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ANTIFUNGAL POTENTIAL OF ALCOHOLIC PLANT EXTRACTS IN REDUCING FUNGAL CONTAMINATION IN PHOENIX DACTYLIFERA TISSUE CULTURE

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Abstract

This study was conducted to investigation was carried out to evaluate the efficacy of certain plant extracts derived from pomegranate peels and black seeds in combating fungal issues in tissue culture, exploring their potential as natural substitutes for chemical treatments.

The study was conducted in the fungi lab, University of Anbar, 2022-2023. In this study, three types of fungi were isolated from the plant tissues. Alcoholic extracts of pomegranate peels and black seed were used at different concentrations 0, 5, 10, 15 and 20% to inhibiting the growth of fungi isolated from tissue culture in PDA medium.

The study results showed the isolation of three types of fungi associated with culture of plant tissues in vitro (*Alternaria alternata*, *Asperigellus niger* and *Fusarium solani*). The results showed the highest rate of growth inhibition at a 20% concentration, the alcoholic extracts of pomegranate peel and black seeds of isolates of fungi *A. alternata, A. niger and F. solani* were (73.89 and 73.63)%, (74.99 and 74.78)%, (77.42 and 78.00)%, respectively. The extracts from black seed and pomegranate peels exhibit the most effective fungistatic properties. These plants possess medicinal qualities, including antioxidant, antimicrobial, and anti-inflammatory attributes, largely attributed to their polyphenolic compounds. So that, can used these extracts to control on pathogen fungi to plant.

Keywords: Alcoholic extracts, Fungi, Tissue culture, Pomegranate peel.

كفاءة المستخلص الكحولي لقشور الرمان والحبة السوداء في تقليل التلوث الفطري في الزراعة النسيجية في بادرات نخيل التمر

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الخلاصة

تمت هذه الدراسة للتحقق من فعالية استخدام مستخلصات نباتية معينة مشتقة من قشور الرمان وبذور حبة السوداء في مكافحة الفطريات الملوثة للزراعة النسيجية، واستكشاف إمكانية استخدامها كبدائل طبيعية للعلاجات الكيميائية. تمت الدراسة في مختبر الفطريات بجامعة الأنبار، للفترة من 2022–2023. في هذه الدراسة، تم عزل ثلاثة أنواع من الفطريات من أنسجة النبات نخيل التمر. تم استخدام مستخلصات كحولية من قشور الرمان وبذور حبة السوداء بتراكيز مختلفة 0، 5، 10، 15، و20% لمنع نمو الفطريات المعزولة من الزراعة النسيجية لبادرات نخيل التمر في وسط PDA. أظهرت نتائج الدراسة عزل ثلاثة أنواع من الفطريات مرتبطة بالزراعة النسيجية للنبات في الاطباق وهم (*Alternaria alternata*, *Asperigellus niger* and *Fusarium solani*). أظهرت النتائج بان للمستخلصات الكحولية من قشر الرمان وبذور حبة السوداء عند تركيز 20%، أعطت أعلى نسبة تثبيط لنمو الفطريات (*A. alternata*, *A. niger* and *F. solani*) بنسب (73.89 و73.63)%، (74.99 و74.78)%، (77.42 و78.00)% على التوالي. اظهر مستخلص بذور حبة السوداء وقشور الرمان خصائص مثبطة لنمو الفطريات الأكثر فعالية. هذه النباتات تمتلك خصائص علاجية مهمة تشمل مضادة للأكسدة ومضادة للأمراض الميكروبية والمضادة للالتهابات، وترجع هذه الخصائص إلى حد كبير إلى مركباتها البوليفينولية. بحيث يمكن استخدام هذه المستخلصات للسيطرة على نمو الفطريات الضارة للنبات.

كلمات مفتاحية: المستخلص الكحولي، الفطريات، قشر الرمان، الزراعة النسيجية.

Introduction

Date palms (*Phoenix dactylifera*) are susceptible to a large number of agricultural pests, totaling up to 280, including fungal, bacterial, phytoplasma, and various insect pests (18). Leaf spot diseases in date palms are common in most date palm cultivation areas worldwide, caused by numerous fungi and are prevalent in neglected orchards. This disease becomes more widespread during seasons with increased rainfall (3 and 18). Fungi rank among the foremost agents responsible for significant agricultural production losses (29). While chemical means remain the most effective approach to

manage fungal plant diseases when compared to alternative methods, their excessive and indiscriminate usage has led to an escalation in resistance issues (20). Therefore, in recent years has been an increased interest many researchers in the therapeutic use of plant extracts isolated from medicinal herbs for several reasons, including their effectiveness, safety, and economic feasibility (17 and 18). These botanical pesticides, of plant origin, are non-toxic to plants, quickly biodegradable, and many types of these plant products are capable of reducing the density of disease-causing agents affecting aerial parts, thus inhibiting disease development (4). These plant extracts do not harm the environment and are safe for both humans and animals (20). Many studies have highlighted the effectiveness of these natural plant products against various disease-causing agents. In a 1997 study by Dushynet and Botryl, the effects of 11 different plant extracts on the growth of the fungal pathogen *Asperigellus solani* were examined (10).

As a result, the cultivation of medicinal plants and their utilization in the pharmaceutical industry has increased. These plants can either be wild, found in various environments, or cultivated in specific locations (18 and 29). It has been discovered that a single plant contains more than one active compound that works synergistically in a balanced formula to treat diseases. This advantage is not present in manufactured drugs, and some natural compounds, like hyoscyamine from the Hyocyamus muticus plant, cannot be replicated in a laboratory despite their importance (4 and 7). Most plant extracts affect target sites different from those typically impacted by antibiotics. Such results are of exceptional significance, particularly in the treatment of resistant fungal pathogens (21 and 32).

Furthermore, the study of the effectiveness of extracts from various medicinal plants, especially those with antimicrobial properties including the Nigella sativa plant, commonly known as the black seed plant, is a herbaceous plant that contains Nigellon, a crystalline substance derived from its seeds (4 and 21). Nigellon, the natural active compound found in a plant, is famed for its robust antioxidant attributes, effectively countering free radicals (2). The plant's seeds are black, possess a distinct aromatic fragrance and taste, and contain over 4% volatile oils, 30% fixed oils, and 20% protein. These seeds are abundant in essential fatty acids and act as antioxidants. Extracts from the plant have been harnessed to combat various harmful microorganisms in humans and animals, primarily due to the presence of Thymoquinone and Thymol (4 and 8). Furthermore, the phenolic compounds within black seeds directly inhibit these microorganisms (4). Multiple studies have verified the efficacy of these plant extracts, demonstrating their toxicity to various pathogens, as evidenced by studies conducted by (19 and 25).

In recent years, the pomegranate from the Punica granatum L. tree has gained substantial attention for its vital role in human nutrition and health. Research publications on pomegranate have doubled, attributed to its rich array of beneficial compounds such as flavonoids, soaps, tannins, anthocyanins, alkaloids, resins, terpenes, and others (4 and 21). These compounds showcase robust antifungal, antiviral, and antibacterial properties. Phenolic compounds, specifically, are

renowned for their ability to combat chelate metal ions, free radicals, and enhance antioxidant activity, thereby disrupting oxidation processes and serve as a crucial defense against free radicals (24). Studies have demonstrated the effectiveness of pomegranate extracts in inhibiting fungal growth at higher rates, surpassing the performance of chemical fungicides, as seen in aqueous extracts from pomegranate peels, which achieved a 75% inhibition of Alternaria alternata, isolated from apple fruits (25). It's worth noting that the efficacy of these plant extracts can vary based on the solvent used, the specific plant part employed for extraction, and the growth stage of the plant (19). The aim of this study was evaluate the efficacy of certain plant extracts derived from pomegranate peels and black seeds in combating fungal issues in tissue culture, exploring their potential as natural substitutes for chemical treatments.

Materials and Methods

Isolation and identification of fungi: The fungi that were connected to the issue were separated once signs of fungal contamination to *Phoenix dactylifera* became evident. This was done by utilized sterile distilled water to repeatedly wash the plant parts in order to remove any remaining tissue culture media, followed by drying with sterile filter paper. Next, these pieces (three) were placed into a sterile culture medium (PDA) and supplemented with 250 mg/l of the antibiotic Chloramphenicol. The Petri dishes were then incubated at (25±2) ºC for three days. Afterward, the fungi that were extracted from the tissue culture were identified and purified using references such as (5, 9 and 11).

Creating extracts with alcohol: The extracts were made in accordance with the (15) technique for making plant extracts. To create these extracts, separatelym 100 g of pomegranate peels and 100 g of black seed powder were used. In each case, 500 mL of 98% ethanol alcohol were added, thoroughly mixed, and placed at room temperature (25°C) for 24 hours in the laboratory. After this incubation period, the solution underwent filtration using Whatman No 1 filter paper. Afterwards, the filtered liquid was concentrated in a rotary evaporator at 40°C to remove the solvent. It was further dried in a petri dish at room temperature until it formed a highly viscous concentrate. This resulting concentrated substance was later blended with 50 mL of distilled water (considered the stock solution) to produce the concentrations applied in the study. To prepare a 5%, 10%, 15%, and 20% concentration, mix 0.5 mL, 1 mL, 1.5 mL and 2 mL of the stock solution with 9.5mL, 9 mL,8.5mL and 8 mL of the solvent respectively, while 0% without adding plant extract.

Detection of certain chemical components within the analyzed plant extracts:
PH detection: Utilize 5 grams of the powdered plant, combining it with 25 milliliters of 95% ethyl alcohol. Heat the mixture in a water bath at 100°C for one minute, then proceed to filter the solution before integrating 100 milliliters of hydrochloric acid. For phenol detection: introduce a 1% aqueous solution of ferric chloride to an equal portion of the aqueous extract. Presence of phenols manifests as a bluish-green precipitate, as described by (16).

To identify saponins in the plant used in the study: we employed a sealed container that held the aqueous extract. A positive result for saponin detection is the presence of thick foam on the extract's surface which persistent for long time, as described by Harborne in 1984.

For detecting tannins in the plant material: We followed the method outlined by (16): Utilize 5 grams of the powdered plant, combining it with 25 milliliters of 95% ethyl alcohol. Heat the mixture in a water bath at 100°C for one minute, then proceed to filter the solution before integrating 100 milliliters of hydrochloric acid. For phenol detection: introduce a 1% aqueous solution of ferric chloride to an equal portion of the aqueous extract. Presence of phenols manifests as a bluish-green precipitate, as described by (16).

Examining how various concentrations of plant extracts impact the inhibition of fungal growth within the PDA medium: To detection the effectiveness of concentrations 0, 5, 10, 15, 20% of black seed and pomegranate extracts. Each concentrations of extracts taking individual 250 ml of sterilized and cooled PDA medium was combined with the extracts. The resulting culture medium, adjusted to the necessary concentration for each extract, was then transferred into sterile petri dishes. with a 9 cm diameter. In the center of each dish, a 0.5 cm diameter disc from *Alternaria alternate, Aspergillus niger* and *Fusarium solani* isolated separately, which had been grown on PDA culture media for 7 days, was inoculated using a sterile cork piercing. This process was repeated four times, and a comparative treatment involved inoculating the center of the dish with a disc of 0.5 cm in diameter, cultured separately on PDA culture media without the use of extract, and fungi that were isolated only from tissue culture. After that, the dishes were kept in an incubator set at 25 ± 2ºC. Once the fungus growth in the control treatment reached the plate's edge, the radial growth of the fungi separated from the tissue culture was calculated by taking the average of two perpendicular diameters from the plate's bottom. Using (1) equation as a guide, the complete experiment was conducted for each element and their interactions.

$$\text{Inhibition percentage} = \frac{\text{The radial growth rate in comparison} - \text{The radial growth rate in the treatment}}{\text{The radial growth rate in comparison}} \times 100$$

Impact of the interplay between the extract type and different quantities of the analyzed extracts from black seeds and pomegranate skins: To make a 5% concentration from combination of pomegranate peels and black seeds, mix 5 mL of each extract with 95 mL of the solvent (totaling 100 mL). To make a 10% concentration from combination two extract, mix 10 mL of each extract with 90 mL of the solvent (totaling 100 mL). To make a 15% concentration from combination two extract, mix 15 mL of each extract with 85 mL of the solvent (totaling 100 mL). To make a 20% concentration, mix 20 mL of each extracts with 80 mL of the solvent (totaling 100 mL), then followed the procedure above.

Statistical analysis: The factorial experiments were two components were included in the study, which was done using a completely randomized design (C.R.D.): extracts at different concentrations and fungus obtained from tissue culture. At a significance level of 0.01 the averages were compared using the Least Significant Difference (L.S.D.) method (31).

Results and Discussion

Chemical identification of certain compounds in plant extracts: The results indicate that the analysis of pomegranate peels and black seeds revealed the presence of saponin, tannins, and phenols, yielding a positive outcome, as indicated in Table 1.

Table 1 Detection of certain active chemicals in the rinds of and black seeds and pomegranate fruits using chemical methods.

No.	Active compounds	Pomegranate peels	Black seed
		Alcoholic extract	
1	Tannins	+	+
2	Saponins	+	+
3	Phenols	+	+
Acid function		6.5	6

(+) Finding is positive.

The findings highlight the significance of the active chemicals in the pomegranate peel and black seed alcoholic extracts as well as their active role in preventing the growth of fungus. Alcohol is more effective than water at extracting the active chemicals, according to a 2003 study by (14). The fact that these extracts generally contain the majority of the active chemicals and have anti-fungal activity may account for their efficiency. According to (6) these tannin and phenolic compounds have antifungal properties. Its ability to combine with cell proteins and precipitate them, altering their nature and functioning as an appropriate solvent for fatty substances, is what makes it unique. Essentially, it breaks down living cells' membranes, allowing their internal components to emerge and causing the fungal and bacterial cells to perish. This effect is further enhanced by the positive chemical detection of phenols, saponins, and tannins (4).

Isolation and identification of fungi: The results are shown in figure 1 the proportion of fungus that were separated from tissue cultures. *Alternaria alternata* had the highest occurrence, accounting for 55%, followed by *Aspergillus niger* at 29%. *Fusarium solani* had the lowest occurrence, with only 16%.

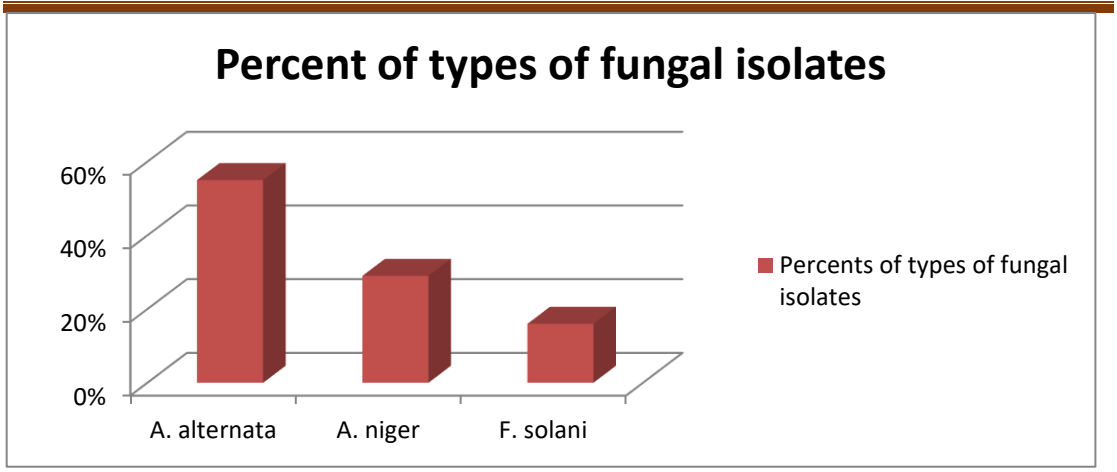

Figure 1 Show the types and percentages of fungi isolated from cultures of date palm tissues.

The impact of concentration on the inhibition of fungal growth isolated from date palm tissue cultures: Table 2 demonstrates the impact of alcoholic extracts derived from pomegranate peel and black seeds at various concentrations in the study. There were significant variations between them based on the type of extract and concentration. The results reveal that, at a 20% concentration, the alcoholic extracts of black seed and pomegranate peel exhibited the highest inhibition of *A. alternata* fungus growth, with percentages of 73.89% and 73.63%, respectively, which were significantly different from other concentrations in the study. Additionally, the most substantial growth inhibition for the *A. niger* fungus occurred at the 20% concentration for both black seed and pomegranate peel extracts, reaching 74.99% and 74.78%, respectively. Similarly, the highest inhibition of *F. solani* fungus growth was observed at the 20% concentration for black seed and pomegranate peel extracts, recording percentages of 77.42% and 78.00%, respectively as show in the figures 2, 3, and 4. Lower concentrations were found to be ineffective in achieving the desired results.

Table 2 The impact of concentration on the inhibition of fungal growth isolated from date palm tissue cultures *in vitro*.

Extract type	Concetration %	*A. alternata*		*A. niger*		*F. solani*	
		Fungus average diameter (cm)	Inhibition ratio (%)	Fungus average diameter (cm)	Inhibition ratio (%)	Fungus average diameter (cm)	Inhibition ratio (%)
Pomegranate	**0**	9.00	0.00	9.00	0.00	9.00	0.00
	5	6.65	24.87	6.32	28.45	6.23	31.54
	10	4.67	47.56	5.61	42.33	4.45	53.13
	15	3.60	64.11	4.25	51.89	3.15	66.22
	20	2.35	73.89	2.45	74.99	2.35	77.42
Black seed	**0**	9.00	0.00	9.00	0.00	9.00	0.00
	5	6.12	28.12	7.14	27.36	6.87	34.56
	10	4.65	48.76	5.25	42.33	4.65	53.87
	15	3.55	64.32	4.65	52.78	3.35	67.98
	20	2.45	73.63	2.85	74.78	2.65	78.00
L.S.D.		**0.352**	**3.821**	**0.198**	**2.236**	**0.643**	**2.243**

Effect of the combination of two extract (pomegranate peels and black seeds) at varying concentration in preventing fungus isolated from date palm tissues from growing: The findings in table 3 show the impact of various concentrations of the combination two extracts (black seed and pomegranate peels) on the preventing activity of isolated fungi. The inhibitory effects varied depending on the concentration levels, which included 0%, 5%, 10%, 15%, and 20%. The most significant inhibition of *A. alternata* was observed at a 20% concentration, reaching 74.66%, showing substantial variance from concentrations of 15%, 10%, 5%, and 0%, which yielded inhibition rates of 64.12%, 47.56%, 26.32%, and 0.00%, respectively. Similarly, *A. niger* exhibited the highest inhibition rate when exposed to a 20% concentration, recording 72.94%. This result differed significantly from concentrations of 15%, 10%, 5%, and 0%, which produced inhibition rates of 52.58%, 42.45%, 27.54%, and 0.00%, respectively.

Furthermore, the highest inhibition rate for *F. solani* was achieved at a 20% concentration, with a percentage of 76.12%, significantly different from 15%, 10%, 5%, and 0% concentrations, which showed inhibition rates of 66.78%, 53.12%, 33.45%, and 0.00%, respectively.

Table 3 The impact of interaction on the in vitro growth inhibition of fungus isolated from date palm tissue cultures.

	A. alternata		***A. niger***		***F. solani***	
Concentration (%)	**Fungus average diameter (cm)**	**Inhibition ratio (%)**	**Fungus average diameter (cm)**	**Inhibition ratio (%)**	**Fungus average diameter (cm)**	**Inhibition ratio (%)**
0	9.00	0.00	9.00	0.00	9.00	0.00
5	6.31	26.32	7.45	27.54	6.56	33.45
10	4.73	47.56	5.54	42.45	4.64	53.12
15	3.70	64.12	4.27	52.58	3.25	66.78
20	2.84	74.66	2.87	72.94	2.46	76.12
L.S.D.	0.357	2.781	0.265	1.612	0.411	1.645

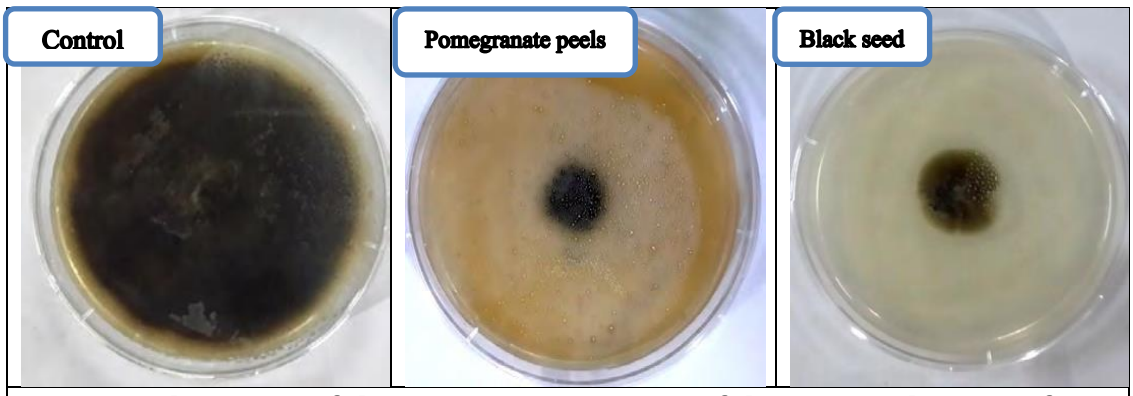

Figure 2 The impact of the (20%) concentrations of the examined extracts from pomegranate peels and black seeds on their ability to hinder the growth of *A. alternata*.

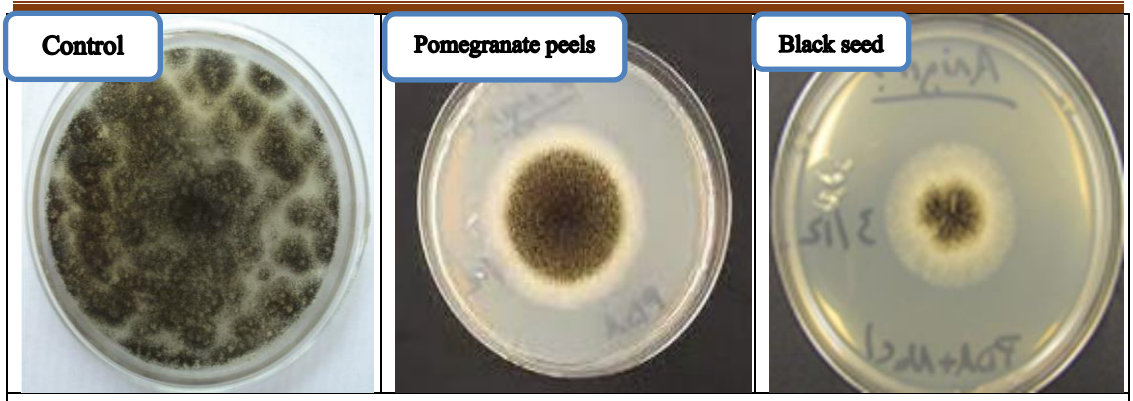

Figure 3 Effect of the (20%) concentrations of the examined extracts from pomegranate peels and black seeds on their ability to hinder the growth of *A. niger*.

Figure 4 Effect of the (20%) concentrations of the examined extracts from pomegranate peels and black seeds on their ability to hinder the growth of *F. solani*.

Presently, it is widely acknowledged that plants contain a multitude of biologically active chemical compounds that work together synergistically, imparting a wide range of bioactive properties (20). Among these compounds, phenolic compounds are the most prevalent and exhibit potent antimicrobial, particularly antifungal, characteristics. The current research reveals that extracts from black seed and pomegranate peels exhibit the most effective fungistatic properties (4 and 7). These plants possess medicinal qualities, including antioxidant, antimicrobial, and anti-inflammatory attributes, largely attributed to their polyphenolic compounds (26). Numerous studies have also confirmed the strong biocidal effects of phenolic acids, flavonoids, and terpenes (22, 27 and 28). (27) demonstrated in their research that polyphenols such as rosemarinic acid, catechin, vanillin, chlorogenic acid, quercetin, and p-coumaric acid are present in the highest concentrations. The antioxidant capacity of the examined extracts is closely associated with their total phenolic and flavonoid content (28). The present studay reveal that the alcoholic extracts of black seeds and pomegranate peel at a 20% concentration exhibited the highest inhibition of *A. alternata*, *A. niger* and *F. solani*. growth.

These results agree with other findings by (4), which demonstrated that preventing fungus isolated from tissue culture from growing using black seed extract resulted in varying proportions of inhibitory activity based on the concentration used, where used high concentrations of plant extracts recorded a high inhibition rate in the fungus growth (4). Furthermore, this study aligns with a study conducted by (20) who

noted that extracts from sag and tansy plants, showed a significant inhibitory effect against the tested fungus at a dose of 20%. They came to the conclusion that, depending on the kind of fungus, the concentration, and the type of extract, the plant extracts suppressed the growth of fungal infections' mycelia. The efficacy of plant extracts, such as those from pomegranate peel and black seed, in restraining fungi isolated from tissue culture may stem from these plants' possession of chemical compounds with antimicrobial properties. When introduced into the culture medium, these compounds are released and alter the medium's natural characteristics, rendering it less conducive to the growth of pathogenic fungi. The inhibition likely results from the presence of specific components in the extracts, such as phenols, saponins, and tannins, which possess anti-fungal properties. The mechanism behind this inhibition involves these compounds making direct contact with microorganisms, disrupting their plasma membranes (4 and 5). Additionally, these compounds can penetrate cell membranes and obstruct active enzyme sites essential for the growth and reproduction of the fungi within their cells (4 and 20). This ultimately hinders the growth of pathogenic fungi, possibly by interfering with metabolic processes necessary for their development. This is in line with findings by (6). (4) highlighted that pomegranate peels are rich in secondary metabolites, including sterols, saponins, triterpenes, alkaloids, glycosides, and tannins. Furthermore, (13) isolated a peptide antifungal compound known as Pomegranin, which effectively inhibits the mycelium growth of Fusarium oxysporum and Botrytis cinerea. Additionally, it's important to emphasize that a plant belonging to a particular genus possesses a unique chemical composition, and its substantial medicinal worth doesn't imply that all other plants within the same genus share the same characteristics (12). In fact, some research has indicated that even within the same species but from different sources, there can be notable variations in chemical composition, leading to differences in bioactive effects and potency (23 and 30). Agrona Also, the results of this study show that lower concentrations of extracts of plant (such as black seed and pomegranate peel extracts) recorded a low inhibition rate in the fungus growth isolated from tissue culture. These concentrations were found to be ineffective in achieving the desired results due to the low levels of active compounds within them.

Conclusions: The appeal of employing natural substances for controlling pathogens is significant. The use of plant extracts sourced from pomegranates and black seeds holds promise as an effective method for restraining the fungi growth derived from in vitro cultures tissues of date palm. This investigation showcases the capacity of natural compounds obtained from these plants to combat fungal pathogens that pose a threat to the well-being and vigor of date palm trees. This is due to the presence of bioactive components in pomegranate and black seed extracts, such as polyphenols and flavonoids, which are recognized for their antifungal properties and have the potential to function as eco-friendly alternatives to synthetic fungicides. The utilization of plant extracts for countering fungal pathogens aligns with the increasing interest in sustainable and environmentally friendly solutions for agricultural challenges. This approach has the potential to lessen the dependence on synthetic

chemical fungicides, which can have adverse effects on both the environment and human health.

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