2010). Epidemiology of superficial fungal infections. *Clinics in Dermatology* **28**: 197-201.

Ameh, S. J., Obodozie, O. O., Inyang, S. U., Abubakar, S. M. and Garba, M. (2010). Current phytotherapy - A perspective on the science and regulation of herbal medicine. *Journal of Medicinal Plants Research* **4**(2): 72-81.

Ang-Lee, M. K., Moss, J. and Yuan, C. S. (2001). Herbal Medicines and Perioperative Care. *JAMA* **286**: 208-215.

Ayisi, N. K. and Nyadedzor, C. (2003). Comparative in vitro effects of AZT and extracts of *Ocimum gratissimum, Ficus polita, Clausena anisata, Alchornea cordifolia* and *Elaeophorbia drupifera* against HIV-1 and HIV-2 infections. *Antiviral Res* **58**(1): 25-33.

Bala, S., Weaver, J. and Hastings, K. L. (2000). Clinical relevance of preclinical testing for allergic side effects. *Toxicology* **209**: 195-200.

Banso, A. and Ngbede, J. E. (2006). Phytochemical screening and *in vitro* antifungal properties of *Fagara zanthoxyloides*. *Int Journal Food, Agric and Environ* **4**: 8-9.

Barnes, J. (2003). Pharmacovigilance of Herbal Medicines: A UK Perspective. *Drug Safety* **26**: 829-851.

Basile, A., Sorbo, S., Giordano, S., Ricciardi, L., Ferrara, S., Montesano, D., Castaldo, R., Cobianchi, M., Vuotto, L. and Ferrara, L. (2000). Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. *Fitoterapia* **71**: 110-116.

Bellew, S. G., Quartarolo, N. and Janniger, C. K. (2004). Childhood warts: an update. *Cutis* **73(6)**: 379-384.

Bergbrant, I.-M. and Brobeg, A. (1994). *Pityrosporum ovale* culture from the forehead of healthy children. *Acta Derm Venereol (Stockh)* **74**: 260-261.

Bickers, D. R., Lim, H. W., Mergol, D., Weinstock, M. A., Goodman, C., Faulkner, E., Gould, C., Gemmen, E. and Dall, T. (2004). The Burden of Skin Diseases. A joint project of the American Academy of Dermatology Association and the Society for the Investigative Dermatology. *J Am. Acad Dermatol* **55**: 490-500.

Boakye, A. D. (2008). Prevelance of Allergies in Ghana. Retrieved 30 March, 2012, from http://www.glofal.org/members/pdf/13.pdf.

Borman, A. M., Campbell, C. K., Fraser, M. and Johnson, B. A. (2007). Analysis of the dermatophyte species isolated in the British Isles between 1980 and 2005 and review of worldwide dermatophyte trends over the last three decades. *Med Mycol* **45**: 131-141.

Burt, S. A. and Reinders, R. D. (2003). Antibacterial activity of selected plant essential oils against *Escherichia coli*. *Letters in Applied Microb* **36**: 162-167.

Carroll, J. A. (1996). Common bacterial pyodermas: Taking aim against the most likely pathogens. *Postgraduate Medicine.* **100**: 311-322.

Chami, F., Chami, N., Bennis, S., Bouchikhi, T. and Remmal, A. (2005). Oregano and clove essential oils induce surface alteration of *Saccharomyces cerevisiae*. *Phytother Res* **19**: 405-408.

Chatora, R. (2003). An overview of the traditional medicine situation in the African Region. *African Health Monitor* **4**(1): 4-7.

Che, C. T., Wang, J. Z., Chow, M. S. S. and Lam, W. K. C. (2013). Herb-Herb Combination for Therapeutic Enhancement and Advancement: Theory, Practice and Future Perspectives *Molecules* **18**: 5125-5141.

Choudhary, N. and Sekhon, S. B. (2011). An overview of advances in the standardization of herbal drugs. *J Pharm Educ Res* **2**(2): 55.

Conway, P. (2002). Tree Medicine: A comprehensive guide to the healing power of over 170 trees. London, Judy Piatkus (Publishers) Ltd.**:** 2173-2177.

Cordell, G. A. (2000). Biodiversity and drug discovery - a symbiotic relationship. *Phytochemistry* **55**: 463-480.

Cowan, M. M. (1999). Plants as antimicrobial agents. *Clinical Microbiology Reviews* **12**(4): 564-582.

CPMR (2014). CPMR Newsletter. Mampong-Akuapem, Centre for Plant Medicine Research **1:** 1-2.

Cushnie, T. P. and Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *Int J of Antimicrob Agents* **26**: 343-356.

Dagnew, M. B. and Erwin, G. (1991). Epidemiology of common transmissible skin diseases among primary school children in North-West Ethiopia. *Trop. Geogr. Med.* **43**: 152-155.

Dahl, L. A. (1994). Dermotophytosis and the immune response. *J Am Acad Dermatol.* **31**: 34-41.

De Smet, P. A. G. M. (2002). Herbal remedies. *N. Engl. J. Med.* **347**: 2046-2056.

Debbiea, S., Graemeb, L., Pierrec, D., Elizabeth, W. and Kelvine, C. (2012). Pharmacovigilance of herbal medicine. *J of Ethnopharmacol* **140**: 513-518.

Dhiman, A., Nanda, A., Amaad, S. and Narasimhan, B. (2011). In vitro antimicrobial activity of methanolic leaf extract of *Psidium guajava* L. *J Pharm Bioallied Sci.* **3**(2): 226-229.

Doe, T. P., Asiedu, A., Acheampong, J. W., Christopher, M. E. and Payne, R. (2001). Skin diseases in Ghana and the UK. *Int J Dermatol* **40**: 323-326.

Donald, M. M. and McCullough, L. (2009). Evaluating the evidence in "Evidence-Based" integrative medicine programmes: Herbal Medicines. *Academic Medicine.* **84**(9): 1229-1234.

Drake, L. A., Dinehart, S. M. and Farmer, E. R. (1996). Guidelines of care for superficial mycotic infections of the skin: *Tinea corporis*, *Tinea cruris*, *Tinea faciei*, *Tinea manuum*, and *Tinea pedis*. Guidelines/Outcomes Committee. *American Academy of Dermatology.* **34**: 282-286.

Dubey, S., Ganeshpurkar, A., Bansal, D. and Dubey, N. (2013). Experimental studies on bioactive potential of rutin. *Chronicles of Young Scientists* **4**: 153-157.

Duron, R. R., Almaguer, L. C., Garza-Juarez, A. D. J., Salazar-Cavaroz, M. D. and Waksman-De-Torres, N. (2009). Development and validation of thin-layer chromatographic methods for quality control of medicinal products. *Acta Chromatographica* **21**(2): 203-215.

Dutta, B. K., Rahman, I. and Das, T. K. (2000). In vitro study on antifungal property of common fruit plants. *Biomedicine* **20**(3): 187-189.

Dweck, A. C. (2008). A review of Guava (*Psidium guajava*). Retrieved 14th May, 2011, from http://www.dweckdata.com/Published_papers/Psidium_guajava.pdf.

Dwyer, D. E. and Cunningham, A. L. (2002). *Herpes simplex* and *Varicella-zoster* virus infections. *Med J Aust* **177**(5): 267-273.

Ebi, G. C. (2001). Antimicrobial activities of *Alchornea cordifolia*. *Fitoterapia* **72**: 69-72.

Edzard, E. (2005). The efficacy of herbal medicine –an overview. *Fundamental & Clinical Pharmacology* **19**: 405-409.

EMEA (2008). Reflection paper on markers used for quantitative and qualitative analysis of herbal medicinal products and traditional herbal medicinal products. London, European Medicine Agency. **EMEA/HMPC/253629/2007**.

Ernst, E. (2002). Toxic heavy metals and undeclared drugs in Asian herbal medicines. *Trends Pharmacol Sci* **23**: 136-139.

Evans, W. C. (2002). Trease and Evans Pharmacognosy. Edinburgh,, W.B. Saunders.**:** 147-162.

Firenzuoli, F. and Gori, L. (2007). Herbal Medicine Today: Clinical and research issues. *eCAM* **4**(S1): 37-40.

Folashade, K. O., Omoregie, H. E. and Ochogu, P. A. (2012). Standardization of herbal medicines - A review. *Int J Bio and Cons* **4**(3): 101-112.

Foster, K. W., Ghannoum, M. A. and Elewski, B. E. (2004). Epidemiologic surveillance of cutaneous fungal infection in the United States from 1999 to 2002. *J Am Acad Dermatol*(50): 748-752.

Friedlander, S. F., Aly, R., Krafchik, B., Blumer, J., Honig, P., Stewart, D., Lucky, A. W., Gupta, A. K., Babel, D. E., Abrams, B., Gourmala, N., Wraith, L. and Paul, C. (2002). Terbinafine in the treatment of Trichophyton *Tinea capitis*: A randomized, double-blind, parallel-group, duration-finding study. *Pediatrics* **109**: 602-607.

Funatogawa, K., Hayashi, S., Shimomura, H., Yoshida, T., Halano, T., Ito, H. and Hirai, Y. (2004). Antibacterial activity of hydrolyzable tannins derived from medicinal plants against *Helicobacter pylori*. *Microbiol Immunol.* **48**: 251-261.

Furniss, B. S., Hannaford, A. J., Smith, W. G. P. and Tatchel, R. A. (1989). Textbook of practical organic chemistry. Essex, Addison Wesley Longman**:** 200-208.

Gagniera, J. J., Boonc, H., Rochona, P., Moherd, D., Barnesg, J. and Bombardier, C. (2006). Recommendations for reporting randomized controlled trials of herbal interventions: explanation and elaboration. *Journal of Clinical Epidemiology* **59**: 1134 - 1149.

Gayosoa, C. W., Limab, E. O., Oliveirac, V. T., Pereirac, F. O., Souzac, E. L., Limac, I. O. and Navarrod, D. F. (2005). Sensitivity of fungi isolated from onychomycosis to *Eugenia caryophyllata* essential oil and eugenol. *Fitoterapia* **76**: 247-249.

George, N. J., Obot, I. B., Ikot, A. N., Akpan, A. E. and Obi-Egbedi, N. O. (2010). Phytochemical and antimicrobial properties of the leaves of *Alchornea cordifolia*. *E-Journal of Chemistry* **7**(3): 1071-1079.

GHP (2007). Ghana Herbal Pharmacopoeia. Accra, STEPRI.**:** 11, 92, 112.

Giacomoni, P. U., Mammonea, T. and Teri, M. (2009). Gender-linked differences in human skin. *Journal of Dermatological Science* **55**: 144-149.

GNDP (2010). Standard Treatment Guidelines. Accra, Ghana, Ministry of Health **2:** 179.

Goldman, P. (2001). Herbal medicines today and the roots of modern pharmacology. *Ann Intern Med.* **135**: 594-600.

Goncalves, J. L., Lopes, R. C., Oliviera, D. B., Costa, S. S., Miranda, M. M., Romanos, M. T., Santos, N. S. and Wigg, M. D. (2005). In vitro anti-rotavirus activity of some medicinal plants used in Brazil against diarrhea. *J Ethnopharmacol* **99**: 403-407.

Grice, E. A. and Segre, J. A. (2011). The skin microbiome. *Nature Reviews of Microbiology* **9**: 244-274.

Guo, R., Canter, P. H. and Ernst, E. (2007). A systematic review of randomised clinical trials of individualised herbal medicine in any indication. *Postgrad Med J* **83**: 633-637.

Hamilton, A. C. (2004). Medicinal plants, conservation and livelihoods. *Biodiversity and Conservation* **13**: 1477-1517.

Haslam, E. (1996). Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J Nat.Prod.* **59**: 205-215.

Havlickova, B., Czaika, V. A. and Friedrich, M. (2008). Epidemiological trends in skin mycoses worldwide. *Mycoses* **51**(4): 2-15.

Hay, R., Bendeck, S. E., Chen, S., Estrada, R., Haddix, A., McLeod, T. and Mahé, A. (2006). Skin Diseases, World Bank and Oxford University Press **6:** 707-721.

Heinrich, M. (1996). Plants as antidiarrhoeals in medicine and diet. Proceedings from a joint meeting of the Society for Economic Botany and the International Society London. Kew, UK, Royal Botanic Gardens**:** 17 -30.

Heinrich, M., Ankli, A., Frei, B., Weimann, C. and Sticher, O. (1998). Medicinal plants in Mexico: healers consensus and cultural importance. *Social Science and Medicine* **47**: 1859-1871.

Hussin, N. M., Muse, R., Ahmad, S., Ramli, J., Mahmood, M., Sulaiman, M. R., Shukor, M. Y. A., Rahman, M. F. A. and Aziz, K. N. K. (2009). Antifungal activity of extracts and phenolic compounds from *Barringtonia racemosa* L. (Lecythidaceae). *Afr J Biotech* **8**: 2835-2842.

ICH (1997). Validation of Analytical Procedures: Methodology. ICH Q2b, FDA. **62 (May):** 27463.

Ioannidis, P. A. J., Evans, J. W. S., Gøtzsche, C. P., O'Neill, T. R., Altman, G. D., Schulz, K. and Moher, D. (2004). Better reporting of harms in randomized trials: An extension of the CONSORT statement. *Ann Intern Med.* **141**: 781-788.

Jindal, A. and Kumar, P. (2011). Antimicrobial flavonoids from *Tridax procumbens* Nat Prod Res. Retrieved 18th June, 2012, from http://www.tandfonline.com/doi/abs/10.1080/14786419.2011.617746.

Jones, H. E. (1998). Immune response and host resistance of humans to dermatophyte infection. *J Am Acad Dermatol*(28): 12-18.

Khullar, N. (2010). Antimicrobials from plants and their use in therapeutics and drug discovery. *The IIOAB Journal* **1**(3): 31-37.

Klancnik, A., Piskernik, S., Jersek, B. and Mozina, S. S. (2010). Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. *J Microbiol Methods* **81**: 121-126.

Konning, G. H., Agyare, C. and Ennison, B. (2004). Antimicrobial activity of some medicinal plants from Ghana. *Fitoterapia* **75**: 65-67.

Kruse, S. O. and Sultan, K. (2009). Stability Testing of Herbal Medicinal Products. Retrieved 6th July, 2011, from http://www.iptonline.com/articles/public/Diapharm%20article%20NP.pdf.

Kunle, O. F., Egharevba, H. O. and Ahmadu, P. O. (2012). Standardization of herbal medicines - A review. *International Journal of Biodiversity and Conservation* **4**(3): 101-112.

Larhsini, M., Oumoulid, L., Lazrek, H. B., Wataleb, S., Bousaid, M., Bekkouche, K. and Jana, M. (2001). Antibacterial activity of some Moroccan medicinal plants. *Phyto Res* **15**: 250-252.

Lin, J., Puckree, T. and Mvelase, T. P. (2002). Anti-diarrhoeal evaluation of some medicinal plants used by Zulu traditional healers. *J Ethnopharmacol* **79**: 53-56.

Lundberg, L., Johannesson, M., Silverdahl, M., Hermansson, C. and Lindberg, M. (1999). Quality of life, health-state utilities and willingness to pay in patients with psoriasis and *atopic eczema*. *Br J Dermatol* **141**(6): 1067-1075.

Lutterodt, G. D. (1992). Inhibition of Microlax-induced experimental diarrhoea with narcotic-like extracts of *Psidium guajava* leaf in rats. *J Ethnopharmacol* **37**: 51-157.

Mahule, A., Rai, P., Ghorpade, D. S. and Khadabadi, S. (2012). *In vitro* antifungal activity of the ethanol fractions of *Argyeria nervosa* (Burm. f.) Boj. leaves *Indian Journal of Natural Products and Resources* **3**: 48-54.

Manjamala, A., Varghese, S. S., Haridas, A. and Berlin, V. M. (2012). In-vitro antimicrobial activity of *Psidium guajava* L. leaf extracts against clinically important pathogenic microbial strains. *Asian J Pharm Clin Res* **5**: 139-145.

Manjamalai, A., Varhese, S. S., Aiswarya, H. and Grace, V. M. B. (2012). Antifungal, Anti-inflammatory and GC – MS Analysis for Bioactive Molecules of *Tridax procumbens* L. leaf. *Asian Journal of Pharmaceutical and Clinical Research* **5**: 139-145.

Marriott, J. F., Wilson, K. A., Langley, C. A. and Belcher, D. (2006). Pharmaceutical compounding and dispensing., Royal Pharmaceutical Society of Great Britain 156-157.

Martin, K. W. and Ernst, E. (2004). Herbal medicines for treatment of fungal infections: a systematic review of controlled clinical trials. *Mycoses* **47**: 87-92.

Mathers, C. D., Lopez, A. D. and Murray, C. J. L. (2001). The Burden of Diseases and Mortality by Condition: Data Methods and Results for 2001. Global Burden of Disease and Risk Factors. Geneva, World Health Organisation**:** 707.

Mavar-Mangaa, H., Haddada, M., Pieters, L., Baccelli, C., Penge, A. and Quetin-Leclercq, J. (2008). Anti-inflammatory compounds from leaves and root bark of *Alchornea cordifolia* (Schumach. & Thonn.) Mull. Arg. *Journal of Ethnopharmacology* **115**: 25-29.

Mavar Manga, H., Brkic, D., Marie, D. E. P. and Quetin-Leclercq, J. (2004). *In vivo* anti-inflammatory activity of *Alchornea cordifolia* (Schumach. & Thonn.) Mull. Arg. (Euphorbiaceae). *J Ethnopharmacol* **92**: 209-214.

McKay, A. D. and Aldridge, R. D. (2006). Skin Toxicology. Fundamental toxicology. . G. J. H. Worthand Duffus, J. H., The Royal Society of Chemistry.

Meazza, G., Dayan, F. E. and Wedge, D. E. (2003). Activity of quinones on Colletotrichum species. *J. Agric. Food Chem* **51**(13): 3824-3828.

Medina, M. L. and Pagano, F. L. (2003). Characterization of *Psidium guajava* pulp "criolla roja". *Revista de la Facultad de Agronom´ıa de La Universidad del Zulia, (LUZ)* **20**: 72-76.

Mitra, S. K. and Kannan, R. (2007). A note on unintentional adulterations in ayurvedic herbs. *Ethnobotanical Leaflets* **11**: 11-15.

Moshihuzzaman, M. and Choudary, M. I. (2008). Protocols on safety, efficacy, standardization and documentation of herbal medicine. *Pure Appl. Chem.* **80**(10): 2195-2230.

Mshana, N. R., Abbiw, K., Addae-Mensah, I., Adjanohoun, E., Ahyi, M. R. A., Ekpere, J. A., Enow-Orock, E. G., Gbile, Z. O., Naomesi, G. K., Odei, M. A., Odunlami, H., Oten-Yeboah, A. A., Sarpong, K., Sofowora, A. and Tackie, A. N. (2000). Traditional medicine and pharmacopoeia. Contribution to the revision of ethnobotanical and floristic studies in Ghana., Organization of African Unity/ Scientific, Technical and Research Commission **1**

Muhammad, S. and Shinkafi, M. A. (2007). In-Vitro Activity of the Extracts of Some Nigerian Plants used as Chewing Sticks on Human Odontopathogens. *Int. Jor. P. App. Scs.* **1**(2): 25-31.

Nair, R. and Chanda, S. (2007). In-vitro antimicrobial activity of *Psidium guajava* l. leaf extracts against clinically important pathogenic microbial strains. *Braz. J. Microbiol. vol.38 no.3 São Paulo July/Sept. 2007* **38**(3): 452-458.

NCCLS (2003). Methods for dilution antimicrobial susceptibility tests for bacteria. Approved Standard. Wayne, Pennsylvania, USA, National Committee for Clinical Laboratory Services. **6:** M7-A6.

Ngane, N. N., Biyiti, L., Amvam Zollo, P. H. and Bouchet, P. (2000). Evaluation of antifungal activity of extracts of two Cameroonian Rutaceae: *Zanthoxylum leprieurii* Guill. et Perr. and *Zanthoxylum zanthoxyloides* Waterm. *J Ethnopharmacol* **70**: 335-342.

Nichols, R. L. (1999). Optimal treatment of complicated skin and skin structure infections. *J Antimicrob Chemother* **44**((Suppl A)): 19-23.

Nyarko, K. A., Asiedu-Gyekye, J. I. and Sittie, A. A. (2005). A manual of harmonised procedures for assessing the safety, efficacy and quality of plant medicines in Ghana, Ghana National Drugs Programme**:** 108.

Odebiyi, O. O. and Sofowora, E. A. (1978). Phytochemical screening of Nigerian medical plants II. *Lloydia* **41**: 2234–2246.

Odebiyi, O. O. and Sofowora, E. A. (1979). Antimicribial alkaloids from a Nigerian chewing stick (*Fagara zanthoxyloides*). *Planta Med.* **36**: 204-207.

Ogata, M., Hoshi, M., Urano, S. and Endo, T. (2000). Antioxidant activity of eugenol and related monomeric and dimeric compounds. *Chem Pharm Bull* **48**: 1467-1469.

Ogunbiyi, A. O., Owoaje, E. and Ndahi, A. (2005). Prevalence of skin disorders in school children in Ibadan, Nigeria. *Paediatric Dermatol.* **22**(1): 6-10.

Okeke, N. I., Ogundaini, A. O., Ogungbamila, F. O. and Lamikanra, A. (1999). Antimicrobial spectrum of *Alchornea cordifolia* leaf extract. *Phytother. Res.* **13**: 67-69.

Oladele, A. T., Dairo, B. A., Elujoba, A. A. and Oyelami, A. O. (2010). Management of superficial fungal infections with *Senna alata* ("alata") soap: A preliminary report. *Afr J Pharm and Pharmacol* **4**(3): 98-103.

Pandey, A. and Shweta, M. (2011). Antifungal properties of *Psidium guajava* leaves and fruits against various pathogens. *Journal of Pharmaceutical and Biomedical Sciences* **13**: 1-16.

Parasuraman, S., Thing, G. S. and Dhanaraj, S. A. (2014). Polyherbal formulation: Concept of ayurveda. *Pharmacogn Rev* **8**: 73-80.

Pasay, C., Mounsey, K. and Stevenson, G. (2010). Acaricidal activity of eugenol based compounds against scabies mites. *PLoS One* **5**: e12079.

Patwardhan, B. and Mashelkar, R. A. (2009). Traditional medicine-inspired approaches to drug discovery: can Ayurveda show the way forward? *Drug Discovery Today* **14**: 804-811.

Pawar, V. C. and Thaker, V. S. (2006). In vitro efficacy of 75 essential oils against *Aspergillus niger*. *Mycoses* **49**: 316-323.

Pesewu, G. A., Cutler, R. R. and Humber, D. P. (2008). Antibacterial activity of plants used in traditional medicines in Ghana with particular reference to MRSA. *J. Ethnopharmacol.* **116**(1): 102-111.

Pinto, E., Vale-Silva, L., Cavaleiro, C. and Salgueiro, L. (2009). Antifungal activity of the clove essential oil from *Syzygium aromaticum* on *Candida, Aspergillus* and *Dermatophyte* species. *J Med Microb* **58**: 1454-1462.

Rabe, T. and van Staden, J. (1997). Antibacterial activity of South African plants used for medicinal purposes. *J Ethnopharmacol.* **56**(1): 81-87.

Rios, J. L. and Recio, M. C. (2005). Medicinal plants and antimicrobial activity. *J Ethnopharmacol* **100**: 80-84.

Rotimi, V. O., Laughton, B. E., Bartlet, J. E. and Mesadomi, H. A. (1988). Activities of Nigerian chewing stick extracts against *Bacteroides gingivalis* and *Bacteroides melaninogenicus. Antimicrob. Agents Chemother* **32**: 598-600.

Santas, J., Almajano, M. P. and Carbó, R. (2010). Antimicrobial and antioxidant activity of crude onion (Allium cepa, L.) extracts. *Int. J. Food Sci Technol.*. **45**: 403-409.

Sathian, B., Sreedharan, J., Ahmad, M., Baboo, N. S., Abhilash, E. S. and Rajesh, E. (2009). Clinical trials in medical research. *Journal of GMC-Nepal* **2**(2): 28-36.

Savin, R. (1996). Diagnosis and treatment of *Tinea versicolor*. *J Fam Pract* **43**: 127-132.

Seebacher, C., Bouchara, J.-P. and Mignon, B. (2008). Updates on the epidemiology of dermatophyte infections. *Mycopathologia* **166**: 335-352.

Senekal, M. (2010). Current resistance issues in antimicrobial therapy. *CME*. **28**(2): 54-57.

Severson, J., Evans, T. Y. and Lee, P. (2001). *Human papilloma virus* infections: epidemiology, pathogenesis, and therapy. *J Cutan Med Surg* **5**(1): 43-60.

Shai, L. J., McGaw, L. J., Aderogba, M. A., Mdee, L. K. and Eloff, J. N. (2008). Four pentacyclic triterpenoids with antifungal and antibacterial activity from *Curtisia dentata* (Burm.f) C.A. Sm. leaves. *J Ethnopharmacol* **119**: 238-244.

Sharma, M. C. and Sharma, M. (2010). Phytochemical and pharmacological screening of combined *Mimosa pudica* Linn and *Tridax procumbens* for in vitro antimicrobial activity. *International Journal of Microbiological Research* **1**(3): 171-174.

Silva-Lizama, E. (1995). *Tinea versicolor*. *Int J Dermatol 1995* **34**(9): 611-617.

Simaan, J. A. (2009). Herbal medicines, what physicians need to know. *The Lebanese Medical Journal* **57**(4): 215-217.

Simmons, A. (2002). Clinical manifestations and treatment considerations of *Herpes simplex* virus infection. *J Infect Dis* **186**(Supp 1): S71-77.

Sofowora, A. (1993). Medicinal plants and traditional medicine in Africa, Spectrum Books Limited, Ibadan**:** 150-156.

Songlin, L., Quanbin, H., Chunfeng, Q., Jingzheng, S., Chuen, L., Cheng, X. and Hongxi, X. (2008). Chemical markers for the quality control of herbal medicines: An overview. *Chinese Medicine* **3**: 7-23.

Stern, J. L., Hagerman, A. E., Steinberg, P. D. and Mason, P. K. (1996). Phlorotannin-protein interactions. *J Chem Ecol* **22**: 1887–1899.

Suresh, P., Ingle, V. K. and Vijaya Lakshmi, V. (1992). Antibacterial activity of Eugenol in comparison with other antibiotics. *J Fd Sci Technol* **29**: 254-256.

Taddel, A. and Rosas Romero, A. J. (2000). Bioactivity studies of extracts from *Tridax procumbens. Phytomedicine* **7**: 235-238.

Taiwo O., Xu H.X. and S.F., L. (1999). Antibacterial activities of extracts from Nigerian chewing sticks. *Phytother. Res.* **13**: 675-679.

Taylor, R. S. L., Edel, F., Manandhar, N. P. and Towers, G. H. N. (1996). Antimicrobial activities of southern Nepalese medicinal plants. *J Ethnopharmacol* **50**: 97-102.

Thakur, L., Ghodasra, U., Patel, N. and Dabhi, M. (2011). Novel approaches for stability improvement in natural medicines. *Phcog Rev* **5**: 48-54.

Thorne, S., Paterson, B., Russell, C. and Schultz, A. (2002). Complementary/alternative medicine in chronic illness as Informed Self-Care Decision Making. *International Journal of Nursing Studies* **39**: 671-683.

Tona, L., Kambu, K., Mesia, K., Cimanga, K., Apers, S., De Bruyne, T., Pieters, L., Totte, J. and Vlietinck, A. J. (1999). Biological screening of traditional preparations from some medicinal plants used as antidiarrhoeal in Kinshasa, Congo. *Phytomedicine* **6**: 59-66.

Trent, J. T., Federman, D. and Kirsner, R. S. (2001). Common bacterial skin infections. *Ostomy Wound Management.* **47**: 30-34.

Tsuchiya, H., Sato, M., Miyazaki, T., Fujiwara, S., Tanigaki, S., Ohyama, M., Tanaka, T. and Linuma, M. (1996). Comparative study on the antibacterial activity of phytochemical flavones against methicillin-resistant *Staphylococcus aureus. J. Ethnopharmacol* **50**: 27-34.

Udopa, S. L., Udopa, A. L. and Lalkarni, D. R. (1991). Influence of *Tridax procumbens* on lysl oxidase activity and wound healing. *Planta Med* **57**: 325-327.

Valerio, L. G. and Gonzales, G. F. (2005). Toxicological aspects of the South American Herbs Cat's Claw (*Uncaria tomentosa*) and Maca (*Lepidium meyenii*): A Critical Synopsis. *Toxicol Rev* **24**: 11-35.

Van Vuuren, S. F. and Viljoen, A. M. (2008). *In vitro* evidence of phyto-synergy for plant part combinations of *Croton gratissmus* (Euphorbiaceae) used in African traditional healing. *Journal of Ethnopharmacology* **119**: 700-704.

Van Vuuren, S. F. and Viljoen, A. M. (2011). Plant based antimicrobial studies-methods and approaches to study the interaction between natural products. *Planta Medica* **77**: 1168-1182.

Volleková, A., Kostálová, D., Kettmann, V. and Tóth, J. (2003). Antifungal activity of *Mahonia aquifolium* extract and its major protoberberine alkaloids. *Phytother. Res.* **17**: 834-837.

Wächtera, G. A., Hoffmannb, J. J., Furbacherb, T., Blakec, M. E. and Timmermanna, B. N. (1999). Antibacterial and antifungal flavanones from *Eysenhardtia texana*. *Phytochemistry* **52**(8): 1469-1471.

Wagner, D. K. and Sohnle, P. G. (1995). Cutaneous defenses against dermatophytes and yeasts. *Clin Microbiol Rev* **8**: 317-335.

Weinstock, M. A. and Gardstein, B. (1999). Twenty-year trends in the reported incidence of *Mycosis fungoides* and associated mortality. *Am J Public Health* **89**(8): 1240-1244.

WHO (2000). General guidelines for methodologies on research and evaluation of traditional medicine, World Health Organisation.

WHO (2001). World Medical Association Declaration of Helsinki. Ethical Principles for Medical Research Involving Human Subjects. *Bulletin of the World Health Organization* **79**: 4.

WHO (2002). WHO Traditional Medicine Strategy 2002–2005., World Health Organisation. **EDM/TRM**.

WHO (2002). WHO Traditional Medicine Strategy 2002 -2005. Geneva, World Health Organisation. **EDM/TRM**.

WHO (2004). Guidelines for the clinical study of Traditional Medicines in the WHO African Region Brazzaville, WHO Regional Office for Africa**:** 57-66.

WHO (2007). Stability testing of active pharmaceutical ingredients and pharmaceutical products, World Health Organisation. **QAS/06.179:** 1-7.

Williamson, E., D. T. Okpako and F. J. Evans (1996). Pharmacological methods in phytotherapy research. Chichester, John Wiley & Sons Ltd, England. **1:** 131-154.

Wu, Y. H., Su, H. Y. and Hseih, Y. J. (2000). Survey of infectious skin diseases and skin infestations among primary school students of Taitung County, Eastern Taiwan. *J. Formos Med. Assoc.* **99**: 128-134.

Xu, H. X., Zeng, F. Q., Wan, M. and Sim, K. Y. (1996). Anti-HIV triterpene acids from *Geum japonicum. J Nat Prod* **59**: 643-645.

Yamada, J. (2004). The anti-fungal and anti-bacterial effects of *Psidium guajava* on fungi *Microsporum gypseum*, *Trichophyton mentagrophytes*, bacteria *Staphylococcus aureus*, and *Staphylococcus epidermidis*. Retrieved 7th May, 2011, from http//eis-web1.byuh.edu.

Yi-Zeng, L., Peishan, X. and Kelvin, C. (2004). Quality control of herbal medicines. *J Chromatography B* **812**: 53-70.

Zeng, Z.-P. and Jiang, J.-G. (2010). Analysis of the adverse reactions induced by natural product-derived drugs. *British Journal of Pharmacology* **159**: 1374-1391.

Zhang, W. J., Chen, B. T., Wang, C. Y., Zhu, Q. H. and Mo, Z. (2003). Mechanism of quercetin as an antidiarrheal agent. *Di Yi Jun Yi Xue Xue Bao* **23**: 1029-1031.

Zhao, W., Wolfender, J.-L., Hostettmann, K., Xu, R. and Qin, G. (1998). Antifungal Alkaloids and Limonoid derivatives from *Dictamnus dasycarpus*. *Phytochemistry* **47**: 7-11.

Zheng, W. F., Tan, R. X., Yang, L. and Liu, Z. L. (1996). Two flavones from *Artemisia giraldii* and their antimicrobial activity. *Planta Med* **62**: 160-162.

APPENDIX I

Certificate of Ethical Clearance

REPUBLIC OF GHANA
(MINISTRY OF HEALTH)

CENTRE FOR SCIENTIFIC RESEARCH INTO PLANT MEDICINE

WHO COLLABORATING CENTRE FOR RESEARCH & DEVELOPMENT OF TRADITIONAL MEDICINE

Local Tel: 03427-2204; / 22103 (0342)-199628 Tel/Fax: 03427-22103/ (0342)-199628
E-mail: [illegible] Website: www.csirpm.org

In case of reply, the number and date of this letter should be quoted

P. O. Box 73,
Mampong-Akuapem
Eastern Region, Ghana

My Ref. No......

Your Ref. No......

TO WHOM IT MAY CONCERN

Dear Sir/Madam,

ETHICAL CLEARANCE

I declare that the attached Research Protocol from Mr. Kwesi Prah Thomford entitled CLINICAL EFFECTIVENESS AND SAFETY STUDIES ON EAF -2011, a polyherbal formulation for the management of superficial mycoses, has been reviewed by the Ethical Committee on Human Research for the Centre for Scientific Research into Plant Medicine.

The research, which involves subjugation of humans as research objects had been judged to be relevant, designed in accordance with accepted scientific practices and norms, as well as -particularly -in harmony with universally accepted international standards and ethical practice in its use of human persons as subjects of research and is in the opinion of the reviewers likely to be successful in achieving its objectives.

The Committee also reports that the researcher has designed purpose specific informed consent forms which are simple, properly designed and user friendly in order to protect the interests of human subjects. Enabling their understanding of all implications of consent to participate.

Prof. Dominic Adotei Edoh
EXECUTIVE DIRECTOR

APPENDIX II

Total Signs and Symptoms Score (TSSS)

The Total Signs and Symptoms Score (TSSS) used in assessing participants during follow up was adapted from Friedlander, *et al.*, (2002). This is a scale that assigns numerical weights to selected characteristics of the disease. In this study the signs graded were

- Desquamation/ Scaling
- Vesicles/ Pustules
- Erythema
- Pruritus

Each participant was graded based on the investigator's evaluation of the selected sign using the scores shown in Table 7.1. The sum of all the scores was calculated as the TSSS using the patient score sheet in Appendix IV.

Table 7.1: Grading scale for the assessment of the signs selected

SCORE	DEFINITION
3.0	Severe
2.0	Moderate
1.0	Mild
0.0	Absent

APPENDIX III

Demographic and Disease Characteristics Questionnaire

Dear Respondent,

This questionnaire is designed to gather information about fungal skin diseases and persons infected with them. The information you provide will be held in strict confidentiality and is expected to increase our understanding of the disease as well as improve future products that may be developed for the condition.

Please Tick as appropriate

1. Sex: Male ☐ Female ☐

2. Age: ☐

3. Occupation: …… (specify)

4. How long have you had these rashes:

   Less than 3 months ☐

   3-12 months ☐

   Over 1year ☐

5. Is this the first time the rash is occurring:

   Yes ☐ No ☐

6. ***If No***, how many times have the rashes recurred:

   Once ☐

   Twice ☐

   More than Twice ☐

7. Do you have any immediate relations with similar infection?

   Yes ☐ No ☐

APPENDIX IV

Participant Score Sheet

The score sheet was used in recording data from participants after the completion of the informed consent forms and throughout the follow up period. The sheet was used together with the grading scale in Appendix I.

Participant ID:

Age:

Sex:

	Baseline	Day 14	Day 28	Day 56	Day 90	Relapse
Pruritus						
Vesicles						
Erythema						
Desquamation						
Papular						
Total Signs and Symptoms (TSSS)						
Microscopy						
Culture						

APPENDIX V

Reference Range for Safety Parameters

The reference ranges used in the study during the safety assessments are listed in Table 7.2. The ranges are the recommended reference from the World Health Organisation

Table 7.2: Reference ranges for safety parameters analysed

Parameter	Reference ranges
Liver Function	
Alkaline Phosphatase (ALP)	98-279 U/L
Alanine Aminotransferase (ALT)	Males Up to 40 U/L Females Up to 32 U/L
Aspartate Transaminase (AST)	Male Up to 38 U/L Females Up to 31 U/L
Albumin (ALB)	34-48 g/dl
Gamma Glutamyl Transferase (GGT)	Male 11 to 51 U/L Females 7 to 33 U/L
Renal Function	
Urea	2.49-7.49 mmol/l
Creatinine	Male 61.8-123.7 µmol/l Females 53-97.2 µmol/l
Haematology	
White Blood Count (WBC)	4.0-10.0
Red blood Cells (RBC)	3.80-6.50
Haemoglobin (HB)	11.5-17.0
Haematocrit (HCT)	37.0-54.0
Platelets (PLT)	150-500

Source: (WHO, 2004)

APPENDIX VI

Adverse Drug Report Sheet Checklist of Possible Side Effects

The check list below was used as part of the safety evaluation. All participants were taken through the questionnaire at each follow up period. The Adverse report sheet was used together with the Toxicty Grading Scale which were adapted from the WHO document on the guidelines for clinical study of Traditional Medicines for the WHO African Region, (2004).

Table 7.3 Adverse Drug Report Sheet

Day:	**0**	**1**	**2**	**3**	**4**	**5**	**6**
Nervous system							
Drowsiness							
Nervousness							
Insomnia							
Nightmares							
Shakiness							
Numbness							
Tinnitus							
Blurred vision							
Unpleasant taste							
Thirst							
Cardiovascular:							
Fast heartbeat							
Irregular heartbeat							
Respiratory:							
Cough							

Chest pain							
Stuffy nose							
Gastrointestinal:							
Heartburn							
Abdominal pain							
Diarrhoea							
Constipation							
Intestinal wind							
Black stools							
Genito-urinary:							
Dysuria							
Nocturia							
Dark urine							
Change in sexual ability/desire							
Mucocutaneous:							
Blister formation							
Pruritus							
Easy bruising							
Dry mouth							
Others (specify):							
Jaundice							

Credit: (WHO, 2004)

APPENDIX VII

Patient Consent Form

EFFICACY AND SAFETY ASSESSMENT OF TOPICALLY ADMINISTERED MEDICINAL PLANT EXTRACT *EAF-2011*

(To be translated into appropriate local language)

To whom it may concern.

I,

Mr./Mrs./Miss…….Age…..…Sex……do hereby consent to give permission onto Prof./Dr./Mrs./Miss……..……to be included and carry research on me in the intended research protocol as explained to me and understood by me. I have understood the patient information sheet after reading/hearing it. I understand that the research involves me taking a herbal medicine for treating Skin infections. I have also been made to understand the implications, benefits and risks of participating in the trial. I accept the tests, treatments and any other procedures (e.g. photography) to be carried out and the risks involved. I have made this decision freely, and understand that I am under no obligation to participate in the study. I understand that I have the right to withdraw from the research at any time, for any reason without penalty or harm. Although I do not have to give reason for withdrawing, I agree to inform the investigators if I experience an adverse effect from the treatment. If I withdraw, I am made to understand that I will be cared for by the doctors like any other patient. Data/biological samples will be coded and remain confidential (name not disclosed). I also agree that I will make myself available for subsequent reviews and monitoring as director by the investigator, there will be no financial cost to me, not even my transportation to- and -from the clinic during the course of the study. I expect that my participation in the study will be held in strict confidence. All the above conditions have been explained to me in the ……..language, in which I am fluent by Prof/Dr/Mr./Mrs. …….

Signature or thumbprint of patient *(or parent/guardian if patient is aged under 18yrs)*……

Date: …….

Signature of Witness……

Date…….

Name and address of Witness…….

I certify that I have fully explained the trial to the above patient, and that I have not put them under any pressure to participate.

Signature of Investigator…….

Name……

APPENDIX VIII

Calibration Curves for the Standard Flavonoids Analysed

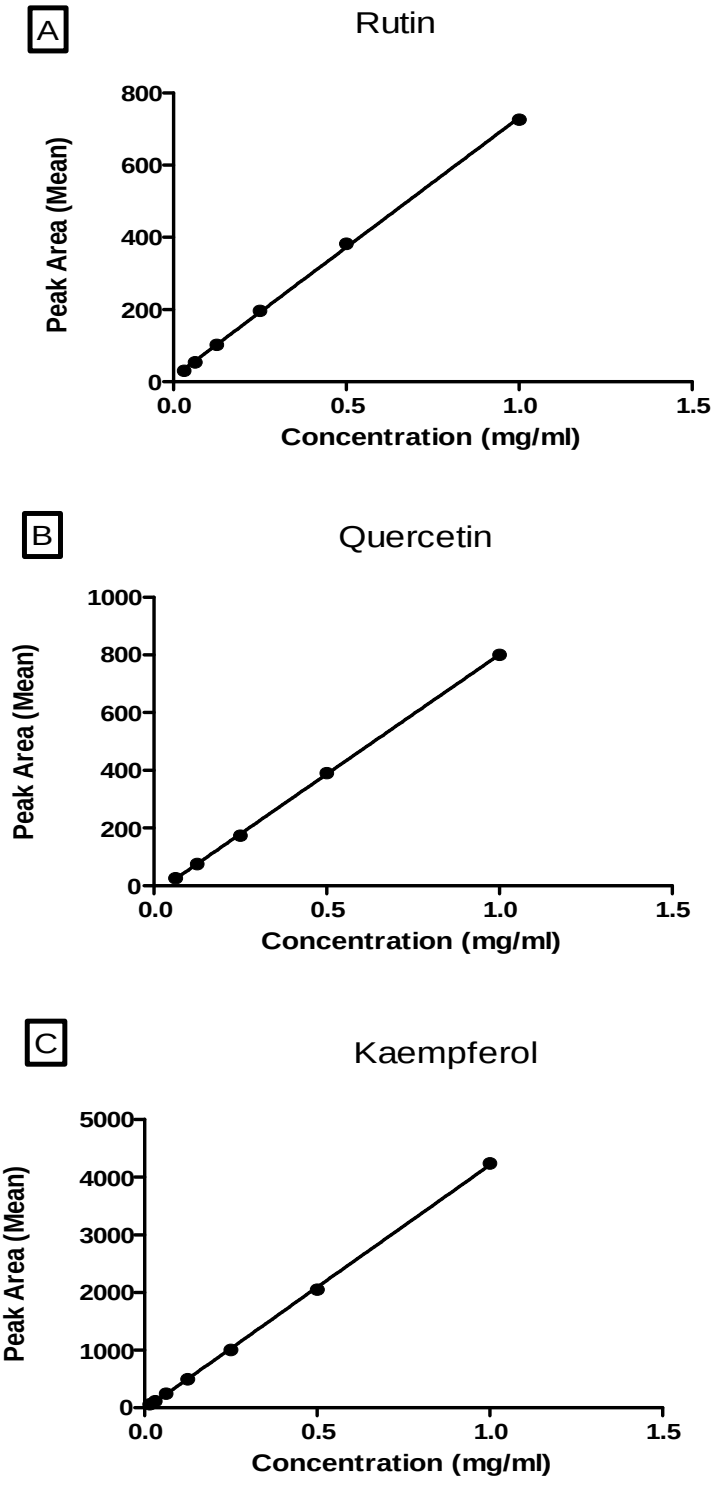


Figure 7.1: Calibration curves for rutin (A), quercetin (B) and kaempferol (C). Correlation coefficient (r^2) obtained for all samples tested <0.998.

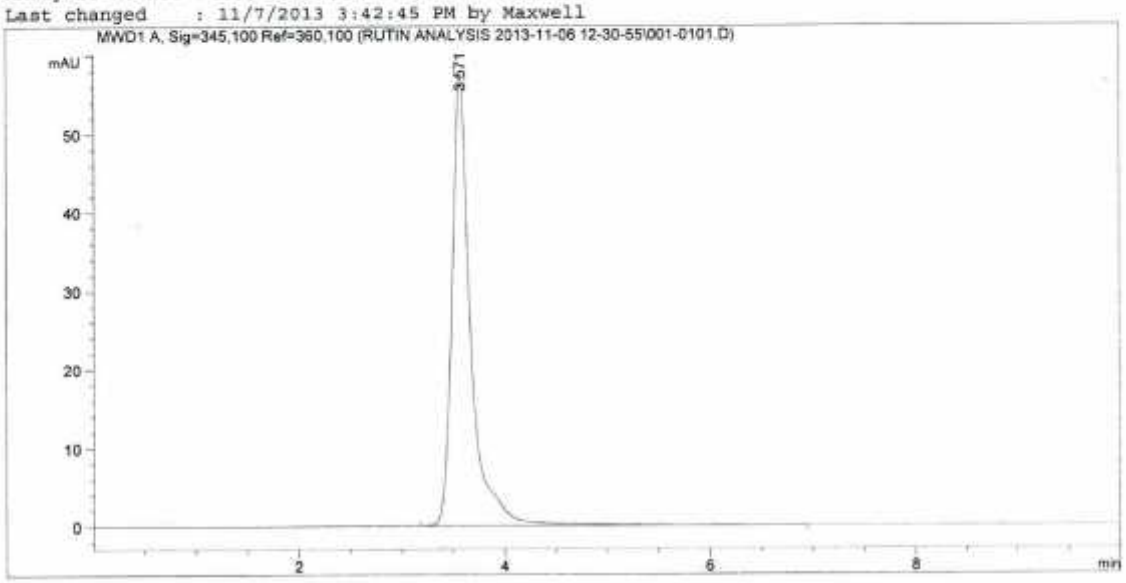
APPENDIX IX

Chromatographic Fingerprint for Rutin

Data File C:\CHEM32\1\DATA\RUTIN ANALYSIS 2013-11-06 12-30-55\001-0101.D
Sample Name: 1mg/ml

Acq. Operator : Maxwell
Seq. Line : 1
Acq. Instrument : CSRPM HPLC
Location : Vial 1
Injection Date : 11/6/2013 12:31:13 PM
Inj : 1
Inj Volume : 10.0 µl

Acq. Method : C:\CHEM32\1\DATA\RUTIN ANALYSIS 2013-11-06 12-30-55\RUTIN.M
Last changed : 11/6/2013 12:30:41 PM by Maxwell
Analysis Method : C:\CHEM32\1\DATA\CAP000024.D\DA.M (RUTIN.M)
Last changed : 11/7/2013 3:42:45 PM by Maxwell



Fraction Information

Fraction collection off

No Fractions found.

Area Percent Report

Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=345,100 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.571	BBA	0.1817	726.39807	57.38038	100.0000
Totals :				726.39807	57.38038	

Figure 7.2: HPLC fingerprint for the standard flavonoid rutin

APPENDIX X

Chromatographic Fingerprint for Quercetin

Data File C:\CHEM32\1\DATA\RUTIN ANALYSIS 2013-11-07 13-21-23\004-0401.D
Sample Name: Quece 0.125mg/ml

Acq. Operator	: Maxwell	Seq. Line	: 4
Acq. Instrument	: CSRPM HPLC	Location	: Vial 4
Injection Date	: 11/7/2013 1:54:47 PM	Inj	: 1
		Inj Volume	: 10.0 µl
Acq. Method	: C:\CHEM32\1\DATA\RUTIN ANALYSIS 2013-11-07 13-21-23\RUTIN.M		
Last changed	: 11/6/2013 3:14:50 PM by Maxwell		
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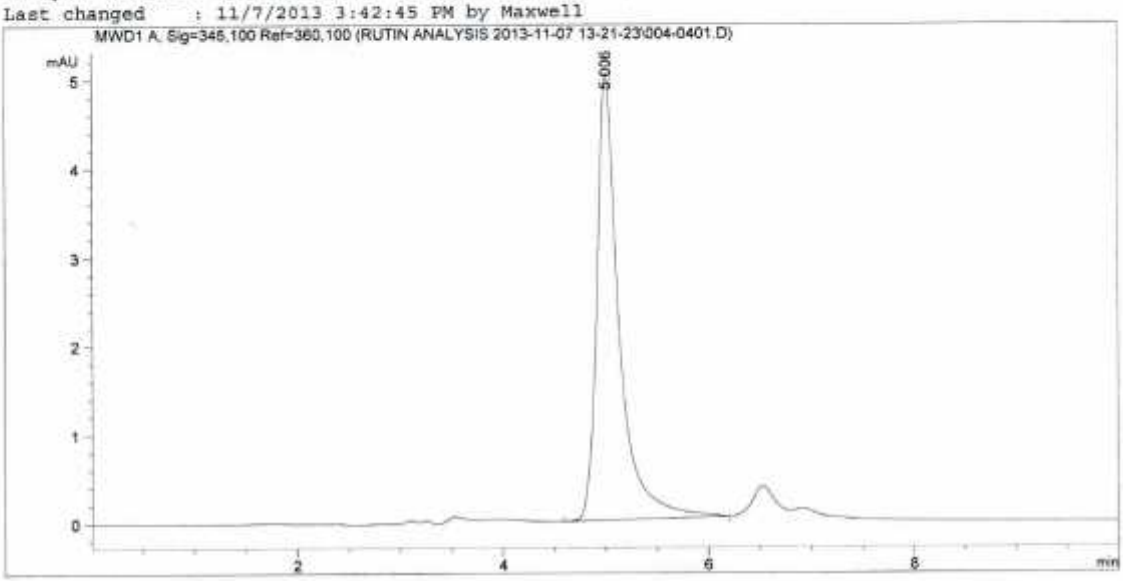
Last changed	: 11/7/2013 3:42:45 PM by Maxwell		



Fraction Information

Fraction collection off

No Fractions found.

Area Percent Report

Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=345,100 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.006	BB	0.2154	75.75788	5.03930	100.0000
Totals :				75.75788	5.03930	

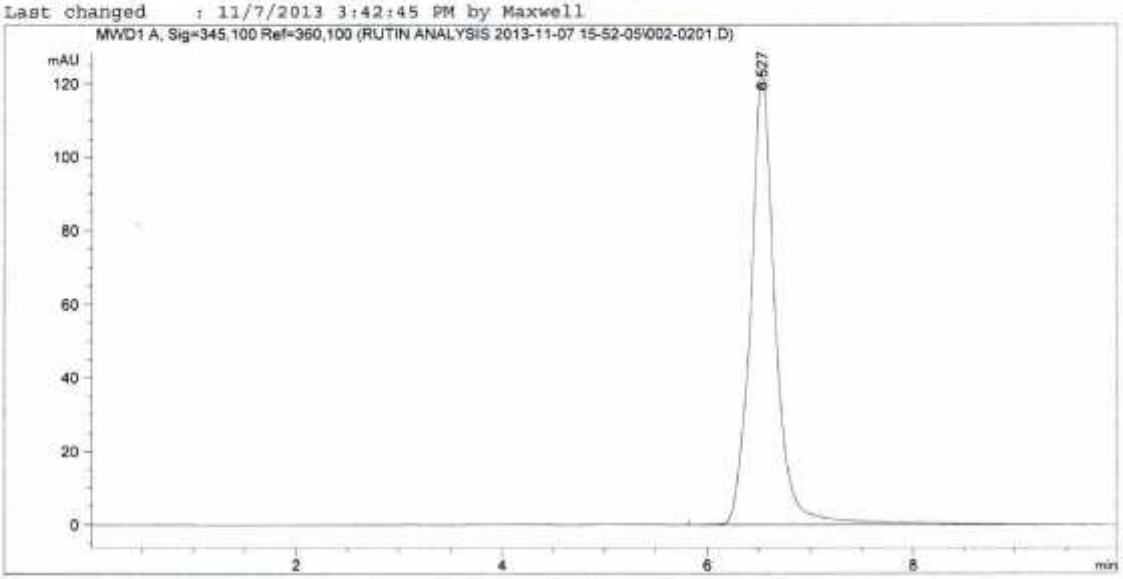
Figure 7.3: HPLC fingerprint for the standard flavonoid quercetin

APPENDIX XI

Chromatographic Fingerprint of Kaempferol

Data File C:\CHEM32\1\DATA\RUTIN ANALYSIS 2013-11-07 15-52-05\002-0201.D
Sample Name: Campeferol 0.5mg/ml

Acq. Operator	: Maxwell	Seq. Line	: 2
Acq. Instrument	: CSRPM HPLC	Location	: Vial 2
Injection Date	: 11/7/2013 4:03:24 PM	Inj	: 1
		Inj Volume	: 10.0 µl
Acq. Method	: C:\CHEM32\1\DATA\RUTIN ANALYSIS 2013-11-07 15-52-05\RUTIN.M		
Last changed	: 11/6/2013 3:14:50 PM by Maxwell		
Analysis Method	: C:\CHEM32\1\DATA\CAP000024.D\DA.M (RUTIN.M)		
Last changed	: 11/7/2013 3:42:45 PM by Maxwell		



Fraction Information

Fraction collection off

No Fractions found.

Area Percent Report

Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: MWD1 A, Sig=345,100 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.527	BBA	0.2406	2048.43530	122.79375	100.0000
Totals :				2048.43530	122.79375	

Figure 7.4: HPLC fingerprint for the standard flavonoid kaempferol

APPENDIX XII

Chromatographic Fingerprint for *Alcornea cordifolia*

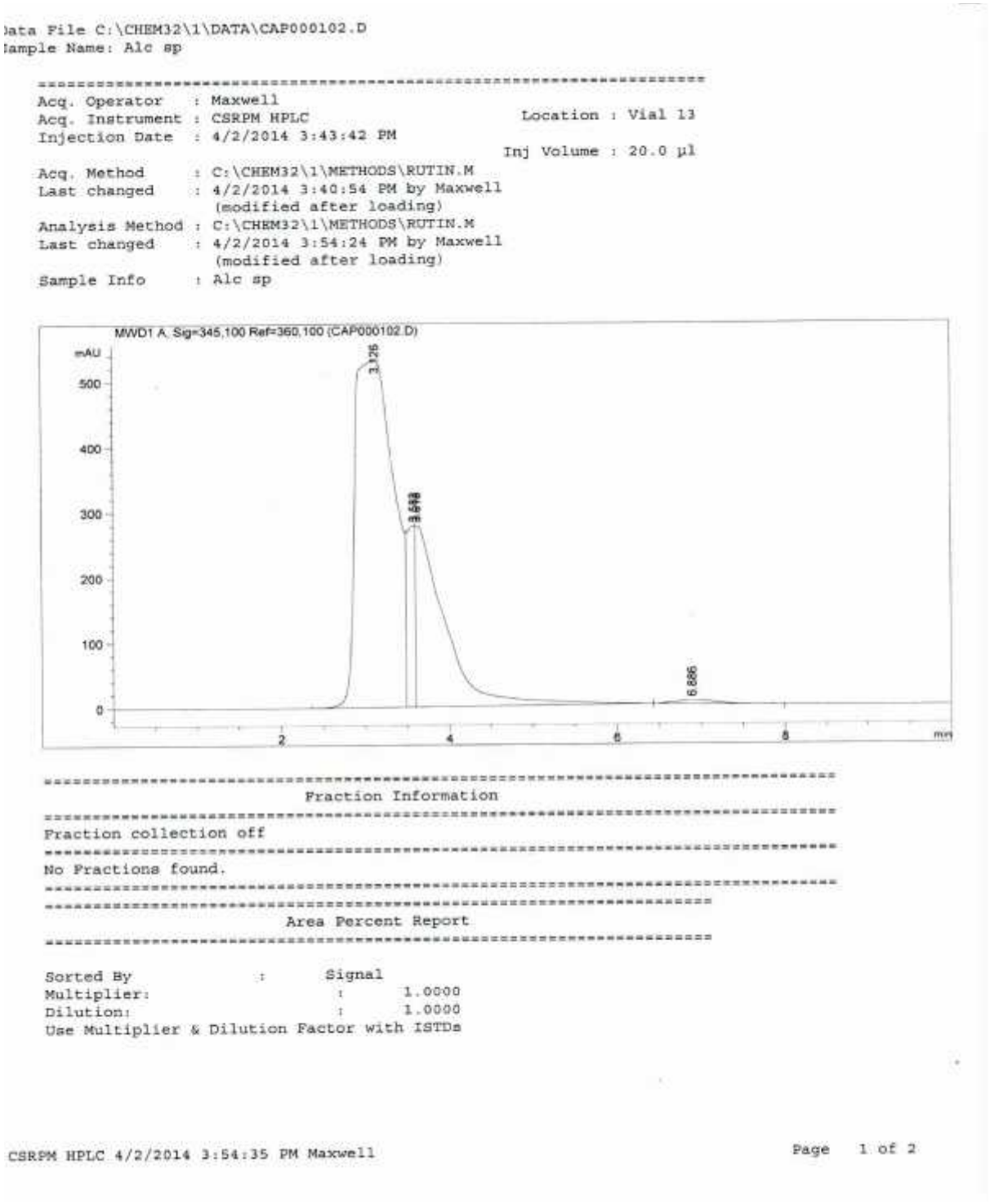


Figure 7.5: Chromatograms for *Alchornea cordifolia* after HPLC analysis

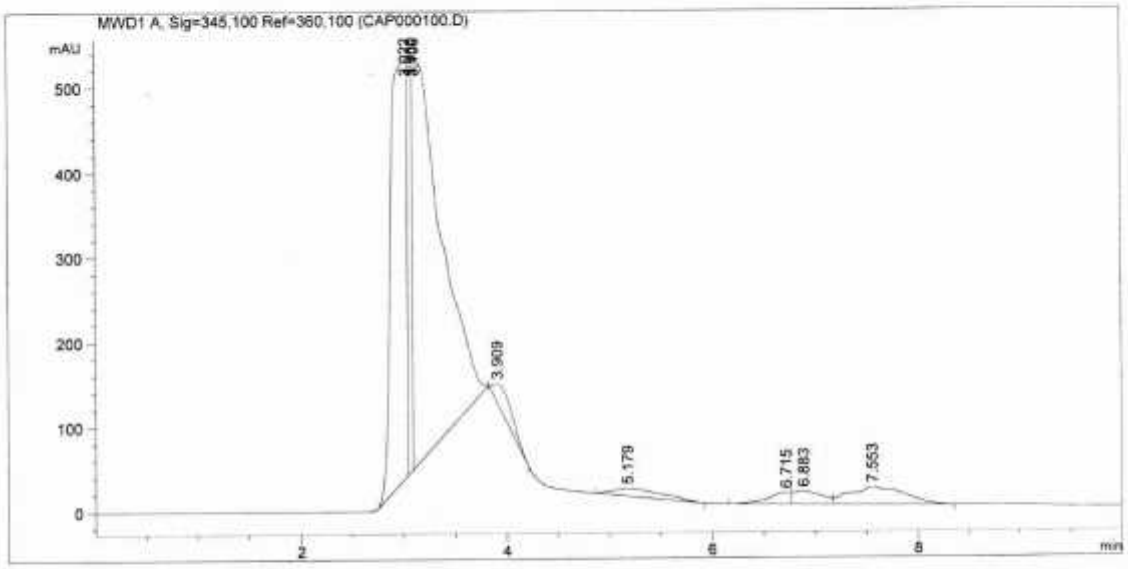
APPENDIX XIII

Chromatographic Fingerprint of *Eugenia caryophyllata*

Data File C:\CHEM32\1\DATA\CAP000100.D
Sample Name: Eugenia sp

Acq. Operator : Maxwell
Acq. Instrument : CSRPM HPLC Location : Vial 11
Injection Date : 4/2/2014 3:13:44 PM Inj Volume : 20.0 µl

Acq. Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 3:06:23 PM by Maxwell
(modified after loading)
Analysis Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 3:24:25 PM by Maxwell
(modified after loading)
Sample Info : Eugenia sp



Fraction Information

Fraction collection off

No Fractions found.

Area Percent Report

Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

CSRPM HPLC 4/2/2014 3:24:35 PM Maxwell Page 1 of 2

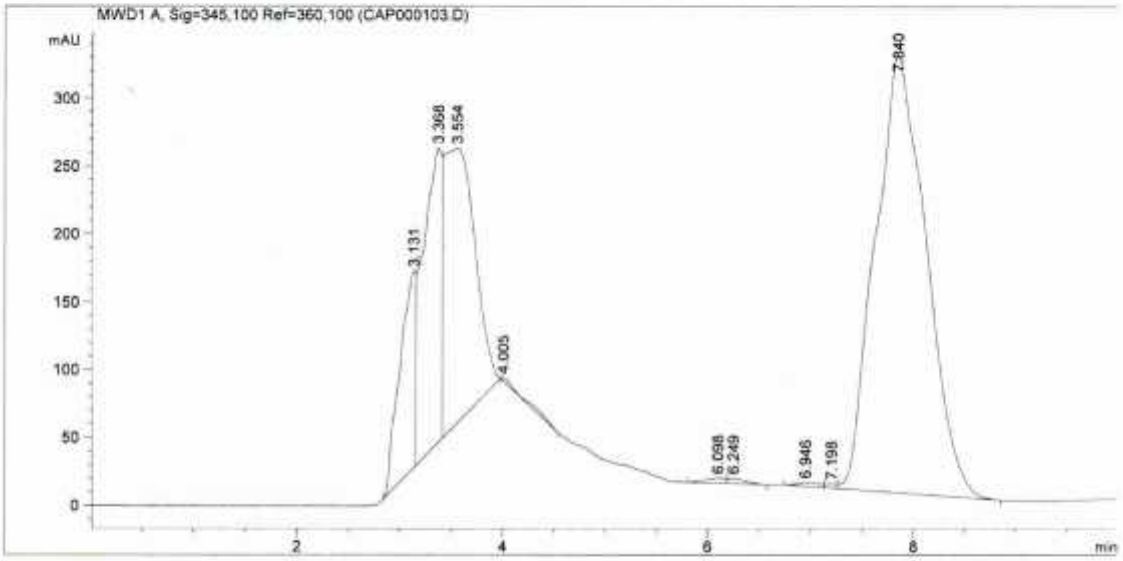
Figure 7.6: Chromatograms for *Eugenia caryophyllata* after HPLC analysis

APPENDIX XIV

Chromatographic Fingerprint of *Zanthoxylum zanthoxyloides*

Data File C:\CHEM32\1\DATA\CAP000103.D
Sample Name: Far sp

Acq. Operator : Maxwell
Acq. Instrument : CSRPM HPLC Location : Vial 14
Injection Date : 4/2/2014 3:57:37 PM
Inj Volume : 20.0 µl
Acq. Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 3:54:37 PM by Maxwell
(modified after loading)
Analysis Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 4:08:19 PM by Maxwell
(modified after loading)
Sample Info : Far sp



Fraction Information

Fraction collection off

No Fractions found.

Area Percent Report

Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

CSRPM HPLC 4/2/2014 4:08:29 PM Maxwell Page 1 of 2

Figure 7.7: Chromatograms for *Zanthoxylum zanthoxyloides* after HPLC analysis

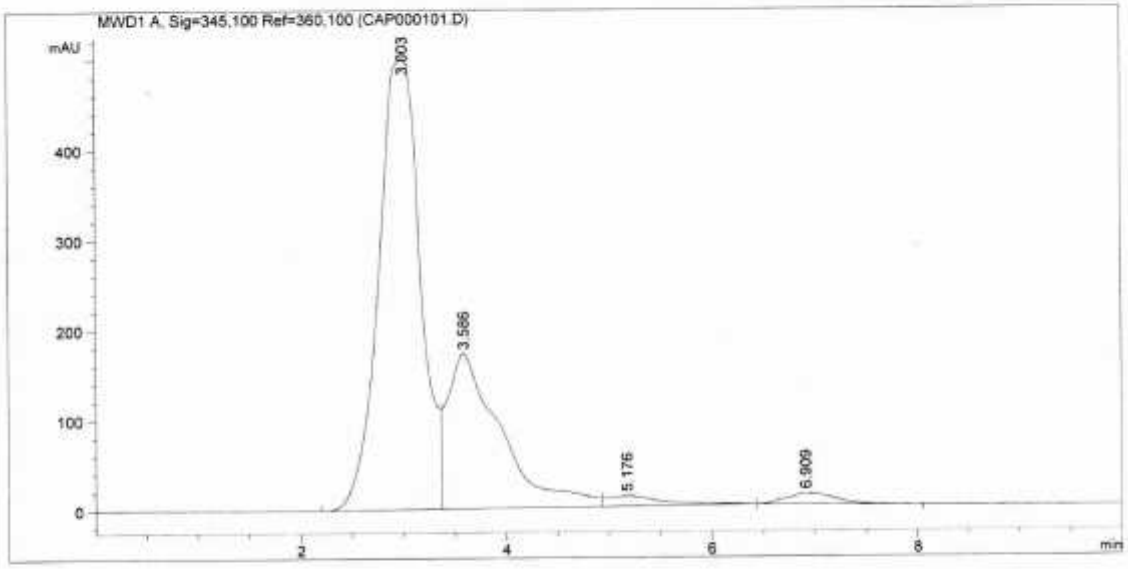
APPENDIX XV

Chromatographic Fingerprint of *Psidium guajava*

Data File C:\CHEM32\1\DATA\CAP000101.D
Sample Name: Psi sp

Acq. Operator : Maxwell
Acq. Instrument : CSRPM HPLC Location : Vial 12
Injection Date : 4/2/2014 3:30:01 PM Inj Volume : 20.0 µl

Acq. Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 3:24:37 PM by Maxwell
(modified after loading)
Analysis Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 3:40:42 PM by Maxwell
(modified after loading)
Sample Info : Psidium sp



Fraction Information

Fraction collection off

No Fractions found.

Area Percent Report

Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

CSRPM HPLC 4/2/2014 3:40:52 PM Maxwell Page 1 of 2

Figure 7.8: Chromatograms for *Psidium guajava* after HPLC analysis

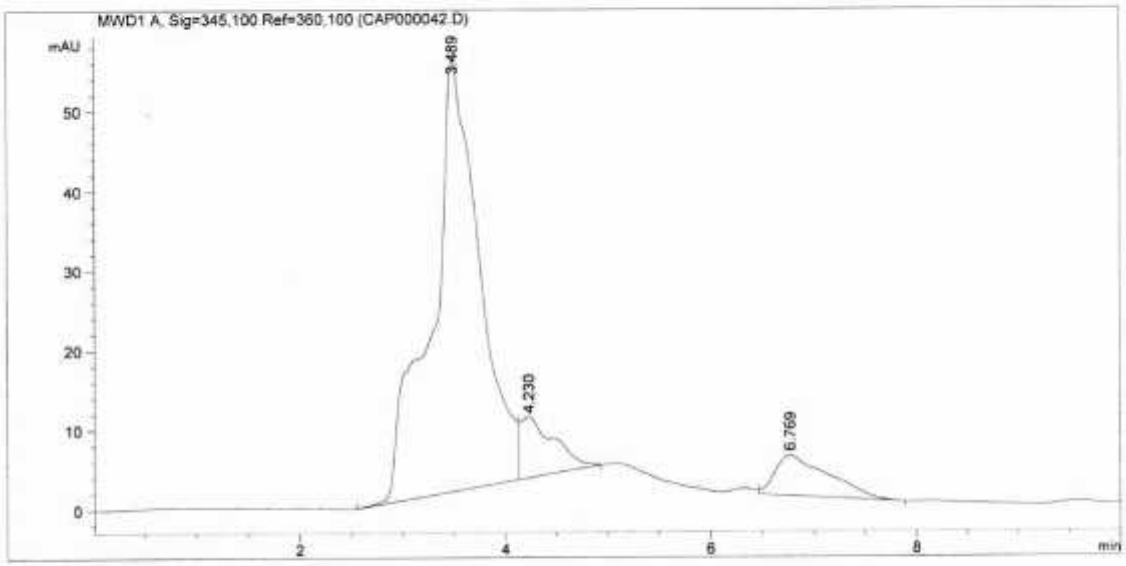
APPENDIX XVI

Chromatographic Fingerprint of *Tridax procumbens*

Data File C:\CHEM32\1\DATA\CAP000042.D
Sample Name: Tri 2

Acq. Operator : Maxwell
Acq. Instrument : CSRPM HPLC Location : Vial 2
Injection Date : 1/22/2014 4:37:13 PM
Inj Volume : 10.0 µl

Acq. Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 1/22/2014 4:33:31 PM by Maxwell
(modified after loading)
Analysis Method : C:\CHEM32\1\DATA\CAP000042.D\DA.M (RUTIN.M, From Data File)
Last changed : 1/22/2014 4:48:04 PM by Maxwell
Sample Info : analysee



Fraction Information

Fraction collection off

No Fractions found.

Area Percent Report

Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

CSRPM HPLC 4/2/2014 3:10:52 PM Maxwell Page 1 of 2

Figure 7.9: Chromatograms for *Tridax procumbens* after HPLC analysis

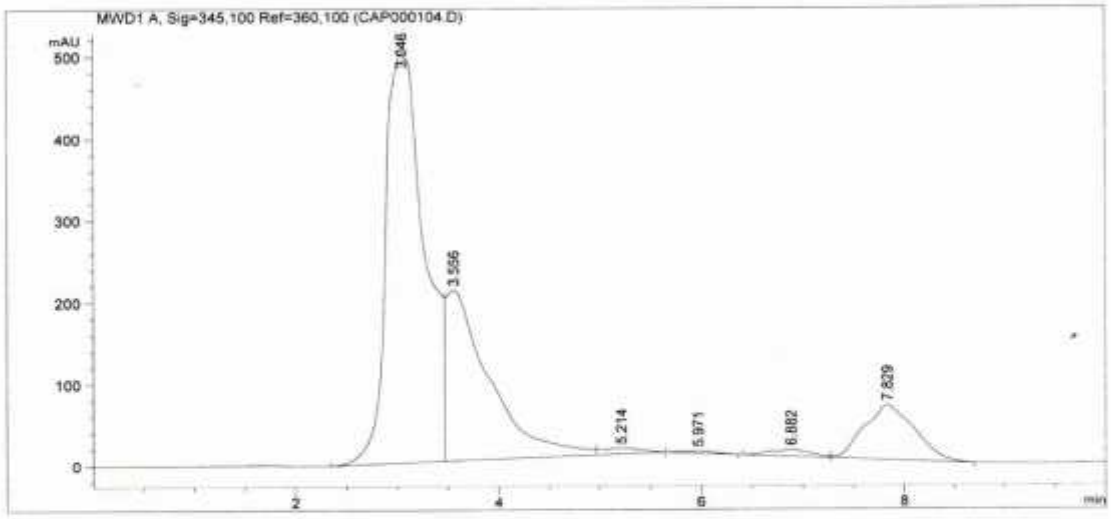
APPENDIX XVII

Chromatographic Fingerprint for *EAF-2011* at Baseline

Data File C:\CHEM32\1\DATA\CAP000104.D
Sample Name: Ont 1 sp

Acq. Operator : Maxwell
Acq. Instrument : CSRPM HPLC Location : Vial 15
Injection Date : 4/2/2013 4:10:10 PM Inj Volume : 20.0 µl

Acq. Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 4:08:31 PM by Maxwell
(modified after loading)
Analysis Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 4:20:52 PM by Maxwell
(modified after loading)
Sample Info : Ont sp



Fraction Information

Fraction collection off

No Fractions found.

Area Percent Report

Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

CSRPM HPLC 4/2/2014 4:21:00 PM Maxwell Page 1 of 2

Figure 7.10: Chromatographic fingerprint for the ointment *(EAF-2011)* at the baseline after HPLC analysis

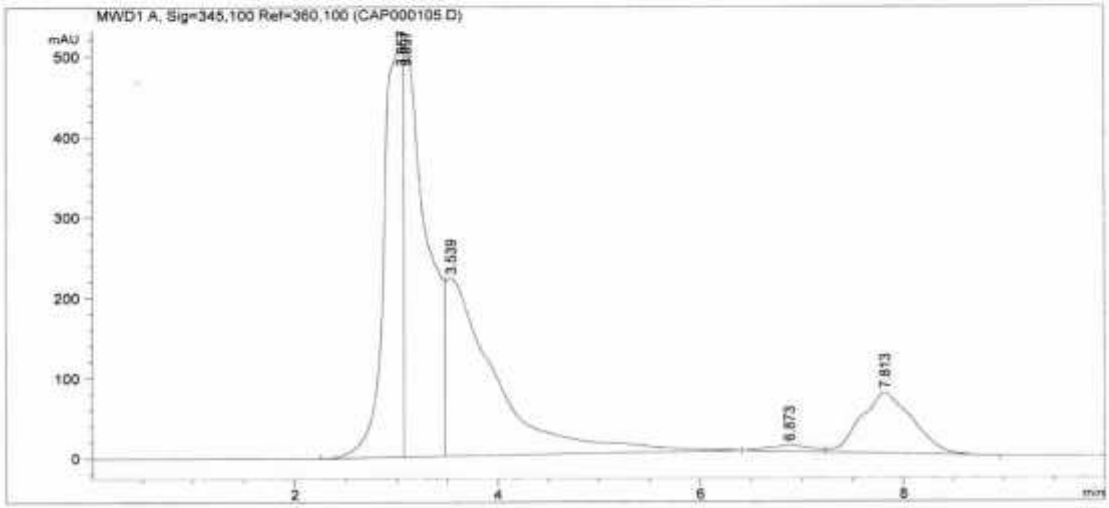
APPENDIX XVIII

Chromatographic Fingerprint of the *EAF-2011* at Month 6

Data File C:\CHEM32\1\DATA\CAP000105.D
Sample Name: Ont 2 sp

Acq. Operator : Maxwell
Acq. Instrument : CSRPM HPLC Location : Vial 16
Injection Date : 4/8/2013 4:22:33 PM Inj Volume : 20.0 µl

Acq. Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 4:21:03 PM by Maxwell
(modified after loading)
Analysis Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 4:33:15 PM by Maxwell
(modified after loading)
Sample Info : Ont 2 sp



Fraction Information

Fraction collection off

No Fractions found.

Area Percent Report

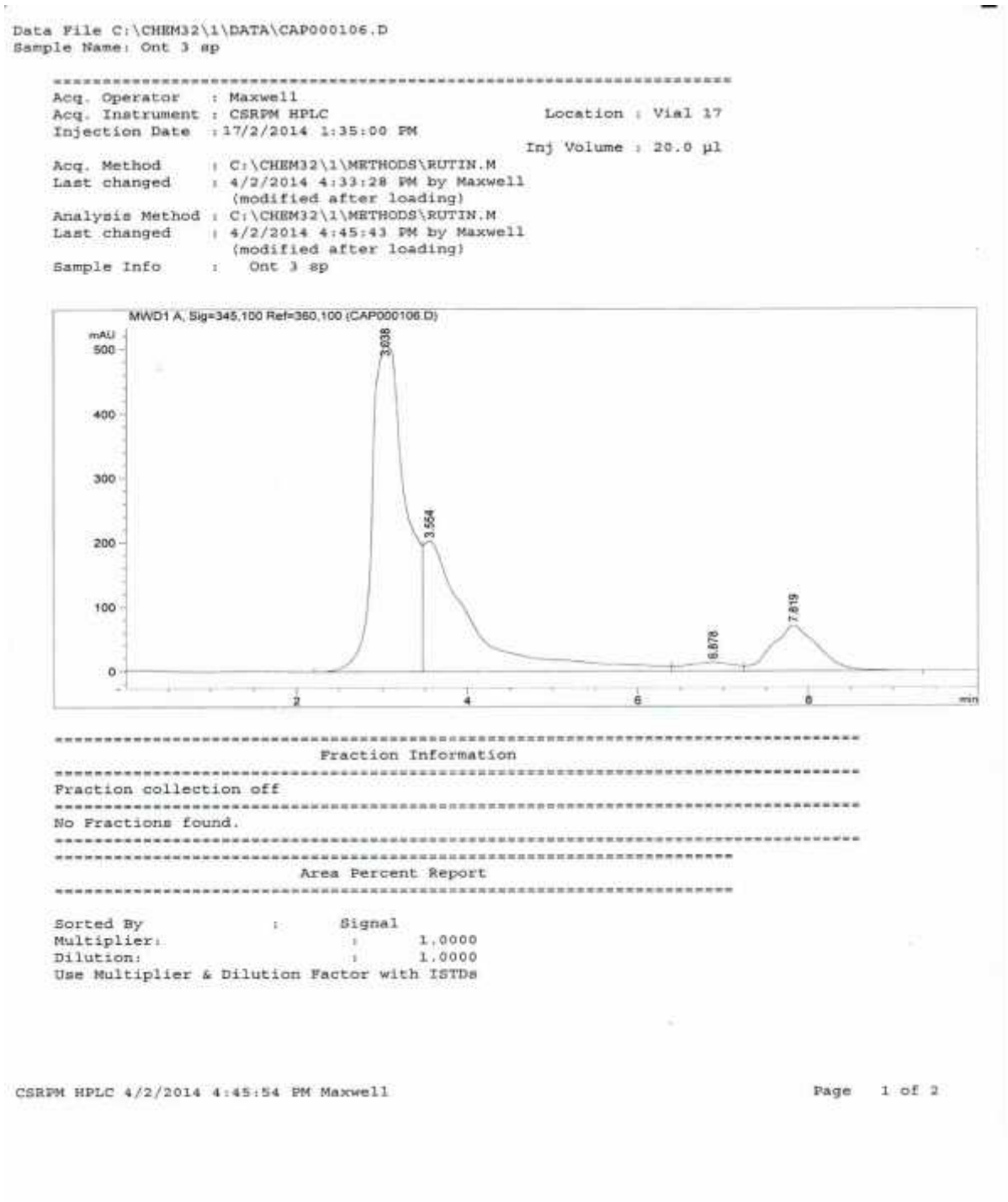
Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

CSRPM HPLC 4/2/2014 4:33:25 PM Maxwell Page 1 of 2

Figure 7.11: Chromatographic fingerprint for the ointment *(EAF-2011)* at month 6 after HPLC analysis

APPENDIX XIX

Chromatographic Fingerprint of the *EAF-2011* at Month 12



Data File C:\CHEM32\1\DATA\CAP000106.D
Sample Name: Ont 3 sp

Acq. Operator : Maxwell
Acq. Instrument : CSRPM HPLC Location : Vial 17
Injection Date : 17/2/2014 1:35:00 PM
Inj Volume : 20.0 µl

Acq. Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 4:33:28 PM by Maxwell
(modified after loading)
Analysis Method : C:\CHEM32\1\METHODS\RUTIN.M
Last changed : 4/2/2014 4:45:43 PM by Maxwell
(modified after loading)
Sample Info : Ont 3 sp

Fraction Information

Fraction collection off

No Fractions found.

Area Percent Report

Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

CSRPM HPLC 4/2/2014 4:45:54 PM Maxwell Page 1 of 2

Figure 7.12: Chromatographic fingerprint for the ointment *(EAF-2011)* at month 12 after HPLC analysis

APPENDIX XX

Pictures for Some Participants in the Clinical Study

Samples photographs from some participants who received the herbal treatment are shown below. The pictures were obtained after their consent.

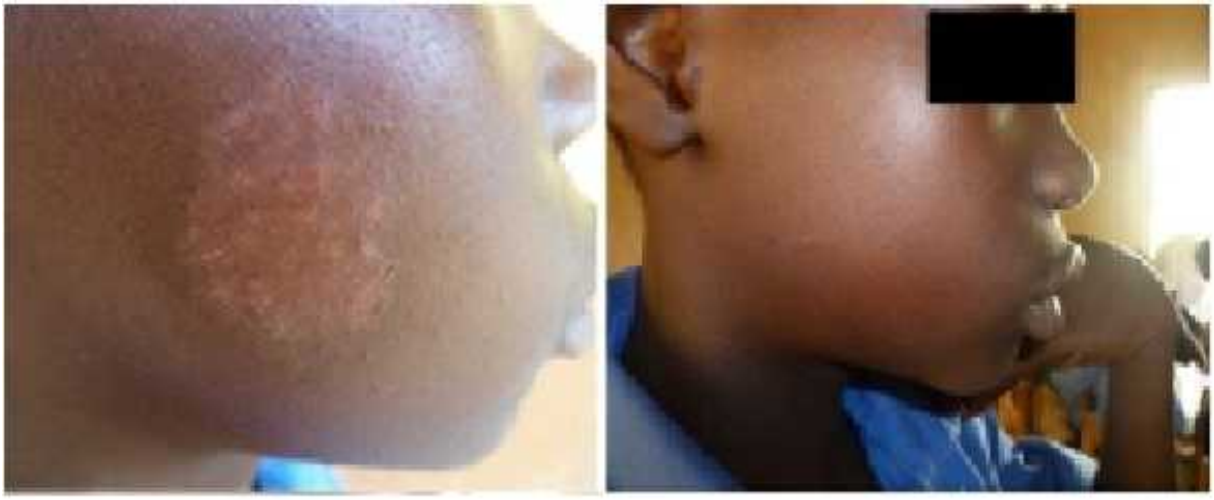


Figure 7.13: A participant with a *Tinea coporis* before treatment shown on the left and after the treatment shown on the right.

Figure 7.14: A participant with *Tinea coporis* before treatment (left) and at the end of the study (right)

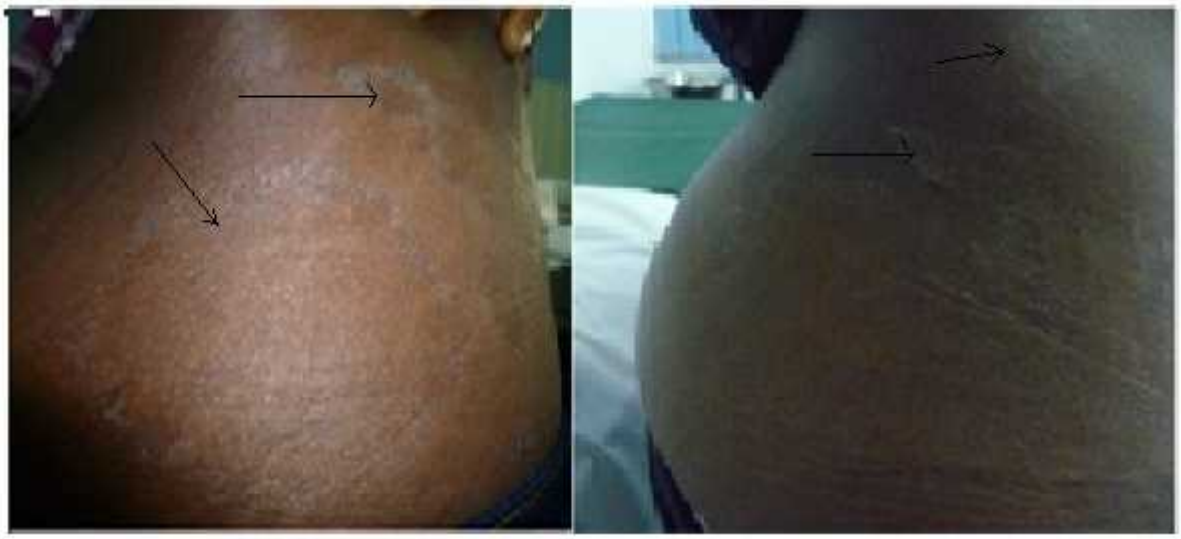


Figure 7.15: A participant with *Tinea coporis* on the gluteus shown by the arrows before treatment (left) and at the end of the study (right)

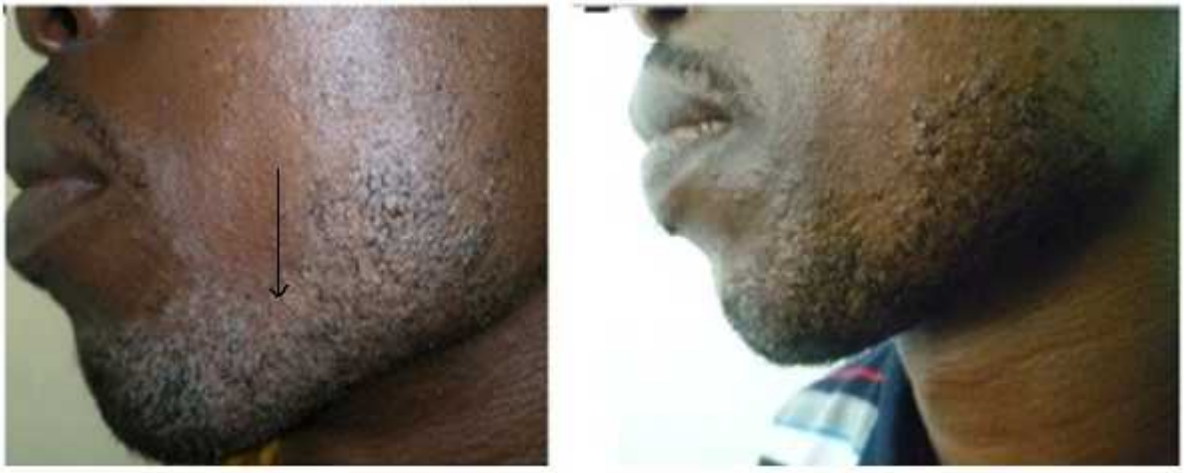


Figure 7.16: A participant with a *Tinea barbae* before treatment indicated by the arrow (left) and at the end of the study (right)

Figure 7.17: A participant showing *Tinea coporis* with kerions and a secondary infection indicated by the arrow before treatment (left) and at the end of the study (right)

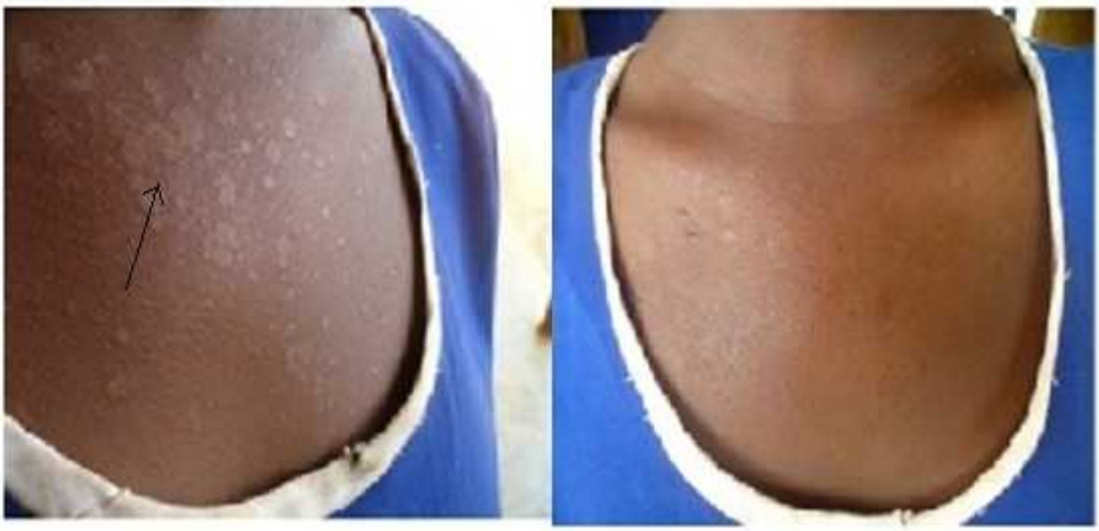


Figure 7.18: A participant with *Pityriasis versicolor* before treatment (left) shown by the arrow and at the end of the study on the right.

APPENDIX XXI

Sample Photomicrographs from the Organs of Rats after the Chronic Toxicity Study

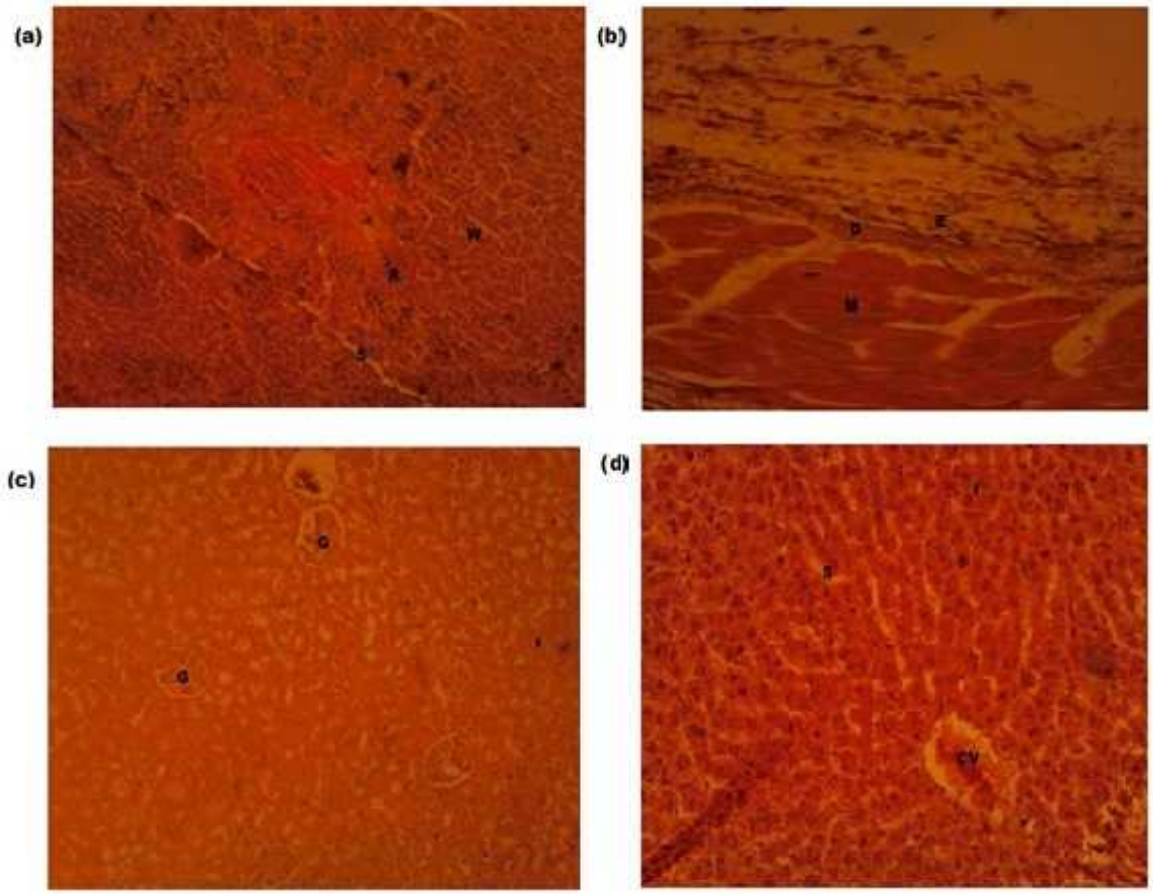


Figure 7.19: Photomicrographs for the spleen (a), skin (b), kidney (c) and liver (d) of rats in the control group. Animals in this group did not receive any treatment. (CV-central vein, D-dermis, E-epidermis G-glomerulus, I-interstitium, R-red pulp, W-white pulp, S-sinusoids and M- muscle tissue)

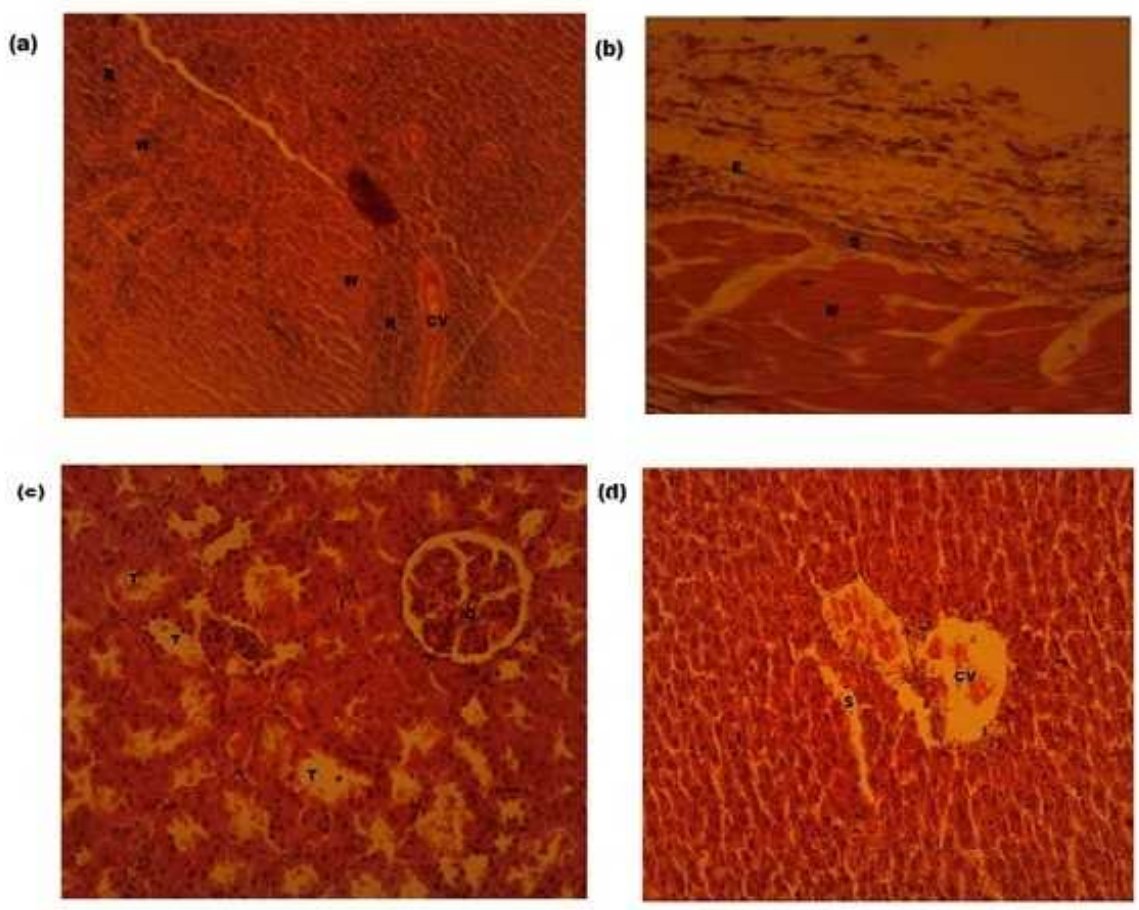


Figure 7.20: Photomicrographs for the spleen (a), skin (b), kidney (c) and liver (d) of rats in the 2% ($^{w}/_{w}$) *EAF-2011*. (CV-central vein, D-dermis, E-epidermis G-glomerulus, I-interstitium, R-red pulp, T-tubules, W-white pulp, S-sinusoids and M- muscle tissue). Magnification x400

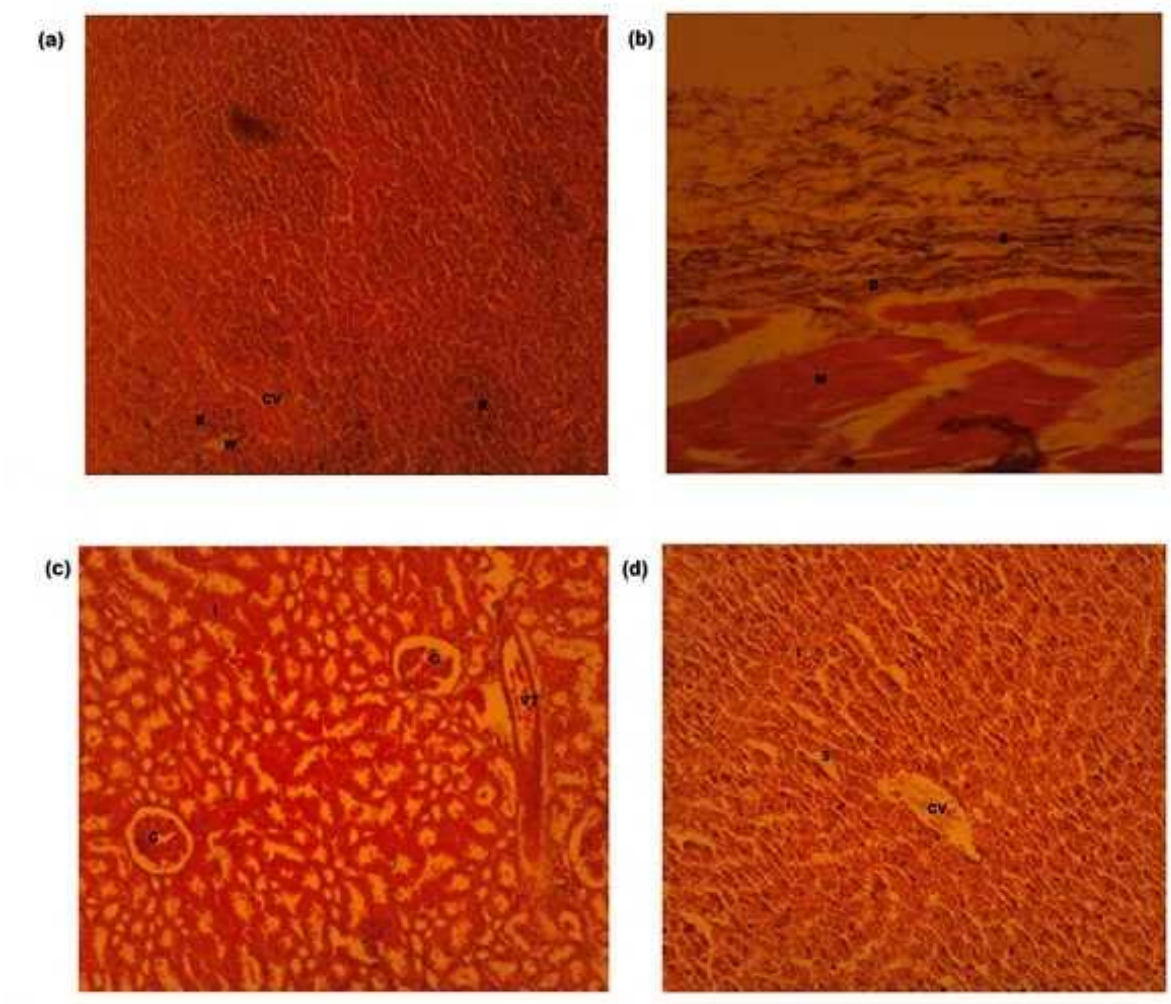


Figure 7.21: Photomicrographs for the spleen (a), skin (b), kidney (c) and liver (d) of rats treated with 5% ($^{w}/_{w}$) *EAF-2011*. (CV-central vein, D-dermis, E-epidermis G-glomerulus, I-interstitium, R-red pulp, W-white pulp, S-sinusoids and M- muscle tissue, VT-vascular tissue). Magnification x400

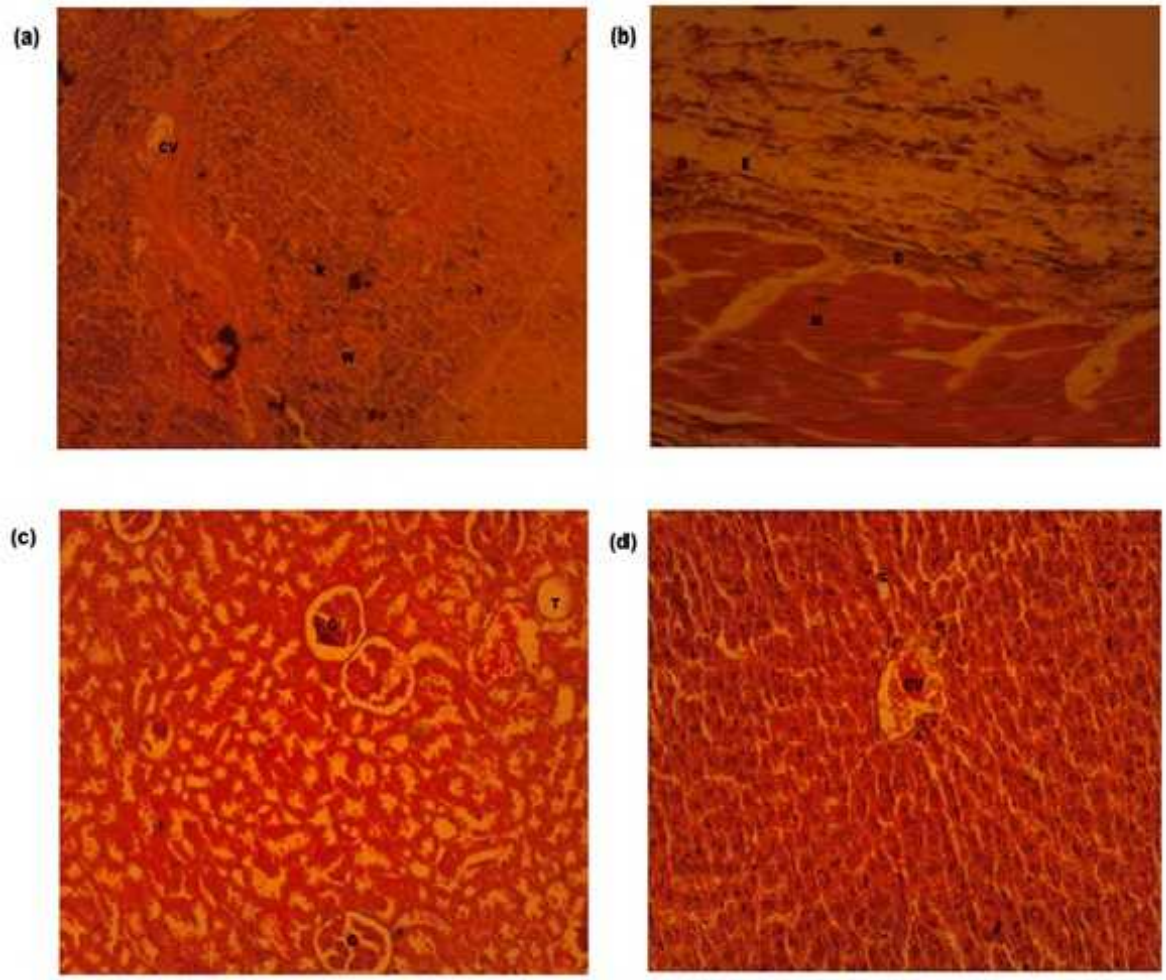


Figure 7.22: Photomicrographs for the spleen (a), skin (b), kidney (c) and liver (d) of rats treated with 10% ($^{w}/_{w}$) *EAF-2011*. (CV-central vein, D-dermis, E-epidermis G-glomerulus, I-interstitium, R-red pulp, T-tubules, W-white pulp, S-sinusoids and M- muscle tissue). Magnification x400

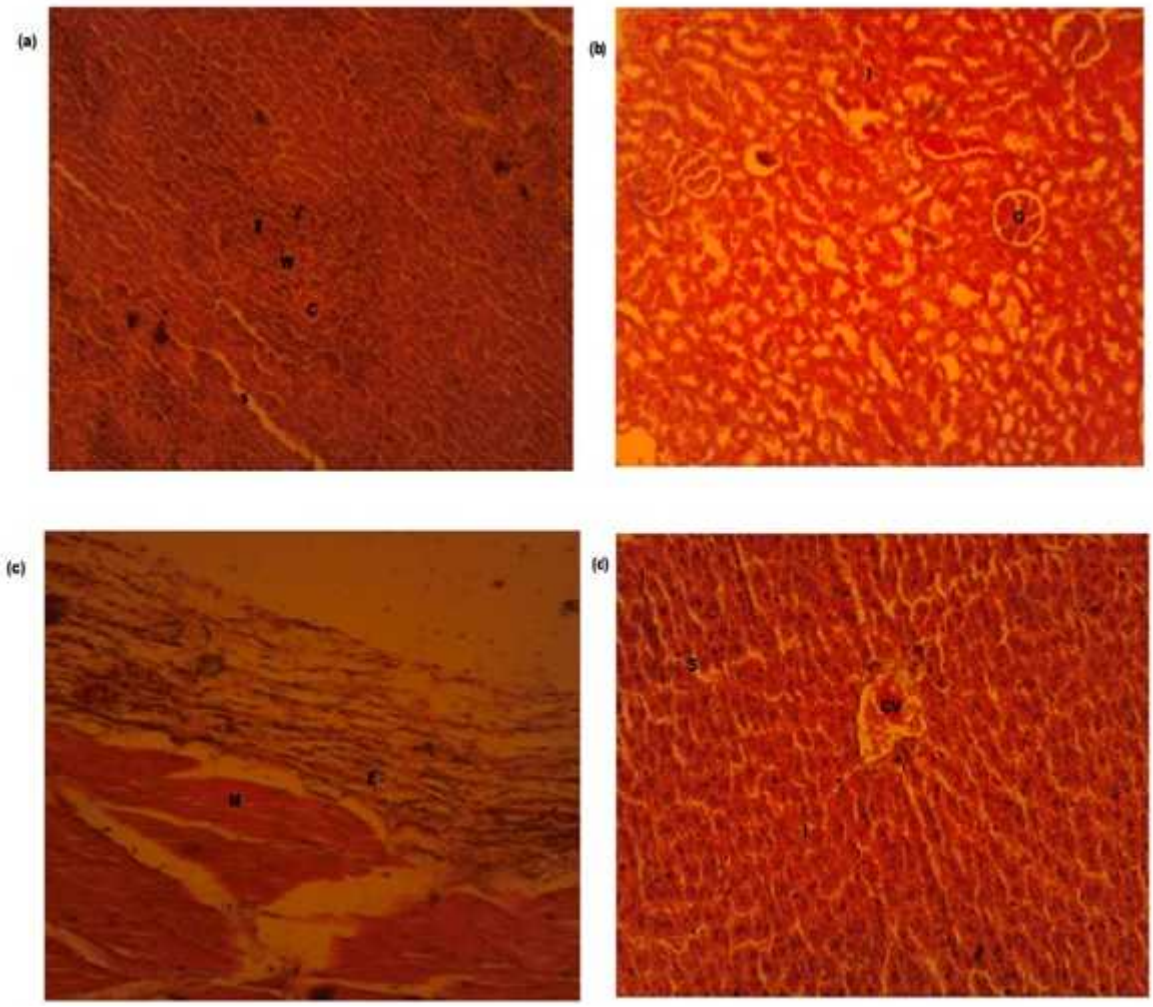


Figure 7.23: Photomicrographs for the spleen (a), kidney (b), skin (c) and liver (d) of rats treated with the reformulated herbal product. (C/CV-central vein, E-epidermis G-glomerulus, I-interstitium, R-red pulp, W-white pulp, S-sinusoids and M- muscle tissue). Magnification x400