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Essential Oils Composition, Antibacterial Activity and Toxicity Study of *Artemisia* Species Growing in Ethiopia

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Abstract

Artemisia essential oils have been used in flavors, scents, mice repellents, cleansers, beauty care products, fragrances, and conventional pharmaceuticals. This work analyzed the phytoconstituents, *in vitro* antibacterial activity, and toxicity of essential oils of three *Artemisia* species growing in Wolaita, Ethiopia. GC-MS has been used to analyze chemical composition. *In vitro* antibacterial activity was assessed by disc diffusion and broth dilution techniques. The MTT assay was used to conduct toxicity studies. The main components of the essential oil extracted from *Artemisia* afra Jacq. ex Willd. were camphor (19.2%), followed by 1,8-cineole (17.8%), artemisia ketone (15.2%), artemisyl acetate (6.9%), and camphene (6.6%). In the *Artemisia* annua L. oil, the main constituents were camphor (21.8%), artemisia alcohol (18.1%), santolina triene (11.6%), -copaene (9.1%), sabinene (4.5%), and δ-cadinene (4.0%). In addition, the main phytochemicals of *Artemisia abyssinica* Sch.Bip. ex A.Rich. oil were yomogi alcohol (39.3%), followed by cis β-farnesene (10.9%), β-selinene (6.7%), β-copaene (5.2%), and nerolidol (5.1%). The essential oils of *A. afra*, *A. annua*, and *A. abyssinica* showed significant antibacterial activity at low molar concentrations, with MIC values of 0.31, 0.15, and 0.62 µL against Staphylococcus aureus and 0.62, 0.31, and 1.25 µL against *E. coli*, respectively, compared with standard broad-spectrum gentamicin (0.25 µL). *A. afra*, *A. annua*, and *A. abyssinica* oils were showed weak toxicity to Vero cell lines with IC_{50} values of 178.47 µg/mL, 183.86 µg/mL, and 187.46 µg/mL, respectively. This result suggests that EOs are promising antibacterial agents given their weak toxic effects on normal cell lines at low concentrations.

Keywords: *Artemisia afra*; *Artemisia annua*; *Artemisia abyssinica*; Essential oils; Antibacterial activity; Toxicity study

Introduction

In primary health care, medicinal plants have played a crucial role. They offer rich raw materials for novel bioactive compounds in new drug discovery and development research [1]. Genus *Artemisia* (Asteraceae) comprises 500 species. They are widely used in many parts of the world, either alone or in combination with other plants, as herbal remedies for various human diseases, notably in the treatment of malaria [2]. *Artemisia* essential oils (EOs) have been used for flavors, fragrances, mice repellents, detergents, cosmetics, perfumes, and traditional medicine [3]. *Artemisia afra* Jacq. ex Willd., 'Agguppiya' (Wolaitigna), 'Kapani' (Oromffa), and 'Kodo' (Guragigna), is one of the *Artemisia* species growing in Ethiopia [4,5], and is traditionally used in the treatment of headaches, eye diseases, ringworm, hematuria, stabbing pain, hemorrhoids, mumps, smallpox, malaria, neuralgia, colitis, and liver disorders, infertility, febrile illness, common cold, epilepsy, roundworm, and stomach pains [5,6].

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Artemisia annua L. is a fragrant annual herb that can reach a height of 2 m [3]. Chinese traditional medicine treats malaria, hemorrhoids, and fever [7]. An experimental basis, *A. annua* is cultivated in Ethiopia and several other African countries [4]. *A. annua* yields artemisinin, and its derivatives are potent antimalarial drugs [8]. *A. annua* has been shown to have growth inhibitory effects on parasites, viruses, fungi, and bacteria. It also possesses anti-inflammatory and anticancer properties and treats osteoarthritis, leukemia, and hepatoma [9].

Artemisia abyssinica Sch.Bip. ex A.Rich., "Chikugn" (Amharic), is an aromatic, grey, and silky-hairy plant. It has antioxidant, antileishmanial, and antitrypanosomal properties in its EOs [3]. It is well-known as a stimulant and an analgesic. It is also used to treat intestinal problems, infectious diseases, and leishmaniasis in traditional medicine [10]. The whole herb can be used to address tonsillitis, and an infusion is used to treat colds and influenza. In folk medicine, the plant has also been used as an anthelmintic, antispasmodic, antirheumatic, and antibacterial agent [11].

The *Artemisia* species are widely used in traditional medicinal practice in Ethiopia. However, comprehensive studies on the above species have not been carried out yet. Thus, this study aimed to look into the essential oil compositions, *in vitro* antibacterial activities, and cytotoxicity of three *Artemisia* species from Ethiopian flora.

Methods

Plant Collection

Aerial parts of *A. afra*, *A. annua*, and *A. abyssinica* were collected from Wolaita, Ethiopia, in December 2021. A Botanist, Shambel Alemu, authenticated collected plant specimens at the National Herbarium of Ethiopia, Addis Ababa University, Ethiopia (Voucher codes: M001/12, M005/12, and M003/12, respectively). Freshly collected plant samples were washed in tap water to remove unwanted foreign materials, then grounded into powder using a mechanical grinder. The powdered samples were subjected to extraction by hydrodistillation.

Essential Oil Extractions

A powder of *A. afra*, *A. annua*, and *A. abyssinica* (500 g each) was separately extracted successfully by hydrodistillation using a Clevenger-type apparatus for 6 hours. The EOs were separated from the aqueous layer using a separatory funnel and dried with anhydrous magnesium sulphate ($MgSO_4$).

Gas Chromatography-Mass Spectrometry

A Gas Chromatography-Mass Spectrometry (GC-MS) examination was performed by GC (7890A, Agilent-Technologies, USA) coupled with MS (5977B, Arrange Agilent-Technologies). The GC column wasan HP-5MS with a length of 30 m, a diameter of 250 m, and a film thickness of 0.25 m coated with 5% phenyl and 95% methylpolysiloxane stationary phase. The mass spectrometer was linked with a computer-fed mass spectra data bank. About 2 µL of the essential oil in chloroform was injected through an autosampler and analyzed with the HP-5MS column. The carrier gas was helium streaming at a 1 mL/min rate. The injector temperature was fixed at 230 °C, and the injection mode was changed to spilled mode with a ratio of 10:1. The initial oven temperature was 40 °C with a 5 minute hold time and raised to 250 °C at 6 °C per minute. It was held at this temperature for 20 minutes, and the total run time was 6 minutes. Mass spectra were recorded in electron impact mode at 70 eV, scanning from 40 to 500 m/z in 0.5 seconds. Phytochemicals in the samples were identified based on comparing their retention time (min), retention index relative to the alkanes of the C7-C30, and mass (m/z) spectral patterns with those spectral databases of authentic compounds stored in the National Institute of Standards and Technology (NIST) library. Finally, each component's calculated retention index (RI*) was evaluated based on the retention time and given retention index (RI) [12].

Antibacterial Activity

The assay of EOs was evaluated by the disc diffusion method as previously described [13,14], with minor modification, against two human pathogenic bacteria strains (*S. aureus* 25923 and *E. coli* 20922). Sterile discs were impregnated with 2.5, 5, 10 µL of oil in 10 µL of dimethylsulfoxide (DMSO), then were put on cultured plates. A disc containing DMSO and gentamicin was used as a control. The treated Petri dishes were incubated at 37°C for 24 h. The antibacterial effects of EOs were determined by measuring the growth inhibition zone around the discs. The experiments were carried out in triplicate.

Determination of Minimum Inhibitory Concentrations

The minimal inhibitory concentration (MIC) was assessed using the broth dilution method [13]. The EOs were dissolved in 10% dimethyl sulfoxide and added to the Mueller Hinton broth to grow the bacteria. Final concentrations of EOs (5 to 0.015 µg/mL) were prepared and dispersed in 96-well microplates. In each test, the media was added to each growth control well. The MIC values of EOs samples were recorded as the lowest concentration of the EOs that inhibited bacterial growth after incubation for 24 h at 37 °C. The antibiotic gentamicin was used as a positive control, and the medium without EOs was used as a negative control.

Cell lines

The toxic effect of EOs on the cells was assessed using the kidney normal cell lines of monkeys (VERO cells) obtained from the national animal health diagnostic and investigation center, Ethiopia. The cells were grown in RPMI-1640 media supplemented with 10% FBS, 2 raM glutamine, 100 units/mL penicillin, and 100 g/mL streptomycin. The cells were cultured at 37°C in a humidified 5% CO2 incubator.

Toxicity Studies by MTT Assay

The *in vitro* cytotoxic effect of EOs was investigated using the MTT assay as described previously [15,16]. The Vero cell lines were seeded at a density of $2x10^4$ cells/well on a 96-well flat-bottom microtiter plate and allowed to grow for 24 h at 37°C in a CO_2 incubator. The culture medium was replaced with a new medium after 24 h of incubation. Then, the cells were treated with the concentration of EOs (100 to 0.78 µg/mL half-fold serial dilution) for 24 h at 37°C in a CO_2 incubator. The culture medium was replaced with a fresh medium after 24 h of incubation. The plate was then incubated for 4 hours at 37°C in a CO_2 incubator with 10 µL of MTT working solution (5 µg/mL in phosphate buffer solution) added to each well. The medium was then aspirated, and the produced formazan crystals were solubilized in 50 µL of 5% DMSO per well in a CO_2 incubator for 30 minutes at 37°C. Finally, the dissolved formazan crystals (purple color) were quantified using the ELISA plate reader at 530 nm. The result was expressed as an IC_{50} value. IC_{50} values were obtained from a dose-response curve of the percentage of cell viability (Y-axis) versus log EOs concentration (µg/mL) (x-axis) by using GraphPad Prism Version 8.0. All experiments were carried out in triplicate.

Results

Chemical compositions analysis

The EOs were obtained by hydrodistillation with a percentage yield of 1.5, 2.6, and 1.2% (v/w) from the areal parts of *A. afra*, *A. annua*, and *A. abyssinica*, respectively. A total of 25 chemical compositions were determined in the GC-MS analysis of *A. afra* EOs, accounting for 99.7% of the overall compositions. Oxygenated monoterpenes were the primary chemical ingredients, followed by sesquiterpenes (24%), monoterpenes (20%), oxygenated sesquiterpenes (8%), and others (8%) (Figure 1). The major individual constituents in *A. afra* oils were camphor 1(19.3%), followed by 1,8-cineole 2 (17.8%), *artemisia* ketone 3 (15.2%), artemisyl acetate 4 (8.9%), and camphene 5 (6.6%). The remaining compounds were identified at 0.4 to 3.5% (Table 1, Figure 2). The previous reports revealed that the EOs of *A. afra* grown in Ethiopia differed in major chemical constituents from different localities [3,17] and other parts of the world [18,19].

The GC-MS analysis of *A. annua* EOs has identified 24 chemical components, which accounted for 99.9% of the overall compositions. Oxygenated monoterpenes (37.5%), monoterpenes (25.0%), sesquiterpenes (20.8%), oxygenated sesquiterpenes (4.2%), and others (12.5%) were the chemical classes of *A. annua* oil (Figure 3). The main individual constituents were camphor 1 (21.8%), *artemisia* alcohol 6 (18.1%), santolina triene 7 (11.6%), α-copaene 8 (9.1%), camphene 5 (4.5%), sabinene 9 (4.4%), and δ-cadinene 10 (4.0%). The remaining compounds were observed from 0.4 to 3.5% (Table 2, Figure 4). The major components of the EOs of *A. annua* were camphor, *artemisia* ketone, 1,8-cineole, and α-pinene from species growing in different geographic locations [20]. Our results were comparable to previously reported EOs of *A. annua* originating from Ethiopia [17] and other parts of the world [21,22].

In the same analysis, *A. abyssinica* EOs revealed 21 compositions, accounting for 99.1% of the overall EO content. Sesquiterpenes (57.1%) were the most abundant chemical constituents in *A. abyssinica* EO, followed by oxygenated monoterpenes and monoterpenes (14.3%), oxygenated sesquiterpenes (9.5%), and others (4.8%) (Figure 5). The principal individual chemical constituents were yomogi alcohol 11 (39.3%), followed by cis- β-farnesene 12 (10.9%), β-selinene 13 (6.7%), germacrene D 14 (5.4%), β-copaene 15 (5.2%), and nerolidol 16 (5.1%). The presence of other chemical components ranged from 0.8 to 3.7% (Table 3, Figure 6). The EO compositions of *A. abyssinica* reported from different localities of Ethiopia were consistent with our results [23,24]. But, the main chemical components of *A. abyssinica* EOs in the case of Europe and different African countries are varied.

Antibacterial Studies

Many studies have been conducted on the antimicrobial activity of natural products, including EOs of certain plants, due to the current emergence of antibiotic drug resistance and undesirable side effects [25]. According to research, the primary chemical constituents of EOs and their synergistic effects may be responsible for their bioactivity. The antibacterial activities of EOs were associated with oxygenated terpenoids such as alcohols, ketones, and monoterpene hydrocarbons [26]. The assay of studied EOs displayed inhibition of growth of tested bacterial stains in different concentrations. The EOs extracted from *A. afra*, *A. annua*, and *A. abyssinica* had zones of inhibition on *S. aureus* ranging from (23.5±0.95-10.9±1.10 mm), (25.2±0.64-15.0±0.80 mm), and (11.2±1.10-3.0±0.12 mm), respectively, when compared to the standard broad-spectrum

Table 1. Chemical composition of the EOs of *A. afra*

NO	Compounds	*A. afra*		
		RI	RI*	*% Area*
1	Santolina triene	903	904	2.2
2	α-Pinene	917	921	2.9
3	Camphene	955	952	6.6
4	Sabinene	975	978	2.4
5	p-Cymene	1024	1023	1.9
6	Artemisia ketone	1063	1060	15.2
7	1,8-Cineole	1070	1068	17.8
8	Artemisia alcohol	1071	1071	2.9
9	α-Thujone	1104	1103	2.5
10	β-Thujone	1115	1112	0.7
11	Camphene hydrate	1135	1134	2.2
12	Camphor	1150	1149	19.2
13	Borneol	1166	1163	0.9
14	Artemisyl acetate	1180	1178	6.9
15	Geraniol	1259	1234	1.4
16	Methyl cinnamate (Z)	1317	1315	2.3
17	2-Phenylpropanoate	1353	1350	1.0
18	α-Copaene	1375	1372	1.5
19	α-Patchoulene	1464	1460	1.2
20	δ-Muurolene	1475	1472	0.7
21	Germacrene D	1478	1475	1.5
22	β-Selinene	1480	1479	1.2
23	γ-Muurolene	1492	1491	1.4
24	Davanone B	1569	1568	1.2
25	β-Eudesmol	1672	1671	2.0
	Total identified			99.2

RI= retention index reported; RI*= retention index calculated

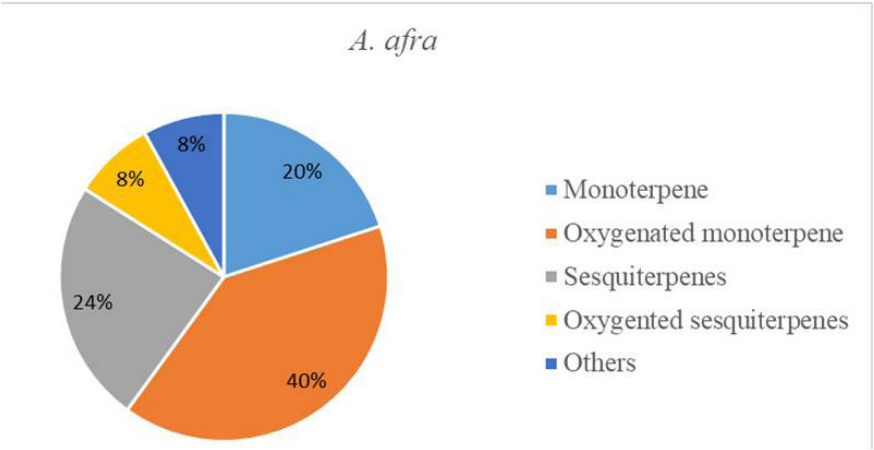

Figure 1. Classes of chemical constituents EOs of *A. afa* species

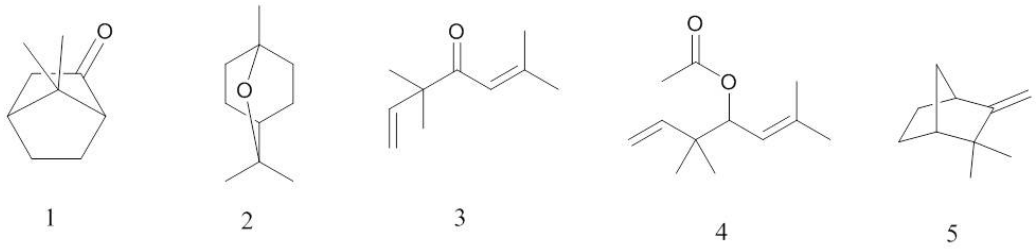

Figure 2. Major chemical constituents EOs extracted from *A. afa*

Table 2. Chemical composition of the EOs of *A. annua*

NO	Compounds	*A. annua*		
		RI	RI*	Area (%)
1	Santolina triene	903	904	11.6
2	α-Pinene	917	921	1.5
3	Camphene	955	952	4.5
4	Sabinene	975	978	4.4
5	p-Cymene	1024	1023	1.7
6	Limonene	1029	1027	1.1
7	1,8-Cineole	1070	1068	1.3
8	Artemisia alcohol	1071	1071	18.1
9	Linalool	1086	1083	0.9
10	Camphene hydrate	1135	1134	1.5
11	Camphor	1150	1149	21.7
12	Terpinen-4-ol	1164	1166	0.8
13	Borneol	1166	1163	0.8
14	D-Carvone	1218	1217	0.7
15	α-Thujenal	1269	1268	0.4
16	Cuminal	1273	1270	0.5
17	Lavandulol acetate	1290	1291	1.3
18	Lebaicone	1314	1312	0.3
19	α-Copaene	1375	1372	9.1
20	Cis-β-Farnesene	1447	1443	1.8
21	β-Selinene	1480	1479	1.3
22	γ-Cadinene	1505	1503	0.3
23	δ-Cadinene	1513	1510	1.9
24	Davanone	1593	1591	1.2
	Total identified			99.9

Table 3. Chemical composition of the EOs of *A. abyssinica*.

NO	Compounds	*A. abyssinica*		
		RI	RI*	Area (%)
1	α-Pinene	917	921	1.1
2	Yomogi alcohol	1001	1004	39.3
3	p-Cymene	1024	1023	2.9
4	Camphor	1150	1149	0.8
5	Geraniol	1259	1234	4.6
6	Cosmene	1298	1297	1.3
7	Bornyl acetate	1307	1307	3.2
8	β-copaene	1430	1429	5.2
9	Aromadendrene	1444	1442	0.8
10	Cis-β-Farnesene	1447	1443	10.9
11	α-Himachalene	1450	1448	1.8
12	α-Patchoulene	1464	1460	1.9
13	δ-Muurolene	1475	1472	3.7
14	Germacrene D	1478	1475	5.4
15	β-Selinene	1480	1479	6.7
16	ε-Muurolene	1496	1494	1.8
17	γ-Cadinene	1505	1503	1.3
18	Nerolidol	1512	1510	5.1
19	Trans-Calame-nene	1520	1518	1.3
20	Davanone B	1569	1568	0.8
21	Davanone	1593	1591	1.4
	Total identified			99.1

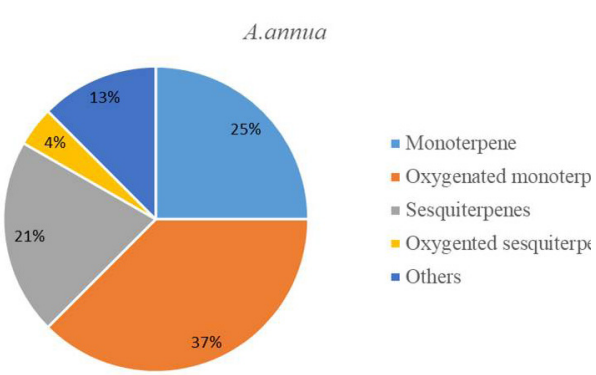

Figure 3. Classes of Chemical constituents EOs *A. annua*.

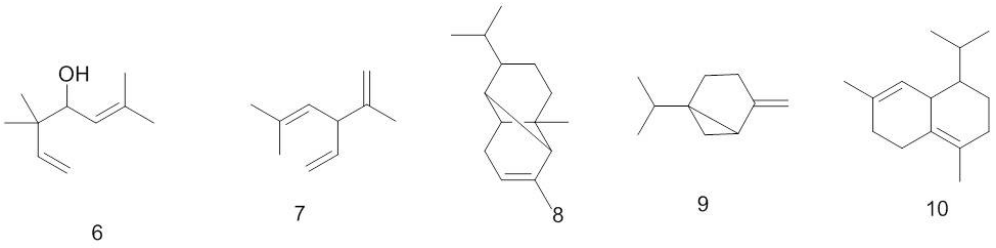

Figure 4. Major chemicals constituents of EOs extracted from *A. annua*

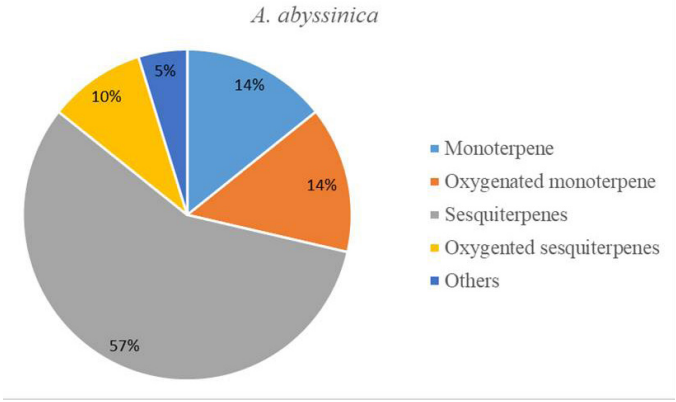

Figure 5. Classes of Chemical constituents EOs of *A. abyssinica.*

antibiotic gentamicin (28±0.12 mm). When compared to the standard broad-spectrum antibiotic gentamicin (24.18 ±0.01 mm), the EOs extracted from *A. afra*, *A. annua*, and *A. abyssinica* had zones of inhibition on *E. coli* ranging from (11.1±0.76–4.7±1.09 mm), (19.4±0.80–6.3±0.60 mm), and (13.8±0.76–6.5±0.86 mm), respectively. At low molar concentration, *A. afra*, *A. annua*, and *A. abyssinica* EOs displayed remarkable antibacterial activity with MIC values of 0.31, 0.15, and 0.62 µL against *S. aureus* and 0.62, 0.31, and 1.25 µL against *E. coli*, respectively, compared with standard broad-spectrum gentamicin (0.25 µL) (Figure 7).

Toxicity study

According to the previous report [16], EOs with IC_{50} values between 10 and 50 g/mL have significant cytotoxic activity, while those with IC_{50} values of between 50-100, 100-200, and 200-300 g/mL have moderate, weak, and very weak cytotoxic qualities, respectively.

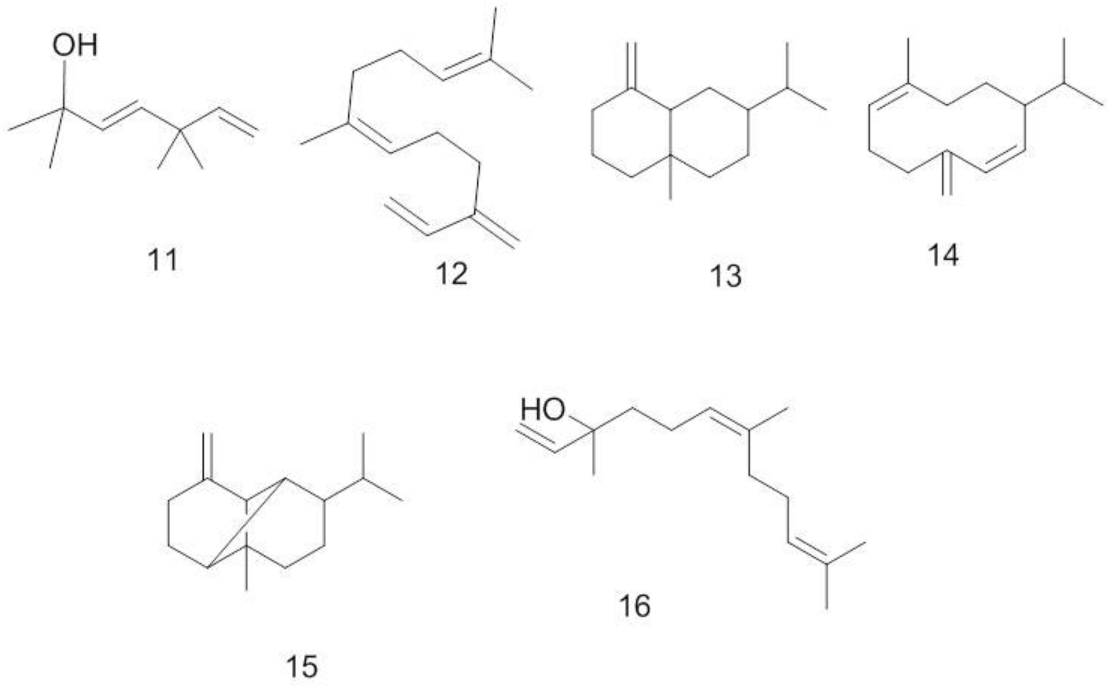

Figure 6. Major chemicals constituents of EOs extracted from *A. Abyssinica*

Table 4. Zone of inhibitions (mm) of EOs of three *Artemisia* species and control (genamicin)

EOs	Concentrations (µL)	Zone of inhibitions (mm)	
		S. aureus	*E. coli*
Artemisia afra	10	23.5 ± 0.95	11.1 ± 0.76
	5	18.0 ± 0.90	9.06 ± 1.35
	2.5	10.9 ± 1.10	4.7 ± 1.09
Artemisia annua	10	25.2 ± 0.64	19.4 ± 0.80
	5	20.0 ± 0.90	14.5 ± 0.51
	2.5	15.0 ± 0.80	6.3 ± 0.60
Artemisia abyssinica	10	11.2 ± 1.10	13.8 ± 0.76
	5	8.2 ± 0.72	9.9 ± 0.90
	2.5	3.0 ± 0.12	6.5 ± 0.86
Gentamicin	5	28 ± 0.12	24± 0.12

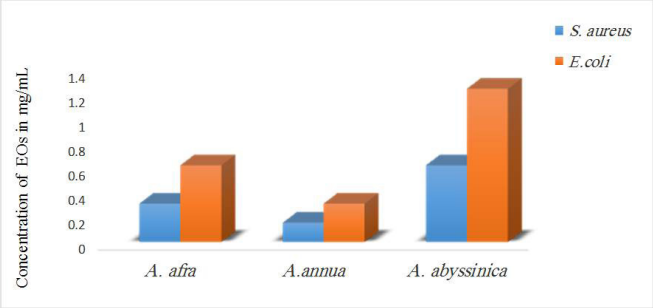

Figure 7. MIC value of EOs of three *Artemisia* species against two bacterial strains

In addition, IC_{50} values greater than 300 g/mL do not indicate cytotoxicity. The cytotoxicity study of the EOs of *A. afra*, *A. annua*, and *A. abyssinica* displayed weak toxicity on the Vero cell line, with IC_{50} values of 178.47 µg/mL, 183.86 µg/mL, and 187.46 µg/mL, respectively.

Discussion

During the last decade, several authors have evaluated the antimicrobial activity of *Artemisia* species and some of its main components. This study emphasized chemical compositions and *in vitro* antibacterial activity of EOs of three *Artemisia* species growing in Ethiopia. The findings revealed that the compositions of EOs obtained from three *Artemisia* species are terpenes (monoterpenes and sesquiterpenes) and oxygenated terpenes. The chemical composition of EOs of *A. afra*, *A. annua*, and *A. abyssinica* are mainly dominated by irregular monoterpenes; yogomi alcohol, artemisyl acetate, *artemisia* ketone, and *artemisia* alcohol. Yogomi alcohol is a principal constituent reported from *A. abyssinica* species, suggesting this compound could be a marker constituent for Ethiopian *A. abyssinica* species. The tested *Artemisia* EOs showed significant antibacterial effect against two bacterial strains (*S. aureus* and *E. coli*); while comparing the activity with standard broad-spectrum gentamicin, which indicated their effectiveness against infectious diseases caused by the microorganisms. The primary chemical constituents in the EOs might be responsible for their antibacterial activity. According to the cell viability assay on the Vero cell line, cytotoxicity increased with increasing concentrations or cytotoxic effects in a concentration-dependent manner.

Conclusions

The oxygenated monoterpenes and sesquiterpene content found in the tested EOs obtained from three *Artemisia* species might be responsible for the observed bioactivity. Hence, the EOs could serve as good ingredients in the development of products for infectious diseases and suggest the potential use of EOs as medicine, which would corroborate the traditional benefits of the plant because of their weak cytotoxic effect at lower concentrations and antibacterial activities. However, further in vivo studies on safety should be carried out.

Conflict of Interests

None.

Acknowledgments

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