



Appraisal of the Constituent Plant Materials in a Ghanaian Antifungal Herbal Product; An in vitro Interactive Combination Analysis and a Pilot Clinical Study to Determine Efficacy.

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Abstract

Evidence based use of herbal medicines has a positive implication for both users and society. In this study, component plant materials of a traditional Ghanaian polyherbal skin product comprising *Eugenia caryophyllata*, *Zanthoxylum zanthoxyloides* *Tridax procumbens*, *Psidium guajava* and *Alchornea cordifolia* and used in the management of superficial skin infections was evaluated to establish their contribution to the overall therapeutic activity of the product. Each of the five (5) plants was subjected to an *in vitro* antimicrobial assay using the microtitre broth technique followed by an interactive combination assay for plants demonstrating noteworthy antimicrobial activity ($MIC \leq 1.0$ mg/ml). Test strains included *Staphylococcus aureus*, *Candida albicans*, *Trichophyton rubrum*, *Epidermophyton floccosum* and *Microsporum canis*. *Eugenia caryophyllata*, *Zanthoxylum zanthoxyloides* and *Alchornea cordifolia* showed better activity than *Psidium guajava*, *Tridax procumbens* and the Total Crude Extract (combination of the 5 extracts). The binary combination of *Eugenia caryophyllata* and *Alchornea cordifolia* indicated synergistic and additive activity against all the test strains. An improved biological activity was also observed when a mixture of the two (2) plants at a ratio of *Eugenia caryophyllata* 60% (w/w) and *Alchornea cordifolia* 40% (w/w) was assayed. A follow up pilot clinical study established that this new recipe was clinically effective but of lower therapeutic effect compared to the original product. In conclusion, the original formulation of the product may be preferred because of the shorter duration of treatment which reduces the risk of harms and cost of treatment.

Keywords: Antimicrobial screening; Dermatophytes; Herbal medicine; Interactive combination study; Polyherbal product; Traditional Ghanaian Medicine

Introduction

The use of herbal medicines and their products among the Ghanaian populace is on the increase. Generally, socio-religious compatibility, affordability and accessibility have been well discussed as factors accounting for this preference. The absence of toxicity and better efficacy are also factors that have been reported as attributable for this trend in usage, although these two

theories are very contentious. The related issues about the toxicity and unproven efficacy of herbal medicines has increased the calls for the scientific validation of all herbals. Practitioners therefore have an obligation in the formulation and use of herbal medicines; especially when the products are made from two or more plant materials, that there is a reliance on scientific evidence for such multiple combinations [1].

The 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 and existence directly linked to the conservation of their flora [2]. The con-

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ervation of medicinal plants is thus a very important 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). The protection of such resources is critical for a country like Ghana where about 135,395 hectares of forest cover is lost each year through unregulated human activities [3].

The implications for users of such multi-component herbal products are also 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 [4]. A clear demonstration of the benefits of such combinations must therefore 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 reduced cost of products.

The practice of herbal medicine in Ghana is undergoing a lot of modernisation to increase the rational use of herbal medicines through the scientific validation of the practice and its products. This study reports on one of these steps at ensuring that herbal products are evidenced based. The product under study is a Ghanaian polyherbal product and has been used for the management of superficial mycoses for more than 20 years without any data about it. The formulation which originated from a Ghanaian traditional healer is prepared as a combination of five (5) plants: *Eugenia caryophyllata*, *Zanthoxylum zanthoxyloides*, *Tridax procumbens*, *Psidium guajava* and *Alchornea cordifolia*. The product has been previously evaluated clinically in a randomised control trial with a 10% (w/w) concentration of the product showing better activity than Whitfield's ointment [5]. However, the use of multiple plant materials for the product was of great concern because of the issues currently being raised about the conservation of botanicals, the cost to users and ultimately its safety and efficacy profile. In view of this, an *in vitro* antimicrobial assay was undertaken to establish the contribution of each plant to the overall biological activity of the product and propose a new recipe with similar or better activity than the original formulation. This recipe was then tested in a pilot human trial to establish its efficacy and safety.

Materials and Methods

Preparation of Plant Materials

The plants used in the original product comprised the dried leaf of *Alchornea cordifolia* (Schum. & Thonn.) Muell.Arg. (Eu-

porbiaceae) 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). All plant parts were dried under shade for two weeks at an ambient temperature after authentication by a taxonomist at the Centre for Plant Medicine, Mampong-Akwapem and voucher specimen deposited at the institution's herbarium (Table 1.0). Hydro-ethanolic extracts of the materials were obtained by macerating 1.0 kg of the powdered plant material in 5.0 litres of 70% (v/v) ethanol for 3 days and then filtering. Ethanol was recovered using the Rotary evaporator and the remaining fluid extract lyophilised to obtain a dry powder. The original formulation for the product was also obtained by combining the five (5) plant materials using a proprietary formula to obtain a combination labelled as the Total Crude Extract (TCE).

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 [6]. The microorganisms chosen for analysis were selected based on their dermatological relevance. Microorganism strains that were used are of the American Type Culture Collection (ATCC) strains. Three dermatophytes and one yeast organism with dermatological importance were selected for the assays and included: *Trichophyton rubrum* (ATCC 10218), *Epidermophyton floccosum* (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.

Determination of MIC of Plant Extracts and Total Crude Product

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 (TCE). Using aseptic manipulation, 100 μ l of Phosphate Buffer 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 inoculum size of 1×10^6 colony forming units/ml (CFU/ml) were introduced. A volume of 100 μ l 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 suitable conditions. Ketoconazole (Sigma

Aldrich, USA) was used as the reference agent for the fungal strains and Ciprofloxacin (Sigma Aldrich, USA) for the bacterial strain.

Detection of Microbial Activity

Testing for bacterial and fungal growth after incubation was done by adding 40 μ l (0.04 % w/v) of *p*-iodonitrotetrazolium chloride (INT) (Sigma Aldrich, USA) to each well of the plate. The plates were subsequently incubated again for 2-4 hrs for the bacterial strain and 24-36 hrs 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.

Table 1 List of Plants used in the Product, Local Ghanaian Name, Common Names and Voucher Specimen Number

Plant Material	Local Ghanaian Name	Common Name	Voucher Specimen Number
<i>Alchornea cordifolia</i> (Schum.&Thonn.) Muell.Arg. (Euphorbiaceae)	Akan: <i>Ogyama/Agyama</i>	Christmas tree	CSRPM 368
(Thumb) (Myrtaceae)	Ga: Gbo Ewe: Avovlo/Ahame Akan: Pepre	Cloves	CSRPM 001CM
<i>Eugenia caryophyllata</i> (Linn) (Myrtaceae)	Akan: <i>Oguawa/Eguaba</i>	Guava	CSRPM 50
<i>Psidium guajava</i> (Linn) (Myrtaceae)	Ga: <i>Gowa</i> Ewe: Goa Ewe: Fomiz-ibge	Coat but-ton	CSRPM 256
<i>Tridax procumbens</i> (Linn) (Asteraceae)	Akan: <i>Okanto</i> Ga: <i>Haatso</i> Ewe: <i>Xesti</i>	Candlewood,	CSRPM 330
<i>Zanthoxylum zanthoxyloides</i> (Lam) (Rutaceae)		Senegal prickly-ash	

Selection of Plant Extracts with Significant Antimicrobial Activity

Individual plant extracts showing significant antimicrobial activity defined as MIC of < 1.00 mg/ml against half of the test microorganisms were then selected for an interactive combination study.

Interactive Combination Studies

The potential synergistic, additive, non-interactive (indifferent) or antagonistic interaction between the selected plants extracts

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 (\sum 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 \sum FIC = FIC (i) + FIC (ii). Results were classified as either synergistic (≤ 0.50), additive (0.50-1.00), indifferent (>1.00-4.00) or antagonistic (>4.00) [7].

The combinations with notable interactions, defined as synergistic activity for more than half of the test, 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 interactions. 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 [8]. Positive and negative controls were included in all assays which were also undertaken in duplicate and the mean values noted.

Pilot Clinical Study of the Reformulated Product

Interventional Products: Based on the outcome of the *in vitro* interactive study, a reformulated product with a new recipe was developed and labelled *RF-2016*. The strength of this product was 5% (w/v). The reformulated product *RF-2016* served as the test product and the original formulation *EAF-2011* as the control.

Ethical Considerations and Trial Design

The protocol employed for the study, the consent form and the patient information sheet were reviewed and approved by the ethics committee for human research of the Centre for Plant Medicine Research, Mampong-Akuapem prior to trial initiation. The trial was performed in accordance with the Declaration of Helsinki and Good Clinical Practice (WHO, 2001). Written in-

formed consent from every study subject was obtained prior to the trial-related activities; the consent forms were retained by the investigators.

A pilot trial in which subjects were randomised in a ratio of 2:1 for the 5% (w/w) RF-2016 and 10% (w/w) EAF-2011 respectively was undertaken. Randomisation was achieved by making participants pick (without replacing), from 15 folded papers with 10 labelled for 5% (w/w) RF-2016 and 5 labelled for 10% (w/w) EAF-2011. Allocation was done to attain this ratio at the end of a 15th recruitment.

Inclusion and Exclusion Criteria

Participants included in the study were males and females between the ages of 8 and 45 years, clinically diagnosed with any of the superficial fungal infections. Exclusion criteria also comprised any individual who had been diagnosed kidney or liver dysfunction, pregnant women, immunocompromised patients and individuals on any orthodox medications that had the potential to affect the outcome of the trial such as corticosteroids and immunomodulating agents. Acutely ill-individuals were also exempted from the study.

Assessment of Efficacy and Drug Related Toxicity

Primary assessment of effectiveness and classification of therapeutic response for each participant was done using a clinical score. The assessment employed 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. In this case pruritus, desquamation, erythema and the presence of vesicular/popular features were used. Clinical effectiveness of the products was defined as TSSS of 0 i.e. the absence of any signs and symptoms from the participants. Adverse effects that may be associated with the product was monitored using the WHO adverse reaction checklist [9].

Results

Minimum Inhibitory Concentrations of Plant Extracts Screened

All 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 2.0). The five (5) plant extracts also failed to show any significant activity against *S. aureus*. However, the activity demonstrated by *Alchornea cordifolia* (MIC: 1.563 mg/ml),

Zanthoxylum zanthoxyloides (MIC: 1.563 mg/ml) and *Eugenia caryophyllata* (MIC: 1.563 mg/ml) was better than Total Crude Extract (MIC: 3.125 mg/ml) used in the formulation of the final product.

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 2.0. Results for the binary and a triple combination are also reported in Table 3.0 together with their sum of Fractional Inhibitory Concentration (\sum FIC) for the binary mixtures.

The combination of *Eugenia caryophyllata* and *Zanthoxylum 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 \sum FIC of 4.0. *Z. zanthoxyloides* and *A. cordifolia* were antagonistic in effect when tested against *C. albicans* (\sum FIC: 59.94) and non-interactive against the four (4) fungi and bacterial strains (Table 2.0). The combination of *A. cordifolia* and *E. caryophyllata* was synergistic in effect against *S. aureus* and additive against *M. canis*, *C. albicans* and *E. floccosum* from the \sum FIC (Table 3.0). A non-interactive effect was noted when the combination was tested against *E. floccosum*. 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.

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. The combination demonstrated synergistic activity against *S. aureus*, *C. albicans*, *M. canis* and *T. rubrum* (Figure 1.0). An additive effect was recorded in the test against *E. floccosum*. The other combinations were also synergistic or additive in effect when tested against *C. albicans*. 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. floccosum* and the additive activity of *A. cordifolia* 60% (w/w) with *E. caryophyllata* 40% (w/w) against *M. canis*.

Table 2 Average MIC (mg/ml) for the plant extracts screened using the micro-dilution assay

Plant Extract	<i>T. rubrum</i>	<i>E. floccosum</i>	<i>M. canis</i>	<i>C. albicans</i>	<i>S. aureus</i>
<i>A. cordifolia</i>	3.125	0.0781*	0.0781*	0.0781*	1.563
<i>T. procumbens</i>	25.00	3.125	3.125	1.563	6.25
<i>Z. zanthoxyloides</i>	6.250	0.0391*	0.0781*	0.0391*	1.563
<i>P. guajava</i>	3.125	0.078*	3.125	1.563	6.25
<i>E. caryophyllata</i>	0.0781*	0.0391*	0.0781*	0.0781*	1.563
Ketoconazole	15 × 10 ⁻²	85 × 10 ⁻³	56 × 10 ⁻³	1.05 × 10 ⁻²	
Ciprofloxacin	-	-	-	-	25 × 10 ⁻³
Total Crude Extract	1.563	3.125	0.0781*	3.125	3.125

* Indicates plant extracts with significant antimicrobial activity

Table 3 MIC (mg/ml) [ΣFIC] for binary combinations of *A. cordifolia*, *Z. zanthoxyloides* and *E. caryophyllata* at a ratio of 1:1

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

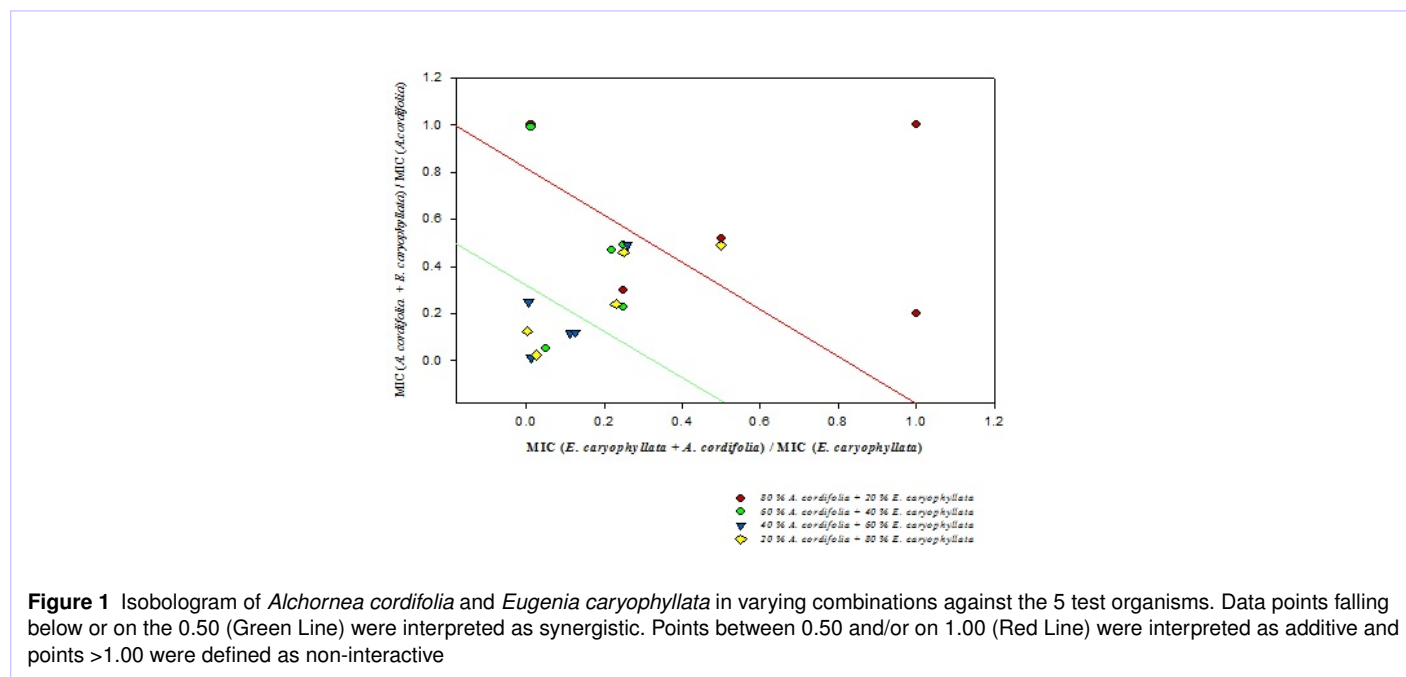


Figure 1 Isobologram of *Alchornea cordifolia* and *Eugenia caryophyllata* in varying combinations against the 5 test organisms. Data points falling below or on the 0.50 (Green Line) were interpreted as synergistic. Points between 0.50 and/or on 1.00 (Red Line) were interpreted as additive and points >1.00 were defined as non-interactive

Pilot Clinical Study

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. Demographical data of subjects is summarised as table 4.0.

Table 4 Demographical data of participants involved in the study

	Control (10% <i>EAF-2011</i>)	Reformulated Product (5% <i>RF-2016</i>)
Age (SD)	14.40 (3.96)	11.50 (4.31)
Sex		
Males (%)	4 (80)	8 (80)
Females (%)	1 (20)	2 (20)

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. This group had 5 (100.0 %) of the treated subjects achieving complete cure by day 56 compared to 1 (10.0 %) for

the reformulated product. The percentage cure for the latter increased to 6 (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 figure 2.0. The study also had two (2) participants from the reformulated product dropping out due to loss on follow up.

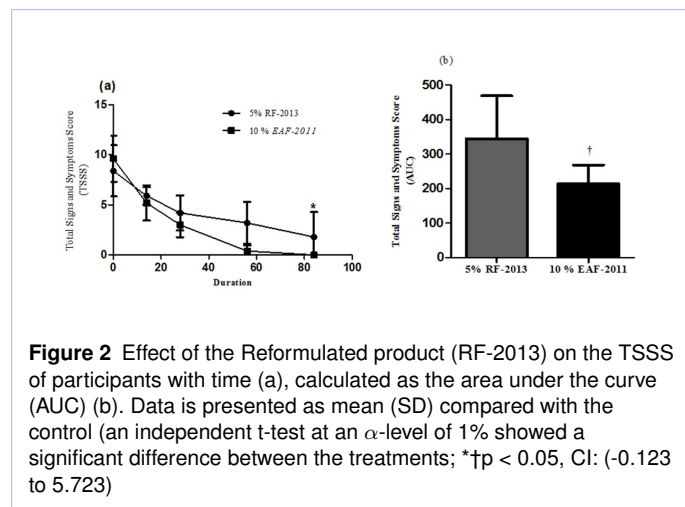


Figure 2 Effect of the Reformulated product (RF-2013) on the TSSS of participants with time (a), calculated as the area under the curve (AUC) (b). Data is presented as mean (SD) compared with the control (an independent t-test at an α -level of 1% showed a significant difference between the treatments; *†p < 0.05, CI: (-0.123 to 5.723)

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.

Discussion

Although herbal medicines continue to receive praise for their potential in meeting the health needs of developing countries, there are still valid concerns about these health products. Underlying these concerns is the lack of valid scientific evidence to back their therapeutic use. This situation is rife in developing countries like Ghana where the evidence for use of most botanical agents is still based on folklore. A shift towards an evidenced-based practice will be very beneficial to all stakeholders.

This report sought to validate the recipe for the formulation which is used to treat skin infections especially those of fungal origin. 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. *Eugenia caryophyllata* proved most efficacious with significant activity against all the fungal strains tested. *Zanthoxylum*

zanthoxyloides and *Alchornea cordifolia* also showed significant activity. These findings confirm other documented evidence on the antimicrobial activity of the three (3) plants [10–12].

On the contrary, although there have been reports indicating the antifungal activity of *Psidium guajava* and *Tridax procumbens* against the test strains [13, 14], in this report the activity shown could not be described as significant; Typical factors such as the variability in chemical constituents can be responsible for this difference in activity.

Significantly, the *in vitro* activity of the individual 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 [15, 16].

Minimum inhibitory concentrations reported in Table 2.0 also showed the binary combinations as generally having better activity than that of the triple combination of *Eugenia caryophyllata*, *Zanthoxylum zanthoxyloides* and *Alchornea cordifolia*. 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*.

The therapeutic effect for the combination of *Eugenia caryophyllata* and *Alchornea cordifolia* was also demonstrated to be optimum when the two plant extracts are combined in the ratio of 60 % (w/w) and 40 % (w/w) respectively. This combination proved most efficacious as shown in Figure 1.0. The 2 plants at that ratio was synergistic against *Staphylococcus aureus*, *Candida albicans*, *Microsporum canis* and *Trichophyton rubrum* and could be recommended as the most suitable combination despite showing only additive effect against *Epidermophyton floccosum*.

The mixture of *Alchornea cordifolia* 40 % (w/w) and *Eugenia caryophyllata* 60 % (w/w) was thus proposed as the new recipe for the product based on the synergistic activity demonstrated against most of the microorganisms tested.

In the pilot clinical study of this new formulation, participants attaining the primary outcome of complete cure for the 10 % (w/w) EAF-2011 was 5 (100.0 %) compared to the 6 (60.0 %) for the 5 % (w/w) RF-2016. This cure rate for RF-2016 increases to 75.0 % when analysis is done without the withdrawals from the study. The change in TSSS is indicated by the plot (Figure 2.0a) and the area under the curve (Figure 2.0 b). However, the time taken to achieve the primary outcome (Figure 2.0a) will make the 10 % (w/w) EAF-2011 the preferred treatment. The

shorter time of exposure to the therapeutic agent reduces the risk of harms and the cost of treatment for patients.

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). The therapeutic effect observed could be concentration dependent as observed in the clinical trial of the original product (*EAF-2011*) 5. The results of this interactive study indicate the potential for an improvement of the product when the combination of *Eugenia caryophyllata* 60 % (w/w) and *Alchornea cordifolia* 40 % (w/w) is used as the new recipe for the formulation.

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