

Identification of Bioactive Compound, Phytochemical Analysis and Antimicrobial Activity of *Lippia adoensis* var. *koseret* from Ethiopia

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Abstract

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 focus of the present paper was to evaluate antibacterial activities of methanol, petroleum ether, chloroform, ethyl acetate, butanol and aqueous extracts of leave of *L. adoensis* var. *koseret* against *S. aureus*, *E. coli* and *P. aeruginosa* at two concentration level (5 and 10mg/ml) and Phytochemical and Thin Layer Chromatography analysis of the extracts showed antibacterial activity. The antibacterial assay was carried out using agar well diffusion method. Broth dilution method was used to determine the minimum inhibitory concentration. Phytochemical analysis was carried out using the standard phytochemical assays. Thin Layer Chromatography analysis of the methanol, petroleum ether and ethyl acetate extracts of the leaves was carried out using the solvent system Hexane: Ethyl acetate (in 7:3 ratios). The results of antibacterial assay revealed that methanol, petroleum ether and ethyl acetate extracts active against *E. coli* and *P. aeruginosa*. Among the tested microorganism *S. aureus*, was resistant to all extracts. The phytochemical screening revealed the presence of various chemical compounds like alkaloids, flavonoids, tannins and tannins. While, carbohydrate and amino acid were absent. Thin Layer Chromatography analysis of the methanol, petroleum ether and ethyl acetate fraction of the leaves of *Lippia adoensis* var. *koseret* revealed bands indicating the presence of various compounds that could act as potential antimicrobial agents.

Keywords: Antimicrobial assay; Minimum Inhibitory Concentration; Photochemical analysis; Thin Layer Chromatography.

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1. INTRODUCTION

Ethiopia has been described as one of the most unusual and important source of biodiversity in the world (USAID 2008). Medicinal plants are the oldest form of health care known to mankind (Buli, *et al.*, 2015). According to WHO, herbal medicines serve the health needs of about 80 percent of the world's population, especially for millions of people in the vast rural areas of developing countries (WHO, 2000).

The Ethiopian flora is estimated to contain between 6500 and 7000 species of higher plants of which about 12 percent are endemic. Ethiopia is also a home for many language, culture and beliefs that has intern contributed to high diversity of traditional knowledge and practice of the people which among others include the use of medicinal plants. More than 95 percent of traditional preparations in the country are of plant origin (Gupta *et al.*, 2010; Demisew & Dagne, 2001).

Lippia adoensis is included under family Verbenaceae that is a large family with about 70 to 80 genera and over 3,000 species; distributed throughout the world mainly in the tropics and temperate regions. In Ethiopia, the family is represented by 9 genera and 30 species. *Lippia* is a genus with 200 species in tropical Africa and America (Terblanché & Kornelius, 1996). Most of these species are traditionally used in the indigenous systems of medicine for the treatment of a variety of human ailments. The majority of them have been used for the treatment of stomach ailments, cardiovascular troubles, coughs, colds and asthma, tranquillizing remedy, prevention of gastritis, headache (Mamun *et al.*, 2013; Conde *et al.*, 2011). Some of the species exhibited strong antioxidant (Ara & Nur, 2009), antidiabetic (Balamurugan & Ignacimuthu, 2011), insecticidal (Okonkwo & Ohaeri, 2013).

Two varieties are recognized in Ethiopia, the wild variety (var. *adoensis*) and the cultivated variety (var. *koseret sebsebe*). *Lippia adoensis* var. *koseret* Sebsebe (Figure 1A), locally known as *koseret*, is known in cultivation so far only in the central and southern highlands of the Ethiopia with altitudinal range 2000-2700m above sea level. Traditionally, the dried leaves are used as one of the ingredients in the preparation of spiced butter (Nigist & Sebsebe, 2009). The fragrant leaves are used by the Gurage and Oromo tribes as one of the condiments in the preparation of spiced butter. The special taste and flavor of the “Gurage Kitfo” is attributed to the oils imparted by the leaves (Akihisa *et al.*, 1992).



Fig-1: *Lippia adoensis* var. *koseret* (A) *Lippia adoensis* var. *adoensis* (B)

The leaves of *L. adoensis* var. *koseret* are used in Ethiopian traditional medicine for the treatment of various skin diseases including eczema and superficial fungal infections (Tadeg, *et al.*, 2004), also for food flavoring agent and preservative (Sishu, *et al.*, 2005). The dried leaves powdered together with barley are consumed to get relief from stomach complaints (Megersa, *et al.*, 2013). The recent study conducted against human pathogenic bacteria and fungi, indicated that the alcohol based extracts (methanol and ethanol) showed stronger antimicrobial activity than water extract of leaf of *Lippia adoensis* var. *koseret* (Buli *et al.*, 2015).

Scientific studies dealing with the pharmacological activities of this particular species is nonexistent except one study that investigated the antioxidant activity of the volatile oil and the major terpenoids therein. Scientific study proved the traditional use of *Lippia adoensis* Var. *Koseret* to retain the freshness of cooking butter and avoid rancidity, which could be associated to the radical scavenging activity. The essential oil of *L.adoensis* var. *koseret* possesses a significant radical scavenging property when assessed in the DPPH (diphenylpicrylhydrazyl) assay. As free radical oxidative stress is implicated in the pathogenesis of a variety of human diseases including inflammation, the traditional uses of the plant for the treatment of various kinds of inflammatory skin diseases is partly justified (Abegaz, *et al.*, 1993). This study is expected to fill knowledge gap and provide base line evidence based information about antimicrobial activity of this medicinal plant against

In Ethiopia, the wild variety *Lippia adoensis* var. *adoensis* (Figure 1.1B), the wild variety locally known as *kesse*, 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 (Akihisa *et al.*, 1992). It has lemon-like odour. In different parts of the country people use the leaf to flavor milk, butter and also as one of the ingredients to prepare spiced red pepper, locally known as *berebere* (Nigist & Sebsebe, 2009). And also used for washing kitchen utensils to impart fresh and spicy fragrance (Hedberg & Edwards, 1989).

common pathogenic bacteria and bioactive compounds present in this herbal medicinal plant. Further Identification of the bioactive compounds or marker compounds present in this plant would be useful for the development of the active constituent in to a drug.

2. MATERIAL AND METHODS

2.1. Collection and preparation of plant material

Fresh leaves of *L. adoensis* var. *koseret* were collected from South nations and nationalities people region of Ethiopia, Wendo Genet city in the month of July, 2018 and identified by botanist at Shashemene Botanical Garden, Ethiopia. The voucher specimen was deposited at the Herbarium, Shashemene Botanical Garden. The leaves were transported by polyethylene bag. In the laboratory, the leaves were washed three times under running tap water followed by rinsing once with distilled water and then air-dried. The leaves were then ground into fine powder using electric grinder and kept in air tight polyethylene bags until further extraction began (Yoshida, *et al.*, 2006).

2.2. Preparation of extract

2.2.1 Extraction and fraction preparation

Lippia adoensis var. *koseret* was sequentially extracted with methanol, petroleum ether, chloroform, ethyl acetate, butanol and aqueous part using soxhlet apparatus. The solvent was evaporated under reduced pressure and the fractions were then placed in vacuum oven at 40 °C to remove any residual solvent. The resulting semisolid mass of each fraction was stored until further use.

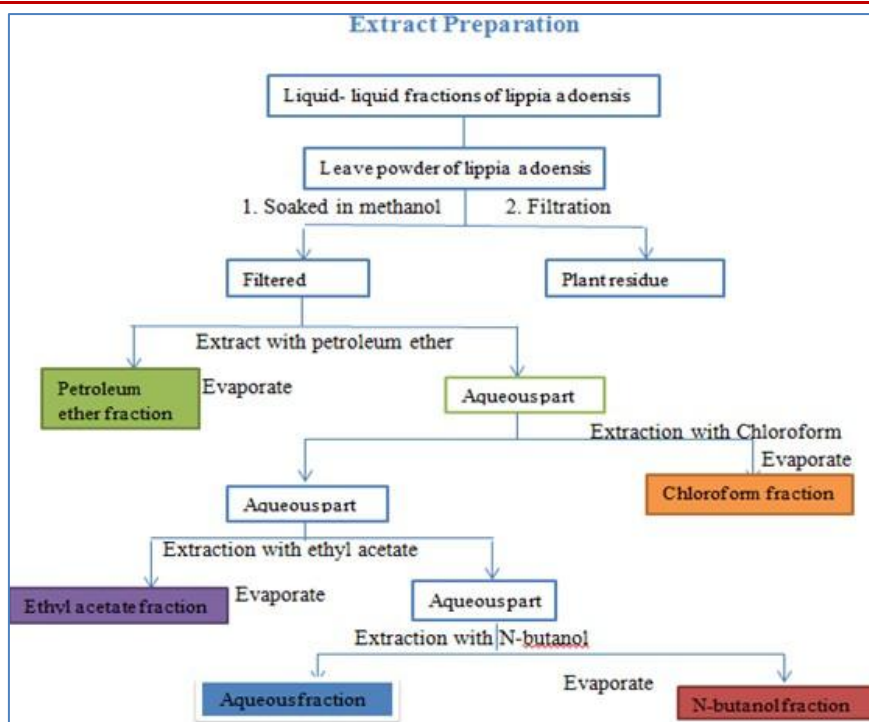


Fig-2: Extraction and fraction preparation

Suspensions of the leaf crude extracts of *L. adoensis* var. *koseret* obtained using the aforementioned extraction solvents were prepared at concentrations of (5 and 10 mg/ml) for agar well diffusion method (Boakye *et al.*, 1977). The tests were conducted at two concentration levels. The various solvent extracts thus prepared were re-extracted with DMSO and aliquoted and stored in 4°C till further use. The results are average of triplicate tests. The procedure followed for the antibacterial tests are described below.

2.3 Antimicrobial Assay

2.3.1 Growth and preservation of Test strains

The screening for antibacterial activity of *L. adoensis* var. *koseret* leaf extract was carried out by using three bacterial pure cultures: *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. The bacteria's were incubated in broth with the help of sterile inoculation loop in Laminar air flow. These pre culture broths were stand overnight in a rotary shaker at 37°C, after this cultures were maintained in freeze for further use.

2.3.2 Preparation of growth media

Nutrient agar was used for preparation of medium for growth of above said organisms. Nutrient agar (6.3 gm with 225 ml of distilled water) was taken for preparation of growth media. Prepared nutrient agar was autoclaved at 121°C and 15 lb. pressure and then poured on to petriplates under sterile conditions. After solidifying it was kept in incubator at 24 hours for checking of contamination, followed by using the plates for further antibacterial susceptibility test of the isolated strains.

2.3.3 Screening of antibacterial activity of leaf extracts

The leaf extract of *L. adoensis* var. *koseret* was determined using agar well diffusion method. DMSO was used as a negative control during the whole test on bacteria. The inoculums of the respective bacteria were streaked on to agar plates using a sterile swab 4 times with *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* in such a way as to ensure thorough coverage of the plates and a uniform thick lawn of growth following incubation. Wells of 6 mm in diameter were formed on to agar plates using a sterile corn borer. The wells were filled with extracts (50 µl) each using sterile micropipette tips and the plates were allowed to stay for 1 to 2 hours at room temperature. Finally, the plates were then incubated at 37°C for 14 to 16 hours. The resulting diameters of zones of inhibition were measured and recorded in mm. The effects were compared with that of the standard antibiotic ampicillin loaded in the well at a concentration of 10 mg/ml.

2.3.4 Determination of Minimum Inhibitory Concentration

Broth dilution method was used to determine the minimum inhibitory concentration (MIC) of 5 mg/mL stock extracts of *L. adoensis* var. *koseret* using ELISA plate. Nutrient broth was first prepared in a usual fashion and sterilized by autoclaving. 100 µl of extracts were added to the wells which contained 100 µl of nutrient broth and 10 µl of bacterial culture was added. The mixture of the nutrient broth, the extracts and bacterial culture were thoroughly mixed.

Ampicillin and DMSO was used as a positive and negative control respectively. The plates were incubated for 17 hours at 37°C. Once incubation has done 5 µl of Resazurin dye was added on to each well and left for one hour. The yellow color has changed into pink and blue colors, indicated viable and dead cells respectively. All the tests were conducted in triplicate and the average of the three measurements was used to present the results.

2.4 Qualitative estimation of phytochemicals

2.4.1 Phytochemical screening

The phytochemical research based on ethnopharmacological information is generally considered an effective approach in the discovery of new anti-infective agents from higher plants (Duraipandiyar *et al.*, 2006). The plant extracts were screened for the presence of reducing sugars, alkaloids, saponins, tannins, flavonoids, phenols, amino acid, according to established procedures (Njoku & Obi, 2009; Trease & Evans, 1989; Harborne, 1973).

Alkaloids: 1 ml of the filtrate was treated with a few drops of Mayer's reagent and another ml was similarly treated with Dragendorff's reagent. Turbidity or precipitation with both reagents was taken as preliminary evidence for the presence of alkaloids (Okwu & Okwu, 2004; Schultes, 1978).

Saponins: 2 gm of powdered sample was boiled in 20 ml of distilled water in a water-bath and filtered. 10 ml of filtrate was mixed with 5 ml of distilled water and shaken vigorously for the formation of a stable persistent froth (Njoku & Obi, 2009).

Flavonoids: 5 ml of dilute ammonia solution was added to a portion of aqueous filtrate of plant extract followed by addition of concentrated H₂SO₄. The development of yellow color was taken as an indication of the presence of flavonoid (Harborne, 1998).

Tannins: 0.5 g crude extract was stirred with 10 ml of distilled water and filter. The addition of FeCl₃ reagent to the filtrate resulting brownish green or blue black color was taken as evidence for the presence of tannins (Usman & Osuji, 2007).

Phenols: for 2 ml of extract 3 drops of a mixture of 1 ml 1% FeCl₃ and 1 ml 1% K₃Fe(CN)₆ were added. Formation of green blue color was taken as an indication of the presence of Polyphenols (Rasool, *et al.*, 2010; Okwu & Okwu, 2004).

Reducing sugars: To 0.5 ml of extract was stirred with 1 ml of water and addition of 5-8 drops of Fehling solution was added at hot and brick red precipitate were observed.

Amino acid/ protein: 5 ml of extract was added to Ninhydrin reagent and boiled in water bath and formation of purple color was taken as an indication for the presence of amino acid.

2.5 TLC analysis

2.5.1 Preparation of TLC plate

TLC is a very convenient and simple way of screening plant (Moreira, *et al.*, 2005) and can be successfully employed in the target directed isolation of active compounds. The plates for TLC were prepared with silica gel G (2:1 ratio) and dried in an oven at 110°C for activation. After activation and drying of plates spotting of plates was carried out.

2.5.2 Spotting of plate

10 µl of the test solution was applied twice on a pre coated TLC plate and developed in the solvent system in a twin trough chamber. The spot made by capillary should not be more than 2 mm in diameter.

2.5.3 Solvent preparation

The mobile phase in required ratio was put into the TLC chamber and allowed to saturate for 45 minutes. TLC plate was placed in TLC glass chamber and allowed the mobile phase to move through adsorbent phase up to a sufficient distance.

2.5.4 Chromatogram analysis

The plate developed chromatogram was visualized in visible and UV light (254 nm and 365 nm). The resolution front (RF) values of each band were recorded according to the formula. Resolution front (RF) = Distance traveled by the sample / Distance traveled by the solvent.

2.5.5 Fractionation by TLC

Approximately 10 mg of the dried extracts, obtained from the evaporation of the fresh extracts to dryness at 40 °C under vacuum, were dissolved in 1 ml of methanol, ethyl acetate and petroleum ether, which they were already showed antimicrobial activity. TLC separation was carried out on silica gel G (2:1 ratio) plates (20 × 20 cm, 0.5 mm thickness) with a developing solvent of Hexane and ethyl acetate (7:3 v/v). Under UV-light, six bands were observed for methanol extract and four bands were observed for ethyl acetate and petroleum ether extracts and scraped separately from the TLC plate. The compounds in each band were extracted with methanol (100%) and filtered before LC-MS detection.

2.5.6 Isolation and purification of active compound

TLC plate was developed and required fractions scrapped and collected in Eppendorf tube. Mixtures dissolved in extract solvents. Centrifuged the solution at 4000 rpm for 5 minutes Supernatant was collected and filtered. The solvent was evaporated and again checked on TLC plate. Anti-microbial assay was checked using agar well diffusion method. Further

procedure followed for antimicrobial assay was the same as described above for solvent leaf extract.

3. RESULT AND DISCUSSION

3.1 Antimicrobial assay

The solvents used for extraction and reconstitution of the extracts were methanol, petroleum ether, chloroform, ethyl acetate, butanol and aqueous.

Chloroform, butanol and aqueous extracts, none of these solvent extracts showed significant activity against tested bacteria. *Staphylococcus aureus* was insensitive strain of all the bacteria used in this study. The results of the antimicrobial screening assay of the crude and fractions extracts of *L.adoensis* var. *koseret* against tested bacteria with varying zone of inhibition are shown in the table

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

Extracts	Concentration (mg/mL)	Zone of inhibition in (mm)		
		Bacterial Strains		
		S.aureus	E. coli	P. aeruginosa
Methanol	5	-	11 ± 1	10 ± 0
	10	-	14.333 ± 0.577	11 ± 1.732
Petroleum ether	5	-	10 ± 1	10 ± 0.577
	10	-	12.666 ± 2.309	11.666 ± 0.577
Ethyl acetate	5	-	11 ± 0.577	10 ± 0
	10	-	14 ± 1,732	16.333 ± 1.154
Positive control (8 µl) Ampicillin	-	24± 3	23 ± 3	26.666 ± 0.577
Negative control	-	ND	ND	ND

ND= Not detected; (-) = No antimicrobial activity; Values are mean of inhibition zone (mm) ± S.D of three replicates

As can be seen from the result, Anti-microbial activity of *L.adoensis* var. *koseret* based on the extraction solvent used the concentration of the extract and species of the tested microorganisms. All the three

leave extracts namely Methanol, Ethyl acetate and Petroleum ether extracts showed significant activity at both concentration levels (5 and 10 mg/ml) against the organisms tested.

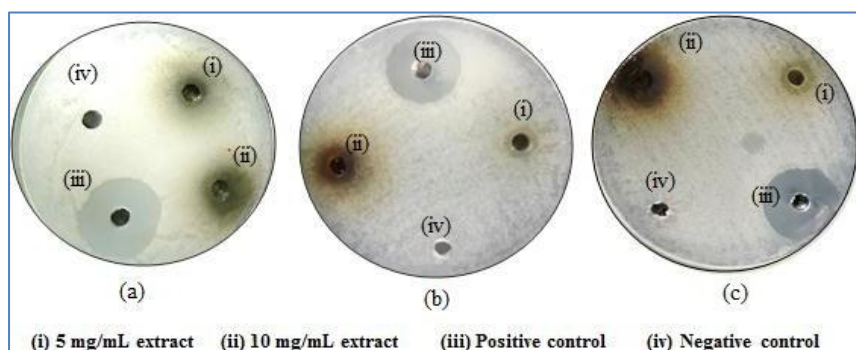


Fig-3: Agar dilution assay of different extracts against *E. coli*: (a) Methanol extract, (b) Petroleum ether, and (c) Ethyl acetate

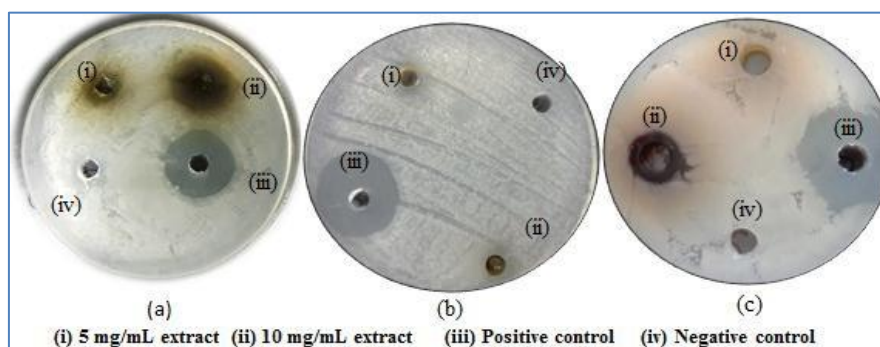


Fig-4: Agar dilution assays of different extracts against *P. aeruginosa*: (a) Methanol extract, (b) Petroleum ether, and (c) Ethyl acetate

Specifically, Methanol and Ethyl acetate gives better activity against *E. coli* at 10 mg/ml and Ethyl acetate is the most active against *P. aeruginosa* at 10

mg/ml. While, petroleum ether extract showed relatively lower antibacterial efficacy against the tested organisms.

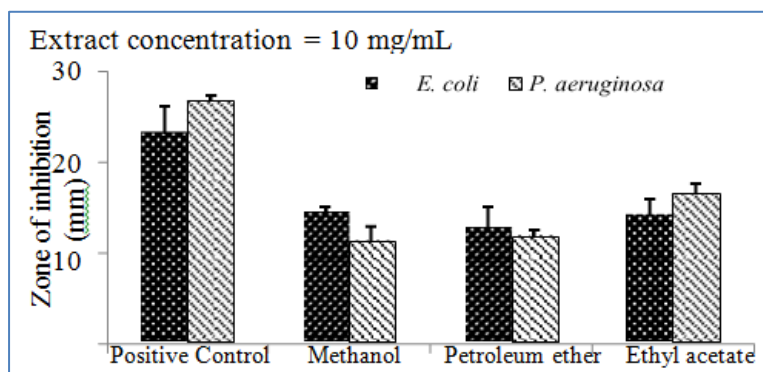


Fig-5: Antimicrobial activity of Methanol, Petroleum ether and Ethyl acetate extracts of *L. adoensis* var. koseret at 10 mg/ml concentrations against *E.coli* and *P. aeruginosa*

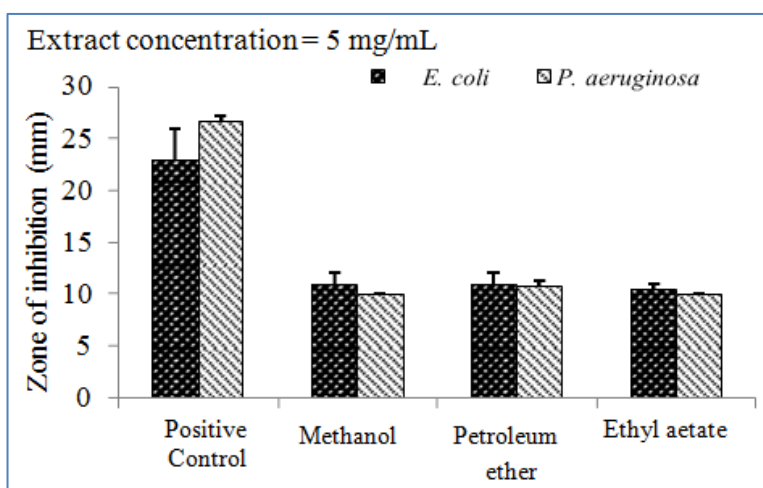


Fig-6: Antimicrobial activity of Methanol, Petroleum ether and Ethyl acetate extracts of *L. adoensis* var. koseret at 5mg/ml concentrations against *E.coli* and *P. Aeruginosa*:

Several reports (Buli *et al.*, 2015; Teka *et al.*, 2015; Tadeg *et al.*, 2005) indicated that *S.aureus* was the most resistant bacteria while *E.coli* was insensitive bacteria and non-polar fractions showed highest antibacterial activity than polar fractions. The present finding gain support from the work of (Aminzare *et al.*, 2017) who have worked on *E.coli* strain, demonstrated that *E.coli* was sensitive bacteria to methanolic extract and inhibition zone diameter of methanol extract against *E.coli* was 14 ± 0.8 . In all the three solvent extracts of plant tested for antibacterial activity, the zone of inhibitions increased with an increase in concentration i.e. Stronger activity was observed at 10 mg/ml than lower concentrations. In the ethyl acetate based extract, high antimicrobial activities were observed against *P. aeruginosa*. The highest average

(16.333 ± 1.154 mm) zone of inhibition was observed in ethyl acetate extract at the concentration of 10mg/ml against *P. aeruginosa*. 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 *E.coli* and *P. aeruginosa* (Table 1).

3.2 Minimum Inhibitory Concentration

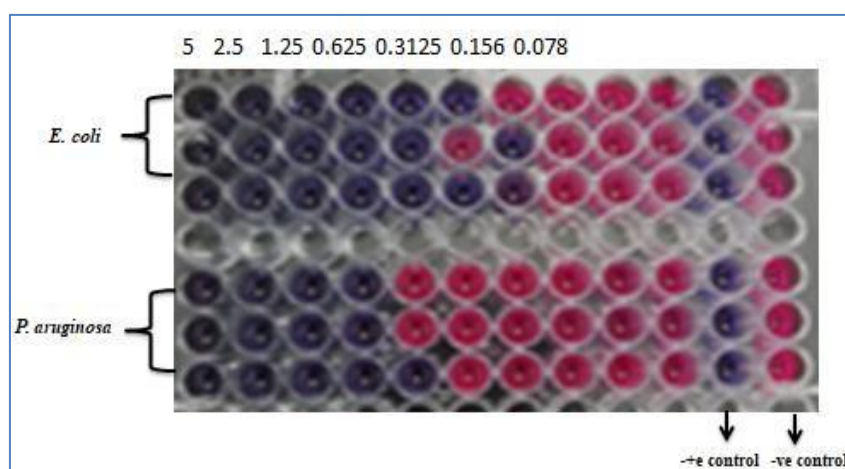
Based on the initial antimicrobial screening assay, Methanol, Ethyl acetate and Petroleum ether solvent extracts were selected for further studies for the determination of MIC, because they were showed activities against the tested bacterial strains. The MIC of the extracts is shown in the table below.

Table-2: Average minimum inhibitory concentrations of *L. adoensis* var. *koseret* in three different extraction solvents

Extract	Minimum inhibitory concentration ($\mu\text{g/mL}$)	
	<i>E. coli</i>	<i>P. aeruginosa</i>
Methanol	143 \pm 0	520 \pm 0
Ethyl acetate	143 \pm 0.059	520 \pm 0.1804
Petroleum ether	1250 \pm 0	1250 \pm 0

The MIC value indicated that all three extracts of *Lippia adoensis* var *koseret* are potent against the tested bacterial strains. The results were in agreement with the initial antimicrobial screening test results. Methanol and Ethyl acetate based extracts showed very lower MIC value as compared with Petroleum ether. The lowest MIC value demonstrated was 143 $\mu\text{g/ml}$ for

Methanol and Ethyl acetate extracts against *E.coli* while 520 $\mu\text{g/ml}$ against *P. Aeruginosa*. The MIC of petroleum ether based extract was 1250 $\mu\text{g/ml}$ against *E.coli* and *P. aeruginosa*. All the three extracts had exhibited bactericidal effects against *E.coli* and *P. aeruginosa*.

**Fig-7: Minimum Inhibitory Concentration of *L. adoensis* var. *koseret* of methanol extract at 5mg/ml**

3.3 Qualitative phytochemical analysis

The results of the qualitative phytochemicals analysis showed that the leaf extracts of *L. adoensis* var. *koseret* also indicated the presence of tannins, flavonoids, polyphenols, alkaloids and saponins while

in the case of ethyl acetate alkaloids was not detected and tannins were absent in petroleum ether extract Table 4. Amino acid and carbohydrates were absent in all the three extracts.

Table-3: Phytochemicals analysis of three different extracts

Phytoconstituents	Methanol	Petroleum ether extract	Ethyl acetate extract
Alkaloids	++	++	--
Phenolics	++	++	++
Tannins	++	--	++
Flavonoids	++	++	++
Saponins	++	++	--
Carbohydrate	--	--	--
Amino acid	--	--	--

(--)= absent, (++)= present,

The phytochemicals are known to have antimicrobial activity (Usman & Osuji, 2007; Ebana *et al.*, 1995). The presence of phenolic compounds indicates that the plants are antimicrobial agent (Rasool *et al.*, 2010; Okwu & Okwu, 2004). These components make phospholipid bilayer membrane sensitive, and then cause an increase in membrane permeability, where compounds may disrupt membrane, lose cellular integrity and eventual lead to cell death (Moreira *et al.*,

2005). It can be suggested that the presence of phenols, alkaloids, flavonoids, saponins in three solvent extracts with, methanol, petroleum ether and ethyl acetate of leaves of *L. adoensis* var. *koseret* may be considered as one of the reason for a good antibacterial property shown by the leaf extract.

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 (Schultes, 1978).

3.3. TLC analysis

TLC analysis confirmed the presence of various bioactive components which were characterized by the distance they travel in a particular TLC system and their appearance (color) after visualization of the

spots. Absolut methanol, petroleum ether and ethyl acetate extracts displayed a series of constituents on the chromatogram. The non-polar fraction showed quite similar fingerprint compared to the polar fraction. The result of TLC analysis of the methanol, petroleum ether and ethyl acetate fractions of the leaf extract showed that the chromatogram revealed 6 bands under UV corresponding to various compounds present in the methanol fraction of leaf extract. In addition, 4 bands were seen under UV in the petroleum ether and ethyl acetate fractions of leaf extract. All of the compounds were visualized under UV 345nm Figure 8.

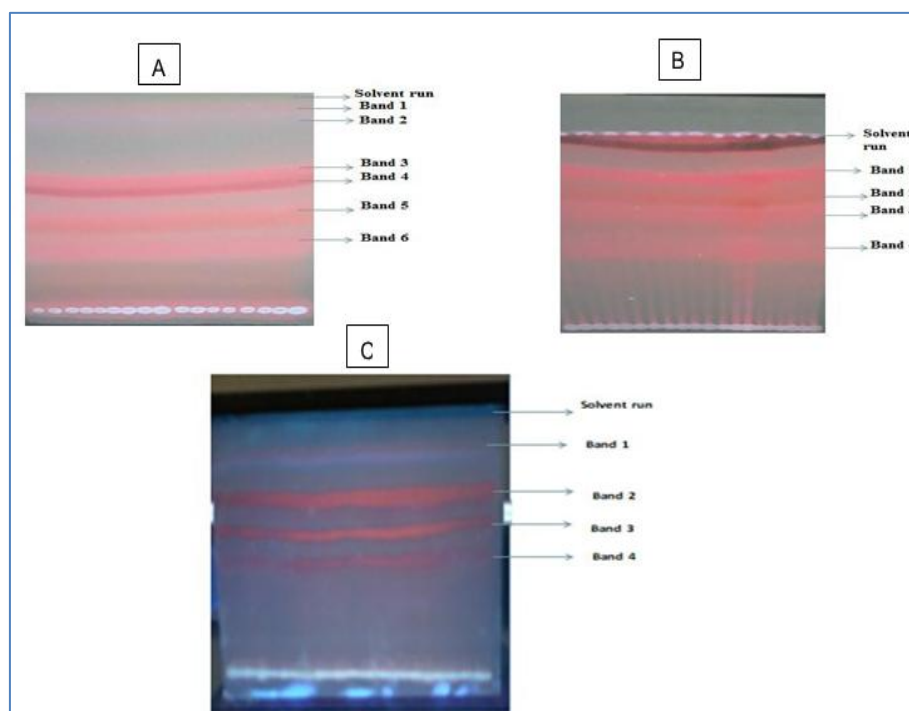


Fig-8: TLC profile of the (A), Methanol (B), Petroleum ether (C), ethyl acetate extracts of leaf after derivatization

The Rf values calculated for each compound in methanol, petroleum ether and ethyl acetate extracts are depicted in Table 4.

Table-4: Rf value of different spots of the extracts

Extracts	Spot number	Distance of solvent front (in cm)	Rf value
Methanolic extract	1	16.9	0.38
	2		0.5
	3		0.56
	4		0.59
	5		0.69
	6		0.79
Ethyl acetate	1	16.5	0.49
	2		0.58
	3		0.7
	4		0.8
Petroleum ether	1	16.2	0.31
	2		0.44
	3		0.56
	4		0.77

The presence of phytochemicals was further strengthened by TLC profiling of leaf extract which showed different bands representing various constituents. This was very well in line with the TLC autobiography and phytochemical screening of the leaf extracts of *L. adoensis* var. *koseret* which showed presence of compounds. Many of the compounds present in the sample could act as potential antimicrobial agents.

3.4 Antimicrobial assay of the TLC spots

After isolation of active compounds from TLC analysis each bands were subjected to antimicrobial assay using agar well diffusion method. The agar well diffusion assay of the bands from methanol and ethyl acetate extracts exhibited antimicrobial activity against *E. coli* at 5mg/ml concentration level. *E. coli* was sensitive against the bands from methanol and ethyl acetate Figure (9). While the bands from petroleum extract was devoid of any antimicrobial activity which in agreement with the initial antimicrobial screening result illustrated that the petroleum ether fraction were less in their activity compared to the polar fractions.

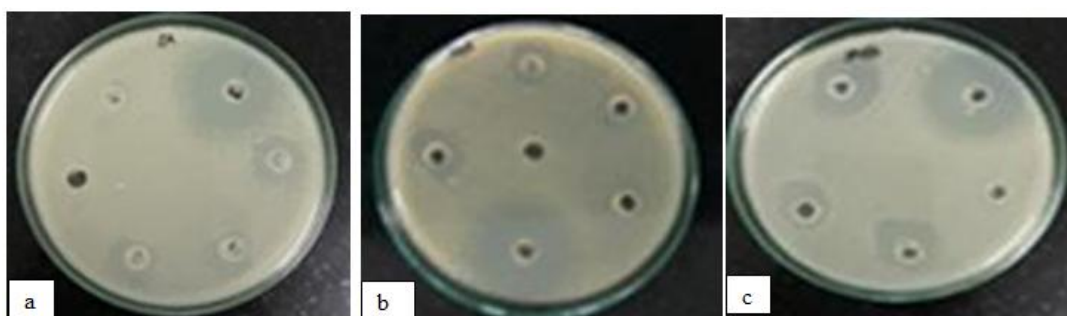


Fig-9: Antimicrobial activity of different spots of methanol and ethyl acetate extract (a) compounds from Ethyl acetate extract (b) Active compounds from Methanol extract

The first three bands from methanol and ethyl acetate extract showed similar result against *E. coli*. On the other hand the fourth and the last bands of methanol extract showed relatively higher antibacterial activity (12 ± 1.98 and 12.5 ± 0.707 mm) against *E. coli*. 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 same zone of inhibition (Table 5).

Table-5: Antimicrobial activity of different spots of methanol and ethyl acetate extracts of *L. adoensis* var. *koseret*

Extract	Number of spots	Zone of inhibition (mm)	
		Bacterial species	
		<i>E. coli</i>	Positive control
Methanol	1	11.5 ± 0.707	24 ± 0
	2	11.5 ± 0.707	24 ± 0.707
	3	11.5 ± 1.414	24 ± 0
	4	12 ± 1.98	24 ± 0
	5	11.5 ± 0.707	24 ± 1.414
	6	12.5 ± 0.707	24 ± 0.707
Ethyl acetate	1	11.5 ± 0.707	24 ± 0
	2	10.5 ± 0.707	24 ± 0.707
	3	11.5 ± 0.707	24 ± 0

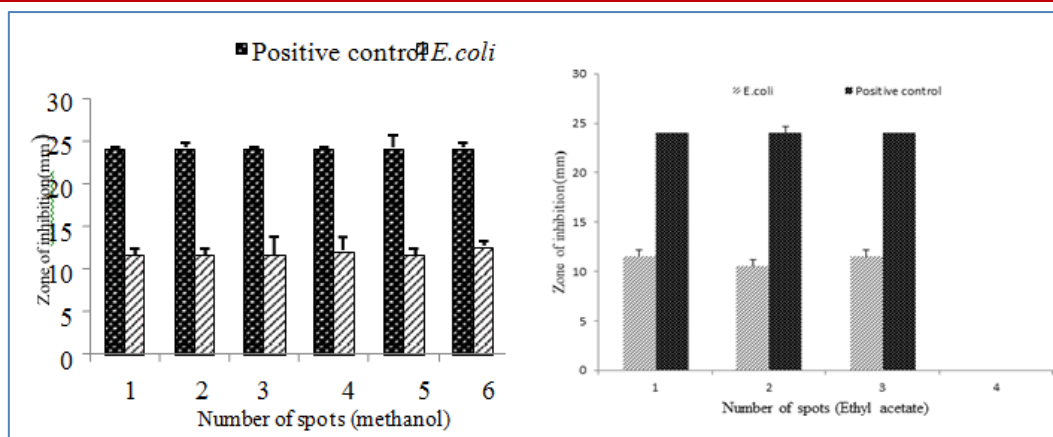


Fig-10: Antimicrobial activities of spots of Methanol and Ethyl acetate extract against E.coli

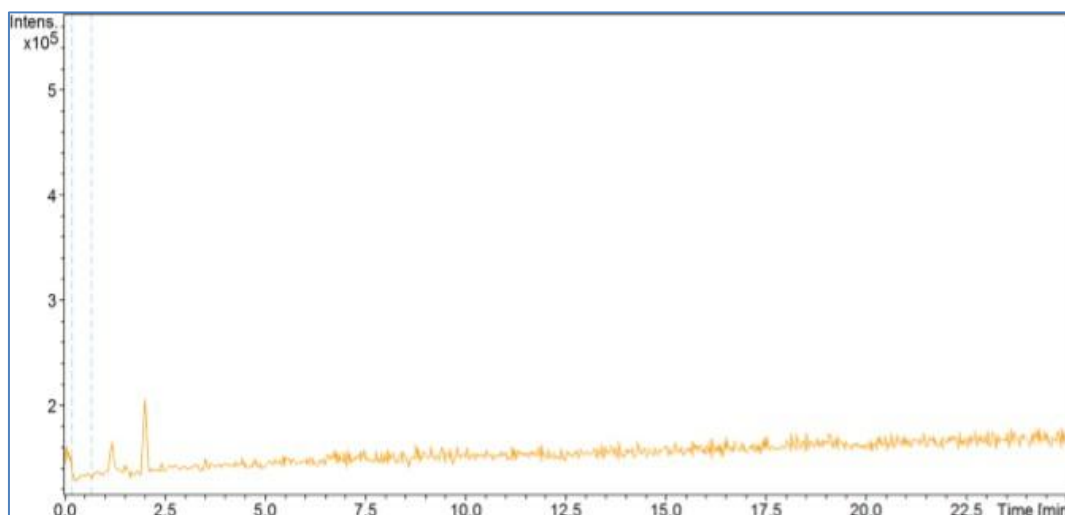
3.5 LC-MS analysis

The purified compound from the TLC analysis were identified and characterized using LC-MS seven dominant peaks were selected for methanol extract and

six peaks for ethyl acetate extract. The identification of the compounds was carried out using database search by comparing the molecular weight of the analysed peaks with standards molecular weight.

Table-6: LC-MS Spectral information of identified Peaks of methanol (Y-1) extract of Lippia adoensis var. koseret

Peak No	Molecular Formula	Molecular weight of standard	Molecular weight of peaks
1	C7H7LiS	130.137	130.1369
2	C6H5F6I	317.999	317.9990
3	C14H18F4N2O3	338.298	338.2979
4	C39H56CIN5O9	774.343	777.3431
5	C44H52CIFN2O7	775.344	775.3445
6	C41H53CIF3N7O7	848.350	848.3539
7	C39H45CIN10O8S	849.355	849.3553



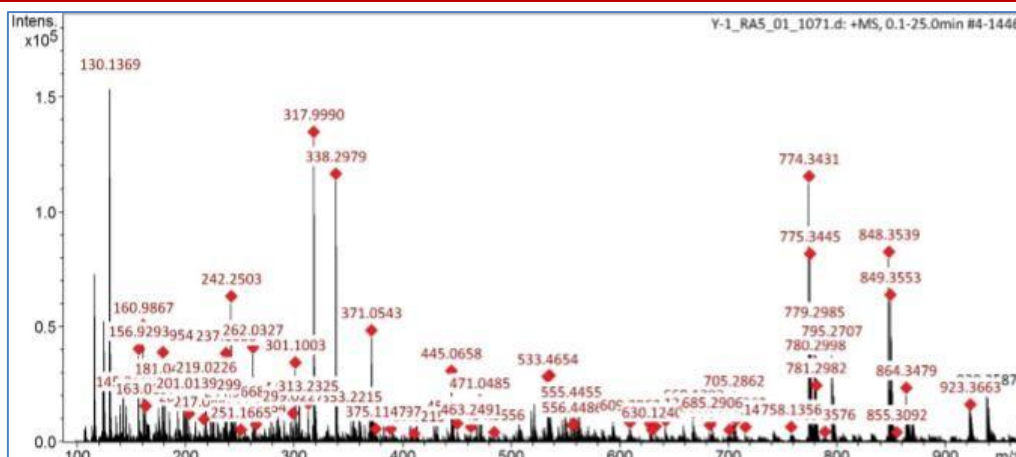


Fig-11: LC-MS result of the spots from Methanol (Y-1) extract of *Lippia adoensis* Var. *koseret*

Table-7: LC-MS Spectral information of identified Peaks of Ethyl acetate(Y-2) extract of *Lippia adoensis* var. *koseret*

Peak No	Molecular Formula	Molecular weight of standard	Molecular weight of peaks
1	C3H14O5	130.140	130.1404
2	C10H10Br2N2	318.008	318.0075
3	C1113C6H20N2O5	338.307	338.3071
4	C56H97NO4	848.374	848.3773
5	C22H17N11O22P2	849.378	849.3787
6	C46H54ClFN2O10	849.380	850.3775

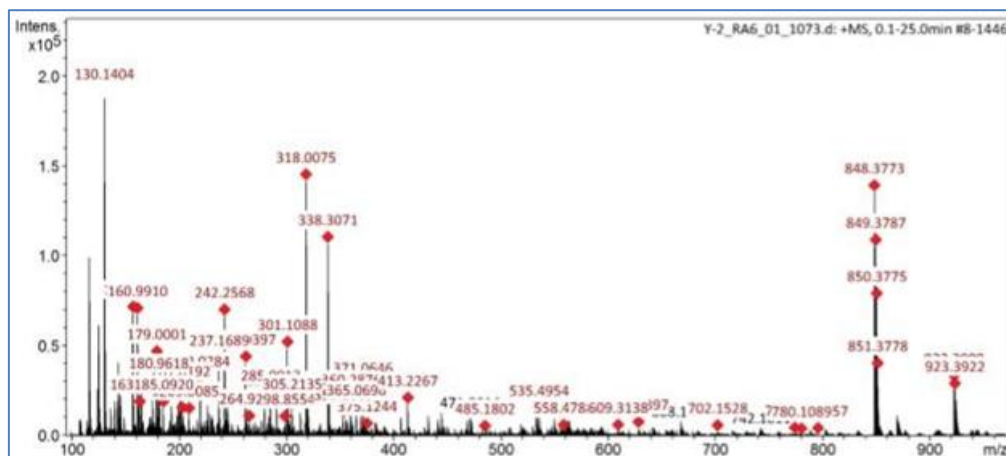
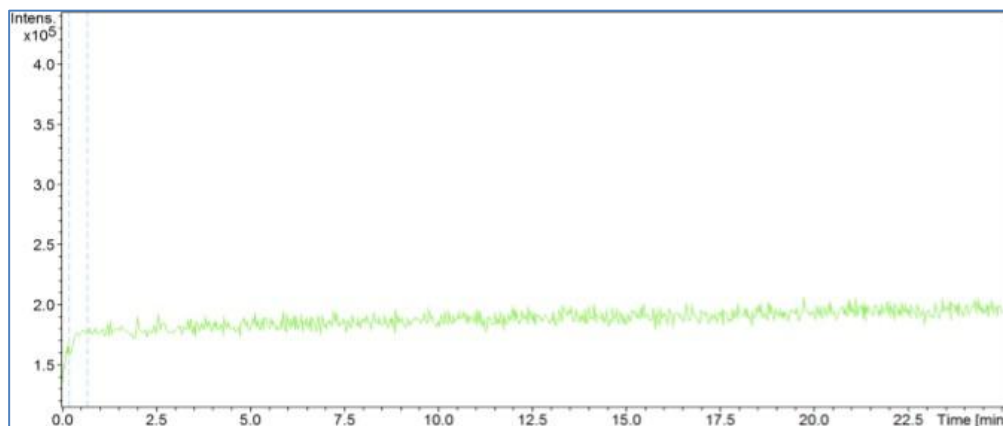


Fig-12: LC-MS result of the spots Ethyl acetate (Y-2) extract of *Lippia adoensis* Var. *koseret*

4. CONCLUSION

To conclude, 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. Methanol, petroleum ether and ethyl acetate extracts showed significant antimicrobial activity against *E. coli* and *P. aeruginosa* while *S. aureus* was the most resistant microorganisms in all assay methods. It was understood from the present study that the extract of *Lippia adoensis* Var. *koseret* contained many phytochemicals as revealed by phytochemical studies, TLC analysis and further characterization using LC-MS may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development. Thus, the present study has authenticated the usefulness of the *Lippia adoensis* Var. *koseret* for medicinal purposes. These species could also be seen as potential sources of useful drugs due to their rich contents of phytochemicals.

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