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Scientific Research

Investigation of the inhibitory, fungicidal and interactive effects of the aqueous extract of Calotropis procera on *Alternaria alternata*, *Alternaria solani*, *Saccharomyces cerevisiae*, and *Fusarium solani* "*in vitro*"

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ABSTRACT

Calotropis procera from the Apocynaceae family is called Stabregh in Iran. This plant is found in tropical areas and southern coasts in Iran and North Africa, Middle East Asia, and Southeast Asia on coastal sand dunes. In this study, the *C .procera* extract was separated using water solvent. The antifungal effect of *C. procera* aqueous extract on *Alternaria alternata*, *Alternaria solani*, *Saccharomyces cerevisiae*, and *Fusarium solani* was investigated. The antifungal activity was evaluated through, disk diffusion agar (*Kirby-Bauer)*, well diffusion agar, minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC). In the combined mode (interaction) of *C. procera* aqueous extract with nystatin antibiotic was investigated. In general, *Saccharomyces cerevisiae* and *Alternaria solani* were the most sensitive and resistant fungal strains with the highest and the lowest diameter of inhibition zone, respectively. So that the diameter of the inhibition zone for *Saccharomyces cerevisiae* and *Alternaria solani* in the presence of 80 mg/ml extract concentration was equal to 13.20 and 8.20 mm, respectively. In the interaction of *C. procera* aqueous extract with nystatin antibiotic, synergistic mode was observed for all fungal strains. The MIC and MFC values for *S. cerevisiae* were 32 and 128 mg/mL, respectively. The MIC and MFC results for *Alternaria solani* were found to 256 and > 512 mg/mL, respectively.

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1. Introduction

The increasing spread of diseases caused by microorganisms, on the one hand, and their resistance to common therapeutic antibiotics, on the other hand, have caused major problems in the treatment system of various societies, especially in developing countries. Therefore, researchers all over the world are looking for new sources of medicine with appropriate effectiveness and as little as possible side effects on human health. The spread of infections caused by pathogenic microorganisms, as well as the lack or reduction of the effectiveness of common antibiotics in recent years, has caused a large part of the research conducted by researchers to find new antimicrobial compounds in order to investigate the antifungal and antibacterial activity of medicinal plants alone or in combination with Antibiotics should be used for Reich therapy. The results of various researches show that the compounds of medicinal plants have both a direct (only) effect and can increase the performance of drugs and antibiotics in order to reduce the dosage and also their harmful effects.[1-7].

Astbaraq with a scientific name*Calotropis procera* It belongs to the Asclepiadaceae family and is one of the medicinal plants that can be found in the provinces of Khuzestan, Bushehr, Hormozgan, Sistan Baluchistan, etc. This medicinal plant has different names in different regions, such as Ghallab and Kharek. From the point of view of botany, it is a shrub that has a height of 3 to 4 meters and its leaves are 15 cm long and 11 cm wide. This plant is also found in other parts of the world such as West Asia, Africa, South America and hot and humid regions. Chemically, the leaves and young branches of the asterberg plant have compounds such as calotropin and calotropagenin. So far, little research has been done on the effect of this plant on its antimicrobial activity. In traditional medicine, the plant is used to treat wounds, epilepsy and spleen problems. In India, the plant is traditionally used to treat stomach ulcers, tumors, and leprosy[8-11].

In spite of the fact that the favorable activity of some medicinal plants in infections caused by pathogenic microorganisms has been proven in various studies, but little research has been done on the interaction between common therapeutic antibiotics and medicinal plants, both in vitro and in vivo. Therefore, the investigation of drug interactions including synergy (synergism) and antagonism (antagonism) is one of the important matters in the field of pharmaceutical sciences, and this has led to the complementary treatment including the simultaneous use of herbal compounds and common therapeutic antibiotics, including new treatment methods in The field is the treatment of diseases caused by microbial infections[12-14].

The aim of this experimental research is to evaluate the antifungal activity of the aqueous extract of Astbraq Ber*Alternaria alternata* ،*Alternaria solani* ،*Saccharomyces cerevisiae* and *Fusarium solani* In outdoor conditionswas, and its interaction with the antibiotic nystatin was also investigated.

2- Materials and methods

1-2- Preparation of astrabrag plant and extraction

This laboratory research was conducted in the year 1400 in the Food Microbiology Laboratory, Department of Food Industry Science and Engineering, Faculty of Animal Science and Food Industry, Khuzestan University of Agricultural Sciences and Natural Resources.

After collecting the leaves of Astbaraq from Gatund city (Khuzestan province), the scientific name of the plant was confirmed by the engineering department of horticultural sciences, Faculty of Agriculture, Khuzestan University of Agricultural Sciences and Natural Resources (herbarium code: 319 KHAU). After cleaning, removing contamination and washing the surface with cold water, the collected leaves were dried at room temperature on a metal net for 3 days. The dried leaves were powdered by laboratory mill. The powder of the obtained leaves was passed through a sieve for the uniformity of the particle size. The extracting process from the leaves of asterberg was carried out according to the method of Sureshjani et al. (2013). 100 grams of powdered leaves were mixed with 1500 grams of distilled water and placed in a 2-liter Erlenmeyer flask on a shaker for 48 hours. After 48 hours, the mixture of water and leaves was passed through a clean net cloth and the pulp of the leaves was separated well. The obtained extract was passed through Whatman filter paper and centrifuged (3000 rpm) for 5 minutes for clarification. The aqueous extract was poured into glass containers and dried in an oven at 40 degrees Celsius. To remove any microbial contamination, the obtained powder was sterilized under UV light for 30 minutes. Aqueous extract obtained from Astbaragh plant was stored in a dark container covered with aluminum foil in a refrigerator (4 degrees Celsius) until the antifungal tests were performed.[15].

2-2- Evaluation of the antifungal activity of the extract of asterberg

Among the 4 antimicrobial methods to evaluate the antifungal activity of the aqueous extract of Astbaragh Bar*Alternaria*

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alternata ،*Alternaria solani* ،*Saccharomyces cerevisiae*And*Fusarium solani* used. The performed methods included: disk diffusion agar (Kerby-Boer), diffusion in agar with the help of a well (well agar), minimum inhibitory concentration (broth macrodilution) and minimum lethal concentration. Antifungal methods are briefly explained below.

1-2-2- Diffusion disk agar (Kirby-Boer)

In the agar disk diffusion method, sterile blank paper disks with a diameter of 5 mm were used. In this method, first, different concentrations of water extract of asterberg (20, 40, 60 and 80 mg/ml) were prepared. Sterile blank discs were soaked in the mentioned concentrations for 30 minutes. 100 microliters of standard fungal strains were cultivated on the surface of sabrose dextrose agar medium. Disks containing different concentrations of water extract of astragalus were gently placed on the surface of the culture medium using sterile forceps. A blank disc without extract was also placed in the middle of the microbial container as a control sample. For the purpose of pre-release, the cultured microbial containers were kept at refrigerator temperature for one hour. After one hour, the microbial containers were kept in the incubator at 27°C for 72 hours. After this period of time, the diameter of the halo of microbial growth was measured using a ruler and the data was recorded in millimeters.[16].

2-2-2- Agar well

In order to perform the agar well test, first, 5 mm diameter wells were dug in Saberose Dextrose Agar culture medium with the help of a sterile Pasteur pipette. The ends of the created wells were closed by a drop of molten agar. It is worth mentioning that 5 wells were created on the surface of each Petri dish (4 corresponding to the concentrations of 20, 40, 60 and 80 mg/ml of the aqueous extract of Astabarak and 1 for control). 100 microliters of standard fungal strains were cultivated on the surface of sabrose dextrose agar medium. The microbial Petri dishes were kept in a greenhouse at 27°C for 72 hours. After 72 hours, the diameter of the growth halo around the wells was accurately measured with a ruler and reported in millimeters.[17].

3-2-2- Minimum inhibitory concentration

12 sterilized laboratory tubes according to Figure 1 were used to evaluate the minimum inhibitory concentration of the aqueous extract of asterberg. In this method, first, a concentration of 512 mg/ml was prepared (5.12 g of the extract was mixed with 9.5 of Sabrose dextrose broth culture medium and 0.5 ml of dimethyl sulfoxide). The next concentrations which included 256, 128, 64, 32, 16, 8, 4, 2 and 1 mg/ml were prepared using dilution in liquid. 2 laboratory tubes were considered as negative control and positive control. 100 microliters of each of the standard fungal strains was inoculated into each of the prepared concentrations. After inoculation, the cultured tubes were placed in a greenhouse at 27 degrees Celsius for 72 hours. After this period of time, the tubes were visually evaluated by comparing with the control tubes. The first tube in which no turbidity was observed (fungal strain did not grow) was considered as the minimum inhibitory concentration.[18].

Figure 1- The minimum inhibitory concentration of the aqueous extract of *Calotropis procera*.

4-2-2- Minimum lethal concentration

The minimum lethal concentration was determined according to the method of Rahmati Junidabad and Alizadeh Behbahani et al. (1400). In this method, surface culture was carried out from the minimum inhibitory concentration tube to higher concentrations in the amount of 100 microliters on the surface of Sabrose dextrose agar culture medium. After that, the cultured Petri dishes were kept in a greenhouse at 27 degrees Celsius for 72 hours. The first petri dish in which no colony was observed was reported as the minimum lethal concentration[19].

3-2- Investigating the interaction of the aqueous extract of asterberg with the antibiotic nystatin

In order to investigate the interaction of antibiotic nystatin with aqueous extract of asterberg, the method of Sosni Gharibund et al. (2018) was used. In this method, in short, to evaluate the interaction (synergism or reduction) of the aqueous extract of the leaves of asterberg in combination with the antibiotic nystatin, the concentration under inhibition was used. In this research, the inhibitory concentration was

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used, which was 1.2, the minimum inhibitory concentration[20].

4-2- Statistical analysis

The tests of this research were carried out in 3 repetitions and the results were reported as "mean ± standard deviation". From analysis of variance One-way and Duncan's post hoc test ($p < 0.05$) were used for data analysis.

3. Results and Discussion

Due to the resistance of pathogenic microorganisms as well as the desire of consumers, today's human tendency is to use natural compounds to treat diseases. In the present study, the antifungal activity of the aqueous extract of asterago against fungi*Alternaria alternata*، *Alternaria solani*، *Saccharomyces cerevisiae*And *Fusarium solani*Checked out. The results of the anti-fungal effect of the aqueous extract of asterago using the agar disk diffusion method are shown in Figure 2.

According to the results, the anti-fungal effect of the aqueous extract of asterberg was dependent on the concentration and the type of fungus under investigation. With the increase in the concentration of the aqueous extract of the asterisk, an increase in the diameter of the non-growth halo was observed for the fungal strains. The results showed that at a concentration of 20 mg/ml of the aqueous extract of Astabreq, the antifungal effect for*Alternaria solani* And *Fusarium solani*It was not observed, but the diameter of the halo of non-growth for*Alternaria alternata* And *Saccharomyces cerevisiae*It was equal to 6.30 and 7.70 mm, respectively. at a concentration of 40 mg/ml for all fungal strains except*Alternaria solani*An aura of lack of growth was observed. At the concentrations of 60 and 80 mg/ml, the aqueous extract of asterbeg led to the prevention of the growth of all fungal species. generally,*Saccharomyces cerevisiae*And*Alternaria solani* respectively, the most sensitive and resistant fungal strains with the highest and the lowest diameter of the halo of non-growth were the water extractp<0.05). so that the diameter of the non-growth halo for*Saccharomyces cerevisiae*And*Alternaria solani* In the presence of the concentration of 80 mg/ml extract, it was equal to 13.20 and 8.20 mm, respectively. In Figure 3, a view of the antifungal effect of asterberg extract on fungi*Fusarium solani* It is shown by the agar disk diffusion method.

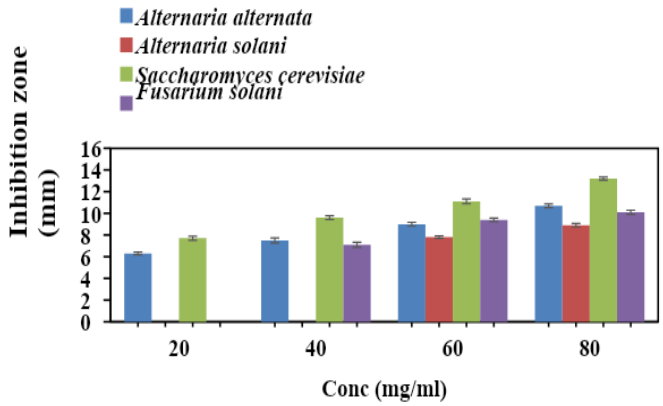

Figure 2. Antifungal activity of *Calotropis procera* extract based on disc diffusion agar method.

Figure 3. The antifungal effect of *Calotropis procera* on *Fusarium solani*.

The results of the present study showed that the findings of the agar well antimicrobial test were in line with the results of the agar disk diffusion test, and the antifungal effect of the aqueous extract of Astbaragh was dependent on the type of mushroom strain and the concentration of the extract (Figure 4). The antifungal effect of the extract (diameter of non-growth halo) increased significantly with increasing its concentration. In addition, all concentrations of the extract were effective against the fungal strains. No growth halo diameter for fungi*Alternaria alternata*، *Alternaria solani*، *Saccharomyces cerevisiae*And *Fusarium solani*At the concentration of 80 mg/ml of the extract, it was equal to 12.90, 11.10, 14.80 and 11.40 mm, respectively. In this regard, strains*Saccharomyces cerevisiae* With the highest diameter of the aura of lack of growth and strain*Alternaria solani* With the smallest diameter of the non-growth halo, they were respectively the most sensitive and resistant fungal species to the extract ($p<0.05$). Comparing the results of the agar disk diffusion and agar well tests showed that the average diameter of the halo of non-growth in the agar well test was significantly higher than that of the agar disk diffusion antifungal method. The reason for this has been attributed by many previous researchers to the direct contact between the antimicrobial agent and the microorganism in the diffusion method in the well. In the agar disk diffusion method, there is indirect contact between the antimicrobial substance and the microorganism, and in fact, the antimicrobial substance needs to penetrate from the paper disk to the surface of the microbial culture medium [12, 21, 22].

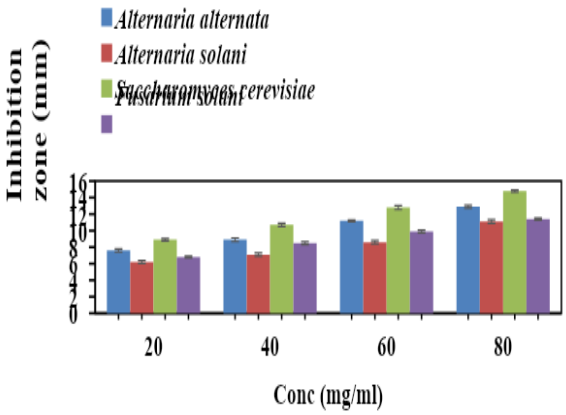

Figure 4. Antifungal activity of *Calotropis procera* extract based on well diffusion agar method.

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The results of the antimicrobial effect of the extract using the minimum inhibitory concentration (MIC) and minimum lethal concentration (MFC) methods are presented in Table 1. According to the results, the lowest MIC and MFC in line with the results of other antifungal methods belong to*Saccharomyces cerevisiae*which showed the highest sensitivity among fungal strains to the extract. AmountsMIC To*Alternaria alternata*، *Alternaria solani*، *Saccharomyces cerevisiae*And *Fusarium solani* It was equivalent to 32, 128, 16 and 128 mg/ml, respectively. While the valuesMFC For these strains, 128, >512, 128 and 512 mg/ml were obtained, respectively.

Table 1. Antifungal activity of *Calotropis procera* extract based on MIC and MFC methods

Fungi species	MIC (mg/ml)	MFC (mg/ml)
Alternaria alternata	32	128
Alternaria solani	128	> 512
Saccharomyces cerevisiae	16	64
Fusarium solani	128	512

The results of the antimicrobial effect of nystatin fungicide and its interaction with the aqueous extract of asterberg are shown in Figure 5. The results showed that nystatin is effective against all fungi and the most effect is on*Saccharomyces cerevisiae*(21.8 mm) was observed (Figure 5). The interaction of nystatin with jashir extract improved the antimicrobial activity of nystatin. So that the average diameter of the halo of nystatin growth is 16.60, 14.70, 21.80 and 18.10 mm for*Alternaria alternata*، *Alternaria solani*، *Saccharomyces cerevisiae*And *Fusarium solani* It increased to 19.20, 15.20, 22.80 and 19.100 mm in the presence of nystatin + water extract of asterberg, which shows the synergistic effect between the extract and nystatin.

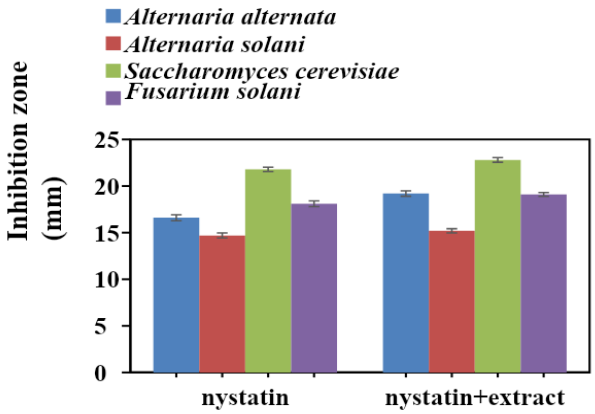

Figure 5. The mean inhibition zone diameter (mm) of nystatin and its interaction with *Calotropis procera* extract on some fungal species.

The antifungal effect of phenolic compounds occurs through disruption of the fungal cell membrane [23]. In the research carried out by Nena et al. (2013), it was found that the diameter of the halo of non-growth of the extract of asterberg for

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pathogenic fungal strains was on average 10.5 to 30 mm.[24]. Yasmin et al. (2008) confirmed the antimicrobial effect of the methanolic extract of asterberg plant[25]. Karim et al. (2008), in another study, also proved the antimicrobial effect of the ethanolic extract of the leaves of asterberg.[26]. Considering that plant extracts are rich in terpene compounds, the antimicrobial effect of plant extracts can also be attributed to these compounds. Terpene compounds with mechanisms including mitochondrial membrane disruption, inhibition of electron transfer (proton pump inhibition) and ATPase inhibition [23, 27, 28].

4- The final conclusion

The results of the present study showed that the aqueous extract of Astebraq has an antifungal effect against*Alternaria alternata*، *Alternaria solani*، *Saccharomyces cerevisiae*And *Fusarium solani*Was. Antifungal activity of asterberg extract increased with increasing extract concentration. According to the antifungal results of the extract in exogenous conditions, it seems that the aqueous extract of asterberg can be used as a natural antifungal agent to inhibit the growth of pathogenic fungi in fresh fruits and vegetables. However, more research needs to be done in this field.

5- Appreciation and thanks

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6- Resources

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Evaluation of the antifungal effect of Froriepia subpinnata essential oil on Aspergillus niger (black mold) and Botrytis cinerea (gray mold) grape poisoning agent: A study" in vitro". *Journal of Food Science and Technology (Iran), 17*(108), 75-83.

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چکیده

کالوتروپیس پروسرا از خانواده Apocynaceae در ایران استبرق نامیده می شود. این گیاه در مناطق گرمسیری و سواحل جنوبی ایران و شمال آفریقا، آسیای خاورمیانه و آسیای جنوب شرقی روی تپه های ماسه ای ساحلی یافت می شود. در این پژوهش آزمایشگاهی، عصاره استبرق با استفاده از حلال آب استخراج شد. اثر ضد قارچی عصاره آبی استبرق بر سویه‌های *آلترناریا آلترناتا*، *آلترناریا سولانی*، *ساکارومایسس سرویزیه* و *فوزاریوم سولانی* بررسی شد. فعالیت ضد قارچی عصاره استبرق با روش‌های دیسک دیفیوژن آگار (کربی-بوئر)، انتشار در چاهک آگار، حداقل غلظت مهارکنندگی و حداقل غلظت کشندگی ارزیابی شد. در حالت ترکیبی (برهمکنش) عصاره آبی استبرق با آنتی بیوتیک نیستاتین مورد بررسی قرار گرفت. بطور کلی، *ساکارومایسس سرویزیه* و *آلترناریا سولانی* به ترتیب با بالاترین و کمترین قطر هاله عدم رشد، حساس‌ترین و مقاوم‌ترین سویه‌های قارچی نسبت به عصاره آبی استبرق بودند. بطوریکه قطر هاله عدم رشد برای *ساکارومایسس سرویزیه* و *آلترناریا سولانی* در حضور غلظت ۸۰ میلی‌گرم در میلی‌لیتر عصاره به ترتیب برابر با ۱۳/۲۰ و ۸/۲۰ میلی‌متر بود. در حالت ترکیبی (برهمکنش) عصاره آبی استبرق با آنتی‌بیوتیک نیستاتین برای تمامی سویه‌های قارچی حالت هم‌افزایی مشاهده شد. حداقل غلظت مهارکنندگی و حداقل غلظت کشندگی برای سویه *ساکارومایسس سرویزیه* به ترتیب ۱۶ و ۶۴ میلی‌گرم بر میلی‌لیتر بود. حداقل غلظت مهارکنندگی و حداقل غلظت کشندگی برای *آلترناریا سولانی* به ترتیب ۱۲۸ و > ۵۱۲ میلی‌گرم بر میلی‌لیتر بود.