Antifungal Effects of Combined Extracts of *Euphorbia abyssinica* and *Coleus species*

Abstract

Although, different plant extracts have frequently been used in folklore medicines to cure different ailments, the hidden truth behind their activity and efficacy is still to be fully unravelled**.**

Aim:To Evaluate the effects of combined 50% methanol extracts of *Euphorbia abyssinica (Desert Candle)*, and *Coleus species* on *Candida albicans, Trichophyton* mentagrophytes, *Microsporum gypseum* and *Epidermophyton floccossum.*

Study Design: The completely randomized block design, two way analysis of variance was used and Duncan's New Multiple Range Test, for mean separation.

Place and Duration of Study: The research was carried out in the Department of Microbiology, University of Nigeria Nsukka, Enugu State, Nigeria, between April 2011 and August 2012.

Methodology: All the fungal strains used in the research were collected from the University of Nigeria Teaching Hospital Enugu, Nigeria. Susceptibility testing was done using pour-plate method, while the checkerboard and Time kill assays were employed to evaluate the efficacy of the combinations.

Results: The individual plant extracts inhibited all the fungal strains tested at different concentrations. *Coleus species extracts* proved to be more potent in activity than *Euphorbia abyssinica* extracts. The combination inhibited the test fungi for more than two weeks. In the Time Kill assay, the combinations showed synergy on *E. Floccossum* only. It showed additive or antagonistic activity on the rest of the fungi tested. The Checkerboard combination showed synergy on *T. Mentagrophytes, M. gypseum,* and *E. foccossum. E. foccosum* was the most susceptible of the fungi tested while *C. albicans* was the least susceptible. The control drug voriconazole also inhibited all the fungi tested. Significant antifungal activity (P=0.05) was observed in the checkerboard assay than in the Time Kill assay.

Conclusion: The results justify the folklore claims that these plants have a wide range of curative uses, suggesting that they can be used as alternative sources of agents for the treatment of resistant fungal infections.

Keywords: *Coleus species, Euphorbia abyssinica* Combined, Extracts, Antifungal Effects, Checkerboard, Time kill.

Introduction

Fungal infections such as onychomycoses, disseminated infections associated with opportunistic pathogens like *C. albicans,* dermatophytosis, (invasion of keratinized tissues – skin, hair and nails – of humans and other animals) caused by three anamorphic fungal genera (*Epidermophyton, Microsporum* and *Trichophyton*), have reportedly increased worldwide[1], and so, have become a public health concern. Recently life-threatening and potentially fatal fungal infections have emerged in immune-compromised people [2] with increasing drug resistance recorded in several cases, which were previously susceptible to the normal synthetic antifungal agents.

The spread of antifungal drug resistance is equally becoming a public health challenge globally [3, 4] ; and the situation has been exacerbated by global travel and distribution of food products[5], innate random mutations [6, 7], acquisition of resistance genes from other microorganisms [8, 9] , wide spread indiscriminate use of antimicrobials [10,11,12,13,5] as pesticides [4]; or, in animal feed [14,15], as food preservatives [16, 17], and for treatment of infected patients. To add to the problem of resistance, treatment failure, and toxicity [9], most synthetic drugs are unaffordable to most people in rural and less developed areas of the world [18]. For the latter, their existence and survival history would be incomplete without a mention of the role plants as sources of food and/or medicines [18, 19, 20]. Plants are naturally endowed with primary and secondary metabolites that are incidentally very important nutrients or medicines to man and livestock [19, 21, 22]. Thus, as research reports on medicinal plants accumulate, there is gradual replacement of synthetic drugs (now notorious for failure in treatment of resistant pathogens and in general toxicity) [9] from the pharmaceutical shops with herbal remedies.

Application of combinations of herbs to cure certain diseases is common in ethno-medicine and has formed the basis for experimentation on combinations of therapies as solution to extensive drug resistance by microbes [23]. Thus, multiple drug resistance (MDR) inhibitors or resistance modifying agents work synergistically to modify the resistance phenotype in microorganisms [24]. The search for such compounds in plants can give a leeway to the treatment of drug resistant infections as alternative to overcoming the problem of resistance [25].

Euphorbia abyssinica and *Coleus species* are perfect examples of medicinal plants with a wide range of activity against pathogenic microoganisms. The native population of *Kendem* in Cameroon often use these two plants [20] to prevent loss of blood after childbirth [20], treat cuts [20], pruritus

[20], superficial infections of the body [20], and diseases of the air ways [20]; also as antispasmodic, and anti-histamine, as well as constricting and releasing tension from smooth muscles [26,20].

E. abyssinica is an evergreen, cactus-like plant that has been classified in the family *Euphorbiaceae* [20].

The word *Coleus* was originally coined from the word *Coleos* in Greek, which also is referred to as "sheath" [27]. *Coleus species* belong to the genus Plectranthus or mint group of sweet smelling fragrance plants that were formally classified in the labiatae, currently, the lamiaceae family of plants [27]. Even though, the above original account of these plants was given by João de Loureiro in the period between1717-1791 [27], in other parts of the world, Kendem in Cameroon for example, it is given different descriptions. There, it is described as Osem antuoh, meaning "Toad's skin" [28].

In ethnomedicine, the traditional doctors in this locality use decoctions from the plant to treat generalized systemic and or superficial skin diseases [28].

The purpose of this research therefore was to extract *Euphorbia abyssinica* and *Coleus species* using a mixture of Ethanol and water (50/50%V) and evaluate the effects that the different combinations of the extracts will have on some selected fungi strains.

2. Materials and methods

2.1. Collection and Preparation of Plant Extracts

The stem-bark of *Euphorbia abyssinica* and whole plants of *Coleus species* were collected from Kendem village in the southern Cameroon. The specimens were authenticated at the Department of Botany, and the research carried out in the Department of Microbiology in the University of Nigeria, Nsukka. The specimens were thoroughly rinsed under running tap water and then cut into tiny pieces and air-dried in the dark. They were pulverized in a mortar, the powder weighed and stored in plastic bags. The powdered materials were then extracted using the method described by Tarh *et al*. [29].

2.2. Test Organisms

The test fungi used were obtained from the Department of Medical Microbiology, University of Nigeria Teaching Hospital Enugu, Nigeria. They were subcultured, purified and their identity reaffirmed by slide culture, staining and biochemical tests.

2.3. Susceptibility Testing of Fungi by Pour-plate Method

The susceptibility testing of fungi was done using pour-plate method as described by Tarh and Iroegbu, [30]. A 2.0 mL amount of a 1000 mg/mL reconstituted plant extract was pipetted into sterile glass test tube containing 18mL of molten Sabouraud Dextrose Agar (SDA) at about 45°C. The mixture was swirled carefully for the contents and agar to homogenize, thereafter, 100 µl of the standard fungal inoculums was seeded onto each tube. Again they were thoroughly mixed, then contents of each tube poured into a sterile Petri dish and allowed to set before incubating at 25-35°C. A culture plate without the extract served as the positive control for growth while another plate containing 2.0 mL of 16 µg/mL voriconazole as the negative control. As soon as growth was observed at the positive control plates the test plates were checked for growth daily and the period of inhibition of growth was recorded in days.

2.4. Checker Board Assay

The 50% methanol extracts were further evaluated in combination using the Checker Board assay method described by Tarh and Iroegbu, [20]. Solutions of the plant extracts were prepared, each in sabouraud broth, and diluted using the continuous variation model, that is, by serially reducing the concentration by 10% with broth down to concentrations below the MIC. Then 2.0 mL of each dilutions of *Euphorbia abyssinica* was put into the tubes in the columns such that while the concentrations of the extract changed 10% serially from column to column, the concentration along each column remained the same. The solutions of *Coleus species* extract were similarly distributed into the tubes in the rows such that while the concentrations of the extract vary from one row to the next, the tubes in each row contained the same concentration of the *Coleus species* extract. Consequently each tube received a combination of the two extracts at different ratios. Each of the tubes was then inoculated with 0.1 mL of the standardized microorganisms (fungi) and all the mixtures were incubated aerobically at 25 -35^{o}c observing daily for appearance of growth.

The MICs of the combinations were then recorded and the fractional inhibitory concentration (FIC), for each extract, was calculated as MIC of extract in the combination divided by MIC of single extract. FIC index was also calculated using the formula, FIC index = Σ FIC *Euphorbia* + FIC *Coleus*. FIC index value of 1 indicates additive interaction, < 1, synergy, >1< 2, Indifference and >2, antagonism [20].

The Isobologram data generated from the results of the interactions of plant extracts in combination, using MIC data directly as well as the calculated FICs, were plotted as the first points which no

122 growth occurred. This resulted in a plot or graph called an “isobole”. Any points which fell on a
123 straight line between the x and y axes were considered as additive. A curved deviation to the left of
124 the additive line was an indication of synergy, while antagonism was indicated by a curved deviation
125 to the right of the additive line [20].

126 **2.5. Time Kill Assay**

127 The effects of 50% methanol extracts of *Euphorbia abyssinica and Coleus species* were evaluated
128 by a kinetic time kill assay using the macrobroth dilution technique as described by Tarh and
129 Iroegbu, [20]. The extracts were reconstituted in 20% Dimethyl Sulfoxide (DMSO) and appropriately
130 diluted to the required concentrations. The inoculum size was determined according to the type of
131 fungus, (e.g. 1 $x10^6$ for *Candida albicans*; and1 $x10^5$ for dermatophytes). About 1.00 mL of the
132 extract was added to 9 ml of Sabouraud dextrose broth, seeded with the appropriate concentrations
133 of the test fungus to achieve concentrations equivalent to 0.5 x MIC, 1 x MIC, 2 x MIC, or 4 x MICs
134 values. Two sets of control tubes were included for each experiment. One set was seeded with the
135 organism in broth without extract, and the other set contained broth without organism and extract.
136 The control drug voriconazole was similarly diluted. All the fungal cultures were incubated at 35°C
137 for ≥ 48 hours. Immediately after inoculation of the tubes, aliquots of 100 µL of the negative control
138 tubes contents were taken, serially diluted in saline and seeded on nutrient agar plates to determine
139 the zero hour counts. The same was done for the tubes which contained the test fungi after 0 hour, 6
140 hours, 12 hours, 24 hours and 48 hours, respectively. After incubation, the emergent colonies were
141 counted and the mean count (CFU) of each test organism was determined and expressed as $\log_{10}$.
142 The Minimum Lethal Concentrations (MLCs) of the extract were the lowest concentrations that gave
143 99.9% to 100% killing.

144 In the interaction study, plant extracts were reconstituted in 20% Dimethyl Sulfoxide (DMSO) and
145 then combined using the continuous variation method to obtain a concentration range which
146 included the MIC obtained with the individual plant extracts as well as sub-inhibitory concentrations.
147 Then 0.1 mL of the standardized inoculums was put in to 9.9 mLs of the diluted plant extracts.
148 Inoculated tubes of Sabouraud Dextrose broth were included as positive controls, Tubes of
149 Sabouraud Dextrose broth only were included as negative controls while other tubes containing the
150 MICs of the plant extract alone were also included in the tests. A volume of 100 µL from the tubes
151 containing fungi without plant extract were withdrawn immediately after inoculation, serially diluted

and seeded on the already prepared Sabouraud Dextrose agar plates to determine the zero-hour count. The tubes were incubated at 25- 35 ^{o} C for > 48 hours, during which aliquots of 100 µl were withdrawn at intervals of 15 minutes, 1 hour, 6 hours, 12 hours, 24 hours, 48 hours after inoculation, diluted and plated for colony counts.

The means of two separate tests counts were determined and expressed as Log_{10} CFU. The interactions were considered synergistic if there were decreases of ≥ 2 log_{10} CFU/mL in colony counts after incubation periods by the combination compared to the most active single agent. Additivity or indifference was described as a < 2 log_{10} CFU/mL change in the average viable counts after the incubation periods for the combination, in comparison with the most active single drug. Antagonism was defined as a ≥ 2 log_{10} CFU/mL increases in colony counts after the incubation periods by the combinations compared to that of the most active single extract alone [30]. All the experiments were performed in quadruples and the data collected from four repeated experiments, was analyzed using the Randomized Complete Block Design (Two-way analysis of variance). Duncan's New Multiple Range Test was used to separate the means that were significantly different.

3. Results

3.1. Testing the Susceptibility of the fungi by Agar plate Method.

It was observed that there was considerable activity of the extract combinations against the fungi tested. This was indicated by long periods of growth inhibition (above two weeks) observe with all the fungal strains tested. (Table 1)

3.2. Checkerboard assay method of evaluating the antifungal effects of interactions between *E. abyssinica* (E) and *Coleus species* (C) Extracts

In the study reported here, the susceptibility pattern seen with the fungi tested showed that *E. floccossum* was significantly inhibited than all the other fungi tested. This synergy was observed in the isoboles as indented points away from the additive line to the left. *Candida albicans* showed some significant level of antagonism to the various combinations tested. This was seen as points of indentations distant away from the additive line to the right.

The Combined effect was synergistic against *T. mentagrophytes.* This was seen at Fractional Inhibitory Concentrations (FIC) of Euphorbia 0.2 / FIC of Coleus 0.7 mg/mL with FIC Index of 0.9 mg/mL, and at FIC of Ea 0.1 / FIC of Cs 0.8 mg/mL and FIC Index of 0.9 mg/mL (Fig.1)

The synergistic effects observed with *M. gypseum* were at four different combinations of *E. abyssinica* (Ea) and *Coleus species (Cs)* extracts proportions viz; at FIC of Ea 0.8 / FIC of Cs 0.1 mg/mL, FIC Index 0.9 mg/mL, At FIC of Ea 0.7 / FIC of Cs 0.2 mg/mL, FIC Index 0.9 mg/mL, at FIC of Ea 0.4 / FIC of Cs 0.5, FIC Index 0.9 mg/mL and at FIC of Ea 0.3 / FIC of Cs 0.6 mg/mL, FIC Index 0.9 mg/mL respectively (Fig. 2).

The plant extract proportions that showed synergy against *E. floccossum* incude: FIC Ea of 0.6 / FIC of Cs 0.1 mg/mL, FIC Index 0.7 mg/mL, FIC of Ea 0.6 / FIC of Cs 0.2 mg/mL, FIC Index 0.8 mg/mL, FIC of Ea 0.6 / FIC of Cs 0.3 mg/mL, FIC Index 0.9 mg/mL and at FIC of Ea 0.1 / FIC of Cs 0.8 mg/mL, FIC Index 0.9 mg/mL (Fig. 3).

3.3. Time-kill assay method of evaluating the antifungal effects of interactions between *E. abyssinica* (E) and *Coleus species* (C) Extracts

In the assay method, the effect of interactions were compared to that of the most efficacious plant extract singly. If the interactions were able to reduce the viable cell counts to more than 2 $\log_{10}$ CFU/mL, this was accepted as synergistic but if there were increases in the viable cell counts which were more than 2 $\log_{10}$ CFU/mL, then this was antagonism.

The antifungal activity of combined hydro alcohol extracts of *Coleus species* and *E. abyssinica* was evaluated by exposing the test fungi to various combined proportions of the extracts at different time intervals, which included; 0 hour, 6 hours, 12 hours, 24 hours and 48 hours. The test fungi viable cell counts were standardized to contain $1x10^6$ for the yeasts and $1x10^5$ for the moulds.

The more potent single plant extract observed was *Coleus species.*

The effect of *Coleus species* extract on *Candida albicans* and *Trichophyton mentagrophytes* cells, showed that, the extract at MIC and at double the MIC concentrations decreased the cell counts to about 0.05 log10 by the 48 hours (Fig. 5 & 6). This same double MIC (15.6 mg/mL), killed *M. gypseum* cells in 6 hours (Fig. 7).

However, when *Coleus species* and *E. abyssinica* extracts were combined, they exhibited no synergistic interactions against *Candida albicans, Trichophyton mentagrophytes* and M. gypseum (Fig. 5, 6 & 7).

In 48 hours, *Coleus species (*the more active of the plant extracts*)* at MIC of 0.98 mg/mL, decreased *E. floccosum viable* cell counts from $1x10^5$ CFU to $0.97\log_{10}$. However, by doubling the MIC to 1.96 mg/mL, the fungicidal effect became prominent against *E. floccosum cells,* which were all inhibited in 3hours. The 1µg/mL of the control drug inhibited the fungal cells in 48 hours (Fig. 8).

On combining the two plant extracts, and comparing the activity observed with *Coleus species* extract alone, the interactions showed synergistic effects against *E. floccosum.*

This was detected by the effects observed with the following interactions; Adding 1:8 proportions i.e 0.098 mg/mL of Ea with 0.78mg/mL of Cs to yield 0.878 mg/mL of these extracts decreased the cell counts from $1x10^5$ to 2.0 $\log_{10}$ CFU in 48 hours. In the same trend, combining 0.59 mg/mL of Ea with 0.098 mg/mL of Cs to give 0.688mg/mL and 0.59 mg/mL of Ea with 0.196 mg/mL of Cs to get 0.786 mg/mL i.e. 6:1 and 6:2 combinations respectively, eradicated the viable cells within 48 hours. However, combining 6:3 proportions i.e. 0.59mg/mL of Ea and 0.29 mg/mL of Cs to get 0.88 mg/mL, dropped the cell count to 1.0 $\log_{10}$ and to 0.3 $\log_{10}$ in 24 hours and 48 hours respectively. The cell counts were all in this case reduced beyond 2 $\log_{10}$, signifying synergy (Fig. 8).

Table 1: Duration of Fungal Growth Inhibition in Weeks by 100mg/mL of Combined Extracts of *Euphorbia abyssinica* and *Coleus species*

	Fungal species / growth inhibition in weeks			
	C. albicans	***T. mentagrophytes***	***M. gypseum***	***E. floccossum***
Plant Extract Combination	**>1**	**>2**	**>2**	**>2**
Voriconazole16µg/mL	**>2**	**>2**	**>2**	**>2**

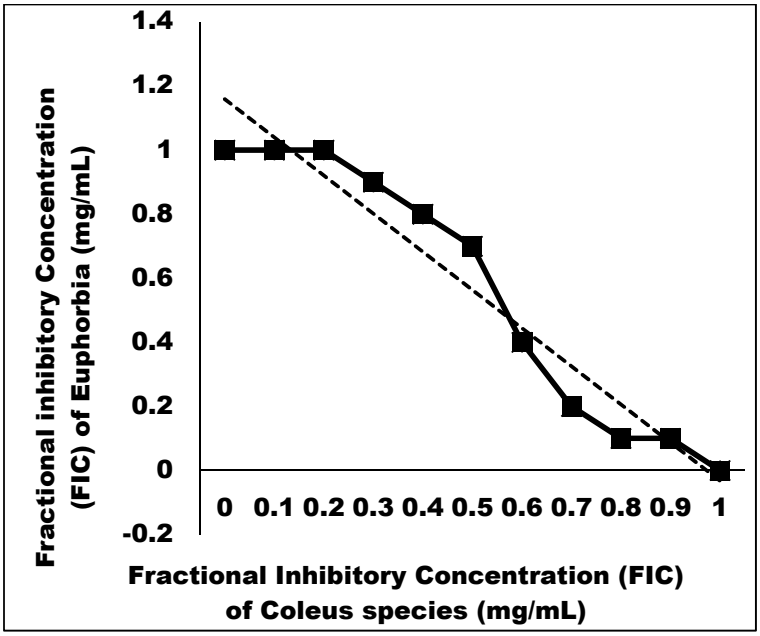
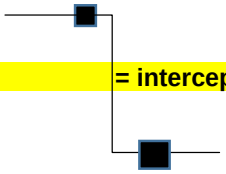


Figure 1: The response effect of *T. mentagrophytes* to Combined Extracts of *E. abyssinica* and *Coleus species*.



= intercept of the interaction between Extracts

---------- = Combined effect not better than the more active single plant extract

Synergy was observed in the isoboles as indented points away from the additive line to the left while antagonism was seen as indentations distant away from the additive line to the right

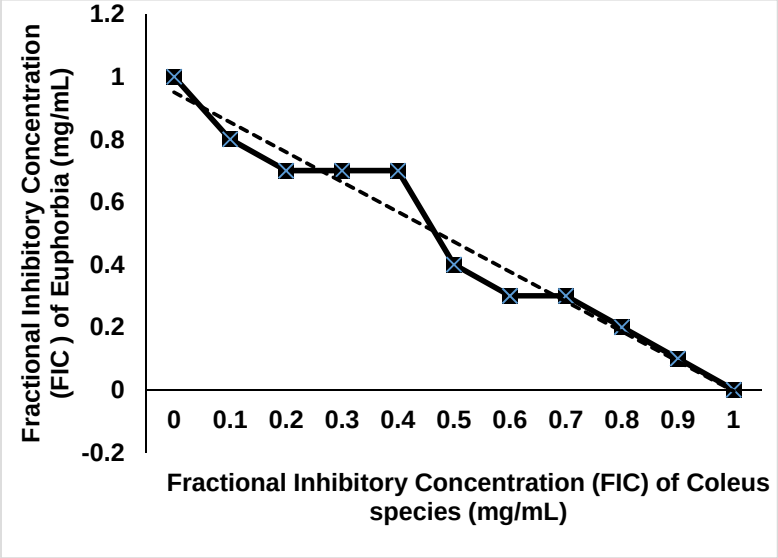


Figure 2: The response effect of *M. gypseum* to Combined Extracts of *E. abyssinica* and *Coleus species*.

= intercept of the interaction between Extracts

---------- = Combined effect not better than the more active single plant extract

Synergy was observed in the isoboles as indented points away from the additive line to the left while antagonism was seen as indentations distant away from the additive line to the right

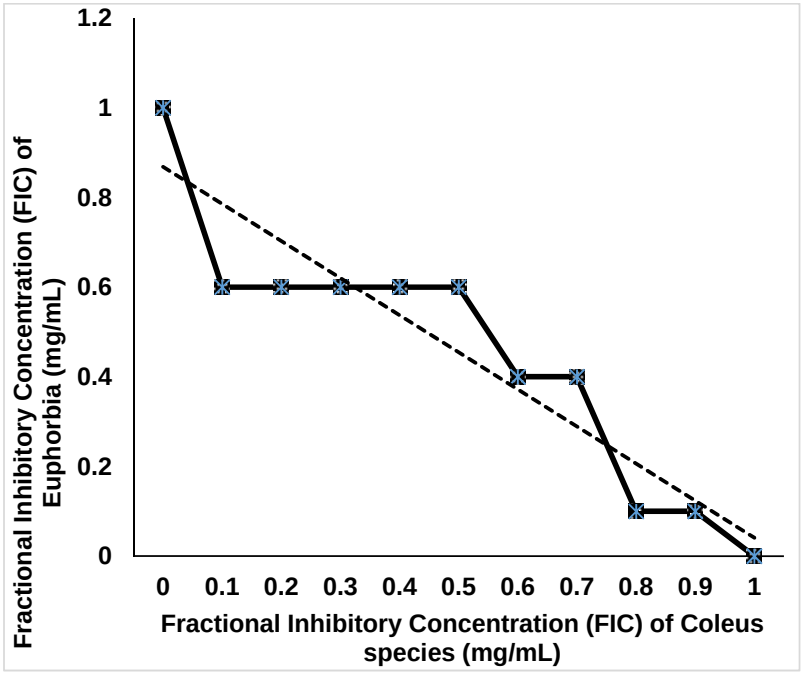
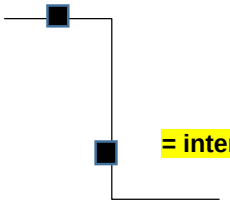


Figure 3: The response effect of *E. floccosum* to Combined Extracts of *E. abyssinica* and *Coleus species*.



= intercept of the interaction between Extracts

---------- = Combined effect not better than the more active single plant extract

Synergy was observed in the isoboles as indented points away from the additive line to the left while antagonism was seen as indentations distant away from the additive line to the right

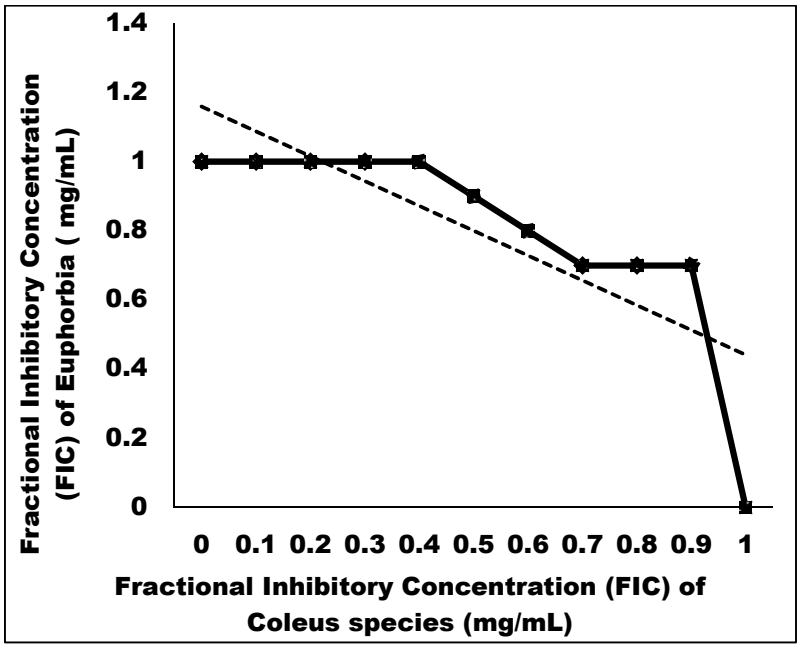


Figure 4: The response effect of *Candida albicans* to Combined Extracts of *E. abyssinica* and *Coleus species*.

= intercept of the interaction between Extracts

---------- = Combined effect not better than the more active single plant extract

Synergy was observed in the isoboles as indented points away from the additive line to the left while antagonism was seen as indentations distant away from the additive line to the right

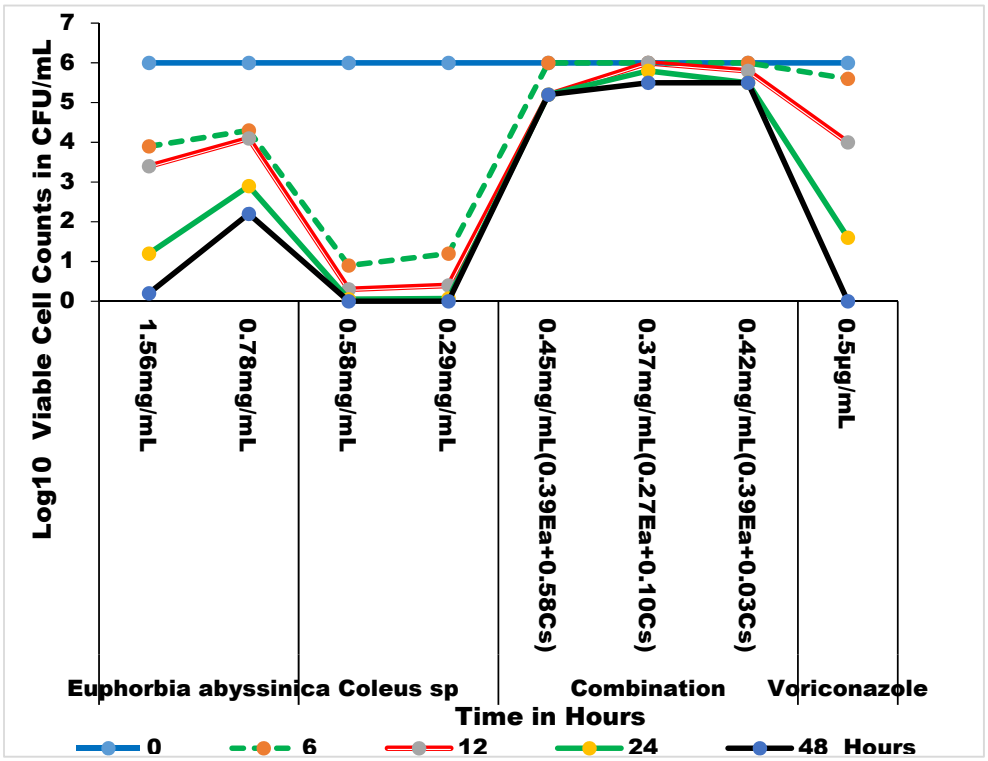
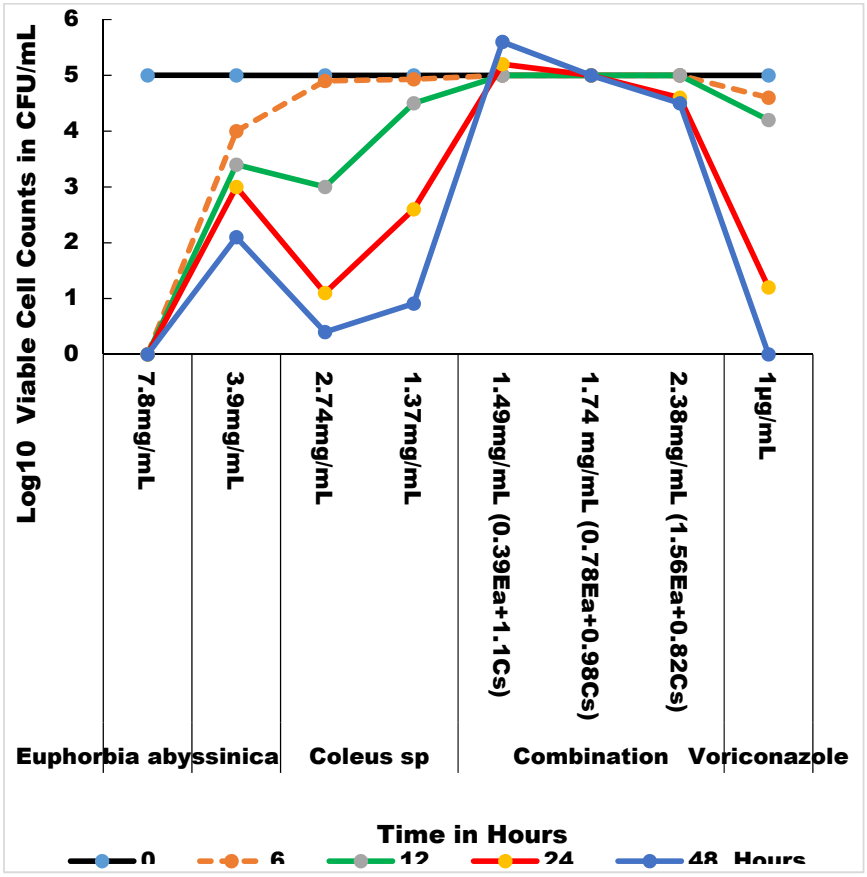


Figure 5: Effect of Time on the Reduction of Viable Cell Counts of *Candida albicans* by the combined extracts;
Ea=*Euphorbia abyssinica*, Cs=*Coleus species*

Interactions that reduce the number of viable cells above 2 $\log_{10}$ CFU/mL, were accepted as synergistic but if there were increases in the viable cell numbers which were more than 2 $\log_{10}$ CFU/mL, then this was antagonism.



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317 **Figure 6: Effect of Time on the Reduction of Viable Cell Counts of *Trichophyton***
318 ***mentagrophytes* by the combined extracts;**
319 **Ea=*Euphorbia abyssinica*, Cs=*Coleus species***
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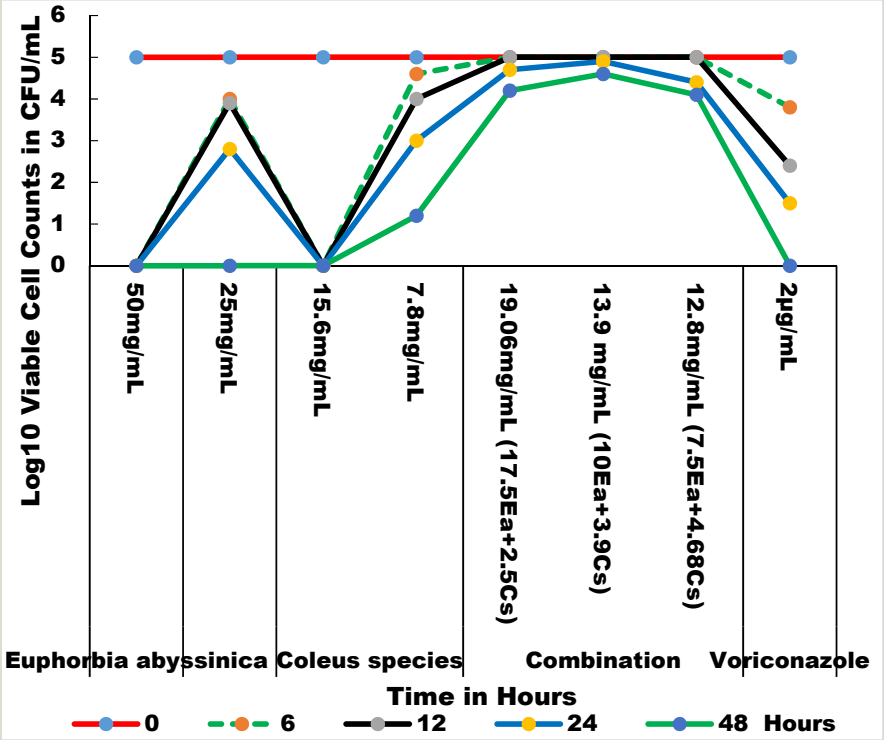

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329 **Figure 7: Effect of Time on the Reduction of Viable Cell Counts of *Microsporum gypseum* by**
330 **the combined extracts;**
331 **Ea=*Euphorbia abyssinica*, Cs=*Coleus species***
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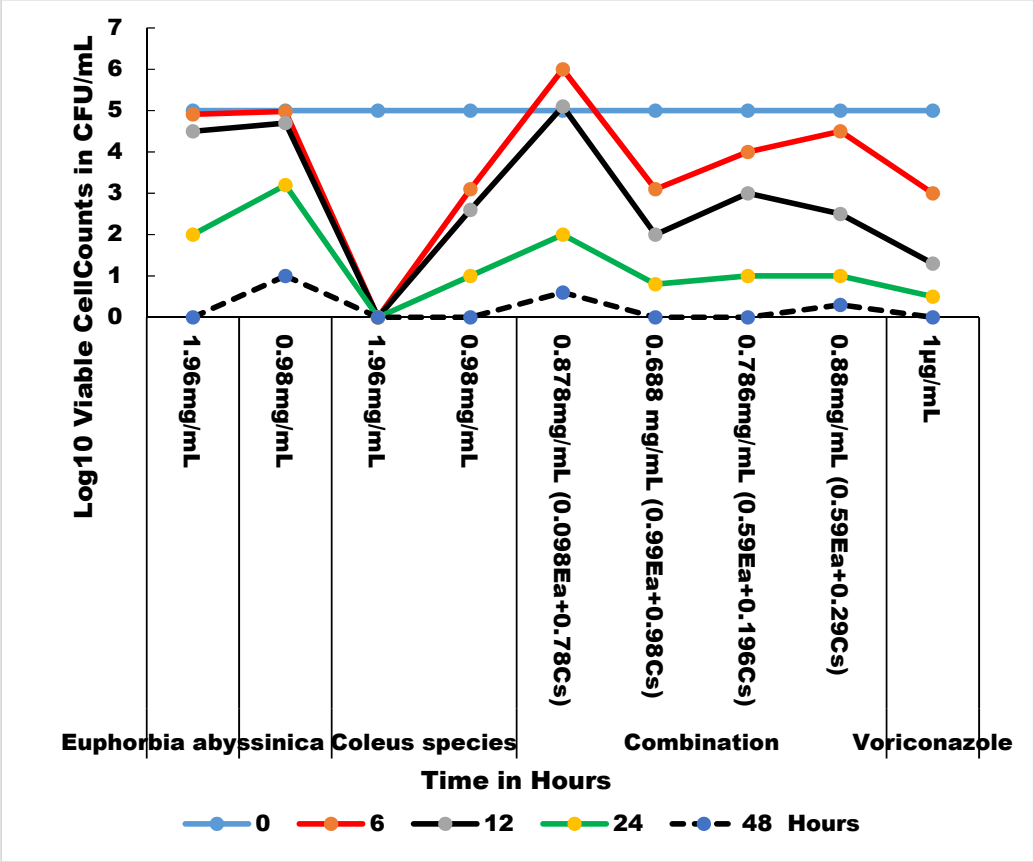

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340 **Figure 8: Effect of Time on the Reduction of Viable Cell Counts of *E. floccossum* by the**
341 **combined extracts;**
342 **Ea=*Euphorbia abyssinica*, Cs=*Coleus species***
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345 **4. DISCUSSION**

346 In ethno medicine, plant extracts are often used in different combinations, whose quantifications are
347 still problematic till today. However, they still remain the preferred method of treatment in most local
348 and under developed areas of the world where the orthodox drugs are note easily available [30].
349 Understanding the hidden truth behind the unusual potentials and efficacies of these plant extracts,
350 was the aim of the study reported here. The Kinetic Time Kill and Checkerboard assay were used to
351 evaluate the antifungal activity of single components as well as the interactions of different
352 proportions of *E. abyssinica* and *Coleus species* extracts. This was to determine whether
353 combinations of the two plant extracts, after interaction, could produce an effect that could be
354 synergistic, antagonistic or additive against the fungi tested. An additive, effect is observed when the
355 combined effect is not more than the individual effect of the most active plant extract. In such a case

even lower concentrations than those obtained with the individual extracts alone equally yield the same effect [31].

For drug interactions which result in synergistic effects (agonists), the concentration of each drug in the combinations may not necessarily need to be up to that obtained when the drugs are used singly. If reduced quantities of the each interacting drug or of a particular component alone can react, they will still produce the required synergy. At times, the observed effect with some drug interactions may indicate that the activities of the reactant (s) have decreased and this effect is termed sub-additive. [31].

Many different methods have been used by different authors to evaluate and represent the effects of drug combinations; and an example of such representation is the isobole, a curve produced by Loewe in 1957. He plotted a graph using the doses of two drugs, one on the 'Y' and the other as the 'X' axes. The individual drug concentrations that could interact, when given in combination, to produce an effect (synegy, antagonism, etc) were seen on the rectangular plot as points which he called the "isobole" [31].

However, when in-vivo, drugs, regardless of the fact that they are administered in combination, or singly, may be encountered by the plasma proteins and other natural components, present in the human system. The evaluation of the activity and effects of the dose of each agonist, gives better information, especially about those agonists which use different modes of action with different receptors to produce synergy. These types of agonists, termed "similar and independent" by Bliss [32], do not interfere with each other since their binding sites are independent of one another.

Studies of this sought try to address some doubts which may arise about the response produced by ion of two agonists; whether their interactions will be additive, synergistic or antagonistic when compared to the single drugs effects.

In this study, *Coleus species* extracts, one of the single components used in the interaction study, showed a significantly level of activity (P=0.05) than the second counterpart (*Euphorbia abyssinica* extract). A contributory factor could be that the *Coleus species* was used as a full-spectrum plant extracts, which means that the entire chemical profile available in the flowers and all other parts together with the roots is present in the final medicinal form [33].

In comparing the methods used in the study, the results indicated that the Agar diffusion, method produced the best response than the Kinetic Time kill and the Checkerboard assays. This was

386 indicated by the synergistic effects produced by the combined plant extracts against the fungal
387 species tested. Tarh and Iroegbu [20] observed that the Kinetic Time kill and the Checkerboard
388 assays are dependent on predetermined MICs of the single extracts, and this could at times not be
389 hundred percent reliable, due to the fact that MIC values can be affected by confounding, bias,
390 inaccuracy and lack of precision in the variables used.
391 In the checkerboard assay the antifungal activity observed with interaction between *E. abyssinica*
392 and *Coleus species* extracts indicated that the two plant extracts are agonists' in-vitro. The fractional
393 inhibitory concentrations (FIC) of both extracts indicated that there was synergy against *T.*
394 *mentagrophytes* and *M. gypseum* at FIC indices of 0.9 mg/mL, respectively. This was also seen
395 against *E. floccossum* at FIC indices of 0.7, 0.8 and 0.9 mg/mL. The effect of the plant extracts at
396 different combinations was indifferent against *Candida albicans*.
397 In the kinetic Time- kill assay, Synergy was significantly observed against *E. floccossum* with more
398 than 2 $\log_{10}$ reduction in the number of viable cells counted within 48hours. Interacting lower
399 concentrations of 0.688 mg/mL and 0.786 mg/mL, killed the cells in 48 hours, while higher
400 concentrations of 0.878 mg/mL and 0.880 mg/mL decreased the cell counts to2.0 $\log_{10}$ and 0.3 $\log_{10}$
401 in 48 hours, respectively. The lower combinations showed better effects because at higher
402 concentrations, the plant extracts could present some unwanted adverse side effects.
403 However, in comparison, the Checkerboard assay showed a more significant sensitivity pattern
404 (P=0.05) in this study than the Time kill Assay. The plant extract combinations inhibited the growth of
405 the three molds tested, but the effects against the yeast *C. albicans* were the reverse because, no
406 synergy was observed at all the combinations tested.
407 The response effects observed between the above plant extracts interactions and the fungi tested,
408 could have resulted from so many factors, both environmental, human as well as the innate changes
409 exerted in the kinetics of one drug by the other. The observation of a diminished effect or inactivity
410 in- vitro is not a confirmation that the same scenario will be observed when the drug is administered
411 internally. This is so because some components of the body may play some roles when the drug
412 gets into the system. These interactions between the tissues and the drugs may also cause changes
413 that may affect the activity and effects of others that use the same receptor type [34]. e.g Calcium,
414 magnesium and aluminum ions, which are components of some antacids can calcify and crystallize
415 metal-tetracycline and render it less absorbable [34]. Drugs that are taken orally, pass through the

digestive tract and are encountered by digestive enzymes prior to their absorption in to the blood. This condition may cause a vast amount of the drug to be lost through the quick metabolic activity of the hepatic system – the so-called "first pass effect" [34]. Competitive Inhibition can also occur amongst the drugs because some of them extensively bound to plasma proteins and, therefore, competition for binding sites, on the receptor, may result in an inadequate serum concentration, of the antibiotic being reached, with consequent failure of therapy [34].

Toxicity test which evaluates the lethal dose (LD50) may present a better picture of the drug effect *in vivo*. Better still, the quanta dose-effect or dose-response curve that displays the percent of animals that respond to the drug i.e the hyperbolic curve described by the equation $E = E_{max}D/(D + C)$ where E is the effect, D is dose and C the constant, which is equal to the dose needed for a half-maximal response, a measure of drug potency, often denoted as ED_{50} or D_{50} [31] can be used.

The wide range of antimicrobial activity observed by other researchers with these two plants has also been confirmed in this research work. Extracts of *E. abyssinica* and *Coleus species* in combination, were able to inhibit the growth of both yeasts and molds. There was no observable significant difference (P=0.05) in the response pattern seen with the different fungal strains used in the study. There have been reports of the same pattern of antimicrobial effects of alcohol extracts of *Coleus species* by Jay, [35] and Tarh and Iroegbu, [30].

5. Conclusion

In this study, the effects of the interactions observed with two plant extracts (*E. abyssinica* and *Coleus species)*, showed that the plant extracts inhibited all the fungi tested, though not at all the combinations. This provides novel information about the antifungal potentials of the above two plant extracts against drug resistant pathogens. It remains to be determined if the effects and interactions observed with the crude extracts used in this study would be reproduced with purified plant extracts or indeed with the isolated active ingredients. Further investigations on the mechanism of synergistic action of these plants are necessary if they must be considered as alternative sources of broad spectrum drugs for antifungal therapy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

REFERENCES

[1] Shivakumar SP, and Vidyasagar GM. J. Appl. Pharmaceut. Sc. Antifungal screening of 61 folkloric medicinal plant extracts against dermatophytic fungi *Trichophyton rubrum* 5 (05); 2015: 038-044

[2] Shanmugam K, Gnanaprakasam AR and Manivachagam C. Antifungal activity of different crude extracts of leaves of *Madhuca indica.* Int. J. Nat. Prod. Res. 2014; 4(3): 88-95

[3] Chiller, T. The Rise in Antifungal Resistance *Medscape* April 04, 2016

[4] Revie NM, Iyer KR, Robbins N, Cowen LE. Antifungal drug resistance: evolution, mechanisms and impact. Curr. Opin. Microbiol. 2018; 12 ; 45 : 70-76. doi: 10.1016/j.mib.2018.02.005. [Epub ahead of print]

[5] Imperial College London. "Resistance to antifungal drugs could lead to disease and global food shortages."ScienceDaily.ScienceDaily,17May,2018. www.sciencedaily.com/releases/2018/05/180517143604.htm

[6] Robbins N, Tavia C and Cowen LE. Molecular Evolution of Antifungal Drug Resistance Ann. Rev. Microbiol. 2017; 71:753-775.

[7] Castanheira M, Deshpande LM, Davis AP, Rhomberg PR, Pfaller MA. Monitoring antifungal resistance in a global collection of invasive yeasts and molds: application of CLSI epidemiological cutoff values and whole genome sequencing analysis for detection of azole resistance in *Candida albicans*. Antimicrob. Agents Chemother. 2017;. 61:e00906-17. https://doi.org/10.1128/AAC.00906-17.

[8] Cowen LE, Sanglard D, Howard SJ, Rogers PD and Perlin DS. Mechanisms of Antifungal Drug Resistance. Cold Spring Harb. Perspect. Med. 2015; doi: 10.1101/cshperspect.a019752 originally published online November 10, 2014

[9] Sanglard D. Emerging Threats in Antifungal-Resistant Fungal Pathogens. *Front. Med.* 2016; 3:11. doi: 10.3389/fmed.2016.00011

[10] Azevedo M-M, Faria-Ramos I, Cruz LC, Pina-Vaz C, and Rodrigues AG. Genesis of Azole Antifungal Resistance from Agriculture to Clinical Settings *J. Agric. Food Chem.*2015*;* 63, 34, 7463-7468 DOI: 10.1021/acs.jafc.5b02728

[11] Saravanasingh K, George FP and Ramamurthy M. A study on Antibacterial and antifungal activities of extracts of Medicinal plant *Aegle marmelos* Int. J. Adv. Res. Biol. Sci. 2016; 3(2): 321-328

[12] Tarh, JE and Iroegbu, CU. Microbiological Evaluation of Combinations of Extracts of *Euphorbia abyssinica* and *Coleus species* for Antibacterial Activity Indian E-J. Pharmaceut. Sc. 2017a; 03[01]

[13] Wiederhold NP. Antifungal resistance: curr. Trends Fut. Strat. Combat. Infect. Drug Resist. 2017:10 249–259 http://dx.doi.org/10.2147/IDR.S124918

[14] Mie A, Helle RA, Gunnarsson S, Kahl J, Guyot EK, Ewa R, Gianluca Q and Grandjean P. Human health implications of organic food and organic agriculture: A comp. Rev. Env. Health. 2017; 16:111.1-22. DOI 10.1186/s12940-017-0315-4

[15] Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ. Worldwide emergence of resistance to antifungal drugs challenges human health and food security.*Sc.* 2018; 360, (6390): 739-742. DOI:10.1126/science.aap7999

[16] Baljeet SY, Simmy G, Ritika Y and Roshanlal Y. Antimicrobial activity of individual and combined extracts of selected spices against some pathogenic and food spoilage microorganisms Int. Food Res. J. 2015; 22(6): 2594-2600

[17] Salas ML, Mounier J, Valence F, Coton M, Thierry A and Coton E. Antifungal Microbial Agents for Food Biopreservation—A Review Microorganisms. 2017; 5: 37 doi:10.3390/microorganisms5030037

[18] Tarh, JE and Iroegbu, CU. *In- vitro*Evaluation of Anti-fungal Effects of *Euphorbia abyssinica* (Desert Candle) Extracts, *J. Adv Med. Pharmaceut.Sc.* 2017b; *13(1): 1-10.*

[19] Padalia H, Anjali T, Chanda S. Antimicrobial activity of some medicinal plant extracts and its synergistic interaction with some antibiotics *J. Pharm. Res. 2016;10(5):211-220*

[20] Tarh, JE and Iroegbu, CU. *In-vitro* Anti-Bacterial Activity of Extracts of *Euphorbia abyssinica* (Desert Candle) Stem-Bark and Latex. *J. Adv Microbiol.* 2017*C; 2(4): 1-13*

[21] Sukandar EY, Neng FK, Pratiwi W, Deani A. Antibacterial Interaction of Combination of Ethanolic Extract of *Zingiber Officinale* Var Rubrum Rhizome, *Boesenbergia Pandurata* Rhizome, and *Stevia Rebaudiana* Leaves With Certain Antibiotics Against Infectious Mouth Microbial. *Asian J Pharm Clin Res. 2016; 9 (1) :332-335.*

[22] Donkor, A-M, Oduro-Mensah D and Konona-Ang P M. *In vitro* Antibacterial Activity of PEG Formulations of Crude Extracts of *Cleome viscosa*, *Tamarindus indica* and *Euphorbia hirta.* Res. J. Microbiol. 2016; 11(6) 202-207.

[23] Dry RJ, Mi Y and Saez-Rodriguez J. Looking beyond the cancer cell for effective drug combinations. Gen. Med. (2016) 8:125

[24] Ranjan S, Bhavya K, Muntaj Sk, Basumata G, Rajesh M. Synergistic Effect of Some Medicinal Plants and Antibiotics Against Few Pathogenic Bacteria *Intern. J. Biol. & Pharmaceut. Res.* 2012; 3(8): 1000-1004.

[25] Sanjeev R, Bhavya K, Muntaj Sk, Basumata G, Rajesh M, Sanjeev R. Synergistic Effect of Some Medicinal Plants and Antibiotics Against Few Pathogenic Bacteria *Int. J. Biol. & Pharmaceut. Res.* 2012; 3(8): 1000-1004.

[26] Yashaswini S and Vasundhara M Coleus (*Plectranthus barbatus*) – A Multipurpose Medicinal Herb. The 12th Invest in ME Research Conference June,2017, Part 2 http://core.kmi.open.ac.uk/display/1034673

[27] Mariya Paul AR and Suresh KD. On the High value Medicinal plant*, Coleus forskohlii Briq. Hygeia.J.D.Med*. 2013; 5(1):69-78.

[28] Biqiku L, Lupidi G, Petrelli D and Vitali LA. Antimicrobial Activity of Single and Combined Extracts of Medicinal Plants from Cameroon**.** IOSR-JPBS 2016; 11 (4) Ver. IV: 86-90.

[29] Tarh JE Okafor JI and. Iroegbu, CU. Evaluation of Extracts of *Coleus* Species For Antibacterial Activity. *Afr. . Biotech.* 2015; 14(2): 125-132.

[30] Tarh JE and Iroegbu, CU. Evaluation of Anti-fungal Activity of Coleus Species Extracts, Int. J. Curr. Res. Biosci. Plant Biol, 2017d; 4(1), 131-138.

[31] Tallarida RJ. Drug Combinations: Tests and Analysis with Isoboles. Curr Protoc Pharmacol, 2016; 72: (9) 19.1–9.

[32] Bliss CI. The toxicity of poisons applied jointly. *Ann Appl Biol,* 1939; 26**:**585–615.

[33] Saraswati JKK and Avinash KN**.** Analytical Techniques for Phytochemicals Screening and Bioactivities of Some *Coleus*. Species: A Review. J. Pharm. Sci. & Res. 2016; 8(4), 227-237

[34] Richens A. Drug interactions and lethal drug. *J. Clin. Pathol.* 1975; s3-9: 94-98

[35] Jay Whole Plant Cannabis Extracts: The Important Combination of THC and CBD September 27, 2016 https://www.yerba.org.