

Full Length Research Paper

***In vitro* evaluation of the antibacterial activities of the methanol, aqueous and n-hexane extracts of *Ocimum lamiifolium* from Ethiopia**

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***Ocimum lamiifolium* (local name Dama Kesse, Amharic) is a medicinal plant in Ethiopia. Its leaves are squeezed and sniffed to treat coughs and colds. They are also used to treat eye infections and to stop nose bleedings. In the present study, leaves of *O. lamiifolium* were collected from their growing habitats. Dried leaf powders were extracted using methanol, distilled water and n-hexane. 25, 50, and 100 mg/ml doses of the extracts made in Tween 80 (2%) were screened for their antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Shigella boydii* using disk diffusion assay. The inhibition zones due to the methanolic extract ranged from 0 (in *S. aureus* due to 25 mg/ml) to 12 mm (in *E. coli* due to 100 mg/ml). Inhibition zones due to the aqueous extract ranged from 8 mm in *S. aureus* and *S. boydii* to 12 mm in *S. boydii* at concentrations of 25 and 100 mg/ml, respectively. The n-hexane extract at 25 mg/ml resulted in inhibition zone that ranges from 7 mm (against *S. aureus*) to 11 mm (against *E. coli*) at 50 and 100 mg/ml doses. The minimum inhibitory concentration of *S. boydii* and *E. coli* was 10 mg/ml due to all the extracts. The minimum inhibitory concentrations on *S. aureus* were 10, 20 and 50 mg/ml due to the aqueous, n-hexane and methanolic extracts, respectively. *P. aeruginosa* was minimally inhibited at 10 mg/ml due to the methanol and aqueous extracts and 15 mg/ml due to the n-hexane extract. The methanol, aqueous, and n-hexane extracts of *O. lamiifolium* leaf extracts inhibited the test bacteria with significantly higher levels of inhibition zones than the negative control (T80). The positive controls (Tetracycline and Chloramphenicol) also showed significantly higher inhibition zones than the 100 mg/ml concentration of the extracts and T80 except that Chloramphenicol failed to inhibit *S. aureus* and *P. aeruginosa*. However, combination of Chloramphenicol with plant extracts raised their inhibition zones from zero to 23 and 25 mm in *S. aureus* and *P. aeruginosa*, respectively.**

Key words: *Ocimum lamiifolium*, antibacterial activity, methanolic extract, aqueous extract, n-hexane extract.

INTRODUCTION

The genus *Ocimum* (Lamiaceae) consists of about 30 species distributed in the tropics and subtropics of the

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Old and New worlds, with some species cultivated in temperate areas. *Ocimum lamiifolium* Hochst.ex Benth(local name Tossign, Amharic) is mostly found in clearings and edges of primary and secondary mountain forests and bushlands, tall grasslands, abandoned fields, at altitudes between 1200 and 2900 m. Traditionally, the fresh leaves are squeezed and the juice is sniffed to treat cough and cold. The juice is also used as eye rinse to treat eye infections. At the same time, the crushed leaves are put in the nostrils to stop nose bleeding (Asfaw and Demissew, 2009).

Biologically, different extracts of the genus *Ocimum* are known for their antibacterial (Nakamura et al., 1999; Nascimento et al., 2000; Adebolu and Oladimeji, 2005; Adiguzel et al., 2005; Ahmad and Aqil, 2007; Goyal and Kaushik, 2011; Patil et al., 2011; Sneha et al., 2011; Prasannabalaji et al., 2012), antifungal (Amadioha, 2001), and antioxidant (Hakim et al., 2008) activities. *O. lamiifolium* extracts are also known to have antibacterial, antifungal, insecticidal and insect repellent (Dagne, 2009), antiinflammatory (Kashyap et al., 2011) activities. The chemical composition of essential oils of six *Ocimum* species from East Africa including *O. lamiifolium* were majorly phenyl propane derivatives or terpenoids, including methyl eugenol, 1, 8-cineole, camphor, bornyl acetate, germacrene-D, E-myroxide, germacrene-B, caryophyllene oxide and p-cymene (Kashyap et al., 2011). In another study by Tchoumboungang et al. (2014), 85.7% of *O. lamiifolium* essential oils were monoterpenes [sabinene (33.8%), (Z)- β -ocimene (17.2%), terpinen-4-ol (8.4%) and others] and sesquiterpenes (8.7%) [β -caryophyllene (5.6%), germacrene D (1.1%), (E)- β -farnesene (1%) and others]. Sabinene is known to have inhibition effects against Gram negative and Gram positive bacteria (Wiert, 2006; Uinoiseau et al., 2010). In addition, eugenol, a component of *Ocimum* has antibacterial and antihelmintic activities (Adebolu and Oladimeji, 2005). Components of *Ocimum basilicum* like apigenin, linalool and ursolic acid, exhibit a broad spectrum of antiviral activity and are used as remedies for treating disorders such as viral ocular, respiratory and hepatic infections (Chiang et al., 2005). The aim of the present study, however, was to test the antibacterial activities of the methanol, aqueous, and n-hexane extracts of the leaves of *O. lamiifolium*.

MATERIALS AND METHODS

Dried and powdered leaves of *O. lamiifolium*, methanol (Reagent chemical Services Ltd., United Kingdom), n-hexane (Uni-Chem Chemical Reagents), nutrient agar (Oxoid LTD., Basingstoke, Hampshire, England), Muller-Hinton agar (Oxoid LTD., Basingstoke, Hampshire, England), sulfuric acid (SDFCL Fine Chemical Ltd., Mumbai, India), Tween 80 (Uni-Chem Chemical Reagents), sodium chloride (Nike Chemical, India), cotton swab (Nataso, India), tetracycline (Oxoid Ltd., United Kingdom), chloramphenicol (Oxoid Ltd., United Kingdom), barium chloride (BDH Chemicals Ltd. Poole, England), an autoclave (Express autoclave, Dixons surgical Ltd.), petri dishes, and distilled water (Biomedical Laboratory, Addis

Ababa University, Ethiopia).

Plant collection and identification

O. lamiifolium leaves were collected from their natural habitats Central and North East Ethiopia. The plants were not flowering during the period of collection. The collected specimens were authenticated by botanists from the National Herbarium of Addis Ababa University and voucher specimens were deposited at the same herbarium of Addis Ababa University.

Extraction of plant

Collected leaves of *O. lamiifolium* were washed by distilled water and subjected to shade drying at 25°C. Then the dried leaves were pulverized to get coarse powder. 100 g of the powder was added to 1 L (1:10, w:v) of three solvent types, namely, methanol (absolute), n-hexane (absolute), and distilled water and each mixture was shaken for 48 h at 120 rotations/min. The solutions were filtered by Whatman No. 1 filter paper. Finally, the methanol and hexane extracts were concentrated under vacuum in a rotary evaporator (Büchi Laboratoriums-Tchnik AG CH-9230 Flawil/Schweiz) to give gummy residues and the aqueous extracts using a lyophilizer (Bioblock Scientific, Illkirch Cedex, France). The crude extracts were then weighed and the yield of the each extract was calculated as 17.8, 12 and 6.7% (methanol, aqueous and n-hexane extract, respectively).

Bacterial strains

Clinical isolates of *Staphylococcus aureus*, *Shigella boydii*, *Escherichia coli*, and *Pseudomonas aeruginosa* were obtained from the Ethiopian Public Health Institute (EPHI). These isolates were screened for their susceptibility towards different doses of the different extracts of *O. lamiifolium* as well as two standard antibiotics [Tetracycline (30 μ g/disk) and Chloramphenicol (30 μ g/disk)]. In order to perform the antimicrobial screening, the bacterial isolates were cultured overnight at 37°C on Nutrient Agar medium. Colonies collected from each 24 h old bacterial culture were diluted in sterile saline and the optical density was adjusted in comparison with 0.5 McFarland' scale to prepare a standardized inoculum (1.5×10^8 cfu/ml). The bacteria from saline solutions were spread on Müller Hinton Agar plates using sterile cotton swabs.

The paper disc diffusion technique was applied to determine the antimicrobial activities of the tested plant extracts. Sterile paper discs (5 mm in diameter) immersed in stock solutions containing 25, 50 and 100 mg/ml prepared in 2% Tween 80 of plant extracts were placed on the surface of inoculated Nutrient Agar plates. Plates were then incubated for 24 h at 37°C, and diameters of the inhibition zones were recorded. All assays were applied in triplicates and the results are given as means \pm standard error of the mean.

Determination of minimum inhibitory concentration (MIC)

MIC is the lowest concentration of an antimicrobial that inhibits the visible growth of microorganisms after overnight incubation (Yilmaz, 2012). MICs were defined as the lowest concentration of the aqueous, methanol and n-hexane extracts of *O. lamiifolium* inhibiting visible growth of the bacteria. On the other hand, the MBC was defined as the lowest concentration of the extracts of *O. lamiifolium* required to kill all the test bacteria (Yilmaz, 2012). The MIC was determined using agar dilution method which is described by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and

Table 1. Minimum inhibitory concentrations (MIC) of *Ocimum lamiifolium* leaf extracts.

| Bacterial species | Gram type | MIC (mg/ml) | | |
|----------------------|-----------|------------------|-----------------|------------------|
| | | Methanol extract | Aqueous extract | n-Hexane extract |
| <i>S. boydii</i> | - | 10 | 10 | 10 |
| <i>S. aureus</i> | + | 50 | 10 | 20 |
| <i>E. coli</i> | - | 10 | 10 | 10 |
| <i>P. aeruginosa</i> | - | 10 | 10 | 15 |

Infectious Diseases (ESCMID, 2000). The following procedure was followed to determine the MIC; 20 ml agar was used in 9-cm Petri dishes for agar dilution. Nineteen-milliliters molten agar was added to 1 ml of each plant extract to make the total volume 20 ml. Müller Hinton agar was prepared as recommended by the manufacturer. The sterilized agar was set to cool to 50°C in a water-bath. Extracts of *O. lamiifolium* were prepared into doses of 5, 10, 15, 20, 25, 50 and 100 mg/ml in 25 to 30 ml containers. Nineteen-milliliters of molten agar was added to each container and mixed thoroughly, and finally poured into pre-labeled sterile Petri dishes on a level surface. The plates were allowed to dry at room temperature so that no drops of moisture remain on the surface of the agar.

Bacterial suspensions were prepared in 0.85% normal saline and were standardized by 0.5 McFarland standards to 1.5×10^8 colony forming units (CFU)/ml. The inocula were inoculated on the dry plates. The inoculum spots were then allowed to dry at room temperature before inverting the plates for incubation. Finally, the plates were incubated at 37°C in air for 18 h. The MIC (the lowest concentration of the extracts that completely inhibited visible growth) was judged by the naked eye.

Determination of susceptibility test of bacteria towards standard antibiotics and their combinations with *O. lamiifolium* extracts

Susceptibility of the test bacteria towards Chloramphenicol, Tetracycline and their combinations with the leaf extracts of *O. lamiifolium* was determined according to the classification indicated by Bauer et al. (1966). Based on this literature, inhibition zones due to Chloramphenicol (30 µg) can be classified as resistant (≤ 12 mm), intermediate (13 to 17 mm), and sensitive (≥ 18 mm) and zones of inhibition for Tetracycline (30 µg) are interpreted as resistant (≤ 14 mm), intermediate (15 to 18 mm), and sensitive (≥ 19 mm).

RESULTS

Antibacterial activities of *O. lamiifolium* leaf extracts

The methanol extract inhibited the test bacteria in a dose dependent manner (Figure 1). At 25 mg/ml, it did not inhibit *S. aureus* while the rest bacteria were inhibited by this dose with inhibition zones just below 10 mm. The 50 mg/ml concentration of the methanol extract on the other hand inhibited all the bacteria with mean inhibition zones ranging from 6 mm (*S. aureus*) to over 10 mm (*E. coli*). At 100 mg/ml concentration, the methanol extract inhibited three of the test bacteria with mean inhibition zones above 10 mm and *S. aureus* with mean inhibition zone close to 10 mm. The antibacterial activity of the methanol

extract was generally lower than that of Tetracycline and Chloramphenicol. However, it was better than Chloramphenicol in inhibiting *S. aureus* and *S. boydii*. The aqueous extract inhibited all the test bacteria at 25, 50 and 100 mg/ml doses minimally inhibiting *S. aureus* and *E. coli* each with mean inhibition zones of 8 mm at 25 mg/ml dose and maximally *S. boydii* (12 mm) at 100 mg/ml. The aqueous extracts too were generally less effective than Tetracycline and Chloramphenicol although Chloramphenicol resistant strains (*S. aureus* and *P. aeruginosa*) were sensitive to these extracts. Like that of the aqueous extract, the n-hexane extract inhibited all the test bacteria at the three dose levels, *S. aureus* being inhibited minimally (7 mm) at 25 mg/ml and *E. coli* being inhibited maximally (11 mm) at 100 mg/ml. In general, the trend of inhibition of the test bacteria by the three extracts of *O. lamiifolium* showed that the aqueous extract is the best followed by its methanol and n-hexane extracts, respectively.

Determination of the MIC

The MIC concentrations of *O. lamiifolium* leaf extracts ranged from 10 to 50 mg/ml (Table 1). The 50 mg/ml concentration of its methanol extract inhibited all the bacteria and its 10 mg/ml inhibited 75% of them. The aqueous extract, on the other hand, inhibited all the bacteria at a concentration of 10 mg/ml and the n-hexane extract inhibited the microorganisms with a range of MICs from 10 to 20 mg/ml of which the 20 mg/ml inhibited the entire, 15 mg/ml inhibited 75%, and 10 mg/ml inhibited 50 percent of them.

Antibacterial effects of the combinations of Tetracycline (30 µg/ml) and Chloramphenicol (30 µg/ml) with *O. lamiifolium* leaf extracts at 100 mg/ml

Chloramphenicol (30 µg) resulted in inhibition zones of 31 and 33 mms in *S. boydii* and *E. coli*, respectively (Figure 2). On the contrary, it did not inhibit *S. aureus* and *P. aeruginosa*. Tetracycline (30 µg) inhibited *S. aureus* with inhibition zone of 11 mm and the rest bacteria with inhibition zones above 19 mm. Combination of these antibiotics to *O. lamiifolium* extracts at a dose of 100

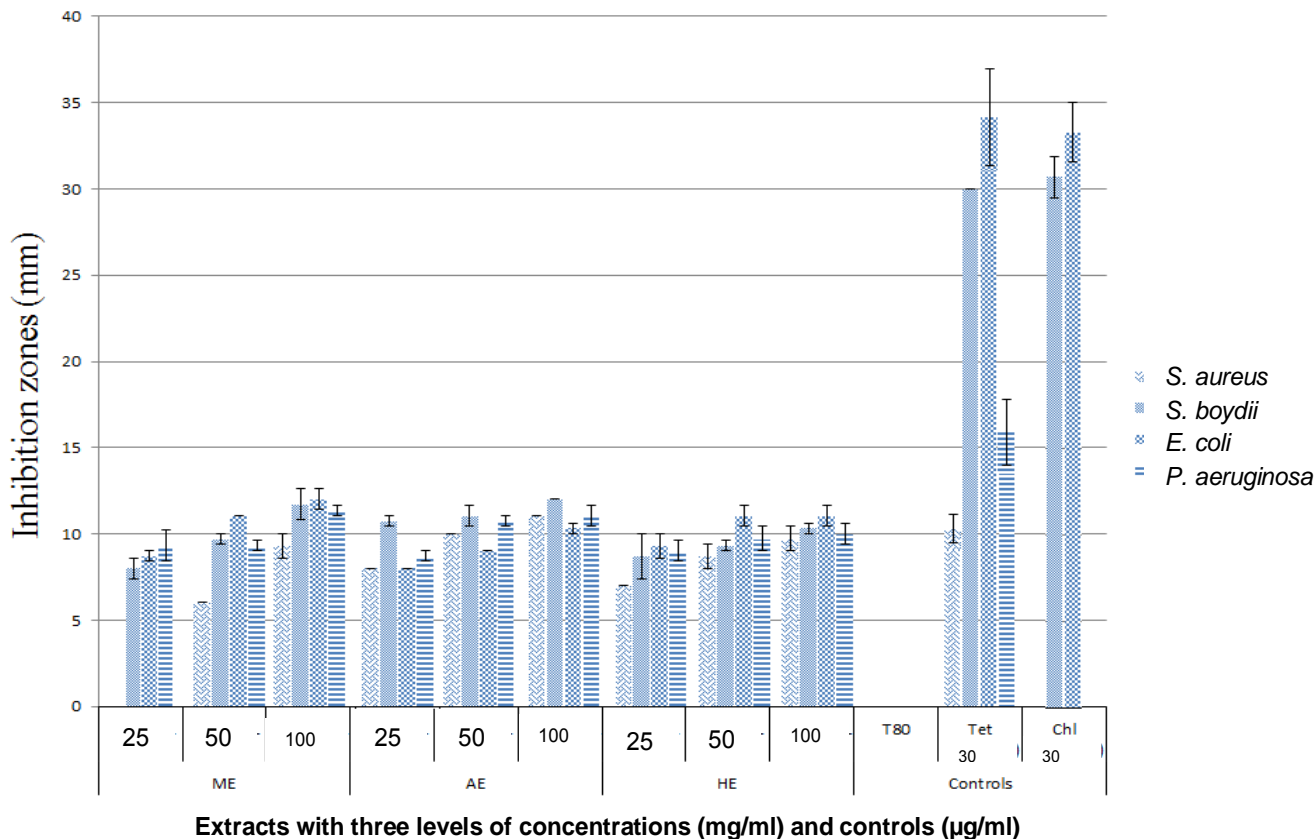


Figure 1. Inhibition zone (mm) of *O. lamiifolium* leaf extracts against Gram positive and negative bacteria. Tet: Tetracycline; Chl: Chloramphenicol; T80: Tween 80; ME: Methanol extract; AE: Aqueous extract; HE: n-hexane extract.

mg/ml, however, increased the inhibition zones of the test bacteria. Inhibition of *S. boydii* and *E. coli* due to Chloramphenicol surpassed inhibition due to the combination of Chloramphenicol and plant extracts (100 mg/ml) (Figure 2). On the contrary, inhibition of *S. aureus* and *P. aeruginosa* was found to be higher than either caused by Chloramphenicol or extracts. In the same manner, inhibition of *S. boydii* and *E. coli* by Tetracycline were higher than inhibition by combination of plant extracts (100 mg/ml) and Tetracycline while *S. aureus* and *P. aeruginosa* were more sensitive to the combinations than individual parts. Generally, combining standard drugs with plant extracts boosted the inhibition of *S. aureus* and *P. aeruginosa* than that of either the drugs or the extracts.

Differences in zones of inhibition among the methanol, aqueous, and n-hexane extracts of *Ocimum lamiifolium* at concentrations of 100 mg/ml

Differences in inhibition zones due to the different extracts of *O. lamiifolium* against the test bacteria are presented in Table 2.

DISCUSSION

Inhibition was concentration dependent in all of the bacteria with *S. boydii* being the most sensitive bacterium followed by *E. coli*, *P. aeruginosa* and *S. aureus* in decreasing order of sensitivity. Similar findings were demonstrated in a study by Gebrehiwot and Unakal (2013) where *E. coli* was the most sensitive followed by *S. aureus* and *P. aeruginosa*, respectively to the aqueous and ethanol extracts of *O. lamiifolium*. This antibacterial activity may be due to the occurrence of antibacterial active components like eugenol and sabinene in the extracts (Adebolu and Oladimeji, 2005; Wiart, 2006; Uinoiseau et al., 2010). In the present study, *S. aureus* and *P. aeruginosa* were found to be resistant to Chloramphenicol may be due to the ability of these bacteria to inactivate Chloramphenicol by enzymes coded by the *cat* genes or the ability of *P. aeruginosa* to inactivate Chloramphenicol by Chloramphenicol acetyltransferase enzyme and decreased outer membrane permeability or active efflux of this drug (Byarugaba, 2009). *S. aureus* was resistant to tetracycline may be due to its abilities like active efflux of the antibiotic and ribosome protection or modification of

Table 2. Inhibition zones of *Ocimum lamiifolium* extracts at different concentrations.

| Extract | Concentration | Mean inhibition zone (mm) ± SEM | | | |
|----------|---------------|---|----------------------------------|----------------------------------|------------------------------------|
| | | <i>S. aureus</i> | <i>S. boydii</i> | <i>E. coli</i> | <i>P. aeruginosa</i> |
| ME | 25 | 0.00 ± 0.00 | 8.00 ± 0.58 ^{*d} | 8.67 ± 0.33 ^{*d} | 9.33 ± 0.88 ^{*d,e} |
| | 50 | 6.00 ± 0.00 ^{*d,e} | 9.67 ± 0.33 ^{*d} | 11.00 ± 0.00 ^{*d} | 9.33 ± 0.33 ^{*d,e} |
| | 100 | 9.33 ± 0.67 ^{*d,e} | 11.67 ± 0.88 ^{*d} | 12.00 ± 0.58 ^{*d} | 11.33 ± 0.33 ^{*d,e} |
| AE | 25 | 8.00 ± 0.00 ^{*a,d,e} | 10.67 ± 0.33 ^{*d} | 8.00 ± 0.00 ^{*d} | 8.67 ± 0.33 ^{*d,e} |
| | 50 | 10.00 ± 0.00 ^{*a,d,e} | 11.00 ± 0.58 ^{*d} | 9.00 ± 0.00 ^{*d} | 10.67 ± 0.33 ^{*d,e} |
| | 100 | 11.00 ± 0.00 ^{*d,e} | 12.00 ± 0.00 ^{*d} | 10.33 ± 0.33 ^{*d} | 11.00 ± 0.58 ^{*d,e} |
| HE | 25 | 7.00 ± 0.00 ^{*a,d,e} | 8.67 ± 1.33 ^{*d} | 9.33 ± 0.67 ^{*d} | 9.00 ± 0.58 ^{*d,e} |
| | 50 | 8.67 ± 0.67 ^{*d,e} | 9.33 ± 0.33 ^{*d} | 11.00 ± 0.58 ^{*d} | 9.67 ± 0.67 ^{*d,e} |
| | 100 | 9.67 ± 0.67 ^{*d,e} | 10.33 ± 0.33 ^{*d} | 11.00 ± 0.58 ^{*d} | 10.00 ± 0.58 ^{*d,e} |
| Controls | Tet | [#] 11.33 ± 0.88 ^{*a,b,c,d,e} | 30.00 ± 0.00 ^{*a,b,c,d} | 34.00 ± 3.06 ^{*a,b,c,d} | 16.00 ± 2.08 ^{*a,b,c,d,e} |
| | Chl | 0.00 ± 0.00 | 30.67 ± 1.20 ^{*a,b,c,d} | 33.33 ± 1.67 ^{*a,b,c,d} | 0.00 ± 0.00 |
| | T80 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |

ME: Methanolic extract; AE: aqueous extract; HE: n-hexane extract; ^aME; ^bAE; ^cHE; ^dT80; ^eChl; T80: Tween 80; Tet: Tetracycline (30 µg/disk); Chl: Chloramphenicol (30 µg/disk); *Significantly higher inhibition than; [#]Not significantly different from b and c at 50 mg/ml and a, b and c at 100 mg/ml extracts.

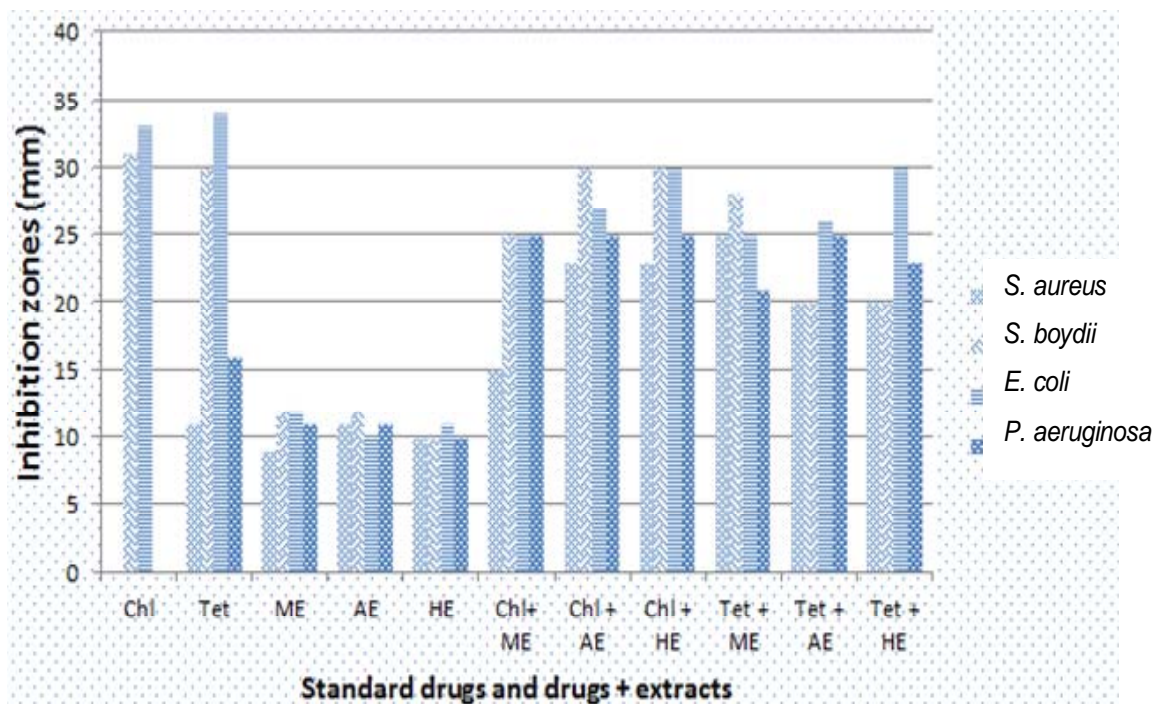


Figure 2. Antibacterial activities of the combinations of Tetracycline (30 µg) and Chloramphenicol (30 µg) with *O. lamiifolium* extracts with dose levels of 100 mg/ml. Tet: Tetracycline; Chl: Chloramphenicol; ME: Methanol extract; AE: Aqueous extract; HE: n-hexane extract.

the antibiotic (Byarugaba, 2009). All the extracts of *O. lamiifolium* showed better inhibition than the negative control (Tween 80) and were less effective than

Tetracycline and Chloramphenicol. However, the leaf extracts were more effective than Chloramphenicol against *S. aureus* and *P. aeruginosa*. On the other hand,

these extracts were less effective than Chloramphenicol against *S. boydii* and *E. coli*. Application of the aqueous extract inhibited even the most resistant bacterium (*S. aureus*) at the lowest concentration (25 mg/ml).

E. coli and *S. boydii* were the most sensitive to the extracts followed by *P. aeruginosa* and the least sensitive of all was *S. aureus* showing that the Gram negative bacteria were more sensitive to the plant extracts than the Gram positive one (*S. aureus*). The results confirmed that *O. lamiifolium* extracts are important to inhibit *S. boydii* and *E. coli*, followed by *P. aeruginosa*, and least effective against *S. aureus*. The aqueous extract of *O. lamiifolium* seem to be effective than its methanol and n-hexane extracts against these bacteria. This result clearly distinguishes the importance of the aqueous extract which contains the most effective components to inhibit bacterial growth contradicting to the finding by Goyal and Kaushik (2011) where the methanolic extract of *Ocimum sanctum* L. showed comparatively higher activity than other organic and aqueous extracts. On the other hand, this result agrees with the work of Gebrehiwot and Unakal (2013) where the aqueous extract was found to be more effective than its ethanol extract against *S. aureus*, *E. coli* and *P. aeruginosa*. Generally, differences in activities of *O. lamiifolium* extracts may be due to the differences in their chemical compositions which are determined by different factors such as climate, plant nutrition, stress (Carson and Hammer, 2011), fertilizer application (Duke, 2009), plant organs used, plant developmental stage, plant origin, chemotypes, and methods used (Zuzarte et al., 2011).

Sometimes, the effectiveness of antibiotics can be increased by coupling them with plant extracts (Kekuda, 2012). In the present study, combination of Tetracycline (30 µg/disc) to the methanol extract of *O. lamiifolium* (100 mg/ml) increased the sensitivity of *S. aureus*. On the other hand, combination of Chloramphenicol (30 µg/disc) with all the extracts increased the sensitivity of *S. aureus* and *P. aeruginosa*. The implication of this finding is that the use of plant extracts in combination with less effective antibiotics can increase the susceptibility of bacteria to these antibiotics and can be solutions to bacterial resistance to antibiotics.

The majority of the dosages of all the extracts of *O. lamiifolium* inhibited the test bacteria with inhibition zones significantly higher than that of tween 80. The positive control (Tetracycline) on the other hand inhibited all the bacteria with inhibition zones significantly higher than all the extracts except the 50 mg/ml and the 100 mg/ml concentrations of the methanol, aqueous, and n-hexane extracts of *O. lamiifolium* which resulted in inhibition zones on *S. aureus* which were not significantly different from that exerted by Tetracycline. This leads to the conclusion that the aqueous, methanol and n-hexane extracts of *O. lamiifolium* have comparable activities with Tetracycline against *S. aureus*.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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