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Antibacterial activity of the methanol, aqueous and n-hexane extracts of *Lippia adoensis*

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The fresh leaves of the wild plant (*Lippia adoensis* var. *adoensis*) are used for washing wooden and ceramic utensils to give fresh and clean smell. The dried leaves of the cultivated variety, *L. adoensis* var. *koseret*, are also used to flavor butter often added to flavor 'kitfo' (minced meat) dish. The dried leaves powdered together with barley are eaten to get relief from stomach complaints. These properties of the plant may be associated with its antibacterial and antioxidant activities. Leaves of *L. adoensis* collected from the wild habitats were shade dried and ground into fine powder. Powder was extracted using the solvents by mixing them in (1:10 w/v) ratio and shaking for 48 h. After filtering, the methanol and n-hexane extracts were dried over a rotary evaporator and the aqueous extract in a lyophilizer. The extracts were then made in concentrations of 25, 50 and 100 mg/mL by Tween 80 (T-80) (2%). Sterilized disks impregnated by these extracts were applied on cultures of bacteria (*Shigella boydii*, *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*) incubated overnight and inhibition zones measured. The aqueous extract inhibited all the test bacteria at lower doses followed by the methanolic and n-hexane extracts and *S. aureus* was found to be more resistant to the plant extracts than the rest of the test bacteria. The methanol, aqueous and n-hexane extracts showed significantly higher levels of inhibition zones with increases in concentration when compared with the negative control (T-80). The positive controls (tetracycline and chloramphenicol) also showed significantly higher inhibition zones than the 100 mg/mL concentrations of the extracts and T-80 except that chloramphenicol failed to inhibit *S. aureus* and *P. aeruginosa.* However, the resistance of these bacteria against chloramphenicol was curved by mixing them with plant extracts. The aqueous extract was the best followed by the methanol and n-hexane extracts in decreasing order. *Sh. boydii* was the most, *S. aureus* the least sensitive bacteria towards leaf extracts of *L. adoensis*. Combination of leaf extracts to antibiotics increased the sensitivity of *S. aureus* and *P.aeruginos* which were more resistant to the antibiotics.

Key words: Ethiopia, *Lippia adoensis*, inhibition zone, methanolic extract, aqueous extract, n-hexane extract, antibacterial activity.

INTRODUCTION

Lippia adoensis (Verbenaceae) is endemic to Ethiopia and Eritrea. It usually grows in disturbed areas and forest margins in the afromontane regions between altitudes of 1600 and 2650 m. The fresh leaves of the wild plant (*L.adoensis* var. *adoensis*) are used for washing wooden and ceramic utensils to give fresh and clean smell. The dried leaves of the cultivated variety, *L. adoensis* var. *koseret* Sebsebe, are used to flavor butter often added to

flavor 'kitfo' (minced meat) dish. The dried leaves powdered together with barley are eaten to get relief from stomach complaints (Asfaw and Demissew, 2009). These properties of the plant are associated with its antibacterial and antioxidant activities.

The chemical composition of the essential oil of the wild and the cultivated varieties of *L. adoensis* contains l-linalool, d-limonene, perillaldehyde, piperitenone, citral α, sesquiterpene hydrocarbons, germacrene D, α-copaene, β-cadinene α- and β-caryophyllene (Abegaz et al., 1993). Linalool is responsible for antifungal (Ahmad et al., 2006; Cseke et al., 2006), antibacterial (Ahmad et al., 2006), antilieshmanial (Ahmad et al., 2006), antimicrobial, spasmolytic and anesthetic (Peter, 2004) activities. Similarly, perillaldehyde has antimicrobial activity (Cseke et al., 2006) and citral has antimicrobial (Ahmad et al., 2006) nature by desrupting cell membranes. Thus, the objective of the present work was to test the antibacterial activity of the aqueous, n-hexane and methanol extracts of the wild variety of *L. adoensis.*

MATERIALS AND METHODS

Dried and powdered leaves of *L. adoensis,* methanol (Reagent chemical Services Ltd., United Kingdom), n-hexane (Uni-Chem Chemical Reagents), nutrient agar (Oxoid LTD., Bsingstoke, Hampshire, England), Müller-Hinton agar (Oxoid LTD., Bsingstoke, Hampshire, England), sulfuric acid (SDFCL Fine Chemical Ltd., Mumbai, India), T-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) were the materials used.

Plant collection and identification

L.adoensis leaves were collected from plants growing in their natural habitats from South Wollo and North Shewa (Central and North East Ethiopia) since these species are common in this floristic region (Asfaw and Demissew, 2009). The collection was done during June 2013. The collected specimens were authenticated by experts from Natural Herbarium of Addis Ababa University. After identification, voucher specimens were deposited at Natural Herbarium of Addis Ababa University with the voucher number (dd_3/2013).

Extraction of plant materials

Leaves of these plants were washed by distilled water and subjected to shade drying in order to avoid loss of volatile compounds by direct sun light. Then, the shade dried leaves were pulverized using a blender and passed through sieve size of 0.6 mm in diameter to get fine powder. One hundred grams of the powder was added to 1 L (1:10, w:v) of three solvent types which were: methanol (absolute), n-hexane (absolute) and distilled water, and each mixture was kept in a shaker for 48 h. Then, the solutions were filtered by Whatman no. 1 filter papers. Finally, the methanol and n-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 lyophylizer (Bioblock Scientific, Illkirch Cedex, France). The crude extracts were then weighed and the yield of each extract was calculated and found to be 14.2, 13.6 and 2% (methanol, aqueous and n-hexane extracts respectively). The extracts were dissolved in T-80 (2%) and distilled water (98%) (Kongcaharoensuntorn et al., 2007) before any antimicrobial test.

Bacterial strains

Staphylococcus aureus, *Escherichia coli, Pseudomonas aeruginosa* and *Shigella boydii* obtained from the Ethiopian Food and Nutrition Research Institute (EFNRI) were screened for their susceptibility towards different doses of the different extracts of *L. adoensis* as well as two standard antibiotics [tetracycline (30 µg/disk) and chloramphenicol (30 µg/disk)]. In order to perform the antimicrobial screening, the bacterial isolates were cultured overnight at 37°C on Nutrient agar. Colonies collected from each twenty-four hours 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 x 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% T-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 means were calculated using IBM SPSS statistics package 20.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (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 Infectious Diseases (ESCMID, 2000). The following procedure was followed to determine the MIC: twenty-milliliter volumes of agar were used in 9-cm Petri dishes for agar dilution MICs and Nineteen-mL volumes of molten agar were added to 1-mL volumes of each plant extract to make the total volume 20 mL.

Müller-Hinton agar prepared as recommended by the manufacturer was set to cool to 50°C in a water-bath. Extracts of *L. adoensis* were prepared into doses of 5, 10, 15, 20, 25, 50 and 100 mg/mL in 25-30 mL containers. 19-mL of molten agar were 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 to avoid drops of moisture 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 x 10^8 colony

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Table 1. Antibacterial activities of leaf extracts of *L. adoensis*.

Plant extract	Concentrations	Diameter of inhibition zone (mm)			
		Gram positive bacteria	Gram negative bacteria		
		S. aureus	*Sh. boydii*	*E. coli*	*P. aeruginosa*
ME	25 mg/mL	-	-	10.7± 0.7	-
	50 mg/mL	4.0 ± 2.0	12.3 ± 0.7	10.3± 0.3	6.0 ± 0.0
	100 mg/mL	6.0 ± 0.0	12.7 ± 0.3	10.3± 0.3	11.0 ± 1.0
AE	25 mg/mL	10.0 ± 0.0	10.0 ± 0.0	10.0±0.0	8.7 ± 0.7
	50 mg/mL	10.0 ± 0.0	10.0 ± 0.0	10.3 ± 0.3	9.3 ± 0.3
	100 mg/mL	10.3 ± 0.3	10.3 ± 0.3	11.0 ± 0.6	10.3 ± 0.3
HE	25 mg/mL	-	-	7.3 ± 1.3	-
	50 mg/mL	-	9.0±0.0	8.7 ± 0.9	-
	100 mg/mL	9.0±0.0	9.0±0.0	9.0 ± 0.6	8.0 ± 0.0
Controls	T80	-	-	-	-
	Tet (30 µg/mL)	10.3 ± 0.9	30.0 ± 0.0	34.0 ± 3.1	16.0 ± 2.1
	C (30 µg/mL)	-	30.7 ± 1.2	33.3 ± 1.7	-

ME= Methanol extract; AE = Aqueous extract; HE = n-hexane extract; (-) = No inhibition; T= Tween 80; C = Chloramphenicol.

Table 2. Minimum inhibitory and bactericidal concentrations (MIC/MBC) of *L. adoensis* leaf extracts.

Bacterial species	Gram type	MIC (MBC) (mg/mL)		
		ME	AE	HE
Sh. boydii	-	20 (25)	10 (15)	20 (25)
S. aureus	+	20 (25)	10 (15)	100 (100)
E. coli	-	20 (25)	10 (15)	15 (20)
P. aeruginosa	-	20 (25)	10 (15)	100 (100)

ME= Methanol extract; AE= Aqueous extract; HE= n-hexane extract.

forming units (CFU) per mL. Plates were inoculated during 30 min with standard inoculum, to avoid changes in inoculum density. The inocula (bacterial suspensions) (1 mL ≅ 10^4 CFU) 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.

Statistical analysis

The data are expressed as the mean ± SEM for each group. The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered significant at $p < 0.05$.

RESULTS

Determination of the antibacterial activities of the leaf extracts of *L. adoensis*

The antibacterial activities of the extracts are presented in Table 1. The aqueous extract showed the strongest inhibition (11 mm diameter) against *E. coli* and for the rest test bacteria at 100 mg/mL concentration (10.3 mm diameter). Although weaker than the aqueous extract, the methanol extract inhibited all the test bacteria at concentrations >50 mg/mL. The n-hexane extract how-ever was found to be the weakest extract inhibiting all the test bacteria at 100 mg/mL concentration. *E. coli* was the most susceptible bacterium followed by *Sh. boydii* and the least was *S. aureus*. The positive control, tetracycline inhibited all the test bacteria and chloramphenicol inhibited only *Sh. boydii* and *E. coli*. *P. aeruginosa* and *S. aureus* which were less inhibited by the *L. adoensis* leaf extracts also were found to be resistant to chloramphenicol. The negative control (2% T-80) however did not inhibit any of the bacteria.

Determination of the MIC and minimum bactericidal concentration (MBC) of *L. adoensis* leaf extracts against the test bacteria

The MIC/MBC concentrations of *L. adoensis* leaf extracts against the test bacteria are shown in Table 2. The concentration 10/15 mg/mL was found to be the least and 100/100 mg/mL the highest MIC/MBC exerted due to the aqueous and n-hexane extracts respectively,

Table 3. Antibacterial activities of the mixtures of tetracycline (30 µg) and chloramphenicol (30 µg) with leaf extracts at 100 mg/mL.

Tetracycline, chloramphenicol and their mixtures with 100 md/mL leaf extracts	Mean zones of inhibition of each extract on bacteria (mm)			
	S. aureus	*Sh. boydii*	*E. coli*	*P. aeruginosa*
ME	6	12.7	10.3	11
AE	10.3	10.3	11	10.3
HE	9	9	9	8
Chloramphenicol	-	29.7	33.3	-
Tetracycline	11.3	30	34	16
Tetracycline + ME	20	20	30	25
Tetracycline + AE	20	15	20	25
Tetracycline + HE	15	20	20	20
Chloramphenicol +ME	21	30	30	22
Chloramphenicol + AE	20	28	25	22
Chloramphenicol + HE	20	30	30	25

suggesting the strong antibacterial activity of the aqueous extract when compared with the methanol and n-hexane extracts. The methanol extract inhibited bacterial growth and survival at 20/25 mg/mL for all the tested bacteria, the aqueous extract at 10/15 mg/mL and the n-hexane extract in a range of 15/20 mg/mL (*E. coli*) to 100/100 mg/mL (*S. aureus* and *P. aeruginosa*).

Antibacterial activities of *L. adoensis* leaf extracts in combination with standard antibiotics

As shown in Table 3 the results of the mixtures of tetracycline (30 µg/mL), chloramphenicol (30 µg/mL) and *L. adoensis* leaf extracts (100 mg/mL) resulted in mean inhibition zones greater than those resulting from the antibiotics. The minimum inhibition zones raised from zero (in *S. aureus* and *P. aeruginosa* due to chloramphenicol alone) to 20 mm (in *S. aureus* due to combination of chloramphenicol with the aqueous and n-hexane extracts). The combination of tetracycline with plant extracts too raised the minimum inhibition zones from 11.3 mm (tetracycline alone against *S. aureus*) to 15 mm (in *S. aureus* and *Sh. boydii* due to combinations of tetracycline with n-hexane and aqueous extracts, respectively).

Differences in zones of inhibition among the methanol, aqueous and n-hexane extracts of *Lippia adoensis* leaves at concentrations of 10 mg/mL

The methanolic, aqueous and n-hexane leaf extracts of *L. adoensis* were tested for their antibacterial activities against *Sh. boydii*, *S. aureus*, *E. coli* and *P. aeruginosa* (Table 4). The methanolic extract concentrations of 50 and 100 mg/mL inhibited *Sh. boydii*, with mean inhibition zones significantly higher than the aqueous and n-hexane extracts as well as the negative control (T-80). It also inhibited *S. aureus* with significantly higher inhibition zone as compared to the n-hexane extract and *P. aeruginosa* with zone of inhibition significantly higher than that of the aqueous and n-hexane extracts. The aqueous extract of *L. adoensis* leaves significantly inhibited all the bacteria at concentrations of 25 and 50 mg/mL as compared to the n-hexane extract. The n-hexane extract was found to be the least effective of all except that it significantly inhibited *S. aureus* at 100 mg/mL than that of the methanol extract. Tetracycline showed significantly higher inhibition zones than those exerted by the leaf extracts and the negative control except the aqueous extract of *L. adoensis* against *S. aureus*. Thus, the aqueous extract with the tested concentrations was strong enough to control *S. aureus* with some com-parison with tetracycline. Tetracycline also resulted in significantly higher inhibition zones towards *S. aureus* and *P. aeruginosa* than that exerted by chloramphenicol. On the other hand, chloramphenicol showed significantly higher inhibition zones towards *Sh. boydii* and *E. coli* only when compared with all the plant extracts and T-80. Finally T-80 was found to inhibit none of the test bacteria.

DISCUSSION

The percentage inhibition of bacteria using the extracts was concentration, extract type and Gram type dependent. The aqueous extracts were the best and the n-hexane extracts the least to inhibit the bacteria. This agrees with the finding by Henao et al. (2011) in which the aqueous extracts of *L. alba* were found to inhibit 100% of three cultures of *Helicobacter pylori* at concentrations of 0.5, 5, 25, 50 and 100 mg/mL. Furthermore, inhibition was concentration dependent in all of the bacteria with *S Sh. boydii*, being the most sensitive bacterium followed by *E. coli*, *P. aeruginosa* and *S. aureus* in decreasing order of susceptibility. Most of these bacteria were also found to be susceptible to the methanolic extract of *Lippia citriodora* (Akroum et al., 2009). Application of the aqueous extracts *L. adoensis* leaves

Table 4. Inhibition zones of *L. adoensis* extracts at different concentrations.

Extract	Concentration	Mean inhibition zone (mm) ± SEM			
		Sh. boydii	***S. aureus***	***E. coli***	***P. aeruginosa***
ME	25	0.00±0.00	0.00±0.00	10.67±0.67*^{d}	0.00±0.00
	50	12.33±0.66*^{bcd}	4.00±2.00*^{cd}	10.33±0.33*^{d}	6.00±0.00*^{bc}
	100	12.67±0.33*^{bcd}	6.00±0.00*^{d}	10.33±0.33*^{d}	11.00±1.00*^{d}
AE	25	10.00±0.00*^{cd}	10.00±0.00*^{cd}	10.00±0.00*^{d}	8.67±0.67*^{cd}
	50	10.00±0.00*^{d}	10.00±0.00*^{cd}	10.33±0.33*^{d}	9.33±0.33*^{cd}
	100	10.33±0.33*^{d}	10.33±0.33*^{d}	11.00±0.58*^{d}	10.33±0.33*^{d}
HE	25	0.00±0.00	0.00±0.00	8.67±0.88*^{d}	0.00±0.00
	50	9.00±0.00*^{d}	0.00±0.00	9.00±0.58*^{d}	0.00±0.00
	100	9.00±0.00*^{d}	9.00±0.00*^{ad}	9.33±0.33*^{d}	8.00±0.00*^{d}
Controls	Tet	30.00±00*^{bcd}	#10.33±0.88*^{bcde}	34.00±3.06*^{bcd}	16.00±2.08*^{bcde}
	Chl	30.67±1.20*^{bcd}	0.00±0.00	33.33±1.67*^{bcd}	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; [a] ME of *L. adoensis;* [b] AE of *L. adoensis;* [c] HE of *L. adoensis;* [d] T-80; [e] chloramphenicol; T-80 = Tween 80; Tet = tetracycline; Chl = chloramphenicol; *= Significantly higher inhibition than; # = Not significantly different from b at 50 and 100 mg/mL extracts.

inhibited even the most resistant bacterium (*S. aureus*) at low concentrations, 10 mg/mL. This is agreeable with the mechanisms of application of these plants by traditional healers in Ethiopia, who use water extracts to treat different ailments. The bacteria (*E. coli* and *Sh. boydii*) were the most susceptible to the extracts followed by *P. aeruginosa* while the least susceptible of all was *S. aureus* showing that the Gram-negative bacteria were more susceptible to the extracts as compared to the Gram-positive one.

Unlike the effectiveness of tetracycline in inhibiting all the test bacteria, chloramphenicol was not able to inhibit the visible growth of *P. aeruginosa* and *S. aureus*. In the present study, both tetracycline and chloramphenicol were used to inhibit the test bacteria by coupling them with the methanol, aqueous and n-hexane extracts of *L. adoensis* leaves. The test bacteria showed different levels of sensitivity against the positive controls, tetracycline and chloramphenicol.

According to Bauer et al. (1966), zones of inhibition for chloramphenicol (30 µg) are inter-preted as follows: resistant (≤ 12 mm); intermediate (13-17 mm); and sensitive (≥ 18 mm). In the same document, zones of inhibition for tetracycline (30 µg) are interpreted as: resistant (≤ 14 mm); intermediate (15-18 mm); and sensitive (≥ 19 mm). Thus in this study, *Sh. boydii* and *E. coli* were sensitive and *S. aureus* and *P. aeruginosa* resistant to chloramphenicol. On the other hand, *S. boydii* and *E. coli* were sensitive, *P. aeruginosa* intermediate and *S. aureus* resistant to tetracycline. However, combination of these antibiotics with plant extracts increased their effect on the bacteria. In the case of chloramphenicol mixed with 100 g/mL extracts, all the bacteria became sensitive with inhibition zone ranges of 20 (*S. aureus* due to chloramphenicol mixed with the aqueous and n-hexane extracts) to 30 mm (in *Sh. boydii* and *E. coli* due to combinations of chloramphenicol and the methanolic and n-hexane extracts). Then again, combination of plant extracts with tetracycline discs (30 µg) resulted from intermediate (15 mm due to combination of tetracycline and n-hexane and aqueous extracts against *S. auereous* and *Sh boydii* respectively) to sensitivity zone sizes (the rest combinations against all the bacteria with maximum inhibition zone of 30 mm against *E. coli* due to combination of tetracycline and the methanolic extract). The implication of this finding is that use of plant extracts in combination with less effective antibiotics can increase the succeptibility of the bacteria to these antibiotics and can be solutions to bacterial resistance to antibiotics.

The methanol extract was significantly effective in inhibiting *Sh. boydii* and *S. aureus* when compared with the aqueous and n-hexane extracts. However, it was the aqueous extract which was significantly effective in inhibiting *E. coli* and *P. aeruginosa* as compared to the n-hexane extract. Generally, the aqueous extract was found to be the most effective in inhibiting the bacteria followed by the methanol and n-hexane extracts respectively. Tetracycline showed significantly higher inhibition zones than those exerted by the leaf extracts and the negative control except the aqueous *L. adoensis* against *S. aureus*. Thus, the aqueous extract with the tested concentrations was strong enough to control *S. aureus* with some comparison with tetracycline. Tetracycline also resulted in significantly higher inhibition zones towards *S. aureus* and *P. aeruginosa* than that exerted by chloramphenicol. Chloramphenicol on the other hand showed significantly higher inhibition zones towards *Sh. boydii* and *E. coli* only when compared with all the plant extracts and T-80. Finally T-80 was found to inhibit none of the test bacteria.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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