South Asian Journal of Research in Microbiology

2(1): 1-8, 2018; Article no.SAJRM.43988

Antibacterial Property of Ethanolic Leaf Extract of *Eucalyptus citriodora* Hook on Clinical and Typed Isolates of *Escherichia coli*

D. Muhammed[1*], E. O. Dada[2] and A. A. Alo[2]

[1]Department of Applied Biology, Federal Polytechnic of Oil and Gas, P.M.B. 5027, Bonny Island, Rivers State, Nigeria.
[2]Department of Microbiology, Federal University of Technology, P.M.B. 704, Akure, Ondo State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author DM designed the study, performed the statistical analysis, wrote protocol and wrote the first draft of the manuscript. Authors DM, EOD and AAA managed the analyses of the study. Authors DM and EOD managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/SAJRM/2018/v2i129234
Editor(s):
(1) Dr. Ana Claudia Coelho, Assistant Professor, Department of Veterinary Sciences, University of Tras-os-Montes and Alto Douro, Portugal.
Reviewers:
(1) Ahmed Mohammed Abu-Dief Mohammed, Sohag University, Egypt.
(2) Jean-De-Dieu Tamokou, University of Dschang, Cameroon.
(3) Izharul Hasan, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/26477

Original Research Article

Received 05 July 2018
Accepted 18 September 2018
Published 30 September 2018

ABSTRACT

The emergence of antibiotic resistance bacteria, necessitated the discovery and development of alternative therapy to bacterial infections. This work assessed the inhibitory property of ethanolic leaf extract of *Eucalyptus citriodora* against clinical isolate and *E. coli* ATCC 35218. Agar well diffusion test was used to determine the antibacterial activity of the extract on both isolates of *E. coli.* For both isolates, no zone of inhibition was observed at concentrations of the extract between 50-150 mg/ml, but at 200-500 mg/ml, there were significant (P>0.05) zones of inhibitions that ranged between 4.2 – 13.7 and 4.7 – 15.4 mm for clinical isolate and *E. coli* ATCC 35218 respectively. The susceptibility of both isolates to conventional antibiotics revealed ciprofloxacin (10 ug) having the highest inhibitions against both isolates (17.3 mm and 13.9 mm) respectively, followed by gentamycin (14.4 mm and 10.8 mm). The clinical isolate was resistant to amoxicillin (30 ug), while *E. coli* ATCC 35218 was susceptible (4.3 mm). The MIC of the extract for both isolates

**Corresponding author: E-mail: mdanjuma20@gmail.com;*

was 200 mg/ml while the MBCs were 300 and 350 mg/ml respectively. The inhibitory activities of the extract (15.4 mm and 13.7mm) at 500 mg/ml concentration is comparable with ciprofloxacin (17.3 mm and 13.9 mm) at a concentration of 10 µg, while that of Gentamycin at 30 µg (14.4 mm and 10.8 mm) is comparable with concentration of the extract at 450 mg/ml (13.7 mm and 11.3 mm). The zones of inhibitions produced by the extract increased with increasing concentrations. This study revealed the potency of *E. citriodora* ethanolic leaf extract as a future herbal candidate to treat infection caused by *E. coli* at high concentrations of the extract.

Keywords: Eucalyptus citriodora; antibacterial; antibiotics resistance; Escherichia coli.

1. INTRODUCTION

Eucalyptus is a genus name 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]. *Eucalyptus citriodora* Hook (family: Myrtaceae) is a tall, evergreen plant which is cultivated for essential oil, fuel, timbers and medicinal purposes [2]. The leaves of *E. citriodora* produce fragrant volatile oil with antibacterial, anti-inflammatory, antiseptic, analgesic, deodorant, diuretic, expectorant activities [3,4]. The leaves contained many bioactive components such as phenolic compounds, flavonoids, sesquiterpenes, aldehydes, ketones and tannins. The essential oil from this plant is widely used in cosmetics, food, and pharmaceutical industries.

Multidrug-resistance (MDR) by bacteria has become a public health issue, which is estimated to cause maximum deaths by the year 2050 along with increasingly high health expenses. A rise in antimicrobial resistance has been reported in *E. coli* worldwide, resulting in complications and treatment issues [5]. According to Tule and Hassani [6], *E. coli* isolates from neonates without any prior exposure to antibiotics are highly resistance to antibiotics like ampicillin (100%) and co-trimoxazole (96%). Also, Purohit et al. [7], evaluated the prevalence of antibiotic resistance of ampicillin, cefoxitin, nalidixic acid, polymyxin-B etc. on commensal *E. coli* isolates from human, animals, as well as water by disk diffusion method and reported that commensal *E. coli* from all sources displayed resistance to all the antibiotics tested except polymyxin-B. The incidence of antibiotics resistance in human isolates was higher compared to that of water or animals. Nahla et al [8], reported an increase in multi-drug resistant phenotypes of *E. coli* to third-generation cephalosporins as well as colistin.

Escherichia coli is ubiquitous and present in both animals and the environment [5]. It is Gram negative, facultatively anaerobic, rod-shaped, coliform commonly found in the intestinal tract of warm-blooded animals including humans. It is the most common cause of food and water-borne human diarrhoea worldwide, leading to deaths especially in young children. It is the leading cause of urinary tract infections (UTIs), bloodstream infections, wounds infections, otitis media and other complications in humans. *E. coli* accounts for more than 50% of UTIs in outpatients.

Previous antibacterial studies showed that essential oil of *Eucalyptus* species had antibacterial effect on the growth of Gram negative and Gram-positive bacteria especially *Escherichia coli* CIP54127 and *E. coli* isolated from urine [9] and *Staphylococcus aureus* [10]. This study determined the antibacterial effect of ethanolic leaf extract of *Eucalyptus citriodora* against clinical isolates and *E. coli* ATCC 35218.

2. MATERIALS AND METHODS

2.1 Plant Leaf Collection

E. citriodora leaves were collected in the month of November, 2017 from Kogi State University, Anyigba, Nigeria. It was identified and authenticated by an expert in the Department of Biological Sciences of Kogi State University, Anyigba. The voucher specimen number of the plant Bio/ FUTA/ 70 was left in the herbarium of Federal University of Technology, Akure, Ondo State, Nigeria.

2.2 Extraction of the Leaves

The method of Dada and Oloruntola, [11] 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 (500 g) of the pulverized leaf powder was dissolved in 4500 ml of 75% ethanol

for 72 hours and then filtered using Millipore (pore size 0.7 µm) filter paper. The filtrate was concentrated to recover the crude extract using rotary evaporator at a reduced temperature of 40°C.

2.3 *In vitro* Assay

The ethanolic leaf extract of *E. citriodora* was reconstituted with dimethyl sulphoxide (DMSO) of 30% and using sterile distilled water, different concentrations were prepared as followings: 500, 450, 400, 350, 300, 250, 200, 150, 100 and 50 mg/ml [12].

2.4 Preparation of McFarland Turbidity Standard Inoculum of *Escherichia coli*

The clinical isolate and *E. coli* ATCC 35218 were collected from Microbiology Laboratory, University of Ibadan Teaching Hospital, Ibadan, Nigeria. The standard inocula of both *E. coli* were used for the study. This was prepared using the method of Cheesebrough [13] to quantify the density of bacterial cells.

2.5 Antibacterial Sensitivity Test

Antibacterial activity test of the extract of *E. citriodora* was carried out using the method of CLSI [14]. Clinical isolate and *E. coli* ATCC 35218. Using sterile pipette, 0.1 ml of the bacterial suspension (1 x 10^6 cfu/ml) was taken and aseptically dispensed into sterile Mueller – Hinton Agar (MHA) plates. The petri dishes were carefully swirled in a clockwise direction to ensured that bacteria suspension was well spread on MHA. The plates were left to stand for 40 minutes. Using a sterile cork borer of 6 mm diameter, 3 wells were aseptically bored on each plate at the distance of 30 mm between opposite wells each and the edges of the plate. Aseptically, 0.1 ml each of the different concentrations of the extracts were then introduced into each well in the petri dishes using sterile pipette. A control well was at the center with 0.1 ml of the reconstituted agents (30% DMSO). The plates were incubated at 37°C for 24 hours. The zones of inhibitions were measured using a caliper. The study was repeated three [3] times and the average values were taken, as the result of the zones of inhibitions of both isolates for different concentrations of the extracts.

This was repeated for standard antibiotics (produced in England by Oxoid) such as ciprofloxacin (fluoroquinones), gentamycin (aminoglycosides), tetracycline (dexycyclines), amoxicillin (aminopenicillins), ofloxacin (quinolone) and nalidixic acid (guinolones).

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

Cheesbreugh [13] dilution method was adopted to determine MIC and MBC of the extract. Extract of different concentrations (500, 450, 400, 350, 300, 250, 200, 150, 100, 50, 25, 12.5, and 6.25 mg/ml) were prepared. MHA was prepared and 5 ml was pipetted into sterile test tube and 0.1 ml 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 a sterile pipette, 1 ml of the various concentrations of extract prepared was dropped into each test tube containing the broth culture clinical isolate and *E. coli* ATCC 35218. The mixture was incubated at 37°C for 24 hours. 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 a test tube that showed no growth during MIC assay and streaked on an agar plate that is free of leaf extract, incubated at 37°C for 24 hours. The least concentration at which no growth was observed was noted as the MBC.

2.7 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. RESULTS

3.1 Percentage Yield of the Ethanolic Leaf Extract of *Eucalyptus citriodora*

Percentage yield of the ethanolic leaf extract of *Eucalyptus citriodora* was 9.37% (46.83/500 g) (Table 1).

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

The sensitivity pattern of clinical isolate and *E. coli* ATCC 35218 to ethanolic leaf extract of *E. citriodora* revealed an inhibitory effect of increasing concentrations of the extract. For both isolates, no zone of inhibition was observed at concentrations of the extract between 50-150 mg/ml, but at concentrations between 200-500 mg/ml, the zones of inhibitions were 4.2 – 13.7 and 4.7 – 15.4 mm for clinical isolate and *E. coli* 35218 respectively (Fig. 1).

3.3 Antibiotics Sensitivity Pattern of *E. coli*

The sensitivity test of *E. coli* 35218 and clinical isolates to conventional antibiotics (Fig. 2), revealed that, ciprofloxacin (10 ug) had the highest zones of inhibition against E. *coli* 35218 and clinical isolates (17.3 mm and 13.9 mm respectfully), followed by gentamycin (14.4 mm and 10.8 mm), tetracycline (13.9 mm and 7.8 mm), ofloxacin (13.0 mm and 8.3 mm), amoxicillin (4.3 mm and 0.0 mm) and nalidixic acid (10.8 mm and 5.4 mm). However, clinical isolate was resistant to amoxicillin (30 ug), while *E. coli* 35218 was susceptible (4.3 mm).

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

The MIC of the extract for *E. coli* ATCC 35218 and clinical isolate was 200 mg/ml while the MBCs were 300 and 350 mg/ml respectively for *E. coli* ATCC 35218 and clinical isolate.

3.5 Comparative Zones of Inhibitions of Conventional Antibiotic with *E. citriodora* Leaf Crude Extract

The result of the comparative zones of inhibitions of the conventional antibiotics with the extract (Fig. 3) revealed that, in both *E. coli* ATCC 35218 and clinical isolate, the inhibitory activities of the extract (15.4 mm and 13.7 mm) at 500 mg/ml concentration could be compared with ciprofloxacin of concentration of 10 ug (17.3 mm and 13.9 mm). Gentamycin (14.4 mm and 10.8 mm) compared with concentration of the extract at 450 mg/ml (13.7 mm and 11.3 mm), tetracycline (13.9 mm) and ofloxacin (13.0 mm) could be compared with the concentration

Table 1. Percentage yield of ethanolic leaf extract of *Eucalyptus citriodora*

Plant species	Plant part	Weight of powder (g)	Volume of solvent (ml)	Yield (g)	% Yield
E. citriodora	Leaf	500	4500	46.83	9.37

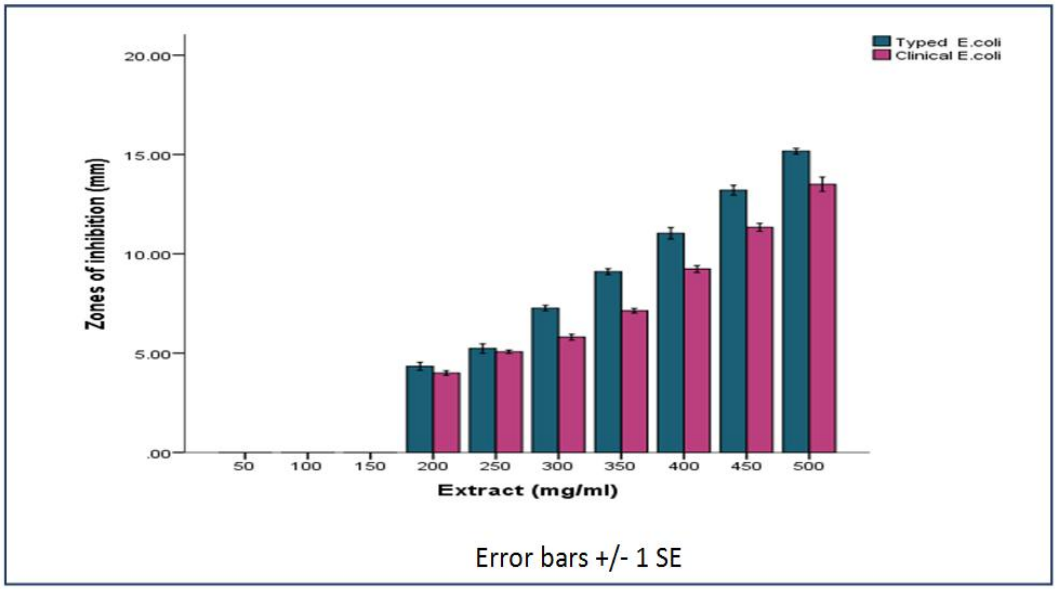

Fig. 1. Antibacterial activities of *E. coli* ATCC 35218 and clinical isolate to the extract

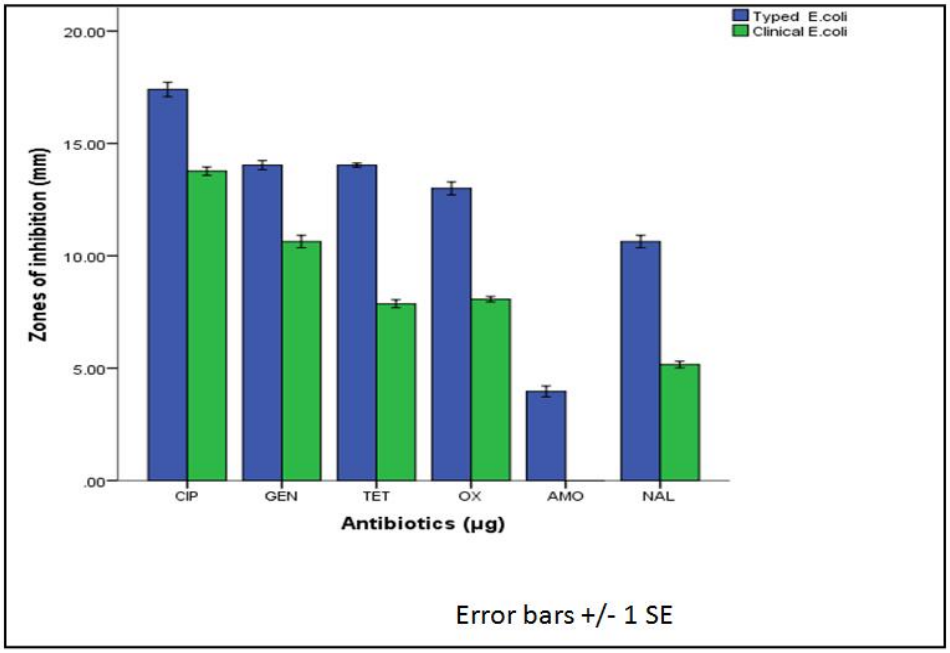

Fig. 2. Sensitivity of *coli* ATCC 35218 and clinical isolate to commercial antibiotics

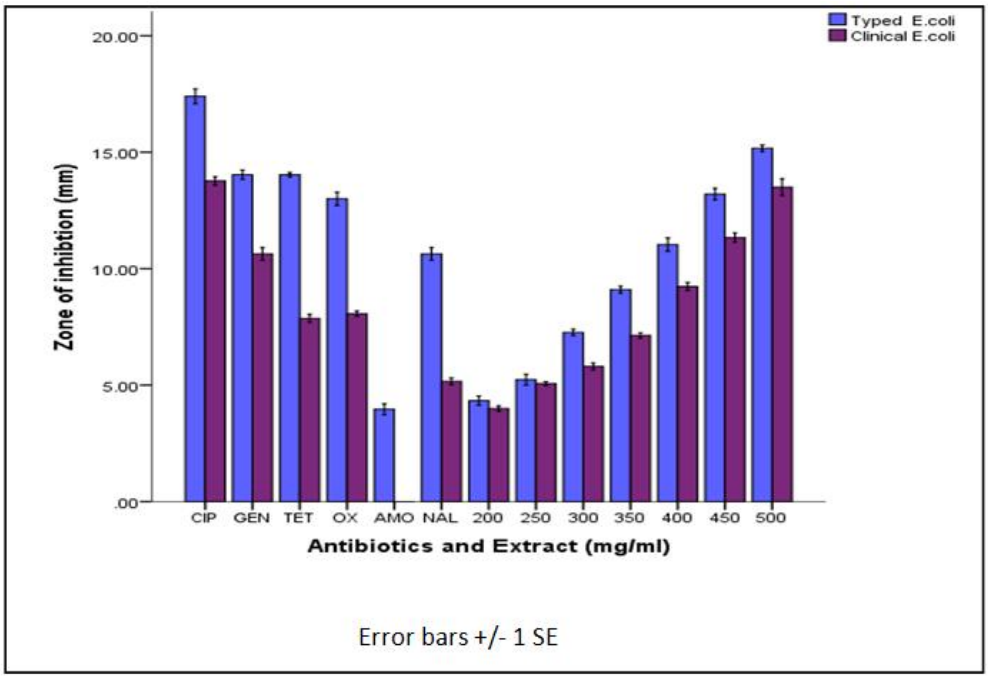

Fig. 3. Comparative study of the susceptibility of the clinical isolate and *E. coli* 35218 to the plant extract and commercial antibiotics

of the extract at 450 mg/ml (13.7 mm) for *E. coli* ATCC 35218. While tetracycline (7.8 mm) and ofloxacin (8.3 mm) could be compared with extract concentrations of 350 and 400 mg/ml (7.2 mm and 9.3 mm respectful) for clinical isolate. Amoxicillin (4.3 mm) could be compared with extract at the concentration of 200 mg/ml (4.7 mm) for *E. coli* 35218. Nalidixic acid

(10.8 mm) could be compared to extract concentration at 400 mg/ml (11.1 mm) for *E. coli* ATCC 35218, while for clinical isolate, nalidixic acid (5.4 mm) could be compared with extract concentration of 250 mg/ml (5.1 mm).

4. DISCUSSION

The extract of *E. citriodora* displayed antibacterial activities against both isolates of *E. coli*, but at high concentrations. This agreed with report of similar study by Tolba et al. [15], that, the zones of inhibitions of *E. citriodora* oil extract increases with increasing concentrations and that *E. coli*, was extremely sensitive to the oil extract (26 ± 0.0 mm). The zones of inhibitions observed might be due to report advanced by Evans [16], that alkaloids occur in plants in association with characteristic acids. This acid could be probably responsible for the zones of inhibitions observed.

Lack of inhibitions observed on both isolates at low concentrations (50-150 mg/ml) could be due to the absence of some bioactive components in the extract. This corroborated the report of Tolba et al. [15], who stated that antibacterial activity of many essential oils, and in particular *Eucalyptus* species, is related to the presence of some compounds such as alcohols, aldehydes, alkenes, esters. The lack of inhibitions observed at low concentrations could suggest that infection caused by *E. coli* might not be treated with a low concentration of *E. citriodora* extract. This disagreed with Dickson et al. [17], who reported that at low concentrations (≥50mg/ml), the aqueous extract of *Eucalyptus* might be effective in the treatment of diseases caused by virulent strains of *E. coli*. Clinical isolate was more resistance to the plant extract compared with *E. coli* 35218. This resistance could be due to report advanced by Yaya et al. [18], that the membrane of this strain was impervious to the active components contained in the extract at those concentrations. Also, lipopolysaccharides and phospholipids cell wall of the isolate could block the penetration of the extract inside the cell cytoplasm.

Also, the antimicrobial activity of the *Eucalyptus citriodora* Hook essential oil could be due to the two major compounds: citronellal and citronellol. However, the zones of inhibitions observed at high concentrations might be due to the presence of tannins and other bioactive components in the extract. This agreed with the report advanced by Dickson et al. [17], that the presence of tannins in plant suggest its medicinal value because tannins have potential antibacterial and antiparasitic effects. This also agreed with the report of Amabye et al. [19], that tannins are known to be made up of phenolic compounds and phenols which have been used extensively as disinfectants, and action of tannins might be due to protein denaturation.

The obtained values of MIC and MBC for both isolates was higher than that reported by Luqman et al. [20]. Also, Tyagi and Malik, [21], reported low value of MIC for *Eucalyptus globules* (4.5 mg/ml) on *E. coli.*

The antibacterial sensitivity of both isolates to ciprofloxacin, ofloxacin, nalidixic acid, tetracycline and gentamycin is unexpected and this could be due to lack of previously exposure of this strain of *E. coli* to those antibiotics. This disagreed with the report of Lucia et al. [22], who observed resistance of *E. coli* strain to these antibiotics. With the exception of gentamycin that displayed inhibition (11.4 mm). The sensitivities of the isolates to ciprofloxacin and gentamycin are expected, this agreed with the similar result advanced by Ahmed et al. [23], that ciprofloxacin and gentamicin revealed high sensitivities against *E. coli* isolates with 80 and 66.66% sensitivity respectively, these sensitivities were higher than that of the current study and this might be due to the strain of *E. coli* involved. This also agreed with the report of Reuben and Owuna [24], that 78.9% of *E. coli* isolates displayed sensitivity to ciprofloxacin and same percentage was observed for gentamicin. The susceptibility displayed by *E. coli* isolates to ciprofloxacin and gentamicin in this study suggested their effectiveness in the treatment of infections caused by *E. coli*. The sensitivity of *E. coli* isolates to tetracycline in this study was unexpected been the most commonly prescribed antibiotic in the hospital and also the most readily available in the communities without prescription. This disagreed with the report of Reta et al. [25]. The resistant recorded for amoxicillin in this study agreed with the report of Kindu [26]. This is expected because of easy accessibility and low cost of the antibiotics. The resistance could also be due to reasons advanced by Todar [27], that antibiotics resistance develops when microorganisms are exposed to effective doses of antibiotics within a shorter period or when the organisms are exposed to smaller concentrations of the antibiotics over a longer period of time. According to Abdel-Rahman et al. [28], DMSO has no antimicrobial activity against the test

organisms, that is why we considered it as control in the analysis.

Findings from comparative zones of inhibitions of the extract with antibiotics revealed that, in both isolates, the concentration of the extract at 500 mg/ml which showed highest zones of inhibition could be compared favourably with ciprofloxacin that also showed the highest inhibition. Similarly, the least inhibition displayed by the extract at a concentration of 200 mg/ml could be compared favourably with amoxicillin that displayed lowest activity.

5. CONCLUSION

This study has shown the future herbal potency of *E. citriodora* leaf extract as a candidate for the 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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/26477