

Anticandidal effects of aqueous and callus extracts of *Artemisia aucheri*

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ARTICLE INFO

Article history:

Received 3 February 2014

Accepted 27 March 2014

Available online 1 June 2014

Keywords:

Artemisia aucheri,

Callus,

Candida albicans,

Anticandidal

ABSTRACT

The use of herbal products in the prevention of the growth of pathogens have been widely studied. Problems in the treatment of infections caused by resistant strains of fungi are indicator of the need for a more in-detailed study of herbal remedies. *Artemisia aucheri* is used as a medicinal plant with anti-microbial properties, and as an astringent. Plant tissue culture is an important technique for the production of secondary metabolites. The aqueous and methanol extracts (AE and ME) were prepared from the resulting callus. In the agar well diffusion method in a concentration of 2500 mg/ml of AE, the growth inhibition zone diameter (IZD) of clinical isolates of *C.albicans* was greater than that of fluconazole and both standard strains of *C.albicans* (PTCC 1167 , 5027) was greater than the positive control. The results of the effect of AE showed that has the greatest effect on *C.albicans* 1167. The mean growth IZD of AE at the concentrations of 1000, 1500, and 2000 (mg ml⁻¹) were 5, 10, and 18 mm, respectively. Based on the results of this study, the concentration of 1000 (mg ml⁻¹) callus AE had no significant effect on any of the strains. The results of the anticandidal effect extracts showed that the ME has the greatest effect on the standard strains. The AE showed significant antifungal effects against the tested clinical isolates. The results obtained from the tube dilution method confirmed the above results. Different concentrations of extracts showed that with increasing concentration the anticandidal activity also increased.

1. Introduction

In recent years, production of the secondary metabolites through the cultivation of plant cells has been an important approach in cell culture studies. In some cases, the amount of secondary metabolites *in vitro* culture was higher than in the whole plant or sometimes cultured cells produced metabolites that are not produced in the original plant (Ziaratnia et al., 2009). Cell

and plant organism cultures may be the cause of possible changes in the production of chemical compounds because organs and plant cells have the capacity to produce a variety of secondary metabolites. The most important secondary metabolites include alkaloids, glycosides, tannins, essential oils, antibiotics, steroids, and hormones (Iranbakhsh et al., 2010). These metabolites are the primary product of the metabolism of plants and are produced by

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photosynthesis under different ecological conditions. Different mutations in the primary metabolic pathway create new compounds which are toxic to herbivores and pathogenic microbes (Farzaneh *et al.*, 2006). Recently, secondary metabolites, which are produced as inactive precursors stored in plant tissue, have been under scrutiny due to their inhibitory properties of pathogenic microorganisms (Das *et al.*, 1999). The emergence of different species of fungi such as *Candida* and dermatophytes has resulted in the researchers' search for new treatment methods to combat them. One of the major treatment challenges is dealing with infectious agents because of their rapid spread and outbreak. A large number of commercial antibiotics are used worldwide for the control of infections and infectious diseases and long-term use of antibiotics has led to the emergence of drug-resistant microbes (Derakhshan *et al.*, 2011). This is a reason for the growing use of natural herbs as they have low risk, available, and inexpensive medication as compared to synthetic antibiotics (Vasanayake *et al.*, 2002).

Candidiasis is undoubtedly one of the most common opportunistic fungal infections in humans and its acute or chronic infections can be seen on the skin, nails, vaginal mucosa, lung, bronchial, and gastrointestinal tract. *Candida albicans* is the most important clinical factor that is the normal inhabitant of the gastrointestinal tract and vaginal mucosa (Whiting, 2010). *Artemisia aucheri* is a shrub of 20-25 cm height found abundantly in Iran. In Iranian traditional medicine, it is used as an astringent with antiseptic, anti-microbial, anti-parasitic, and anti-poisoning properties (Mahboubi & Ghazian Bidgoli, 2009). From this medicinal plant, an extract called santonin which has long been the most famous gastrointestinal vermifuge (Zargari, 1994). Previous studies have identified 17 major components of the essential oil of this plant. Their findings have also shown that this plant has 6 types of monoterpenes. The main components of the essential oil of *Artemisia* are geranyl acetate, alpha-citrate, and geranyl linalool (Mahboubi & Ghazian Bidgoli, 2009).

Gundidza (1993) studied the antifungal activity of the essential oil obtained from the aerial stalks of *Artemisia* on several fungi such as *Aspergillus niger*, *Candida albicans*, *Geotrichum candidum*, and *Penicillium citrinum*. It was found that this oil has a strong

inhibitory effect on the growth of these fungi (Farzaneh *et al.*, 2006). Suresh *et al.* (2010) observed antifungal activity in 2 species of *Artemisia* named *A.abrotanum* and *A.pallens*. The most antifungal activity of their extract was observed on *Candida albicans*, *Saccharomyces cerevisiae*, and *Trichosporon beigeli* yeasts (Suresh *et al.*, 2010). Lopes-Lut *et al.* (2008) observed antifungal activity in the essential oil of 7 species of *Artemisia*. They found that the resulting essence, in addition to having antibacterial effect on *Escherichia coli*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*, had significant effects on *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus niger*. The study by Mahboubi and Ghazian Bidgoli (2009) showed that *Artemisia sieberi* is a potential source of biological compounds and its antifungal effect is greater than its antibacterial effect. Due to numerous reports on the antimicrobial properties of *Artemisia aucheri* and the possibility of production of secondary metabolites through tissue culture and callus production, the present study was performed. This study aimed to perform *in vitro* culture of *Artemisia aucheri*, produce callus, and evaluate the antifungal effects of its aqueous and methanol extracts.

2. Material and Methods

2.1. Preparing the callus

First, the *Artemisia aucheri* seeds were obtained from the Medicinal Plants and Natural Resources Research Center of Isfahan province, Iran. Then, the seeds were rinsed with water using a mild detergent. Subsequently, they were placed in 70% ethanol for 1 minute and in a solution of sodium hypochlorite 1.5% for 10 minutes. Finally, they were rinsed 3 times with double distilled water and were sterilized. Petri dishes were carefully washed, the bottom of each petri dish was covered with a layer of filter paper, and 20 ml of distilled water was added. Then, the petri dishes were placed in autoclave with caution and sterilized. Complying with all aseptic conditions, the petri dishes were taken into laminar air flow cabinet, their lids were opened next to the gas flame, and the seeds were sterilized and transferred into petri dishes.

Then the petri dishes were transferred to the culture room and kept in the dark at $25 \pm 2^\circ\text{C}$ (Gharehmatossian *et al.*, 2012). After a week,

seedlings were detected. When the seedlings grow up to about 3-4 cm in size, in compliance with aseptic conditions, the petri dish lids were opened under laminar airflow hood near a flame and the seedlings were transferred to Murashige and Skoog medium (MSO). In order to produce callus, different amounts of naphthaleneacetic acid (NAA) and benzylaminopurine (BAP) hormones were added to the MSO medium and 4 mediums were prepared with different ratios of hormones (Table 1).

Under aseptic conditions and laminar air flow hood near the flame and using a sterile surgical blade, one part of the plant produced was divided into several parts in a glass. Each part was separately placed on a medium containing hormones and then transferred to the culture room. After two weeks, the produced calluses were removed from the culture and dried under vacuum and high temperature, and extraction was performed using 2 ml methanol and water. After filtration, methanol and aqueous extracts were kept in a refrigerator for subsequent evaluation (Gharehmatossian et al., 2012). For dilution of the extract, 10% Dimethyl sulfoxide was used.

2.2. Preparation of suspensions from *Candida albicans*

Standard *Candida albicans* (ATCC 62061, PTCC 1167 and 5027) were purchased from the Iranian Industrial Collection of Bacteria and Fungi and two clinical isolates, the cause of vaginal candidiasis (*b-21*) and the other the cause of nail candidiasis (*C-593*), were purchased from the Fungi and Yeast Bank of Alireza Khosravi, Faculty of Health of Tehran University. The fungi were cultured in Sabouraud dextrose agar (SDA) medium (Himedia, Mumbai, India), and kept at a temperature of about 26°C. A dilution equivalent to 0.5 McFarland (1.5×10^8 cfu/ml) was prepared in Sabouraud dextrose broth (SDB) medium (Himedia, Mumbai, India) for the following steps. To evaluate the antifungal effects of aqueous and methanol extracts of *Artemisia aucheri* callus, agar well and disk diffusion and tube dilution methods were used. In these methods, sterile distilled water was used as negative control and 0.02 mg/ml fluconazole was used as positive control (Khanafari & Hosseini, 2000).

2.3. Determination of antifungal activity of extracts using agar well diffusion method

In order to perform agar well diffusion method a 24-hour microbial culture of standard *Candida albicans* (PTCC 5027, PTCC 1167, and ATCC 62061) and clinical isolates was prepared equal to 0.5 McFarland.

Then, the obtained suspension was cultured on SDA medium in 4 directions using a sterilized swab. After half an hour, using a sterilized Pasteur pipette, wells with a diameter of 6 mm were created on the medium with 2.5 cm distance from each other. 100 ml of various concentrations of aqueous and methanol extracts of callus were separately added to each well. Sterile distilled water mentioned was used as the negative control and fluconazole antibiotics as positive controls. After 24 hours of incubation at 37°C, the growth inhibition zone around each well was measured using a millimeter ruler. To verify the results, the experiment was repeated 3 times (KhajehKaramoddini et al., 2011).

2.4. Determination of the antifungal effect of the extract using agar disk diffusion method

To perform this method, from a 24-hour microbial culture of standard *Candida albicans* (PTCC 5027, PTCC 1167, and ATCC 62061) and clinical isolates was prepared equal to 0.5 McFarland standard was prepared in the SDB medium. Subsequently, this fungus suspension was cultured on SDA medium in all directions using bacterial lawn method. Discs containing different concentrations (2500, 2000, 1500, 1000 mg/ml) of aqueous and alcoholic extracts of plant and callus were removed using a sterilized forceps and with distance from the plate wall were placed on the medium and inoculated with yeast samples. After incubation for 24 hours at 37°C, the diameter of the inhibition zone around the disk was measured using a millimeter ruler (Khanafari and Hosseini, 2000).

2.5. Determination of the effect of anticandidal extracts using the tube dilution method

To perform this method, 2500, 2000, 1500, and 1000 (mg/ml) dilutions of aqueous and methanol extracts of the plant were prepared in sterile tubes. After preparing the SDB medium in 6 separate sterilized tubes, 1 ml of sterilized

culture medium was poured into the tubes and, in the same conditions, 1 ml of each extract was added to each dilution separately. Then, a suspension equivalent to 0.5 McFarland was prepared from the studied yeast and 1 ml of this suspension was added to the tubes. For each of the tests, tube number 1 was considered as a negative control consisting of *Candida albicans* and medium without any of the extracts. Moreover, tube number 2, containing different *Candida albicans* and fluconazole antibiotics, was considered as positive control. Tubes were incubated for 24 hours at 37°C. After incubation, the first tube that was not turbid was selected and considered as the minimum active concentration of the extract. To identify the effect of yeast extract, the medium from the first transparent tube and one or two tubes after it was inoculated on SDA medium. After 24 hours of incubation at 37°C, if the yeast had grown on medium it was considered as the minimum inhibitory concentration (MIC), and if the yeast growth had stopped, it was considered as the minimum fungicide concentration (MFC) (Khanafari and Hosseini, 2000).

2.6. Statistical Analysis

Statistical analysis was performed using ANOVA test with a completely randomized design with 3 replications and SPSS for Windows (version 16; SPSS Inc., Chicago, IL, USA). The graphs were plotted using Microsoft Excel (Microsoft Corporation, Washington, USA).

3. Results

3.1. The results of the production of *Artemisia aucheri* callus under tissue culture conditions

Based on the results of table 2, it was observed that the percentage of callus production on medium 3 and 4 was the highest. Furthermore, in the same environment, callus necrosis had reached its minimum level; therefore, this environment was used to produce callus and subcultured callus. In environments 1 and 2, callus production was observed with higher percentage of necrosis. The results presented showed that equal proportions were effective in producing callus.

3.2. The results of the effect of aqueous and methanol extracts of *Artemisia aucheri* callus in inhibiting the growth of standard strains of *Candida albicans*

In this study, the aqueous and methanol extracts of *Artemisia aucheri* callus had similar effects on standard *Candida albicans*. In agar well diffusion method, both extracts had a satisfactory effect on standard strains of *Candida albicans*. In the 2500 mg/ml concentration of aqueous extract of *Artemisia aucheri* callus, the growth inhibition zone of both standard strains of *Candida albicans* (PTCC 1167 and PTCC 5027) was greater than the positive control. The results of the effect of aqueous extracts of *Artemisia aucheri* callus showed that it had the greatest impact on *Candida albicans* (PTCC 1167). The mean diameter of the growth inhibition zone of aqueous extract at the concentrations of 1000, 1500, and 2000 mg/ml was 5, 10, and 18 mm, respectively. Inhibition zone diameter of less than 8 mm was acceptable and was not an interpretation for antifungal properties of any plants; therefore, the 1000 mg/ml concentration of aqueous extract of this plant had no significant effect on any of the strains tested. The 1500, 2000, and 2500 mg/ml concentrations of aqueous extracts of *Artemisia aucheri* callus tested on yeast showed that inhibitory activity increased with increasing concentration. The results of the inhibitory effect of aqueous extract of plant callus of *Artemisia aucheri* on standard strains of *Candida albicans*, compared to fluconazole, in agar disk diffusion method was not satisfactory.

The inhibitory effect of the methanol extract of *Artemisia aucheri* callus was greater than that of its aqueous extract. In all tested strains of *Candida albicans*, the inhibitory effect of the methanol extract of *Artemisia aucheri* callus was satisfactory as compared to fluconazole. The diameter of the inhibition zones of the standard strains of *Candida albicans* (PTCC 5027) was greater than that caused by fluconazole. In the two standard strains of *Candida albicans* (ATCC 62061 and PTCC 1167), a growth inhibition zone equal to that of fluconazole antibiotics was observed. The results are presented in Figures 1, 2. In the tube dilution method, the anticandidal effects of methanol extracts of *Artemisia aucheri* callus were

observed more than its aqueous extract in the growth inhibition of standard strains of *Candida* (Table 3).

3.3. The results of the effect of aqueous and methanol extracts of *Artemisia aucheri* callus for inhibition of the growth of clinical isolates of *Candida albicans*

In the well diffusion method, a 2500 mg/ml concentration of aqueous and methanol extracts of plant callus had an inhibitory effect on clinical isolates of *Candida albicans* (C-593) and (b-21), respectively. In the agar disk diffusion method, 2000 mg/ml and 2500 mg/ml concentrations of both aqueous and methanol extracts of *Artemisia aucheri* callus in the two clinical isolates causing nail candidiasis (C-593) and vaginal candidiasis (b-21b) had a greater growth inhibition zone diameter than fluconazole. Results are shown in Figures 3, 4. In tube dilution method, the anticandidal effects of the methanol extract of *Artemisia aucheri* callus was greater than that of its aqueous extract in inhibiting the growth of the clinical isolates tested (Table 4).

4. Discussion

Today, the most of the world considers medicinal plants as a lifesaving medicinal source. With regards to medicinal plants, the 3 issues of selection, reproduction, and preservation of genotypes are of great importance. Through tissue culture and regeneration of medicinal plants in glass environment techniques, we have been able to widely reproduce important and high quality medicinal plants and to produce plants free of pathogenic agents (Chintalwar et al., 2003). Numerous antifungal medications with different formulations are available for treatment. Nevertheless, due to lack of response to treatment, the disease develops into acute or chronic forms, and sometimes, its recurrence is observed. Moreover, due to the need for long-term use of these medications, a relatively wide range of side effects have been reported (Vanasayak et al., 2002). Since the majority of pathogenic fungi are eukaryotes, chemical treatment with antifungal drugs can disrupt the patient's tissue cells (Klepser, 1999). Thus, restrictions are established on the use of such

chemical antifungal compounds. *Candida* is the most common opportunistic pathogen capable of causing cutaneous, mucocutaneous, and systemic disease (Fry and Okarter, 2005). According to previous studies, the *Artemisia aucheri* herb has shown desirable antifungal activity, and thus, its essence is a valuable source of antifungal and antibacterial agents (Mahboubi and Ghazian Bidgoli, 2009). In the present study concentrations of aqueous and methanol extracts of *Artemisia aucheri* and its callus showed that with increasing concentrations of the extract, the anticandidal activity also increases. The charts also show that in the 2500 mg/ml concentration in the well diffusion and disk diffusion methods, a greater diameter of inhibition was observed in both aqueous and methanol extracts when compared at lower concentrations. Based on the growth inhibition zone diameter of callus extracts of *Artemisia aucheri* and its comparison with that of the plant extracts of *Artemisia aucheri*, it can be concluded that tissue culture method was an appropriate technique of increasing secondary metabolites with more antifungal activity in this plant. In the processed callus, the anticandidal effects were increased compared to the mother plant, and it can be said that new compounds are produced in the callus which did not exist in the mother plant. This is consistent with findings of previous research on the use of these hormones to induce callus production.

Acknowledgement

Our sincere appreciation goes to the respected authorities of the Research Laboratory of the Islamic Azad University, Falavarjan Branch.

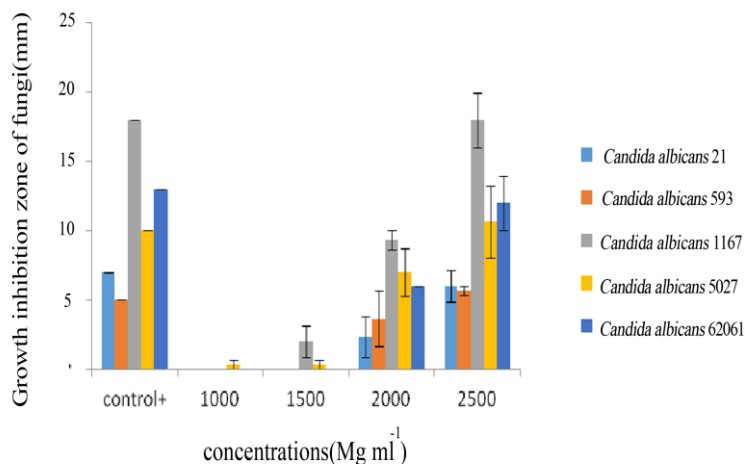


Fig 1. Growth inhibition zone of *Candida albicans* strains in the different concentrations of aqueous extract of *Artemisia aucheri* callus in agar well diffusion method.

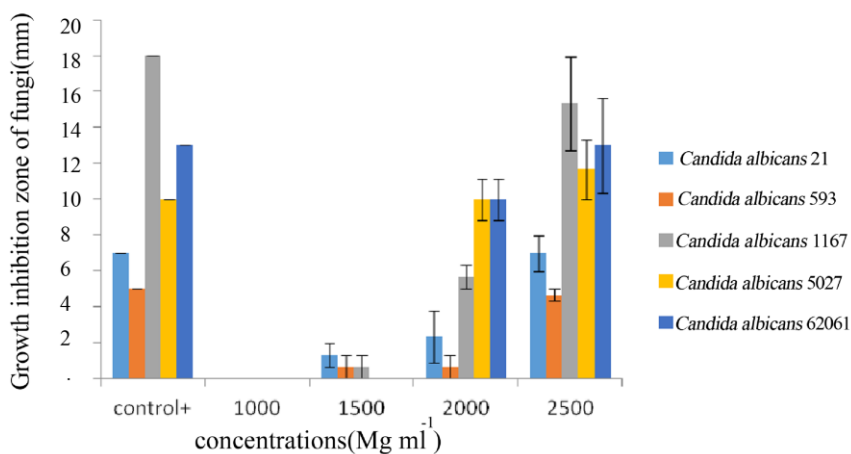


Fig 2. Growth inhibition zone of *Candida albicans* strains in the different concentrations of methanol extract of *Artemisia aucheri* callus in agar well diffusion method.

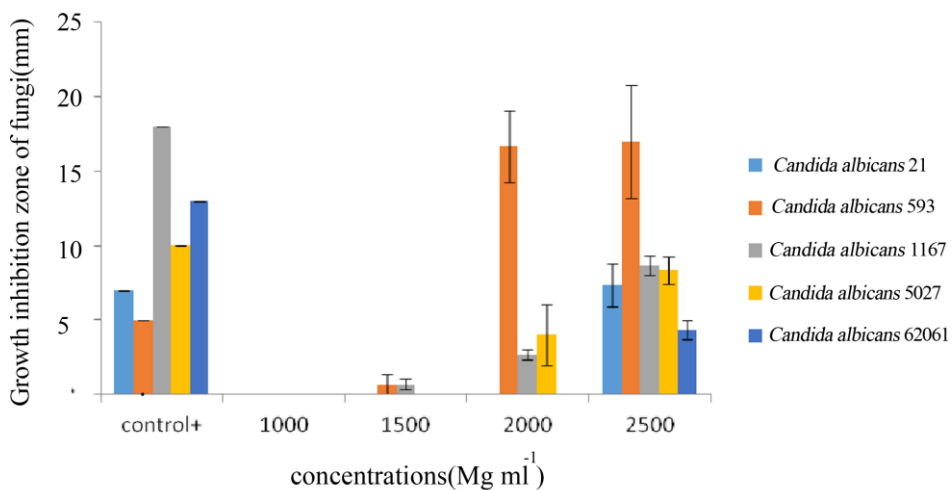


Fig 3. Growth inhibition zone of *Candida albicans* strains in the different concentrations of aqueous extract of *Artemisia aucheri* callus in disk diffusion method.

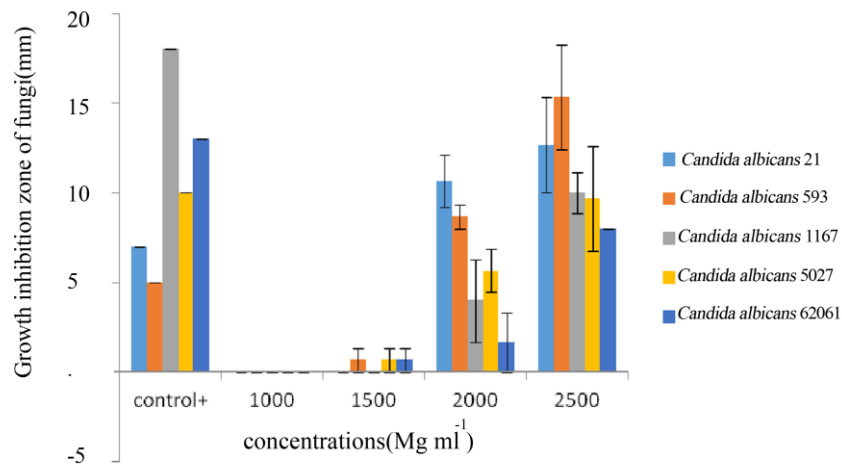


Fig 4. Growth inhibition zone of *Candida albicans* strains in the different concentrations of methanol extract of *Artemisia aucheri* callus in disk diffusion method

Table 1. Murashige and Skoog medium (MSO) with hormones to produce callus

Medium	BAP (mg/l)	NAA (mg/l)
Medium 1	2	1
Medium 2	2	2
Medium 3	1	2
Medium 4	1	1

BAP: Benzylaminopurine; NAA: Naphthaleneacetic acid

Table 2. The results of the effect of different concentrations of NAA and BAP hormones in the production of *Artemisia aucheri* callus

The medium used	Percentage of callus production	Percentage of necrosis
2 mg L ⁻¹ BAP + 1 mg L ⁻¹ NAA + MS	30	70
2 mg L ⁻¹ BAP + 2 mg L ⁻¹ NAA + MS	40	60
1 mg L ⁻¹ BAP + 2 mg L ⁻¹ NAA + MS	80	25
1 mg L ⁻¹ BAP + 1 mg L ⁻¹ NAA + MS	100	10

BAP: Benzylaminopurine; NAA: Naphthaleneacetic acid

Table 3. The minimum inhibitory concentration and minimum fungicide concentration of aqueous and methanol extracts of *Artemisia aucheri* callus on standard strains of *Candida albicans* in the tube dilution method

<i>Candida albicans</i> (ATCC 62061- PTCC 1167-PTCC 5027)	MIC (g/ml)	MFC (g/ml)
Aqueous extracts of <i>Artemisia aucheri</i> callus	1.3-1.6-1.6	1.8-2.1-2.1
Methanol extracts of <i>Artemisia aucheri</i> callus	1.3-1.3-1.5	1.8-2.2

MIC: Minimum Inhibitory Concentration; MFC: Minimum Fungicide Concentration

Table 4. The minimum inhibitory concentration and minimum fungicide concentration of aqueous and methanol extracts of *Artemisia aucheri* callus on clinical strains of *Candida albicans* in the tube dilution method

<i>Candida albicans</i> (C-593, b-21)	MIC (g/ml)	MFC (g/ml)
Aqueous extracts of <i>Artemisia aucheri</i> callus	1.3-1.1	1.8-1.6
Methanol extracts of <i>Artemisia aucheri</i> callus	1.5-1.5	2-1.8

MIC: Minimum Inhibitory Concentration; MFC: Minimum Fungicide Concentration

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