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Experimental Article

Evolution of Antifungal Activity of *Artemisia herba-alba* Extracts on Growth of *Aspergillus* sp. and *Rhizopus* sp.

Eman MG Gebreil[1], Nagwa SA Alraaydi[2], Saleh HM EL-Majberi[2] and Idress Hamad Attitalla[3]*

[1]Department of Microbiology, Faculty of Science, Benghazi University, Libya
[2]Department of Botany, Faculty of Science, Benghazi University, Libya
[3]Department of Microbiology, Faculty of Science, Omar Al-Mukhtar University, Al-Bayda, Libya

Abstract

Plant extracts and their constituents have a long history as antifungal agents, but their use in biotechnology as preservatives, due to the increasing resistance of fungi to fungicides, has been rarely reported in Libya. The aim of this study is to evaluate the antifungal activity of ethanol extract and water extract of the wild native plant *Artemisia herba-alba* against two genera of mold fungi *Aspergillus* sp. and *Rhizopus* sp. This mold fungal causes significant damage to crops in the field or during storage. In this study, a hot ethanol extract was prepared using a device Soxhlet, and water extract hot as well as a cold ethanol extract and cold-water extract aqueous extract three concentrations (25% - 50% - 75%) of plant extracts were used on the tested fungi. All extracts showed an effect on the tested fungi. The concentrations of (75% - 50%) of the extracts had an effect on the tested fungi, while most concentrations of 25% of the extracts did not record any effect on the tested fungi. The hot ethanol extract of *the Artemisia herba-alba* plant was more effective than the other extracts. *Aspergillus* sp. was recorded with the highest inhibitory zone (0.73 mm).

More Information

***Address for correspondence:**
Idress Hamad Attitalla, Department of Microbiology, Faculty of Science, Omar Al-Mukhtar University, Al-Bayda, Libya, Email: idress.hamad@omu.edu.ly

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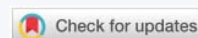
Keywords: Antifungal activity; *Artemisia herba-alba*; *Aspergillus* sp.; *Rhizopus* sp.



Introduction

For many years, people have utilized plants for therapeutic purposes. Globally, there has been a surge in interest in ethnopharmacy as a source of pharmacologically active chemicals, especially in the hunt for medications to combat multi-resistant microorganisms. Additionally, because of their availability and economic status, plants constitute the primary source of medicine in some impoverished nations for the treatment of infectious diseases. Nonetheless, despite the significant number of novel antibiotics derived from natural or semi-synthetic resources being brought to market, only around 20% of the world's plant species have been subjected to pharmacological or biological testing [1]. Scientists are trying to devise new means to increase food production with the world population expanding rapidly. Unfortunately, severe crop loss is still inevitable owing to plant diseases, particularly those caused by phytopathogenic fungi. Application of the synthetic fungicides has been considered to be one of the cheapest and most common approaches for control. However, these chemicals usually take long timelines to be degraded completely causing heavy toxicity to human beings, domestic animals, etc [2]. In this work, we try to introduce some medical plants from Libyan growing in Libya as wild plants like *Artemisia herba-alba* family Asteraceae as herd perennial growing in arid areas, is endemic in the south of Benghazi in Libya and in other rest of the Mediterranean. The parts of plants that are most often used for medicinal purposes are fruits and/or seeds, though other parts of the plants can be used, roots or leaves [1]. The mold fungi that have not been selected in this study, *Aspergillus* sp. and *Penicillium* sp., are two common worldwide spread fungi belonging to class Ascomycetes. Both fungi live in soil as saprophytes, which are called soil-borne fungi or soil inhabitants, causing damage to wounded ribbing fruits in fields or stores through rotting symptoms as well as infecting vegetables. *Rhizopus* sp. is also a severe mold and causes the rotting of fruits and vegetables, especially in humid storage. This genus belonging to class Zygomycetes is found all the time in soil and air as spores. The extensive use of artificial antifungal compounds and fang fungicides to control mold fungi has great hazardous effects on the environment and human health due to the toxicity effect from residual remaining compounds. For this reason, nowadays we are forcing and concentrating on safe alternative natural antifungal compounds extracted from medical plants.

Literature review

Evaluate the antifungal activity of aqueous extract and essential oil of *Artemisia herba-alba* on some specific storage fungi. This plant has been selected on the basis of its ethnobotanical uses. Different concentrations of aqueous extract (20, 25, and 30%) and essential oil (0.15, 0.175, 0.200, and 0.250%) were evaluated in vitro for their antifungal activity against *Fusarium* spp. and *Alternaria* spp. The concentration of 30% of extracts inhibits the growth of Alternaria while 0.025 % of essential oil recorded a good antifungal activity. On the other hand, the aqueous extracts showed better efficacy than essential oil on *Fusarium* spp. and *Alternaria* spp. Therefore the *Artemisia herba-alba* aqueous extract and its richness of secondary metabolites (flavonoids, alkaloids, tannins, saponins, and steroids) [3].

In another study, the inhibitory efficacy of aqueous extract of *Artemisia herba-alba* and *Borago officinalis* leaves was evaluated in a type of bacterium that posits a gram stain, including the isolated Staphylococcus bride the results showed efficacy against microbial for both extracts. The bacteria are under study some of the active compounds have been detected in the leaves of *Artemisia herba-alba* and *Borago officinalis* these leaves were found to contain (tannins, alkaloids, resins, soaps, phenols, flavonoids, and (Glycoside). Also, the sensitivity of this isolation was studied for some antibiotics, and most of them were resistant to Ceftriaxone, Amikacin, Imipenem, and Ciprofloxacin, with a rate of 100%. The results showed synergism efficacy when mixing the antibiotic Ceftriaxone, Amikacin, Imipenem, and Ciprofloxacin with each of the aqueous extracts of the blocks *Artemisia herba-alba* and *Borago officinalis* on a different bacterium under study [4].

Additionally, it was discovered that one study aimed to identify the phenolic compounds from *Artemisia herba-alba* in order to assess their in vitro antibacterial and antioxidant properties. Using 100% ethanol, absolute methanol, and distilled water, the maceration procedure was used to extract phenolic chemicals. The quantification of polyphenols and flavonoids was performed using the Folin-Ciocalteu reagent and the aluminum trichloride method, respectively. The evaluation of the antioxidant activity of the extracts was carried out by the FRAP, the DPPH• radical trapping, and the neutralization of the hydrogen peroxide technique. The lipid peroxidation was assessed by thiobarbituric acid reactive substances. In addition, the antibacterial activity of the three extracts was tested on *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 33862, *Escherichia coli* ATCC 2592, and *Pseudomonas aeruginosa* ATCC 27853 bacteria using agar diffusion and agar incorporation methods. The results showed that the methanolic extract was highly rich in polyphenols and flavonoids. Also, the reducing power $CE50 = 249.88 \pm 6.07$ μg/ml and the inhibition capacity of the DPPH• radical $CI50 = 34.71 \pm 0.96$ μg/ml were significantly higher ($p < 0.05$) than the ethanolic and aqueous extracts. Also, a highly significant inhibitory potential of lipid peroxidation was obtained with the methanolic extract ($MDA = 66.97 \pm 3.61$ μmol/g tissue). However, a highly significant hydrogen peroxide scavenging effect was obtained from the ethanolic extract. A better antibacterial activity was obtained with the methanolic and ethanolic extracts [5] of *Artemisia herba-alba* (Asso.) either as essential oils or as extracts. Tests using DPPH, β-carotene bleaching, and metal chelating power have demonstrated the antioxidant effects of essential oils, organic, and aqueous extracts of the same plant against free radicals. This plant's chemicals interact with components of fungal cells, including chitin, cell walls, membrane ergosterol, and eukaryotic nuclei, thereby interrupting their formation and conferring tremendous effectiveness. It is commonly known that sesquiterpene lactones reduce the hyphal development of fungal infections. This plant appears to have strong biological properties and may offer an alternative to current treatments for a variety of oxidative and viral illnesses [6].

Materials and methods

Plant sampling

The aerial parts of *Artemisia herba-alba* shrubs were collected from the Tamimi region east of Libya in November 2022. The collected samples were identified in the herbarium of the Botany Department, Faculty of Science, Benghazi University. The collected aerial parts were left to dry in an open-air process for about 7 days prior to the extraction. The dried plants were blended to produce fine powder to be used in the preparation of plant extracts [7].

Preparation of extracts

The dried and ground plant material 30 g of the dried powdered was placed in 70 ml of 96% ethanol and hot extracted using soxhlet for 3 h. The resulting solution was filtered and concentrated under reduced pressure (Rotatory evaporator). The residues were weighed and calculated as the percentage of yield (%) and then stored in a tightly closed container. 30 g of the dried powdered plant material was cold extracted by soaking in 70 ml of 96% Ethanol and 30 ml of distilled water for 24 h with frequent shaking. The extracts were filtered and evaporated under reduced pressure by using a Rotary evaporator. The obtained residues were filtered with filter paper 0.1 mm and dissolved in their solvent after that kept in a tightly closed container for further analysis [8].

The hot water extract was obtained by soaking (30 g) of plant powder in 70 ml distilled water and left for about 30 min. The extract was poured and concentrated under vacuum pressure; the obtained residues were filtered with filter paper 0.1mm. The cold water extract was obtained by maceration (30 g) of dried plant powdered in 70 ml distilled water for 24h then the residues were filtered with filter paper 0.1mm, following which, different concentrations (25% - 50% -75%)

of each extract were prepared [7]. Figure 1 show the Soxhlet-prepare the hot ethanol extract of the *Artemisia herba-alba* and Figure 2 show the Rotary evaporator – evaporation of hot ehhanol extracts of *Artemisia herba-alba*.

Figure 1: Soxhlet- prepare the hot ethanol extract of the *Artemisia herba-alba.*

Figure 2: Rotary evaporator – evaporation of hot ehhanol extracts of *Artemisia herba-alba.*

Collection of fungal samples

The fungi, selected in this study include *Aspergillus* sp. and *Rhizopus* sp.. Samples of (infected) vegetables onions and infected wet bread were randomly collected from the local market in Benghazi city.

Isolation of fungi

Isolation of *Aspergillus* sp. onto Agar Plates: Sterile cotton swabs were used by dipping the swab into sterile distilled water, then scraped off the surface of the infected onion area to collect fungal spores *[7]*. The sample was then transferred onto the surface of a Potato Dextrose Agar (PDA) by gently swabbing the surface. The plates were incubated at 25-30°C for 3 days to allow fungal growth [7].

Isolation of *Rhizopus* sp. onto agar plates: Using sterile tweezers, the collected sample of bread was transferred immediately onto a sterile petri dish containing the chosen agar medium potato dextrose agar (PDA). The inoculated petri dish was incubated at an appropriate temperature (typically between 25 °C – 30 °C) for fungal growth.

Preparing the fungal suspension

After harvesting the tested fungi (*Aspergillus* sp., *Rhizopus* sp.) after growth, if researchers are working with an agar culture, water should be added to the fungal colony, and a pipette is used to suspend the spores into the liquid. Once the fungal culture was ready, the liquid was typically turbid due to the presence of fungal spores. A sterile pipette or tube was used to transfer a known volume of the fungal culture into a clean, sterile container. If necessary, sterile water could be added to dilute the suspension to the desired concentration [7]. After preparing the fungal suspension, it may be necessary to dilute it to obtain a specific spore concentration for the experiment. The required concentration of fungal spores was determined, typically measured as colony-forming units (CFUs) or *spores per milliliter* (spores/mL) for experiments (Koh, et al. 2012). Dilution is advised if the fungal suspension is too concentrated, to start by making a 1:10 dilution. For example, by mixing 1 mL of the fungal suspension with 9 mL of sterile water or saline. The fungal suspension was stored at 4°C for 24 hours before use [9].

The antimicrobial activity experiments

The antimicrobial activity of the plant extracts was determined using the agar well diffusion method, where potato dextrose agar (PDA) plates were inoculated with the test fungal suspension (*Aspergillus* sp., *Rhizopus* sp.) when relieving a 1:10 concentration, on each plate of 7 wells that were made by sterile standard cork borer. Each well was filled with the same quantity (1 ml) of the different concentrations (25% - 50% - 75%) of studied plant extracts and the plates were then incubated for 48-72 h at 28 °C; three replicates of each plate were prepared. Antifungal activity was assessed by measuring the diameter of the growth-inhibition zone (GIZ) in mm for the test organisms compared to the control. The inhibition zone diameter for individual microorganisms was measured with a ruler in millimeters [10].

Results

Identification of isolated fungi

***Aspergillus* sp.:** On the surface of the Petri dish, *Aspergillus* sp. forms a circular colony with a distinct and smooth border. The colony initially appeared white or light yellow in color, but as it matured, it gradually turned darker, eventually becoming black. This dark coloration is due to the production of conidia, the fungal spores. The surface texture of the colony was slightly rough or velvety, with some areas appearing denser than others. From the underside of the Petri dish, the colony showed a lighter color at first, but as the fungus continued to grow, the black pigmentation of the conidia became visible, covering the lower surface as well. The edges may appear more defined, with some radial patterns depending on the growth conditions.

Under the microscope, *Aspergillus* sp. revealed a network

of fine, branching hyphae. These hyphae were relatively thick-walled and appear septate, forming a mycelial structure. The conidiophores, which are the structures that produce the conidia, are typically long and erect, bearing dark, spherical, or oval-shaped conidia at their tips. These conidia were densely packed and contributed to the black color of the colony. The conidiophore structures were well-defined and arranged in clusters, often giving the colony a spiky or brush-like appearance. [11].

***Rhizopus* sp.:** When observing *Rhizopus* sp. on a Petri dish, it was noticed that the colony typically started off as a round or somewhat irregular shape. Initially, the colony appeared white or light gray, becoming denser as it matured. Over time, the center of the colony began to darken, turning brown or grayish-brown, while the edges often remained lighter. The surface of the colony looked cottony or covered with a light layer of white mycelium, with some areas appearing denser, particularly in the central region. From the underside of the Petri dish, it was seen that the colony started out as white or very pale. However, as growth progressed and spores began to form, the color changed to dark brown or black in the central areas. This color change was due to the formation of spores at the tips of the reproductive structures. The edges may have appeared smoother or less defined than on the top, but as time passed, the color spread more from the center toward the edges [12].

When examining the sample under the microscope, *Rhizopus* presented as thick, long hyphae known as mycelium. These hyphae are non-septate, meaning the cells were continuous without cross-walls. The hyphae grew branching and intertwining to form a complex network. At the tips of these hyphae, swollen structures called "sporangiophores," which bear the sporangia were observed. The sporangia are round or oval sacs that contain spores. Initially, the spores were transparent or white, but as they matured, they turned darker. The sporangia, which appeared as swollen structures at the tips of the hyphae, eventually ruptured, releasing the spores into the air. This process helps the fungus spread and propagate. Figure 3 Show *Aspergillus* sp. & Figure 4 *Rhizopus sp*. Under compound light microscope.

Figure 3: *Aspergillus* sp

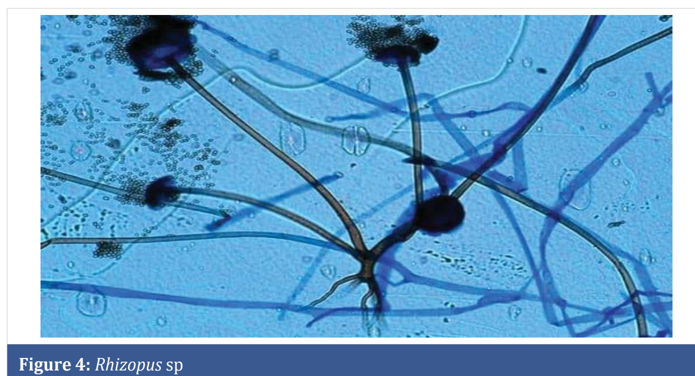


Figure 4: *Rhizopus* sp

Effect of *Artemisia herba-alba* Ethanol Extract (Cold) on Growth of Two Fungal Genera *Aspergillus* sp. and *Rhizopus* sp.

All *Artemisia herba-alba* ethanol extract (Cold) concentrations had different degrees of antifungal activity against the tested fungi, the highest antifungal activity was recorded for *Artemisia herba-alba* extract in concentration 75% against *Aspergillus* sp. (0.73 mm) whereas, the extract concentration 25% was the slightest effective against the tested fungi (Table 1).

Effect of *Artemisia herba-alba* ethanol extract (Hot) on the growth of two fungal genera *Aspergillus* sp. and *Rhizopus* sp.

All *Artemisia herba-alba* ethanol extract (hot) concentrations had different degrees of antifungal activity against the tested fungi, the highest antifungal activity was recorded for *Artemisia herda-alba* extract in concentration 75% against *Aspergillus* sp. (0.86 mm) whereas, and the extract concentration 25% was the slightest effective against the tested fungi (Table 2). Figure 5 show Effect of *Artemisia herba-alba* ethanol extract (hot-cold) on a. *Aspergillus* sp. b. *Rhizopus* sp.

Table 1: Effect of Artemisia *herba-alba* ethanol extract (cold) on fungal growth.

Extract concentrations (%)	Growth-inhibition zone (mm) of plant pathogenic fungi (Mean ± SD)	
	***Aspergillus* sp.**	***Rhizopus* sp.**
0 Distilled Water	0.00	0.00
25	0.26 ± 0.057	0.2 ± 0.00
50	0.56±0.057	0.33 ± 0.057
75	0.73±0.057	0.46 ± 0.057
Ethanol 96%	0.00	0.00

SD: Standard Deviation; ND: Not Detected

Table 2: Effect of *Artemisia herba-alba* ethanol extract (hot) on fungal growth.

Extract concentrations (%)	Growth-inhibition zone (mm) of plant pathogenic fungi (Mean± SD)	
	***Aspergillus* sp.**	***Rhizopus* sp.**
0 Distilled Water	0.00	0.00
25	0.23 ± 0.057	0.2 ± 0.00
50	0.53 ± 0.057	0.43 ± 0.057
75	0.86 ± 0.00	0.56 ± 0.057
Ethanol 96%	0.00	0.00

SD: Standard Deviation; ND: Not Detected

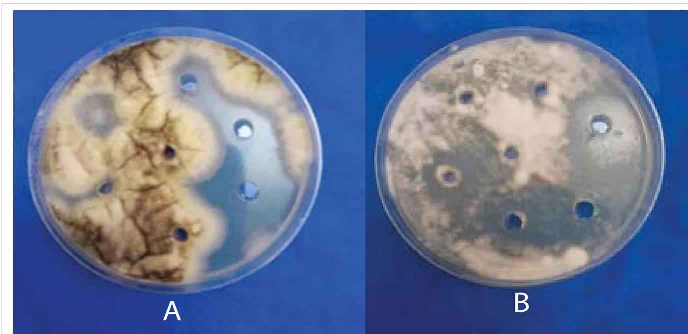


Figure 5: Effect of *Artemisia herba-alba* ethanol extract (hot-cold) on a. *Aspergillus* sp. *b. Rhizopus* sp.

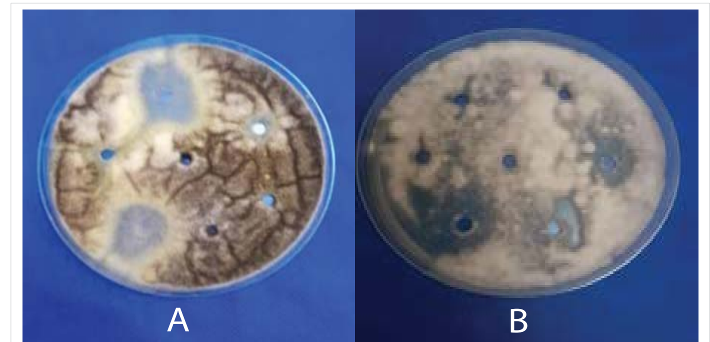


Figure 6: Effect of *Artemisia herba-alba* water extract (hot-cold) on a. *Aspergillus* sp. *b. Rhizopus* sp.

Effect of *Artemisia herba-alba* Water Extract (Cold) on growth of two fungal genera *Aspergillus* sp. and *Rhizopus* sp.

All *Artemisia herba-alba* water extract (cold) concentrations had different degrees of antifungal activity against the tested fungi, the highest antifungal activity was recorded for *Artemisia herba-alba* extract in concentration 75% against *Aspergillus* sp. (0.6 mm) whereas, and the extract concentration 25% was the slightest effective against the tested fungi (Table 3).

Effect of *Artemisia herba-alba* water extract (Hot) on growth of two fungal genera *Aspergillus* sp. and *Rhizopus* sp.

All *Artemisia herba-alba* water extract (hot) concentrations had different degrees of antifungal activity against the tested fungi, the highest antifungal activity was recorded for *Artemisia herba-alba* extract in concentration 75% against *Aspergillus* sp. (0.73 mm) whereas, and the extract concentration 25% was the less influential effective against the tested fungi (Table 4). Generally, tested fungi *Aspergillus* sp. and *Rhizopus* sp. Figure 6 show the effect of Artemisia herba-alba water extract (hot-cold) on a. *Aspergillus* sp. b. *Rhizopus* sp.

Table 3: Effect of *Artemisia herba-alba* water extract (cold) on fungal growth.

Extract concentrations (%)	Growth-inhibition zone (mm) of plant pathogenic fungi (Mean ± SD)	
	Aspergillus sp.	*Rhizopus* sp.
0 Distilled Water	0.00	0.00
25	0.3 ± 0.00	0.16 ± 0.057
50	0.43 ± 0.057	0.33 ± 0.057
75	0.6 ± 0.1	0.43 ± 0.057
Ethanol 96%	0.00	0.00

SD: Standard Deviation; ND: Not Detected

Table 4: Effect of *Artemisia herba-alba* water extract (hot) on fungal growth.

Extract concentrations (%)	Growth-inhibition zone (mm) of plant pathogenic fungi (Mean ± SD)	
	Aspergillus sp.	*Rhizopus* sp.
0 Distilled Water	0.00	0.00
25	0.33 ± 0.057	0.23 ± 0.057
50	0.53 ± 0.057	0.4 ± 0.00
75	0.73 ± 0.057	0.56 ± 0.057
Ethanol 96%	0.00	0.00

SD: Standard Deviation; ND: Not Detected

Discussion

The present study aimed to evaluate the antifungal potential of ethanol and aqueous extracts of *Artemisia herba-alba* (commonly known as white wormwood) against two common fungal species: *Aspergillus* sp. and *Rhizopus* sp. These fungal genera are known for their ubiquitous presence in the environment and for causing various diseases in humans, animals, and plants, as well as being implicated in food spoilage. The results of the study shed light on the antimicrobial properties of *Artemisia herba-alba* and provide important insights into its potential use in alternative antifungal therapies. The findings of this study showed that both ethanol and aqueous extracts of *A. herba-alba* exhibited significant antifungal activity against all two tested fungal species, with the ethanol extract generally displaying stronger inhibition than the aqueous extract. This result is consistent with previous studies that have demonstrated the higher solubility and efficacy of bioactive compounds from *Artemisia* species in alcohol-based solvents compared to water. The antifungal compounds in *A. herba-alba* could include flavonoids, terpenoids, and other phenolic compounds, many of which have been linked to antimicrobial properties [13].

These compounds are better extracted using ethanol, which is a polar solvent that can solvate both hydrophilic and lipophilic bioactive molecules. While the ethanol extract was generally more effective, the degree of inhibition varied across the fungal species *Aspergillus* sp. and *Rhizopus* sp. may possess more robust mechanisms of resistance to the specific compounds present in *A. herba-alba* extract [6].

The varying susceptibility could be attributed to differences in the cell wall composition and metabolic pathways of the fungi. For instance, *Aspergillus* species are known to have thicker chitin layers in their cell walls, which might reduce the penetration of antifungal compounds. As observed, ethanol extracts demonstrated stronger antifungal activity than aqueous extracts. This result is in agreement with studies that report enhanced antifungal effects of plant extracts in alcohol-based solvents. The reason for this can be attributed to the higher extraction efficiency of ethanol in dissolving essential oils, polyphenols, flavonoids, and other bioactive components

with antifungal activity. Aqueous extracts, on the other hand, may not extract as many of these compounds, leading to a less potent antifungal effect. This discrepancy highlights the importance of choosing an appropriate solvent for maximizing the antimicrobial potential of *A. herba-alba* [14].

The antifungal activity of *A. herba-alba* extracts may involve several mechanisms, including disruption of the fungal cell membrane, inhibition of fungal growth by altering cell wall biosynthesis, and interference with fungal spore germination Phenolic compounds, such as flavonoids and tannins, are known to cause oxidative stress in fungal cells by generating reactive oxygen species (ROS), which can damage cellular components. Additionally, terpenoids may interact with cell membrane lipids, disrupting membrane integrity and leading to leakage of intracellular contents. The stronger antifungal activity observed in the ethanol extracts could be a result of a more complex profile of bioactive compounds extracted using alcohol as the solvent. The antifungal properties of *Artemisia herba-alba* suggest that it could be an alternative or complementary source of antifungal agents, particularly in agricultural and medicinal contexts. As the efficacy of synthetic antifungals diminishes due to increasing resistance, plant-based products like those derived from *A. herba-alba* could offer a more sustainable solution. In particular, the higher efficacy of ethanol extracts may support their potential use in the development of natural antifungal formulations, such as topical creams, sprays, or preservatives in food products. However, further research is needed to determine the safety, efficacy, and optimal concentrations of these extracts in practical applications [15].

In conclusion, both ethanol and aqueous extracts of *Artemisia herba-alba* exhibited antifungal activity against *Aspergillus* sp. and *Rhizopus* sp. with the ethanol extract proving more effective. The variation in fungal susceptibility suggests that different fungal species may respond differently to plant-derived compounds. These results support the potential of *A. herba-alba* as a source of natural antifungal agents and highlight the importance of solvent choice in maximizing the antimicrobial efficacy of plant extracts.

Recommendations for using plant extracts to control fungal growth

Finally, based on the study, it is recommended to use plant extracts that have an impact on fungal growth. Modern methods should be employed to extract the maximum possible amount of active substances found in most medicinal plants. This will help avoid the use of chemical fungicides, which cause significant harm to the ecosystem's human and animal health. Additionally, it is advised to conduct more precise studies to identify the active substances and determine the most important one that has a strong effect on fungal growth.

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