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Original Article

Influence of solvent on antimicrobial activity of Carlinae radix essential oil and decoct

Jovana Jović* *, Tatjana Mihajilov-Krstev, Andrea Žabar, Zorica Stojanović-Radić

Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia

** E-mail: jovanajović85@gmail.com*

***Abstract*:**
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Plants of the family Asteraceae are known for their use in ethnopharmacology, available as commercial drugs. In this study, antimicrobial activity of Carlinae radix commercial drug's vinegar decoction and essential oil, dissolved in various solvents (ETOH- ethanol, DMSO- dimethyl sulfoxide and Tween 80- polyoxyethylene sorbitan monolaurate) was tested to investigate the effect of solvents on activity and to compare the results with previous researches. The microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The results showed that antimicrobial activity of the Carlinae radix oil significantly depends on the solvent and that most efficient antimicrobial effect had the essential oil dissolved in 7% ethanol, which points to significant synergistic effect of the oil with this solvent.

Key words: Antimicrobial activity, *Carlina acanthifolia* L. root, synergistic activity, solvent, MIC and MBC

Introduction

Plants of the family Asteraceae are known for their use in ethnopharmacology. Essential oil is present in high amounts in these plants, but in traditional medicine much more frequent way of utilisation is using of decoct. *Carlina acaulis L.* (Asteraceae) is widely spread herb in East Asia and Europe. Commonly used part of this plant in ethnopharmacology is root, known as Carlinae radix.

In traditional medicine, well known use of this drug is in the form of tinctures and decoction against urinary tract infections, skin diseases and wound irrigation (Tucakov, 1971). It was confirmed that inulin and flavonoids from roots have the antitumor, antiviral, antibacterial, antidiabetic, antioxidant and neuroprotective activity (Albulescu *et al*., 2004; Chan *et al*., 2010). Previous investigations on this commercial drug's composition, based on morphological and anatomical features of the dried root material, revealed that in Serbia, Carlinae radix mostly contains roots of *C. acanthifolia*, instead of *C. acaulis* (Đorđević *et al*., 2004; Stojanović-Radić, 2011). Together with this, chemical analyses determined very similar chemical compositions of the oils from both species, suggesting that adulteration of the drugs would not affect biological activities of commercial drug material. Studies of *C. acanthifolia* essential oil showed yield from 1-2%, while chemical analysis identified 11 compounds of the oil (Đorđević *et al*., 2005; Stojanović-Radić, 2011). Carlina

oxide was a major component (98.9±0.9%), which is known as potent antimicrobial compound (Chalchat *et al*., 1996; Wicthl *et al*., 2002). The essential oil showed inhibitory activity on reference strains of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumoniae* and *Candida albicans* (Đorđević *et al*., 2007; Stojanović-Radić *et al*., 2012). Together with essential oil's antimicrobial activity, Stojanović-Radić *et al*. (2012) investigated antimicrobial properties of different root decocts, where vinegar decoct had the highest inhibitory effect on the bacterial strain *S. aureus* ATCC 6538 in comparison to water and wine decocts (Stojanović-Radić *et al*., 2012).

In this study, we tested the antimicrobial activity of vinegar decoct and essential oil isolated from commercial Carlinae radix drug against total of eighteen microbial strains. Among them, one set was made of ATCC (American Type Culture Collection) reference strains, while the second set was comprised of multiresistant clinical isolates from wounds. These strains were chosen in order to explore the effect on common wound pathogens, since this plant is commonly used for wound irrigation. Together with this, the tested oil was dissolved in three different solvents (ETOH-ethanol, DMSO- dimethylsulfoxide and Tween 80-polyoxyethylene sorbitan monolaurate) in order to investigate the influence of solvent to antimicrobial activity and compare the results with previous researches.

Materials and methods

Essential oil isolation

The essential oil was obtained from 100 g of dried root material of the commercial herbal drug Carlinae radix ("Jeligor", Svrljig) by hydro-distillation method, using Clevenger' s apparatus (Clevenger, 1928). The essential oils (1.05–1.60 g per batch) were obtained in the mean yield of 1.05% (w/w). The obtained oils were separated by extraction with freshly distilled diethyl ether and dried over anhydrous magnesium sulphate. The solvent was evaporated under a gentle stream of nitrogen at room temperature in order to exclude any loss of the essential oil and immediately analyzed. When the oil yields were determined, after the bulk of ether was removed under a stream of N_2, the residue was exposed to vacuum at room temperature for a short period to eliminate the solvent completely. The pure oil was then measured on an analytical balance and multiple gravimetric measurements were taken during 24 h to ensure that all of the solvent had evaporated.

Preparation of decoct.

Decoct was prepared from 2 g of the roots, which were cut into small pieces and then extracted with 100 ml of boiling apple vinegar for 10 min, as described previously (Stojanović-Radić *et al*., 2012). After the plant material was filtered off, the obtained extract (decoct) was used as such in the antimicrobial tests.

Microorganisms

Antimicrobial activity assays were performed against eight American Type Culture Collection (ATCC) strains: *Salmonella enteritidis* 13076, *Pseudomonas aeruginosa* 3554, *Enterococcus faecalis* 19433, *Enterobacter aerogenes* 13048, *Proteus mirabilis* 12453, *Clostridium perfringens* 19404, *Klebsiella pneumoniae* 10031 and yeast *Candida albicans* 10231. Multiresistant bacterial strains were isolates from wounds (clinical isolates-CI): *Enterobacter aerogenes, Acinetobacter sp.* (2 clinical isolates), *Proteus mirabilis, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* (3 clinical isolates) and *Klebsiella oxytoca.* Bacterial strains were maintained on the Nutrient agar and yeast on Sabouraud dextrose agar (Microbiological Laboratory, Department of Biology and Ecology, Faculty of Science and Mathematics, Niš).

Antimicrobial activity testing

Antimicrobial activity was evaluated by microdilution method as described previously (Stojanović-Radić *et al*., 2010). Bacterial strains were transfered on new Nutrient agar and *Candida albicans* on Sabouraud dextrose agar, and incubated for 18 h at 37°C. Overnight strains were used to make suspensions in sterile saline solution (0.9% NaCl). Standard turbidity was adjusted to 0.5 McFarland (density of bacterial cells 1.0-1.5 x 10^8 and yeast 1.0-1.5 x 10^7 CFU/mL) and this inoculum size was used to prepare a final colony number of 1-2 x 10^6 (1-2 x 10^5 for yeast) colony forming units (CFU/mL) in a plate with sterile Mueller Hinton broth (MHB).

Carlinae radix essential oil was dissolved in three different solvents: 70% ethanol, DMSO and 0.05% Tween 80. A serial doubling dilutions of the oil in Mueller Hinton broth (Methodology 1) or in the same solvent (Methodology 2) were prepared in 96 well microtiter plate and used for experimental work.

Methodology 1. Microtiter plate wells were filled with 100 µl MHB and 100 µl Carlinae radix oil (dissolved in 70% ethanol) were added into the first wells. Double dilutions were made by transferring 100 µl of the first dilution in subsequent wells (for each microorganism strain) in a concentration range from 0.03 mg ml^{-1} to 70 mg ml^{-1}. Antimicrobial activity was tested in 96-well plates, prepared by dispensing 90 µl of MHB and 1 µl of the inoculum into each well. Then, 10 µl of the appropriate oil dilutions were transferred to wells and initial concentration of the oil was 7 mg ml^{-1}.

Methodology 2. Microtiter plates were filled with different solvents (100 µl in each well): 70% ethanol, DMSO and 0.05% TWEEN 80. Double dilutions were made in solvent by adding Carlinae radix oil (100 µl into the first wells), dissolved in the same solvent, and transferring 100 µl from the first in subsequent wells. Antimicrobial activity was tested like in Methodology 1, with oil concentration range from 0.003 mg ml^{-1} to 7 mg ml^{-1}. Negative controls were solvents in inoculated broth: 7% ethanol, 10% DMSO and 0.005% TWEEN 80. In all tests, positive controls were tetracycline (bacteria) and nystatin (yeast), both in the concentration range from 0.25-512 µg ml^{-1}.

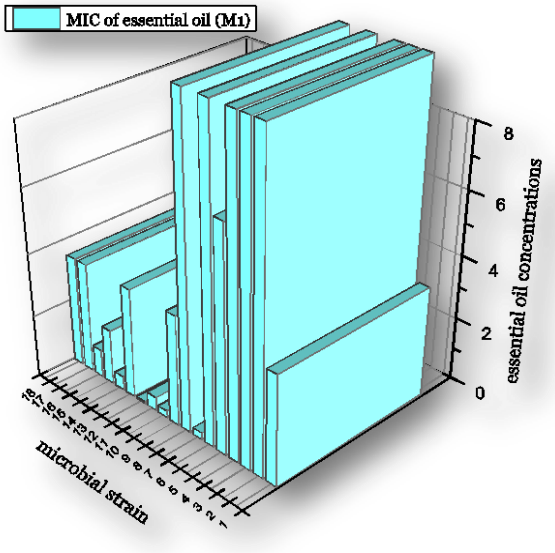
Decoct activity was tested in plates with MHB (100 µl). The first wells (for each microorganism strain) were filled with 100 µl of decoct for making a series of double dilutions and inoculated. Concentration range of decoct was from 0.024% to 50% (v\v). Negative control was commercial apple vinegar in initial concentration of 2% (v\v).

After incubation period of 24 h at 37°C, bacterial growth was determined by adding 20 µl of 0.5% TTC (triphenyl tetrazolium chloride) aqueous solution. MIC (minimum inhibitory concentration) was read as the lowest concentration of oil at which there was no visible growth and red color. The broth from wells without visible growth (100 µl) was transferred to MHA (Mueller Hinton Agar) for 24 h at 37°C. Minimum bactericidal concentration (MBC) was defined as lowest oil concentration killing 99.9% of microorganism cells. Control of microorganisms' growth was inoculated broth (without oil), while non-inoculated wells were included to ensure broth sterility. The experiment was performed in triplicate and the mean values are presented.

Results and Discussion

The results of broth microdilution assay of Carlinae radix essential oil are presented in Table 1.

In methodology 1, the oil showed MIC/MBC activity in the range from 0.055-7.000/1.750-7.000 mg ml^{-1}. The highest microbistatic and microbicidal effect were manifested against reference strain *Pseudomonas aeruginosa* 3554 (MIC/MBC = 0.055/1.750 mg ml^{-1}). Also, significant activity was obtained against clinical isolates *Pseudomonas aeruginosa* (CI 2), *Acinetobacter sp.* (CI 2) (MIC/MBC = 0.219/7.000 mg ml^{-1}) and ATCC strains *Enterobacter aerogenes* 13048 (MIC/MBC = 0.437/1.750 mg ml^{-1}) and *Salmonella enteritidis* 13076 (MIC/MBC = 0.437-3.500 mg ml-1) (Graph 1). Clinical isolates *Klebsiella oxytoca*, *Acinetobacter sp.* (CI 1), *Escherichia coli*, *Pseudomonas aeruginosa* (CI 1) and *Proteus mirabilis* were resistant to the highest tested concentration (7.000 mg ml^{-1}).



Graph 1. Minimum inhibitory concentrations of Carlinae radix essential oil dissolved in ethanol (Methodology 1). The bars above the concentration of 7.00 mg ml^{-1} are placed to represent strains resistant to the highest tested concentration of essential oil. Microbial strain legend: 1- *Enterobacter aerogenes* (clinical isolate), 2- *Acinetobacter sp.* (clinical isolate 1), 3- *Proteus mirabilis* (clinical isolate), 4- *Escherichia coli* (clinical isolate), 5- *Staphylococcus aureus* (clinical isolate), 6- *Pseudomonas aeruginosa* (clinical isolate 1), 7- *Pseudomonas aeruginosa* (clinical isolate 2), 8- *Klebsiella oxitoca* (clinical isolate), 9- *Pseudomonas aeruginosa* (clinical isolate 3), 10- *Acinetobacter sp.* (clinical isolate 2), 11- : *Salmonella enteritidis* 13076, 12- *Pseudomonas aeruginosa* 3554, 13- *Enterococcus faecalis* 19433, 14- *Enterobacter aerogenes* 13048, 15- *Proteus mirabilis* 12453, 16- *Clostridium perfringens* 19404, 17- *Klebsiella pneumoniae* 10031 and 18- *Candida albicans* 10231.

Table 1. Antimicrobial activity of Carlinae radix essential oil dissolved in different solvents

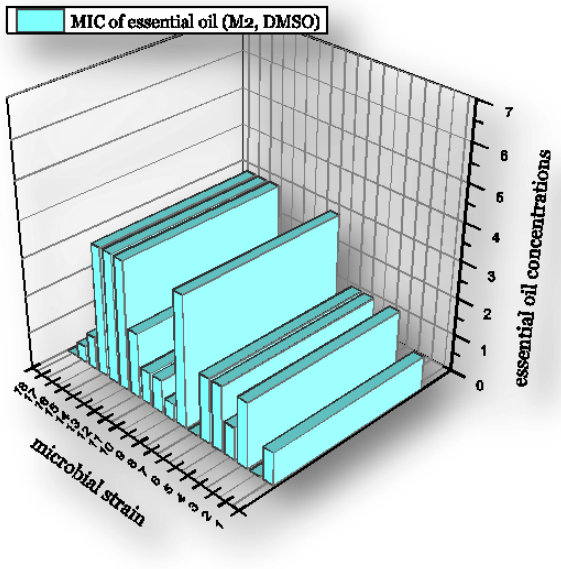
Bacterial/fungal strain	strain type	Carlinae radix oil methodology 1		Carlinae radix oil methodology 2			Negative control			Antibiotic TET, NYST	
		MIC (mg ml^{-1})	MBC (mg ml^{-1})	MIC ETOH (mg ml^{-1})	MIC DMSO (mg ml^{-1})	MIC TWEEN (mg ml^{-1})	ETOH 7 %	DMSO 10 %	TWEEN 0.005 %	MIC (mg ml^{-1})	MBC (mg ml^{-1})
Enterobacter aerogenes	clinical isolate	3.500	>7.000	<0.003	0.875	>7.000	NA	NA	NA	0.002	0.002
Acinetobacter sp.	clinical isolate 1	>7.000	>7.000	<0.003	<0.003	3.500	A	NA	NA	0.008	0.016
Proteus mirabilis	clinical isolate	>7.000	>7.000	<0.003	1.750	>7.000	A	NA	NA	0.004	0.008
Escherichia coli	clinical isolate	>7.000	>7.000	<0.003	0.875	>7.000	NA	NA	NA	0.016	0.016
Staphylococcus aureus	clinical isolate	7.000	7.000	<0.003	1.750	3.500	NA	NA	NA	<0.001	0.001
Pseudomonas aeruginosa	clinical isolate 1	>7.000	>7.000	<0.003	1.750	>7.000	NA	NA	NA	0.016	0.128
Pseudomonas aeruginosa	clinical isolate 2	0.219	7.000	<0.003	<0.003	>7.000	A	A	NA	0.004	0.032
Klebsiella oxitoca	clinical isolate	>7.000	>7.000	<0.003	3.500	7.000	NA	NA	NA	0.002	0.008
Pseudomonas aeruginosa	clinical isolate 3	3.500	3.500	<0.003	0.437	7.000	A	NA	NA	0.001	0.002
Acinetobacter sp.	clinical isolate 2	0.219	7.000	<0.003	0.875	7.000	A	NA	NA	0.008	0.008
Salmonella enteritidis	ATCC 13076	0.437	3.500	<0.003	0.875	7.000	NA	NA	NA	0.001	0.008
Pseudomonas aeruginosa	ATCC 3554	0.055	1.750	<0.003	1.750	7.000	A	NA	NA	0.004	0.032
Enterococcus faecalis	ATCC 19433	3.500	3.500	<0.003	3.500	7.000	NA	NA	NA	0.004	0.004
Enterobacter aerogenes	ATCC13048	0.437	1.750	<0.003	3.500	>7.000	NA	NA	NA	0.001	0.008
Proteus mirabilis	ATCC 12453	1.750	7.000	<0.003	3.500	3.500	NA	NA	NA	0.016	0.032
Clostridium perfringens	ATCC 19404	0.875	7.000	<0.003	0.875	7.000	NA	NA	NA	0.001	0.001
Klebsiella pneumoniae	ATCC 10031	3.500	3.500	<0.003	0.437	7.000	NA	NA	NA	0.001	0.001
Candida albicans	ATCC 10231	3.500	>7.000	<0.003	<0.003	>7.000	A	A	NA	0.016	0.016

Methodology 1- essential oil dissolved in Mueller Hinton broth, Methodology 2- essential oil dissolved in solvents (ETOH- ethanol, DMSO- dimethylsulfoxide and Tween 80- polyoxyethylene sorbitan monolaurate), A-active, NA-not active, TET- tetracycline, NYST- nystatin

Generally, Gram-negative bacterial strains possess higher resistance to external agents, which can be attributed to their characteristic membrane structure (Beveridge, 1999). The results obtained in the present study for *Klebsiella pneumoniae* 10031 and *Candida albicans* 10231, showed higher resistance in comparison with the previous research (Stojanović-Radić *et al*., 2012). In previous investigation, solvent was absolute alcohol which could lead to synergism and, thus, higher activity of oil.

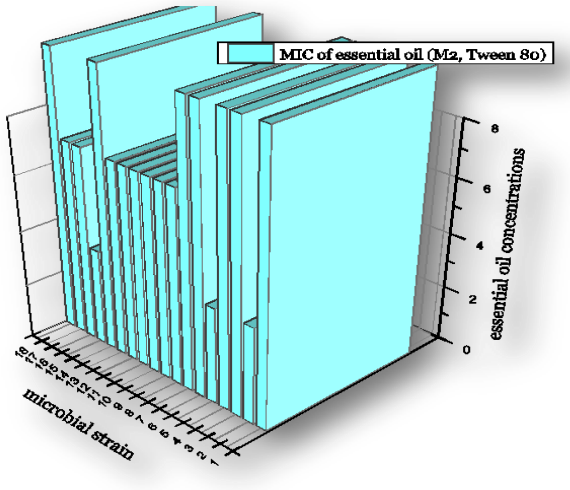
Results of microdilution method (methodology 2) where the essential oil was dissolved in ethanol showed inhibitory activity against all tested strains at surprisingly low concentrations, lower than the tested concentration range (MIC<0.003 mg ml^{-1}), but without bactericidal effect (MBC>7.000 mg ml^{-1}) (Table 1). The results could be explained by the synergism between the antimicrobial components of the oil and ethanol. This method used the same concentration of the solvent in all tested wells of the microtiter plate (7%) in combinations with different concentrations of the oil (0.003 mg ml^{-1} to 7 mg ml^{-1}). In methodology 1, concentration of 7% ethanol was in the first well only and then subsequently two-fold diluted, so we can conclude that at this concentration, ethanol exhibits efficient synergistic action, probably by increasing permeability of the membrane for the active compounds of the essential oil. It is also very important to mention that this solvent (7% ethanol), tested alone against all model microorganisms did not exhibited any inhibitory effect.

In this study, inhibitory effect of the oil dissolved in DMSO ranged from 0.437 to 3.500 mg ml^{-1} (Graph 2). Hilli *et al*. (1997) tested thirteen oils dissolved in DMSO and showed their antimicrobial activity. However, cinnamon oil, which was not dissolved in DMSO had higher activity against the tested strains. The results confirmed that higher concentrations of DMSO indicate antagonistic effect (Hilli *et al*., 1997). 10% DMSO and 0.005% Tween 80 had no activity against our strains (except for *Candida albicans* and *Pseudomonas aeruginosa* clinical isolate 2), as in the previous research (Prabuseenivasan *et al*., 2006). Increased resistance of microorganisms, compared to MIC/MBC of the oil dissolved in ethanol, could be explained by antagonistic action of DMSO with carlina oxide (the main component of the oil) or by the lack of membrane permeability alteration effect.



Graph 2. Minimum inhibitory concentrations of Carlinae radix essential oil dissolved in DMSO (Methodology 2)

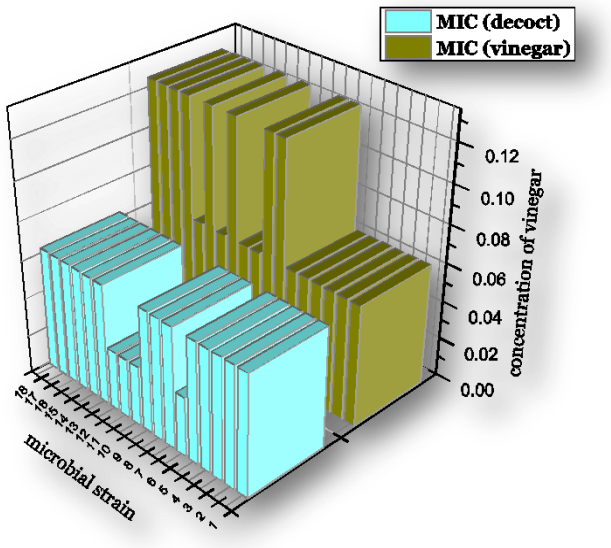
The oil dissolved in Tween 80 had relatively low activity against all tested strains of microorganisms (MIC = 3.500-7.000 mg ml^{-1}) or the strains showed complete resistance. There was not bactericidal activity of oil in these solvents. Baumann *et al*. (2011) confirmed the stimulatory effect of Tween 80 on protein secretion in yeast (*Pichia pastoris*). A source of oleic acid such as Tween 80 enhanced subsequent acid survival of probiotic lactobacilli when added to the growth medium (Corcoran *et al*., 2007).



Graph 3. Minimum inhibitory concentrations of Carlinae radix essential oil dissolved in Tween 80 (Methodology 2). The bars above the concentration of 7.00 are placed to represent strains resistant to the highest tested concentration of essential oil.

Non-ionic detergents are characterized by their uncharged, hydrophilic head groups, so that could be the reason of low oil activity. Antagonistic and synergistic effect is probably reflected by oil's distribution between two phases, water (broth) and the solvent, which influences the effect of oil components on the microorganisms.

Bacterial strains also used in this study were multiresistant isolates from wounds. This set was chosen as model since, according to the literature, decoct of Carlinae radix is used in traditional medicine for rinsing wounds on skin (K o j i ć *et al.*, 1998). Comparison of decoct activity with apple vinegar (control) confirmed that decoct exhibited antimicrobial activity. Apple vinegar concentration was generally higher (or equal to) MIC/MBC of the control when compared to MIC/MBC of decoct. The results showed that decoct caused minimal inhibitory activity at a concentration of 0.781-1.562% v\v and minimal bactericidal activity from 0.781-6.250% v\v. The highest resistance showed *Acinetobacter sp*. (CI 1), *Pseudomonas aeruginosa* (CI 3), *Enterobacter aerogenes* 13048 and *Candida albicans* 10231 (Table 2). In previous studies, apple vinegar decoct showed higher activity against *S. aureus* (MIC = 0.78%, MBC = 3.12%) when compared to water and wine decoct (S t o j a n o v i ć - R a d i ć , 2011). Activity can be attributed to the extracted compounds at low pH.



Graph 4. Minimum inhibitory concentrations of Carlinae radix decoct. The MIC bars of decoct which are at the same values as the MIC of vinegar present the lack of decoct activity (strains 1, 2, 3, 4, 5 and 9).

Table 2. Antimicrobial activity of *Carlinae radix* decoct

Bacterial/fungal strain	strain type	*Carlinae Radix* decoct		Apple vinegar	
		MIC (%)	MBC (%)	MIC (%)	MBC (%)
Enterobacter aerogenes	clinical isolate	1.562	1.562	0.063	0.125
Acinetobacter sp.	clinical isolate 1	1.562	3.125	0.063	0.125
Proteus mirabilis	clinical isolate	1.562	1.562	0.063	0.125
Escherichia coli	clinical isolate	1.562	1.562	0.063	0.125
Staphylococcus aureus	clinical isolate	1.562	1.562	0.063	0.25
Pseudomonas aeruginosa	clinical isolate 1	0.781	1.562	0.063	0.125
Pseudomonas aeruginosa	clinical isolate 2	1.562	3.125	0.125	0.25
Klebsiella oxitoca	clinical isolate	1.562	3.125	0.125	0.25
Pseudomonas aeruginosa	clinical isolate 3	1.562	3.125	0.063	0.063
Acinetobacter sp.	clinical isolate 2	0.781	0.781	0.063	0.125
Salmonella enteritidis	ATCC 13076	0.781	6.25	0.125	0.125
Pseudomonas aeruginosa	ATCC 3554	0.781	3.125	0.063	0.063
Enterococcus faecalis	ATCC 19433	1.562	3.125	0.125	0.125
Enterobacter aerogenes	ATCC 13048	1.562	3.125	0.063	0.063
Proteus mirabilis	ATCC 12453	1.562	1.562	0.125	0.125
Clostridium perfringens	ATCC 19404	1.562	1.562	0.125	0.125
Klebsiella pneumoniae	ATCC 10031	1.562	1.562	0.125	0.125
Candida albicans	ATCC 10231	1.562	3.125	0.125	0.125

Conclusion

Based on the obtained results it can be concluded that the antimicrobial activity of the oil Carlinae radix depends on the solvent. The most efficient inhibitory effect had the oil dissolved in ethanol at 7% concentration, which can be explained by the effect of the ethanol in this concentration on membrane permeability, which leads to increased transfer of the oil's active compounds into the target places on/inside the microbial cell. Vinegar decoct of Carlinae radix exhibited very significant antimicrobial activity, even against all tested multiresistant microbial strains, which justifies its ethnopharmacological utilization as an efficient

wound rinsing agent. As a final recommendation, the oil should be used in combination with ethanol (in the form of tinctures) at minimum 7% of the solvent concentration to achieve highly efficient antimicrobial effect. Also, since the decoct of Carlinae radix shows remarkable antimicrobial properties at very low percentage concentrations, it should be considered as more frequently used wound rinsing agent for the treatment of infected wounds.

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