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Chemical Composition and Antibacterial Activity of Essential Oil of *Callistemon citrinus* from Ethiopia

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Authors' contributions

This work was carried out in collaboration between both authors. Author NA designed the study, performed the statistical analysis and wrote the protocol. Author SY managed the literature searches and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The present study aimed to determine the chemical components and antibacterial activities of essential oil of the leaves of *Callistemon citrinus* collected from Ethiopia.
Methodology: For identification and determination of the phytochemical constituents of the oil, Gas chromatography and Gas-chromatography/Mass-spectrometry analysis was carried out. Agar disc diffusion method was also employed to evaluate the antibacterial activities of the oil.
Results: A total of fifteen phytochemical constituents were identified. The major constituents of the oil were found to be 1, 8-cineole (76.9%) and α-terpineol (15.3%) of the total composition. The *In vitro* antibacterial activity of the oil against *Salmonella typhi, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* exhibited significant growth inhibition. The highest zone of inhibition was obsreved against *Salmonella typhi* (27.93±2.10 mm) followed by *Staphylococcus aureus* (23.83±2.75 mm) at tested dose of 50 mg/ml while the positive control Gentamycine at the same dose showed inhibition zone of (15.00±1.33 mm) and (13.25±1.25 mm) respectively.
Conclusion: Essential oil of Ethiopian *Callistemon citrinus* is reach of biologically active ingredients and posses potent antibacterial activities.

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Keywords: Antibacterial activities; Callistemon citrinus; essential oil; GC-MS analysis; in vitro.

1. INTRODUCTION

The negative side effects and the emergence of multi-drug resistant bacterial pathogens against the existing antibiotics are the current global challenge. Therefore, there is a need to develop safe, highly effective and broad-spectrum antimicrobial agents that possess potent biological activities with novel mode of action [1]. The use of traditional medicinal plants to satisfy the need is thus an alternative and promising hope [2-4]. Reports showed that over 50% of currently available clinical drugs are of natural product origin mainly from plants [5]. Essential oils derived particularly from aromatic plants is taking the upper most growing area of interest in the formulation of alternative antimicrobial drugs because of the potent biological activities they possess [6,7].

Callistemon citrinus, which is commonly known as 'Crimson Bottle Brush' is an evergreen shrub belonging to the Family Myrtaceae [8,9]. It is a woody aromatic tree and the leaf of the plant is commonly used as tea substitute and has a refreshing flavor [10]. Moreover, traditional healers use the aerial parts of the plant for the treatment of different illness. Egyptians are using the plant volatile oils as antimicrobial and antifungal agents. They are also applying it for the treatment of cough, bronchitis and insecticidal purposes [11,12].

Studies on the chemical compositions of essential oil of the leaves of *C. citrinus* from South Africa, Iran, Cameroon and Reunion Island revealed, the major constituent was found to be 1,8-cineole and accounted from 47.9 to 82.0% of the oil [13-16]. It is a volatile monoterpene that have been elucidated with various pharmacological effects and it is commonly used as a marker for medicinal classification of an essential oil [17]. Similarly, *In vitro* antibacterial activity of essential oils of the leaves of South African, Iranian, Egyptian and Reunion Island *C. citrinus* exhibited strong zone of inhibitions against both gram (-) and gram (+) species of pathogenic bacteria [11,16-18].

Becuse of the bright red flowering spike appearance of the plant, it is mostly used as decorating purposes of the house, offices, streets and gardens in Ethiopia. Beyond decoration, traditional healers use the areal part of the plant for the treatment of several complients and it is one of the common homeguarden plant species identified [19]. However, to the best of our knowledge, no research work was done and reported on the biochemical composition and antimicrobial activity of essential oils of *Callistemon citrinus* growing in Ethiopia. This study therefore aimed to identify the major biochemical constituents and determine antimicrobial activities of essential oil of the leaves of the plant from Ethiopia.

2. MATERIALS AND METHODS

2.1 Plant Collection and Authentication

Fresh leaves of *Callistemon citrinus* (var. splendens) were collected from Northern Ethiopia during the beginning of its flowering period in March 2014. Specimen of the plant was identified and authenticated at the Ethiopian National Herbarium, Addis Ababa University. A voucher specimen labeled (FM155) was prepared and deposited in the Herbarium for future references.

2.2 Extract Preparation of the Plant Material

The fresh leaves of the plant were cleaned, cut in to pieces and air dried under shad at room temperature for 10 days. 300 gm of the dried *C. citrinus* leaf was ground in to a coarse powder. It was then hydrodistilled using a Clevenger type apparatus for 3 hours. Essential oil of the plant material was extracted and then subjected to dry over anhydrous Na_2SO_4 to remove any trace water and stored at 4℃ until further biochemical analysis were undertaken.

2.3 Physicochemical Determination of the Oil

Specific gravity, refractive index and optical rotations of the oil were measured using a Fisher and Davidson gravimeter, Abbe's refractrometer and Bellingham and Stainly polarimeter respectively. Acid and saponification values were also carried out according to the standard methods described by Paudyal et al. [20].

2.4 Gas-Chromatography (GC) Analysis of the Oil

The GC analysis of the volatile oil was carried out on an Agilent 6890N GC equipped with flame

ionization detector (FID) and DB-5 column (30 mm x 0.25 mm, 250 µm film thickness). The column oven temperature was hold at 70℃ for 5 minutes followed by a ramp of 5℃/minute to 120℃. Then, a second ramp to 280℃ at 10℃/min. This final temperature of 300℃ was held isothermally for 5 min. Injection and detector temperatures were 250℃ and 300℃ respectively. Helium constant flow of 1.5 ml/min was used as the carrier gas. Diluted sample oil (0.01, V/V in mehtylenechloride) of 1.0 µL was injected manually and in splitless mode. Peaks were measured by electronic integration. n-hexane was run under the same condition for Kováts indices determination.

2.5 Gas-Chromatography/ Mass-Spectrometry (GC/MS) Analysis of the Oil

GC-MS analysis of the oil was performed on an Agilent 6890N GC with 5975 inert MSD system (software; MSD chemstation D.02.00.275 with NIST Library Ver.2.0 d) equipped with a HP 5-MS capillary column (30 mm x 0.25 mm id, film thickness 250 µm). Column and oven temperature program was the same as previously used in GC analysis. An electron ionization system, with ionization energy of 70 eV was used. The ion source and MS transfer line temperatures were set at 150℃ and 280℃ respectivelly. Helium was also used as the carrier gas (1 ml/min) with the scan range of 50 to 1000 amu.

2.6 Identification of Compounds

Identification of compounds done based on the comparison of their retention times to *n*-hexane, compared to published data and spectra of authentic compounds [21,22]. They were further identified and authenticated using their mass spectra compared to the Wiley version 7.0 N libraries and literature data. The results then confirmed by the comparison of the elution order of the components with their relative retention indices on non-polar phases reported in the literature.

2.7 Bacterial Strains

For the antibacterial test of the oil, four bacterial species namely; *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (ATCC 14028) and *Staphylococcus aureus* (ATCC 25923) were used after they identified and separated from a mixed culture according to the standard procedures [23]. Pure culture inocula then prepared by taking well isolated colonies of the same morphology from an overnight agar plate culture. The top of each colony was touched with sterile standared inoculating loop and the actively growing broth culture was adjusted with sterile saline solution to obtain turbidity comparable to that of the 0.5 McFarland standards.

2.8 Antibacterial Screening

Agar disc diffusion method was employed for the determination of antibacterial activities of essential oil of the leaves of *C. citrinus* according to the method described by Berghe and Vlietinck, [24]. The inocula of the bacterial strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 Mc (corresponding to 10^7-10^8 CFU/ml) Farland standard turbidity. Then, each inoculate of the respective bacteria were spread in such a way as to ensure through coverage of the plates and uniform thick lawn of growth following incubation. A sterilized disc of 6 mm diameter was placed on agar plates using a sterile cork borer. Essential oil was diluted in ethanol to the test concentrations of 0.5 mg/ml, 5 mg/ml, 10 mg/ml, 50 mg/ml and 100 mg/ml. Finally, 10 µl of the test oil was applied on the discs and the plates were incubated at 37℃ for 24 hrs. Gentamycin (50 mg/ml) and ethanol were used as positive and negative control standards respectively. Each test were performed in three triplicates and repeated three times. To evaluate the degree of antibacterial activity of the oil, sensitivity test was employed by measuring the diameter of zone of inhibition formed against the test bacteria.

3. RESULTS AND DISCUSSION

3.1 Physicochemical Properties of the Oil

Hydrodistillation of fresh leaves of *C. citrinus* gave a yield of 0.73% (w/w) volatile oil on the dry weight basis. The oil was characterized by strong pungent odor, colorless appearance and soluble in organic solvents. Other physicochemical parameters such as refractive index, density, specific gravity, specific optical rotation, boiling point, acid value, ester and saponification values of the oil were also determined and presented in Table 1.

Table 1. Physicochemical properties of essential oils of the leaves of *C. citrinus*

Parameters	Mean value
Yield	0.73% (w/w)
Color	Colorless
Odor	Pungent
Boiling point	97℃
Solubility	Soluble in organic solvents
Density (g/cm^3)	0.8800
Refractive index (25°C)	1.4549
Specific optical rotation (25℃)	23°.4375'
Specific gravity (23℃)	0.8812 ± 0.01
Acid value	4.37 ± 0.16
Saponification value	14.025 ± 0.236
PH (23.5℃)	4.28
Ester value	9.655 ± 0.396

3.2 Chemical Composition of the Oil

Fifteen compounds which comprised 98.0% of the total oil composition were identified. The main constituents of the oil were found to be 1, 8-cineole (76.9%) and α-terpineol (15.3%) followed by terpinen-4-ol (1.9%) and 2-methylbutyl isobutyrate (1.2%). The oil was dominated by oxygenated monoterpenes and accounted 95.1% of the total oil composition. The remaining constituents are monoterpene hydrocarbons (0.8%), sesquiterpenes (0.5%) and other trace components accounted a sum total of 1.6%. With the Retention index and area percentages, the chemical constituents of the oil are presented in Table 2.

Essential oil extracted from the leaves of Iranian *C. citrinus* identified with 36 components and the major compounds were found to be 1, 8-cineole (34.1%), α-pinene (29.0%) and α-terpineol (10.7%) [16]. Similarly, in the essential oil of the same plant material from South Africa, 24 components were identified with 1, 8-cineole (61.2%) and α-pinene (13.4%) were being the predominant compounds [17]. The volatile oil composition of the leaves of *C. citrinus* from Reunion Island were also evaluated and 1, 8-cineole (68.3%) and α-pinene (18.7%) were found to be the major compounds [18]. Although the essential oil of leaves of *C. citrinus* from different countries have been studied and found with same major components with herein study, there were found with slight variations in the amount yield of the oil and percentage composition of the constituents. This variation could be attributed to the difference in generic, geographical and environmental determinants such as soil type, temperature, weather condition and location [17].

The abundance of 1, 8-cineole in the essential oil of the leaves of *C. citrinus* make the present study similar to the report from Iran, South Africa, Reunion Island and Himalayas [16-18,25]. α-pinene was the second to third abundant compound identified from essential oil of the same plant material in the previous studies, however, it was not detected in the essential oil of Ethiopian *C. citrinus*.

Table 2. Composition of *C. citrinus* leaf essential oil identified by GC, GC-MS analysis method

Compound's name	RI $_{Lit}$	RI $_{Exp}$	Composition (%)
2-methylbutyl isobutyrate	nf	965	1.2
1, 8-cineole	1031	1028	76.9
3-Carene	1009	1012	0.1
α-Terpinene	1018	1015	0.5
Terpinolene	1086	1088	0.2
Fenchol	1117	1119	0.1
Trans-pinocarveol	1133	1131	0.8
pinocarvone	1156	1148	0.1
4-isopropyl-6-methylcyclohex-2-enone.	nf	1139	0.1
Terpinen-4-ol	1177	1177	1.9
α-Terpineol	1189	1183	15.3
5-isopropenyl-2-methyl-2-cyclohexen-1-yl acetat	1362	1366	0.2
1-cyclopentylethanone	933	927	0.2
(-)-Spathulenol	1578	1575	0.2
α-Guaiene	1453	1449	0.3
Total			**98.0**

RI_{Lit} = Retention Index from Literature; RI_{Exp}= Retention Index from experiment; nf = not found
Even-though retention index experimental was not exactly similar with that of described in literature, the fragmentation confirmed these components

Table 3. Antibacterial sensitivity test of *C. citrinus* leaf essential oil (zone of inhibition in mm)

Oil concentration (mg/ml)	Test bacteria			
	Salmonella typhi	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Escherichia coli***
0.5	13.4 ± 1.2	---	---	---
5	19.7 ± 2.1	14.5 ± 1.4	---	11.2 ± 0.8
10	20.7 ± 2.4	15.7 ± 1.5	9.7 ± 1.5	13.8 ± 0.7
50	27.9 ± 2.1	23.8 ± 2.8	16.4 ± 0.5	18.3 ± 0.8
100	39.1 ± 3.6	30.3 ± 2.5	17.0 ± 2.5	21.1 ± 1.3
Gentamycine (50 mg/ml)	15.0 ± 1.3	13.2 ± 1.2	33.3 ± 0.2	35.5 ± 0.7

3.3 Antibacterial Assay

Antibacterial activity of the oil carried out using agar disc diffusion method and different level of dose dependent antibacterial sensitivity were recorded against both gram (+ve) and gram (-ve) species of the test bacteria. The highest antibacterial activity was observed on *S. typhi* (39.13±3.60 mm) followed by *S. aureus* (30.33±2.52 mm) at tested dose of 100 mg/mL and the least effect was recorded on *P. aeruginosa* which was totally showed no response at 0.5 and 5 mg/ml concentrations of the oil. This is in consistence with Oyedeji et al. [17]; Haque et al. [13] study who reported that *S. aureus* was found to be more sensitive and *P. aeruginosa* was relatively resistant bacterial species against essential oil of the same plant material from South Africa and Bangladesh respectively.

Although, plant extracts are known to be more active against gram positive than gram negative bacteria [11,13,26], the present study revealed, essential oil of the leaves of Ethiopian *C. citrinus* exhibited broad spectrum antibacterial effects against both groups of bacteria. However, the highest bacterial growth inhibition of the oil recorded on the gram negative test bacterium; *S. typhi*. The antibacterial action of the oil against the remaining gram negative tested bacteria (*E. coli* and *P. aeruginosa*) in contrary were found lower than the gram positive tested bacterium *S. aureus* (Table 3). The report by Oyedeji et al. [17]; Haque et al. [13] also deduced the same result of herein study in which effect of the same oil against *E. coli* and *P. aeruginosa* were found low and the effect against *S. aureus* were the highest.

The presence of 1, 8-cineole in the essential oil of *C. citrinus* leaf can confirm the medicinal potential of the plant. Because it is used as a marker for medicinal value of essential oils clasiffication [17]. The antibacterial activity demonstrated in the essential oil of this plant material thus could be implicated with the presence of 1,8-cineole and α-terpineol along with 2-methylbutyl isobutyrate, α-terpinene, terpinen-4-ol, and trans-pinocarveol that are found to be major constituents of the oil. Several previous studies reported these constituents to have antimicrobial activities [17,18,27] yet, trace constituents could also play a role in the sum total antibacterial effect of the oil [17].

4. CONCLUSION

Essential oil of the leaves of Ethiopian *C. citrinius* is rich of biologically active compounds that have potent antibacterial activities. During each antibacterial assay, the growth of test bacteria were significantly inhibited. Better zone of bacterial growth inhibition was recorded against the oil than the standard control antibiotic Gentamycin. It is thus, one scientific evidence to elucidate the high potential source of the plant in the formulation of the near future new antibacterial agents.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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