

Antimicrobial Activity of *Lippia adoensis* var. koseret Against Human Pathogenic Bacteria and Fungi

Gemechu Ameya Buli^{1,*}, Abdella Gure Duga², Engda Dessalegn³

¹Department of Medical Laboratory Science, College of Medicine and Health Sciences, Arba Minch University, Arba Minch, Ethiopia

²Wondo Genet Colleges of Forestry and Natural Resources, Hawassa University, Hawassa, Ethiopia

³Department of Chemistry, Hawassa College of Education, Hawassa, Ethiopia

Email address:

gemechuameya@gmail.com (G. A. Buli), abdellag66@yahoo.com (A. G. Duga), engidae@yahoo.com (E. Dessalegn)

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Abstract: *Background:* *Lippia adoensis* var. koseret is a well known medicinal plant endemic to Ethiopia. It has been traditionally used to treat different infectious diseases and also in food preparation as condiment. The aim of the current study was to evaluate antibacterial and antifungal activities of water, ethanol and methanol based crude extracts of *L. adoensis* var. koseret against selected human pathogenic bacteria and fungi. *Methods:* Crude extracts of *L. adoensis* var. koseret were extracted by maceration method. Disc diffusion assay of the extracts were carried out in four different concentrations against three different bacteria species (*Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli*) and two clinical isolated fungal species (*Candida albicans* and *Aspergillus flavus*) by using Kirby-Baur disk diffusion method. Agar dilution method was used to determine the minimum inhibitory concentration, the minimum bactericidal and fungicidal concentrations of the extracts against similar microorganisms. *Results:* Water-based extract of *L. adoensis* var. koseret exhibited significantly less antimicrobial activity as compared to ethanol and methanol crude extract against tested isolates of bacteria and fungi ($P < 0.05$); while, there was no significant difference between ethanol and methanol extracts. Among the tested microorganism *S. aureus*, was the most sensitive of all whereas *C. albicans* was the most resistant microorganism to alcohol based extract of *L. adoensis* var. koseret. The minimum inhibitory concentration of *L. adoensis* var. koseret ranged from 3.12 to 25mg/ml in the alcohol based extracts but it was higher in the water-based extract. The lower bactericidal concentration (5.20 mg/l) and the highest fungicidal concentration (37.50 mg/ml) were observed in methanol based extracts against *S. aureus* and *C. albicans*, respectively. *Conclusions:* Antimicrobial activity of *L. adoensis* var. koseret varies with extraction solvent and tested microorganisms.

Keywords: Antibacterial Activity, Antifungal Activity, Crude Extract, *L. adoensis* var. koseret

1. Introduction

Medicinal plants are the oldest form of healthcare known to mankind [1]. They had been used by all cultures throughout history and were essential part of the advance of modern civilization [2]. Regardless of the enormous advances observed in modern medicine, plants still play important role to in health care and considerable percent of modern pharmaceutical drugs contain plant ingredients. However, only a small proportion of medicinal plants are examined for bioactive compounds [3].

Lippia adoensis is one of the five indigenous *Lippia* species in Ethiopia where it occurs as an erect woody shrub

up to 1-3m tall. It is endemic medicinal plant and cultivated variety commonly found in home gardens in different regions of Ethiopia with altitudinal range of 1600-2200m [4]. Two varieties are recognized in Ethiopia, the wild variety (var. *adoensis*) and the cultivated variety (var. *koseret sebsebe*). *L. adoensis* var. *koseret sebsebe*, locally known as koseret, is widely grown in the central and southern highlands of the country. Traditionally, the dried leaves are used as one of the ingredients in the preparation of spiced butter [5] and also for food flavoring agent and preservative [6].

The leaves of *L. adoensis* are used in Ethiopian traditional medicine for the treatment of various skin diseases including eczema and superficial fungal infections [7]. The dried leaves powdered together with barely eaten to get relief from

stomach complaints [8]. A previous study showed that essential oils of this medicinal plant possess a significant radical scavenging property. Its free radical oxidative stress is implicated in the inhibition of pathogenesis of a variety of inflammatory diseases [9]. The chemical compositions of *L. adoensis* var. koseret, investigated so far are essential oils. Linalool is the major component, and appreciable amount of sesquiterpene hydrocarbons (germacrene, α -copaene, β -cadinene, and, β -caryophyllene) and uncommon monoterpene ketone, 2-methyl-6-methylene-2, 7-octadien-4-one (ipsdienone), were also found in the essential oil [10].

Due to the increasing incidence of antibiotic resistance in bacteria and fungi, there always is a need for new and effective therapeutic drugs [11]. This necessitates continued efforts to look for alternative antimicrobial drugs. The purpose of the current study was to evaluate antibacterial and antifungal activities of water, ethanol and methanol based crude extracts of *L. adoensis* var. koseret against selected human pathogenic bacteria and fungi.

2. Methods

2.1. Collection and Extraction of Plant Materials

Fresh leaves of *L. adoensis* var. koseret were collected from Botanical garden of Hawassa College of Education, Hawassa, South Ethiopia in October, 2013 and identified by taxonomist at the Biology Department, College of Natural Sciences of Addis Ababa University, Ethiopia. The voucher specimen was deposited at the Herbarium, Biology department Addis Ababa University. The leaves were transported by polyethylene bag followed by sprinkling water on the leaves and piercing the bag at several points in order to allow air circulation. In the laboratory, the leaves were washed three times under running tap water followed by rinsing once with sterile distilled water and then air-dried.

The leaves were then ground into fine powder using electric grinder followed by the preparation of suspensions in ethanol, methanol and water. Twenty five grams of the fine powder was suspended in 250ml of each of three solvents separately in sterilized screw capped 500ml glass beakers. The suspensions were kept in orbital shaker for 12hrs at room temperature. The suspensions were filtered using Whatman No. 1 filter paper and were evaporated to remove the solvent under vacuum in Rotary evaporator kept at 40 °C. The powdered crude extracts obtained from the three solvents were weighed and separately dissolved in distilled water to prepare the respective stock solutions of 200mg/ml and stored in deep freezer at a temperature of -20 °C until further use [12, 13].

2.2. Bacterial and Fungal Isolates

The screening for antibacterial and antifungal activities of *L. adoensis* var. koseret leaf crude extracts was carried out by using three bacterial pure cultures: *Staphylococcus aureus* (ATCC-25923), *Enterococcus faecalis* (ATCC-29212) and *Escherichia coli* (ATCC-25922) and two clinical fungal

isolates of *Candida albicans* and *Aspergillus flavus*, obtained from Ethiopia Public Health Laboratory. Mueller Hinton agar (MHA) medium and Sabouraud's dextrose agar (SDA) were used to carry out the screening for antibacterial and antifungal activity, respectively.

2.3. Disc Diffusion Assay

Suspensions of the leaf crude extracts of *L. adoensis* var. koseret obtained using the aforementioned extraction solvents were prepared in distilled water at concentrations of 10, 20, 40 and 80 mg/ml for disc diffusion assay. Diffusion discs of approximately 6mm diameter were prepared from Whatman No. 1 filter paper by using puncher and sterilized by autoclaving and dried in oven. Then 10 μ l of the extracts from each of concentrations was impregnated on sterile disc using sterile micropipette tips followed by air drying and then stored at 4°C in separate sterile containers. Then disc diffusion assay was carried out using Kirby- Baur disk diffusion method according to [14]. Gentamycin (10 μ g mlG) and ketoconazole disc (10 μ g mlG) were used as positive control for bacteria and fungi, respectively while blank discs impregnated with each solvents were used as negative control. All the tests were conducted in triplicate and the average of the three measurements was used to present the results.

2.4. Determination of Minimum Inhibitory Concentration

Agar dilutions method was used to determine the minimum inhibitory concentration (MIC) of *L. adoensis* var. koseret extracts. From 200mg/ml of stock plant extract, two fold serial dilutions was used to prepare suspensions of 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml and 1.56mg/ml concentrations in molten MHA and SDA after cooling to 45°C. The resulting suspensions were poured into sterile Petri dishes under aseptic condition and let to solidify. After the plates were solidified, they were inoculated with diluted suspension of each microorganism at 0.5 McFarland standards. The plates were then incubated at 37 °C for 24 hrs, and 27 °C for 48hrs in the case of bacteria and fungi, respectively. The minimum dilution of the leaf crude extracts completely inhibiting the growth of each organism was taken as the MIC [14].

2.5. Determination of the Minimum Bactericidal and Fungicidal Concentration

In order to determine minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC); the MIC, the concentration preceding to MIC and one more concentration between this two concentrations were used. Then, those three concentrations were adjusted in nutrient broth and Sabouraud's dextrose broth in 250ml conical flask and inoculated with the respective test microorganism in test tube and incubated at 37 °C for 24 hrs and 48hrs at 27 °C for bacteria and fungi, respectively. Then, sterile plates of MHA and SDA were inoculated by transferring a loopful of broth medium inoculated with the respective test microorganism followed by incubation at 37°C for 48hrs and at 27°C for

72hrs for bacteria and fungi, respectively. MHA and SDA plates without the leaf extract and inoculated with the respective test microorganism served as control. All the tests were done in triplicates [14].

2.6. Statistical Analysis

For each assay, all the measurements were replicated three times and the results were presented as mean \pm SD. One way ANOVA followed by Tukey's test was used to compare extraction solvents and the difference in the sensitivity of the test microorganisms using the statistical package for social sciences (SPSS) version 16 and P-value \leq 0.05 was considered as statistically significant.



Figure 1. Disc diffusion assay against *S. aureus* (The right) and agar dilution (The left) of determining antimicrobial activity of *L. adoensis* var. *koseret* against test microorganisms.

Disc diffusion assay of ethanol based extracts exhibited antimicrobial activity against all the tested organisms at 80 mg/ml. *S. aureus* exhibited antimicrobial activity at 20 mg/ml while *C. albicans* was only inhibited at the concentration of 80mg/ml while *E. coli*, *E. faecalis* and *A. flavus* were inhibited by ethanol based extract at 40mg/ml and above. In the methanol based extract, high antimicrobial activities were observed. The highest average (20.33mm) zone of inhibition

3. Results

In the disc diffusion assay of *L. adoensis* var. *koseret*, water-based extracts had antimicrobial activity only against *S. aureus* and *E. faecalis* at the concentration of 80 mg/ml. *E. coli* and both the tested fungal species (*C. albicans* and *A. flavus*) were resistant to water-based extracts at 80mg/ml. In general, water-based extract of *L. adoensis* var. *koseret* showed significantly ($p < 0.5$) lower antimicrobial activity than the ethanol and methanol based extracts (Table 1).

was observed in methanol based extract at the concentration of 80 mg/ml against *S. aureus* (Figure 1). On the other hand, more or less similar results were obtained against the other tested microorganisms. Positive and negative controls were also carried out to monitor antimicrobial activity of the medicinal plant. All negative controls showed no antimicrobial activities whereas positive gives different zone of inhibition for different microorganisms (Table 1).

Table 1. Antimicrobial activity of *L. adoensis* var. *koseret* extract obtained using three different solvent at four different concentrations.

Extraction solvents	Extract concentration	Inhibition zone (mm)				
		Bacterial species			Fungal Species	
		<i>S. aureus</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>A. flavus</i>	<i>C. albicans</i>
Water	10 mg/ml	-	-	-	-	-
	20 mg/ml	-	-	-	-	-
	40 mg/ml	-	-	-	-	-
	80 mg/ml	9.00 \pm 1.00	-	8.33 \pm 0.57	-	-
Ethanol	10 mg/ml	-	-	-	-	-
	20 mg/ml	8.33 \pm 0.57	-	-	-	-
	40 mg/ml	9.66 \pm 0.57	8.33 \pm 0.57	8.33 \pm 0.57	8.33 \pm 0.57	-
	80 mg/ml	13.00 \pm 1.00	9.00 \pm 1.00	10.33 \pm 0.57	12.00 \pm 1.00	8.66 \pm 1.15
Methanol	10 mg/ml	11.33 \pm 1.15	-	-	-	-
	20 mg/ml	13.00 \pm 1.00	-	-	-	-
	40 mg/ml	17.66 \pm 0.57	8.33 \pm 0.57	10.00 \pm 1.00	9.66 \pm 1.52	-
	80 mg/ml	20.33 \pm 0.57	11.33 \pm 1.15	13.33 \pm 0.57	12.33 \pm 0.57	8.33 \pm 0.57
Positive control		22.33 \pm 0.57	20 \pm 1.00	17.00 \pm 1.00	22.33 \pm 0.57	23.66 \pm 0.57
Negative control		-	-	-	-	-

(-) = No antimicrobial activity; Values are mean of inhibition zone (mm) \pm S.D of three replicates

In the determination of MIC against the tested microorganisms, *E. coli* and *C. albicans* were not inhibited by water extract at 100mg/ml. In the contrary, the other tested

microorganisms were inhibited at 100 mg/ml in water extract. Like in the case of disc diffusion assay, the tested microorganisms were more resistant to water-based extracts

than to the ethanol and methanol based extracts.

The MIC of ethanol based extract was 6.25mg/ml against *S. aureus* while it was 25mg/ml in the case of *E. coli* and *C. albicans*. The MIC for *E. faecalis* and *A. flavus* was 12.50mg/ml. In the methanol based extract, the MIC was

3.12 mg/ml in the case of *S. aureus* while *C. albicans* was the most resistant microorganism. In the case of the other tested microorganisms, there was no significant difference between alcoholic extracts (Table 2).

Table 2. Average minimum inhibitory concentrations, minimum bactericidal and fungicidal concentrations of *L. adoensis* var. *koseret* in three different extraction solvents.

Assay methods	Extract solvent	MIC, MBC and MFC (mg/ ml)				
		Bacterial species			Fungal species	
		<i>S. aureus</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>A. flavus</i>	<i>C. albicans</i>
MIC	Water	100.00	*	100.00	100.00	*
	Ethanol	6.25	25.00	12.50	12.50	25.00
	Methanol	3.12	12.50	12.50	12.50	25.00
MBC/MFC	Water	100.00	*	*	*	*
	Ethanol	9.37	29.17	20.83	20.83	29.17
	Methanol	5.21	22.92	20.83	18.75	37.50

(*) = No inhibitory or bactericidal/fungicidal activity at 100mg/ml, MIC=Minimum inhibition concentration, MBC=Minimum bactericidal concentration, MFC= Minimum fungicidal concentration

Like in the case of disc diffusion and MIC assay, the extracts exhibited bactericidal and fungicidal activities to varying extents. Water-based extracts had exhibited bactericidal effects only against *S. aureus* at the concentration of 100mg/ml (Table 2). The lowest MBC (5.20 mg/ml) was recorded in the case of methanol based extracts against *S. aureus* while higher values were recorded in all the tests against all other microorganisms included in the screening (Table 2).

4. Discussion

In current study, of all the extracts obtained using the three extraction solvents at four different concentrations, methanol based extracts at the concentration of 80 mg/ml exhibited the highest antimicrobial effects against all the tested microorganisms although the extent of inhibition across the microorganisms was variable. Among the tested bacterial species, *S. aureus* was the most sensitive while *E. coli* was the most resistant to the extracts obtained using all the three solvents. Likewise, from the two fungal species, *A. flavus* was more sensitive than *C. albicans* in all cases. Similar results were observed by 80% methanol extract of this medicinal plant against similar bacteria species but with a little difference [7, 15]. Among the extraction solvents, methanol was the best followed by ethanol.

One of the differences between the current study and those studies may be the concentration of methanol. In the previous two studies, 80% methanol was used while in the current study absolute methanol was used. So, the results may have been influenced due to the differences in the polarity and concentrations of the alcohol. Penetration of alcohols increases with dilution with water which in turn improves the phytochemical extraction power of the alcohol [16]. Besides, the amount of active ingredient in a plant can vary with factors like the variety of plant, the geographic location, the season and time of harvest, soil conditions, storage conditions, and the method of preparation.

Extracts in both ethanol and methanol had significantly higher antimicrobial activities against tested microbes than water extract. This is due to alcohols are more efficient in cell walls degradation which have non-polar character and cause polyphenols to be released from cells. Enzyme polyphenol oxidase is also inactivated in methanol and ethanol extract [17]. In addition, alcohols were found easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material [18] and nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds which are most often obtained through initial alcoholic extraction [19].

E. coli, Gram negative bacteria are more resistance to tested microorganism because they have an outer membrane that is made up of high density lipopolysaccharides that serves as a barrier to many environmental substances including antibiotics [20]. They also have innate multidrug resistance to many antimicrobial compounds owing to the presence of efflux pumps in meticulous [21]. But successively numerous phytochemicals were shown to act as potential efflux pump inhibitors with antimicrobials for Gram-positive bacteria [22, 23]. Hence, these are the possible reason why all selected medicinal plant showed lower antimicrobial activity than the others.

Phytochemical screening tests carried out on *L. adoensis* also indicated the presence of tannins, flavonoids, polyphenols, alkaloids and saponins which are the main antimicrobial phytochemical [15]. Mechanism of action of chemical compounds of medicinal plants mediate their effects on the microorganisms is almost the same to conventional drugs those already well understood for the chemical compounds. This indicates herbal medicines do not differ greatly from conventional drugs in terms of how they work and medicinal plants can be as effective as conventional [24].

In the current study, antimicrobial activity of *L. adoensis* var. *koseret* extract in methanol exhibited high antimicrobial

activity against *S. aureus*. At concentration of 80 mg/ml this extract showed comparable antimicrobial activity with positive control, Gentamycin (10 µg mlG) against *S. aureus* while no growth inhibition was observed in the case of all the negative controls. On the other hand this medicinal plant showed a low antimicrobial activity against *C. albicans* in all assay methods and similar finding was observed with Yared *et al.*, (2014). Generally, all three tested assay methods were support each others in current study.

Medicinal plants are commonly used in treatment of various diseases and scientific verification should be done to enhance trust of consumer and to show as they are effective for what they needed. Then this study is expected to fill knowledge gap and provide base line evidence based information about antimicrobial activity of this medicinal plant against common pathogenic and opportunistic microorganisms. In addition to this it also gives some information for traditional medicine practitioners to develop their profession that improve the quality of the health service they render. Traditional use of *L. adoensis* var. koseret by the local people in treating various types of infectious diseases specially caused by *S. aureus* was supported by this study. This study has some limitation like lack of phytochemical analysis, fractional extraction and addition pharmaceutical tests that should be carried out to confirm patient safety. Therefore, other additional studies are needed to acknowledge traditional medicine practitioners practice.

5. Conclusion

Antimicrobial activity of *L. adoensis* var. koseret differs based on the extraction solvent used, the concentration of the extract and the species of the tested microorganism. Ethanol and methanol extracts showed significant antimicrobial activity against *S. aureus* while *E. coli* and *Candida albicans* were the most resistant microorganisms in all assay methods.

Abbreviations

ATCC: American type culture collection; MBC: Minimum bactericidal concentration; MFC: Minimum fungicidal concentration; MHA: Mueller Hinton agar; MIC: Minimum inhibition concentration; SD: Standard deviation; SDA: Sabouraud's dextrose agar

Authors' Contributions

GA: participated in the design of the study, coordinate and involved in data collection and experimental work, analyzed the data and drafted the paper. AG and ED participated in the analysis and revised subsequent drafts of the paper. All authors read and approved the final manuscript.

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