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Bactericidal and brine shrimps toxicity of essential oils from *Aframomum Melegueta* [K. Schum]

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Aframomum melegueta **(Roscoe) K. Schum, family of Zingiberaceae, is a tropical tree with spicy edible fruit. This plant has both medicinal and nutritive values. There is paucity of literature on the toxicity and bioactivity of the essential oils from this plant from Nigeria. Essential oils were extracted from the leaves, stems, roots (rhizomes) and seeds of the plant through hydro-distillation using the Clevenger-type apparatus. The toxicity of the volatile oils was determined using the brine shrimp toxicity assay at concentrations of 10.0, 100.0 and 1000.0 ppm and the median lethal concentration (LC_{50}) was calculated using Finney's probit analysis. The antimicrobial assay was carried out using the cup plate agar diffusion method. Five bacteria consisting of three strains of Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis* and *Salmonella typhi*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were screened. The stems' essential oil displayed the highest toxicity (LC_{50} = 0.2 µg/mL) followed by the seeds' essential oil (LC_{50} = 0.3 µg/mL), while LC_{50} of the essential oil from the leaves offered the least toxicity (LC_{50} = 17.5 µg/mL) in brine shrimp toxicity assay. All essential oils showed moderate inhibition of the tested bacteria at 1mg/mL but showed no inhibition below 125 µg/mL concentration. The high brine shrimps' toxicity and bactericidal activity of the essential oils is indicative of their potentials as sources of pharmaceuticals or natural pesticides.**

Key words: Essential oils, brine shrimps toxicity, bactericidal activity, *A. melegueta*

INTRODUCTION

Essential oils are complex mixtures of natural compounds or secondary metabolites which may contain 20 to 60 components at different concentrations (Ekundayo, 1986; Bakkali et al., 2008; Miguel, 2010; Rubiolo et al., 2010). They often composed of large groups of organic compounds with diverse functionality. However, common constituents are hydrocarbons (terpenes and sesquiterpenes) and oxygenated compounds (Alcohols, aldehydes lactones, acids, phenols, oxides, lactones, ethers, and esters) (Poumortazavi and Hajimirsadeghi, 2007).

It is believed that essential oils take part in plants' chemical defence systems. This suggests that they appeared as plant's reaction to attacks (Bassole and Juliani, 2012). In view of the fact that plants are stationary and could not run from attacks, they have developed alternative methods to fight for their existence. Some produced thorns, hairs, or thick corticle, while others

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produce chemicals that render them toxic, unpalatable or nauseating (Dosumu, 2008). Precisely, essential oils help to protect plants from bacterial, fungal, and other microbial infections. Leaf volatiles serve to dissuade herbivory by marauding insects and animals, while floral volatiles attract pollinators; including animals which are essential for pollination and seed dispersal, and repel others which are harmful. The same compounds that have served plants very well in their chemical warfare also are useful to man in many applications. Thus, essential oils have been used as perfumes, flavours and in medicinal preparations for centuries. Currently, over 1,000 different chemical components have been identified in essential oils. The synergistic effect of all the components make up the peculiar fragrance observed in any essential oil (Bauer et al., 2001). In fact, the absence of one component may alter the odour of oil completely. Such components are the character-impact compounds (George-Nasamanto and Cori, 1971).

Major components of essential oils are found to reflect on the biophysical and biological features. The degree of their effects is dependent on their concentration when tested singly, or comprised in the essential oils used wholly. It is therefore unclear whether the potency of essential oils depends on the synergistic functions of its various components as commonly believed (Ipek et al., 2005). However, there is a strong argument that, the activity of the main components is modulated by other minor constituents (Franzios et al., 1997; Santana- Rios et al., 2001; Hoet et al., 2006). This is expected because all components of the essential oils play a role in defining the density, texture, colour and cellular distribution (Cal, 2006). Thus, for biological purposes, it is more informative to study the entire oil rather than some of its components because the concept of synergism seems more meaningful (Bakkali et al., 2008).

Essential oils are used in cosmetic industry, in sanitary products, dentistry, in agriculture as food preservers and additives, and as natural medicines (Burt, 2004). Moreover, essential oils are used for massages in mixtures with vegetal oil or in baths but most frequently in aromatherapy (Bauer and Garbe, 1985). In all, essential oils play an important role in the protection of plants, and useful to man as fragrance, food and medicine. Most of the people living in rural African settlements rely on medicinal plant preparations use in treatment of any kind of diseases. The preparations are either made by natives themselves or obtained from traditional medicine practitioners. In Nigeria, the alternative therapy, that is, the use of natural herbs for treatment of diseases is now becoming well accepted even among the urban population and the elites as well. In advanced societies, the relatively recent enhancement in the preference for natural products has led to a renewal of scientific interest in essential oils (Nychas, 1995).

The composition of essential oils from a particular species of plant may differ due to geographical sources (Arras and Grella, 1992; Cosentino et al., 1999; Jerkovic et al., 2001; Kokkini et al., 1997). Therefore, there is need for continued research into local plant species from different climes. However, many essential oil-bearing plants in Nigeria have not been investigated for Brine shrimps' toxicity and pharmacological activity. As a result, the bactericidal activity and brine shrimps' toxicity of the essential oils from the leaves, stems, rhizomes and seeds of *Aframomum melegueta* K. Schum grown in Nigeria was investigated.

MATERIALS AND METHODS

General experimental procedures

All chemicals used in this research were of 'analar' grade, obtained from Sigma-Aldrich sales agents in Nigeria. Materials and apparatus used were: an all-glass Clevenger distiller, a 2000 ml heating mantle, 2000 ml round bottom flasks, Pasteur pipette, dimethylsulphoxide (DMSO) and brine shrimps (*Artemia salina*) eggs. The GC-MS spectra were obtained on Agilent 6890N GC coupled with MS-5973-634071 series running on Agilent-Chemstation retention time locking software (Agilent Technologists, USA) as reported earlier (Owokotomo et al., 2014).

Collection of plant materials

A. melegueta plants were harvested at the farm in Akure, Nigeria. Identification and authenticated was achieved at the Herbarium of the Forest Research Institute of Nigeria, Ibadan. Voucher number was *FHI109020.*

Extraction of essential oils and analyses

The leaves, stems and roots of *A. melegueta* were carefully separated and washed. They were then subjected to hydro-distillation separately for 3 to 5 h using a Clevenger-type apparatus. The oil samples were collected into sample vials and kept in the refrigerator before GC-MS analysis.

Essential oils were analyzed by Agilent (USA) 6890N GC coupled with MS-5973-634071 Series. The capillary column used was DB-1 (fused-silica) [30.0m (length) X320.00µm (diameter) X1.00µm (film thickness). Helium was used as the carrier gas at constant flow rate of 1.0 ml/min and average velocity of 37 cm/s; the pressure was 0.78 psi. The initial column temperature was set at 100°C (5 min) then increased to 250°C at the rate of 5°C/min. The injector was the split type (50:1) and volume injected was 1.0 µL. The chromatograms integrated using Chem-Station software and the compositions of the essential oils were ascertained by comparing the GC-MS data with (NIST 02) library spectra and data from literature (Robelo et al., 2003).

Brine shrimps toxicity assay of the extracted essential oils

The brine shrimps toxicity of the essential oils was determined using assay described by Krishnaraju et al. (2005) with minor modifications.

Hatching of *Artemia salina* (Brine shrimps)

A plastic container (aquarium) with two compartments was used as the hatching vessel. Holes were made into divider to allow water

Table 1. Major Bioactive Constituents of *A. melegueta.*

Plant part extract	Predominant bioactive constituent	Percentage occurrence	Terpenoid subclass
Leaf	Myrtenyl acetate	29.06	Monoterpene ester
	Limonene	19.45	Monoterpene olefins
	γ-elemene	8.84	Sesquiterpene
Stem	Caryophyllene oxide	19.70	Sesquiterpene
	Myrtenyl acetate	14.70	Monoterpene ester
	β-eudesmene	10.83	Monoterpene
Seed	α-caryophyllene	48.78	Sesquiterpene
	β-caryophyllene	32.50	Sesquiterpene
Rhizome	Myrtenyl acetate	22.70	Monoterpene ester
	Pinocarvyl acetate	11.50	Monoterpene ester
	Cyperene	8.96	Sesquiterpene
	Caryophyllene	5.97	Sesquiterpene

circulation between the compartments. The container was then flooded with fresh sea water. Two spatulas of brine shrimps were added to one side and covered with a booklet in order to produce a dark environment for proper hatching. Other side of the aquarium was left exposed to light. The aquarium was allowed to stay undisturbed for two days, when the hatched brine shrimps swam across the divider holes to the side is exposed to light.

Sample preparations and stationing of the brine shrimps

Essential oil solutions were prepared by dissolving 20.0 mg of the essential oils separately in 0.3 ml of dimethylsulphoxide (DMSO) and 1.7 ml of sea water (1000.0 ppm). Additional concentrations of 100.0 ppm and 10.0 ppm were prepared through serial dilution.

Sea water (3.0 ml) was transferred into the specimens' vials in triplicates. Then, 0.5 ml of each prepared concentration was introduced into the vials and ten brine shrimps were put into each specimen vial and control vial. All vials were topped with sea water up to 5.0 ml and left open for 24 h.

Statistical analysis

Finney's probit analysis was used to determine the LC_{50} of each essential oil and the percentage mortality calculated using, the equation:

$$\% \text{ Mortality} = \frac{\text{No. of dead } \textit{nauplii}}{\text{Initial No. of live } \textit{nauplii}} \times 100$$

Antimicrobial activity assay of the essential oils

The antimicrobial assay was carried out using cup plate diffusion method (Washington and Sutter, 1980). Five strains of bacteria were used in this study. The bacteria were of three strains Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis* and *Salmonella typhi*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). All microbes were of clinical isolates obtained from the Department of Pharmaceutical Microbiology, University of Ibadan, Nigeria. Nutrient agar (Oxiod Laboratories, UK) was used as a medium of growth of the bacteria. The agar was poured in sterile Petri dishes and was allowed to solidify. Overnight broth cultures of micro-organisms were used to seed different agar plates, one organism per plate.

Wells of approximately 5 mm in diameter were made on the agar medium, using a sterile cork borer. The plates were turned upside down and the wells labelled with a marker. Each well was then filled with 0.2 ml of the prepared essential oil solutions. Gentamicin (Oftalmiso Laboratories, Spain) was included as the control drugs for the anti-bacteria activity. The plates were incubated at 37°C for 24 h. Tests were conducted in triplicates and zones of inhibition (mm) were expressed as the mean. Data were analysed using descriptive statistics

RESULTS AND DISCUSSION

Predominant bioactive constituents

The constituents of the essential oils were analysed and reported earlier (Owokotomo et al., 2014). The major constituents of the essential oils of *A. melegueta* are presented in Table 1.

Brine shrimps toxicity

The essential oils of *A. melegueta* (Roscoe) K. Schum were screened for brine shrimps lethality and the toxicity was determined by LC_{50} (median lethal concentration) using Finney probit analysis. The LC_{50} ranges from 0.2 to 17.5 µg/ml (Table 2). The LC_{50} for the essential oils of *A. melegueta* stems and that of the seeds were the lowest (0.2 and 0.3 µg/ml, respectively), while that of the leaves of *A. melegueta* was the highest (17.5 µg/ml). These

Table 2. Toxicity of the essential oils on brine shrimps (*Artemia salina*) (10) in triplicate.

Essential oil	No. of dead Shrimps; Conc.1000 ppm	%Mortality	No. of dead Shrimps; Conc.100 ppm	%Mortality	No. of dead Shrimps; Conc.10 ppm	%Mortality	LC_{50} µg/ml
AFL	6, 5, 7	60	4, 1, 2	23.3	0, 2, 4	20.0	17.5
AFSD	10,10, 10	100	9, 10, 9	93.3	8,7,7	73.3	0.3
AFR	10,10, 10	100	7, 9, 7	76.7	4 ,6 ,4	46.7	9.5
AFSM	10,10,10	100	9,10,9	96.6	8, 8, 7	76.7	0.2

Key: AFL=*A. melegueta* leaf, AFSM = *A. melegueta* stem, AFR = *A. melegueta* rhizome, AFSD = *A. melegueta* seed essential oils.

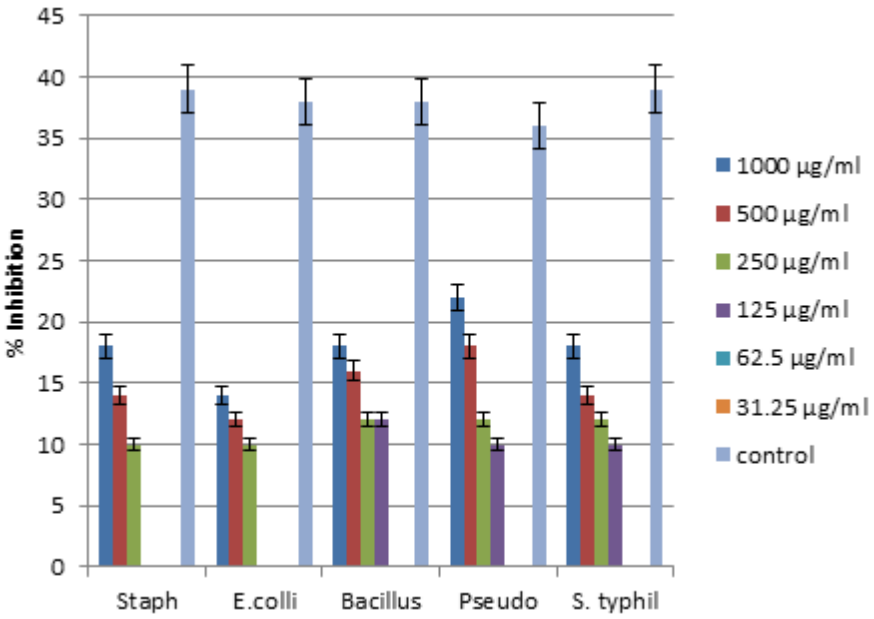

Figure 1. Bacteria growth profile of *A. melegueta* seeds' essential oil.

values were low which indicated that, the essential oils were toxic and thus, contained cytotoxic and pesticidal properties. The degrees of toxicities of the essential oils were directly proportional to the concentration. Maximum mortality (100.0%) of the brine shrimps was recorded at 1000.0 ppm and the least mortality (20.0%) also recorded at 10.0 ppm of solution of the essential oils.

Their toxicity has further corroborated literature reports on the cytotoxicity of plant essential oils (Bakkali et al., 2008). According to Carson et al. (2002), essential oils seem to have no specific mechanism or cellular target because of the high number of compounds present in any sample. They are lipophilic, thus, they pass through the cell wall and cytoplasm membrane, and disrupt the structure of their different layers. Cytotoxicity which appears includes membrane damages.

Antibacterial activity

The plants' essential oils were active against all bacteria strains (*S. aureus, B. subtilis, S.typhi, E. coli,* and *P. aeruginosa*) but inferior compared with inhibition ability of the antibiotic agent, gentamicin (10.0 µg/ml) (Figures 1 to 4). The essential oils were more effective within the concentration range of 1000.0 to 250.0 µg/mL against all micro-organisms. There was marked reduction in the antimicrobial activity of the oils at 62.0 µg/mL. *E. coli* and *S. aureus* seem to be less susceptible when compared to *B. subtilis, S. typhi* and *P. Aeruginosa.*

According to Belletti et al. (Krishnaraju et al.,2005), several essential oils, as well as some of their components such as caryophyllene and caryophyllene oxide, limonene, α-pinene, β-pinene and β-caryophyllene have displayed significant antimicrobial activity against bacteria and yeasts. Therefore, the antimicrobial results observed in this investigation could be related to the presence of myrtenyl acetate, limonene, Caryophyllene oxide, caryophyllene, Pinocarvyl acetate, γ-elemene and Cyperene which are the major constituents of these essential oils. The activities of these compounds were probably modulated by other minor components present

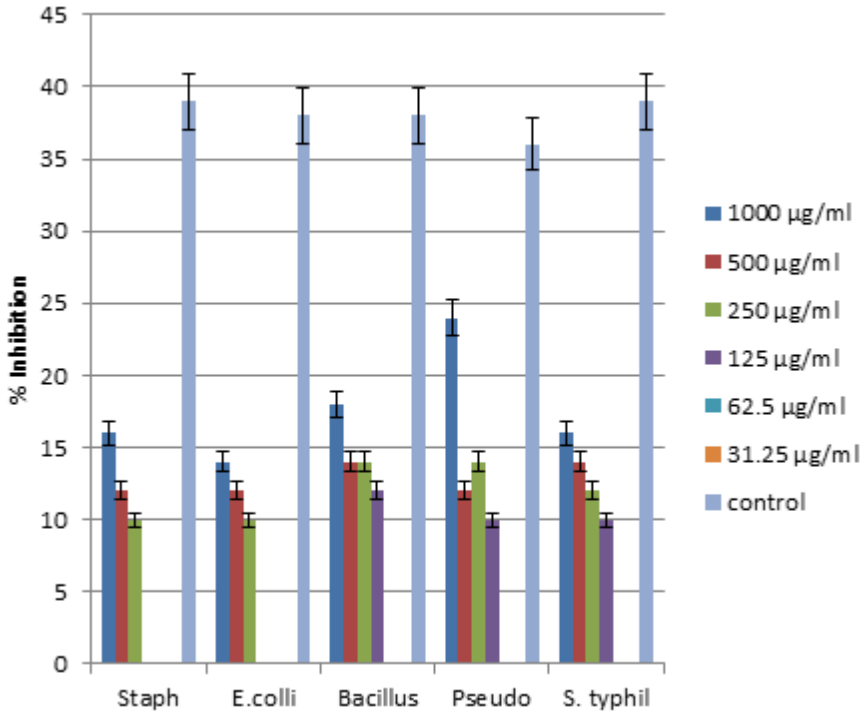

Figure 2. Bacteria growth profile of *A. melegueta* rhizomes' essential oil.

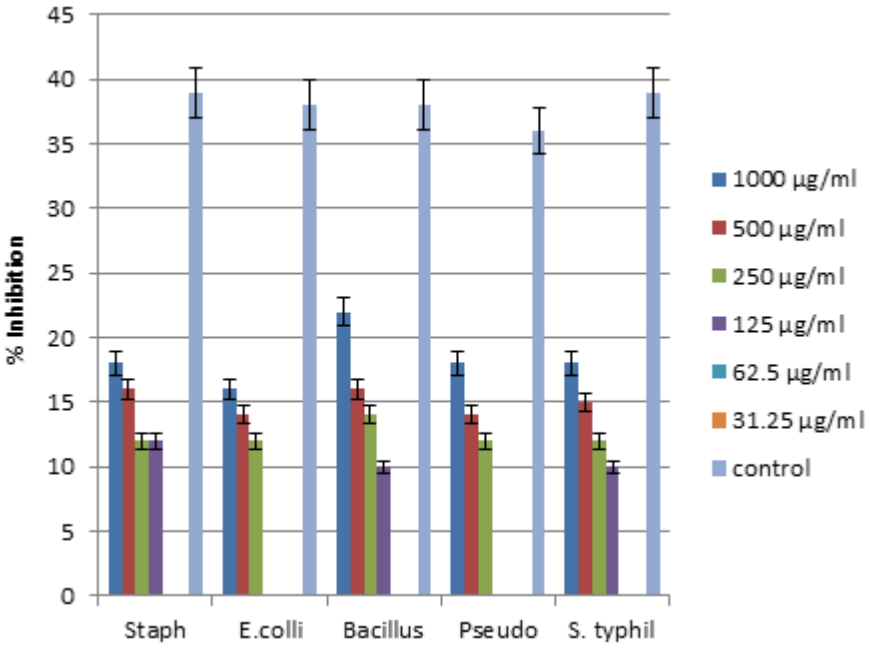

Figure 3. Bacteria growth profile of *A. melegueta* stems' essential oil.

in the essential oils.

Conclusion

Results from this work revealed that essential oils from the leaves, stems, rhizomes (roots) and seeds of *A. melegueta* were lethal to *Artemia salina.* They exhibited 100% mortality at 1000 ppm. *A. melegueta* stem and seed were the most potent with LC_{50} of 0.20 and 0.30 µg/ml respectively, while the leaves essential oil

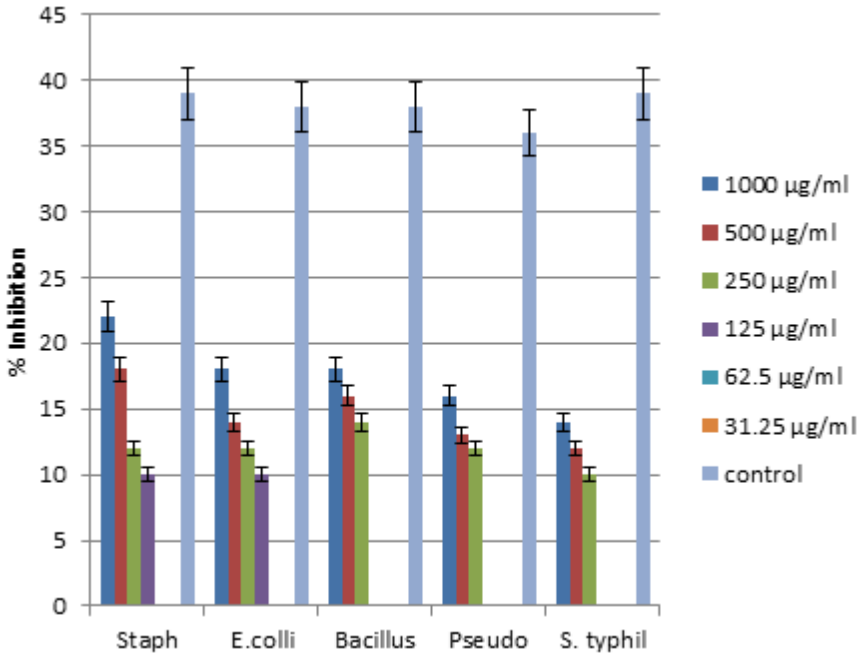

Figure 4. Bacteria growth profile of *A. meleguata* leaves' essential oil.

presented the least brine shrimps toxicity of LC_{50} 17.5 µg/ml.

The antibacterial activity of the essential oils showed that, these were effective against the pathogenic organisms. The activities compared the standard antibiotic and gentamicin, but high enough to support the ethno-medical usage of the plant.

The results suggest that the essential oils of *A. melegueta* (Roscoe) K. Schum may provide a source of natural products, which act as natural antibacterial and pesticidal agents. This research also support and rationalise the use of the plant in African ethno-medicine as used in Nigeria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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