Phytochemical Screening and *in –vitro* Antimicrobial Activity of *Monodora myristica* Seed Extract on Selected Human Pathogens

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Abstract: *Monodora myristica* is a spice whose various parts are used traditionally for the treatment of a variety of ailments. The purpose of this study was to determine the phytochemical constituents and in-vitro antimicrobial activity of methanolic seed extract of *M. myristica* against *Staphylococcus aureus, Esherichia coli* and *Candida albicans*. Plant seeds were collected, dehulled, air-dried, blended and extracted with methanol using soxhlet apparatus. The result of the qualitative phytochemical screening showed the presence of phenolic compounds, flavanoids, terpenoids, saponins, alkaloids, tannins and cardiac glycoside. Gas chromatography-mass spectrometry (GC/MS) analysis of the extract revealed the presence of twenty-one different compounds. The first compound detected was formic acid at a retention time of 6.032 minutes while the last compound detected was7-Acetyl-2-hydroxy-2 methyl-5-isopropylbicyclo [4.3.0.] nonane, at a retention time of 0.72 minutes. The extract exhibited increased antimicrobial activity with increasing concentration. The mean inhibition zone diameter (IZD) at 80mg/ml, 40 mg/ml, 20 mg/ml, 10 mg/ml extract concentrations were 25.8mm, 22.5mm, 20.5mm and 15.0mm for *E .coli*, 22.5mm, 21.0mm, 17.8mm, 15.0mm for *S. aureus* and 24.5, 20.5, 17.0, 18.0mm for *C. albicans*. The MIC ranged between 2.5 and 3.5 mg/ml while the MBC values ranged between 3.0 and 3.5 mg/ml

Key word: Antimicrobial activity, antimicrobial resistance, bioactive compounds, medicinal plants, *Monodora myristica*.

INTRODUCTION

Antimicrobial resistance is a global problem and a challenge to public health. The search for new antimicrobial agents is on-going and has extended to the field of medicinal plants (Miltra, 2014). Medicinal plants have attracted attention due to their wide range of applications in ethnomedicine (Alshehri, 2020). Medicinal plants have been reported to contain bioactive compounds known as phytochemicals which confer health benefits on humans and can be used to develop novel chemotherapeutic agents (Florence and Jeeva, 2016). Phytochemicals are present in different parts of plants; leaves, sterms, barks, roots, inflorescence, flowers, fruits and seeds (Sathya *et al.*, 2018). Spices are among such medicinal plants with beneficial natural ingredients usually added to foods. They impact aroma and taste of foods due to the presence of essential/volatile oils that comprise terpenes and terpenoids along with various aliphatic hydrocarbons, acids, alcohols, etc. (Omar *et al.*, 2016).

Monodora myristica is a tropical perennial tree of the Annonacea family of flowering plant that is widely distributed in Sub-Saharan Africa (Bunrubai *et al.*, 2007) and some parts of Central and South America. (Ekeanyanwu and Etienajirhevwe, 2012). The odour and tatse of *M. myristica* seeds is similar to that of nutmeg hence it is commonly called African nutmeg. Indigenous Nigerian names include: Ehuru (Igbo), Ariwo (Yoruba), Erhe (Urhobo), Iwor (Itsekiri), Ikposa (Bennin), and Guijija dan miya (Hausa) (Ekeanyanwu, 2013).

For traditional medicinal purposes, seeds are used as stimulant to relieve constipation, relieve toothache, dysentery, diarrhea, dermatitis, fever, control passive uterine hemorrhage in women immediately after child birth, headache, eye diseases and as vermifungal (Ezenwali *et al.*, 2010; Adesomogu *et al.*, 1991; Odoh *et al.*, 2004; Ekeanyawu *et al.*, 2010; Udeale, 2000 ; Onyenibe, 2015).

There is dearth of scientific information investigating the acclaimed ethno-medicinal uses of some Nigerian indigenous medicinal plants and their chemical constituents which has led to their neglect and underutilization. The objectives of this study were to determine the chemical composition, and in-vitro antimicrobial activities of methanolic seed extract on some selected human pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Knowledge of the bioactive compounds and antimicrobial activities of *M. myristica* seed extract will help in understanding its ethno-medicinal use, reveal health promoting chemical constituents that are still underutilized and lead to the discovery of new drug candidates and precursors which can be modified by the application of chemical synthesis.

MATERIALS AND METHODS

Collection and Identification of Plant Materials: African nutmeg (*M. myristica*) seeds were purchased from Sapele market, Delta State. Identification and authentication of the plant materials was done by Prof. Henry Adewale Akinnibosun of the Department of Plant Biology, University of Benin (UNIBEN) and Voucher number UBH-M350 was assigned to it.

Preparation of Extract: The seeds were washed with water to removed dust and rinsed with distilled water. The seeds were dehulled and air dried for two-weeks and thereafter blended into powder form using a dry electric blender (SAISHO, Model-S-748). After which approximately 250 g of the seed powdered was extracted with 2000 ml of methanol, using the Soxhlet apparatus. The extract was then concentrated to dryness at 55^{o}C using a thermostatically controlled water bath (Habamu *et al.*, 2010).

Preliminary Phytochemical Screening of Extract: The method described by Okah and Okwute (2020) with slight modification was used to screen the crude extract for the presence of alkaloid, steroids, phenols, flavanoids, glycosides, saponins, tannin and terpenoids.

Analysis of the crude extraact by Gas Chromatography-Mass Spectrophotometry (GC-MS)

The GC-MS analysis was carried out according to the method described by Isahq *et al.* (2015). GC-MS analysis was done at Leedex Laboratories Lagos, A SHIMADZU GCMS-QP 2010 Plus system was used. The GC-MS was operated under the following conditions: Column oven temperature: 60^{0}C; Injection temperature: 250^{0}C; Injection mode: split; Pressure: 100.0 kPa; Total flow: 9.7 ml/min; Column flow: 2.16 ml/min; Linear velocity: 37.9 cm/sec; Purge flow: 3.0 mL/min; and Split ratio: 2:1. The generated chromatogram was recorded. The identification of the components was carried out using the peak enrichment technique of reference compounds and computer matching with those of NIST.05 library mass spectrum.

Antimicrobial assay of the extract: Clinical isolates of *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* were obtained from the Medical laboratory of University of Benin Teaching Hospital, Benin-City. Isolates were characterized and identified using Gram staining, Cultural characterization using selective or indicative media and biochemical characterization as described by Hamburger, and Hostettmann, (2001). The pure isolates of each of the test organisms were inoculated in sterile slants containing Nutrient agar and potato dextrose agar and refrigerated at 4℃. Overnight broth cultures of the selected pure clinical isolates of *S. aureus*, *E. coli* and *C. albicans* were obtained, diluted with sterile distilled water and standardized by comparing the turbidity with 0.5 McFarland turbidity standard (Murray *et al.*, 2016). A sterile cotton swab was dipped into test tubes of each of the standardized organisms, rotated several times and pressed firmly on the inside wall of the test tube to remove excess fluid.

Dried surfaces of prepared Mueller Hilton agar plates were inoculated with the test bacteria (*S. aureus* and *E. coli*) by streaking the swab over the entire agar surface while surfaces of petri dishes containing potato dextrose agar were streaked with the fungus *C. albicans*. Wells of 7 mm in diameter were made onto the uniformly streaked Mueller Hinton agar/Sabouraud dextrose agar plates.

Preparation of Different Concentrations of Extract

A stock concentration of 100mg/ml of extract was prepared by dissolving 1.0g of extract in 10ml of 10% Tween 80. Different concentrations (80mg/ml, 40mg/ml, 20mg/ml, and 10mg/ml) of extract were made in sterile universal containers from the stock respectively**.** Each well was filled with 0.1 ml of the extract at varying concentrations (80mg/ml, 40mg/ml, 20mg/ml, and 10mg/ml). The same quantity of Tween-80 (10%) served as negative control while 1.0 µg/ml of Ciprofloxacin and 10 µg/ml of fluconazole were used as positive controls for bacteria and fungi respectively. All plates were incubated in an upright position. However, bacteria plates were incubated overnight at 37^{o}C and fungal plates were incubated at room temperature (25^{o}C) for 72 hr. The absence or presence of growth was observed on the plates and the diameter of clear zone was measured in mm and recorded. The experiments were done in duplicates and the mean zones of inhibitions calculated (CLSI, 2017)

Determination of MICs of the selected antimicrobial agent: The MIC's of the extracts that showed activity against the organisms were determined by the modified broth dilution method described by Cheesbrough, (2006). Varying concentrations of the extract ranging from 0.5 – 5.0mg/mL were constituted in 10ml of Mueller-Hinton broth in sterile capped tubes. Exactly 100 µL of the overnight broth culture of each test organisms diluted one in hundred-fold (1:100), corresponding to 0.5 McFarland turbidity standard (1x10^{8}CFU/ml) were added. In each round of experiment, a tube without the drug but with same volume of broth and inoculum served as controls. The same experiment was repeated for the fungal isolate but Potato dextrose broth was used in place of Mueller-Hinton broth. All tubes were appropriately incubated (37^{o}C for 24 hours for bacteria while fungal plates at room temperature (20-25^{o}C) for 72 hours). After incubation, tubes were observed for growth by examining for turbidity. In all cases, the lowest concentration of the antimicrobial substance at which there was no observable bacterial or fungal growth was recorded as the MICs.

Determination of Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) of the Extracts: The tubes with no visible growth following MIC determination were plated onto fresh Nutrient agar and PDA plates using a flamed wire loop to determine the MBC/MFC. MIC experimental tubes with concentrations higher than the MIC concentrations were considered. All plates were appropriately incubated. The MBC/MFC was taken for the minimum tube concentration in which no growth of bacterial/fungal growth was observed after plating out and appropriate incubation (Cheesebrough, 2006).

Statistical Analysis: Data analysis was carried out using Microsoft excel, Spss and Graphpad prism applications. All data were summarised by descriptive (mean, mean ± standard error of mean, etc.) into table charts and graphs and inferential (ANOVA, Tukeys multiple comparison) statistics at 0.05 significance levels (Ogbeibu, 2005).

RESULTS

The result of the qualitative phytochemical screening of the methanolic seed extract of *M. myristica* showed the presence of important phytochemicals as shown in Table 1.

Table 1: Phytochemical Constituents in *M. myristica* seeds

Phytochemical	Result
Tannin	+
Steroid	-
Terpenoids	+
Alkaloid	+
Saponin	+
Phenolic compounds	+
Flavonoid	+
Cardiac Glycoside	+

Key + = Positive – Present, - = Negative –Absent

Results of the gas chromatography- mass spectrometry (GC-MS) analysis are shown in Table 2 and Figure 1. The chromatogram reveals twenty-one chemical groups of compounds registered by each peak. The first peak detected the first compound (formic acid) at a retention time of 6.032 minutes while the last peak detected the last compound (7-Acetyl-2-hydroxy-2 methyl-5-isopropylbicyclo [4.3.0.] nonane) at a retention time of 0.72 minutes. The compound with the highest % height was acetic acid (22.94% height, peak 2) while glycerin recorded the highest % area (44.24 area%, peak 9).

Table 2: GC-MS identified constituents of methanol seed extract of *M. myristca*

Peak No.	Retention Time(Mins.)	Peak Area (%)	Height (%)	Compound Name	Compound Formula	Mol. Wt.
1	6.032	0.15	0.59	Formic acid	CH_2O_2	46
2	6.279	8.39	22.94	Acetic acid	$C_2H_4O_2$	60
3	6.986	0.12	0.48	Aziridine, 1-ethenyl-	C_4H_7N	69
4	7.490	0.15	0.60	Pyridine	C_5H_5N	79
5	7.652	0.24	1.02	2,3- Butanediol	$C_4H_{10}O_2$	90
6	7.754	2.09	8.45	2,3- Butanediol	$C_4H_{10}O_2$	90
7	8.255	0.93	3.31	1,3- Propanediol	$C_3H_8O_2$	76
8	9.083	2.93	3.13	L- Lactic acid	$C_3H_6O_3$	90
9	12.637	44.24	7.94	Glycerin	$C_3H_8O_3$	92
10	13.531	0.21	0.53	4H- Pyran-4-one, 2,33-dihydro-3,5- dihydroxy-6-methyl	$C_6H_8O_4$	144
11	14.515	0.42	1.09	Bicyclo[3.1.0]hexan-3-ol,4-methylene-1-(1-methylethyl),-(1.alpha.,3.alpha.,5.alpha.)	$C_{10}H_{16}O$	152
12	16.248	0.21	0.52	5-Heptene-2-one,6-methyl-	$C_8H_{14}O$	126
13	16.669	10.24	19.21	2-Oxabicyclo [2.2.2] Octan-6-ol, 1,3,3,-trimethyl-	$C_{12}H_{20}O_3$	212
14	16.900	0.51	1.20	5-Heptene-2-one,6-methyl-	$C_8H_{14}O$	126
15	17.996	6.79	8.97	Bicyclo(3.1.1)heptanes-2,3-diol,2,6,6-trimethyl	$C_{10}H_{18}O_2$	170
16	18.483	18.59	16.01	9-Octadecenoic acid (Z)-,methyl ester	$C_{19}H_{36}O_2$	296
17	19.067	1.32	1.29	Methyl stearate	$C_{19}H_{38}O_2$	298
18	19.794	0.74	0.69	Octanoic acid,7-oxo-	$C_8H_{14}O_3$	158
19	23.207	0.40	0.41	1-(7-Hydroxy-1,6,6-trimethyl-10-oxatricyclo[5.2.1.0(2,4)]dec-9yl)ethanone	$C_{14}H_{22}O_3$	238
20	23.950	0.63	0.65	Gingerol	$C_{17}H_{26}O_4$	294
21	24.175	0.72	0.97	7-Acetyl-2-hydroxy-2 methyl-5-isopropylbicyclo [4.3.0.] nonane	$C_{15}H_{26}O_2$	238
	100.0	100.0				

%Height

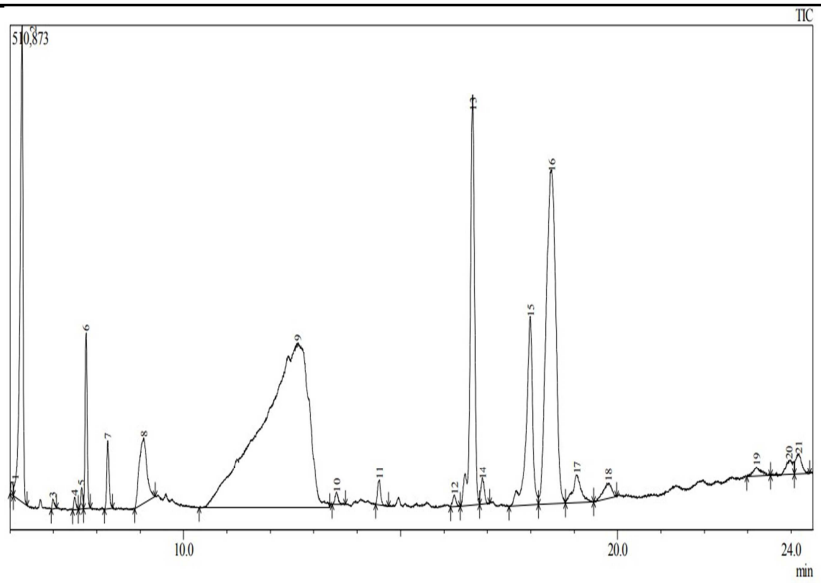

Figure 1: GC-MS sample chromatogram of the methanol seed extract of *M. myristica*

The mean inhibition zone diameter (IZD) at 80mg/ml, 40 mg/ml, 20 mg/ml, 10 mg/ml extract concentrations are 25.8mm, 22.5mm, 20.5mm and 15.0mm for *E .coli*, 22.5mm, 21.0mm, 17.8mm, 15.0mm for *S. aureus* and 24.5, 20.5, 17.0, 18.0mm for *C. albicans* as shown in figure 2.0. The zone of inhibition recorded against all the tested organisms in the study increased with increase in concentration of the extract, with the highest concentration (80mg/ml) exhibiting the highest inhibition zone diameter. In all concentration of the extract considered, the positive controls (ciprofloxacin and fluconazole) were observed to show higher inhibition zone diameters. Tween 80 used as negative control had no inhibition zone diameter

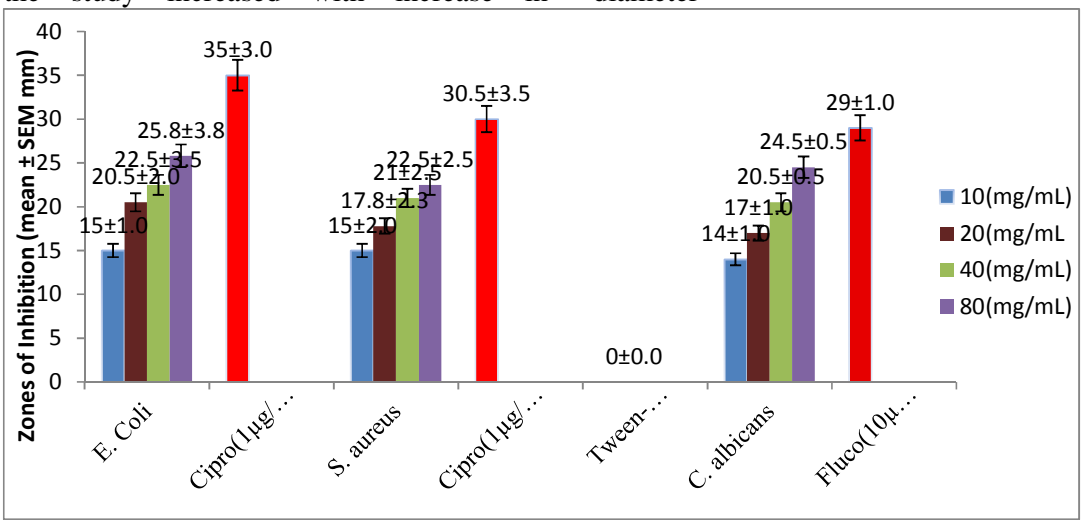

Figure 2: Antimicrobial activities of the methanol extract of *M. myristica* at different concentrations against test organisms.

The MIC ranged between 2.5 and 3.5 mg/ml while the MBC values ranged between 3.0 and 3.5 mg/ml as shown in table 3.0.

Table 3: Minimum inhibitory concentrations (MICs) and Minimum Bactericidal/Fungicidal Concentrations (MBCs/MFCs) of the methanol extract of *M. myristica* against the Test Organisms

Organisms	MICs	MBCs/MFCs (mg/ml)
Esherichia coli	2.5	3.0
Staphylococcus aureus	3.0	3.0
Candida albicans	3.5	3.5

DISCUSSION

The result of the qualitative phytochemical screening of the methanolic seed extract of *M. myristica* revealed the absence of steroids, while tanins, terpenoids, alkaloid, saponin, phenols, flavonoid and cardiac glycoside were present. This result is in agreement with those reported by Enabulele *et al.* (2014).

The GC-MS analysis revealed the presence of twenty-one (21) compounds, some of which have never been reported as constituents of *M. myristica* seeds. The identified compounds belong to the following categories of chemicals: organic acids, terpenoids, saturated fatty acids, unsaturated fatty acid esters, alkaloids, flavonoids, alcohols and phenolic compounds. Some of the identified compounds are in line with those reported by other researchers: acetic acid and oleic acid (Ezeuko *et al.*, 2017), 1, 3, 3-trimethyl-2-oxobicyclo [2.2.2.] octan-6-ol (Edewor and Kazeem, 2016) and gingerol (Feyisayo and Oluokun, 2014).

The health benefits of some of the identified compounds have been reported. Organic acids such as formic acid, acetic acid and lactic acid improve digestion, nutrient digestibility, intestinal health and promotes growth performance in animals. Organic acids have antimicrobial activity based on their ability to cross the cell membrane, due to the lipophilic nature of their undissociated forms, modifying proton and associated anion concentrations of the cytoplasm of microorganisms, consequently, purine bases and essential enzymes are negatively affected and bacteria viability decreases (Dibner and Buttin, 2002; Warnecke and Gill, 2005).

Aziridine is an alkaloid with proven antitumor and antimicrobial activity. Aziridiines are powerful alkylating agents and their in-vivo potency is based primarily on toxicity to microbes (Dembitsky *et al.*, 2013).

Pyridine is an aromatic compound with antihypertensive, antihistaminic, anticoagulant, anti-inflammatory, antibacterial, antifungal, antivial, antitubercular, antidiabetic and antimalaria activity (Prathima *et al.*, 2013).

Glycerin reduces pressure in the eye. This supports the traditional use of African nutmeg seeds for the treatment of eye diseases.

Propanediol and butanediol are aliphatic primary alcohols and are used as excipient in many drug formulations to increase the solubility and stability of drugs. Propanediol works as a humectants and emollient in the skin. This supports traditional the use of African nutmeg seeds in the treatment of dermatitis.

Gingerol is a phenolic compound with multiple biological activities including antioxidant, anti-inflammatory, antimicrobial, anticancer, neuroprotective, cardiovascular protective, respiratory protective, antiobesity, antidiabetic, antinausea and antiemetic activities (Mao *et al.*, 2019).

Methyl esters such as 9-Octadecenoic acid and methyl stearate are unsaturated fatty acids with antimicrobial, anti-inflammatory, antiandrogenic and anemiagenic properties (Rajeswari *et al.*, 2013; Lee *et al.*, 2009).

Octanoic acid is a saturated fatty acid with antibacterial and antioxidant activity (Mokhtar *et al.*, 2017).

Bicyclo (3.1.1.) heptanes-2, 3-diol, 2,6,6-trimethyl- is a terpenoid with antimicrobial activity. 4H- Pyran-4- one, 2,33- dihydro-3,5- dihydroxy-6- methyl-, is a flavonoid with antimicrobial, anti-inflammatory and antiproliferative activities. 1,3,3-Trimethyl-2-Oxabicyclo [2,2,2] octan-6- ol or eucalyptol a natural, organic compound which is a colorless liquid is used as an ingredient in many brands of mouthwash and cough suppressant (Edewor and Kazeem, 2016).

The antimicrobial activity of an antimicrobial agent is evident by the presence of growth inhibitory zones on seeded agar plate (Bairy *et al.*, 2002). The IZDs recorded for the methanol seed extract of *M. myristica* as well as Ciprofloxacin and fluconazole (positive controls) shows good activity against the test organism when compared to Tween-80 (negative control) which showed no activity. Extracts were considered active at zone of inhibition of >7 mm due to the diameter of the cork borer used to make the agar wells (Ndukwe *et al.*, 2005; Usman *et al.*, 2005). The zone of inhibitions recorded in this study varied with the tested organism and increased with an increase in the concentration of the extract, with the highest concentration (80mg/ml) exhibiting the highest inhibition zone diameter. However, the extract concentrations were less than those observed with the reference antibacterial drug (ciprofloxacin) and antifungal agent (fluconazole). This disparity is due to the pure nature of the standard control drugs, whereas the extract is still in its crude state which requires a lot of purification in order to isolate the active compounds (El-Mahmood, 2009). Tween 80 which was used as negative control had no inhibition zone diameter which clearly revealed that it wasn't a contributing factor to the activity of the plant extract. The zones of inhibition recorded in this study are similar to those reported by Benneth *et al.* (2022) who reported zones of inhibition of 24.0mm, 17.90mm, 14.50mm and 11.50mm for *S. aureus* and 23.50mm, 17.41mm, 13.90mm and 6.57mm for *E. coli* at extract concentrations of 30mg/ml, 20 mg/ml, 10 mg/ml, 5.0 mg/ml respectively.

The extract exhibited antibacterial and antifungal activities. The MIC and MBC/MFC result shows that bacteria are more susceptible to the plant extract than fungi. *E. coli* is more susceptible to the bacteriostatic and bacteriocidal activity of the plant extract than *S. aureus*. The extract also exhibited fungicidal and fungistatic activity against *C. albicans* as shown in table 3.0. This result is in agreement with those reported by Benneth *et al.* (2022) who reported *E. coli* to be more susceptible than *S. aureus* and Nwaiwu and Imo, (1999) who reported antifungal activity of *M. myristica* against mycelial growth of three foodborne fungi; *Aspergillus fumigatus, Aspergillus nidulans* and *Mucor hiemalis*. The MIC and MBC values are quantitative indices used to measure the effectiveness of an antimicrobial agent against microorganisms and are of importance in fixing benchmark of effective dose concentration of antimicrobial agents (Bairy et al., 2002, Vinothkumar *et al.*, 2012). The lower the MIC value, the more potent the antimicrobial agent, conversely, the higher the MIC value, the less potent the antimicrobial agent (Dowe *et al*., 2016)

CONCLUSION

The result of the phytochemical screening of the methanolic seed extract of African nutmeg showed the presence of important phytochemicals which can be prototypes and lead compounds for drug discovery. The antimicrobial activity result shows that the seed extract has good potentials as an antibacterial and antifungal agent and further provide a rationale for the use of the seed extract in traditional medicine practice in Nigeria for the treatment of human infections.

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