



Investigating the inhibition of the growth of spoilage fungi causing apple's rot and mold using *Elettaria cardamomum* essential oil

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ABSTRACT

Considering the sensitivity of apple fruit to many pests and diseases and the sensitivity of consumers to synthetic pesticides, the use of plant essential oils has increased to increase the lifespan of various horticultural products, including apples. In this research, total phenol, total flavonoid and antioxidant properties of *Elettaria cardamomum* essential oil were investigated. In addition, the antimicrobial property of this essential oil was evaluated on a number of fungi that cause spoilage of apple fruit, including *Penicillium expansum*, *Botrytis cinerea*, and *Alternaria alternata*. The phenol and flavonoid content of *E. cardamomum* essential oil was equal to 69.60 mg of gallic acid per gram of essential oil and 27.40 mg of quercetin per gram of essential oil, respectively. The amount of antioxidant property of *E. cardamomum* essential oil in DPPH and ABTS free radical inhibition method was obtained as 57.30% and 63.60%, respectively. In the investigation of the antifungal property, the largest inhibition zone was observed in *P. expansum* by disk diffusion and agar well methods, and the minimum inhibitory concentration for *P. expansum* and *B. cinerea* fungi was 8 mg/ml and minimum fungicidal concentration was 64 mg/ml. Considering the high antioxidant and antifungal properties of *E. cardamomum* essential oil, it can be used as a suitable alternative to synthetic fungicides.

1- Introduction

Apples are one of the most popular fruits in the world, rich in various vitamins and minerals, and widely cultivated across the globe. Preliminary studies have suggested that regular apple consumption may reduce the risk of colorectal cancer, prostate cancer, and lung cancer. While apple peels contain a significant amount of unspecified phytochemicals with antioxidant properties. Fungi such as *Penicillium expansum*, *Botrytis cinerea*, and *Alternaria alternata* are prevalent disease-causing agents of apples, affecting both field cultivation and post-harvest storage. The incidence and impact of fungal diseases significantly compromise apple quality, both for fresh consumption and fruit processing, altering the properties of the fruit and its derived juice. These contaminating agents, if left unidentified and unaddressed promptly, can lead to widespread apple spoilage, resulting in substantial direct economic losses for growers [1-3].

Numerous pesticides are employed with remarkable efficacy in combating fungal diseases and safeguarding crops. However, the extensive use of synthetic pesticides in fruit production clearly highlights their inherent limitations and detrimental effects on both the environment and human health [4]. Consumers prioritize purchasing fruits that are untreated with pesticides, free from defects and diseases, and safe for consumption. Consequently, the fresh produce industry faces a pressing need to identify alternative solutions to synthetic fungicides [5]. Today, driven by the growing consumer demand for natural alternatives, essential oils derived from plants are increasingly being employed in food preservation practices. These plant-based essences hold immense promise as natural

preservatives due to their potent antimicrobial and antioxidant properties, complex composition, diverse modes of action, and ability to mitigate the development of resistance among pathogenic agents [6-8]. Green cardamom (*Elettaria cardamomum*) is a plant from the ginger family, 30 to 50 centimeters tall, that grows in cold and mountainous regions. Green cardamom is more widely used in Iran than other types of cardamom due to its spicy flavor and is one of the spices used in various dishes [9]. Various studies have reported the antimicrobial and antioxidant effects of green cardamom [9-11]. Therefore, the aim of this study was to investigate the phenolic and flavonoid compounds and antioxidant properties of green cardamom essential oil and its inhibitory power in preventing the growth of postharvest fruit decay and mold fungi in apple.

2- Materials and Methods

2-1- Preparation of Essential Oil

After obtaining green cardamom, 50 g of the ground plant was extracted using the hydro-distillation method with the aid of a Clevenger apparatus. Finally, after removing the excess water, the essential oil was stored in dark containers at 4°C [12].

2-2- Measurement of Total Phenols

Total phenols of the essential oil were measured using gallic acid standard and Folin-Ciocalteu reagent. Briefly, 0.1 mL of the essential oil (5 mg/mL) was added to 1 mL of 10% v/v Folin-Ciocalteu reagent. The solution was mixed for 5 min. Then, 0.3 mL of 10% sodium carbonate solution was added to it, and after keeping the solution for 2 h, the absorbance of the sample was measured at a wavelength of 760 nm. Gallic acid standards of 0-0.5 µg/mL were used. The amount of total phenols was reported as mg

gallic acid equivalent (GAE) per g essential oil [13].

2-3- Measurement of Total Flavonoids

In this method, 0.5 mL of green cardamom essential oil (1 g/10 mL) was mixed with 1.5 mL of methanol, 0.1 mL of aluminum chloride (10% methanol), 0.1 mL of potassium acetate, and 2.8 mL of distilled water. After incubating the sample for 30 min, the absorbance was measured at a wavelength of 415 nm, and the total flavonoid content was reported as mg quercetin equivalent (QE) per g essential oil [14].

2-4- Determination of Antioxidant Activity of Essential Oil

2-4-1- Measurement of DPPH Radical Scavenging Activity

In this method, 50 μ L of essential oil or control sample was mixed with 5 mL of 0.12 mM ethanolic DPPH solution. The samples were then incubated for 30 min at room temperature in the dark, and their absorbance was measured at a wavelength of 517 nm. DPPH radical scavenging activity was calculated using the following formula [15]:

$$\text{Free radical scavenging (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

2-4-2- ABTS Radical Scavenging Activity

ABTS radicals were prepared by mixing aqueous ABTS solution and potassium persulfate. Then, 0.1 mL of essential oil or control sample was mixed with 3.9 mL of ABTS radical cation. Finally, the absorbance of the samples was recorded at a wavelength of 734 nm. [16]:

$$\text{ABTS- activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

2-5- Determination of Antimicrobial Activity of Essential Oil

2-5-1- Disc Diffusion Assay

In the disc diffusion assay, each strain was cultured on the surface of Sabouraud dextrose agar (SDA) plates. After drying the

inoculated plates, discs impregnated with essential oil were placed on the agar plates. After incubating the plates at 27°C for 72 h, the antifungal activity was measured as the zone of inhibition in millimeters [17].

2-5-2- Agar Well Diffusion Assay

After surface inoculation of strains on Sabouraud dextrose agar (SDA) plates, 6 mm diameter wells were created in them. Then, 20 μ L of the prepared essential oil was added to the wells. After incubating the plates at 27°C for 72 h, the diameter of the no-growth zones around each well was measured in millimeters [18].

2-5-3- Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

To determine the minimum inhibitory concentration (MIC), the essential oil was first sterilized using 0.22 μ m filters. Serial dilutions were prepared in 10 mL test tubes from a concentration of 512 mg/mL. Then, 20 μ L of the microbial suspension was mixed with each of the essential oil concentrations and the growth of the strains was observed visually by turbidity after 72 h at 27°C. The first tube without turbidity was reported as the MIC.

To determine the minimum bactericidal concentration (MBC), 20 μ L aliquots from each of the turbid-free tubes were inoculated on Sabouraud dextrose agar (SDA) plates. After incubation at 27°C for 72 hours, the concentrations without growth were determined as the MBC [19].

2-6- Statistical Analysis

The experiments in this study were performed in triplicate. The results were analyzed using SPSS software version 18. One-way ANOVA and Duncan's test were used to determine the significance of differences between means at a 95% confidence level ($p < 0.05$).

3- Results and Discussion

The phenol and flavonoid contents of green cardamom essential oil are presented in Figure 1. As can be observed, the total phenol content was determined to be 69.60 milligrams of gallic acid equivalent (GAE) per gram of essential oil, and the total flavonoid content was found to be 27.40 milligrams of quercetin equivalent (QE) per gram of essential oil. According to research conducted in various studies, a significant portion of secondary plant metabolites are phenolic and flavonoid compounds. These compounds possess various biological properties, including antimicrobial and antioxidant activities [20]. The total phenol and flavonoid contents of green cardamom ethanol extract were reported to be 15.33 milligrams of gallic acid equivalent (GAE) per gram and 18 milligrams of Rutin

Equivalent (RE) per gram, respectively [21]. Also, the total phenol content of various green cardamom extracts has been reported to range from 13.1 to 63 milligrams of gallic acid equivalent (GAE) per gram of dry matter [10]. The total phenolic content of green cardamom extract has been reported to be approximately 45 mg gallic acid equivalent per gram of dry weight. This compound is thought to play a role in the antioxidant properties of green cardamom [11]. It is noteworthy that variations in the content of phenols and flavonoids can be attributed to various geographical and climatic factors in the plant growth region, including soil type, temperature, water availability, altitude, plant genotype, and extraction and measurement methods [22, 23].

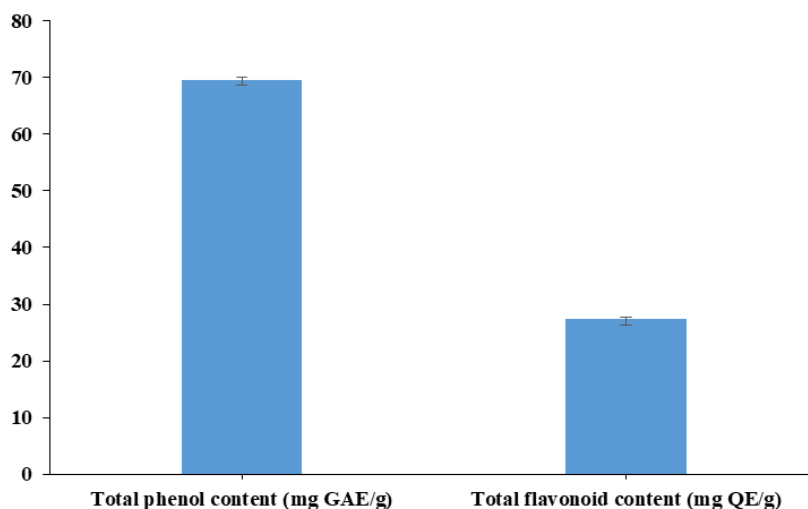


Fig. 1 Total phenol and flavonoid contents of *E. cardamomum* essential oil

Also, the antioxidant activity of green cardamom essential oil was also determined using two methods: DPPH and ABTS free radical scavenging assays. The results showed 57.30% and 63.60% inhibition, respectively (Figure 2). The free radical scavenging activity of cardamom extract has

been attributed to the hydrogen donating ability of its constituents [11]. The antioxidant activity of green cardamom essential oil has been demonstrated using two methods: ABTS radical scavenging assay and ferric reducing antioxidant power (FRAP) assay [10]. The DPPH free radical scavenging activity of green cardamom essential oil was reported to be 43% [9].

Sharafati et al. (2017) reported the antioxidant activity of green cardamom extract to be 45.7%, which was attributed to the phenolic compounds present in the plant. These researchers stated that the difference in the levels of antioxidant properties of plant extracts and essential oils can be due to the presence of different compounds in extracts and essential oils, which are affected by factors such as geographical location, temperature, plant growth stage, harvest time, genetic factors, and environmental factors related to the plant [21]. The free radical scavenging of cardamom extract has been attributed to the hydrogen donating ability of its constituents [11]. The antioxidant activity of green cardamom essential oil has been demonstrated using two methods: ABTS radical scavenging

assay and ferric reducing antioxidant power (FRAP) assay [10]. The DPPH free radical scavenging activity of green cardamom essential oil was reported to be 43% [9]. Sharafati et al. (2017) reported the antioxidant activity of green cardamom extract to be 45.7%, which was attributed to the phenolic compounds present in the plant. These researchers stated that the difference in the levels of antioxidant properties of plant extracts and essential oils can be due to the presence of different compounds in extracts and essential oils, which are affected by factors such as geographical location, temperature, plant growth stage, harvest time, genetic factors, and environmental factors related to the plant [21].

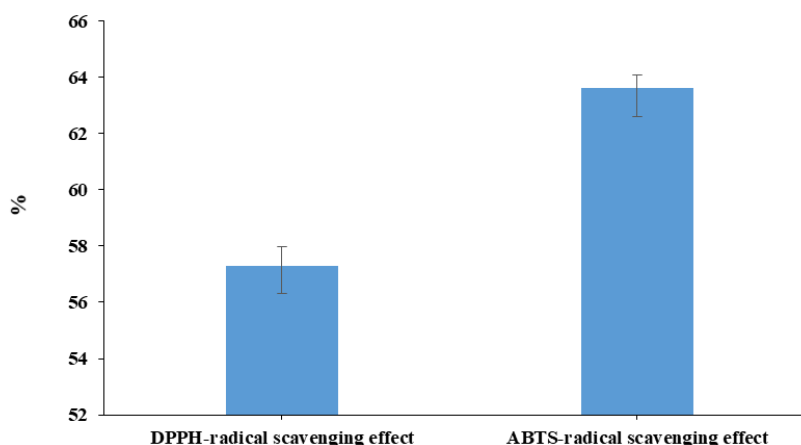


Fig. 2 Antioxidant activity of *E. cardamomum* essential oil

The results of the inhibition zone diameters in the disc diffusion method are shown in Figure 3. It was observed that there was no significant difference between the diameters of the inhibition zones created in the fungi *P. expansum* and *B. cinerea*, but the diameters of the inhibition zones created in these fungi

were significantly different from those created in *A. alternata*. The strongest antifungal effect with a diameter of 12.80 mm was observed in *P. expansum* and the weakest effect with a diameter of 10.10 mm was observed in *B. alternata*.

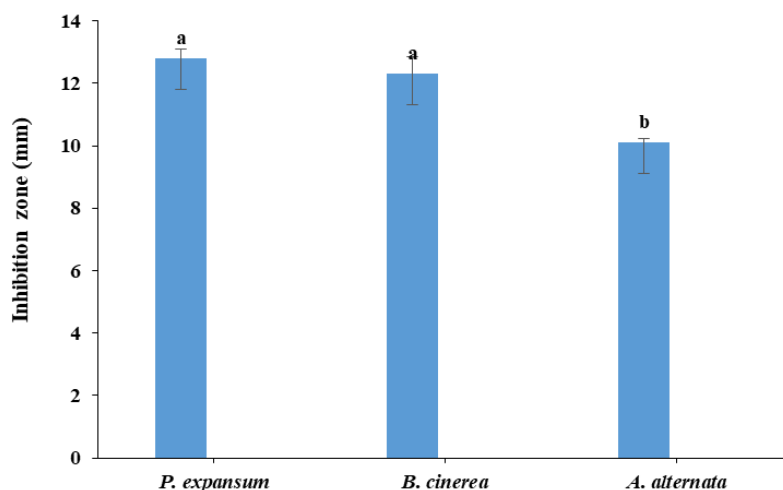


Fig. 3 Antifungal activity of *E. cardamomum* essential oil based on disk diffusion agar (DDA) method

The results of the agar well assay inhibition zone diameters are shown in Figure 4. It was observed that similar to the disc diffusion method, there was no significant difference between the diameters of the inhibition zones

created in the fungi *P. expansum* and *B. cinerea*, but there was a significant difference between the diameters of the inhibition zones created in these two fungi and the inhibition zone created in *A. alternata*.

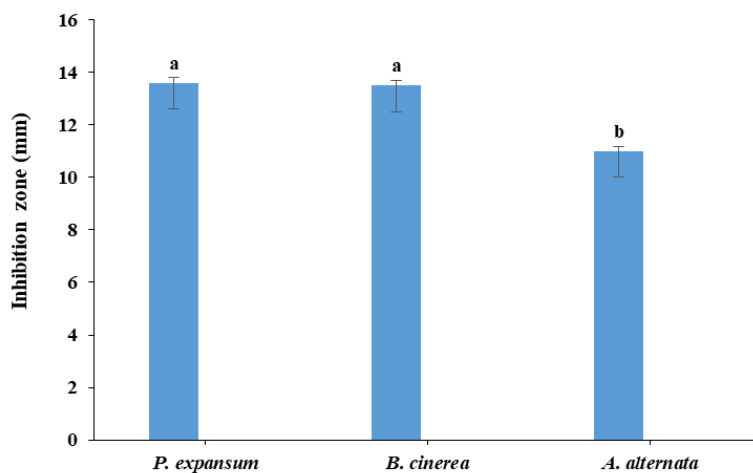


Fig. 4 Antifungal activity of *E. cardamomum* essential oil based on well diffusion agar (WDA) method

The results of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) are shown in Table 1. The MIC of green cardamom essential oil for the fungi *P. expansum* and *B.*

cinerea was 8 mg/mL, while the MIC for *A. alternata* was 16 mg/mL. The MFC of the essential oil for the fungi *P. expansum* and *B. cinerea* was 64 mg/mL, while the MFC for *A. alternata* was 256 mg/mL.

Table 1 Antifungal effect of *E. cardamomum* essential oil based on minimum inhibitory concentration and minimum fungicidal concentration methods

Microorganism	MFC (mg/ml)	MIC (mg/ml)
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<i>P. expansum</i>	8	64
<i>B. cinerea</i>	8	64
<i>A. alternata</i>	16	256

As observed in the results, the inhibition zones in the agar well assay were larger than those in the disc diffusion method. This is likely due to the better and easier diffusion of the essential oil and, consequently, its greater impact in the agar well assay [24]. Various mechanisms have been reported for the antimicrobial properties of essential oils. For instance, plant essential oils can increase membrane permeability. As a result, by penetrating the membrane, they lead to membrane swelling and cell death. In addition, the antifungal activity of plant essential oils has been attributed to the active compounds present in them, including ketones, aldehydes, and phenols. These active compounds, due to the presence of an aromatic nucleus and a phenolic OH group in their structure, can form hydrogen bonds with the SH groups in the active sites of enzymes, leading to the deactivation of fungal enzymes. In addition, plant essential oils, due to their low molecular weight and high lipophilicity, are easily absorbed by hydrophobic mycelia and inhibit the growth of fungal cells [6]. It is important to note that the variations in antimicrobial properties observed for a particular plant across different studies can be attributed to differences in the constituents of their extracts and essential oils. These differences can arise due to factors such as variations in climate, soil conditions, and harvesting stages [25]. The antimicrobial and antioxidant effects of green cardamom essential oils and extracts have been reported

in various studies [26]. Singh et al. (2008) attributed the antioxidant and antimicrobial effects of green cardamom essential oils and extracts to the presence of phenolic compounds within them [27]. Kapoor et al. (2008), in their investigation of the antifungal properties of green cardamom essential oil against various fungi, including *Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus oryzae*, emphasized that increasing the concentration of the essential oil led to enhanced inhibitory power. They attributed this antifungal activity to the phenolic compounds present in green cardamom [28]. The results of the present study were consistent with other studies conducted on the antimicrobial effect of essential oils and plant extracts [29-40].

4- Conclusion

This research has demonstrated that green cardamom essential oil is rich in phenolic and flavonoid compounds, which play a significant role in its antioxidant and antifungal properties. The antifungal efficacy of green cardamom essential oil in inhibiting and controlling the growth of apple fruit-infecting fungi, coupled with the growing preference for natural alternatives, makes this bioactive compound a promising substitute for synthetic pesticides in managing horticultural crop pests. However, further studies are recommended to identify the specific constituents of the essential oil, purify them to determine the most potent compound responsible for its antimicrobial activity, and conduct a more in-depth

investigation of the essential oil's antimicrobial mechanism of action.

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بررسی مهار رشد قارچ‌های عامل فساد و کپک زدگی پس از برداشت میوه سیب با استفاده از اسانس هل سبز

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اطلاعات مقاله	چکیده
تاریخ‌های مقاله: تاریخ دریافت: ۱۴۰۳/۳/۳ تاریخ پذیرش: ۱۴۰۳/۴/۶	با توجه به حساسیت میوه سیب به بسیاری از آفت‌ها و بیماری‌ها و حساسیت مصرف‌کنندگان به آفت‌کش‌های سنتزی، استفاده از اسانس‌های گیاهی جهت افزایش طول عمر محصولات مختلف باغبانی از جمله سیب افزایش یافته است. در این پژوهش میزان فنول کل، فلاونوئید کل و خاصیت آنتی‌اکسیدانی اسانس هل سبز مورد بررسی قرار گرفت. علاوه بر این، خاصیت ضد میکروبی این اسانس بر تعدادی از قارچ‌های عامل فساد و کپک زدگی پس از برداشت میوه سیب از جمله <i>Alternaria alternata</i> و <i>Botrytis cinerea</i> ، <i>Penicillium expansum</i> مورد ارزیابی قرار گرفت. میزان فنول و فلاونوئید کل اسانس هل سبز به ترتیب برابر با ۶۹/۶۰ میلی‌گرم گالیک اسید در گرم اسانس و ۲۷/۴۰ میلی‌گرم کوئرستین در گرم اسانس بود. میزان خاصیت آنتی‌اکسیدانی اسانس هل سبز در روش مهار رادیکال آزاد DPPH و ABTS به ترتیب ۵۷/۳۰ و ۶۳/۶۰ درصد به دست آمد. در بررسی خاصیت ضد قارچی بیشترین قطر هاله عدم رشد در روش دیسک دیفیوژن و چاهک آگار در <i>P. expansum</i> مشاهده شد و حداقل غلظت مهارکنندگی برای قارچی‌های <i>P. expansum</i> و <i>B. cinerea</i> برابر با ۸ میلی‌گرم در میلی‌لیتر و حداقل غلظت کشندگی در این قارچ‌ها برابر با ۶۴ میلی‌گرم در میلی‌لیتر بود. با توجه به خواص آنتی‌اکسیدانی و ضد قارچی بالای اسانس هل سبز، می‌توان از آن به عنوان جایگزین مناسبی برای قارچ‌کش‌های سنتزی استفاده کرد.
کلمات کلیدی: اسانس هل سبز، خاصیت آنتی‌اکسیدانی، اثر ضد قارچی، میوه سیب، عفونت پس از برداشت.	
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