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Antimicrobial and Therapeutic Potentials of the Ethylacetate Fractions of Crude Methanolic Extract of *Monodora myristica* Seed

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Authors' contributions

The study was done in collaboration between the authors. Author EEN carried out the experimental work (which was his bachelor of Pharmacy degree research work) and also wrote the manuscript while author GCE supervised the work. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: The study was designed to evaluate the antimicrobial properties and potential therapeutic applications of *Monodora myristica* seed.
Study Design: Antimicrobial evaluation design.
Place and Duration of Study: The study was conducted at the Pharmaceutical/Medicinal Chemistry research laboratory, Faculty of Pharmaceutical sciences, University of Nigeria, Nsukka between January 2000 and February 2001.
Methodology: Standardized solutions of ethylacetate-soluble and ethylacetate-insoluble fractions of the methanolic extracts of *Monodora myristica* seed were evaluated for antimicrobial activity against some gram-positive bacteria, some gram-negative bacteria, a yeast and a mould using the agar disk method.
Results: The ethylacetate-soluble fraction showed significant activities against the gram-positive

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bacteria (*Bacillus subtilis*), gram-negative bacteria (*Klebsiella pneumoniae* and *Escherichia coli*), the yeast (*Candida albicans*) and the mould (*Aspergillus niger*) with most of the activities comparable to those of the controls. Its activity against *K. pneumoniae* was greater than that of the control while its activity against *E. coli* was equal to that of the control. Its activities were also greater than those of the ethylacetate-insoluble fraction against most of the test organisms except against *C. albicans* where its activity was less than that of the ethylacetate-insoluble fraction. The activity of the ethylacetate-insoluble fraction against *K. pneumoniae* was also equal to that of the control. However both the ethylacetate-soluble and ethylacetate-insoluble fractions had no activity against the gram-positive bacteria (*Staphyllococus aureus*) and the gram-negative bacteria (*Pseudomonas aeruginosa* and *Salmonella typhi*). Again, compared to the controls, the activities of both fractions against *C. albicans* and *A. niger* were not as strong as those against the other sensitive gram positive and gram negative organisms.

Conclusion: The ethylacetate-soluble fraction of *M. myristica* seed has greater antimicrobial activities than the ethylacetate-insoluble fraction. The activities of the ethylacetate-soluble fraction were comparable to those of the controls, being greater against *K. pneumonia* and equal against *E. coli*.

Keywords: Antimicrobial properties; therapeutic potential; Monodora myristica seeds; Ibo-Nigeria folkloric medicine.

1. INTRODUCTION

Antimicrobial agents are substances that kill microorganisms or inhibit their growth. The use of herbs and spices to achieve this is known to have been a common practice for thousands of years [1]. *Monodora myristica* is one of the antimalarial plants in Ibo-Nigeria folkloric medicine with claims of antimicrobial properties. It has been reported to possess high antifungal properties [2]. The *Monodora myristica* plant is an ornamental tree with a height of up to 30m high, dense foliage and spreading crown. It flowers from September to April at which time the new leaves appear. The fruits are produced between April and September. They are about 15 cm in diameter, green, round, woody and are suspended in a long stalk. The pulp is white and contains numerous seeds of about 1.5 cm long [3]. It grows widely in Cameroon, Nigeria and other West and sub-saharan African countries. It is called 'Efuru' or 'Ehuru' in Ibo-Nigeria, 'Gujiya dan miya' in Hausa-Nigeria and 'Abo lakoshe', 'arigbo' or 'eyi naghose' in Yoruba-Nigeria [1]. This study is a follow up of a previous study by the authors on the antimicrobial screening of twenty (20) plants used as antimalarial remedies in Ibo-Nigeria folkloric medicine in which *Monodora myristica* was observed to possess high antimicrobial properties [4]. That study was done to find out if these twenty antimalarial herbs or any of them had antimicrobial properties that could be of benefit to modern medicine and pharmacotherapy.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant materials

This consisted of the seeds of *M. myristica*. These seeds were collected in September at Nsukka in Enugu state of Nigeria and were identified by Mr Paulinus Ugwu and Mr J.E Ekekwe both of Botany department of University of Nigeria, Nsukka. They were then prepared by cutting, sun-drying and milling. The powdered forms were then used in the experiments.

2.1.2 Reagents

Sulphoric acid (Loba Chemie, India), Chloroform (Loba Chemie, India), Ammonia solution (Griffin & George), Ferric chloride (Loba Chemie, India), Fehling's solution 1 and 11(Loba Chemie, India), Ethylacetate (Analar BDH Ltd England), Hydrochloric acid (Loba Chemie, India), Glacial acetic acid (Analar BDH Limited England), Aluminium chloride, Ethanol, Bromine water, Mayer's reagent, Distilled water, Sodium hydroxide, Tollen's reagent, 2,4-dinitrophenylhydrazine, Acetic anhydride (Pharmacos Ltd, England), acetic acid (Fluka Chemie GMBH, Switzerland), silica gel GF_{254}.

2.1.3 Solvents

Methanol, Ethylacetate (Analar BDH Ltd England), Methylethylketone (MEK),

MEK/Hexane, Dimethyl sulphoxide (DMSO) (Sigma Aldrich Co), Chloroform (Loba Chemie, India) and Ethanol (Analar BDH Ltd England).

2.1.4 Instrumentation

Uniplan TLC spreader (Burkle Ltd, Germany), Chromatographic tank (Corning Ltd, USA), Aluminium plates, Silica plates, Separating funnel, Evaporating dish (Corning Ltd, USA), Rotary evaporator (Barloworld scientific Ltd, UK), Water bath (Medica instrument, MFG.Co.India), Capillary tubes, Test tubes, Conical flasks, Measuring cylinders, Beakers, Pipettes, Funnels, Filter papers, Weighing balances (Ohaus corp. Pine brook, NJ USA), Glass chromatoplates, UV lamp (Genlab Ltd, UK), Bunsen burner and Spatula.

2.1.5 Microbiological materials

Broth cultures of test organisms [gram-positive bacteria (*B. subtilis, S. aureus*), gram-negative bacteria (*K. pneumoniae, P. aeruginosa, S. typhi, E. coli*), the yeast (*C. albicans*) and the mould (*A. niger*)], Sterile Petri dishes, Sterile Cork borer, Sterile Forceps, Inoculation loop, Incubator (Genlab Ltd, UK), Autoclave (Desco Ltd, USA), Indelible Marker, Lighter, Nutrient agar, Paper strip. The control drugs were research grade Penicillin G (Teva), Chloramphenicol (Biochem) and Nystatin (Teva). The above organisms were clinical isolates obtained from the microbiology laboratory of the University of Nigeria, Nsukka. The cultures of the organisms were maintained on nutrient agar slants at 4℃ and were re-identified by biochemical tests according to the methods described in [5,6].

2.2 Methods

M. myristica seeds were sun-dried, milled and extracted by cold maceration with 95% methanol. The solvent was recovered by evaporating the resulting solution to dryness under reduced pressure using a rotary evaporator. This methanolic extract was further fractionated with ethylacetate to obtain an ethylacetate-soluble fraction and an ethylacetate-insoluble fraction. Penicillin G, chloramphenicol and nystatin were used as controls for the screening. Solutions of the ethylacetate fractions together with solutions of the controls were standardized to 10 mg/ml solution in dimethyl sulphoxide solutions [7]. These standardized solutions of the extracts including the controls were then evaluated for anti-microbial activity against some gram-positive bacteria (*B. subtilis, S. aureus*), gram-negative bacteria (*K. pneumoniae*, *P. aeruginosa*, *S. typhi, E. coli*), the yeast (*C. albicans*) and the mould (*A. niger*) using the agar ditch method. This process is illustrated with the flow chart in Fig. 1.

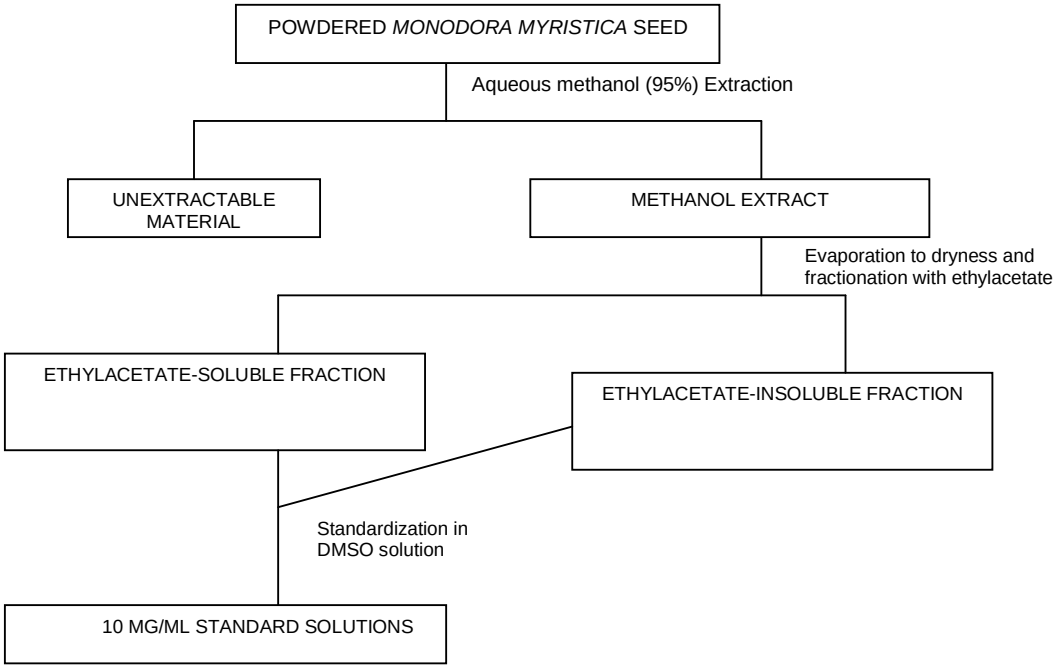

Fig. 1. Flow chart for the extraction and fractionation of *M. myristica* seeds

3. RESULTS

The results showed that the ethylacetate-soluble fraction had significant activities against *B. subtilis*, *K. pneumonia, E. coli*, *C. albicans* and *A. niger* with most of the activities comparable to those of the controls. Its activity against *K. pneumonia* was greater than that of the control while its activity against *E. coli* was equal to that of the control. Its activities were also greater than those of the ethylacetate-insoluble fraction against the test organisms except against *C. albicans* where it had near-equal activity with the ethylacetate-insoluble fraction. The activity of the ethylacetate-insoluble fraction against *K. pneumonia* was also equal to that of the control. However both the ethylacetate-soluble and ethylacetate-insoluble fractions had no activity against *S. aureus*, *P. aeruginosa* and *S. typhi*. Again, compared to the controls, the activities of both fractions against *C. albicans* and *A. niger* were not as strong as those against the other sensitive gram positive and gram negative organisms. These results are shown in Table 1, Table 2 and Fig. 2.

4. DISCUSSION

The foregoing study showed that the ethylacetate-soluble fraction of *M. myristica* seeds was generally more active than the ethylacetate-insoluble fraction against the various organisms used for the study. Its activities were comparable and in some cases equal to those of the controls. These results corroborate the rationale behind *M. myristica's* variety of uses in Ibo-Nigerian folkloric medicine [8,9]. Against *B. subtilis*, the ethylacetate-soluble fraction showed appreciable antimicrobial activity greater than that of the ethylacetate-insoluble fraction but slightly lower than that of the control/pure drug. The activity of the ethylacetate-insoluble fraction was however fairly appreciable. *Bacillus* organisms are free-living, gram-positive rods that are aerobic and spore-forming. Several of the species produce potent toxins that can be lethal in experimental animals [10]. They are ubiquitous, being found in soil, water, dust and air. They are largely seen as non-pathogenic and as contaminants when isolated in the bacteriology laboratory but occasionally they can be responsible for significant disease. Infections with *bacillus* organisms have also been associated with intravenous drug abuse, operative procedures, traumatic wounds, burns, hemodialysis and prosthetic heart valves and are characteristically resistant to the penicillins and cephalosporins [11]. These situations may however benefit therapeutically from *M. myristica* seed which has shown appreciable activity against *B. subtilis*.

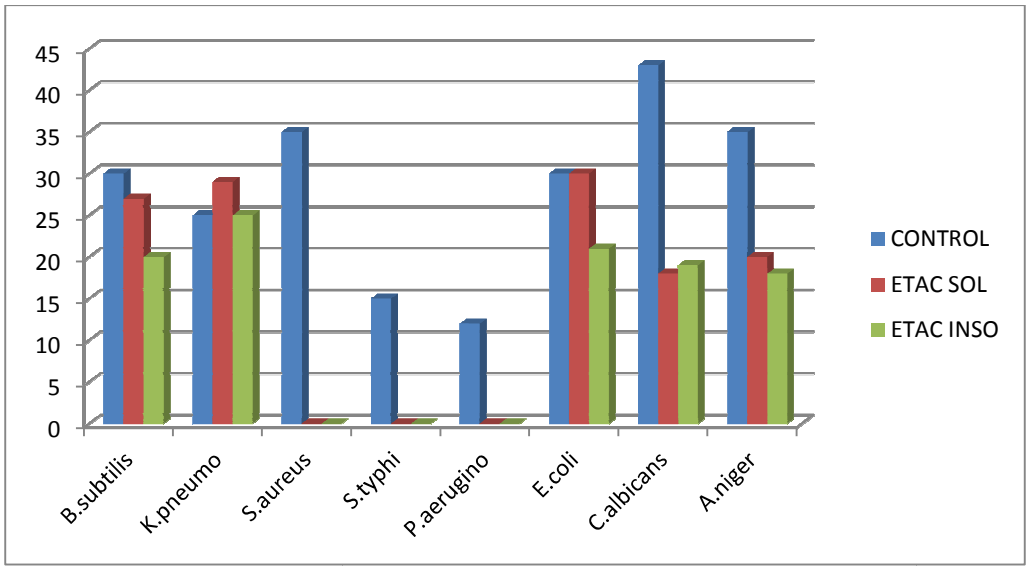

Fig. 2. Bar chart comparing the relative activities of the *M. myristica* fractions with that of the control drugs

Key: ETAC SOL = Ethylacetate soluble fraction, ETAC INSO = Ethylacetate insoluble Fraction, CONTROL = Control/Pure drugs, B. Subtilis = Bacillus subtillis, K. pneumo = Klebsiella pneumoniae S. aureus = Staphylococcus aureus, S. typhi = Salmonella typhimurium, P. aerugino = Pseudomonas aeruginosa, E. coil = Eschericia coli, C. albicans = Candida albicans, A. niger = Aspergilus niger

Table 1. Results of inhibition zone diameter measurements

S.N	Plant	Fraction used	Concentration (mg/ml)	Inhibition zone diameter (mm)							
				B. sub	***K. pneu***	***S. aur***	***S. typhi***	***P. aeru***	***E. coli***	***C.alb***	***A. niger***
1	*Monodora myristica* seed	ETAC soluble	10	27	29	-	-	-	30	18	20
2	*Monodora myristica* seed	ETAC insoluble	10	19	25	-	-	-	21	19	18
3	Penicilline G	P.D	10	30	25	35	15	-	30	-	-
4	Chloramphenicol	P.D	10	30	21	31	12	12	25	-	-
5	Nystatin	P.D	10	-	7	-	-	13	12	43	35

Key: ETAC = Ethylacetate, PD = Pure drug, B. Sub = Bacillus subtillis, K. pneu = Klebsiella pneumoniae S. aur = Staphylococcus aureus, S. typhi = Salmonella typhimurium, P. aeru = Pseudomonas aeruginosa, E. coil = Eschericia coli, C. alb = Candida albicans, A. niger = Aspergilus niger

Table 2. Percentage activities of the *M. myristica* fractions relative to those of the control drugs

S/N	Organisms	Percentage activities of the *M. myristica* fractions relative to those of the control drugs (Fraction activity ÷ Control drug activity × 100)	
		Ethylacetate soluble fraction (%)	**Ethylacetate insoluble fraction (%)**
1	*Bacillus subtilis*	90	63
2	*Klebsiella pneumoniae*	116	100
3	*Staphyllococcus aureus*	0	0
4	*Salmonella typhimurium*	0	0
5	*Pseudomonas aeruginosa*	0	0
6	*Escherichia coli*	100	70
7	*Candida albicans*	42	44
8	*Aspergillus niger*	57	51

NOTE: For table 2, the control drug with the highest Inhibition zone diameter was used for the corresponding organism in the calculation

Against *K. pneumoniae*, the ethylacetate-soluble fraction gave an activity greater than that of the control while the ethylacetate-insoluble fraction gave an activity equal to that of the control drug. This is of very clinical importance considering the problem associated with the treatment of *Klebsiella* infections. *K. pneumonia* is a gram-negative bacillus, one of the few that cause primary lobar pneumonia, a non-motile and encapsulated organism and an important nosocomial pathogen accounting for up to 10% of hospital acquired infections [12]. Multi-drug resistant strains have become endemic in many hospitals with the persistence of the organism associated with the continued use of large quantities of antibiotics and with the establishment of intestinal carriage among asymptomatic patients [13-15]. *Klebsiella* is resistant to ampicillin, carbenicillin with strains resistant to cephalothin, chloramphenicol, tetracycline and gentamicin increasing in frequency probably due to the acquisition of multidrug-resistant R factors. However almost all strains of the organism remain sensitive to Amikacin which has been reserved for only gentamicin-resistant organisms [16,17]. The high activity of *M. myristica* seed against the *Klebsiella* organism could make it a potential source of alternative and effective antimicrobial compound against the organism.

Both fractions showed no activity against *S. aureus* as in the preceding study [4]. *S. aureus* is a highly resistant gram-positive, non-spore forming bacterium with a high prevalence in both communities and hospitals. The high morbidity and mortality associated with it as well as the economic consequences and the virtual absence of a non-human reservoir makes the organism a major subject of epidemiologic studies [18].

Both fractions also showed no activity against *S. typhi* as in the preceding study [4]. *S. typhi* is the causative agent of typhoid fever (enteric fever). It is a non-spore forming gram-negative enterobacteria rod. Selection of antimicrobials for the treatment of *Salmonella* infections has been complicated by the emergence of *Salmonella* strains that are resistant to multiple antimicrobials [19-21]. This resistance is transferred from organism to organism on plasmids that carry genetic determinants of resistance (R factors).

Against *P. aeruginosa*, both fractions also showed no activity in contrast to the result in the preceding study [4], where crude *M. myristica* seed extract showed appreciable anti-*pseudomonas* activity. This may need further confirmation. *P. aeruginosa* is a gram-negative aerobic and flagellated rod belonging to the family of *pseudomonadeceae*. It is cosmopolitan in distribution and is sometimes present as part of the normal microbial flora of man. It rarely causes disease in normal healthy persons despite being a common human saprophyte. However, disease process as a result of infection by it begins with some alteration or circumvention of normal host defenses which may involve a disruption in the integrity of physical barriers to bacteria invasion such as the skin or mucous membranes or their circumvention as in the case of intravenous lines, urinary catheters or endotrachael tubes [22]. The pathogenesis of the infection from this organism is multifactorial as suggested by the large number of potential virulence factors it produces and the broad spectrum of diseases it causes. The incidence and relative frequency of hospital acquired infections from it has also been reported to be on the rise [23].

Both fractions also showed appreciable activity against *E. coli*. The activity of the ethylacetate-soluble fraction was equal to that of the control here and greater than that of the ethylacetate-soluble fraction. The activity of crude *M. myristica* seed extract was greater than that of the control in the preceding study [4], indicating its potential therapeutic usefulness in infections caused by *E. coli* like urinary tract infections (UTIs), bacteremia, neonatal meningitis, traveler's diarrhea etc. *E. coli* belong to the family of *enterobacteriaceae* which is a diverse group of gram-negative non-spore-forming *bacilli*, many of which are pathogenic to man, other animals and plants. They are aerobic but can grow under anaerobic conditions and so are facultative anaerobes [17]. Many members of this group including *E. coli* possess plasmids which are extrachromosomal genetic elements on which genes expressing virulence properties are carried. Some of these plasmids called R factors encode for resistance to multiple antibiotics. Heavy use of antibiotics in hospitals favors the selection of R factor containing strains which might contribute to the increased antibiotic resistance of resident flora. Other R factor genes encode for the conjugal transfer of plasmids from organism to organisms even members of different species causing widespread outbreaks of nosocomial infections that have involved hundreds of patients, many institutions and several bacteria species. *E. coli* is the most common cause of urinary tract infections (UTIs),

comprising more than 90% of infections arising outside the hospital [17]. It is also the leading cause of gram-negative bacteremia in adults and treatment of these infections could benefit from the high antimicrobial activity of *M. myristica* seed extract which has shown appreciable antimicrobial activity against it.

Both fractions again showed activity against *C. albicans* with the ethylacetate-insoluble fraction showing greater activity than the ethylacetate-soluble fraction. These activities were however much less than those of the controls. *C. albicans* is a fungi confined to human and animal sources. They are normal commensals of man and are found on diseased skin, enteric gastrointestinal tract, expectorated sputum, the female genital tract and urine of patients with indwelling foley catheters. Interestingly it rarely colonizes normal skin but damaged skin becomes rapidly colonized with *C. albicans* [24]. Incidence of diseases due to *C. albicans* has increased in frequency over the last 50 years with a relatively large number of manifestations. These may therapeutically benefit from the appreciable antimicrobial activity of *M. myristica* seed. This strong antifungal activity of *M. myristica* shown in this study corroborates the results of other works that also demonstrated its antifungal properties and its use in the preservation of Okro and other crops in Ibo-Nigerian agriculture.

Both fractions also showed activity against *A. niger*, with the ethylacetate-soluble fraction showing greater activity than the ethylacetate-insoluble fraction. These activities were also much less than those of the controls. *A. niger* is a mould that is ubiquitous in nature. It causes aspergillosis, a disease that describes an illness attributed to the antigenic stimulation, colonization or tissue invasion by *aspergillus*. The disease is acquired by inhalation of airborne spores (conidia) which are small enough (2.5 – 3.0 micrometer) to reach the alveoli or to gain entrance to paranasal sinuses with diverse clinical manifestations. Exposure to aspergillosis is nearly universal but the disease is uncommon. When it occurs it could be invasive resulting in serious infections that may require surgical excision of infected body parts to contain the disease e.g. invasive aspergillosis of the brain and paranasal sinuses, non-invasive sinus colonization and possibly aspergillosis of prosthetic cardiac valve which can aid response to chemotherapy, though prognosis in *aspergillus* endocarditis remains dreadful [25]. *M. myristica* seed could be therapeutically relevant in these conditions.

The study in summary has shown that the ethylacetate-soluble fraction of *M. myristica* seed possess appreciable antimicrobial properties which are greater than those of the ethylacetate-insoluble fraction. However further in-vivo and in-vitro tests may be needed to confirm these results.

5. CONCLUSION

The ethylacetate-soluble and the ethylacetate-insoluble fractions of *M. myristica* seeds possess appreciable antimicrobial activities. However the ethylacetate-soluble fraction showed greater antimicrobial activities than the ethylacetate-insoluble fraction. The activities of the ethylacetate-soluble fraction were comparable to those of the controls, being greater against *K. pneumonia* and equal against *E. coli*.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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