Inhibitory effects of the volatile oils of *Callistemon citrinus* (Curtis) Skeels and *Eucalyptus citriodora* Hook (Myrtaceae) on the acetylcholine induced contraction of isolated rat ileum

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Abstract: Using steam distillation method, the volatile oils of *Eucalyptus citriodora* and *Callistemon citrinus* were obtained and their chromatographic profiles examined in hexane: ethylacetate (4:1; 7:3) and hexane-chloroform (7:3). The effects of the volatile oils on acetylcholine (Ach) induced contraction of the rat isolated ileum were investigated based on the ethnomedicinal use of the volatile oil of *E. citriodora* in treating diarrhoea.
Relative to the weight of the fresh leaves (200g in each case), *E. citriodora* produced 0.75% of the volatile oil while the *C. citrinus* yielded 0.5%. Combination of hexane-ethylacetate (4:1) gave the best resolution of the constituents as *E. citriodora* produced six major spots while *Callistemon citrinus* produced three. The concentration-dependent contractions of the ileum produced by the increasing concentration of the Ach were observed to be remarkably attenuated in the presence of the volatile oils at 5 and 10 mg/ml. At 5mg/ ml, the volatile oils of *E. citriodora* and *C. citrinus* independently reduced the Ach maximum contraction to 74.11 ± 12.4 and 19.05 ± 5.17% respectively. At 10mg/ml, the volatile oils further significantly ($P<0.05$) inhibited the contraction induced by the Ach. The results obtained validated the ethnomedicinal use of the volatile oils particularly that of *E. citriodora* in reducing ilea contractions occasioned by diarrhoea. However, *C. citrinus* volatile oil seems to be more effective.

Keywords: *Callistemon citrinus,* diarrhoea, *Eucalyptus citriodora*, volatile oils.

INTRODUCTION

Essential or volatile oils are odoriferous materials produced by some plants. They are naturally found in many plant families like compositae, lamiaceae, poaceae and myrtaceae etc. Among the volatile oil containing species in myrtaceae family are the clove, *Syzigium aromaticum*, *Eucalyptus* species and bottle brush, *Callistemon* species. The oils of *Syzigium aromaticum*, *Eucalyptus* species are among the officially recognized volatile oils in the Pharmacy world (Evans, 1989) while that of *Callistemon* is yet to receive such attention. *Eucalyptus* is one of the world's most important and most widely planted genera (Emilia, 1996) with more than 111 different species (Boland and Brophy, 1991). One of the four species grown in Nigeria is *E. citriodora* Hook (Gill, 1992). The members of the species are used in forestry, as ornamentals, and sources of volatile oils (Evans, 1989). Medicinally, the barks and the leaves are used as antiseptic, stimulating expectorant and are used to treat colds, influenza, toothaches, snake bites as well as diarrhoea (Newall *et al*, 1996). In addition, in herbal medicine practice in Nigeria, clove (*Syzigium aromaticum)* flowers are usually used alongwith some other medicinal plants to relieve intestinal spasms or colitis particularly in children in many communities where the supply of modern drug distribution is inadequate. In ethno medicinal practice in Edo and Delta parts of Nigeria, the oil *Eucalyptus* is used to relieve diarrhoea (Gill, 1997). However, the use of pure volatile oils alone as antispasmolytic agents is not common.

As diarrhoea is characterized by uncontrolled contraction of the intestinal muscles, this work was carried out to examine the ethnomedical claimed use of the volatile oil of *Eucalyptus citriodora* alongside that of *Callistemon citrinus* Curtis on acetycholine-induced contraction of the isolated rat ileum.

MATERIALS AND METHODS

Collection and extraction of plant materials

Fresh leaves of *Eucalyptus citriodora* Hook and *Callistemon citrinus* (Curtis) Skeels were collected in October, 2008 between the hours of 8-9 am in the University of Benin, Ugbowo Campus, Benin City. Early morning collection of the plants was made to ensure high yield of the oils. The plants were authenticated by Professor Idu, Plant Taxonomist, Department of Botany, Faculty of Science, University of Benin, Benin City. Shortly after collection, the volatile oils of the fresh leaves (200g each) were separately obtained by steam distillation method for 3-4 h as specified in the African Pharmacopoeia (1986). Extraction was taken as complete when an almost constant volume of oil persisted for about 30 minutes. The volume of the volatile oils obtained were measured, weighed and stored in a refrigerator maintained at 4^{o}C until required.

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Drugs and chemicals used

These included acetylcholine, chloroform, n-hexane, ethylacetate (Sigma-Aldrich), sodium chloride (NaCl) , sodium hydrogen carbonate ($NaHCO_3$), D-Glucose, sodium dihydrogen phosphate (NaH_2PO_4), potassium chloride (KCl), magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$) (BDH Chemicals).

Thin-layer chromatography of the volatile oils

Thin layer chromatographic profiles of the volatile oils of the two plants were carried out by separately dissolving 0.2 ml in 0.5 ml of re-distilled acetone. The acetone solution of each oil was carefully spotted on commercial plates of silica gel 60 F_{254} and developed in hexane – ethyl acetate (7:3); hexane - chloroform (7:3) and hexane – ethyl acetate (4:1). After development, the plates were sprayed with 12% Sulphuric acid and heated in the oven maintained at 110°C for 3-5 minutes. The retention factors (R_f) values of the spots were calculated and the respective colours were noted.

Animals

Mature albino rats weighing between 160-170 g were obtained from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City where they had access to animal feeds (Ewu Feeds) and water *ad libitum.* Approval for use of the animals was obtained from the Faculty of Pharmacy Ethical Committee on the use of animals for experiments.

Preparation of rat ileal tissue

The animals were anaesthetized via chloroform inhalation in a gas chamber for 3-5 minutes after which they sacrificed. The abdomen was opened using a dissecting set and the ileum dissected out carefully and transferred into a Petri dish containing Tyrode's solution of the following composition: sodium chloride 40g, sodium hydrogen bicarbonate 5g, D-glucose 5g, sodium dihydrogen phosphate 0.25g, potassium chloride 1g, magnesium chloride 0.5g, and calcium chloride 1.32g in 5L of distilled water. The lumen of the ileum was flushed with a 5ml syringe and freed of the mesenteries by careful cutting with a scissors. Rat ileum measuring 1-2cm was cut and a thread attached at both ends of the tissue and suspended in 50 ml capacity organ bath containing the physiological salt solution with the aid of a tissue holder. This was connected to a unirecorder (Gemini 7050) via an isometric transducer.

Baseline was set with 7N tension (0.7g weight) as the 0 tension i.e. calibration of the equipment. The speed was maintained at 5.0mm/min while the sensitivity was 6.0. The organ bath was adequately aerated with 95% O_2 and 5% CO_2 while the temperature was maintained at 36.0 ± 1°C. The tissue was allowed to equilibrate for 45 minutes.

Administration of the drugs

Dose responses were obtained with 0.001-1 mg/ml acetylcholine with a contact time of 30 s resting time of 1 min 30 s (2min. time cycle) was allowed. The response of the ileum to the acetylcholine was regarded highest when further increase in the concentration was no longer matched with further contraction. The response by each drug administered was expressed as the percentage of the highest contraction produced by the highest concentration as shown below:

$$\text{Percentage response (\%)} = {}^{\text{Response}}/_{\text{Highest response}} \times 100$$

Effects of the volatile oils were determined by administering 5mg of the oil and allowed 10 min contact time after which the acetylcholine concentrations were administered with the 2 min contact time. Similar procedure was carried out with 10 mg of the oils and responses of the ileum to the various concentrations were recorded. The baseline obtained during equilibration at 0.7g tension was used to calculate the force of contraction. Concentration-response curves were plotted for acetylcholine alone, acetylcholine with *Eucalyptus* oil, and acetylcholine with *Callistemon* oil using final bath concentration (FBC) calculated by dividing each concentration of the acetylcholine by 50 ml. The experiment for each volatile oil was carried out four different times and the average taken.

Statistical analyses were carried out using analysis of variance (ANOVA) in Graphpad InStat 3 statistical packages.

RESULTS

Yields of the volatile oils from the plants

After 3 hours of steam distillation, the fresh leaves (200g) of *E. citriodora* yielded 1.5ml (0.75%) of the volatile oil while *C. citrinus* produced 1ml (0.5%). One (1) ml of *E. citriodora* was observed to weigh 0.9703 g while one (1) ml of *C. citrinus* oil weighed 0.9470 g.

Thin layer chromatographic results

The chromatographic analyses of the two volatile oils revealed that the mixture of hexane-ethyl acetate (8:2) gave the best resolutions of the major components of the two oils. Unlike in the other two solvent systems (hexane-ethyl acetate, 7:3; hexane-chloroform, 7:3), after developing the plates, the volatile oil of *E. citriodora* showed six (6) different spots while the oil of *C. citrinus* showed three spots (table 1).

Effects of the volatile oils on the isolated rat ileum

Acetylcholine was used to stimulate contraction of the tissues. The contractions were observed to increase with increase in the concentrations of acetylcholine administered. The acetylcholine produced the lowest ileal contractions at a concentration of 4ng/ml final bath concentration (FBC) while the highest contraction EC_{max}

Table 1: Chromatographic analyses of the volatile oils *E. citreodora* and *C. citrinus* in Hexane-Ethylacetate (4:1)

Volatile oils	No of major spots	Colour	RF values
Eucalyptus citreodora	6	1. Light brown 2. Light brown 3. Light brown 4. Brown 5. Violet 6. Light brown	0.24 0.57 0.67 0.71 0.80 0.93
Callistemon citrinus	3	1. Light brown 2. Light brown 3. Yellow	0.67 0.75 0.79

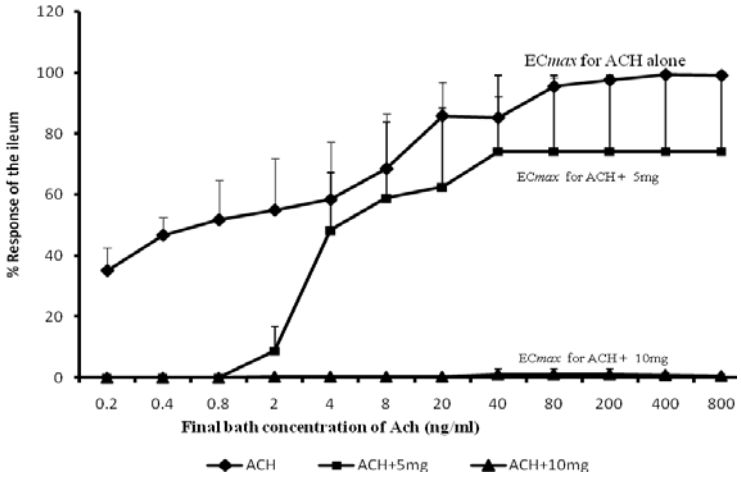

Fig. 1: Inhibitory effect of the volatile oil of *Eucalyptus citriodora* on Acetylcholine induced contractions of rat ileum.

was obtained at 16000 ng/ml FBC. The effects of the volatile oils were monitored by the extent to which each concentration attenuated the contractions induced by acetylcholine. At a concentration of 5mg, the volatile oils of *E. citriodora* completely inhibited the contractions produced by 4-16 ng/ml of acetylcholine. The contraction of 54.88 % with 40 ng/ml acetylcholine was reduced to 8.72 ± 8.145% by the volatile oil. Similarly, the highest contraction (100%) produced by the ACH was reduced to 74.11%.

At 10mg concentration of the oil, the contractions produced by acetylcholine were almost completely abolished (fig. 1). The inhibitory effects of the volatile oil on ACH induced rat ileum contractions were observed to be significant ($P<0.05$).

Similarly, the volatile oil of *C. citrinus* produced significant inhibition on the contractions of the isolated rat ileum. The contraction of 38.45% with 16 ng/ml ACH alone was reduced to 1.265% in the presence of 5 mg of the volatile oil. At 16000 ng/ml of the ACH which earlier gave the highest contraction, in the presence of the 5mg of the volatile oil, the highest contraction obtained was 19.05%. At 10mg, the contraction produced by the highest concentration of acetylcholine was reduced to 11.56 % (fig. 2). The variations were also observed to be significant at $P<0.05$.

DISCUSSION

The yields of the volatile oils obtained from the two plants were observed to vary. This can be attributed to the turgidity of the cells containing the volatile oils. The yields of the oil can also vary based on the plant part, the location of the oil in a particular organ, time of collection as volatile oil containing plants collected in the afternoon may likely produce lower oil quantities than those collected in the morning or late in the evening probably due to the effect of evaporation.

The variations in the TLC results indicated that the constituents of the oils may vary to some extent. Although, there were some spots that had similar colours and closely related R_f values, the quantities of such may not be the same. The solvent systems used in resolving the constituents determine to a large extent the nature of separation one is likely to have in a particular chromatographic work. The combination of hexane-ethyl acetate (4:1) in this work produced the best resolution in

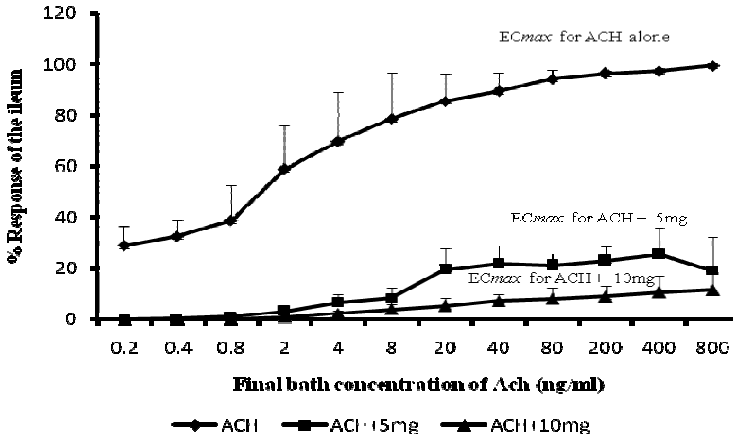

Fig. 2: The inhibitory effects of volatile oil *Callistemon citrinus* on Acetylcholine induced contraction of rat ileum.

that each of the oils produced the maximum number of spots (6 major spots for *E. citriodora* and 3 for *C. citrinus*). The significance of this information is that for anytime the species of *Eucalyptus* and *Callistemon* leaves are subjected to chromatographic evaluation using hexane- ethyl acetate (4:1) there must be 6 major spots for the former and 3 major spots for the latter with their corresponding colours and of course the R_f values. These can be used to evaluate the authenticity of these volatile oils alongwith their respective refractive indices, densities and optical rotations.

However, the spots observed did not mean that these are the only constituents each of the volatile oil contains. There were others that could occur in minute quantities difficult to detect on a TLC plate.

Like other secondary metabolites found in medicinal plants, volatile oils are sometimes responsible for various biological activities exhibited by medicinal plants that may contain them. The volatile oil yielded by the fresh leaves of *Ageratum conyzoides* L. (Compositae) has been reported to possess larvicidal and insecticidal activities (Ayinde and Odigie, 2001; Gbolade *et al.*, 1999), while some volatile oils obtained from other medicinal plants have been reported to exhibit remarkable insect repellent activities against *Simulium damnosum* S.L., the vector of human onchocerciasis (Aisien *et al.*, 2004). Also, the volatile oils obtained from the fresh leaves of *Cymbopogon citratus* L. (Poaceae) and *Ocimum gratissimum* L. (Lamiaceae) as well as fresh orange and grape (*Citrus sinensis* and *C. maxima*, Rutaceae respectively) peels have been scientifically established as potent antimicrobial agents against some bacteria and funDgi (Onawunmi and Ogunlana, 1986; Ahonkhai and Ayinde, 2005). In addition, some volatile oil containing plants like *Mentha villosa, O. gratissimum* and *Alpinia zerumbet* have been reported to show hypotensive effects in laboratory animals (Lahlou, 2000; Lahlou *et al.*, 2003; Interaminense, 2005). On the effects of the volatile oils on the intestinal motility, Bezarra (2000) reported the myorelaxant and the antispasmodic effects of *Alpinia speciosa* Schum on rat ileum while the antispasmodic effects of *Satureja hortensis* L. (Lamiaceae) has also been reported in literature (Hajhashemi, 2000).

The volatile oils of *Eucalyptus* species have been reported to vary in chemical compositions.

For instance, *E. globulus* was reported to be richer in 1,8-cineole, *Z*-citral and alpha-citral were more abundant in *E. staigeriana* compared with other monoterpenes while *E. citriodora* contains low amounts of citreole and citronellol with high concentration of citronella (Maciel *et al.*, 2009, Batish *et al.*, 2006). Its volatile oil was reported to possess activities against a wide range of micro-organisms (Emilio, 1996). Also, the volatile oils *Callistemon citrinus* and *C. viminalis* were reported to contain high concentrations of 1,8-cineole (61.2% and 83.2%) and alpha-pinene (13.4% and 6.4%) respectively (Oyedeji *et al.*, 2009). The volatile oils of the *E. citriodora* and *C. citrinus* were shown in this work to exhibit dose related relaxant effect on the isolated ileum as indicated in their respective inhibition of the contractions produced by ACH there by significantly reducing the EC_{max} produced by the compound. The activities observed were related and this could be due to the fact that the plants belong to the same family Myrtaceae. Clove flower (*Syzigium aromaticum*) also belongs to this family and it has a common use in various localities where it is usually infused in water and administered to infants to reduce colic pains. Its volatile oil has been reported to be potent in relaxing in relaxing ileal and tracheal muscle contraction (Reiter and Brandt, 1985).

Since the volatile oils significantly inhibited the contraction produced by the Ach, it is possible that they

both antagonise the activities of the muscarinic receptors in the rat ileum. Based on the results obtained, the *Callistemon* oil can be said to be more potent than that of *Eucalyptus.*

This work has established the probable medicinal applications of volatile oils of these plants as anti-spasmodic agents as they effectively inhibited Ach-induced contraction of the rat ileum. However their applications may require use of appropriate pharmaceutical adjuvant to reduce or prevent any toxicity.

REFERENCES

African Pharmacopoeia (1986). General methods of analysis, compiled by Organization of African Unity/Scientific Technical Research Commission (OAU/STRC), Volume 2, No.3, First Edition; Lagos, Nigeria, pp.118-120.

Ahonkhai I and Ayinde BA (2005). Antimicrobial activities of some volatile oils against some pathogenic organisms. *Journal of Pharmacy and Bioresources*; **2**(2): 56-59.

Aisien MSO, Imasuen AA, Wagbatsoma VA and Ayinde BA (2004). Preliminary evaluation of the repellent activity of some plant essential oils against *Simulium damnosum* S.L., the vector of human onchocerciasis. *Int. J. Trop. Insect Sci.,* **24**(2):196-199.

Alfonso R. Gennaro, John E. Hoover; Remington (2000). The Science and Practise of Pharmacy, 20th Edition Gopsons Papers Ltd., Noida.

Ayinde BA and Odigie F (2001). Larvicidal properties of the volatile oil of *Ageratum conyzoides* L. (Compositae) against *Culex* specie mosquito larvae. *Nigerian Journal of Applied Science,* **19**: 23-25.

Batish DR, Singh HP, Setia N, Kaur S, and Kohli RK (2006). Chemical composition and inhibitory activity of essential oil from decaying leaves of *Eucalyptus citriodora Z. Naturforsch.* **61c**: 52-56.

Bezerra MAC, Leal-Cardoso JH, Coelho-de-Souza AN, Criddle DN and Fonteles MC (2000). Myorelaxant and antispasmodic effects of the essential oil of Alpinia speciosa on rat ileum. *Phytother Res.*, **14**(7): 549-551.

Boland J and Brophy JJ (1991). *Eucalyptus* leaf oils: Uses, Chemistry, Distillation and Marketing. ACIAR/CSIRO, Australian Centre for International Agricultural Research; APN (eds.); Inkata Press Melbourne, pp.237-243.

Elliot WR and Jones DL (1984). Encyclopaedia of Australian Plants suitable for cultivation. Volume 4, Lothian Publications, Melbourne.

Emilio L (1996). Bioactive acylphloroglucinol derivatives from *Eucalyptus* species Phytochemistry. *The International Journal of Plant Biochemistry & Molecular Biology,* **41**(1): 7-22.

Evans WC (1989). Trease and Evans' Pharmacognosy, 13th Edition, Bailliere Tindall, London, p.204.

Gbolade AA, Onayade OA and Ayinde BA (1999). Insecticidal activity of *Ageratum conyzoides* L. volatile oil against *Callosobruchus maculatus* F. in seed treatment and fumigation laboratory tests. *Insect Sci. Applic*., **19**(2/3): 237-240.

Gill LS (1992). Ethnomedical uses of plants in Nigeria. Uniben Press, Benin City, p.170.

Hajhashemi V, Saraei H, Ghannadi AR and Mohseni M (2000). Antispasmodic and anti-diarrhoeal effect of *Satureja hortensis* L. essential oil. *J. Ethnopharmacol*., **71**(1-2): 187-192.

Interaminense LF, Leal-Cardoso JH, Magalhaes PJC, Duarte GP and Lahlou S (2005). Enhanced hypotensive effects of the essential oil of *Ocimum gratissimum* leaves and its main constituent eugenol, in DOCA-Salt Hypertensive Concious Rats. *Planta Med*., **71**: 376-378.

Lahlou S (2000). Hypotensive and bradycardiac action of *Mentha x villosa* essential oil. *Planta Med.,* **67**: 638-643.

Lahlou S, Interaminense LF, Leal-Cardoso JH and Duarte GP (2003). Antihypertensive effects of the essential oil of *Alpinia zerumbet* and its main constituents Terpenen -4-ol in DOCA-Salt hypertensive conscious rats. *Funda. Clin. Pharmacol*., **17**(3): 323-330.

Maciel MV, Morais SM, Bevilaqua CML, Silva RA, Barros RS, Sousa RN, Sousa LC, Brito ES and Souza-Neto MA (2010). Chemical composition of *Eucalyptus* spp. essential oils and their insecticidal effects on *Lutzomyia longipalpis. Veterinary Parasitology*, **167**(1): 1-7.

Newall CA, Anderson LA and Phillison JD (1996). Herbal medicines (A guide for Healthcare professionals). First Edition. The Pharmaceutical Press, London, p.108.

Onawunmi GO and Ogunlana EO (1986). Antimicrobial activities of the oil from lemon grass *Cymbopogon citratus* Staff. *In*: Drug production from Natural Products (Edited by Adesina SK), Medex Publication, Lagos, Nigeria, pp.194-206.

Oyedeji OO, Lawal OA, Shode FO and Oyedeji AO (2009). Chemical composition and antibacterial activity of the essential oils of *Callistemon citrinus* and *Callistemon viminalis* from South Africa. *Molecules* **14**(6): 1990-1998.

Reiter M and Brandt W (1985). Relaxant effects of the essential oil of *Eucalyptus citriodora* on tracheal and ileal smooth muscles of the guinea pig. *Arzneimittelforschung.,* **35**(1A): 408-414.