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*Full Length Research Paper*

# Anti-bacterial and brine shrimps lethality studies of the essential oils of *Crassocephalum crepidioides* (Benth S. More) grown in south west Nigeria

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**Interest in plants' essential oils has grown recently due to increased use of natural products in medicine and cosmetics. In spite of its long use, there has been little prior study on the bioactivity and toxicity of the essential oils of *Crassocephalum crepidioides* (Benth S. More). Thus in this work, the anti-bacterial and brine shrimps' lethality of the essential oils obtained through hydro-distillation were evaluated. The oils were analyzed by gas chromatography and gas chromatography/mass spectrometric techniques. The toxicity of the oils was considered using the brine shrimps' lethality assay at concentrations of 10, 100 and 1000 ppm. The antimicrobial assay was carried out using the agar diffusion method. The bacteria were three strains of Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis* and *Salmonella* Typhi) and two strains of Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The main constituents of the leaves essential oil of *C. crepidioides* were α-caryophyllene (10.29%) and β-cubebene (13.77%), while the stem essential oil were mainly thymol (43.93%) and 4-cyclohexybutyramide (20.94%). The results of the brine shrimps' lethality assay suggest that the essential oil of the stem ($LC_{50}$ = 9.10 µg/mL) was just as toxic as the essential oil of the leaves ($LC_{50}$ = 9.2 µg/mL). The essential oils were active against all the bacterial strains but low when compared with the standard antibiotic, Gentamicin.**

**Key words:** Essential oils, toxicity, antibacterial activity, *Crassocephalum crepidioides,* Nigeria.

## INTRODUCTION

Medicinal plants are very useful for human health. A number of studies have shown that medicinal plants could be ethical phyto-medicine for recovering from illnesses. Many plants that are used as landscape plants

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for decorative and esthetic applications have also been found useful to human health (Cetin et al., 2010; Yigit et al., 2016).

Essential oils (EOs), the volatile and odorous constituents of many plants, have been used through ages as medicines in the management of diseases. However, by the middle of the 20th century, application of essential oils had been reduced almost entirely to use as perfumes, in cosmetics and in food flavouring, while their use in pharmaceutical preparations had drastically declined (Edris, 2007).

Prior to the discovery of penicillin, scientists had been actively engaged in studying the bactericidal and bacteriostatic actions of essential oils. The discovery of antibiotics, however, shifted interests so drastically from these studies (Nagy and Tengerdy, 1967). Besides, huge success in organic synthesis leading to the productions of many analogs of phytochemicals in commercial quantities led to gradual neglect of plant derived medicines.

As a result of the recent call by the World Health Organization (WHO) to reduce the level of salt in processed foods, it becomes imperative that other additives be introduced to maintain the safety of foods. EOs provide such a new scope for new methods of making food safe with a natural or 'green' image (Burt, 2004). The use of combinations of EOs and their isolated components may provide new approaches to increase the efficacy of EOs in foods, taking advantage of their synergistic and additive effects (Bassolé and Juliani, 2012).

Essential oils have also found applications in animal health. Antibiotic ionophores have been very successful in reducing energy and protein losses in the rumen, but the use of antibiotics in animal feeds is facing reduced social acceptance. For this reason, scientists have become interested in evaluating other alternatives to control specific microbial populations to modulate rumen fermentation. Essential oils have been identified as a plausible alternative (Calsamiglia et al., 2007).

In a recent report (Dobre et al., 2011), the essential oils of Oregano, clove bud and white thyme were found to show higher activity against bacteria in direct contact method, having a greater inhibition diameter than the reference control, streptomycin 50 mg/ml. The antibacterial activity of 21 plant essential oils against six bacterial species (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus vulgaris*) was evaluated by Seenivasan et al. (2006). The result showed that cinnamon, clove, geranium, lemon, lime, orange and rosemary oils exhibited significant inhibitory effect. Cinnamon oil showed promising inhibitory activity even at low concentration, whereas aniseed, eucalyptus and camphor oils were least active against the tested bacteria.

Several *in vitro* studies have been published confirming the effect of essential oil and their major compounds on plant and human pathogenic fungi. In a review (Vidyasagar and Nuzhat, 2013), essential oils of 50 species from 18 family of plants were found to possess varying but significant antifungal activity. Essential oil from an *Ocimum* genus, *Ocimum micranthum* showed a dose-dependent antifungal activity against pathogenic and food spoiling yeasts (Morris et al., 1979). In another investigation, essential oil of another genius of *Ocimum*, *Ocimum gratissimum* inhibited the growth of four candida species (Nakamura et al., 2004). Analysis of the ultra-structure of the yeast cells revealed changes in the cell wall and in the morphology of some sub-cellular organelles. The essential oil obtained from the fresh leaves of *Zanthoxylum alatum* which consisted of linalool, 2-decanone, -fenchol, 2-tridecanone, $\beta$-phellandrene, sabinene and $\alpha$-pinene as the main components, exhibited potent antifungal activity against A*lternaria alternata*, *Alternaria brassicae* and C*urvularia lunata*. The essential oil also showed significant antibacterial activity against *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus* and *Escherichia coli*.

Orafidiya et al. (2002) investigated the efficacy of *O. gratissimum* essential oils in the treatment of acne. *O. gratissimum* oil in comparison with 10% benzoyl peroxide and a placebo, over a period of 4 weeks for the reduction of acne lesions in a population consisting mainly of students suggests that *O. gratissimum* oil was significantly more active than benzoyl peroxide ($P < 0.05$), while 2.0% oil in cetomacrogol had similar activity as compared to the reference product. In a review (Cavanagh and Wilkinson, 2005), Lavender oil (primarily *Lavandula angustifolia*) was found to be active against many species of bacteria, including those resistant to antibiotics such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus.* Also, investigations into the antibacterial properties of a range of Lavandula oils support the anecdotal use of lavender oils as antibacterial agents, and demonstrated that some oils which had not been previously investigated (e.g. *L*. *heterophylla*) displayed good antibacterial activity against a range of bacteria.

Essential oils from the flower and petals of palmarosa (*Cymbopogon martini*), evening primrose (*Primula rosea*), lavender (*Lavandula angustifolia*) and tuberose (*Polianthus tuberosa*) were tested for their antibacterial activities against Gram-positive and negative bacteria. Different concentrations of each essential oil ranging from 10 to 100% were tested. Both Gram-positive and negative bacteria were found susceptible to the studied essential oils. With increase in concentration of essential oil, increase in the zone of inhibition was observed. Thus, dose-dependent response was clear for each essential oil (Lodhia et al., 2009).

In a recent publication, the essential oils of *Coriandrum sativam* (L.) in India were found effective against both Gram positive (*Staphylococcus aureus*) and negative (*Enterobacter aerogenes, Klebsiella pneumoniae*, *Vibrio cholerae* and *Salmonella typhi*) bacterial strains using

Agar well diffusion method (Suganya et al., 2012). Similarly, oregano and thyme essential oil showed antibacterial activities against one Gram-positive strain (*Bacillus cereus* CCM 2010) and two Gram-negative strains (*Pseudomonas aeruginosa* CCM 1960; *Escherichia coli* CCM 3988) of bacteria in a further attestation of the potentials of plants' essential oils as remedy for many diseases (Kacaniova et al., 2012).

Antimicrobial (antibacterial and antiviral) activities of five essential oils (EOs) extracted from the aerial parts (leaves and flowering tops) of three species growing in the north of Morocco: *Origanum elongatum*, *Thymus capitatus* and *Mentha suaveolens*, were investigated. Chemical analyses of the oils revealed that thyme EO predominantly contains carvacrol, *p*-cymene, γ-terpinene and β- caryophyllene. Oregano EO was mainly constituted by carvacrol, thymol, *p*-cymene and γ-terpinene. While, mint EO was characterized by piperitenone oxide (-)-isopulegol and limonene. All extracts exhibited antibacterial activity at different levels against strains reported as the causal agents of foodborne diseases, but a low antiviral activity was observed (El Moussaoui et al., 2013).

The antimicrobial activity of essential oils of leaves, bark and wood of *Cassia bakeriana* Craib against aerobic and anaerobic oral bacteria was evaluated using microdilution method, the same was done in the cell viability test carried out with Vero cells. Oils were very active with minimum inhibitory concentrations between 62.5 and 125.0 µg/mL for most of the tested bacteria, including *Streptococcus mutans*, the main etiological agent of dental caries (Cunha et al., 2013). Also, recent studies on the antifungal activity of clove essential oil (EO), obtained from *Syzygium aromaticum*, indicates that clove oil consisting mainly of eugenol as the active principle, have considerable antifungal activity against clinically relevant fungi, including fluconazole-resistant strains (Pinto et al., 2009).

Persistent research into local plants to verify their usefulness especially in the maintenance of human health remains a necessity. This is due to the fact that anti-malarial plants used by the Kenyah Dyale people of Borneo in Indonesia were better antibiotics than control plants without such use in tradition (Leaman et al., 1995). It means that the same plant species from different geographical locations may be different in chemical composition which determines their bioactivities.

Synthetic drugs usually provide effective antibiotic therapy for bacterial infections but there is an increasing problem of antibiotic resistance and a continuing need for new solutions (Karen and Edzard, 2003). Essential oils from spices and herbs are regarded as the most promising alternatives, because they do not cause microbial resistance due to the diversity of mechanisms of action (Dobre et al., 2011). Thus in this work, the anti-bacterial and brine shrimps lethality studies of the essential oils of *Crassocephalum crepidioides* (Benth S. More) grown in south west Nigeria was investigated.

## MATERIALS AND METHODS

### Collection of plant materials

*C. crepidioides* plants were harvested at an abandoned cocoa farm near the Federal university of technology, Akure, Ondo State, Nigeria. The plant was identified and authenticated at the Herbarium of the Forest Research Institute of Nigeria, Ibadan with voucher number FHI109021.

### Extraction of essential oils

The plant parts were carefully separated, washed and then subjected to hydro-distillation separately for 2-3 h using an all glass Clevenger-type apparatus (Clevenger, 1928). The Clevenger apparatus was modified with graduated column to enable direct estimation of oil yield. The weight of fresh plant material was 1 kg for each plant part. The oil yield was calculated in percentage of volume per weight (v/w) of plant samples. The oil samples were stored in air-tight containers at 0°C before GC-MS analysis without any further treatment.

### Gas chromatography/mass spectrophotometric (GC/MS) analysis

The essential oils were analyzed using Agilent (USA) 6890N GC Coupled with MS-5973-634071 Series. The capillary column type was DB-1 (fused-silica) [30.0 m (length) x 320.00 µm (diameter) x 1.00 µm (film thickness)]. The carrier gas was helium 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 (held for 5 min) to final temperature of 250°C at a rate of 5°C/min. The injector was the split type and set at 50:1, and volume injected was 1.0 µL. The chromatograms were auto-integrated by Shem-Station and the constituents were identified by comparison of the GC-MS data with (NIST02) library spectra and data from literature (Adams, 1995).

### Toxicity assay of the essential oils

Toxicity of the essential oils was conducted using the brine shrimps (*Artemia salina)* lethality assay as described by Krishnaraju et al. (2005).

An improvised aquarium of plastic container was filled with fresh sea water prior to the introduction of two spatulas of brine shrimps to one side. The aquarium was then left for two days until the hatched brine shrimps swam across the divider to the side exposed to light.

Stock solution was prepared by emulsifying 20.0 mg of the essential oils separately in 0.3 mL of dimethylsulphoxide (DMSO) and the volume was made up with 1.7 ml of fresh sea water to equal 1000.0 ppm concentration. After this, serial dilution was done to obtain two additional concentrations of 100.0 and 10.0 ppm.

### Stationing of the brine shrimps

Fresh sea water (3.0 mL) was transferred into the specimens' vials prepared in triplicates. Then, 0.5 mL of each prepared concentration was introduced into the specimen vials followed by

**Table 1.** Toxicity of the essential oils on brine shrimps (*Artemia salina*).

| 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 |
|---|---|---|---|---|---|---|---|
| CRSL | 10,10,10 | 100 | 6,7,7 | 66.7 | 4,5,7 | 53.33 | 9.2 |
| CRSS | 10,10,10 | 100 | 8,7,7 | 73.3 | 6,5,4 | 50.00 | 9.1 |

CRSL = *C. crepediodes* leaf; CRSS = *C. crepediodes* stem.

the introduction of ten brine shrimps into every specimen vial including the control vial. Finally, every specimen vial was topped up with sea water until it reached 5.0 mL. All the vials containing the shrimps were left opened for 24 h.

#### Statistical analysis

Finney's probit analysis was used to determine the $LC_{50}$ of each essential oil. The toxicity is expressed by $LC_{50}$ which is defined as concentration of extract that kills 50% of the shrimps within 24 h. Percentage mortality was calculated as number of dead *nauplii* divided by initial number of *nauplii* (10) multiplied by 100.

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

Toxicity of the extract against the brine shrimps was determined by a statistically significant decrease in the survival of brine shrimps exposed to plant extract, relative to the survival of shrimps in the control.

#### Antimicrobial activity assay of the essential oils

The antimicrobial assay was carried out using the agar diffusion method (Washington and Sutter, 1980). The bacteria were three strains of Gram positive bacteria (*Staphylococcus aureus, Bacillus subtilis* and *Salmonella* Typhi) and Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). All the microbes were clinical isolates obtained from the Department of Pharmaceutical Microbiology, University of Ibadan, Nigeria.

Nutrient agar was used as the medium of growth of the bacteria [Oxiod Laboratories, UK]. The agar was poured in sterile Petri dishes and 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 surface of the agar medium using a sterile cork borer.

The plates were turned upside down and the wells labeled with a marker. Each well was then filled with 0.2 mL of the prepared essential oil solutions. Gentamicin (Oftalmiso Laboratories, Spain) was used as the control drug for the anti-bacteria assay. The plates were incubated at 37°C for 24 h and the zones of inhibition were measured at the end of the incubation period. Tests were conducted in triplicates and zones of inhibition (mm) were expressed as the mean.

## RESULTS AND DISCUSSION

### Essential oils' composition

The major compositions of the essential oils were determined and reported earlier (Owokotomo et al., 2011). The most abundant constituents of the leaf essential oil of *C. crepidioides* were α-caryophyllene and β-cubebene, while the dominant constituents of the stem essential oil were thymol and 4-cyclohexylbutyramide.

### Toxicity of the essential oils on brine shrimps (*Artemia salina) nauplii*

The $LC_{50}$ calculated for the essential oils of the leaf and stem of *C. crepidioides* were quite close, 9.1 (stem) and 9.2 µg/mL (leaf) (Table 1). These values are low and they suggest that the essential oils were toxic. According to Ghisalberti (1993) and McLaughin et al. (1993), it appears that brine shrimps lethality (BSL) is predictive of cytotoxicity and pesticidal activity.

### Antimicrobial activities of the essential oils

The essential oils of the leaves and stems of *C. crepidioides* (Benth. S. More) evaluated for antimicrobial activities against pathogenic strains of Gram positive (*S. aureus, B. subtilis, S.* Typhi*)* and two strains of Gram negative bacteria (*E. coli* and *P. aeruginosa*) (Figures 1 and 2) were active against all the microbes but inferior to the antibiotic agent, Gentamicin (control) and were dose dependent in activity. The essential oils were more effective within the concentration range of 1000.0 to 250.0 µg/mL in all the micro-organisms. There was marked reduction in the antimicrobial activity of the essential oils at 62.0 µg/mL; no inhibition was recorded at 31.25 µg/mL. The stem essential oil with MIC (the lowest concentration of test substance that prevented growth) of 62.5 µg/mL was considered more potent than the leaf essential oil with MIC value of 125.0 µg/mL. The two EOs were generally more active against *B. subtilis* but they were a poorer agent against *E. coli.* This may be that the essential oils were strain-specific in activity. This result is in consensus with a recent report by Santos et al. (2015) on the antimicrobial, cytotoxic and genotoxic effects of essential oil samples from *Eugenia astringens cambess* that indicated the use of essential oil (12.5 mL) together with the antibiotic (amoxicillin, 12.5 µL volume) aided potent inhibition of a strain of *Staphylococcus aureus*.

It has been observed that the major components of

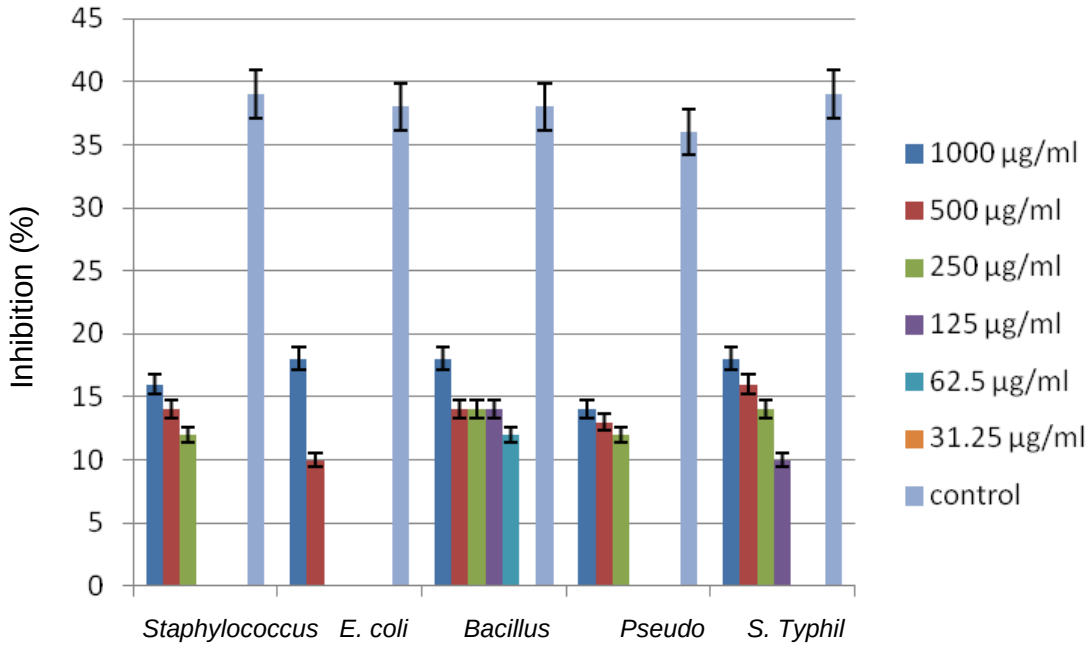


**Figure 1.** Bacteria growth profile of *C. crepidioides* stems' essential oil.

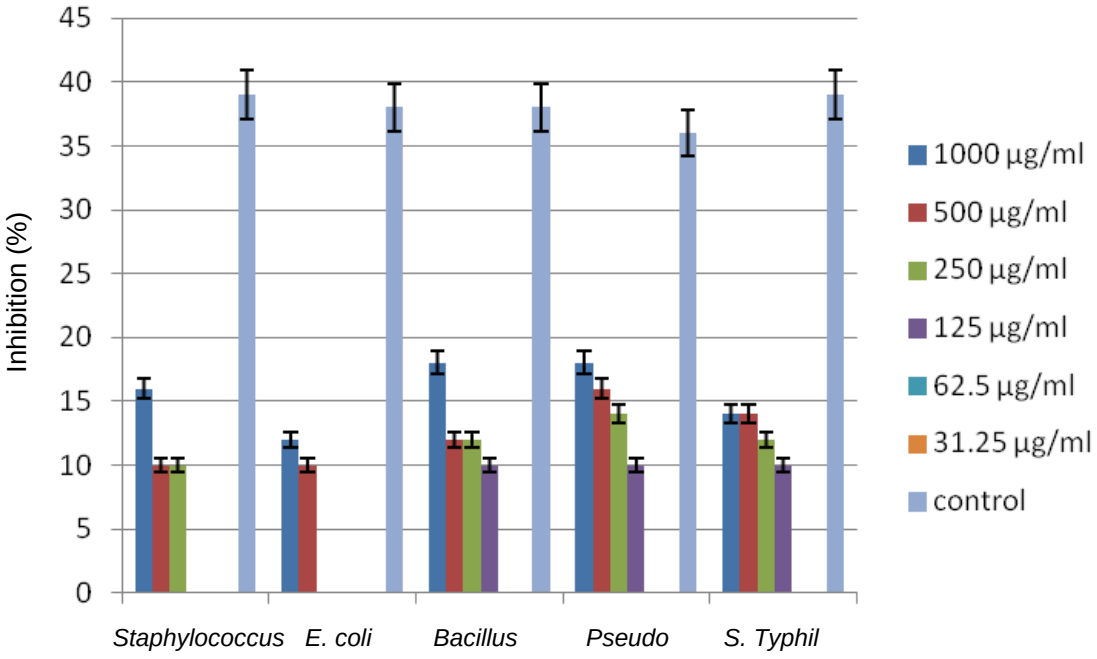


**Figure 2.** Bacteria growth profile of *C. crepidioides* leaves' essential oil.

EOs reflect their biological properties (Ipek et al., 2005). According to Burt (2004), it is most likely that the bioactivity of EO is not attributed to one specific compound or mechanism because EOs components are lipophilic which enables them to partition in the lipids of the microorganisms' cell membrane and mitochondria, disturbing the structures and rendering them more permeable. These lead to leakages of ions and other cell contents if extensive enough, will lead to death of the pathogenic organism. However, the antimicrobial activities have been explained through C-10 and C-15 terpenes with aromatic rings and phenolic hydroxyl

groups abilities to form hydrogen bonds with active sites of enzymes (Meccia et al., 2009). According to Belletti et al. (2004), several essential oils, as well as their components such as caryophyllene and caryophyllene oxide, limonene, α-pinene, β-pinene and β-caryophyllene have displayed significant antibacterial activity against bacteria and yeasts. Therefore, the antimicrobial results observed in this investigation could be related to the presence of thymol and caryophyllene which are the major constituents of these essential oils. The bio-activity of these compounds was probably modulated by other minor components present in the essential oils.

## Conclusion

The essential oils of *C. crepedioides* were lethal to *Artemia salina,* exhibiting 100% mortality at 1000 ppm. The $LC_{50}$ of 9.2 µg/mL (Leaves' EO) and $LC_{50}$ of 9.1 µg/mL (stems' EO) were considered low and imply high toxicity which is predictive of cytotoxicity and pesticidal activity. The antibacterial activity of the essential oils showed that they were effective against the pathogenic organisms. The activity was below that of the standard antibiotic, gentamicin but high enough to support the use of the plant in African traditional medicine, especially in south west Nigeria.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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