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Original Research Article

IN VITRO COMPARATIVE STUDY OF ANTIBACTERIAL ACTIVITY OF ETHANOLIC LEAF EXTRACTOF *EUCALPYTUS citriodora* HOOK WITH SELECTED ANTIBIOTIC ON CLINICAL AND TYPED ISOLATES OF *Escherichia coli.*

ABSTRACT

This study was aimed to compare the antibacterial activity of ethanolic leaf extract of *Eucalyptus citriodora* with selected antibiotics. 30% dimethyl sulphoxide (DMSO0 was used in the control well. The bacteria used were clinical isolate of *E. coli* and typed *E. coli* of ATCC 35218. Standard antibiotic such as ciprofloxacin, chloramphenicol, amoxicillin, gentamycin, tetracycline and nalidixic acid were used. Standard method was used to determined MIC and MBC of the leaf extract and the inhibitory efficacy of the leaf extracts was compared with commercial antibiotic on both isolates of *E. coli* using standard method. The extract shows the inhibitory effect of increasing concentration. For both isolates, no zone of inhibition was observed at concentration of the extract between 50-150mg/ml, but at higher concentration between 200-500mg/ml, there was a significant increase ($P<0.05$) in zones of inhibition that rages between 4.2 – 13.7 and 4.7 – 15.4mm for clinical and typed isolates respectively. The susceptibility of the both isolates to conventional antibiotic indicated that ciprofloxacin (10ug) had the highest inhibition against both isolates (17.3mm and 13.9mm respectfully), followed by gentamycin (14.4mm and 10.8mm), The clinical isolate was resistant to amoxicillin (30ug), while typed isolate was susceptible (4.3mm). The MIC of the extract for both isolates was 200mg/ml while the MBCs were 300 and 350mg/ml respectively. The result of the comparative zones of inhibition of the conventional antibiotic with leaf extract show that, in both isolates of *E. coli*, the inhibitory activity by the extract (15.4mm and 13.7mm) at 500mg/ml concentration can be compared favorably with ciprofloxacin of concentration of 10ug (17.3mm and 13.9mm). Gentamycin (14.4mm and 10.8mm) can be compare favorable to concentration of the extract at 450 mg/ml (13.7mm and 11.3mm). This study revealed the potency of *E. citriodora* ethanolic leaf extract as a future herbal candidate to treat infection cause by *E. coli*.

Keywords: *Eucalyptus citriodora,* Comparative, Antibacteria, Antibiotics and Activity.

1.0 INTRODUCTION

The word *Eucalyptus* is a genus name that emanated from the Greek word *Eucalyptus*, meaning "well-covered," and refers to its flowers that, in bud, are covered with a cup-like membrane Nair *et al.*, (1). According to Rakholiya and Chanda (2) *Eucalyptus citriodora* Hook (family: Myrtaceae) is a tall, evergreen and graceful plant which is cultivated for purpose of essential oil, fuel, timbers and medicinal. Husain and Ali (3), and Kharwar *et al.* (4), Reported that the leaves of *E. citriodora* produce a fragrant volatile oil that have activities like antibacterial, anti-inflammatory, antiseptic, analgesic, deodorant, diuretic, expectorant, in their report, the leaves contain many bioactive components such as phenolic compounds, flavonoids, sesquiterpenes, aldehydes, ketones and tannins. There equally indicated that, this essential oil from this plant is widely used in cosmetics, food, and pharmaceutical industries.

~~Rakholiya and Chanda (2), advanced that antibiotics have been used for treatment of number of bacterial diseases and that this antibiotic is the most important weapon to fight against infectious diseases, but wide use of antibiotics has led to development of resistant strains, which is becoming a global problem. There are alternatives to conventional antibiotics treatment to prevent the spread of infectious diseases such as use of plant extracts.~~ Freitas *et al.*, (5), indicated other strategy as the of combination of active phytochemicals with antibiotics to fight against various drug resistant microorganisms. This research work is aimed at determining the antibacterial activity of the ethanolic leaf extracts of *E. citriodora* and compare the extract to conventional antibiotics.

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2.0 MATERIALS AND METHODS

2.1 Plant leaf Collection

The leaf of *E. citriodora* leaf was collected in the month of November, 2017 from Kogi State University, Anyigba, Kogi State, Nigeria, identified and authenticated by comparing with herbarium specimens by Professor S.S. Usman, in the Department of Biological Sciences, Kogi State University, Anyigba, Kogi State, Nigeria

2.2 Extraction and Phytochemical Screening of the leaves

The method of Dada and Oloruntola, (6) was adopted for extraction. The leaves were washed, air dried at room temperature for three weeks and pulverized using mortar and pestle. Five hundred grams (500g) of the pulverized leaf powder was macerated in 4.5litre of 75% ethanol for 72hours and then filtered using Millipore (pore size 0.7µm) filter paper. By the use of rotary evaporator at reduce temperature of 40^0C, the filtrate was concentrated to recover the extract for further use.

2.3 In vitro Assay

The crud of ethanolic leaf extract of *E. citridora* was reconstituted with dimethyl sulphoxide (DMSO) of 30% and using sterile distilled water, different concentrations were prepared as following s: 500,450,400,350.300,250,200,150,100 and 50mg/ml. (7).

2.4 Preparation of McFarland Turbidity Standard

This standard was prepared using the method of cheesbrought (8) to quantify the density of bacteria cell.

2.5 Preparation of Inoculum of *Escherichia coli* for *In vitro* Assay

The clinical and typed isolate (*E. coli* ATCC 35218) were collected from Microbiology Laboratory, University of Ibadan Teaching Hospital, Ibadan, Nigeria. The standard inocula of both clinical and typed *E. coli* were used for the study.

2.6 Antibacterial Sensitivity Test

Antibacterial activity test was carried out using the method of CLSI (9) on both isolates of *E. coli* to the extract of the ethanolic leaf of *E. citriodora*. Using sterile pipette, 0.1ml of the bacterial suspension (1 x 106 cfu/ml) was taken and aseptically dispensed into sterile petri dishes and Mueller – Hinton Agar was poured aseptically into Petri dishes containing 0.1ml of *E. coli* (both type and Clinical Isolate). The petri dishes were carefully swirled in a clockwise direction to ensure that bacterial were homogenized in MHA. The plates were left to stand for 40 minutes to solidify the medium. Using sterile cork borer (7mm), three wells were aseptically bored on each plate at the distance of 30mm between opposite wells each and the edges of the plate. Aseptically, 0.1 ml each of the different concentration of the extracts was then introduced into each well in the petri dishes using sterile pipette. A control well was in the center with 0.1ml of the reconstituted agents (30% DMSO). The plates were incubated at 37^oc for 24hours. The zones of inhibition were measured using a caliper. The study was repeated three (3) times and average value was taken, as the result of the zones of inhibition of the both isolates for different concentration of the plant extracts.

2.7 Antibiotic Assay

The methods of CLSI (9) was used to compare the inhibitory efficacy of the leaf extracts with commercial antibiotic on both isolates of *E. coli.* Standard antibiotic (ciprofloxacin, chloramphenicol, amoxicillin, gentamycin, tetracycline and nalidixic acid) were used against *E. coli*. With the aid of sterile pipette 0.1 ml of the bacterial suspension (1 x 10^6 cfu/ml) was aseptically introduced into sterile plates. Sterilized MHA was aseptically poured into the plates containing 0.1ml of both isolates of *E. coli*. All the petri dishes were swirled to ensure that the bacterium was distributed evenly in MHA, the plates were allowed to stand for 40 minutes in other to solidify the medium. Using sterile pair of forceps, the antibiotic disc was gently laid aseptically on the plate. The plate was incubated at

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37°c for 24 hours. All the plates were observed for zone of inhibition around the disc. The diameter of the clear zones was measured in millimeters (mm) using a caliper.

2.8 Determination of Minimum Inhibitory Concentration (MIC) & Minimum Bactericidal Concentration (MBC)

Cheesbreugh (8) dilution method was adopted to determine MIC and MBC of the extract. Crude extract of different concentration of the ethanolic leaf of *E. citriodora* (500,450,400,350,300,250,200,150,100,50,25,12.5, and 6.25mg/ml) were prepared. MHA was prepared and 5ml was pipette into sterile test tube and 0.1ml of inoculum of *E. coli* (1 x 10^6 cell/ml) was introduced into each test tube and was properly mixed. With the aid of sterile pipette, 1ml of the various concentration of extract prepared was dropped into each test tube containing the broth culture of both isolates of *E. coli*. The mixture was incubated at 37°c for 24hours. Turbidity measurement using a spectrophotometer was checked for growth in each test tube. High turbidity indicated growth and inhibition of growth was indicated by low turbidity. The concentration in which no growth was observed as shown by cleared broth indicated the minimum inhibitory concentration while the MBC was determined by taking a loopful each from test tube that showed no growth during MIC assay and streaked on agar plate that is free of leaf extract, incubated at 37°c for 24 hours. The least concentration at which no growth as observed was noted as the MBC.

2.9 Statistical analysis

All data were expressed as mean ±S.E. One-way analysis of variance was used to analyze data. $P< 0.05$ was considered significant difference between means (Duncan's multiple range test).

3.0 RESULT

3.1 Antibacterial Activity of Ethanolic Leaf Extract of *E. citriodora*

The sensitivity pattern of both isolates of *E. coli* to ethanolic leaf extract of *E. citriodora* is shown in figure 1. It shows the inhibitory effect of increasing concentration of the extract. For both isolates, no zone of inhibition was observed at concentration of the extract between 50-150mg/ml, but at higher concentration between 200-500mg/ml, there was a significant increased ($P<0.05$) in zones of inhibition that rages between 4.2 – 13.7 and 4.7 – 15.4mm for clinical and typed isolates respectively.

3.2 Antibiotic Sensitivity Pattern of *E. coli*

The result of the susceptibility of the both isolates of *E. coli* to conventional antibiotic is show in figure 2. Ciprofloxacin (10ug) had the highest inhibition against both isolates (17.3mm and 13.9mm respectfully), followed by gentamycin (14.4mm and 10.8mm), tetracycline (13.9mm and 7.8), oflaxacin (13.0mm and 8.3mm), amoxicillin (4.3mm and0.0mm) and nalixidic acid (10.8mm and 5.4). However, clinical isolate was resistant to amoxicillin (30ug), while typed isolate was susceptible (4.3mm).

3.4 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC of the extract for both isolates was 200mg/ml while the MBCs were 300 and 350mg/ml respectively.

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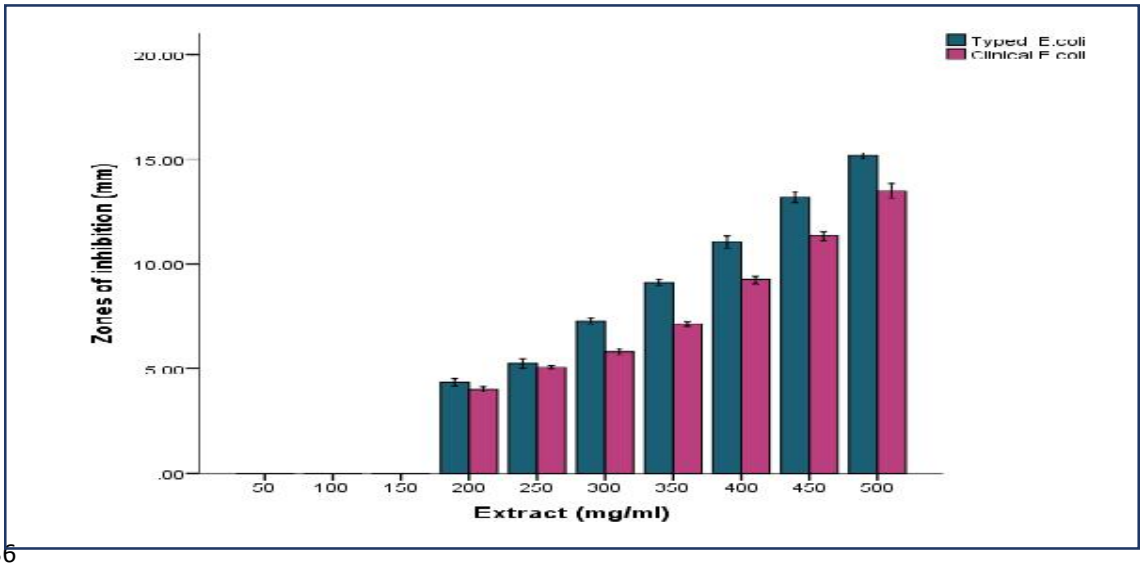

Figure 1: Antibacterial activities of the clinical and typed isolates of *E. coli* to the extract.

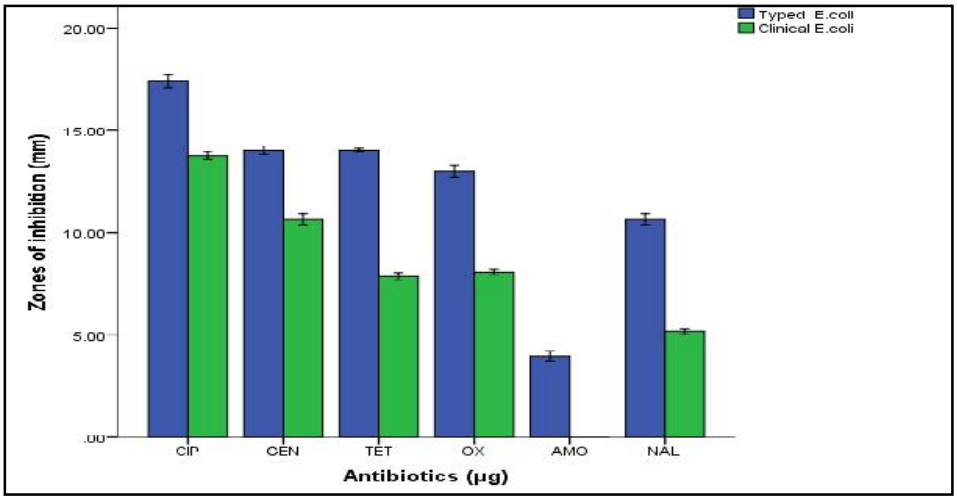

Figure 2: Susceptibility of the clinical and typed isolates of *E. coli* to commercial antibiotic.

3.5 Comparative Zones of Inhibition of Conventional Antibiotic with *E. citiodora* Leaf Crude Extract

The result of the comparative zones of inhibition of the conventional antibiotic with leaf extract is show in figure 3. From the result, it shown that in both isolates of *E. coli*, the inhibitory activity by the extract (15.4mm and 13.7mm) at 500mg/ml concentration can be compared favorably with ciprofloxacin of concentration of 10ug (17.3mm and 13.9mm). Gentamycin (14.4mm and 10.8mm) can be compare favorable to concentration of the extract at 450 mg/ml (13.7mm and 11.3mm), tetracycline (13.9mm) and oflaxacin (13.0mm) can be compare with the concentration of the extract at 450mg/ml (13.7 mm) for typed isolate. While tetracycline (7.8mm) and oflaxacin (8.3mm) can be compare with extract concentration of 350 and 400mg/ml (7.2mm and 9.3mm) for clinical isolate. Amoxicillin (4.3mm) can be favorably compare to extract at the concentration of 200mg/ml (4.7mm) for typed isolate. Nalixidic acid (10.8mm) can be compare to extract concentration at 400mg/ml (11.1mm) for typed isolate, while for clinical isolate, nalixilic acid (5.4mm) is compared favorable to extract concentration at 250mg/ml (5.1mm).

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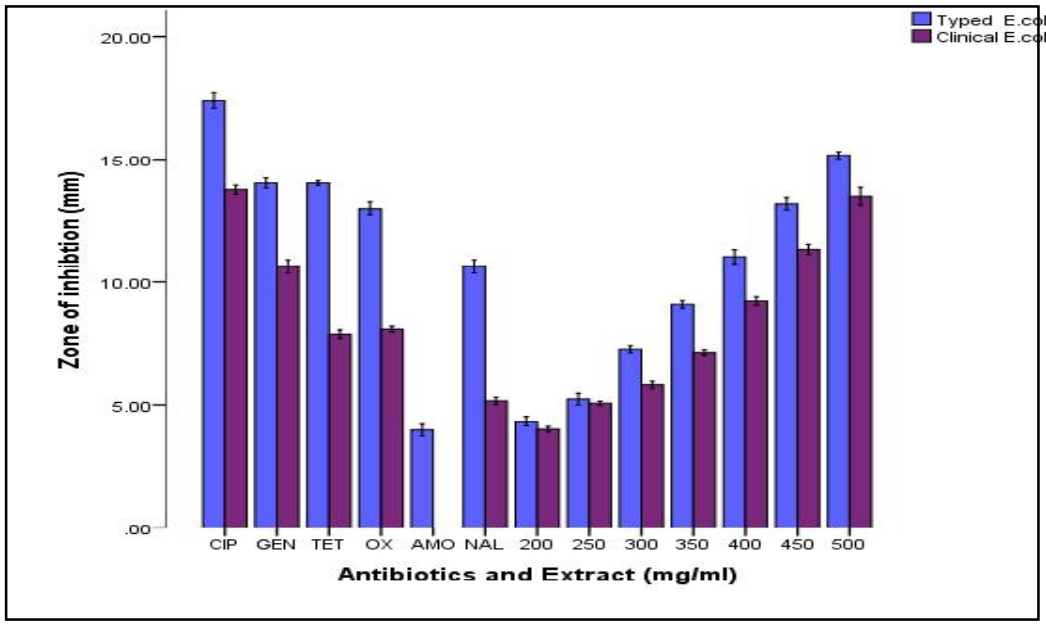

Figure 3: Comparative study of the Susceptibility of the clinical and typed isolates of *E. coli* to the plant extract and commercial antibiotic

4.0 DISCUSSION

The inhibitory pattern of clinical and typed isolates of *E. coli* to the ethanolic leaf extract of *E. citriodora* in this study revealed the sensitivity that show zones of inhibition that varied from concentration to concentration. The extract of *E. citriodora* from this *in vitro* study show to possess antibacterial activity against both isolates of *E. coli*, but at higher concentration. This result agreed to similar report by Tolba *et al*. (10), who reported that, the zone of inhibition of *E. citriodora* oil extract increased with the increasing concentration (10, 20 and 30 µl) and that *E. coli*, was extremely sensitive to the oil extract (26 ± 0.0 mm), however, *P. aeruginosa* and *Enterococcus faecalis* were resistant to the oil extract. The inhibition observed in this study might probably be arrogated to the report presented by Evans, (11), that alkaloids occur in plants in association with characteristic acids. This acid could be probably responsible for the inhibition observed in this study.

Lack of inhibition observed for both isolates at lower concentration (50-150mg/ml) could be due to other bioactive component not tested in this study that could be absent in the extract. This corroborated with the report of Tolba *et al*. (10), who stated that antibacterial activity of many essential oils, and in particular *Eucalyptus* species, is related to the presence of some favorable classes of compounds such as alcohols, aldehydes, alkenes, esters and also that antimicrobial activity of the *Eucalyptus citriodora* Hk essential oil could be due to the two major compounds: citronellal and citronellol. However, the inhibition observed at higher concentration might be due to presence of tannin and other bioactive component in the extract. This is in agreement with the report advanced by Dickson *et al*. (12), that the presence of tannins in plant suggest it to be of medicinal value because tannins have shown potential antiviral, antibacterial and antiparasitic effects. This also concours with the report of Amabye *et al*. (13), that tannins are known to be made up of phenolic compounds and phenols that have been used extensively as disinfectants and that action of tannin may be due to protein denaturation and is found to be non-specific. Furthermore, lack of inhibition observed at low concentration could suggest that infection caused by *E. coli* might not be treated with low concentration of *E. citriodora* extract. This in lined with the report presented by Dickson *et al*. (12), that at low concentration, the crude extract of *E. camaldulensis* was not active against *P. aeroginosa*, but at high concentration, it began to show some activity. The lack of inhibition noticed at low

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concentration(50-150mg/ml) in this study disagreed with another report presented by Dickson *et al*., (12), that at low concentrations (≥50mg/ml), the aqueous extract of *E. camaldulensis* may be effective in the treatment of diseases caused by virulent strains of *E. coli*.

It was observed from this study that clinical isolates were more resistant to the plant extract compare to the typed isolate. This resistant observed in clinical isolate could be attributed to the genetic makeup of the organism. ~~This agreed with the report of Amabye *et al*. (13).~~

The obtained value of MIC in this study was higher than the one reported by Luqman *et al.,* (14) that the MIC, MFC and MBC of *E. citriodora* essential oil ranged from 1.25 mg/ml to 10 mg/ml against pathogenic fungi, 1.25 mg/ml to 5.0 mg/ ml against drug resistant mutants of *C. albicans*, 10 mg/ml to more than 10 mg/ml against human pathogenic bacteria and 1.25 mg/ml to more than 10 mg/ml in drug resistant mutants of *E. coli* and *M. smegmatis.* Also, Tyagi and Malik, (15), reported low values of MIC value for *Eucalyptus globulus* was (4.5 mg/ml and 2.25 mg/ml for *E. coli* and *Staphylococcus aureus*, respectively).

The antibacterial sensitivity of both isolates to ciprofloxacin, ofloxacine, nalixidic acid, tetracycline and gentamycin in this work is unexpected and this might probably due to non-indiscriminate previous exposure of this strain of *E. coli* to those antibiotics. This is in disagreement with the work of Lucia *et al*. (16), who observed resistant against *E. coli* strain isolated from milk originating from Sinjai district, South Sulawesi. With the exception of gentamycin that displayed inhibition (11.4mm) against *E. coli* that agreed with this work. The sensitivity of the isolates to ciprofloxacin and gentamycin were expected, this agreed with the similar result presented by Ahmed *et al*. (17) in Islamabad that ciprofloxacin and gentamicin revealed the highest sensitivity against the *E. coli* isolates with 80 and 66.66% sensitivity respectively, these sensitivities were higher than that of the current study and this might probably be due to the strain of *E. coli* involved. This is also in lined with the report of Reuben and Owuna (18), that 78.9% of *E. coli* isolates showed sensitivity to ciprofloxacin and same percentage was observed for gentamicin. The susceptibility displayed by *E. coli* isolates to ciprofloxacin and gentamicin in this study suggest their effectiveness in the treatment of infections caused by *E. coli*. The sensitivity of *E. coli* isolates to tetracycline in this study was unexpected due to the fact that this is the most commonly prescribed antibiotic in the hospital and also the most easily available in the community without prescription. However, this is contrary to the report of Reta *et al.* (19). The resistant recorded for amoxicillin in this study agreed with the report of Kindu (20). This is expected because of easy accessibility and low cost of the antibiotic and this resistant could probably due to reason advanced by Todar, (21), who reported that antibiotic resistance develops when microorganisms are exposed to effective doses of an antibiotic within a shorter period or when the organisms are exposed to smaller concentrations of the antibiotic over a longer period of time.

Findings from comparative zones of inhibition of the extract to antibiotic revealed that, in both isolates, the concentration of the plant extract at 500mg/ml which shown highest inhibition can be compared favorably with ciprofloxacin that also shown highest inhibition. Similarly, the least inhibition displayed by the extract at concentration of 200mg/ml can be compare favorably with amoxicillin that displayed lowest activity.

4.1 Conclusion

This study provided the information on the future herbal potency of *E. citriodora* leaf extract as a candidate for treatment of *E. coli* infection. Further investigation to determine the pure active components of the leaves extract of the *E. citriodora* responsible for these activities and the effect on long term administration is recommended for further studies.

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