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Effects of Aqueous and Ethanolic Extract of *Rhazya stricta* on Control of Date Contamination Under In Vitro Conditions

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Abstract

Background: Contamination of tissue culture medium is a common problem that causes the loss of some or all cultures. Despite all measures taken in tissue culture laboratories to prevent culture contamination, bacterial and fungi contamination is still a significant problem under in vitro culture. Achieving a suitable method for controlling the contaminants in the in vitro culture medium will be a considerable development in the micropropagation of dates.
Objectives: We aimed to investigate the effects of different aqueous and ethanolic extracts of *Rhazya stricta* on the disinfection of dates under in vitro culture.
Methods: A factorial test was performed based on a completely randomized design with three replications. The study included three factors: Plant organs (leaves and roots), solvent type (ethanolic and aqueous), and extract concentration (0, 25, 50, and 75 mg/mL).
Results: The lowest contamination (38.33%) was seen in the treatment with ethanolic root extract (50 ppm). In comparison, the control and the treatment containing aqueous leaf extract (with concentrations of 0 and 25 ppm) resulted in the highest contamination rate (100%) in tissue culture conditions.
Conclusions: The alcoholic extract of medicinal plants can be used to disinfect the explants and the substrate.

Keywords: Tissue Culture, Medicinal Plants, Disinfection, Explants

1. Background

The science of tissue culture has been widely developed to help create a large number of plants in a relatively short period. For the growth of in vitro plants, there is a need for a culture medium containing mineral salts. Furthermore, the culture of plant tissues and cells requires a variety of compounds, including plant hormones (phytohormones), vitamins, and sucrose (1, 2).

Unfortunately, such a culture medium, rich in nutrients, can also provide the basis for the rapid growth of bacteria and fungi. This type of contamination in the culture medium usually develops rapidly and negatively affects plant growth and ultimately destroys the cultured plant tissue by evacuating nutrients in the culture medium and producing toxic substances. Therefore, the complete disinfection of food culture medium, containers, seeds, tissues, and any means used in culture is unavoidable by heat or filtration in this standard tissue culture system (3-6). Furthermore, any procedure for tissue culture should be performed in the purified air stream under the laminar hood. Despite using these precise measures to maintain sterile conditions, the contamination of cultivated plants can be considered a serious problem that sometimes destroys some plants and even all plants cultivated (6, 7).

Rhazya stricta belongs to the Apocynaceae family and Rauwolfioideae subfamily. It is burned like Esfand in Saudi Arabia. *Rhazya stricta* is a small evergreen plant with

leathery, thick, long, oval, and narrow leaves on both sides. The average length of the leaf is 7 - 10 cm, with a width of 12 cm in the middle. The flowers are white and slightly fragrant. The fruit is 6 cm long and 0.5 cm wide; long seeds appear 1 cm in length. This plant is distributed in India, Afghanistan, Saudi Arabia, and Iran. It is found in the forests of the southeastern subtropical region between Chabahar, Nikshahr, Iranshahr, and Saravan in Iran. This plant has been used as a medicine to treat various diseases in Afghanistan, India, Iran, Pakistan, Qatar, and Saudi Arabia. It has more than 50 different Indole alkaloids, some of which have anti-cancer properties, such as valesiacotamine and tetrahydrozoline. The raw ethanolic extract of *R. stricta* fruit has antimicrobial activity (8, 9).

Methanolic and chloroformic extracts of roots have antimicrobial and antifungal activity against *E. coli*, *Aspergillus flavus*, *A. terreus*, *B. subtilis*, *S. aureus*, and *C. albicans*. Aqueous extract of *R. stricta* has antimicrobial activity against *Neisseria meningitides* (10-16). *Rhazya stricta* and its metabolites help cure cancer, skin diseases, hypertension, rheumatism, sore throat, syphilis, and fever (17). Various studies have found that different parts of *R. stricta* contain many phytochemical elements such as alkaloids, flavonoids, and terpenes (18). Some natural substances in plants have antimicrobial properties and are used as a seasoning in foods in some countries. The vegetable aromatics are used in foods not only for their aroma but also for their food preservation and medicinal properties (19-23).

Different types of antimicrobial chemicals have been used in plant tissue culture. Antibiotics have been extensively tested to prevent or reduce the growth of bacteria in tissue culture (3-5, 24). However, antibiotics are less applied because they are expensive and only work against bacteria. Furthermore, they are less efficacious against various bacteria, are usually unstable, and often produce phytotoxic properties (25-27).

A study (6) reported a method for removing fungal contamination in cyclamen tissue culture that involved immersing cyclamen tissue fragments in imidazole and triazole fungicidal solution or culturing a fragment of cyclamen tissue in the culture medium containing this fungicide. It examined the microbial contamination and its sources in tissue cultures of cassava, hemp, cowpea, and banana in Nigeria. The results indicated that the emergence of bacterial infection was faster than fungal infection in plant tissue culture. Nineteen infectious agents, including 11 bacterial and eight fungal species, were found in tissue culture media. Bacterial infections included *Pseudomonas fluorescens*, *Escherichia coli*, *Proteus* species, *Micrococcus species*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Corynebacterium* species, and *Ironenia* species, and extracted fungi included *Alternaria tenuis*, *Aspergillus niger*, *Aspergillus fumigatus*, *Cladosporium species*, *Saccharomyces*, *Fusarium oxysporum*, *Rhizopus nigracans*, and *Fusarium culmorum* (28-30). The results indicated that plant explants' most transmitted infections included *P. fluorescens*, *Corynebacterium* species, *B. subtilis*, *A. niger*, *R. nigracans*, and *F. camrom* (25 - 39%). The user-transmitted infection included *B. cereus*, *E. coli*, and *S. aureus* (48 - 36%), the workplace-borne contaminants included *E. coli* (12%) and *S. aureus* (16%), and ambient airborne contamination included *Bacillus*, *Streptococcus faecalis*, and *E. coli*. Glove-borne infections included *Corynebacterium*, *B. subtilis*, *S. pneumoniae*, and *S. faecalis*.

Date palm with the scientific name of *Phoenix dactylifera* L. is probably a Phoenician word. This is a monocotyledonous plant of the Palmaceae family, diploid (2n = 36), perennial, evergreen, dioecious, and heterozygous, with a meager growth rate. It is native to tropical and subtropical regions of Africa and South Asia (31, 32).

Dates are essential export products of Iran, and their export has a long history. In Iran, Khuzestan, Hormozgan, Kerman, Fars, Sistan and Baluchestan, and Bushehr provinces produce 99% of Iranian dates. The medicinal importance of dates has been proven with the advancement of medical and nutritional knowledge. Dates contain calcium, which is a major factor in bone strength. It also contains a significant amount of phosphorus, a vital element for the brain (33-35).

It was found that the maximum contamination in palm tissue culture was due to *Aspergillus niger*, *Penicillium* sp., and Alternaria *alternata* with rates of 16.8%, 10%, and 8%, and the minimum contamination was caused by *Trichoderma harzianum* and *Nigrospora* sp. (36).

Two major groups of contaminants were identified in date tissue culture laboratories in Iraq. The first group included fungi, the most predominant of which were *A. niger* and *Alternaria alternate*, and the second group included bacteria belonging to *Bacillus* and *Staphylococcus* genera (37).

Actinomycin at concentrations of 0, 100, 250, 500, and 1000 mg/L has been used at different stages of callus

production, body embryogenesis, branching, and rooting of palm tissue culture to control the contamination of date palm explants. The results indicated that a concentration of 500 mg/L of actinomycin led to contamination-free culture (38).

2. Objectives

This experiment aimed to investigate the antifungal and antibacterial properties of *R. stricta* essential oil and extract in Murashige and Skoog culture media and their effects on the growth of date plants.

3. Methods

To investigate the antifungal and antibacterial properties of *R. stricta* extract in Murashige and Skoog media and their effects on date growth, we conducted a factorial experiment as a completely randomized design with three replications in 2021 at the tissue culture laboratory of the Agricultural Biotechnology Research Institute in University of Zabol.

3.1. Preparation of Aqueous Extract of Rhazya stricta

We soaked 20 g of powdered plant leaves separately in 80% distilled water and ethanol solvents and kept them on a shaker for 24 hours. After one day, the material was filtered with Whatman grade-2 filter paper. A vacuum rotary apparatus removed the solvents from the filtered material. The concentrated extracts were stored in an oven at 40°C for 48 hours until a pure extract was obtained and the solvent was removed entirely. Finally, the obtained extracts were dried and kept in the refrigerator at 4°C until the test after weighing.

3.2. Preparation of Plant Samples

The plant materials used in this experiment included date offshoots prepared from Iranshahr groves (Sistan and Baluchestan Province) and transferred to the laboratory (Figure 1A). Palm roughages were cut and removed using a saw and knife by a longitudinal cut in the sheath (Figure 1B).

3.3. Preparation of Aqueous Extract of Rhazya stricta

We soaked 20 g of powdered plant leaves and roots separately in distilled water solvent and kept them on a shaker for 24 hours. After one day, the material was filtered with Whatman grade-2 filter paper. The concentrated extracts were stored in an oven at 40°C for 48 hours. Finally, the extracts were dried and kept in the refrigerator at 4°C until the test after weighing.

3.4. Preparation of Ethanolic Extract of Rhazya stricta

We soaked 20 g of powdered plant leaves and roots separately in 80% ethanol and kept them on a shaker for 24 hours. After one day, the material was filtered with Whatman grade-2 filter paper. A vacuum rotary apparatus removed the solvents from the filtered material. The concentrated extracts were stored in an oven at 40°C for 48 hours until a pure extract was obtained and the solvent was removed entirely. Finally, the extracts were dried and kept in the refrigerator at 4°C until the test after weighing.

3.5. Culture of Explants

First, Murashige and Skoog (MS) culture medium containing Benzyl Adenine (BA) (0.5 mg/L), 3% sucrose, 9 g/L agar, and pH 5.7 was prepared, and then the *R. stricta* extract was prepared from the aerial organs and root at concentrations of 25, 50, and 70 ppm under the laminar hood. Before freezing, the substrate was added to the culture medium by a 0.45-μ syringe filter. The substrate without plant extract was considered as a control.

3.6. Disinfection of Explants

The explants were washed with dishwashing liquid for disinfection and then rinsed with running water. The samples were then sterilized with 3% sodium hypochlorite for 20 minutes. Of course, sodium hypochlorite was tested at different concentrations, but 3% was better, as high concentrations caused burns and tissue loss. Finally, they were rinsed three times with sterile distilled water and transferred to the culture medium under an isolated laminar hood.

3.7. Storage Conditions of Samples

Before treatments, the samples were stored in a 37°C incubator for three weeks to determine the contamination percentage. Three samples were cultured for each alcoholic and aqueous extract at each concentration, i.e., three replicates per concentration. Samples were

Figure 1. Three-year-old offshoot (A) and the offshoot prepared to be sown in the medium (B)

stored at 20 ± 2°C. Photoperiodic lighting (16 hours of light and 8 hours of dark) and the percentage growth inhibition of bacteria and fungi were measured after one month.

3.8. Data Analysis

The factorial experiment was conducted as a completely randomized design with three replicates. Data analysis was done using SAS software, and the GLM test with a probability level of 5% was used for the mean comparison test. All diagrams were drawn using Excel software.

4. Results

According to Table 1, the simple effects of organ type (leaf or root), type of extract (ethanolic or aqueous), and extract concentration (0, 25, 50, and 75 ppm) on the percentage of contamination of date explants were significant in the culture medium at 5% probability level. The study of dual interactions indicated that the interaction of plant organs×extract was significant at a probability level of 1%, and the dual interaction type of extract×concentration was significant at a probability level of 5%. However, the interaction of organs× extract concentration was not significant. The interaction of the three factors in the test was significant at a probability level of 1% (Table 1).

As shown in Figure 2, there was a statistical difference between different *R. stricta* organs that were examined in the present study. The *R. stricta* leaf extracts for the disinfection of date explants in tissue culture medium caused 40.33% more contamination than the root extract (Figure 2A).

Different solvents had different effects in obtaining *R. stricta* extract for the disinfection of tissue culture medium, as ethanol solvent appeared much more successful than aqueous solvent and could control tissue culture contaminants 58.5% more than aqueous solvent. In the present study, the contamination percentage was 70% in cultures containing aqueous extract and 44.16% in cultures containing ethanol solvent (Figure 2B).

Figure 3A indicates that different concentrations of aqueous and ethanolic extracts significantly affected the contamination of date explants in tissue culture. The highest level of contamination (88.33%) was observed in the control treatment and the lowest (35%) at a concentration of 75 ppm of the extracts. However, there

Table 1. Analysis of Variance of Contamination Percentage of Date Tissue Culture Under the Influence of Aqueous and Ethanolic Extracts of *Rhazya stricta* at Different Concentrations [a]

Sources of Changes	Degree of Freedom	Contamination Percentage
Replication (r)	2	258.33 ^{ns}
Organ (a)	1	4408.33*
Extract type (b)	1	8008.33*
Concentration (c)	3	7275*
Organ × extract	1	675**
Organ × extract concentration	3	208.33 ^{ns}
Extract type × concentration	3	1408.33*
Organ × extract type × concentration	3	341.66**
Test error	-	89.44
Coefficient of variation	-	16.56

[a] * Significant at a 5% probability level; ** Significant at a 1% probability level; ns, non-significant.

was no statistically significant difference between the two concentrations of 50 and 75 ppm (Figure 3A).

As shown in Figure 3B, the lowest level of contamination (38.33%) was obtained in the group disinfected with ethanolic root extract, while the aqueous leaf extract treatment resulted in the highest rate of contamination (88.33%) under tissue culture conditions.

In the present study, the inhibitory effect of aqueous extract was less than that of ethanolic extract, and the ethanolic extract of *R. stricta* at a concentration of 50 ppm (95.33%) had the best effect on the date plant after adding the extract to the tissue culture medium, followed by the aqueous extract at a concentration of 75 ppm (80%). The lowest inhibition of fungal and bacterial growth was observed in the control treatment (9.66%) (Figure 4).

As shown in Figure 5, the highest level of contamination (100%) was obtained in the aqueous leaf extract treatment at concentrations of 0 and 25 ppm (Figure 6A), while the lowest level of contamination (6.66%) was observed in the culture containing ethanolic extract of *R. stricta* root at a concentration of 50 ppm (Figure 6B).

5. Discussion

It was found that bamboo plant explants taken from field and greenhouse conditions and added as 2 mL/L to the culture medium reduced the percentage of culture contamination from 58 to 52% and from 63 to 11%, respectively (39). Some research (40-43) was undertaken to apply different concentrations of turmeric powder, and carbendazim to the MS culture medium to remove contamination of the tissue culture medium. They showed that the application of turmeric powder and carbendazim in MS culture completely controlled the contamination of the culture medium. In the turmeric powder treatment, increasing concentration reduced the contamination percentage.

In many plants cultured in media containing antibiotics, especially gentamicin, the leaves became necrotic and fell off during growth, but they had more rooting than the treatment at the same concentration (44). *Rhazya stricta* leaf and fruit extracts demonstrated antibacterial activity against *S. aureus*, *E. coli*, *P. aeruginosa*, *B. subtilis*, *S. pyogenes*, and *S. typhi* (45). In this research, the leaf aqueous and ethanolic extracts of *R. stricta* were more effective than the root aqueous and ethanolic extracts against contamination by bacteria and fungi. *Rhazya stricta* leaf extract controlled bacterial growth on locally isolated meningococcal strains, increasing with concentration and treatment time (46). In this research, the contamination was controlled by increasing the concentration of ethanolic and aqueous extracts.

Chloroformic and methanolic extracts of *R. stricta* roots exhibited antimicrobial activity against *B. subtilis*, *E. coli*, *S. aureus*, and *P. aeruginosa* (11). The antibacterial activity of *R. stricta* leaf extracts at various concentrations was examined, showing that the organic alkaloid extract was most effective against *E. coli* (10). *Rhazya stricta* chloroformic and methanolic root fractions demonstrated

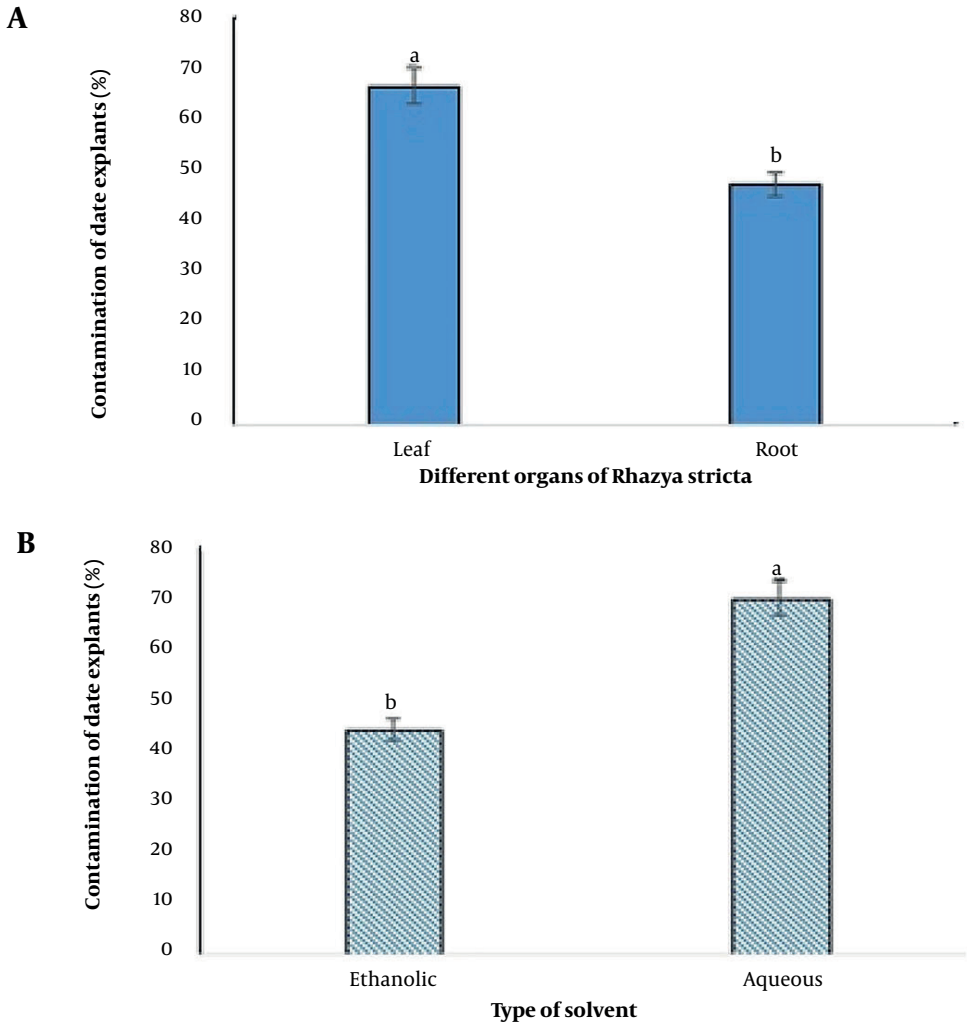

Figure 2. The simple effect of *Rhazya stricta* organ type on the contamination of date explants in tissue culture (A); Simple effect of the solvent type in *R. stricta* extraction (B)

antifungal activities against *A. terreus*, *A. flavus*, and *C. albicans* (47). Another study revealed that fractionated *R. stricta* methanolic and chloroformic samples had antifungal activity against *T. longifusis*, *C. albicans*, *A. flavus*, and *F. solani* (48). In this research, the ethanolic and aqueous root extracts had antimicrobial effects but less than the leaf extract.

Different solvents were used to extract the phytochemical content of *R. stricta* against *S. typhimurium*. The results showed that *R. stricta*, with the help of hydroalcoholic solvent, can be an effective plant in eliminating some bacteria, including *S. typhimurium* (9). This research also showed that the hydroalcoholic extract was more effective than the aqueous extract. Contrary to our results, a study (49) used two antibiotic mixtures to remove contaminants in a tomato culture medium. When both compounds were added to the culture medium, plant growth decreased, and contamination was not eliminated.

The contamination of the culture medium decreased with the addition of an antifungal agent, and the number of leaves was less than in control without fungus and more than in control with fungus (50). The presence of antibacterial agents in the culture medium reduced the contamination and was suitable for plant growth. However, the presence of antibacterial agents disrupts the growth system and causes stress in plant growth, including the number of leaves.

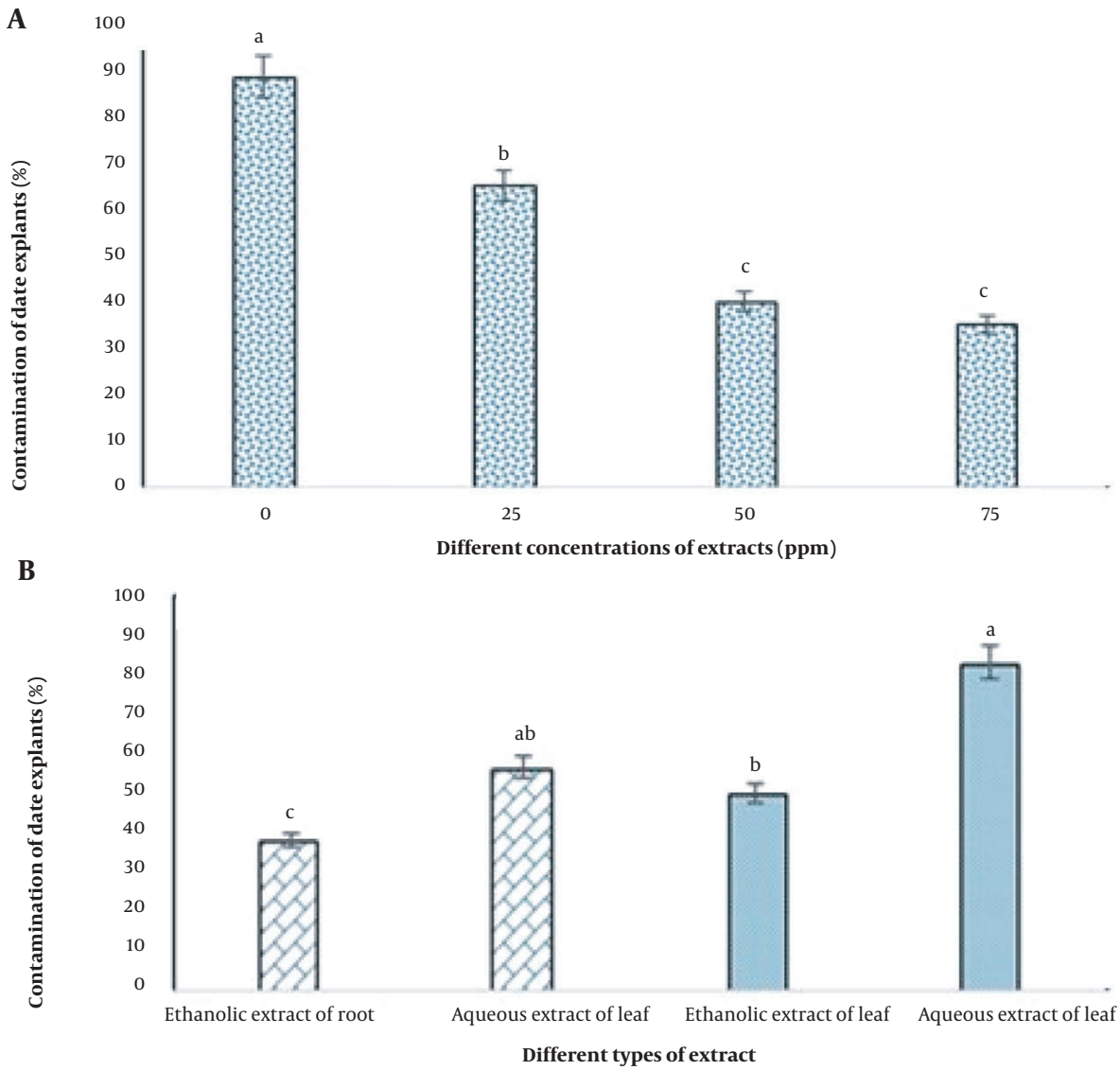

Figure 3. The simple effect of extract concentration on contamination in date tissue culture (A); Organ × solvent interaction in extraction (B)

5.1. Conclusions

When the purpose of tissue culture is the commercial propagation of plants, contamination control and preparation of sterile explants are greatly important. Disinfectants are essential for controlling bacterial and fungal contamination in the in vitro culture. Based on the research results, the best result in controlling contamination in palm tissue culture was obtained from ethanolic root extract, and the worst was obtained from the aqueous leaf extract. In general, the ethanolic extract was more potent than the aqueous extract, and the root extract was more potent than the leaf extract.

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Footnotes

Authors' Contribution: B.FZN. and H.Kh. designed the experiment; R.S. and Z.M. did the experiment; B.FZN., H.Kh.,

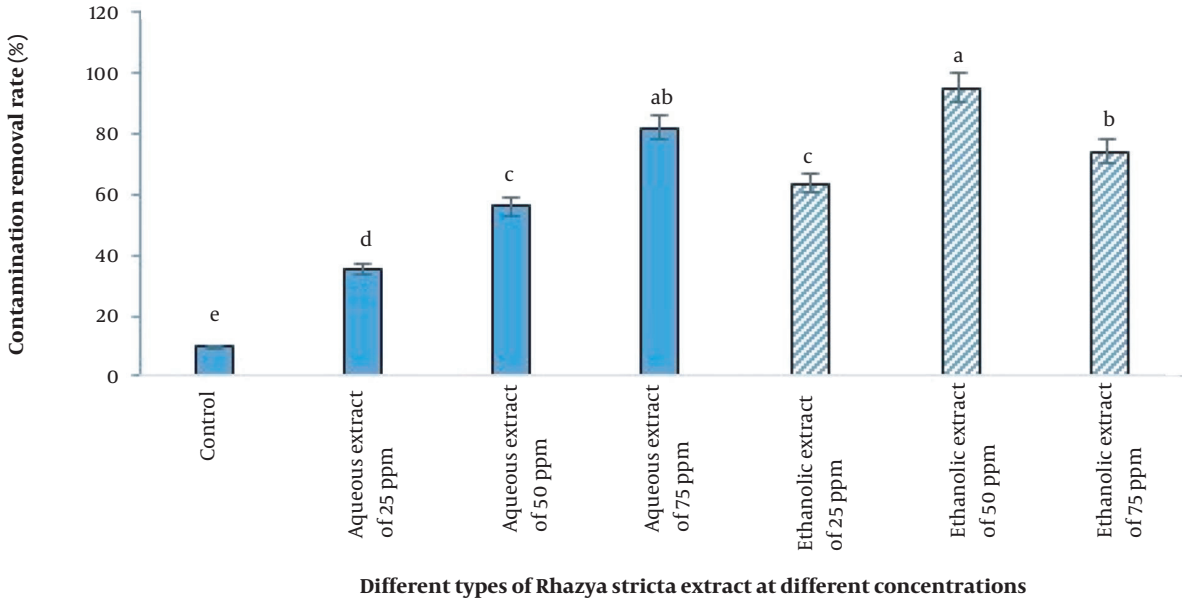

Figure 4. Interaction of various aqueous and ethanolic extracts and different concentrations for disinfection

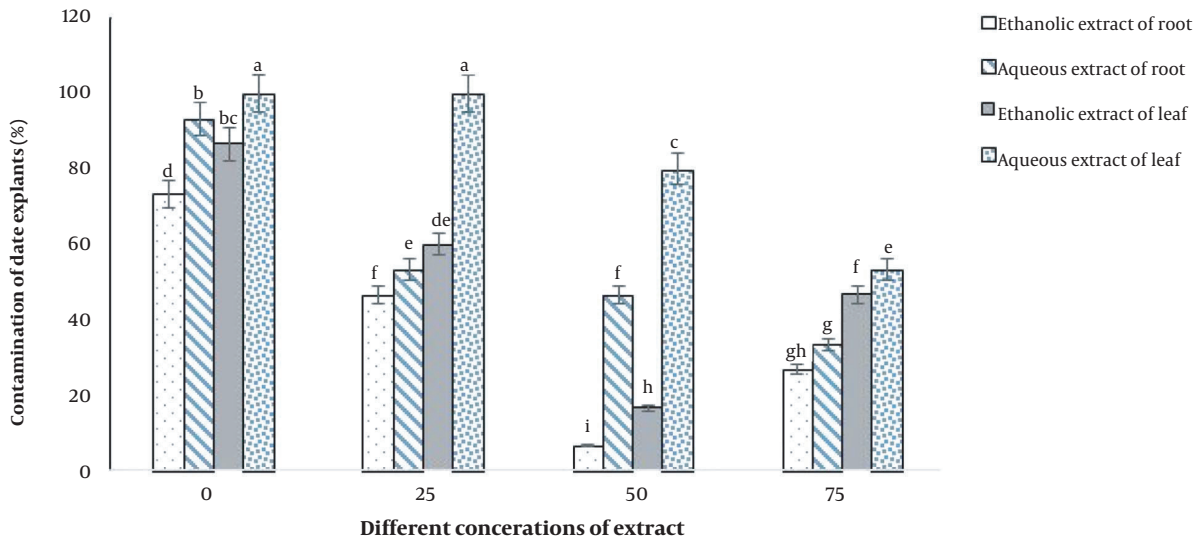

Figure 5. Interaction of organ × type of extract × concentration on the level of contamination of date explants under tissue culture conditions

Figure 6. Sample of bacterial contamination (control) in date tissue culture (A); Sample of grown date offshoots in culture medium containing ethanolic extract of *Rhazya stricta* at a concentration of 50 ppm (B)

and Z.M. wrote the article; H.K. edited the article.

Conflict of Interests: All authors declare no conflict of interest.

Data Reproducibility: All the data are embedded in the manuscript.

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