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Evaluation of antimicrobial activity of five *Vincetoxicum* taxa growing in Turkey

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ABSTRACT: The antibacterial, antifungal and antimycobacterial effects of ethanolic extracts obtained from aerial parts and roots of five *Vincetoxicum* taxa (*V. canescens* subsp. *canescens*, *V. canescens* subsp. *pedunculata*, *V. parviflorum*, *V. fuscatum* subsp. *fuscatum* and *V. fuscatum* subsp. *boissieri*) were tested against two Gram (+) bacterial strains (*Staphylococcus aureus* and *Bacillus subtilis*), three Gram (-) bacterial strains (*Escherichia coli*, *Acinetobacter baumannii* and *Aeromonas hydrophila*), three fungal strains (*Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*) and *Mycobacterium tuberculosis* by using the broth dilution method. Ethambutol, Isoniazid, Ampicillin and Fluconazole were used as reference antimicrobial agents. All tested extracts showed significant antibacterial activity against *A. baumannii* with MIC values ranging from 31.25 to 62.5 µg/ml when compared to reference drug Ampicillin with 125 µg/ml MIC value. Moreover, the extracts obtained from aerial parts of *V. fuscatum* subsp. *boissieri* and roots of *V. canescens* subsp. *pedunculata* were found the most effective extracts against *A. baumannii* with 31.25 µg/ml MIC value.

KEYWORDS: *Vincetoxicum*; antimicrobial activity; plant extract; broth microdilution method; *Acinetobacter baumannii*.

1. INTRODUCTION

Infectious diseases including nosocomial infections caused by various microorganisms have become a major health problem worldwide due to emerging multi-drug resistant strains. Antimicrobial resistant strains lead to serious infections like urinary tract, pneumonia and bloodstream infections [1, 2]. New antimicrobial agents with novel mechanisms of action are needed to fight effectively against infectious diseases [1]. This unmet need resulted from a variety of factors such as manufacturing synthetic agents with high costs, various adverse effects and ability to develop resistance to all classes of antimicrobial agents used against them [1, 3, 4]. Nowadays, one of the most popular way of exploring new antimicrobial agents with low cost is to use medicinal plants as a source [4, 5]. More than 20.000 plant species with medicinal properties are listed by The World Health Organization (WHO) for treating various illnesses like pneumonia, diarrhea, ulcers, colds and bronchitis [6]. Furthermore, according to literature, approximately 40 % of antimicrobial agents are naturally sourced [5].

The genus *Vincetoxicum* N.M. Wolf which is one of the members of Apocynaceae; subfamily Asclepiadoideae [7, 8] comprises nearly 100 species which are distributed throughout Asia, Europe, Japan [9] and North America [10]. Leaves, rhizome and dry seeds of *Vincetoxicum* species have various usages in folk medicine due to medicinal purposes [11]. *V. hirundinaria* Medik, *V. nigrum* (L.) Moench and *V. stocksii* Ali & Khatoon have been traditionally used in different medicinal systems like European and Chinese for the treatment of neurosis, malaria, scrofula, injuries, rupture, fever, scabies, wounds [7, 11, 12] and as expectorant, diuretic, emetic [13, 14], antileishmanial [12], antitumoral [7, 15], laxative and diaphoretic agents [7]. In Pakistan, poultice of *V. stocksii* has been externally used for the treatment of injuries, wounds and cancers [12]. In the field of veterinary medicine, roots of *V. hirundinaria* have been used to treat dropsy and some other illnesses [7]. The plant is known as "dompte-venin" in France and used as emetic and expectorant [16]. Infusion and decoction of the roots and whole plant are used as antidote for poisons in Italy [17], while in Turkey the plant is known as "Kırlangıç kuyruğu or Panzehir otu" and emetic properties of roots were reported [18]. In

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East Anatolia, *V. canescens* (Willd.) Decne. subsp. *canescens* is locally known as "Zilasur or Zehir otu" [19, 20], and crushed parts of the plant have been externally used for the treatment of scabies [19] and fungal infections [19, 20].

Antifungal and antibacterial activities of *V. rossicum* (Kleo.) Barb. and *V. nigrum* [21, 22]; antifungal, antibacterial [12, 23], antileishmanial and antimalarial activities of *V. stocksii* [12] and antioxidant activity of *V. lutea* L. [11] have been determined. Furthermore, cytotoxic [24, 25], antifeedant and growth inhibition [26-28] effects of the genus were also reported.

Phytochemical investigations revealed the presence of triterpenoids [13, 15], phenanthroindolizidine alkaloids [21, 22, 25], steroids [15] and sugars [27, 29] in whole parts of the *Vincetoxicum* species. Moreover, acetophenone, volatile compounds [13], starch [27] and saponins [27, 30] were detected from the roots, and alkanols [15] and tannins [28] were detected from the aerial parts. As another secondary metabolites flavonoids [14, 23] and steroidal glycosides [14, 15] were found in *Vincetoxicum* species.

The genus *Vincetoxicum* is one of the largest genera of the subfamily Asclepiadoideae in Turkey and represented by 10 taxa that three of them (*Vincetoxicum canescens* (Willd.) Decne. subsp. *pedunculata* Browicz, *Vincetoxicum parviflorum* Decne. and *Vincetoxicum fuscatum* (Hornem.) Reichb. subsp. *boissieri* (Kusn) Browicz) are endemic to Turkey [30, 31]. *Vincetoxicum* species growing wild in Turkey were firstly investigated in our previous studies for their phytoconstituents [28], antifeedant activities against *Spodoptera littoralis* and *Leptinotarsa decemlineata* [27, 28] and antifungal activity against *Aspergillus fumigatus* [32]. There is no investigation on antibacterial activities of Turkish *Vincetoxicum* species in the literature. Therefore, the aim of the present study was to evaluate antibacterial, antifungal and antimycobacterial effects of ethanolic extracts obtained from aerial parts and roots of five *Vincetoxicum* taxa (*V. canescens* subsp. *canescens*, *V. canescens* subsp. *pedunculata, V. parviflorum, V. fuscatum* subsp. *fuscatum* (Hornem.) Reichb. and *V. fuscatum* subsp. *boissieri*) against two Gram (+) bacterial strains [*Staphylococcus aureus* (ATCC 25925) and *Bacillus subtilis* (ATCC 6633)], three Gram (-) bacterial strains [*Escherichia coli* (ATCC 25923), *Acinetobacter baumannii* (ATCC 02026) and *Aeromonas hydrophila* (ATCC 95080)] and *Mycobacterium tuberculosis* H37Rv, and three fungal strains [*Candida glabrata* (ATCC 90030), *Candida parapsilosis* (ATCC 22019) and *Candida tropicalis* (ATCC 750)].

2. RESULTS AND DISCUSSION

The antibacterial, antifungal and antimycobacterial activities of ethanolic extracts obtained from different parts of *V. canescens* subsp. *canescens*, *V. canescens* subsp. *pedunculata, V. parviflorum, V. fuscatum* subsp. *fuscatum* and *V. fuscatum* subsp. *boissieri* growing in Turkey were tested against Gram (+) (*S. aureus*, *B. subtilis*), Gram (-) bacterial strains (*E. coli*, *A. baumannii*, *A. hydrophila*), fungal strains (*C. glabrata*, *C. parapsilosis, C. tropicalis*) and *M. tuberculosis*. The percentage yields of ethanolic extracts with further details of the studied plants were given in Table 1. The antibacterial [33] and antifungal [34-36] activities were performed using the broth dilution method assay and antimycobacterial activity [37] was performed using the REMA plate method assay. Ampicillin, Ethambutol, Isoniazid and Fluconazole were used as reference antimicrobial agents. The antibacterial and antifungal activity results were given in Tables 2 and 3, respectively.

The antibacterial activity results revealed that the ethanolic extracts obtained from aerial parts and roots of studied plants exhibited antibacterial activity with different MIC values against all tested bacterial strains when compared to reference antibacterial agents (Ampicillin, Ethambutol, Isoniazid). All tested extracts showed significant antibacterial activity against *A. baumannii* ranging from 31.25 to 62.5 µg/ml MIC values when compared to reference drug Ampicillin with 125 µg/ml MIC value. Especially, the extracts of roots of *V. canescens* subsp. *pedunculata* and aerial parts of *V. fuscatum* subsp. *boissieri* were found the most effective against *A. baumannii* with 31.25 µg/ml MIC value (Table 2).

Against *B. subtilis*, the ethanolic extracts obtained from aerial parts of *V. parviflorum* and roots of *V. canescens* subsp. *canescens* indicated significant antibacterial efficiency with 31.25 µg/ml MIC value while the other tested extracts showed lower antibacterial efficiency with 62.5 µg/ml MIC value. Comparison of MIC values of tested extracts and reference drug demonstrated that tested extracts were not found effective as reference drug Ampicillin (0.9 µg/ml MIC value) (Table 2).

The all studied extracts showed growth inhibitory effect against *M. tuberculosis* with 31.25 µg/ml MIC value except root extracts of *V. fuscatum* subsp. *fuscatum* with 62.5 µg/ml MIC value. However, the effectiveness of the all extracts was found lower than reference antimycobacterial agents Isoniazid and Ethambutol (0.97 and 1.95 µg/ml MIC values, respectively) (Table 2).

The results of antifungal activity studies demonstrated that the all studied plants displayed growth inhibition effects on tested fungal strains with different MIC values ranged from 15.62 to 62.5 µg/ml. When compared the efficiency of the all tested extracts on *C. glabrata*, the ethanolic extract obtained from aerial parts of *V. fuscatum* subsp. *boissieri* found the most effective with 31.25 µg/ml MIC value. Also root extracts of *V. parviflorum* and *V. fuscatum* subsp. *fuscatum* showed the highest efficiency against *C. tropicalis* with the same MIC value (31.25 µg/ml). The root extracts of all studied plants exhibited significant antifungal activity on *C. parapsilosis* with 15.62 µg/ml MIC value, except root extract of *V. canescens* subsp. *pedunculata* (62.5 µg/ml MIC value). The Fluconazole had different MIC values against *C. glabrata*, *C. parapsilosis* and *C. tropicalis* with 3.90, 15.62 and 3.90 µg/ml, respectively. Comparison of MIC values of extracts and reference antifungal agent Fluconazole indicated that the plant extracts exhibited lower efficiency than reference agent (Table 3).

Some of the secondary metabolites including flavonoids, phenolic acids, tannins, saponins, alkaloids, terpenoids, essential oils [38, 39], polysaccharides and sterols [40] were reported to have antimicrobial properties. Choosing the extraction solvent is important to get active component extract. Ethanol was a suitable solvent for phenolics, alkaloids, sterols, terpenoids [39] and broad range of polar constituents [41]. So in the present study ethanol was chosen as an extraction solvent.

In our previous studies, phytochemical screening of these *Vincetoxicum* taxa indicated that the aerial parts of the whole plants contain alkaloids, flavonoids, sugars and cardiactive glycosides, and also three of them (*V. fuscatum* subsp. *boissieri, V. fuscatum* subsp. *fuscatum* and *V. parviflorum*) contain tannins [28], and the roots of the whole plants contain steroidal glycosides, starch and sugars, and one of them (*V. fuscatum* subsp. *boissieri*) contains saponins [27]. Furthermore, presence of phenanthroindolizidine alkaloids in aerial parts of the tested plants were demonstrated by LC/MS/MS analysis [28].

Antimicrobial activity of different *Vincetoxicum* species were examined in a few study [21, 23, 32, 42]. Bazzaz and Haririzadeh (2003) tested antimicrobial activity of methanol extract obtained from total parts of *Vincetoxicum pumilum* Decne. against seven bacterial strains including *B. subtilis*, *S. aureus*, *E. coli*, and a fungus *Candida albicans* by using cylinder plate assay method. They found that the plant extract showed significant antifungal activity against *C. albicans* while there were no effects observed against tested bacteria [42]. Zaidi and Crow (2005) studied various fractions (ethyl acetate, hexane, butanol, chloroform and water) of methanol extract obtained from *V. stocksii* for their antimicrobial activities against 12 fungal strains including *C. albicans* and 12 bacterial strains including *B. subtilis, E. coli* and *S. aureus* by agar well diffusion and disk diffusion assays. The study results indicated that the tested plant exhibited significant antimicrobial activity against *B. subtilis* and *C. albicans* and moderate activity against *E. coli*, while there were no activity observed against *S. aureus* at 200 µg/ml concentration [23]. Mogg *et al.* (2008) evaluated antimicrobial activity of ethanolic extracts obtained from roots, fresh leaves and mature fruits of *V. rossicum* against some fungi and bacteria. They found that the plant had antimicrobial activity, and the root extract also showed greater activity than the leaves [21]. In our previous study, four extracts of increasing polarity (CH_2Cl_2, CH_2Cl_2: MeOH (1:1), MeOH and total EtOH) obtained from root and aerial part of each of the five *Vincetoxicum* taxa evaluated for their antifungal activity against *A. fumigatus* at 1 mg/ml concentration by using agar dilution method. In tested forty extracts, the CH_2Cl_2 extracts of aerial part and root of *V. parviflorum* and root of *V. canescens* subsp. *canescens* were exhibited the highest inhibitory effect against *A. fumigatus* with inhibition value of 45.86% [32]. In the present study the root extracts of all studied plants exhibited significant antifungal activity against *C. parapsilosis*.

Generally, Gram (-) bacterial strains are more resistant than Gram (+) bacterial strains [40, 43], the cell wall of the Gram (-) bacteria are more complex than the Gram (+) bacteria. The outer membranes of Gram (-) and Gram (+) bacteria are arranged differently so the differences are affected penetration of macromolecules during the treatment [44]. Moreover, Gram (-) bacteria are more resistant against natural components than Gram (+) bacteria. The hydrophilic cell wall structure of Gram (-) bacteria contains lipopolysaccharide and this structure inhibits the penetration of hydrophobic oils, steroids and extracts, and accumulation of them in the target cell membrane. This information confirmed why Gram (+) bacteria indicated greater sensitivity against natural products than Gram (-) bacteria [40]. According to the literature this is the first study on antibacterial effect of *Vincetoxicum* genus against Gram (-) nosocomial pathogen *A. baumannii* which becomes a severe healthcare problem worldwide due to its ability to gain resistance to all classes of antimicrobial compounds used against it [45]. This microorganism takes clinical attention in intensive care units (ICU) and also responsible from broad range of infections such as bacteremia, urinary tract, meningitis, bloodstream, ventilator associated pneumonia and infections of surgical wound [2]. In the present study, all tested plants showed stronger antibacterial activity against *A. baumannii* when compared to reference drug Ampicillin. The extract of the root of *V. canescens* subsp. *pedunculata* and aerial part of *V. fuscatum* subsp. *boissieri* were found

the most effective. The findings indicated that the tested plants can be a promising source of antimicrobial agents in the treatment of infections caused by *A. baumannii.*

3. CONCLUSION

The activity of five *Vincetoxicum* taxa on tested microorganisms was indicated that further examination with detailed studies is needed to confirm the effect of them to use these plants as a source of antimicrobial agents for the treatment of infectious disease. Especially, examination of two *Vincetoxicum* taxa (*V. canescens* subsp. *pedunculata* and *V. fuscatum* subsp. *boissieri*) which exhibited the highest effectiveness against *A. baumannii* is required. In a conclusion, further investigations on various microorganisms, animal studies, mode of action mechanisms, toxicity tests, comparison of the efficiency of extracts and active components are another important studies should be done in the future.

4. MATERIALS AND METHODS

4.1. Chemicals

Isoniazid (Sigma, I3377), Fluconazole (Sigma, F8929), Ethambutol (Sigma, E4630), RPMI 1640 Medium (Sigma, R6504), 3-(N-morpholino)-propanesulfonic acid (MOPS, Sigma, M1254), Resazurin sodium salt powder (Sigma R7017), Ethanol (EtOH) and Dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Middlebrook 7H9 broth and casitone, glycerol and oleic acid-albumin-dextrose-catalase were purchased from Becton Dickinson (Sparks, MD, USA). The all solutions were prepared with distilled water and for the experiments freshly prepared solutions were used.

4.2. Microbial strains

Bacterial strains [*Staphylococcus aureus* (ATCC 25925), *Escherichia coli* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Acinetobacter baumannii* (ATCC 02026) and *Aeromonas hydrophila* (ATCC 95080)], fungal strains [*Candida glabrata* (ATCC 90030), *Candida parapsilosis* (ATCC 22019) and *Candida tropicalis* (ATCC 750)] and *Mycobacterium tuberculosis* H37Rv were obtained from the Refik Saydam Hıfzıssıhha Institute, Ankara, Turkey.

4.3. Plant materials

Plant materials were collected from their natural habitats in different regions of Turkey and identified by Dr. Sevda Güzel and confirmed by Dr. Ahmet İlçim, Department of Biology, Faculty of Arts and Science, Mustafa Kemal University (Antakya, Turkey). The dried voucher specimens were stored in the herbarium of the Mustafa Kemal University (Further details were given in Table 1).

4.4. Extraction procedure

The powdered air dried aerial parts and roots were extracted three times with 96% ethanol (500 ml of solvent per 100 g of plant material), sonicated for 30 min. and at room temperature left overnight with shaking [21]. After filtrated with Whatman No.1 filter paper, the solvents were evaporated at 35-40°C under reduced pressure by using vacuum evaporator (Heidolph-Rotar TLR 1000) and the extracts were kept in dark at 4°C.

4.5. Antimicrobial assays

The antimicrobial activities of ethanolic extracts obtained from five *Vincetoxicum* taxa were evaluated by using two different methods. The broth dilution method assay was used for antibacterial [33] and antifungal [34-36] activities and the resazurin microtitre assay (REMA) plate method was used for antimycobacterial activity [37].

2.5.1. Antibacterial assay

Antibacterial activity of the extracts was tested against two Gram (+) bacterial strains: *S. aureus* (ATCC 25925) and *B. subtilis* (ATCC 6633); three Gram (-) bacterial strains: *E. coli* (ATCC 25923), *A. baumannii* (ATCC 02026) and *A. hydrophila* (ATCC 95080). Ampicillin, which is a semi-synthetic penicillin with broad spectrum, was chosen as a reference antibacterial agent. Antibacterial assay was performed using a literature method [33]. Stock solutions of the extracts (2000 µg/ml) were prepared by dissolving 2 mg of the extract in 1 ml of DMSO. The 1 ml of Mueller-Hinton broth was added to the all sterilized tubes, then stock solution of the extract was added to the first tube which contain Mueller - Hinton broth. After that, 1 ml solution was

Table 1. List of plant materials, their origins, voucher references and percentage yields of ethanol extracts.

Taxon	Endemic taxon[a]	Phytogeography[a]	Locality[a]	Altitude (m)	Voucher references	Extracted Part	Yields (%)[b,c]
V. canescens subsp. *canescens* (Willd.) Decne.	-	Irano-Turanian element	C6: Kahramanmaraş, Engizek Mountain, Fallow fields.	1000	MKUH 1283	Aerial part Root	13.78 24.3
V. cancescens (Willd.) Decne. subsp. *pedunculata* Browicz	Endemic	East Mediterranean element	B3: Afyon, Dinar; Kumalar Mountain.	1500-1600	MKUH 1284	Aerial part Root	11.47 20.8
V. fuscatum subsp. *fuscatum* (Hornem.) Reichb.	-	Unknown	B6: Kayseri, Pınarbası, Hınzır Mountain.	1800	MKUH 1315	Aerial part Root	15.51 27.05
V. fuscatum (Hornem.) Reichb. subsp. *boissieri* (Kusn) Browicz	Endemic	Irano-Turanian element	A5: Amasya, Ferhat Mountain.	460	MKUH 1316	Aerial part Root	13.56 24.44
V. parviflorum Decne.	Endemic	Irano-Turanian element	A7: Trabzon.	1200	MKUH 1334	Aerial part Root	11.28 28

[a] Information are based on the Flora of Turkey. [b,c] Extract yields of aerial parts [28] and roots [27] were taken from previous studies.

Table 2. The MIC values (µg/ml) of the tested extracts and reference drugs against bacterial strains and mycobacterial strain.

Taxon	Used part	MIC (µg/ml) values against the tested bacterial strains					
		Staphylococcus aureus (ATCC 25925)	*Bacillus subtilis* (ATCC 6633)	*Acinetobacter baumannii* (ATCC 02026)	*Escherichia coli* (ATCC 25923)	*Aeromonas hydrophila* (ATCC 95080)	*M. tuberculosis* H37RV
V. canescens subsp. *canescens*	Aerial part	125	62.5	62.5	250	125	31.25
	Root	>500	31.25	62.5	125	250	31.25
V. canescens subsp. *pedunculata*	Aerial part	125	62.5	62.5	250	250	31.25
	Root	125	62.5	31.25	250	250	31.25
V. fuscatum subsp. *fuscatum*	Aerial part	125	62.5	62.5	250	125	31.25
	Root	125	62.5	62.5	250	250	62.5
V. fuscatum subsp. *boissieri*	Aerial part	125	62.5	31.25	250	125	31.25
	Root	125	62.5	62.5	250	250	31.25
V. parviflorum	Aerial part	125	31.25	62.5	250	125	31.25
	Root	125	62.5	62.5	125	250	31.25
Reference Drug							
Ampicillin		31.25	0.9	125	15.62	31.25	NT
Isoniazid		NT	NT	NT	NT	NT	0.97
Ethambutol		NT	NT	NT	NT	NT	1.95

The MIC values were determined in duplicate with deviations within one two-fold dilution. NT: Not tested

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Table 3. The MIC values of the tested extracts and reference drug against fungal strains (µg/ml).

Taxon	Used part	MIC (µg/ml) values* against the tested fungal strains		
		Candida glabrata (ATCC 90030)	*Candida tropicalis* (ATCC 750)	*Candida parapsilosis* (ATCC 22019)
V. canescens subsp. *canescens*	Aerial part	62.5	62.5	62.5
	Root	62.5	62.5	15.62
V. canescens subsp. *pedunculata*	Aerial part	62.5	62.5	62.5
	Root	62.5	62.5	62.5
V. fuscatum subsp. *fuscatum*	Aerial part	62.5	62.5	62.5
	Root	62.5	31.25	15.62
V. fuscatum subsp. *boissieri*	Aerial part	31.25	62.5	62.5
	Root	62.5	62.5	15.62
V. parviflorum	Aerial part	62.5	62.5	62.5
	Root	62.5	31.25	15.62
Reference Drug				
Fluconazole		3.90	15.62	3.90

* The MIC values were determined in duplicate with deviations within one two-fold dilution.

transferred from first tube to the subsequent tube, and this process was repeated sequentially for serial dilution. Thus, each tube contained 1 ml of solution in serially descending concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.8, 3.9 and 1.9 µg/ml). This dilution series were also applied for Ampicillin [33]. Standard strains working suspensions were prepared in sterile tubes and turbidity adjusted to match McFarland standard No: 0.5, then 1:20 dilution of each suspension was prepared by using distilled water. 10 µl of prepared bacterial suspension was added in each plate and bacterial concentration of each plate adjusted to $5x10^5$ CFU/ml [46]. The microbial growth effects of tested solvent (DMSO) was also checked with a control test containing inoculated broth and DMSO was added at the same dilutions mentioned above. The results of control test indicated that the tested solvent had no growth effect on bacterial strains. The minimal inhibitory concentration (MIC) value of each extract was determined in duplicate tests.

2.5.2. Antimycobacterial assay

1. Culture medium: For the REMA plate method assay 7H9-S medium was prepared with Middlebrook 7H9 broth including 0.1% casitone, 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase.

2. Resazurin reagent: The resazurin sodium salt powder was used for preparing resazurin reagent. A working solution of resazurin reagent was made in distilled water at 0.01% (w/v) concentration and filtered to sterilize by using 0.22 µm membrane filter (Ministar, Sartorius Stedim Biotech GmbH, Goettingen, Germany); the prepared solution was kept for up to 1 week at 4°C.

3. The REMA plate method: This method was performed in duplicate according to the method mentioned at Nateche *et al.* [37] with minor changes. As the standard strain *M. tuberculosis* H37Rv and as reference antimycobacterial agents Ethambutol and Isoniazid were used. Stock solutions of the studied extracts and reference agents were prepared at 1000 µg/ml concentration in DMSO. 0.22 µm membrane filters were used for filtration procedure of the prepared solutions. In a 96-well microtitre plate, two-fold dilution series of all solutions were made by using 7H9-S (100 µl). The concentrations ranging from 0.12 to 250 µg/ml were tested. A sterility control without inoculum and a growth control containing no antibiotic were added in tested plate. H37Rv inoculum was made by resuspending a loopful of the Lowenstein-Jensen culture medium in a tube containing 5 ml 7H9-S medium with several glass beads and the tube was mixed by vortex during 2 min then waited for 30 min to form sediment. The supernatant was passed to another sterile tube and the turbidity adjusted to match a McFarland standard No.1; this prepared suspension was diluted again in 7H9-S (1:20). All studied plates were inoculated with suspension (100 µl) and put in plastic bags for sealing; then incubated at 37°C. After incubating for 7 days, resazurin working solution (30 µl) was put into each well and then at 37°C the each plate was incubated for 24 h. The results were recorded visually. The resazurin color changes from blue to pink, indicates reducing of resazurin and this was revealed bacterial growth. For having a positive result, the color change exhibiting bacterial growth has to be comparable to that seen in the positive growth control. The MIC values were described as minimum solution concentrations which prevented a full color change of the resazurin from blue to pink.

2.5.3. Antifungal assay

The antifungal activity of the each extract was studied against three fungal strains [*C. glabrata* (ATCC 90030), *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 750)] by using the microdilution broth method [35, 36] which was mentioned at the NCCLS standard document M27-A2 [34]. A synthetic triazole antifungal agent, Fluconazole was chosen as a reference drug. Antifungal assay was carried out in RPMI 1640 Medium which buffered to pH 7.0 with 0.165 M 3-(N-morpholino)-propanesulfonic acid. The each standard strain's working suspension was prepared by a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 medium. Stock solutions of reference antifungal agent and each studied extract were made in DMSO at 1000 µg/ml concentration. For filtration of the prepared solutions 0.22 µm membrane filter was used. Two-fold dilution series of reference antifungal agent and the solutions were prepared in a 96-well microtitre plate by using RPMI 1640 medium (100 µl). The concentrations range from 0.12 to 250 µg/ml were tested. A sterility control without inoculum and a growth control containing no antibiotic were added in each plate. Also 100 µl of the working inoculum suspension was put into each plate. The incubation of the plates was performed in ambient air for 48 h at 35°C. The MIC value was minimum concentration of the each extract which inhibited growth of the organism was visually determined.

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