



EVALUATION OF THE ANTIMICROBIAL PROPERTY OF *Eugenia aromatica* (L. bali) AND *Myristica fragrans* (L.) AGAINST THE GROWTH OF MICROORGANISMS ASSOCIATED WITH DANDRUFF (*Malassezia restricta* AND *Propionibacterium acne*)

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ABSTRACT

This work was aimed at assessing the antimicrobial activities of *Eugenia aromaticum* and *Myristic fragrans* buds and seeds extracts on *Propionibacterium acne* and *Malassezia restricta* causing dandruff infection. The microbial strains were obtained from the Microbiology Laboratory of the Federal teaching hospital, Gombe. The phytochemical constituents of the plant extracts and antimicrobial testing of the extracts on bacterial and fungal strains were carried out using agar well diffusion method. The determination of the minimum inhibitory concentration (MIC) of the extracts were carried out using broth dilution method. Analysis of Variance (ANOVA) test was used to analyze the data obtained to determine if there was significant difference between the aqueous, n-hexane and chloroform extracts used. The phytochemical screening revealed the presence of alkaloids, tannins, saponins, flavonoids, steroids and glycosides in both plant extracts. The result showed that all the extracts were efficient against the growth of *M. restricta* and *P. acne* at all concentration. Aqueous plant extract shows highest zone of inhibition against the tested bacterial and fungal isolate. The minimum inhibitory concentration (MIC) of *M. fragrans* extracts; aqueous, n-hexane and chloroform against *M. restricta* was determined at 0.5mg/ml, 5mg/ml, and 50mg/ml concentration respectively and that of *P. acne* was determined at 5mg/ml for all the plants extracts. The MIC of *E. aromaticum* plant extracts were at 0.5mg/ml, 5mg/ml and 50mg/ml for aqueous, n-Hexane and chloroform extracts respectively against *M. restricta* and *P. acne*. The extract of *M. fragrans* and *E. aromaticum* inhibits the growth of *M. restricta* and *P. acne* to a certain extent. Hence have antimicrobial potential which may be helpful in the management of dandruff infection.

Keywords: Antimicrobial activity; *Eugenia aromaticum*; *Malassezia restricta*; microbial strain; MIC

INTRODUCTION

Dandruff infection causes discomfort and can be an embarrassing situation (Gupta, 2013). Recent estimates showed that about 3.5 billion humans are affected with this infection and the available treatment of therapy for dandruff include the use of chemical agents in the form of shampoos, creams and ointments. It was reported that dandruff is a common scalp disorder that has occurred for centuries and has a prevalence of nearly 50% in the worldwide population. The formation of

dandruff has been studied for decades, but no coincident view has been widely accepted.

However, the development of resistance and failure to prevent reoccurrence of the infection by chemical antifungal agents has been reported (Baroni., *et al*, 2008). Moreover, global estimate indicated that 80% of about 4 billion populations cannot afford the products of the modern Pharmaceutical Industry. The use of biological control strategies involving plant extracts would aid in the management of dandruff infection (Fu y., *et al*, 2009).

There has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This has forced scientists to search for new antimicrobial substances from various sources like the medicinal plants (Sofowora, 2010)

It is well known that plant extracts are safer, contrary to the synthetic drugs which are associated with many side effects. Recent work revealed the potential of several herbs as sources of drugs (Ahn, 2017). Similarly, Balunas M. J and Kinghorn, A.D (2005) has shown that plants represent a potential source of novel antibiotics.

In recent years, scientific research has focused more in discovering and identifying local plant species with bioactive compounds as an essential oil, their characterization, extraction and purification processes and application in drug industries (Osuntokun O.T. and Ogunleye A.J. 2017).

Plants such as *Eugenia aromaticum*, *Myristic fragrans*, *Xylopi aethiopic a*, *Piper igrum* and *Piper guineese* are locally used in the treatment of bacterial and fungal infection. However, these have not been tested scientifically. Evaluation of activities of some of these plants may pave way in our struggle to control infections and other related issues.

MATERIALS AND METHODS

Collection of Microbial Samples

The microbial strains used in this study were pure clinical isolates (*Propionibacterium acne* and *Malassezia restricta*) obtained from the Microbiology Laboratory, Federal Teaching Hospital Gombe. The isolates were tested for viability by sub – culture at 37°C in an incubator for 24 hours prior to microbial testing.

Sterilization of Glass Wares and Working Bench

Glass wares used were properly washed with detergent and sterilized in an autoclave at 121°C for 15 minutes before use. The work was carried out under aseptic condition. The work bench was disinfected with 70% ethanol.

Preparation of Culture Media

Sabouraud Dextrose Agar

The Sabouraud Dextrose Agar (SDA) was prepared according to manufacturer's instruction i.e 65g of the media was weighed using weighing balance. After which the powder was dissolve in 1000ml of distilled water. The suspension was stirred gently until homogenous mixture was obtained. The media was autoclaved at 121°C for 15 minutes and allowed to cool before pouring in to sterilized plates. 20ml of the media was poured in each sterilized plate, because fungi grow slowly more media will be required to be poured into the plates so that the fungi will grow properly without the media drying up.

Nutrient Broth

The nutrient broth agar (NBA) was prepared according to manufactures instruction. i.e. 25g of the media was weighed using weighing balance. After which the powder was dissolved in 1000ml of sterilized distilled water. The suspension was stirred gently until homogenous mixture was obtained. The media was autoclaved at 121°C. 1ml of the media was poured in each sterilized test tube and plugged with cotton wool and capped with aluminum foil.

Collection of Plant Samples

The dry seeds and buds of *M. fragrans* and *E. aromaticum* were purchased from main market in Gombe. The samples were packed separately in clean sterilize polythene bags and brought to the herbarium, Department of Biological Sciences, Gombe State University

for identification and authentication. After identification, the samples were pulverized and stored in an air tight container for further use.

Extraction of plant samples (crude extracts)

Plant extracts were obtained using maceration method. Powdered plant materials were extracted with chloroform, hexane and aqueous separately at room temperature for 48h using muslin cloth. Crude extracts were subjected to dryness in a hot air oven at 3⁰C for 24h. The dried crude extracts were kept at -5⁰C until evaluated. Each of the plant resultant extract was weighed and stored in the refrigerator until use.

Percentage yield was calculated using the formula suggested by Bhattacharjee., *et al.* (2006).

Where:

Extract yield %= $W_1/W_2 \times 100$

w_1 = Net weight of powder in grams after extraction

w_2 =Total weight of wood

Extraction of plant materials with chloroform, aqueous and n-Hexane

Air-dried and powdered *E. aromaticum* and *M. fragrans* were extracted with chloroform (CH₃Cl), aqueous and n-Hexane using Maceration method. The maceration extraction procedure is a semi-continuous process, which has been found to yield an optimal extraction of similar products and prevent loss of some bioactive compounds as a result of heat. The protocol followed was the standard method of extraction published by Current Protocols. 100g of each of powdered *E. aromaticum* and *M. fragrans* were weighed into conical flask. Plant material was soaked with 250ml chloroform, aqueous and n-Hexane at and covered with foil paper. The solvent was stored at room temperature and kept for 24hours. After 24

hours the solutions was filtered using muslin cloth.

Filtered extract was concentrated by using a hot plate at low temperature (40-50⁰C). Dried extract was weighed and expressed in percentage of original sample. All extracts were stored at 40⁰C until used.

Preparation of plant extracts concentration

Stock solution was prepared by dissolving 10g of the solid plant extracts in 100mls of normal saline, making a stock of 100mg/ml. The concentration was prepared from the stock solution using dilution formula as follows:

$$C_1V_1=C_2V_2$$

Where:

C_1 = present concentration

V_1 = volume to use

C_2 = required concentration

V_2 = required volume

A working solution of 500mg/ml, 750mg/ml and 1000mg/ml concentration was used to test for the antimicrobial effect of the extracts. The working concentration was prepared using the stock solution.

Determinations of Antimicrobial Activity of Plants Extract Using Agar Well Method

The crude extracts of the different plant samples were subjected to antimicrobial assay using the Agar Well Diffusion method of (Murray *et al.*, 1995). The antimicrobial assay of the plant extracts was carried out on the test isolates and were inoculated on the surface of freshly gelled sterile nutrient agar and Sabouraud Dextrose agar plates by streaking using sterilized swab stick. Four wells were aseptically bored on each agar plate using a sterile cork borer (6 mm) and wells were properly labelled. Fixed volumes (50 μ l) of different concentrations (500mg/ml, 750 mg/ml and 1000mg/ml) of the extracts (chloroform, aqueous and n-Hexane) were

then introduced into the wells in the plates respectively. A control well was at the centre with 50 μ l of the extracting solvent. The plates were allowed on the bench for 40 minutes for pre – diffusion of the extract to occur and then incubated at 37°C for 24 hours. The resulting zone diameter of inhibition was measured using a transparent ruler calibrated in millimetres (mm). The readings were taken to be the zone diameter of inhibition of the microbial isolates in question at that particular concentration (Koche, et al., 2012).

Minimum Inhibitory Concentration

When there is total inhibition at minimum concentration or least concentration used, then minimum inhibitory concentration (M.I.C) must be carried out, for example if minimum inhibitory concentration is (5g/ml) and it happen that there is total inhibition at this level, then M.I.C test is set. Example, 2.5g/ml, 1.25g/ml and 0.0625g/ml respectively. This is done in order to know where the inhibition starts (Koche, et al., 2012).

Qualitative Phytochemical Screening of Plant Extracts

The main objective of phytochemical analysis was to analyze the presence or absence of different bioactive compound in *E. aromatica* buds and *M. fragrans* seed having anti dandruff agent. These bioactive compound include: alkaloid, tannins, flavonoids, saponinins, phenols, anthraquinones, glycosides, proanthocyanidins and steroid on all the extracts (chloroform, aqueous and n-Hexane) using standard procedures at room temperature as adopted by (Okwu, 2005, Ladipo, et al., 2010).

Test for alkaloids

0.2 g of each plant samples were added in each test tube and 3 ml of hexane were mixed in it, shaken well and filtered. Then took 5 ml of 2% HCL and poured in a test tube having the mixture of plant extract and hexane.

Heated the test tube having the mixture, filtered it and poured few drops of picric acid in a mixture. Formation of yellow color precipitate indicates the presence of alkaloids.

Test for tannins

2 ml of the aqueous extract was added to 2 ml of water; 1 to 2 drops of diluted ferric chloride solution was added. A dark green or blue green coloration indicates the presence of tannins.

Test for flavonoids

0.5 g of plant extract was added in a test tube and 10 ml of distill water, 5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of 1 ml concentrated H₂SO₄. Indication of yellow color shows the presence of flavonoid in each extract.

Test for saponin

1 ml of aqueous extract was added few volume of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth for 20 min.

Test for s steroids

Ten (10 ml) ml of each extract was evaporated. The residue was dissolved in 0.5 ml of hot acetic anhydride; 0.5 ml of the filtrate chloroform was added and then treated with Liebermann Burckhardt reagent. The appearance of blue-green at the interphase, confirms the presence of steroids.

Test for glycosides

2.5 of 50% sulphuric acid were added to 5ml of the extract in a test tube. The mixture was heated in boiled water for 15min, cooled and neutralized with 10% NaOH and 15mls of Fehling's reagent was added and mixture will be boiled. A brick-red precipitate was observed which indicate the presences of glycosides. The screening or analysis was done to test for the presence of the bioactive

compounds such as alkaloid, flavonoids, saponins, tannins, phenol, eugenol, steroids etc. as described by (Evans and Trease, 1989).

Statistical Data Analysis

Data obtained from the studies was subjected to statistical analysis using statistical data package for social science. Analysis of variance (ANOVA) was carried on the data and the mean was separated using Duncan multiple range test (DMRT).

RESULTS

Antimicrobial Sensitivity Testing of Plants Extracts

Zones of inhibition of 3 different treatment of the extracts E. aromatica against the growth of M. restricta

Table 1 represents the of antimicrobial activity of *E. aromatica* against the growth of *M. restricta*. The result shows that the zone of inhibition occurs at all concentrations however *M. restricta* was more sensitive to the aqueous extract of *E. aromatica*. The highest zone of inhibition (64mm diameter) was recorded at 1000mg/ml aqueous extract concentration. The lowest zone of inhibition (41mm diameter) was recorded at 500mg/ml chloroform extract concentration.

Table 1: Zones of inhibition of 3 different treatments of the extracts *E. aromatic* against the growth of *M. restricta*

Concentration (mg/ml)	Mean zone of inhibition (mm) ± Standard error			
	Control	Aqueous	n-Hexane	Chloroform
500	40±0.1 ^a	52±0.07	40±0.08 ^a	41±0.04
750	40±0.1 ^{ab}	56±0.03	43±0.9 ^{ac}	43±1.1 ^{bc}
1000	40±0.1	64±0.2	45±0.07	48±1.3

Values are mean ±standard error (n=3). Mean values with the same superscript in a raw are not significantly different (P<0.05)

MIC of the extracts of E. aromatica against the growth of M. restricta

Table 2 represents MIC of the extracts of *E. aromatica* against the growth of *M. restricta*.

The results show that the MIC of aqueous extracts was found to be in at 0.5mg/ml concentration. The MICs of n-Hexane and chloroform were found at 0.5mg/ml and 50mg/ml concentration respectively.

Table 2: MIC of the extracts of *E. aromatica* against the growth of *M. restricta*

Extracts	MIC	Concentration (mg/ml)						
		500	50	5	0.5	0.05	0.005	0.0005
Aqueous	MIC	-	-	-	+	+	+	+
n-Hexane	MIC	-	-	-	+	+	+	+
Chloroform	MIC	-	-	+	+	+	+	+

Key: Growth: + , No Growth: -

Zones of inhibition of 3 different treatment of the extracts *E. aromatica* against the growth of *P. acne*

Table 3 shows the antimicrobial of *E. aromatica* against the growth of *P. acne*. The zone of inhibition occurs at all concentrations. The working concentrations of the whole

plant extracts exhibit inhibition on *P. acne*. However, the highest zone of inhibition (54mm) was recorded in aqueous extract at working concentration of 1000mg/ml and the lowest zone of inhibition (21mm) was recorded in chloroform extract at working concentration of 500mg/ml.

Table 3: Zones of inhibition of 3 different treatment of the extracts *E. aromatic* against the growth of *P. acne*

Concentration (mg/ml)	Mean zone of inhibition (mm) ± Standard error			
	Control	Aqueous	n-Hexane	Chloroform
500	25±0.05 ^{ab}	34±0.7	23±1.8 ^{ac}	21±0.06 ^{bc}
750	25±0.05 ^a	43±0.07	31±0.9	26±0.4 ^a
1000	25±0.05	54±0.9	33±0.5	29±0.04

Values are mean ±standard error (n=3), Mean values with the same superscript in a raw are not significantly different (P<0.05)

MIC of the Extracts of *E. aromatica* Against the Growth of *P. acne*

Table 4 represents MIC of the extracts of *E. aromatica* against the growth of *P. acne*. The

results show that the MIC of aqueous extracts was found to be at 0.5mg/ml concentration. The MICs n-Hexane and chloroform were found at 0.5mg/ml and 50mg/ml concentration respectively.

Table 4: MIC of the extracts of *E. aromatica* against the growth of *P. acne*

Extracts	Concentration (mg/ml)	Concentration (mg/ml)						
		500	50	50	5	0.5	0.005	0.0005
Aqueous	MIC	-	-	-	-	+	+	+
n-Hexane	MIC	-	-	-	-	+	+	+
Chloroform	MIC	-	-	+	+	+	+	+

Key: Growth: + , No Growth: -

Zones of inhibition of 3 different treatment of the extracts *M. fragrans* against the growth of *M. restricta*

Table 5 represents the of antimicrobial activity of *M. fragrans* against the growth of *M. restricta*. The result shows that the zone of inhibition occurs at all concentrations and

aqueous extract was more potent compare to other extracts however the highest zone of inhibition (66mm diameter) was recorded at 1000mg/ml aqueous extract concentration. The lowest zone of inhibition (38mm diameter) was recorded at 500mg/ml chloroform extract concentration.

Table 5: Zones of inhibition of 3 different treatment of the extracts *M. fragrans* against the growth of *M. restricta*

Concentration (mg/ml)	Mean zone of inhibition (mm) ± Standard error			
	Control	Aqueous	n-Hexane	Chloroform
500	40±0.1 ^{ab}	54±0.04	40±0.9 ^{ac}	38±1.4 ^{bc}
750	40±0.1 ^a	60±2.0	45±0.07 ^b	41±0.4 ^{ab}
1000	40±0.1	66±0.09	48±0.04	45±0.07

Values are mean \pm standard error (n=3). Mean values with the same superscript in a raw are not significantly different (P<0.05)

MIC of the extracts of *M. fragrans* against the growth of *M. restricta*

Table 6 represents MIC of the extracts of *M. fragrans* against the growth of *M. restricta*.

The results show that the MIC of aqueous extracts was found to be at 0.05mg/ml concentration. The MICs of n-Hexane and chloroform were found at 0.5mg/ml and 5mg/ml concentration respectively.

Table 6: MIC of the extracts of *M. fragrans* against the growth of *M. restricta*

Extracts	Concentration (mg/ml)							
	500	50	5	0.5	0.05	0.005	0.0005	
Aqueous MIC	-	-	-	-	+	+	+	
n-Hexane MIC	-	-	-	+	+	+	+	
Chloroform MIC	-	-	+	+	+	+	+	

Key: Growth: + , No Growth: -

Zones of inhibition of 3 different treatment of the extracts *M. fragrans* against the growth of *P. acne*

Table 7 shows the antimicrobial of *M. fragrans* against the growth of *P. acne*. The zone of inhibition occurs at all concentrations. The working concentrations of the whole

plant extracts exhibit inhibition on *P. acne*. However, the highest zone of inhibition (44mm) was recorded in aqueous extract at working concentration of 1000mg/ml and the lowest zone of inhibition (23mm) was recorded in chloroform extract at working concentration of 500mg/ml.

Table 7: Zones of inhibition of 3 different treatment of the extracts *M. fragrans* against the growth of *P. acne*

Concentration (mg/ml)	Mean zone of inhibition (mm) \pm Standard error			
	Control	Aqueous	n-Hexane	Chloroform
500	25 \pm 0.05	34 \pm 0.9	20 \pm 0.6	23 \pm 0.3
750	25 \pm 0.05	40 \pm 0.07	27 \pm 0.02	29 \pm 0.1
1000	25 \pm 0.05	44 \pm 1.0	29 \pm 0.07	33 \pm 0.3

Values are mean \pm standard error (n=3). Mean values with the same superscript in a raw are not significantly different (P<0.05)

MIC of the extracts of *M. fragrans* against the growth of *P. acne*

Table 8 represents MIC of the extracts of *M. fragrans* against the growth of *P. acne*. The

results show that the MICs of aqueous, n-Hexane and chloroform extracts was found to be in at 0.5mg/ml.

Table 8: MIC of the extracts of *M. fragrans* against the growth of *P. acne*

Extracts	Concentration (mg/ml)							
	500	50	5	0.5	0.05	0.005	0.0005	
Aqueous MIC	-	-	-	+	+	+	+	
n-Hexane MIC	-	-	-	+	+	+	+	
Chloroform MIC	-	-	-	+	+	+	+	

Key: Growth: + , No Growth: -

Phytochemical Results of *E. aromaticum* and *M. fragrans*

Table 4.9 shows the results of phytochemical screening carried out on *E. aromaticum* and *M. fragrans*. The result indicates the presence of flavonoids, tannins, alkaloids, steroids, saponin and glycosides in *E. aromaticum* and *M. fragrans* extract. Symbols such as (+), (++) and (+++) indicates slightly detected, moderately detected and highly detected respectively.

Table 9: Phytochemical parameters of plants extracts

Parameters	<i>E.aromatica</i> (Clove)	<i>M. fragrans</i> (Nutmeg)
Flavonoids	+	+
Tannins	++	+
Glycosides	++	+
Alkaloids	+++	++
Steroids	+++	++
Saponin	+	+

Key: Mildly Detected: +

Moderately: ++

Strongly: +++

DISCUSSION

E. aromatica (Clove)

All extracts of *E. aromatica* showed antimicrobial activity against the concerned microbial strains evaluated in this study as shown in Table 1. These results are in consistence with that of Chowdhury *et al.*, (2008) showing that *E. aromatica* extract inhibited the growth of *M. furfur* at 500µg/ml with inhibition zone of 22mm diameter. It also concurred with that of Mansourian *et al.*, (2014) who demonstrated that *E. aromatica* extracts (10 mg/100µl) possessed antifungal activity against *C. albicans*, with an inhibition zone diameter of 29.6 mm. The result is also in line with that Fu *et al.*, (2009) which showed antibacterial activity of the extracts of *E. aromatic* against *P. acne* and *S. epidermidis* with inhibition zone diameter of

24mm and 20mm at 100mg/ml concentration. Duraipandiyan *et al.* (2006), reported that antifungal activity of clove extracts was due to the presence of phenolic components (*eugenol* and *carvacrol*) whereas Alam *et al.* (2008), reported that inhibitory action of clove ethanolic extract was due to reduction in the amount of *ergosterol*, a specific fungal cell membrane component. Viuda-Martos *et al.* (2007), reported antifungal potential of clove extract against food spoilage fungi *Aspergillusflavus* and *A. niger* by agar dilution method. Reportedly, clove extracts possess antioxidant and antimicrobial properties due to the presence of phenolic compounds, such as flavonoids, hydroxybenzoic acids, and hydroxyphenylpropenes (Kumar *et al.*, 2012).

The antimicrobial activities of the clove may be attributed to the high content of *eugenol* in different extracts, confirmed by GC-MS analysis of clove, which inhibits the biosynthesis of ergosterol, a component of the microbial cell membranes. These may lead to the disruption of microbial cell membrane permeability causing cell death (Roome *et al.*, 2008).

M. fragrans (Nutmeg)

M. fragrans extracts inhibited the growth of the all isolates tested; fungi; *M. restricta* and bacteria; *P. acne*. The result of the present study showed that the Chloroform have more activity *M. restricta*. This result is comparable to that of Pooja *et al.* (2012), which showed that the methanol extract of *M. fragrans* have good activity against both the fungal strains, *Candida albicans* (0.237 mg/ml) and *A. niger*.

Chloroform extract was found to be more potent against *P. acne* with 52mm diameter of inhibition respectively. The result is in line with the findings of Shweta *et al.*, (2016), which showed that 95% of chloroform extract inhibit the growth of bacteria; *S. aureus*,

Salmonella spp., *Shigella* and *E. coli* with zone of inhibition of 24, 25, 19 and 30mm diameter respectively.

According Hoareau and Silva, (1999) and Kohinur *et al.* (2019), numerous active compounds including β -pinene, α -pinene, sabinene, safrole, terpinene-t-ol, myristicin, α -terpineol, and α -terpinene isolated from nutmeg seed may be responsible for antifungal and antibacterial activity. Some of this compounds may disturb the energy metabolism of mitochondria in the electron transfer and phosphorylation stage. The energy metabolism in mitochondria is inhibited with disturbance of electron transfer. Electron transfer will reduce oxygen and reduce the function of tricarboxylic acid cycle. Non-existence of phosphorylation stage causes inhibited formation of ATP and ADP.

Cho *et al.*, (2007) reported three chemical compound which may be responsible for the antimicrobial activity, these compound are called lignans and include, erythroaustrobailignan-6 (EA6), meso-dihydroguaiaretic acid (MDA) and nectandrin-B (NB) these compounds were isolated from the methanol extract of *M. fragrans* seeds.

CONCLUSION

Base on the result obtained, it can be concluded that *Eugenia aromaticum* and *Myristic fragrans* buds and seeds extracts showed antimicrobial activity against *Propionibacterium acne* and *Malassezia restricta* and contains phytochemicals which may serve as source of new and effective drugs. Therefore, the study supports the use of *E. aromaticum* and *M. fragrans* in traditional medicine to treat Dandruff infections, as practiced in the past.

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