



## Phytochemical Investigation, Evaluation of The Antibacterial and Antioxidant Activities of the Leaves Extracts of *Leonotis ocymifolia*

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### ABSTRACT

*Leonotis ocymifolia* (Burm. f.) Iwarsson, usually recognized as Ras-kimir or Yeferes Zeng; is a therapeutic plant that belongs to Lamiaceae family. The extracts of this plant were obtained by the maceration method. Phytochemical analysis outlined the existence of alkaloids, saponins, flavonoids, phenols, terpenoids, and tannins in the *n*-hexane solvent; alkaloids, saponins, terpenoids, tannins, flavonoid, and phenols in EtOAc solvent; and alkaloids, flavonoids, steroids, saponins, terpenoids, anthraquinones, phenols and tannins from MeOH solvent extracts of *L. ocymifolia* leaves. The solvent extracts were tested against one Gram-positive bacterial species (*Listeria monocytogen*) and one Gram-negative bacteria (*Klebsiella pneumonia*) using disc diffusion method at (50 mg/mL, 100 mg/mL and 200 mg/mL) concentrations. The results of the invitro antibacterial activity tests were compared with the commercially available antimicrobial agent (chloramphenicol) and Dimethyl sulfoxide was the negative control. MeOH and *n*-hexane fraction revealed more potent antibacterial activities against the growth of the bacterial strains than EtOAc extract. The radical scavenging performance of the extracts were measured by utilizing DPPH. The percentage inhibitions of MeOH, EtOAc, and *n*-hexane extracts were compared with the control ascorbic acid (200 ppm to 1000 ppm) have the value ranges from 88.46% - 95.25%, 77.55% - 91.48%, and 60.61% - 79.19%, respectively. Generally, MeOH was higher in percentage yield and antioxidant activities than EtOAc and *n*-hexane. One compound (1) was isolated from the EtOAc extract using silica gel column chromatography. The structure of the isolated component was characterized by using a combination of spectroscopic techniques such as FTIR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, UV and DEPT-135.

**Keywords:** Medicinal plant, Maceration, Phytochemicals, Antibacterial activities, Antioxidant, *L. ocymifolia*.

### INTRODUCTION

The usage of plants in medication dates to ancient human history, with early humans utilizing them not only for food, shelter, and fragrances but also for treating a variety of ailments. Evidence of medicinal plant use for combating infections can be traced to a Sumerian clay tablet from Nagpur, which is approximately 5,000 years old (Teshahun et al., 2014). Nature has long been a vital source of medicinal compounds, and many modern pharmaceuticals have been derived from natural substances, often inspired by their use in traditional healing practices. Despite the advancements in modern medicine, traditional plant-based remedies continue to be a cornerstone of healthcare, with around 80% of the global population relying on them as their primary form

of treatment.

Traditional medicinal plants contain a variety of compounds that serve as remedies for both chronic and infectious illnesses. Microbiologists are particularly interested in screening these plants for potential new therapeutics. Many of the active ingredients in plant-based drugs are secondary metabolites (Amabye et al., 2015). The medicinal properties of these plants stem from the phytochemical compounds they contain, which produce specific pharmacological effects in the human body. These compounds help combat various ailments and act as antioxidants. Antioxidants are vital for counteracting free radicals within biological cells, as these free radicals can have harmful effects on living organisms (Getachew et al., 2022).

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The Lamiaceae family, also known as Labiatae, comprises around 300 genera and more than 7,500 species, with a wide global distribution (Tekla et al., 2020). The genus name *Leonotis* derives from the Greek words "Leon" (meaning lion) and "ous," "otis" (meaning ear), reflecting the resemblance of its flowers to lion's ears (Scott et al., 2004). *Leonotis ocymifolia* (*L. ocymifolia*) is a shrub with multiple stems that generally reaches a height of about 2.5 meters. Its leaves are short, petiolate, and either oblong-lanceolate or lanceolate, measuring around 5-10 cm long, slightly oblique at the base, with short teeth along the leaf margin. The plant's characteristic orange-red flowers are arranged in compact clusters along the stems. The fruit is composed of four tiny seeds positioned at the bottom of the calyx tube. After flowering, the plant naturally dies, and new growth begins in the spring. It can be propagated through cuttings, rootstock division, or seeds (Gonfa et al., 2021). In Ethiopia, *L. ocymifolia*, locally known as Raskmir or Yeferes Zeng, is used in traditional medicine to treat conditions such as headaches, neck ulcers, swelling (Yineger et al., 2007), hookworm, gout, and leishmaniasis. It is also believed to have Birth control effects, acts as an antiemetic (Lulekal et al., 2014), and is used for general malaise, as well as for expelling parasites in animals (Meniso et al., 2019). Chemical analysis of *L. ocymifolia* leaves from Ethiopian collections bare the existence of five distinct labdane-type diterpene lactones (Habtemariam et al., 1994). In the Delanta Woreda of South Wollo, Ethiopia, local people use the plant's leaves to expel intestinal parasites and treat fever, headache, and malaria, particularly in Woinadega areas. However, a literature review on the chemical and biological properties of *L. ocymifolia* indicated that no systematic studies have been conducted on the phytoconstituents, antioxidants, and antibacterial activities of its leaves. Therefore, this study was carried out to establish the claimed antibacterial and antioxidant properties, as well as to isolate the active secondary metabolites existing in the leaves of *L. ocymifolia*, based on its traditional medicinal use in Ethiopia.

## MATERIALS AND METHODS

### Plant Material:

The leaves of *L. ocymifolia* were gathered from Mesno-amba Kebele, Delanta Woreda, South Wollo, Amhara regional state Ethiopia. After the plant was collected, the specimen was identified and authenticated by Fasil Asfaw (MSc) and stored at the Herbarium of Department of Biology, College of Natural Science, Wollo University, Dessie, Ethiopia. The fresh leaves of *L. ocymifolia* were collected and removed from the unwanted part that was already dried and dead. The plant specimens were cleaned using tap water without

squeezing to eliminate dust particles and then air dried at room temperature for one month.

### Extraction of the Plant Material:

The dried leave of the sample was pulverized into fine dust by using Ririhong grinder (China) and kept in tightly plastic bags for extraction purpose. The dried powdered leave 500 g was soaked with 1200 mL *n*-hexane in a 3 L Erlenmeyer flask for about 72 hours and it was filtered with Whatman filter paper No 1 and the supernatant was put in a separate beaker. The marc (350 g) obtained from the previous process was again soaked with 1000 mL EtOAc and stayed for the same time and it was filtered. The same process was repeated for MeOH (200 g) based on increasing polarity. Then each separated solvent extracts were evaporated under reduced pressure at 40 °C using rotary evaporator. The extracts were weighed, transferred into beakers and kept in the oven at 4 °C for further usage (Bulugahapitiya, 2018).

### Phytochemical Screening of the Extract:

Initial phytochemical examining of secondary metabolites of *n*-hexane, EtOAc and MeOH extracts of *L. ocymifolia* were performed by utilizing standard tests (Altemimi et al., 2017; Goyal, 2014; Meniso et al., 2019; Njoku et al., 2011; Shalligito et al., 2022; Sirak et al., 2021). The data of phytochemicals were analyzed qualitatively as the absence of secondary metabolites (-), slight positive reaction for secondary metabolites (+) and definitive positive reaction for secondary metabolites (++) (Regasa et al., 2018).

### Antimicrobial Activity Test:

The antibacterial effects of plant extracts on various bacterial species were evaluated using the disc diffusion method (Sahalie et al., 2018). This method was chosen for its simplicity, ability to process multiple samples, and suitability for testing specific inhibitors. Primarily, 25 mL of sterile Muller Hinton Agar (MHA) was poured into a petri dish and allowed to cool. The inoculant suspension was adjusted to the 0.5 McFarland turbidity standard, and within 15 minutes, a sterile cotton swab was dipped into the suspension. The swab was gently rotated and pressed against the inside of the tube to remove excess liquid. Then, the swab was used to evenly spread the suspension on the MHA plate. The plates were left to sit at room temperature for 5 minutes.

To prepare the plant extract solutions, 0.5 g of the extract was dissolved in 1.0 mL of Dimethyl sulfoxide (DMSO) to create a 500 mg/mL stock solution. Dilutions of the stock solution (50, 100, and 200 mg/mL) were made by further diluting the stock in 1 mL of DMSO. These extracts were then

applied to filter paper discs (Sahalie et al., 2018). Chloramphenicol at a concentration of 30 µg/mL served as positive control, whereas DMSO was used as the negative control. The discs were placed on the prepared MHA plates, and the plates were allowed to stand undisturbed at 5°C for 2 hours before being incubated at 37°C for 24 hours (Tebeje, 2019). All assays were carried out three times for each bacterial species.

The antibacterial activity of *L. ocymifolia* was tested against one Gram-positive (*L. monocytogenes*) and one Gram-negative (*K. pneumoniae*) bacterium, both obtained from the Dessie International Clinical Laboratory (ICL), using the disc diffusion method at concentrations of 50 mg/mL, 100 mg/mL, and 200 mg/mL. After the incubation period, the zone of inhibition was measured from the edge of each disc using sliding calipers and compared to the controls, with measurements taken using a ruler. The mean inhibition zone and standard error (Mean ± SEM) were determined (Sahalie et al., 2018). Antibacterial activity was considered significant if the inhibition zone exceeded 6 mm.

#### DPPH Radical Scavenging Activity:

The competency of scavenging free radicals by the plant extracts was gaged by using the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method (Mekonnen et al., 2018) and this competency was assessed following the protocol outlined by Alemu et al. (Keshebo et al., 2016) using DPPH. A 0.04% DPPH solution was prepared by dispensing 4 mg of DPPH in 100 mL of methanol (MeOH). Similarly, 10 mg of each crude extract (n-hexane, ethyl acetate (EtOAc), and methanol (MeOH)) from the leaves of *L. ocymifolia* was dissolved in 10 mL of methanol, resulting in a 1000 ppm stock solution for each extract. Stock solutions of ascorbic acid and the crude extracts were prepared by dissolving 10 mg of ascorbic acid and the plant extracts separately in 10 mL volumetric flasks and adding methanol to the mark to make 1000 ppm stock solutions. From these stock solutions, concentrations of 200 ppm, 400 ppm, 600 ppm, 800 ppm, and 1000 ppm were prepared in separate test tubes. After thoroughly shaking the mixtures, they were incubated for 30 minutes in the dark at room temperature. Following incubation, the absorbance of each sample was measured at 517 nm using a UV-T60 spectrophotometer (Keshebo et al., 2016). Ascorbic acid at varying concentrations was used as a positive control, while methanol served as the blank.

#### Fractionation of Ethyl Acetate Extract of *Leonotis ocymifolia* Leaves:

A total of 100 grams of silica gel was measured and mixed with 200 mL of a n-hexane: ethyl acetate (EtOAc) solvent mixture (8:2). The

resulting mixture was then packed into a column. To prepare the sample, 7 grams of the dried EtOAc crude extract was dissolved in 10 mL of the n-hexane: EtOAc (8:2) mixture. This solution was carefully applied to the top of the packed silica gel using a dropper. Elution was carried out first with 400 mL of n-hexane: EtOAc (8:2), followed by 200 mL of n-hexane: EtOAc (7:3). In total, 12 fractions of the EtOAc crude extract from the leaves of *L. ocymifolia* were collected. The isolated compound 1 was then characterized using various spectroscopic methods. UV-Vis and FTIR spectra were obtained with a UV-T60 spectrophotometer and a PerkinElmer Spectrum 65 instrument, respectively, in the range of 4000–200 cm<sup>-1</sup>. Additionally, <sup>1</sup>H- and <sup>13</sup>C-NMR spectra were noted on a Bruker Avance 400 NMR spectrometer at frequencies of 400.13 MHz and 100.60 MHz, respectively.

## RESULTS

### Yields of Solvent Extracts of *Leonotis ocymifolia*:

The extract yields were summarized in **Error! Reference source not found.** . As shown in the table, the total yield of the extract from the plant leaves were about 1.66 %, 4.48 %, and 12.8 % for n-hexane, EtOAc, and MeOH, respectively. Previous studies (Habtemariam et al., 1994) showed that the percentage yield of the extraction of leaves of *L. ocymifolia* varies according to the part of the plant and solvent type used for the extraction.

### Phytochemical Components of the Leaf Extracts:

Phytochemical screening of the three solvent extracts were performed using the standard phytochemical methods to detect the existence and absence of secondary metabolites (Regasa et al., 2018). Intensive indication of all tested secondary metabolites except steroids and anthraquinones were found in the three solvent extracts of the leaves of *L. ocymifolia* (Table 2).

### Analysis of In-vitro Antibacterial Activity:

The antibacterial activity of *L. ocymifolia* was assessed in one Gram-positive (*L. monocytogenes*) and one Gram-negative (*K. pneumoniae*) bacteria using the disc diffusion method at different concentrations (50 mg/mL, 100 mg/mL, and 200 mg/mL). The quantities were expressed as mean ± SEM (n=3); chloramphenicol was the positive control. The results obtained were summarized in Table 3. The n-hexane solvent extract resulted in the highest zone of inhibition as compared to EtOAc and MeOH at all tested concentrations, chloramphenicol being as a positive control. All the solvent extracts inhibit the Gram-negative bacteria (*K. pneumoniae*) and Gram-positive

bacteria (*L. monocytogen*) at all tested concentrations (Fig.1).

**DPPH Radical Scavenging Activities of Leaf Extracts of *Leonotis ocymifolia*:**

Antioxidants are compounds that defend cells against damage induced by free radicals, which are unstable molecules generated by the body due to

environmental pressures and other factors. These compounds are often referred to as free radical scavengers (Mekonnen et al., 2018; Joseph & Raj, 2010). In the present study, the antioxidant activity of n-hexane, EtOAc, and MeOH extracts from the leaves of *L. ocymifolia* was assessed using the DPPH method. The findings indicated that the free radical scavenging activity improved with

**Table 1: Extract yields of the leaves of *L. ocymifolia***

Solvent extracts	Weight (g)	Appearance	Consistency	Yield (% w/w)
n-hexane	8.3	Yellow	Powder	1.66
EtOAc	15.7	Blue black	Semisolid	4.48
MeOH	25.6	Dark green	Semisolid	12.8

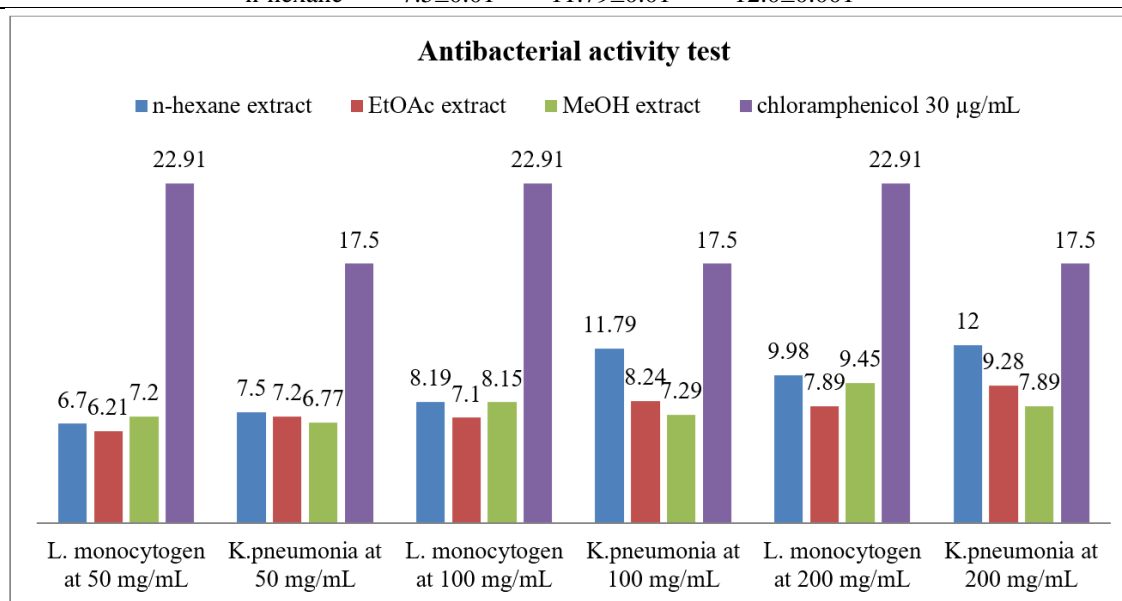
**Table 2: Phytochemical studies of *L. ocymifolia* solvent extracts**

Secondary Metabolite's	Types of tests	n-hexane	EtOAc	MeOH
Saponins	Foam test	+	++	++
Terpenoids	Salkowski's test	+	++	++
Tannins	Ferric chloride test	+	++	++
Flavonoids	Lead acetate solution Test	+	+	+
Steroids	Steroid's test	-	-	+
Alkaloids	Mayer's test	+	++	++
Phenols	Phenol test	+	+	+
Anthraquinones	Anthraquinones test	-	-	+

NB: (+) represent the presence, (++) definite presence and (-) absence of secondary metabolites.

**Table 3: Antibacterial activities of solvent extracts of the leaves of *L. ocymifolia* against *L. monocytogen* and *K. pneumonia*.**

Tested bacteria	Extracted solvent	Concentration in mg/mL			Control, chloramphenicol (30 µg/mL)
		50	100	200	
<i>L. monocytogen</i>	MeOH	7.2±0.03	8.15±0.15	9.45±0.02	22.91±0.04
	EtOAc	6.21±0.02	7.10±0.05	7.89±0.03	
	n-hexane	6.7±0.01	8.19±0.01	9.985±0.05	
<i>K. pneumonia</i>	MeOH	6.77±0.01	7.29±0.04	7.89±0.01	17.5±0.2
	EtOAc	7.2±0.01	8.24±0.03	9.28±0.04	
	n-hexane	7.5±0.01	11.79±0.01	12.0±0.001	



**Fig. 1: The antibacterial activity chart on the solvent extracts of the leaves of *L. ocymifolia* against *L. monocytogen* and *K. pneumonia*.**

concentration ranging from 200 ppm to 1000 ppm. The DPPH inhibition percentages were as follows: for the *n*-hexane extract, 60.61% to 79.19%; for the EtOAc extract, 77.55% to 91.48%; and for the MeOH extract, 88.46% to 95.25% (Table 4).

#### Spectroscopic Characterization of Compound 1:

The IR spectrum of the compound revealed several important features. A broad band at 3439.4234 cm<sup>-1</sup> suggested the presence of hydrogen bonding involving oxygen. The methyl C-H stretching was confirmed by a sharp peak at 2932.2339 cm<sup>-1</sup>. A

sharp peak at 1731.7275 cm<sup>-1</sup> indicated C=O stretching, which is characteristic of a carbonyl group, specifically an ester. The peak at 1237.1116 cm<sup>-1</sup> was indicative of C-O-C stretching, while a peak at 1151.2948 cm<sup>-1</sup> suggested the presence of O-C-C stretching. Additionally, a sharp peak at 754.0306 cm<sup>-1</sup> was associated with a methyl group attached to a quaternary carbon, as well as -C-H stretching. Overall, the IR spectrum demonstrated the presence of an oxygenated carbon, an aliphatic ester group, and a methyl group attached to a quaternary carbon in the isolated compound. The UV spectrum of compound 1 has shown

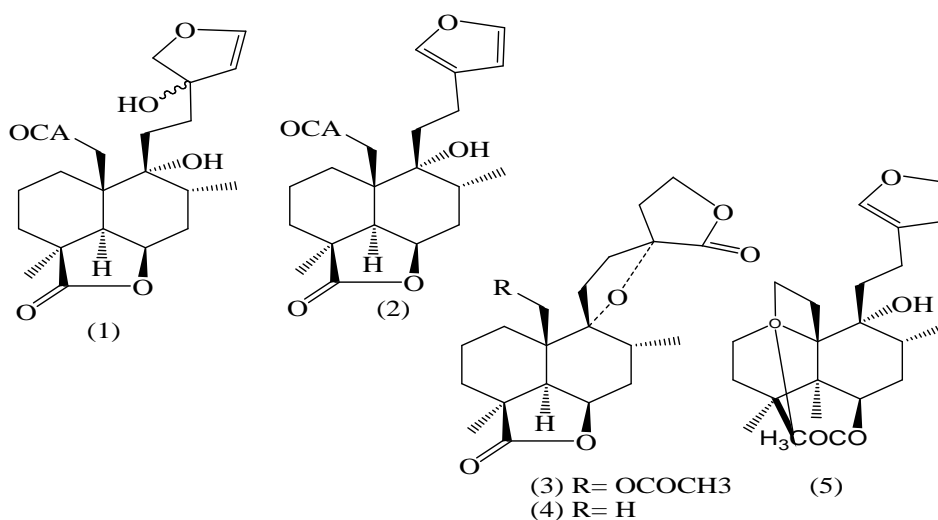


Fig. 1: Diterpenes obtained from the leaves of *L. ocyimifolia*.

Table 4: The antioxidant activities of *n*-hexane, EtOAc and MeOH solvent extracts.

Solvent extract	Concentration in ppm	Absorbance	Scavenging activity in (% w/w)
<i>n</i> -hexane	200	0.8764	60.61
	400	0.6643	70.14
	600	0.5721	74.28
	800	0.4874	78.09
	1000	0.4629	79.19
EtOAc	200	0.4995	77.55
	400	0.4756	78.62
	600	0.3029	86.38
	800	0.2943	86.77
	1000	0.1894	91.48
MeOH	200	0.2566	88.46
	400	0.1594	92.83
	600	0.1501	93.25
	800	0.1372	93.83
	1000	0.1056	95.25
Ascorbic acid (control)	200	0.0799	96.78
	400	0.0698	97.19
	600	0.0690	97.21
	800	0.0129	99.48
	1000	0.0120	99.51
DPPH		2.225	

absorbance peak at 220 nm which may support that the compound has C=O bond, mostly observed in diterpenes (Al-Massarani et al., 2017; Shi et al., 2005). Many saturated diterpene compounds with a skeleton of 20 carbon atoms absorb UV in the 198-220 nm range (Fig. 2).

The  $^{13}\text{C}$  spectrum of compound 1 also strengthens the fact that there were two different chemical shift

regions, from  $\delta$  75- 185 which was for the carbonyl group (oxygenated carbon) region and from  $\delta$  17-46 (C-CH<sub>3</sub> and C-CH<sub>2</sub>) which was for the aliphatic group. The region from  $\delta$  17 to  $\delta$  46 has 15 carbons. The region from  $\delta$  75 to  $\delta$  185 was that of the carbonyl carbon and oxygenated carbon region of compound 1 which contains 5 carbons. Therefore, compound 1 has a total of 20 carbon

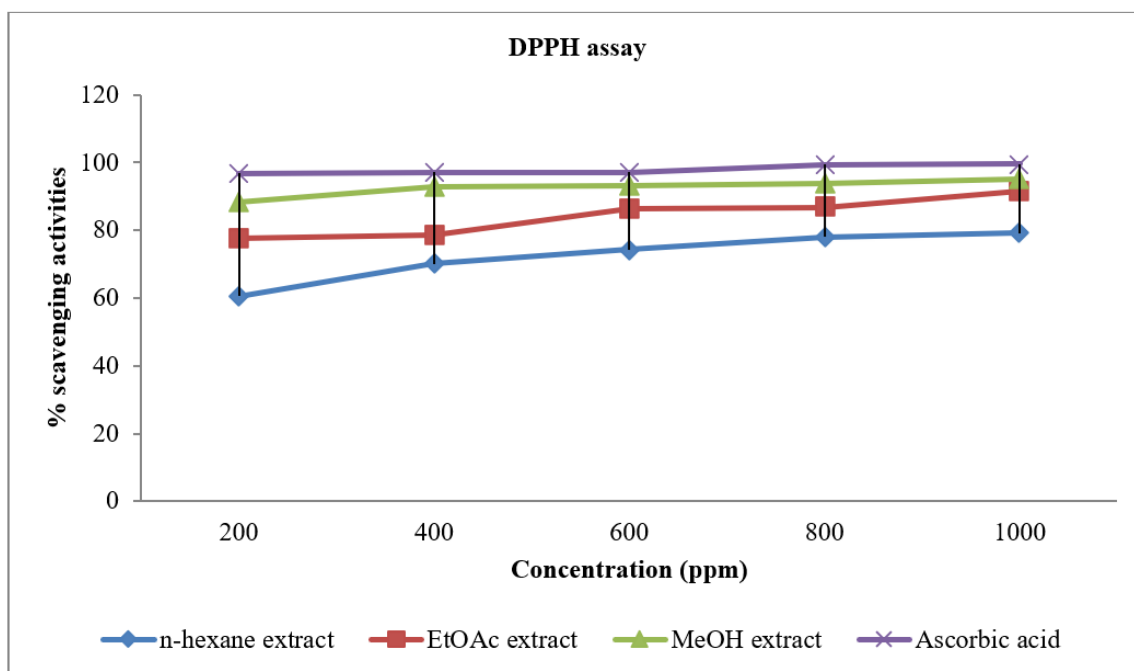


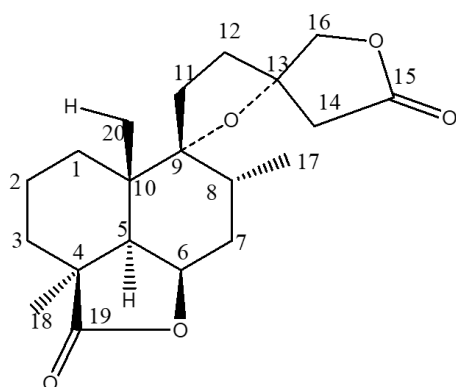
Fig. 3: DPPH radical scavenging activities of MeOH, EtOAc and *n*-hexane extracts of the leaves of *L. ocymifolia*.

Table 5: Comparison of  $^{13}\text{C}$  NMR and  $^1\text{H}$  NMR data of isolated compound and literature.

No	Literature (13S)-9 $\alpha$ , 13 $\alpha$ -Epoxylibbda-6 $\beta$ (19), 16(15)-diol dilactone			
	Carbon $\delta$ (ppm)	Hydrogen $\delta$ (ppm)	Hydrogen $\delta$ (ppm)	Carbon $\delta$ (ppm)
1.	28.2	–	–	27.97
2.	18.1	–	–	17.85
3.	28.9	–	–	28.69
4.	46.1	–	–	44.07
5.	46.1	2.07	2.00	45.83
6.	76.1	4.68	4.65	76.32
7.	29.2	–	–	29.2
8.	31.8	–	–	31.58
9.	92.2	–	–	91.95
10.	39.1	–	–	38.80
11.	31.8	–	–	31.68
12.	37.1	–	–	36.74
13.	88.2	–	–	86.03
14.	43.2	2.96 and 2.56	2.85 and 2.54	41.85
15.	174.7	–	–	175.08
16.	78.8	4.12 and 4.24	4.09 and 4.20	78.72
17.	17.8	0.87	0.82	17.33
18.	23.5	1.28	1.28	22.91
19.	183.6	–	–	184.03
20.	23.2	1.02	1.03	23.32

atoms. The  $^{13}\text{C}$  NMR spectrum data of compound 1 was nearly similar to the previously isolated compound (13S)-9 $\alpha$ , 13 $\alpha$ -Epoxy-labda-6 $\beta$ (19), 16(15)-diol dilactone, (Habtemariam et al., 1994) from the leaves of *L. ocymifolia*.

DEPT-135 NMR showed 17 peaks corresponding to 14 carbons. Compound 1 has three methyl groups at  $\delta$  17.33, 22.91 and 23.32. There were eight methylene groups at  $\delta$  27.97, 17.85, 28.69, 29.2, 31.68, 36.74, 41.85 and 78.72. There are three methine groups in the aliphatic region at  $\delta$  31.58, 45.83 and 76.32. The difference in the number of carbon atoms of the  $^{13}\text{C}$  NMR spectrum and the DEPT-135 NMR spectrum was six. Therefore, compound 1 has six quaternary carbon atoms at  $\delta$  38.80, 44.07, 86.03, 91.95, 175.08 and 184.03. The presence of a carbonyl group in compound 1 was also supported by its  $^1\text{H}$  NMR spectrum. The  $^1\text{H}$  NMR spectrum depicted that there were two separated regions of protons, one in the carbonyl carbon (oxygenated carbon) region around  $\delta$  4 -  $\delta$  7.5 which has 3 protons and the other was the aliphatic region around  $\delta$  (0.8- 3.00) which has 25 protons. Therefore compound 1 has 28 hydrogen. From all the above data, the following structure was suggested for compound 1 (Table 4).



**Fig. 4: Suggested structure of compound 1**

## DISCUSSION

The extraction yield of the leaves of *L. ocymifolia* showed that the percentage yield increased with increasing polarity, likely implies the presence of more polar compounds than non-polar ones. The phytochemical analysis results has given an evidence for the antibacterial and antioxidant activities of the plant because alkaloids are for example used as painkillers, antimicrobial, stimulants, muscle relaxants, anesthetics, antimicrobial, antidiabetic, anticancerous, anti-HIV, antioxidants etc. (Habtemariam et al., 1994). The result aligns well with the findings of previous studies, which identified tannins, saponins, and

alkaloids in the MeOH solvent extract. The various secondary metabolites were also liable for the observed antibacterial and antioxidant activities of the plant.

The antibacterial evaluation results might suggest that the effectiveness of the solvent extracts inhibiting the bacteria was also evaluated at higher concentrations (Oyedemi et al., 2005), as displayed in Table 3. The statistical evaluation revealed that *n*-hexane and EtOAc solvent extracts were shown better inhibition activity against *K. pneumonia* than *L. monocytogenes* at all concentrations. However, MeOH solvent extract was shown better inhibition activity against *L. monocytogenes* than *K. pneumonia* at all concentrations.

Evidence suggests that compounds, particularly those from natural sources, can offer protection against free radicals. Polar solvents like methanol (MeOH) are effective at extracting a wide range of bioactive compounds, while non-polar solvents like *n*-hexane extract only a limited number of compounds (Shalligito & Tesfa, 2022). As a result, *n*-hexane exhibited lower antioxidant activity compared to MeOH and EtOAc, as shown in Table 4. In the DPPH radical scavenging assay, various concentrations of ascorbic acid (200, 400, 600, 800, and 1000 ppm) were used to create a calibration curve. The results demonstrated that the MeOH extract had stronger free radical scavenging activity than both the EtOAc and *n*-hexane extracts.

In conclusion, The extracts of *L. ocymifolia* leaves showed both antibacterial and antioxidant activities and the antioxidant and antibacterial activities of crude extracts increased with increasing their concentrations. This study demonstrated that the leaves of *L. ocymifolia* hold great potential as sources of antibacterial and antioxidant compounds, suggesting their effectiveness as preventive agents in the development of certain diseases.

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## Data availability statement:

Data are available from the corresponding author on reasonable request.

## Declarations:

### Ethics approval and consent to participate:

Not applicable.

### Consent for publication:

The authors have provided their consent for publication.

**Competing interests:**

The authors declare that they have no competing interests.

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