Int J Basic Sci Med. 2017;2(2):106-110 Original Article

http://ijbsm.zbmu.ac.ir

doi 10.15171/ijbsm.2017.20

Study of the Antifungal Activity of Shikonin and Alcoholic – Oily Extracts of Iranian *Arnebia euchroma* L

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Received December 31, 2016
Accepted June 17, 2017
Published online June 30, 2017

Abstract

Introduction: Today regarding drug resistance of fungi and bacteria, many researches have focused on herbal-based medication. As these herbal-based medications can show better adaptivity, the minimum advantage of them compared to synthetic drugs is that they are harmless. This article aimed to study the antifungal effect of alcoholic extract and essence of *Arnebia euchroma* L (Abukhalsa) roots on saprophytic and dermatophytic fungi.

Methods: In this research, the roots were collected from Zagros heights in spring. Then they were dried and 300 mL ethanol was added to each 100 g dried powder. The alcoholic extraction was performed by maceration and the extract was concentrated by distillation in vacuum. The clevenger apparatus was used to extract the essence; then it was extracted by boiling water at vacuum for 4–6 hours. Shikonin was provided in commercial form. The antifungal activities of alcoholic extract, essence and Shikonin were studied and recorded using cylinder test based on the diameter of inhibition zone in Sabouraud-Dextrose agar. Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) were measured by broths macrodilution tests.

Results: The results from cylinder, MIC and MFC tests showed that 30% of shikonin was more effective than root on fungi. Our data demonstrated that alcoholic extract was better than oily extract.

Conclusion: The alcoholic extract had better characteristics than the essence. To confirm the final findings, further researches are required.

Keywords: *Arnebia euchroma* (Abukhalsa), Dermatophytes, Saprophytes, Shikonin

Please cite this article as follows: Sabokbar A, Tabaraie B, Zand Karimi M, Talebi S. Study of the Antifungal Activity of Shikonin and Alcoholic – Oily Extracts of Iranian *Arnebia euchroma* L. Int J Basic Sci Med. 2017;2(2):106-110. doi:10.15171/ijbms.2017.20.

Introduction

One of the most common herbal drugs that are used in traditional medication is Abukhals (*Arnebia euchroma*) from the family of Boraginaceae. This plant is herbaceous, with sharp silver pubes and the flower is cluster shaped with stretched and alternate leaves. One of the most common habitats of this plant is Iran, especially Kerman.[1,2] The root of this plant was used as an ointment for wounds and burnings. It was used in reducing the swellings and had anticancer activity. It caused mild constipation, was used in nourishing the liver, kidneys and spleen, and had vulnerary effect.[3] The new studies have shown that its extracts contain shikonin that is used in treatment of burns and dermatitis,[1] proliferation of skin's stem cells,[3] improving the arthritis, and inhabitation of inflammation by its antibacterial and antifungal effects.[3,4] Two species of this plant, Ordinary and Grandis, have been characterized in Iran. These species are seen in Dena heights in Zagros mountains.[4] This root has 40 cm length and 2 cm depth with needle-shaped leaves that are grown to 10 cm and has bloom in June.[5] The root contains naphthoquinone and the most important and abundant of these compounds are shikonin and alkanin (shikalkin). The analysis of this plant showed that 8.5% (w/w) of this pig-

ment could be extracted.[6-8] Shikonin and alkanin are red and lipophilic pigments seen in most species. The other species of Arnebia such as nobilis, hispidissima, densiflora, and decumbens are found all over the world. [9]

Another substance that is found in Arnebia's root is naphthazarin (5,8-dihydroxy-1,4-naphthoquinone). The other substances such as cycloshikonin, acylshikonin, acetylshikonin, beta and beta dimethyl acrylate, isovalerate, beta acetoxy isovalerate, and arnebin 5,6 were also extracted.[10,11]

In this research, we tried to use Iranian native fungi species to provide logical and significant results. The article aimed to study the antifungal effect of alcoholic extract and essence of *Arnebia euchroma* L (Abukhalsa) roots on saprophytic and dermatophytic fungi.

Methods

Extracts and Essence

The roots were collected from Zagros heights in spring and delivered to Kosha Faravar Giti Institute. After confirmation, the samples were dried in a dark and dry place and then the roots were separated.[3] After drying, 300 mL of ethanol was added to 100 g of dried powder. In order to complete the extraction process, the mixture was placed on shaker for 72 hours.[11] The alcoholic extraction was performed by maceration and the extract was concentrated by distillation in vacuum.[12] The clevenger apparatus was used to extract the essence, then it was extracted by boiling water at vacuum for 4-6 hours. The essence was separated by N-hexane from the water, and anhydride sodium sulfate was used to complete the separation process. The essence was collected in a dark dish, then stored in 4°C.[13] Shikonin was supplied from market in commercial form.

Dry Weight of Extracts

In order to evaluate the antifungal activity of extracts, 5 mL of alcoholic concentrated extracts were added to 3 pre-weighted test tubes and dried after incubation for 24 hours. All tubes were remeasured and the dry weighs were calculated.[12]

Fungal Strains

For this research, the dermatophytic fungal strains including *Trichophyton mentagrophytes* (PTCC5054), *Trichophyton rubrum* (PTCC5143), *Microsporum canis* (PTCC5069), and *Candida albicans* (PTCC5027) and saprophytic strains such as *Aspergillus fumigatus* (PTCC5009) and *Penicillium chrysogenum* (PTCC5076) were provided from Microbial Collection of Iran, Industrial Research Organization.

Preparation of Fungal Suspension

All the fungi were cultured in Dextrose agar. Fresh colonies were diluted by physiological saline and agitated steadily by vortex. The concentration of the suspension was 1.5×10^8 cfu/mL equal to the standard of half McFarland.[14]

Evaluating the Antifungal Sensitivity to Cylinder Test

For this test, prepared fungal suspension was cultured on Sabouraud-Dextrose agar using swap. The sterile cylinders were applied in determined intervals inside the plate (15 mm from plate's wall and 24 mm from center of 2 cylinders). Then, 0.2% of alcoholic extracts and oily extracts as well as 30% of shikonin were added to each cylinder. In one cylinder, ethanol and in the other DMSO were used as negative blanks, and fluconazole was used as positive blank. All the plates were incubated in 25°C for 72 hours and the results were recorded and compared based on the diameter of colonies. All the experiments were repeated 3 times.[14,15]

Determination of MIC and MFC

The Broth microdilution was applied to determine the MIC. In a 96-well plate, 22 wells contained Sabouraud-Dextrose broth. The concentration series of 0.01 to 0.9 mL were prepared for each well and 1.5×10^6 CFU/mL fungi were added to each well. Three wells were selected as blanks for fungal growth, lack of contamination of culture, and contamination of extracts. Finally, all test tubes were incubated in 25°C for 72 hours. The last dilution that did not show any growth was MIC value. All the wells that did not show any growth, were cultured again with Sabouraud-Dextrose agar and incubated in 25°C for 48 hours. The extract dilutions that did not show any fungal growth, were selected as MFC values.[14]

Statistical Analysis

The data was analyzed by SPSS software (20th edition), using independent *t* test and one-way analysis of variance (ANOVA) with P value < 0.05.

Results

The results from cylinder, MIC and MFC tests showed that the effect of 30% shikonin on fungi are more efficient than root. Alcoholic extract was better than oily extract (Figure 1 and Table 1). It is necessary to mention that there is a significant difference among *T. mentagrophytes, T. rubrum, M. canis, C. albicans, A. fumigatus,* and *P. chrysogenum*.

Results of MIC-MFC tests

The results from MIC and MFC tests using broth microdilution are shown in Tables 2 and 3.

Discussion

Herbal drugs have been used since ancient times. Recently regarding the drug resistance of fungi and bacteria, herbal drugs as natural reservoirs have received much attention from researchers. Many of these plants were seen in human and animal food and it was proven that they have no side effects. Type and amount of the metabolites in different parts of plants vary based on ecological conditions.[1,15] European and developed countries are in a period of transition to production of herbal drugs. It has been proven that high dose of these drugs has no adverse effect

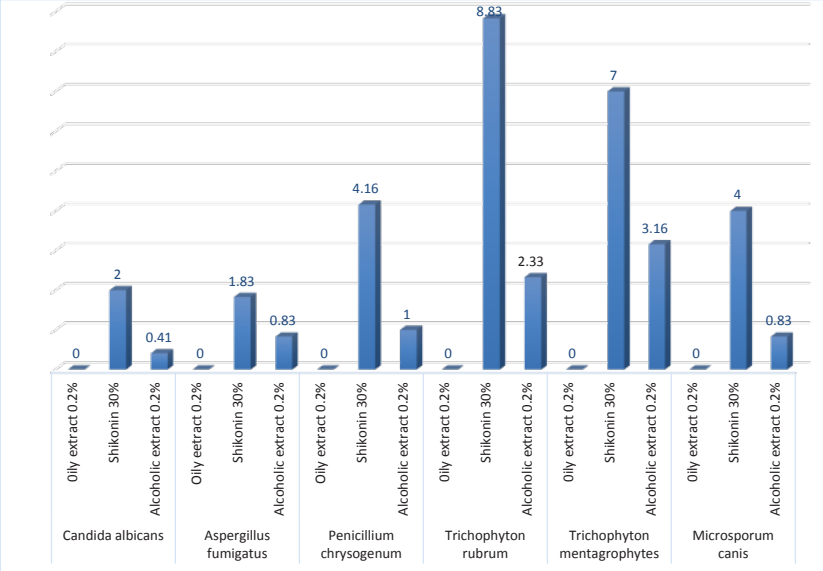

Figure 1. Mean Values of Cylinder Test Results Regarding Different Fungi and Extracts.

on human. This view leads to efforts to provide herbal products and derivatives. Today, production of herbal compounds for treating some microorganism-based illnesses are followed by large pharmaceutical companies. As chemical drugs are synthetic or semi-synthetic, they might be harmful and cause side effects on humans. Iran and other ancient countries such as China, Greece, and Italy have used herbal products to treat the diseases.[16,17]

Baranek et al showed that shikonin had positive effects on inhibition of cells.[18] Haghbeen et al prepared 2 different cultures for *A. euchroma* which had high amounts of pigments. However, antimicrobial experiments showed that they had no effects on gram-negative bacteria and fungi, but optimal effects were observed on gram-positive bacteria.[2] Doulah et al showed that the extracts of other species of *Arnebia* had good antimicrobial effects on gram-negative and gram-positive bacteria.[7] Ashkani Esfahani et al illustrated that extracts of *A. euchroma* compared to sulfadiazine silver had better effects on second-degree burns.[19] Pirbalouti et al determined the antimicrobial activities of the extracts of eight plant species endemic in Iran. The antimicrobial activities of these extracts of 8 Iranian traditional plants were investigated against *Escherichia coli* O157:H7 and *Bacillus cereus*. Most of the extracts showed a relatively high antimicrobial activity against all the tested bacteria.[20]

Nasiri et al showed that *A. euchroma* ointment was an effective treatment for healing burn wounds in comparison with SSD and could be regarded as an alternative topical treatment for burn wounds.[21]

In cylinder test, we studied the antifungal effects of shikonin, alcoholic and oily extracts on saprophytic, dermatophytic and yeast Candida. We found that shikonin had better effects compared to alcoholic and oily extracts, as. In our study, MIC-MFC tests provided different results with previous works. These tests were used in mentioned fungi and good results were obtained. According to these tests, comparing to alcoholic and oily extracts, shikonin had better effects on fungi as shown in figures and tables.

Table 1. Comparison of Cylinder Test Results Between Alcoholic Extract and 30% Shikonin (Compared Means for 2 Independent Populations)

	Group	Mean	SD	*F*	*df*	*P*
Microsporum canis	Alcoholic Extract	0.83	0.42	- 3.07	6	0.022
	Shikonin	4	2.01			
Trichophyton mentagrophytes	Alcoholic Extract	3.16	2.39	-3.009	6	0.024
	Shikonin	7	0.85			
Trichophyton rubrum	Alcoholic Extract	1.59	2.33	- 6.84	6	0.00
	Shikonin	1.03	8.83			
Penicillium chrysogenum	Alcoholic Extract	1	0.38	-5.14	6	0.002
	Shikonin	4.16	1.17			
Aspergillus fumigatus	Alcoholic Extract	0.83	0.19	-2.77	6	0.032
	Shikonin	1.83	0.69			
Candida albicans	Alcoholic Extract	0.41	0.09	-5.704	6	0.001
	Shikonin	2	0.54			

Table 2. MIC–MFC of Fungi Corresponding to Shikonin and Alcoholic and Oily Extracts

Milliliter	Oily Extracts		Alcoholic Extracts		Shikonin	
	MIC	MFC	MIC	MFC	MIC	MFC
Microsporum canis (PTCC5069)	0.8	0.9	0.06	0.08	0.01	0.02
Trichophyton mentagrophytes (PTCC5054)	0.4	0.6	0.04	0.06	0.02	0.04
Trichophyton rubrum (PTCC5143)	0.6	0.8	0.04	0.06	0.01	0.02
Candida albicans (PTCC5027)	0.8	0.9	0.2	0. 4	0.09	0.01
Aspergillus fumigatus (PTCC5009)	0.6	0.8	0. 1	0.2	0.08	0.9
Penicillium chrysogenum (PTCC5076)	0.6	0.9	0.2	0. 4	0.06	0.6

Table 3. Comparison of MIC–MFC Tests Between Alcoholic Extract and 30% Shikonin (Compared Means for 3 Independent Populations)

	Group	Mean	SD	*F*	*P*
	Oily Extract	1000	500		
Microsporum canis	Alcoholic Extract	30	10	11.58	0.009
	Shikonin	5	5		
	Oily Extract	10	10		
Trichophyton mentagrophytes	Alcoholic Extract	60	20	15.85	0.004
	Shikonin	5	5		
	Oily Extract	500	400		
Trichophyton rubrum	Alcoholic Extract	20	10	0.001	0.000
	Shikonin	5	5		
	Oily Extract	100	200		
Penicillium chrysogenum	Alcoholic Extract	50	10	0.000	47.04
	Shikonin	5	5		
	Oily Extract	300	400		
Aspergillus fumigatus	Alcoholic Extract	70	20	0.098	3.5
	Shikonin	5	5		
	Oily Extract	250	100		
Candida albicans	Alcoholic Extract	80	10	0.012	10.8
	Shikonin	5	5		

Conclusion

Our results indicated that 30% shikonin extract had better antifungal effects than alcoholic extracts and essence. The alcoholic extract had better characteristics than the essence. To confirm the final findings, further researches are recommended.

Competing Interests

Authors declare that they have no competing interests.

Ethical Approval

Not applicable.

Acknowledgements

The authors would like to thank Islamic Azad University of Karaj for its important technical supports.

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