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Volatile constituents and antimicrobial activity of *Vinca major* L. subsp. *hirsuta* (Boiss) stearn grown in Turkey

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ABSTRACT: In this study, volatile compounds (VCs) in the essential oil (EO), SPME and SPME of *n*-hexane extract of *Vinca major* subsp. *hirsuta* (Boiss.) Stearn were analyzed by GC-FID/MS instrument. A total of 32, 38, and 26 compounds with in the 98.5%, 98.6%, and 98.6% were identified, respectively. As a result of VCs study, (*Z*)-3-hexenol (36.8%) in the EO, 1,3,5-trimethylbenzene (31,5%) in the SPME, and phenylethyl alcohol (32.8 %) in the SPME of *n*-hexane extract of *V. major* subsp. *hirsuta* were found to be major compounds. Sesquiterpenes (20.6%, and 11.5%) for the HD and SPME, monoterpenes (18.6%) for the SPME of *n*-hexane extract of *V. major* subsp. *hirsuta* were found as the main groups among the terpenic compounds, respectively. Then, the antimicrobial activity of the EO and the solvent extracts (*n*-hexane, acetonitrile, methanol, and water) of *V. major* subs. *hirsuta* against 3 gram negative, 3 gram positive, 1 tuberculosis and 2 fungus were screened. The EO showed the only activity against the *Mycobacterium smegmatis* ATCC607 (MIC, 152 µg/mL) and *Candida albicans* ATCC60193 (MIC, 38 µg/mL). The *n*-hexane extract did not show any activity against all tested microorganisms. The best antimicrobial activity for the acetonitrile, methanol, and water extracts were observed against *M. smegmatis* with 69 µg/mL, 609 µg/mL, and 437 µg/mL MIC values, respectively. None of the extracts were found to be active to *Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, and *Saccharomyces cerevisiae* RSKK 251.

KEYWORDS: *Vinca major* subsp. *hirsuta*; Volatile constituents; Antimicrobial activity; SPME; GC-FID/MS.

1. INTRODUCTION

The genus *Vinca* L. (Apocynaceae) contains perennial subshrubs or herbaceous species distributed from Europe to North-west Africa, and South-west Asia. In the flora of Turkey, four species of the genus *Vinca* grow wildly including *Vinca major* [1, 2]. *Vinca* species are grown as ornamental plants and leaves of the *Vinca major* are used as diuretic, against constipation, appetizing, and antipyretic in Turkey [3]. Antimicrobial [4], antioxidant [5], antidiabetic [6, 7], and anti-diarrheal properties [8] of *Vinca* species extracts were mentioned.

Volatile component analysis for the aerial parts and leaf of *Vinca rosea,* and *Vinca difformis* [4, 9-11] and *in vitro* α-glucosidase, glucoamylase, antimicrobial and anti-proliferative activities of *Vinca rosea, V. major*, *Vinca herbacea*, and *Vinca minor* has been reported, respectively [6, 12]. Phenolic composition and antioxidant activity for the methanol extracts obtained from the flower, leaf, and stem of *V. major* subsp. *hirsuta* had mentioned. Leaf of the plant had given the highest antioxidant activity. Five phenolic compounds were reported in all extracts of *V. major* subsp. *hirsuta* [13]. However, to the best of our knowledge of literature survey, no data about volatile composition in the EO, SPME and SPME of *n*-hexane extract and antimicrobial activities (EO and solvent extracts) of *V. major* subsp. *hirsuta* have been reported up to date. The purpose of this study was to evaluate the extent of the variations for the VCs and antimicrobial activities for the EO and solvent extracts (*n*-hexane, acetonitrile, methanol, and water) obtained from the aerial part of *V. major* subsp. *hirsuta*. This article presents first report on volatile chemical evaluations and antimicrobial activities for the *V. major* subsp. *hirsuta* grown in Turkey.

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2. RESULTS

2.1. Chemical composition of the EO, SPME and SPME of n-hexane extract

Volatile components in the EO, SPME and SPME of *n*-hexane extract of the *V. major* subsp. *hirsuta* were analyzed by GC-FID/MS using Rxi-5MS column. Identification of the VCs made by a typical library search and literature comparison [14-22]. The chemical profile of volatiles, the percentage content, and calculated retention indices of the constituents are summarized in table 1.

Table 1. Volatile compounds of *V. major* subs. *hirsuta* species growing in Turkey.

Compounds	RI*	RI[a]	HD[b]	SPME[c]	SPME[d]
				(%)[e]	
Hexanal	803	800	0.8	-	-
(*Z*)-3-Hexenol	858	861	36.8	1.0	1.5
4-Methyloctane	864	867	-	-	0.5
1-Hexanol	863	869	17.4	-	-
p-Xylene	878	877	-	0.9	-
o-Xylene	894	891	-	0.9	-
2-Heptanol	894	892	-	-	1.1
Cumene	929	929	-	0.1	-
α-Pinene	940	938	0.2	-	0.5
3-Ethyl-2-methylheptane	942	940	-	0.1	-
(*E*)-2-Heptenal	954	956	-	-	0.5
Propylbenzene	958	957	-	1.4	-
4-Methylnonane	961	959	-	0.1	-
1-Ethyl-3-methylbenzene	963	964	-	9.8	-
Benzaldehyde	960	965	-	-	4.0
1-Ethyl-2-methylbenzene	983	980	-	5.1	-
3-Octanone	979	981	0.1	-	-
psi-Cumene	985	983	-	3.5	-
Myrcene	988	989	-	-	1.1
1,3,5-Trimethylbenzene	996	996	-	31.5	-
(*Z*)-3-Hexenyl acetate	1004	1004	12.9	0.5	-
trans-2,4-Heptandienal	1012	1011	-	-	1.0
o-Cymene	1022	1025	-	7.4	0.2
Limonene	1031	1030	0.4	0.3	16.3
Indane	1041	1039	-	1.1	
Benzene acetaldehyde	1052	1056	0.1	-	7.2
1-Methyl-3-propylbenzene	1058	1052	-	2.0	-
1,4-Diethylbenzene	1056	1056	-	1.5	-
γ-Terpinene	1054	1057	-	-	0.5
1-Octanol	1063	1068	0.3	-	-
Acetophenone	1073	1069	-	-	0.5
4-Ethyl-1,2-dimethylbenzene	1078	1078	-	0.5	-
1-Ethyl-2,4-dimethyl-benzene	1083	1086	-	1.5	-
Nonan-2-ol	1097	1096	-	-	2.2
Linalool	1095	1097	1.6	-	-
Nonanal	1100	1101	0.3	-	-
Undecane	1100	1104	-	5.0	-
Phenylethyl alcohol	1117	1124	-	-	32.8
Pentylcyclohexane	1130	1132	-	0.1	
1,2,4,5-Tetramethylbenzene	1131	1131	-	1.5	-
6-Methylundecane	1155	1148	-	0.1	-
1,2,3,4-Tetramethylbenzene	1159	1154	-	1.0	-
2-Methylundecane	1164	1158	-	0.1	-
2-Hydroxy acetophenone	1167	1165	-	4.6	-
p-Methyl acetophenone	1180	1174	-	0.1	-
Methyl salicylate	1195	1197	2.7	4.4	-
Dodecane	1200	1204	-	0.5	-
β-Citronelolol	1228	1225	0.8	-	-
trans-Geraniol	1253	1252	1.0	0.3	-

Table 1 (Continued)**.** Volatile compounds of *V. major* subs. *hirsuta* species growing in Turkey.

Compounds	RI*	RI[a]	HD[b]	SPME[c]	SPME[d]
				(%)[e]	
Ethyl salicylate	1269	1262	0.1	-	-
Tridecane	1300	1303	-	0.1	9.5
Undecanal	1305	1304	-	-	0.5
β-Damascenenone	1380	1386	0.1	-	-
Tetradecane	1400	1402	-	-	12.8
Jasmone	1390	1391	0.4	-	-
trans-(*β*)-caryophyllene	1417	1416	15.8	8.2	-
β-Copaene	1430	1436	0.2	-	
5-Methyltetradecane	1454	1451	-	-	0.8
trans-*β*-Farnesene	1454	1453	0.1	0.1	-
4-Methyltetradecane	1457	1454	-	-	1.6
α-Humulene	1460	1461	2.2	1.2	-
trans-*β*-Ionene	1489	1487	-	-	0.9
Germacrene-D	1484	1487	2.1	1.8	
Hexadecane	1500	1502	-	-	0.7
α-(*E*,*E*) Farnesene	1505	1504	0.2	0.1	-
β-Bisabolene	1509	1508	-	0.1	-
(*Z*)-3-Hexenyl benzoate	1565	1562	0.1	-	-
Spathulenol	1577	1576	0.1	-	-
Caryophyllene oxide	1582	1582	0.3	-	-
α-Bisabolol	1685	1685	-	-	0.2
Hexahydrofarnesyl acetone	1846	1848	0.1	-	-
Nonadecane	1900	1900	-	-	0.6
n-Hexadecanoic acid	1966	1970	0.2	-	-
Ethyl palmitate	1994	1995	-	0.1	-
Heneicosane	2100	2099	0.1	-	0.8
Phytol	2110	2109	0.8	-	-
Linoleic acid	2131	2128	0.1	-	-
Tricosane	2300	2298	0.1	-	0.3

Chemical classes	HD[b]		SPME[c]		SPME of *n*-hex.[d]	
	%[e]	NC[f]	%[e]	NC[f]	%[e]	NC[f]
Monoterpenes	0.6	2	7.7	2	18.6	5
Monoterpenoids	3.5	4	0.3	1	-	-
Sesquiterpenes	20.6	6	11.5	6	-	-
Sesquterpenoids	0.1	1	-	-	0.2	1
Diterpene	0.8	1	-	-	-	-
Terpene related	0.4	2	-	-	0.9	1
Aldehyde	1.2	3	-	-	13.2	5
Alcohol	54.5	3	1.0	1	37.6	4
Aliphatic hydrocarbons	0.2	2	6.1	8	27.6	9
Aromatic hydrocarbons	-	-	61.2	14	-	-
Esters	15.8	4	5	3	-	-
Ketones	0.5	2	4.7	2	0.5	1
Acids	0.3	2	-	-	-	-
Others	-	-	1.1	1	-	-
Total	98.5	32	98.6	38	98.6	26

*Litreture RI values; [a]Retention Index calculated from retention times relative to that of n-alkane series (C_6-C_{30}); [b]HD: Hydrodistillation; [c]SPME: Solid phase microextraction; [d]SPME: Solid phase microextraction of *n*-hexane extract; [e]%: Percentages obtained by FID peak-area normalization; [f]NC: Number of compounds.

2.2. Antimicrobial activities for the EO and solvent extracts

The antimicrobial activities of the EO and solvent extracts of *V. major* subsp. *hirsuta* against seven bacteria (3 gram negative, 3 gram positive, and one no gram) and two fungi were evaluated. After the inhibition diameters were observed in mm, the MIC values (µg/mL) were calculated [15, 23, 24] (Table 2).

3. DISCUSSION

A total of 32, 38, and 26 compounds from HD, SPME and SPME of *n*-hexane extract of *V. major* subsp. *hirsuta* were identified and represented to an average of 98.5%, 98.6%, and 98.6%, respectively. VCs study showed that (*Z*)-3-hexenol (36.8%), hexanol (17.4%), (*Z*)-3-hexenol acetate (12.9%) and *trans*-(*β*)-caryophyllene (15.8%) were found to be major compounds in the EO of *V. major* subsp. *hirsuta*. SPME GC-FID/MS analysis of *V. major* subsp. *hirsuta* gave the 1-ethyl-3-methylbenzene (9.8%), 1,3,5-trimethylbenzene (31,5%), *o*-cymene (8.2%) and *trans*-(*β*)-caryophyllene (7.4%) as main constituent. In addition, limonene (16.3%), phenylethyl alcohol (32.8 %), tridecane (9.5%), and tetradecane (12.8%) were found as the main compounds in the SPME GC-FID/MS analysis of *n*-hexane extract obtained from *V. major* subsp. *hirsuta*. The main volatile components of the *V. major* subsp. *hirsuta* varies depending on the extraction technique used. In general, (*Z*)-3-hexenyl acetate (12.9%, and 0.5%), methyl salicylate (2.7%, and 4.4%), *trans*-Geraniol (1.0%, and 0.3%), *trans*-(*β*)-caryophyllene (15.8%, and 8.2%), *cis*-*β*-farnesene (0.1%, and 0.1%), *α*-humulene (2.2%, and 1.2%), germacrene-D (2.1%, and 1.8%), and *α*-farnesene (0.2%, and 0.1%) were found both in the EO and SPME of *V. major* subsp. *hirsuta*, respectively. (*Z*)-3-Hexenol (36.8%, 1.0%, and 1.5%), and limonene (0.4%, 0.3%, and 16.3%) were present in all three EO, SPME and SPME of *n*-hexane extract of the *V. major* subsp. *hirsuta*, respectively. Only compounds (*Z*)-3-hexenol (36.8%, 1.0%, and 1.5%), and limonene (0.4%, 0.3%, and 16.3%) were found in all three separate samples, respectively. The results showed that no regular increase or decrease for the type or amounts of components depends on the used techniques. The data from the present study demonstrated that sesquiterpene components (20.6%, 11.5%) in the EO and SPME, monoterpene (18.6%) in the SPME of *n*-hexane extract of the *V. major* subsp. *hirsuta* were the predominant compounds among the all terpenes as seen in table 1. Indeed, alcohols (54.5%, 37.6%) in the EO and SPME of *n*-hexane extract, and aromatic hydrocarbons (61.3%) in the SPME of *V. major* subsp. *hirsuta* were the major class compounds, respectively (Table 1).

Table 2. Screening for the antimicrobial activity of the EO and the solvent extracts of *V. major* subsp. *hirsuta.*

Extracts	Stock Sol. (µg/ml)		Microorganisms, inhibition zone (mm) and minimal inhibition concentration (MIC, µg/mL)								
			Gram negative			*Gram positive*			*No Gr.*	*Fungi*	
			Ec	*Yp*	*Pa*	*Ef*	*Sa*	*Bc*	*Ms*	*Ca*	*Sc*
EO	12800	mm	-	-	-	-	-	-	12	10	-
		MIC	-	-	-	-	-	-	152	38	-
n-Hexane	24300	mm	-	-	-	-	-	-	-	-	-
		MIC	-	-	-	-	-	-	-	-	-
Acetonitrile	5500	mm	6	6	6	-	-	-	15	10	-
		MIC	275	275	275	-	-	-	69	137	-
Methanol	97500	mm	-	-	-	-	-	7	9	-	-
		MIC	-	-	-	-	-	2437	609	-	-
Water	69600	mm	-	-	-	-	-	-	10	-	-
		MIC	-	-	-	-	-	-	437	-	-
Amp.	10	mm	10	10	18	10	35	15	-	-	-
		MIC	10	18	128	35	10	15	-	-	-
Strep.	10	mm							35		
		MIC							4		
Flu	5	mm								25	25
		MIC								8	8

Ec: Escherichia coli, *Yp*: *Yersinia pseudotuberculosis*, *Pa*: *Pseudomonas aeruginosa*, *Sa*: *Staphylococcus aureus*, *Ef*: *Enterococcus faecalis*, *Bc*: *Bacillus cereus* 702 Roma, *Ms*: *Mycobacterium smegmatis*, *Ca*: *Candida albicans*, *Sc*: *Saccharomyces cerevisiae*, Amp.: Ampicillin, Strep.: Streptomycin, Flu.: Fluconazole, (-): no activity

In the literature, GC-MS analysis for the chloroform extract of *V. rosea* has given campesterol, stigmasterol and *β*-sitosterol and also its moderate glucoamylase activity (51.87%) reported [4]. Volatile oil analysis obtained from the leaf of *V. rosea* has yielded citronellyl acetate, aliphatic compounds, cadinene, and 2-heptanol [9]. Volatile components for the aerial parts of *V. difformis* extracts were also studied [11]. An essential oil analysis for the leaf of *V. rosea* had given and aldehydes, sesquiterpenes, faty acids, and lochnerol type compounds characterized [10]. The major volatile components of *Vinca herbacea* and *Vinca soneri* were found tetrapentacontane (77.84%), 6-octadecanoic acid (28.85%), respectively. We also identified some similar aliphatic compounds and 2-heptanol [9] in this work. Also, it has been reported that the major phenolic components of the leaves of *V. herbacea* and *V. soneri* are routine trihydrate (1280,25 mg/100g), chlorogenic acid (401.23 mg/100g), respectively [25].

The observed chemovariation of *V. major* subsp. *hirsuta* are in good agreement with the published data obtained from other specie [14, 15 ,18-22, 26, 27] due to the used extraction methods and locality. It is well known that there are many factors (altitude, temperature, land, growing conditions, and season), which can affect the qualitative and quantitative differences in the VCs produced in plant.

Considering the antifungal activity results, the best MIC values for the EO was found as 38 µg/mL against *C. albicans.* Acetonitrile extract was only active to gram negative bacteria *E. faecalis, Y. pseudotuberculosis,* and *P. aeruginosa* in all tested extracts. The most active extract was found to be acetonitrile and second best activity was observed with 69 µg/mL MIC value against *M. smegmatis*. The EO and water extracts were only active to *M. smegmatis*. It has been observed that the best anti-tuberculosis activity was observed for acetonitrile extract against to *M. smegmatis* among the all tested microorganism.

In the literature, *in vitro* α-glucosidase inhibitor activity performed as a preliminary screening for petroleum ether, chloroform, ethyl acetate, methanol, and aqueous extracts of *V. rosea*. In comparison with all the extracts, methanol extract had shown promising activity with IC_{50} values of 77.41 µg/mL for *V. rosea* [6]. Antimicrobial and anti-proliferative activities of *V. major*, *V. herbacea*, and *V. minor* grown in Iran had screened. Endophytic fungi bioactivity of methanol and ethyl acetate extracts (7.8-250 µg/mL) were assessed against a panel of pathogenic fungi and bacteria. Data had shown that both methanol and ethyl acetate extracts from all endophytic isolates had significant cytotoxic effects against the model target fungus *Pyricularia oryzae* [12]. The differences for the biological activity for the plants could be related to the many factors (species, concentration, etc.) that could affect the activity as we observed.

4. CONCLUSION

VCs composition of the *V. major* subsp. *hirsuta* has been analyzed and antimicrobial activities for the EO and solvent extracts were investigated for the first time. (*Z*)-3-Hexenol (36.8%), 1,3,5-trimethylbenzene (31.5%), and phenylethyl alcohol (32.8%) were found to be major compounds of all three methods (EO, SPME, SPME of *n*-hexane extract), respectively. However, linalool, *β*-citronelolol, *β*-damascenenone, α-copaene, spathulenol, caryophyllene oxide, and phytol as terpenic compounds were found only in the EO of *V. major* subsp. *hirsuta*. These clearly showed that various extraction methods that were used in this work gave the identification of different components as in the literature. Sesquiterpenes were found to be major class of component (20.6%) among the terpenes in the EO oil of *V. major* subsp. *hirsuta.* The amount of (*Z*)-3-hexenol (36.8%), 1-hexanol (17.4), and *trans*-(*β*)-caryophyllene (15.8%) were so high that EO of *V. major* subsp. *hirsuta* could be the source for the production of these compounds. In general, the greatest activity was observed for the EO of *V. major* subsp. *hirsuta* against *M. smegmatis* with 38 µg/mL MIC value. Acetonitrile extract was only active to gram negative bacteria (*E. faecalis, Y. pseudotuberculosis,* and *P. aeruginosa*) and water extract was only active to *M. smegmatis* (437 µg/mL, MIC) among the all test microorganism. Therefore, the overall results of observed antimicrobial activities suggest that EO and solvent extracts of *V. major* subsp. *hirsuta* may have promising prospect for pharmaceutical and other industrial applications. In further study, activity guided isolation and purification could be carried out on *V. major* subsp. *hirsuta.*

5. MATERIALS AND METHODS

5.1. Plant material

V. major subsp. *hirsuta* was collected on June 10th, 2018 in the flowering stage from Kanuni campus of Karadeniz Technical University, Trabzon-Turkey [2]. Voucher specimens deposited in the Herbarium of the Department of Biology, Karadeniz Technical University (KTUB-1284).

5.2. Hydrodistillation (HD) procedure for obtaining the essential oil

EO of *V. major* subsp. *hirsuta* obtained from the fresh aerial parts of plant (~100 g) by hydrodistillation in a modified Clevenger-type apparatus with cooling bath (-10 ^{o}C) system (3 h) [yields: 0.055% (w/w)]. The obtained oils dissolved in HPLC grade *n*-hexane (1 mL), dried over anhydrous sodium sulphate, and stored at 4-6 ^{o}C in a sealed brown vial [14, 15].

5.3. Solvent extractions (*n*-hexane, acetonitrile, methanol, and water)

The fresh plant of *V. major* subsp. *hirsuta* (10.0 g) were disintegrated and extracted (x3 times) with HPLC grade *n*-hexane (15 mL), acetonitrile (15 mL), methanol, and water (15 mL) at room temperature. The crude *n*-hexane, acetonitrile, and methanol extracts were filtered through a 0.45 µm filter and concentrated under reduced pressure using a rotary evaporator to give crude *n*-hexane (0.1402 g), acetonitrile (0.2140 g), and methanol (0.2942 g) extracts. The water extract was lyophilized to give crude extract (0.1530 g) [14, 15].

5.4. Solid phase micro extraction (SPME) analysis

The fresh plant samples (1.2 g) and *n*-hexane extract (87 mg) were placed in vials sealed with a silicone-rubber septum cap. A poly dimethylsiloxane/divinyl benzene fiber (Supelco, USA) was used for the absorption of the volatile components. Before the analysis, the fibers were conditioned for 5 min at 250°C in the GC injector. SPME were done at 50°C with incubation time of 5 min, and extraction time of 10 min.

5.5. Gas chromatography-Mass spectrometry (GC-FID/ MS)

EO analysis was carried out using, a Shimadzu QP2010 ultra GC-FID/MS, Shimadzu 2010 plus FID, fitted with a PAL AOC-5000 plus auto sampler and Shimadzu Class-5000 Chromatography Workstation software. The separation was analyzed by means of a Restek Rxi-5MS capillary column (30 mm x 0.25 mm × 0.25 µm) (USA). EO injections to GC-FID/MS was performed in split mode (1:30) at 230 °C. The EO solution (1 µL) in *n*-hexane (HPLC grade) were injected and analyzed with the column held initially at 60ºC for 2 min and then increased to 240ºC with a 3ºC/min heating ramp and the final temperature of 250ºC was held for 4 minutes. Helium (99.999 %) was used as carrier gas with a constant flow-rate of 1 mL/min. Detection was implemented in electronic impact mode (EI); ionization voltage was fixed at 70 eV, scan mode (40-450 *m/z*) was used for mass acquisition [14, 15].

5.6. Identification of volatile constituents

Retention indices of the volatile components of *V. major* subsp. *hirsuta* was determined by Kovats method using *n*-alkanes (C_6-C_{30}) as standards. Volatile compounds were identified by comparisons with literature RI [14-19] and MS compared to existing analytical standards and by matching mass spectral libraries (NIST, Wiley7NL, FFNSC1.2, and W9N11).

5.7. Microorganisms used for antimicrobial activity

The test microorganisms used in the study were obtained from Refik Saydam Hıfzısıhha Institute (Ankara) and are as follows: *Escherichia coli* ATCC35218, *Yersinia pseudotuberculosis* ATCC911, *Pseudomonas aeruginosa* ATCC43288, *Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC29212, *Bacillus cereus* 709 Roma, *Mycobacterium smegmatis* ATCC607, *Candida albicans* ATCC60193, and *Saccharomyces cerevisiae* RSKK 251.The EO and solvent extracts were weighed and the stock solutions were prepared in *n*-hexane, acetonitrile, methanol, and water, respectively. Inhibition diameters were measured by the agar well diffusion method [23, 24] and the MIC value was determined as microgram-milliliter (µg/mL) to the microdilution technics (Table 2).

5.8. Antimicrobial activity assessment (Agar-well diffusion method)

The antimicrobial screening test using agar-well diffusion method as adapted was used earlier [15, 23, 24]. Each microorganism was suspended in Mueller-Hinton broth (Difco, Detroit, MI) and diluted approximately 10^6 colony forming unit (cfu) per ml. They were "flood-inoculated" onto the surface of Mueller Hinton agar, Brain Heart Infusion agar and Potato Dextrose Agar (PDA) (Difco, Detriot, MI) and then dried. Brain Heart Infusion agar was used for *M. smegmatis* and *S. mutans*. For *C. albicans* PDA was used. Five-millimeter diameter wells were cut from the agar using a sterile cork-borer, and 50 µL of the compound substances were delivered into the wells. The plates were incubated for 24-48 h at 36°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. Compound stock solutions were prepared at different concentrations (5.500-97.500 µg/mL) according to the amount of material obtained. The 1/10 dilution of each solvent was used as a control.

5.9. Minimal inhibition concentration (MIC) assay

The antimicrobial properties of the EO and solvent extracts of *V. major* subsp. *hirsuta* were investigated quantitatively in respective broth media by using microdilution method and the minimal inhibition concentration (MIC) values (µg/mL) were examined [24]. The antibacterial activity assays were carried out in Mueller-Hinton broth (MHB) at pH. 7.0±0.2 and 18-24 h at 36 °C incubated. For antifungal activity test were used Yeast Extract Peptone Dextrose (YEPD) broth (pH 6.5±0.2) and 48 h at 36 °C incubated. Brain Heart Infusion broth (BHI) (Difco, Detriot, MI) was used for *M. smegmatis* and incubated for 72 h at 36 °C. The MIC value was defined as the lowest concentration that showed no growth. Ampicillin (10 mg/mL), streptomycin (10 mg/mL) and fluconazole (5 mg/mL) were used as standard antibacterial and antifungal drugs, respectively. The 1/10 dilution of each solvent was used as a control.

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