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Scientific Research

Determination of total phenols and flavonoids, antioxidant potential and antimicrobial activity of hydroalcoholic extract of *Withania somnifera* against fungi cause spoilage postharvest apple and strawberry fruits

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ABSTRACT

In the present study, the hydroalcoholic extract of *Withania somnifera* was extracted and then the content of total phenol (by Folin-Ciocalteu reagent), total flavonoid (by aluminum chloride method), antioxidant activity (based on DPPH and ABTS free radical scavenging methods) and its antifungal effect against *Rhizopu stolonifers*, *Botrytis cinerea*, *Penicillium expansum* and *Alternaria alternata* (fungi cause spoilage postharvest apple and strawberry fruits) were determined based on disk diffusion agar, well diffusion agar, minimum inhibitory concentration, and minimum fungicidal concentration. The amount of phenol and flavonoids in the extract was 53.16 mg GAE/g and 28.20 mg QE/g, respectively. The antioxidant power of the hydroalcoholic extract was 69.80 and 57.15 µg/ml in terms of DPPH and ABTS free radical scavenging, respectively. The results of antimicrobial activity based on disk diffusion agar and well diffusion agar showed that increasing the concentration of the extract caused a significant increase in the diameter of the growth inhibition zone and *Botrytis cinerea* and *Alternaria alternata* were the most resistant and sensitive strains to the extract, respectively. In general, *Penicillium expansum* and *Alternaria alternata* were more sensitive than *Rhizopus stolonifer* and *Botrytis cinerea* with minimum inhibitory and fungicidal concentrations of 16 and 128 mg/ml, respectively.

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1- Introduction

Natural antioxidants present in fruits, tea, vegetables, grains, and medicinal plants are used not only to prevent and treat various diseases caused by oxidative damage, but also to improve the shelf life of horticultural products. Donating hydrogen to highly reactive radicals prevents the formation of free radicals [1, 2].

Plant secondary metabolites or "phytochemicals" are produced by plants for a myriad of functions, from UV protection, protection against pathogens and herbivores, pigmentation to improve pollination chances, and other means of improving plant survival and health, without Used directly, they play a role in vital functions such as growth and reproduction. In the last few decades, there has been a great scientific interest in these compounds and their benefits for human health, as many of them show significant antioxidant and antibacterial activity [3-6].

As consumers are concerned about the negative impact of synthetic chemicals in food, there is a need to find natural products without chemical preservatives. Therefore, there is a growing interest in the use of natural extracts as an alternative to synthetic additives, because these compounds show synergy with other preservation methods, they are considered safe, and they have special properties such as antioxidant, antidiabetic, antimutagenic and antitumor activity. Bacteria appear [7-9].

Paneerbad (*Withania somnifera*) contains a range of diverse phytochemicals that enable it to have a wide range of biological consequences. In preclinical studies, it has shown antimicrobial, anti-inflammatory, anti-tumor, anti-stress, neuroprotective, cardioprotective and antidiabetic properties. In addition, it has shown the ability to reduce reactive oxygen species, modulate mitochondrial function, regulate apoptosis and reduce inflammation and increase endothelial function. Due to these medicinal properties, Paneerbad is a potential drug for the treatment of various clinical conditions, especially related to the nervous system [10]. Paneerbad extract is a complex mixture of many phytochemicals including phenolic compounds and flavonoids. However, the pharmacological effect of Paneerbad root to withanolides[1] attributed [11]. The antioxidant and antimicrobial activity of Paneerbad extract has been shown in various studies [12, 13]. Therefore, this study aims to extract the hydroalcoholic extract of Paneerbad and investigate the amount of total phenol, total flavonoid, antioxidant activity and its antifungal effect on *Rhizopus Astolonifera* ،*Botrytis cinera* ،*Penicillium expansum* And*Alternaria alternata* (Mushrooms causing corruptionAfter harvesting the fruit apple and strawberry).

2- Materials and methods

2- 1- Materials

Folin-Ciocalto identifiers,[2]DPPH ،[3]ABTS, gallic acid and quercetin were obtained from Sigma (USA). Sabro dextrose agar and Sabro dextrose broth cultures were purchased from Merck (Germany). Other chemicals used in this research were of laboratory grade.

2- 2- Extraction of the extract

The hydroalcoholic extract of Paneerbad was extracted using the method of Danani et al. (2017) with the necessary changes. After collecting and drying it in the shade, the dried plant was finely powdered using an electric mill. Next, 5 grams of powdered plant material was mixed with 50 ml of solvent (water:ethanol; 50:50% v/v) in a round bottom glass container and refluxed for about 5 hours at 100°C. The obtained liquid extract was separated from the solid residue by vacuum filtration and concentrated using a rotary evaporator [12].

2- 3- Measuring the amount of total phenol

100 microliters of the extract was mixed with 2.5 ml Folin-Ciocalto phenol reagent (10 times

[1]- Withanolides

[2]- 2,2-diphenyl-1-picrylhydrazyl

[3]- 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

dilution) and kept at room temperature for 5 minutes. Then 2.5 ml of Na solution$_2CO_3$ Saturation was added and the mixture was left for 1 hour at room temperature in a greenhouse. Next, the absorbance of the reaction mixture was read at 725 nm, and the total phenol content of the extract was expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g) [14].

4-2- Determination of total flavonoid content

To determine the amount of total flavonoid, 1 ml of plant extract diluted with 1 ml of 2% AlCl methanolic solution$_3$ mixed. After incubation at room temperature for 15 minutes, the absorbance of the reaction mixture was measured at 430 nm. Quercetin was chosen as a standard and a standard curve (0-50 mg/L) was prepared. Total flavonoid content was expressed as mg quercetin equivalent per gram of extract (mg QE/g) [15].

5-2- Antioxidant activity

2-5-1- DPPH free radical inhibition

The DPPH radical scavenging activity of the extract was measured according to the method provided by Yegangi et al. (2018) [16]. Briefly, a stock solution of the plant extract was prepared in methanol to obtain a concentration of 1 mg/ml. Then the dilutions were prepared in the concentration range of 7.81 to 500 µg/ml. 1 ml of each diluted solution was mixed with 1 ml of methanolic solution containing DPPH radical (0.2 mM). After 30 minutes of storage at room temperature and in the dark, the absorbance was measured at 517 nm wavelength. The control sample contained all the reagents except the extract. The radical scavenging ability was calculated using the following formula:

$I\% = ((A \text{ blank} - A \text{ sample})/A \text{ blank}) \times 100$

Antioxidant activity according to IC_{50} It was shown (the effective concentration of the extract to inhibit 50% of DPPH radicals).

2-5-2- Inhibition of ABTS free radical

The method of Naushad et al. (2021) with minor modifications was used to determine the ABTS radical scavenging activity of the extract [17]. Briefly, an equal volume of a solution of 7 mM ABTS and 2.45 mM $K_2S_2O_8$ It was mixed together and stored at 25°C for 16 hours in the dark. The ABTS radical cation solution obtained was then diluted with methanol to reach an absorbance of 0.7 ± 0.2 at 734 nm. After that, the extract (0.1 ml) was mixed with ABTS radical solution (3.9 ml) and the obtained solution was kept at room temperature for 6 minutes and then its absorbance at 734 nm (As) against the control sample (Methanol; Ac). The ABTS radical scavenging activity of the extract was calculated according to the following formula:

$I\% = ((A \text{ c} - A \text{ s})/A \text{ c}) \times 100$

Antioxidant activity according to IC_{50} (Effective concentration of extract to inhibit 50% of ABTS radicals) was shown.

6-2- Antifungal activity

Antifungal effect of Paneerbad hydroalcoholic extract against*Rhizopus Astolonifera* ،*Botrytis cinera* ،*Penicillium expansum* And*Alternaria alternata* It was checked. The lyophilized fungal cultures were opened under sterile conditions and cultured in sabro dextrose broth for 72 hours at 27°C.

2- 6- 1- Agar diffusion disc[4]

For this purpose, sterile paper discs (with a diameter of 6 mm) were immersed in extract solutions (20, 60, 100 and 140 mg/ml). Then the disc was placed in the center of the medium surface and the plates were kept at 27°C for 72 hours. The diameter of the growth halo around the disc was measured and reported as the antifungal activity of the extract [18].

2- 6- 2- Agar well[5]

In this method, the fungal suspension was spread on sabro-dextrose agar medium in Petri dishes using an L-shaped spreader. Then, several wells with a diameter of 6 mm were created on the surface of the medium and filled with 60 microliters of extract. The Petri dishes were kept at 27°C for 72 hours and the diameter of the inhibitory zones around the wells was determined [19].

2- 6- 3- minimum inhibitory concentration[6] Growth

The minimum inhibitory concentration of the extract was determined by broth macrodilution method in 12 sterile 10 ml test tubes. First, successive dilutions of Paneerbad extract (512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 mg/ml) were prepared in Sabro dextrose broth medium. Then

[4]- Disk diffusion agar (DDA)

[5]-Well diffusion agar (WDA)

[6]- Minimum inhibitory concentration (MIC)

the mushroom extract and suspension were added to the wells. Next, the plate was incubated at 27°C for 72 hours. The minimum inhibitory concentration was determined as the lowest concentration that prevented visible (eye) growth [9].

2- 6- 4- minimum concentration of fungicide[7]

In this method, 100 microliters of the culture medium from each laboratory tube in which turbidity was not observed, was cultured on Sabro dextrose agar. The plate was incubated at 27°C for 72 hours and the lowest dilution that caused complete inhibition of growth was considered as the minimum lethal concentration [20].

7-2- Statistical analysis

The tests of this research were repeated three times. The data were then analyzed using SPSS software (version 26) and one-way variance test[8] were analyzed. from Duncan's test (p<0.05) was used to determine the significant difference between the average data.

3. Results and Discussion

The results of total phenolic and flavonoid content of Paneerbad hydroalcoholic extract are reported in Figure 1. The extract contained 53.19 ± 0.78 mg GAE/g total phenols and 28.20 ± 0.80 mg QE/g total flavonoids.

[7]- Minimum fungicidal concentration (MFC)

[8]- One-way ANOVA

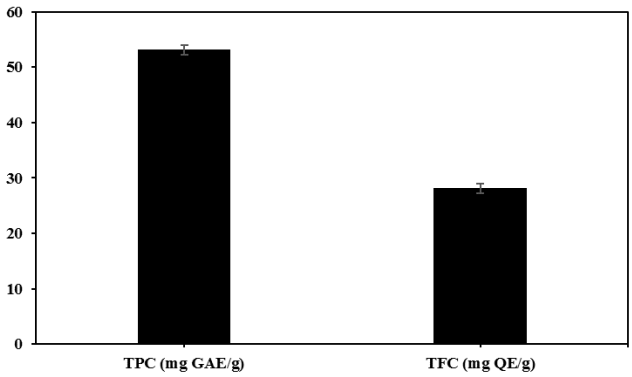

Figure 1. Total phenols (TPC) and flavonoids (TFC) contents of *Withania somnifera* hydroalcoholic extract.

According to Figure 2, the hydroalcoholic extract of Paneerbad had significant antioxidant activity. so that its ability to inhibit DPPH and ABTS free radicals is equal tomg/mlو ٦٩/٨٠ ± ٠/٥٨ mg/ml was 57.15 ± 0.67.

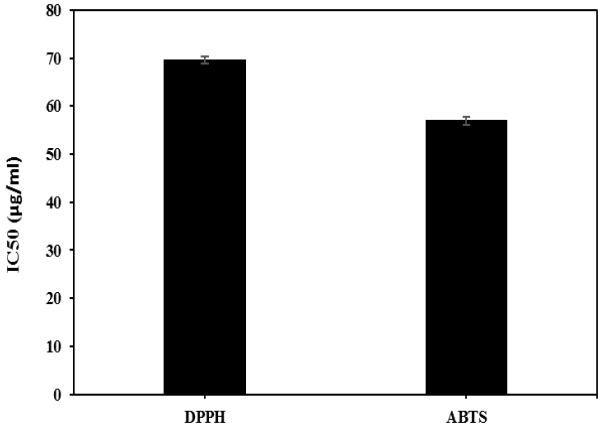

Figure 2. Antioxidant activity of *Withania somnifera* hydroalcoholic extract.

In the study of Danani et al. (2017), extract yield, phytochemical compounds such as total phenol and withanolide content of aqueous extracts and aqueous-alcoholic extracts prepared using two conventional and green methods (ultrasound and microwave) were compared. The antioxidant activity of extracts was also determined using DPPH and ABTS antioxidant assay methods. The yield of the extract, the chemical composition of the extracts (total phenol and withanolide content) and the antioxidant activity of the extracts were dependent on the extraction process and also the solvent composition. The content of total phenol was maximum in the extract prepared with ethanol (35.93 mg GAE/g), followed by water-ethanol (21.15 mg GAE/g) and water (17.63 mg GAE/g). In both DPPH and ABTS methods, ethanolic extracts had the lowest IC value$_{50}$ and they were different in the following order: ethanol < water-ethanol < water [12].

Antifungal results of Paneerbad hydroalcoholic extract based on agar disk diffusion test showed that increasing the concentration of the extract leads to a significant increase in its antifungal effect (diameter of non-growth halo) (Figure 3). The concentration of 20 mg/ml extract does not have any antifungal effect against*Rhizopus Astolonifera* And*Botrytis cinera* Was. In addition, the concentration of 60 mg/ml extract was able to prevent growth*Botrytis cinera* was not However, all studied concentrations have a significant antifungal effect on*Penicillium expansum* And*Alternaria alternata* They were. At the concentration of 140 mg/ml of the extract, the smallest diameter of the lack of growth halo (9.70 mm) corresponds to*Botrytis cinera* and the largest diameter of the aura of non-growth (12.00 mm) to*Alternaria alternata* It was determined that it indicates the resistance and sensitivity of these fungal strains to the hydroalcoholic extract of Paneerbad, respectively.

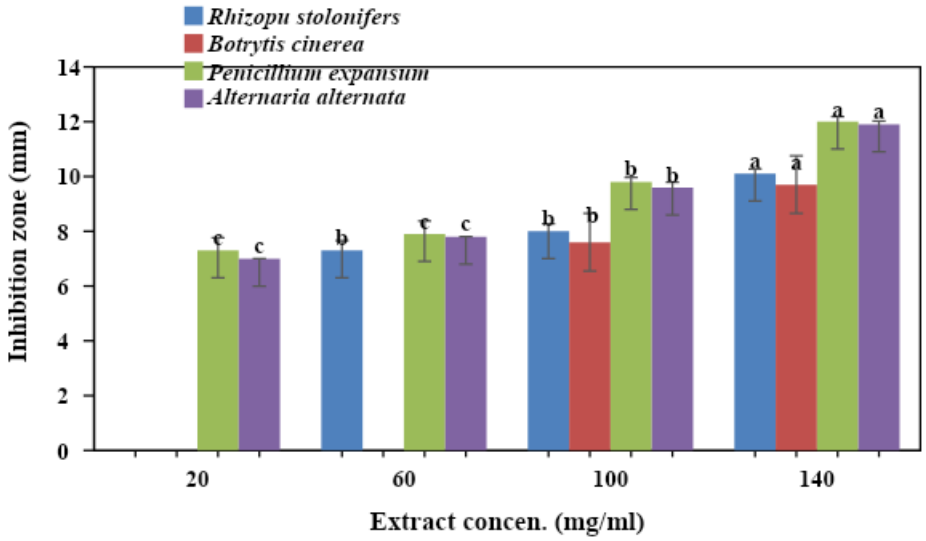

Figure 3. Antifungal activity of *Withania somnifera* hydroalcoholic extract, based on disk diffusion agar (DDA) method.

Similar antifungal results were observed in the agar well test (Figure 4); The diameter of the non-growth halo increased significantly with the increase of the extract concentration from 20 to 140 mg/ml. Furthermore,*Botrytis cinera* And *Penicillium expansum* respectively, with the lowest (10.00 mm) and the highest (12.80 mm) diameter of the non-growth halo at the concentration of 140 mg/ml, the most resistant and sensitive fungal strains were against the extract. Also, it should be noted that the diameter of the non-growth halo in the agar well test was larger than that of the agar disk diffusion method. This state is due to the direct contact of the extract with microorganisms in this method. Whereas, in the antimicrobial disk diffusion agar test, the extract must be diffused from the surface of the disk into the environment to show its inhibitory effect [21, 22].

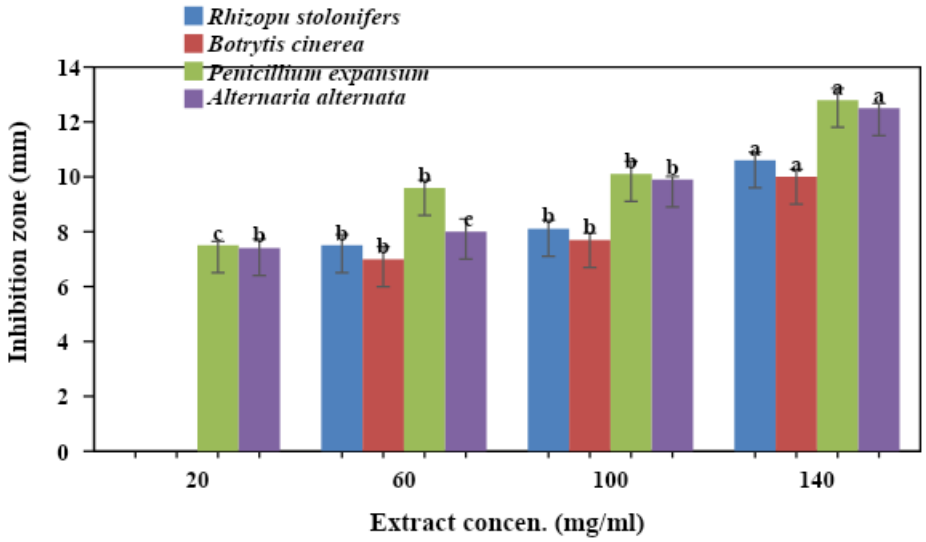

Figure 4. Antifungal activity of *Withania somnifera* hydroalcoholic extract, based on well diffusion agar (WDA) method.

The results of tests of the minimum inhibitory concentration of Paneerbad hydroalcoholic extract for*Rhizopus Astolonifera* ،*Botrytis cinera* ،*Penicillium expansum*

And*Alternaria*It was 64, 64, 16 and 16 mg/ml respectively. The minimum lethal concentration was 512, 512, 128 and 128 mg/ml for the mentioned strains (Figures 5 and 6). It has been reported that due to their hydrophobic nature, essential oils and extracts are easily absorbed by the fungal mycelium, thus inhibiting its growth [23-25].

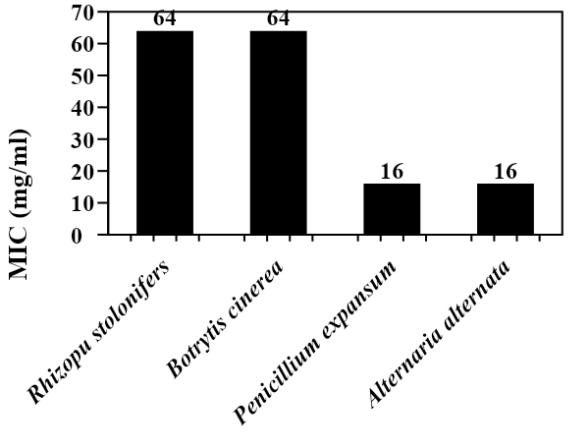

Figure 5. Antifungal activity of *Withania somnifera* hydroalcoholic extract, based on minimum inhibitory concentration (MIC) method.

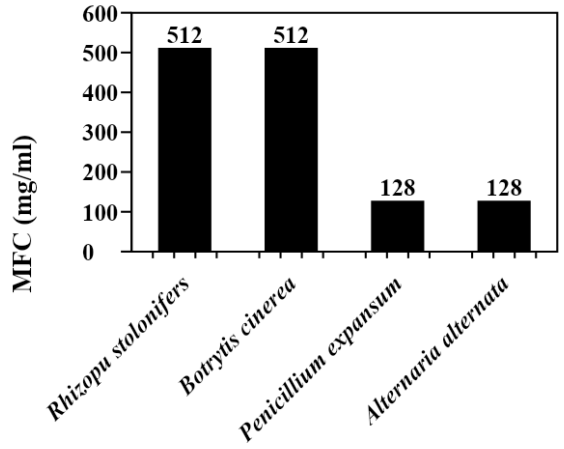

Figure 6. Antifungal activity of *Withania somnifera* hydroalcoholic extract, based on minimum fungicidal concentration (MFC) method.

In the study of Arora et al. (2004), methanolic, hexane and diethyl ether extracts of both leaves and roots of Paneerbad were investigated for antimicrobial activity using agar disk diffusion method. Among the tested extracts, only the methanolic and hexane extracts of both leaves and roots were found to have strong antimicrobial activity. The antimicrobial effect of the extracts can be attributed to the presence of Vitaferin[9] A and attributed with withanolide D. [13]. In addition, Alam et al. (2012) investigated the antioxidant and antimicrobial activity of the methanol extract (20:80; methanol:water) of the root, fruit and leaf of Paneerbad. The antioxidant properties of this plant were tested using DPPH radical inhibition, iron reduction power, iron chelating activity and beta-carotene decolorization inhibition tests. DPPH values, iron reduction power, iron chelating activity and beta-carotene decolorization inhibition for three types of extracts respectivelymg/ml was 801-93-101-73, mM Fe/kg 2-29-26, 0.65-0.22 mg/ml and 79.67-69.87%, the high antioxidant activity of the extract obtained from It shows the leaf of Paneerbad. Antibacterial activity using the agar well diffusion method and against five pathogenic Gram-negative bacteria (*Escherichia coli* ،*Salmonella typhi* ،*Citrobacter freundii* ، *Pseudomonas aeruginosa* And*Klebsiella pneumoniae*) was measured. Leaf extract has the highest activity against*Salmonella typhi* showed (diameter of non-growth halo: 32 mm), while the lowest activity against*Klebsiella pneumoniae* (Diameter of lack of growth: 19 mm) The lowest value of the minimum inhibitory concentration was 6.25 mg/ml, which is compared to*Salmonella typhi* and then 12.5 mg/ml against bacteria*Escherichia coli* was [26]. These results show that the antioxidant capacity of each extract may be related to the concentration of ascorbic acid, anthocyanin and polyphenols. In addition, phenolics, ascorbic acid, and anthocyanins are associated with plant antimicrobial efficacy because they cause hyperacidification at the pathogen plasma membrane interface, potentially leading to disruption of H^+-ATPase is required for ATP synthesis [27]. Jain and Varshani (2011) Diameter of non-growth halo regarding antimicrobial activity of methanolic extract of Paneerbad plant against*Escherichia coli* ، *Pseudomonas aeruginosa* ،*Staphylococcus aureus* ،*Streptococcus mutans* And*Candida albicans* reported 38, 36, 15, 38 and 32 mm respectively. These researchers found that the aqueous extract of Paneerbad has a higher antimicrobial activity compared to the methanolic

[9]- Withaferin

extract [28]. Therefore, ascorbic acid, anthocyanin and polyphenols may be responsible for the significant antimicrobial and antioxidant activity of Paneerbad hydroalcoholic extract. Polyphenols and flavonoids have considerable medicinal importance and may act as antioxidant, antimicrobial and immunomodulatory agents [29].

4 - Conclusion

According to the results of this study, the hydroalcoholic extract of Panirbad is rich in phenolic and flavonoid compounds, which play a very important role in the occurrence of antioxidant activity of this compound. Also, the antifungal activity of the extract was dependent on the concentration and this compound has a significant antimicrobial effect against the strains*Rhizopus Astolonifera* ،*Botrytis cinera* ، *Penicillium expansum* And*Alternaria alternata* shows. In general, the findings of this research show that the hydroalcoholic extract of Paneerbad is a bioactive compound with significant antifungal and antioxidant activity.

5- Appreciation and thanks

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6- Resources

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مقاله علمی_پژوهشی

تعیین فنول و فلاونوئید کل، پتانسیل آنتی‌اکسیدانی و فعالیت ضدمیکروبی عصاره هیدروالکلی پنیرباد بر قارچ‌های عامل فساد پس از برداشت میوه سیب و توت فرنگی

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چکیده

در پژوهش حاضر، عصاره هیدروالکلی پنیرباد استخراج گردید و سپس محتوای فنول کل (به روش معرف فولین-سیوکالتو)، فلاونوئید کل (به روش رنگ‌سنجی کلرید آلومینیوم)، فعالیت آنتی‌اکسیدانی (بر پایه روش‌های مهار رادیکال آزاد DPPH و ABTS) و اثر ضدقارچی آن بر *ریزوپوس استولونیفر*، *بوتریتیس سینرا*، *پنی‌سیلیوم اکسپانسوم* و *آلترناریا آلترناتا* (قارچ‌های عامل فساد پس از برداشت میوه سیب و توت فرنگی) بر اساس روش‌های دیسک دیفیوژن آگار، چاهک آگار، حداقل غلظت مهارکنندگی و حداقل غلظت کشندگی تعیین گردید. میزان فنول و فلاونوئید کل عصاره پنیرباد به ترتیب برابر با ۵۳/۱۶ mg GAE/g و ۲۸/۲۰ mg QE/g بدست آمد. قدرت آنتی‌اکسیدانی عصاره هیدروالکلی بر حسب درصد مهار رادیکال‌های آزاد DPPH و ABTS به ترتیب برابر با ۶۹/۸۰ µg/ml و ۵۷/۱۵ بود. نتایج فعالیت ضدقارچی بر اساس روش‌های دیسک دیفیوژن آگار و چاهک آگار نشان داد که افزایش غلظت عصاره سبب افزایش معنی‌دار قطر هاله عدم رشد گردید. *بوتریتیس سینرا* و *آلترناریا آلترناتا* به ترتیب مقاوم‌ترین و حساس‌ترین سویه‌های قارچی در برابر عصاره بودند. بطور کلی *پنی‌سیلیوم اکسپانسوم* و *آلترناریا آلترناتا* نسبت به *ریزوپوس استولونیفر* و *بوتریتیس سینرا* حساس‌تر بودند، بطوریکه حداقل غلظت مهارکنندگی و کشندگی برای این گروه به ترتیب ۱۶ mg/ml و ۱۲۸ مشاهده گردید.