Journal of Food Science and Technology (Iran)

Homepage:www.fsct.modares.ir

Scientific Research

Antifungal effect of aqueous, hydroalcoholic and ultrasonic extracts of shallot (*Allium stipitatum*) on *Aspergillus niger* and *Penicillium expansum* isolated from moldy cheese.

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ARTICLE INFO

Article History:
Received: 2025/09/23
Review: 2025/12/05
Accepted: 2025/12/14

Keywords:
Shallot (*Allium stipitatum*) extract, Antifungal, MIC (Minimum Inhibitory Concentration), MFC (Minimum Fungicidal Concentration).

DOI: 10.48311/fsct.2026.117596.82927

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ABSTRACT

Contamination of food products by molds is a significant challenge in the food industry. The aim of this study was to investigate and compare the antifungal effects of aqueous, hydroalcoholic (ethanolic), and ultrasonic extracts of the shallot (*Allium stipitatum*) on these two molds. Molds were isolated from spoilaged Iranian white cheese. Aqueous and hydroalcoholic extracts were prepared using distilled water and 70% ethanol by Soxhlet extraction, respectively, and the ultrasonic extract was obtained using an ultrasonic bath at 45°C and 20 kHz for 20 minutes. Antifungal activity of the extracts was evaluated by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC), data were analyzed using two-way ANOVA and Duncan's test with a 95% confidence level. The results demonstrated that aqueous, hydroalcoholic, and ultrasonic extracts exhibited antimicrobial effects against both fungi. ultrasonic extract of shallot had the strongest inhibitory and fungicidal effects against both fungi ($P<0.05$). Additionally, *Penicillium expansum* was more resistant to all extract types compared to *Aspergillus niger*, requiring higher extract concentrations for inhibition and fungicidal effect ($P<0.05$). The differences between the active compounds of shallot aqueous, hydroalcoholic and ultrasonic extracts could indicate the key role of the antifungal compounds present in the extract.

1- Introduction

Fermented dairy products, particularly cheese, hold a significant position in the human diet owing to their wide variety and high nutritional value [1]. Nevertheless, cheese production and storage processes can facilitate the proliferation of various microorganisms, including filamentous fungi, which results in product spoilage, quality degradation, and, occasionally, the production of toxic secondary metabolites (mycotoxins). Fungi belonging to the genera *Aspergillus* and *Penicillium* are recognized as key agents of spoilage in various cheese types. These molds can contaminate a broad spectrum of cheeses, causing undesirable alterations in their flavor, odor, texture, and color. A practical strategy to mitigate this issue involves the application of antifungal compounds during cheese manufacturing or ripening [2,3].

Due to growing public apprehension regarding the consumption of synthetic preservatives and escalating microbial resistance, there has been a significant surge in interest towards natural sources as safe and effective alternatives for controlling microbial spoilage in the food industry [4]. Medicinal plants demonstrate high potential in this domain, primarily due to their diverse bioactive compounds possessing antimicrobial and antioxidant properties [4].

The shallot plant (*Allium stipitatum*), similar to other members of the *Allium* genus, is rich in bioactive compounds. Native to the Middle East and Central Asia, it has historically been employed in traditional medicine for treating various ailments, in addition to its common culinary uses. Recent scientific investigations have also highlighted the numerous pharmacological properties of this plant, including its antibacterial, antiviral, anti-inflammatory, and anticarcinogenic activities [5,6,7]. Organosulfur Compounds, Saponins, and Flavonoids have been identified as the primary compounds responsible for these beneficial properties [8]. Furthermore, other compounds, such as Allyl Propyl Disulfide, Diallyl Disulfide, Diallyl Trisulfide, and Ajoene, have been isolated from shallot; these are typically the degradation products of Alliin [9]. These compounds impart the characteristic aroma of shallot and demonstrate notable antibacterial, antifungal, and antiviral efficacy [10]. Their principal antifungal mechanism involves reacting with thiol (-SH) groups present in microbial proteins and enzymes, thereby disrupting their function. This action subsequently leads to damage to the cell membrane, leakage of cellular contents, and ultimately, cell death [11].

Therefore, the objective of this study was to isolate the dominant cheese spoilage molds and investigate the antifungal efficacy of shallot extracts against them under *in vitro* conditions.

2-Materials and Methods

2.1. Mold Isolation

First, fresh, low-fat Iranian white pasteurized cheese (Doosheh Haraz, Iran) was procured. The package was then opened after disinfection with 70% ethanol and kept under a laboratory hood, protected from secondary contamination, at ambient temperature for 7 days until mold growth became visible. The visible mold on the cheese was aseptically isolated, and the genus and species of the fungi were identified by a university mycologist using the slide culture method under an optical microscope (Nikon, Japan).

2.2. Preparation of Shallot Extracts

Fresh shallot bulbs were authenticated by the Horticulture Department of Sari Agricultural Sciences and Natural Resources University and subsequently transferred to the Chemistry and Feed Analysis Laboratory at Islamic Azad University, Babol Branch. The collected samples were thoroughly washed several times with clean water. After complete water removal, they were sliced and completely dried in a hot air stream at 40°C, away from sunlight. The dried shallot was then powdered using an electric grinder (Best, China) and

sieved (Damavand, Iran) to enhance the extraction efficiency.
A Soxhlet apparatus was utilized for the extraction process (50 g of shallot powder with 450 mL of 70% ethanol (Merck, Germany)). The apparatus was switched off after 3 hours, and the solvent was evaporated using a rotary vacuum evaporator (IKA, Germany). The resulting extract was stored in the refrigerator until the experiments [12]. For the hydroalcoholic extract, 70% ethanol was used instead of distilled water [13].

2.3. Preparation of Ultrasonic Extract

For the preparation of the ultrasonic extract, 10 g of the plant material was mixed with 100 mL of distilled water and subjected to an ultrasonic bath (Easy Elma, Germany) at 45°C and 20 kHz power for 20 minutes. This process facilitated the release of the plant extract through tissue disruption. The extract was then passed through filter paper, and the solvent was evaporated using a rotary evaporator. The final extract was stored away from sunlight in the refrigerator until testing [14].

2.4. Determination of Minimum Inhibitory Concentration (MIC)

The eleven-tube method was employed to determine the minimum concentration of shallot extract required to inhibit fungal growth. All tubes contained Sabouraud Dextrose Broth (Merck, Germany). One milliliter of diluted shallot extract (1:4 with DMSO (Merck, Germany)) was added to the first tube and thoroughly mixed. One milliliter was then transferred from the first tube to the second, and the rest of the tubes were prepared similarly using serial dilution. Finally, 10 µL of fungal suspension was added to all tubes except for the 11th tube (negative control). All tubes were incubated in an incubator (Memmert, Germany) at 25°C for 72 hours. The first tube (containing the lowest concentration of shallot extract) showing no turbidity was considered the Minimum Inhibitory Concentration (MIC). This assay was performed in triplicate [15].

To prepare the fungal suspension, a pure colony was used under completely sterile conditions. The fungus was grown in an incubator, spores were sampled, and transferred into Sabouraud Dextrose Broth medium. Using a spectrophotometer (Unico, China) at a wavelength of 520 nm, the transmittance of the diluent solution (Sabouraud Dextrose Broth) was adjusted to 90%. This equated to a final concentration of 1×10^6 colony forming units (CFU) per milliliter of the suspension [15].

2.5. Determination of Minimum Fungicidal Concentration (MFC)

To determine the Minimum Fungicidal Concentration (MFC), surface plating was performed on Sabouraud Dextrose Agar (Merck, Germany) using samples from the MIC tube and the tube immediately before and after it. All plates were incubated at 25°C for 72 hours. The lowest concentration of the extract that resulted in no mold growth was considered the Minimum Fungicidal Concentration (MFC). This assay was performed in triplicate [15].

2.6. Statistical Analysis

The results of the microbiological assays were presented as the mean ± standard deviation. Mean data analysis was conducted using two-way Analysis of Variance (ANOVA) and Duncan's test at a 95% confidence level ($P<0.05$) using SPSS 20 software. Graphs were plotted using EXCEL 2016.

3-Results

Microscopic examination using the slide culture method revealed that the two molds isolated from the spoiled cheese were *Aspergillus niger* and *Penicillium expansum*.
The microbiological assays demonstrated that the Minimum Inhibitory Concentration (MIC) of the aqueous, hydroalcoholic, and ultrasonic shallot extracts against *Aspergillus niger* was significantly lower ($P<0.05$) than the MIC against *Penicillium expansum*. Consequently, the highest antifungal activity was

consistently observed against *Aspergillus niger* (Table 1).

Table 1. Minimum Inhibitory Concentration (MIC) of aqueous, hydroalcoholic and ultrasonic extracts of shallot (µg/ml)

Extract	*Penicillium expansum*	*Aspergillus niger*
	Mean ± SD	Mean ± SD
Aqueous	100 ± 0.0 ^{Cb}	62.5 ± 25.0 ^{Ca}
Hydroalcoholic	43.75 ± 12.5 ^{Bb}	18.75 ± 7.21 ^{Ba}
Ultrasonic	10.93 ± 3.12 ^{Ab}	5.46 ± 1.56 ^{Aa}

Different capital letters (A, B, …) in each column indicate significant differences (P<0.05).
Different lowercase letters (a, b, …) in each row indicate significant differences (P<0.05).

In the determination of the Minimum Fungicidal Concentration (MFC), it was found that the aqueous shallot extract was capable of killing *Aspergillus niger* at a concentration greater than 100 µg/. The results further indicated that *Penicillium expansum* exhibited a higher survival capability against the shallot extracts compared to *Aspergillus niger* (Table 2).

Table 2. Minimum Fungicidal Concentration (MFC) of aqueous, hydroalcoholic and ultrasonic extracts of shallot (µg/ml) on *Aspergillus niger* and *Penicillium expansum*

Extract	*Penicillium expansum*	*Aspergillus niger*
	Mean ± SD	Mean ± SD
Aqueous	> 100	100 ± 0 ^{C}
Hydroalcoholic	75 ± 28.86 ^{Ba}	37.5 ± 14.43 ^{Ba}
Ultrasonic	21.78 ± 6.25 ^{Ab}	9.37 ± 3.61 ^{Aa}

Different capital letters (A, B, …) in each column indicate significant differences (P<0.05).
Different lowercase letters (a, b, …) in each row indicate significant differences (P<0.05).

4- Discussion

The use of the antimicrobial properties of medicinal plants in the form of extracts and essential oils presents an intelligent strategy for incorporating these natural antimicrobial resources into food products. This approach not only utilizes their antimicrobial characteristics but also allows for the incorporation of the plant's aroma, flavor, and color, if desired [16]. While shallot has traditionally been used as a flavoring agent, its pieces possess relatively weak antimicrobial properties. However, various studies have shown that the extract and essential oil of the plant exhibit significant antimicrobial effects [17].

Although essential oils possess stronger antimicrobial activity, a drawback to their use is the strong odor imparted to the food, which is not always acceptable to the production team. Therefore, using the extract is a more suitable solution. However, the lower antimicrobial properties of extracts limit the extent of this method's application.

In the present study, the antifungal properties of the aqueous, hydroalcoholic, and ultrasonic extracts were evaluated. The results indicated that the ultrasonic extract exhibited the highest antimicrobial effect compared to the other extracts. Specifically, the ultrasonic extract inhibited the growth of *Aspergillus niger* at a concentration of 5.46 µg/mL and the growth of *Penicillium expansum* at a concentration of 10.93 µg/mL.

The antimicrobial activity of the shallot plant is attributed to the presence of compounds that are chemically phenolic and flavonoid in structure, whose antimicrobial properties have been scientifically proven. However, the superiority of the ultrasonic shallot extract in antifungal properties over other extracts has several reasons. The first reason relates to the extraction mechanism: the ultrasonic

extraction method uses high-frequency sound waves, which cause the phenomenon of cavitation. As a result of this phenomenon, bubbles burst in the solvent medium, leading to greater cell wall disruption of the shallot. This results in the release of more bioactive compounds, such as phenolic, flavonoid, and sulfur-containing compounds, from the shallot tissue [18]. The second reason is that the ultrasonic method involves less heat compared to conventional methods, meaning heat-sensitive compounds like allicin are not degraded, and a higher concentration of active substances leads to greater antifungal potency [19].

The ethanol solvent is semi-polar, which facilitates the extraction of a relatively higher concentration of flavonoids, allicin, phenolic compounds, and some sulfur-containing compounds compared to the aqueous extract. The aqueous extract, due to its polar nature, primarily extracts the highest concentration of sugars, proteins, and water-soluble compounds [20]. In other words, ethanol, due to its moderate polarity, has the ability to dissolve both polar and semi-polar compounds, whereas water only extracts highly polar compounds. The main antifungal compounds in shallot, such as allicin and sulfides, are primarily semi-polar or lipophilic [21], which explains the weaker activity of the aqueous extract.

In the species comparison, *Aspergillus niger* showed greater sensitivity to the shallot extracts than the other fungus, while *Penicillium expansum* demonstrated relatively higher resistance. Nevertheless, the pattern of effect was consistent across all cases: the ultrasonic extract showed the highest inhibition, and the aqueous extract showed the lowest growth inhibition. Previous studies have shown that the *Aspergillus* genus is generally less resistant to essential oils and plant extracts than the *Penicillium* genus. For example, studies on the antifungal effect of methanol extract of garlic against *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Penicillium* species showed that the garlic extract had a stronger antifungal effect against the *Aspergillus* genus than the *Penicillium* genus [22,23,24].

The reasons for the higher resilience of *Penicillium* compared to *Aspergillus* against the antifungal properties of the extract have been investigated in prior research. The most significant reason is the difference in the structure and composition of the cell walls of the two fungal genera. The variation in the ratio of chitin, β\betaβ-glucan, mannan, and protein, coupled with a thicker or more resistant cell wall structure in the *Penicillium* genus, can impede the penetration of active compounds from the shallot extract [25]. The second reason is the presence of potent phosphatase, esterase, or oxidase enzymes in the *Penicillium* genus, which degrade and inactivate the active compounds in the shallot extract more effectively than in *Aspergillus* [26]. A third, more theoretical reason suggests that the shallot extract contains polyphenol and flavonoid compounds that exhibit less antifungal activity specifically against the *Penicillium* genus [27].

5- Conclusion

The results obtained from the antifungal assays of different shallot extracts demonstrated that the extraction method significantly impacts the degree of fungal growth inhibition against the tested species. In this experiment: The ultrasonic extract showed the lowest Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values against *Aspergillus niger* and *Penicillium expansum*, indicating the strongest antifungal activity. This strength is attributed to cellular cavitation, leading to greater release of phenolic and sulfur-containing compounds, and the preservation of heat-sensitive components. The aqueous extract exhibited the highest MIC and MFC values, resulting in the weakest antifungal effect. This is due to its inability to extract the important lipophilic and sulfur-containing antifungal compounds present in shallot. The ethanol extract showed a moderate antifungal effect because its semi-

polar nature allows for the extraction of both polar and semi-polar compounds. Overall, the current findings indicate that the ultrasonic extraction method can significantly enhance the antifungal activity of shallot by increasing the yield of its active compounds. Accordingly, the use of this method for preparing plant extracts for natural antifungal applications is recommended. Furthermore, conducting supplementary studies to precisely identify the effective compounds and investigate potential synergy with other common natural antifungal agents could be beneficial in developing effective plant-based antifungal agents.

Funding

The author declares that he / she has not received any funding.

Author Contributions

All activities were carried out by the author.

Competing Interests

The author confirms that he / she has no financial conflicts of interest or competing interests in this study.

Acknowledgements

The authors would like to express their sincere gratitude to the Vice-chancellor for Research and Technology of Agricultural Sciences and Natural Resources University of Khuzestan for supporting this study (1403.38).

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مجله علوم و صنایع غذایی ایران

سایت مجله: www.fsct.modares.ac.ir

مقاله علمی_پژوهشی

اثر ضد قارچی عصاره آبی، هیدروالکلی و اولتراسونیک موسیر بر *آسپرژیلوس نایجر* و *پنی سیلیوم اکسپانسوم* جدا شده از پنیر کپک زده

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اطلاعات مقاله

تاریخ های مقاله :

تاریخ دریافت: ۱۴۰۴/۰۸/۲۰

تاریخ داوری: ۱۴۰۴/۰۹/۱۴

تاریخ پذیرش: ۱۴۰۴/۰۹/۲۳

کلمات کلیدی:

عصاره موسیر،

ضد قارچی،

حداقل غلظت ممانعت از رشد،

حداقل غلظت کشنده.

DOI: 10.48311/fsct.2026.117596.82927

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چکیده

آلودگی مواد غذایی به کپک‌ها، یکی از چالش‌های مهم در صنایع غذایی به شمار می‌رود. هدف از این پژوهش، مطالعه و مقایسه اثرات ضد قارچی عصاره‌های آبی، هیدروالکلی (اتانولی) و اولتراسونیک حاصل از گیاه موسیر، بر رشد این دو کپک بود. کپک مورد مطالعه از فساد پنیر سفید ایرانی کپک‌زده جدا شدند. عصاره‌های آبی و هیدروالکلی موسیر به ترتیب با حلال آب مقطر و الکل اتانول ۷۰ درصد با استفاده از دستگاه سوکسله؛ و عصاره اولتراسونیک با بهره‌گیری از حمام فراصوت در دمای ۴۵ درجه سلسیوس و توان ۲۰ کیلوهرتز به مدت ۲۰ دقیقه به دست آمدند. فعالیت ضد قارچی عصاره‌ها بر اساس تعیین حداقل غلظت ممانعت از رشد (MIC) و حداقل غلظت کشنده (MFC) مورد ارزیابی قرار گرفت و تجزیه‌وتحلیل میانگین داده‌ها با آنالیز واریانس دوطرفه و تست دانکن با سطح اطمینان ۹۵ درصد انجام شد. نتایج نشان داد که عصاره‌های آبی، هیدروالکلی و اولتراسونیک بر روی هر دو قارچ مورد مطالعه اثر ضد میکروبی داشتند. قوی‌ترین MIC و MFC به‌دست‌آمده به ترتیب مربوط به عصاره‌های اولتراسونیک، هیدروالکلی و آبی بود ($P<0.05$). همچنین مشخص شد که *پنی سیلیوم اکسپانسوم* در مقایسه با *آسپرژیلوس نایجر* در برابر همه انواع عصاره‌ها مقاوم‌تر بود و مقادیر بالاتری از عصاره برای مهار رشد و کشتن قارچ لازم بود ($P<0.05$). تفاوت بین ترکیبات مؤثره عصاره‌های آبی، هیدروالکلی و اولتراسونیک موسیر می‌تواند نشان‌دهنده نقش کلیدی ترکیبات ضد قارچی موجود در عصاره باشد.