



Scientific Research

Evaluation of antioxidant potential, total phenol and flavonoid and antimicrobial activity of *Pterocarya fraxinifolia* essential oil on pathogenic bacteria: “in vitro”

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ABSTRACT

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Adding chemical preservatives increases the shelf life of food products, but long-term and indiscriminate use of chemical preservatives increases the resistance of microorganisms and health risks associated with their absorption. Medicinal plants have a great diversity both in the world and in Iran and have the potential to be used as alternatives to chemical compounds. In this study, the antimicrobial effect of *Pterocarya fraxinifolia* essential oil against *Bacillus subtilis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Shigella dysenteriae*, *Enterobacter aerogenes*, and *Salmonella typhi* was investigated by disk diffusion agar, well diffusion agar, minimum inhibitory concentration, and minimum bactericidal concentration. The total phenol and flavonoid content of the essential oil was determined using Folin Ciocalteu and aluminium chloride methods, respectively. The antioxidant activity of the essential oil was evaluated using two methods of inhibiting free radicals DPPH and ABTS. The total phenol and flavonoid content of the essential oil was 38.63 mg of gallic acid/g and the flavonoid content was 19.20 mg of quercetin/g. The essential oil of *P. fraxinifolia* was able to inhibit free radicals DPPH (58.60%) and ABTS (59.80%). The results of the antimicrobial activity of the essential oil by the disk diffusion agar and well diffusion agar methods showed that *B. subtilis* and *E. aerogenes* are the most sensitive and resistant microbial strains to the essential oil, respectively. The minimum inhibitory concentration for these bacteria was 2 and 64 mg/mL, respectively, and the minimum bactericidal concentration was 32 and > 512 mg/mL, respectively.

1. Introduction

The food industry mainly uses synthetic food preservatives to prevent oxidation and microbial contamination of packaged foods. But today, due to the adverse effects of synthetic compounds on health and the environment and the increasing problem of the emergence of drug-resistant strains, the focus of the food industry is towards the use of natural antioxidant and antimicrobial compounds extracted from plants as preservatives. Natural food preservatives are safe, environmentally friendly, and cost-effective [1-6].

Essential oils are volatile, natural, and complex compounds that are formed by aromatic plants as secondary metabolites. They were usually first developed by the Arabs in the Middle Ages by steam or water distillation and are used for mummification, food preservation, and as antimicrobial, analgesic, sedative, anti-inflammatory, antispasmodic, and local anesthetic drugs due to their antiseptic properties, i.e., bactericidal, virucidal, fungicidal, and their medicinal properties and aroma. To date, these features have not changed much, except that more information is now known about some of their mechanisms of action, especially at the antimicrobial level [7]. Essential oils play an important role in protecting plants in nature as antibacterial, antiviral, antifungal, insecticidal, and also against herbivores by reducing their appetite for these plant species. Essential oils are extracted from various aromatic plants and are liquid, volatile, transparent, and rarely colored, soluble in fat and soluble in organic solvents with a density less than water. They can be synthesized by all plant organs such as buds, flowers, leaves, stems, branches, seeds, fruits, roots, wood or bark and are stored in secretory cells, cavities, canals, epidermal cells or glandular trichomes [7].

Pterocarya fraxinifolia is a common coastal tree that is mainly located in lowland areas and is the only representative tree of the genus *Pterocarya* outside of East Asia, with specific

morphological features. Locally, *P. fraxinifolia* provides coastal protection and food for aquatic and terrestrial consumers. *P. fraxinifolia* has been introduced to Europe for gardening and is currently used in forestry. For centuries, *P. fraxinifolia* habitats have been declining, mainly due to conversion to agricultural land and recently due to sand mining and road construction and hydropower plants [8]. *P. fraxinifolia* is a fast-growing tree that is naturally distributed throughout the Black Sea region of western Turkey and is native to the Caucasus from northern Iran to Ukraine [9]. *P. fraxinifolia* is a thick, deciduous tree with a furrowed, dark gray bark, reaching a height of up to 35 meters. This species grows naturally in the provinces of Gorgan, Gilan, and Mazandaran and in recent years, small populations of it have also been found in the provinces of Ilham and Lorestan [10]. However, limited information exists about the antimicrobial and antioxidant properties of its essential oil and extract. Nabavi and colleagues (2008) investigated the antioxidant activity of the methanolic extract of *P. fraxinifolia* and the results of these researchers showed that the extract of *P. fraxinifolia* is capable of inhibiting free radicals [10]. Batooli and colleagues (2016) identified the chemical compounds of the essential oil of *P. fraxinifolia* leaves at different phenological stages in the Gilan region [11]. Given the above, the aim of this study is to extract the essential oil of the *P. fraxinifolia* tree and determine the total phenol content, total flavonoid, and investigate its antimicrobial and antioxidant effect in order to introduce a new type of natural medicinal compounds with antimicrobial and antioxidant properties.

2. Materials and methods

2.1. Materials

Materials of laboratory grade prepared from Merck (Germany) and Sigma (USA) companies were used to perform chemical and microbial tests.

2.2. Essential oil extraction

To prepare the essential oil, the dried plant was powdered and placed in a Clevenger apparatus and the essential oil extraction was performed according to the water distillation method for 3 h. The essential oil was dehydrated by sodium sulfate and the resulting essential oil was stored in clean dark-colored containers until the chemical and microbial tests were performed at a temperature of 4 °C [3].

2.3. Total phenolic content

The total phenol content of the essential oil was measured using the Folin-Ciocalteu reagent. For this purpose, 0.5 mL of essential oil was combined with 2.5 mL of 10 percent Folin reagent and kept at room temperature for 10 min. Then, 2 mL of sodium carbonate was added to the mixture and after a period of 90 min, the absorption of the essential oil sample was read at a wavelength of 765 nm. The total phenol content of the essential oil was reported based on milligrams of gallic acid per gram of essential oil [12].

2.4. Total flavonoid content

To determine the total flavonoid content of the essential oil, the aluminum chloride spectrophotometric method was used. In this method, 0.5 mL of the essential oil was diluted with methanol and the resulting solution was combined with NaNO₂ and AlCl₃ and kept at room temperature for 6 min. After 6 min, 1 M NaOH was added to the solution and its absorption was measured at a wavelength of 510 nm. Quercetin was used as a standard and the total flavonoid content of the essential oil was reported as milligrams of quercetin per gram of essential oil [13].

2.5. Antioxidant Activity

2.5.1. Scavenging of DPPH radicals

To perform this test, 50 µL of the essential oil was combined with 5 milliliters of the methanolic solution of DPPH and after 30 minutes at room temperature, the absorption of

the sample was measured at a wavelength of 517 nanometers. The antioxidant activity was calculated and reported based on the following formula [14]:

$$\text{Antioxidant activity (\%)} = \frac{(\text{Absb} - \text{Abs})}{\text{Absb}} \times 100$$

In this equation, Absb and Abs are the absorption of the control and the sample, respectively.

2.5.2. Scavenging of ABTS radicals

The pre-made ABTS monocation radical was produced in equal amounts by oxidizing the ABTS solution (7 mM) with a potassium persulfate solution (2.45 mM). The mixture was kept in the dark at a temperature of 25 °C for 12 h, and then 1 mL of the resulting solution was diluted in 60 mL of methanol to achieve an absorption of 0.706 at 734 nm. 1 mL of the ABTS cationic radical solution was added to the essential oil and its absorption was measured at a wavelength of 734 nm. The percentage of ABTS radical inhibition by the essential oil was calculated using the equation mentioned in the section on inhibiting free radicals DPPH [15].

2.6. Antimicrobial Activity

The antibacterial activity of the essential oil against the bacteria *Bacillus subtilis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Shigella dysenteriae*, *Enterobacter aerogenes*, and *Salmonella typhi* was investigated according to the methods of agar disk diffusion, agar well, minimum inhibitory concentration, and bactericidal concentration.

2.6.1. Disk diffusion agar

To investigate the antimicrobial effect of the essential oil by the disk diffusion agar method, first, paper disks were impregnated with 20 µL of essential oil. After culturing the microbial suspension on the surface of Mueller Hinton agar medium, the paper disks were placed on the culture medium. Incubation was carried out at a temperature of 37 °C for 24 h and the

inhibition zone around the paper disks was measured in mm [16].

2.6.2. Well diffusion agar

In this method, wells with a diameter of 6 mm at a distance of 20 mm were created by the end of a Pasteur pipette on the surface of Mueller Hinton agar medium. The bottom of the wells was closed by the culture medium agar. 20 μ L of sterilized essential oil by syringe filter (0.22 microns) was gently added to each well. For each of the pathogenic microorganisms with food origin, lawn culture was performed according to 0.5 McFarland suspension. The plates were kept at a temperature of 37 °C and after 24 h, the diameter of the inhibition zone was measured in mm by a ruler and reported [12].

2.6.3. Minimum Inhibitory and bactericidal concentrations

The methods presented by Alizadeh Behbahani and colleagues [3] were used to determine the minimum growth inhibitory concentration and the minimum bactericidal concentration of the essential oil. The dilution method in a 96-well plate was used to determine the minimum growth inhibitory concentration. For this purpose, a concentration of 512 mg per mL of essential oil was prepared and sterilized, and then sequential concentrations of it (1-512 mg/mL) were prepared. Then, 100 μ L of essential oil and 10 μ L of microbial suspension were added to each well and incubated at a temperature of 37 °C for 24 h. Then, 10 μ L of triphenyl tetrazolium chloride reagent was added to each well and the change in red or purple color was visually examined. The absence of red or purple color indicated no microbial growth and in this regard, the lowest concentration at which the bacterium did not grow was considered as the minimum inhibitory concentration of the essential oil.

The pour plate method was used to determine the minimum bactericidal concentration of the

essential oil. In summary, the contents of the wells in which no red or purple color change was observed in the method of determining the minimum inhibitory concentration were cultured on Mueller Hinton agar medium as a pour plate. Incubation was carried out under the above conditions and colony growth was examined. The first concentration that prevented colony formation was reported as the minimum bactericidal concentration of the essential oil.

2.7. Statistical analysis

All results were processed using the one-way analysis of variance method in Minitab software (version 16). The significant difference between the mean results was determined by the Tukey test at the 95% confidence level. The results were reported as mean \pm standard deviation and the tests were repeated three times.

3. Results and discussion

Phenolic compounds, as a major group of phytochemicals, are of high importance due to their antioxidant activity. The essential oil of *P. fraxinifolia* contained 38.63 mg of gallic acid per gram of total phenol and 19.20 mg of quercetin per gram of total flavonoid (Figure 1). Akhbari and colleagues (2017) reported that the methanolic extracts of the leaf and stem of the *P. fraxinifolia* tree contain 137.96 and 255.30 mg of gallic acid per gram, respectively [17]. In addition, it has been reported that the essential oil of the leaf and bark of the *P. fraxinifolia* tree contains 429.51 and 88.53 mg of gallic acid per gram of total phenol and 24.32 and 11.82 mg of quercetin per gram of total flavonoid, respectively [18]. The difference in the results of this study and other research is due to the fact that the age and diversity of the plant, geographical conditions, drying methods, and extraction methods affect the amount and type of chemical compounds of the essential oils [16, 19].

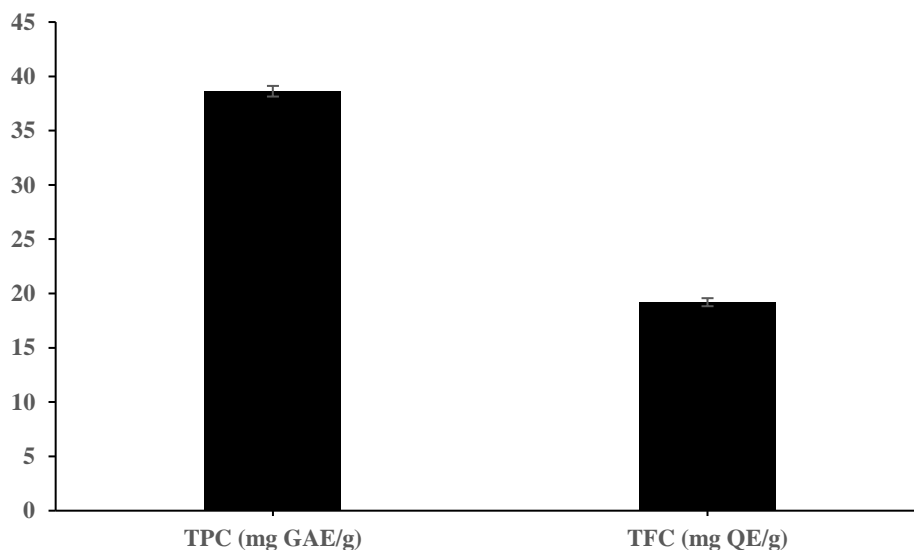


Figure 1. Total phenol content (TPC) and total flavonoid content (TFC) of *P. fraxinifolia* essential oil. GAE = Gallic acid equivalent; QE = quercetin equivalent.

The antioxidant activity of the essential oils is mainly attributed to the synergy between their compounds, and the main compounds are primarily responsible for this positive biological effect of the essential oils [16]. The antioxidant activity of the essential oil based on the inhibition of DPPH and ABTS radicals was observed to be 58.60% and 59.80%, respectively (Figure 2), indicating the strong ability of the essential oil to neutralize free radicals DPPH and ABTS through the mechanism of donating a hydrogen atom or electron [20]. Akhbari and colleagues (2017) reported that the antioxidant activity of the leaf and stem essential oils and the methanolic extracts of the leaf and stem of the *P.*

fraxinifolia tree based on the inhibition of free radicals DPPH are 43.45, 19.25, 39.80 and 15.84 mg/mL, respectively, and these values in the linoleic acid/beta-carotene inhibition test are 18.66, 14.18, 80.99 and 86.74%, respectively [17]. The antioxidant activity of the leaf and bark essential oil of the *P. fraxinifolia* tree based on the inhibition of free radical DPPH, reducing power, inhibition of nitric oxide, and iron chelation has also been reported in the study of Ebrahimzadeh and colleagues [18]. Therefore, it can be inferred that the essential oil of *P. fraxinifolia* has a strong radical inhibition feature along with the potential to end the lipid oxidation reaction. This oil can be used as a natural alternative to artificial antioxidants in food preservation technologies to improve the oxidative stability of many food products.

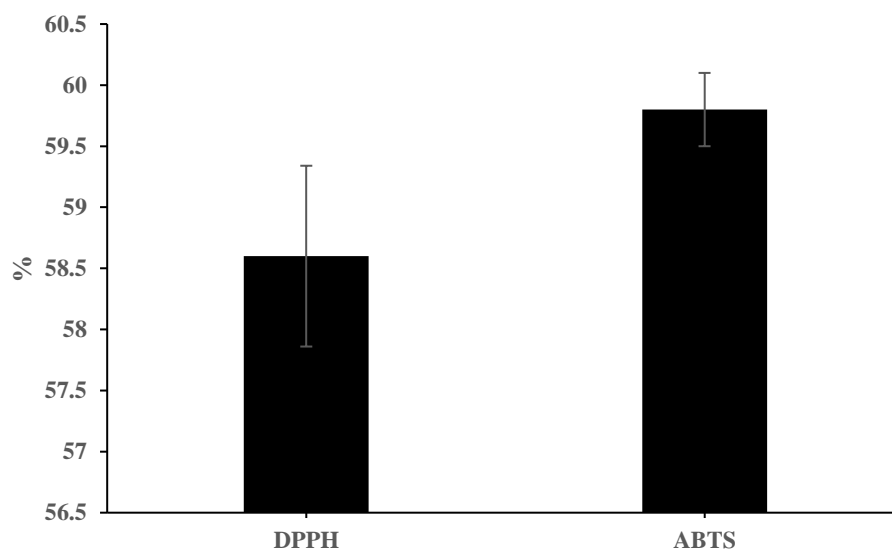


Figure 2. Antioxidant effect of *P. fraxinifolia* essential oil based on DPPH and ABTS radical scavenging methods.

The antimicrobial effect of the essential oil of *P. fraxinifolia* on the studied microorganisms was investigated and the results are presented in Figures 3 and 4 and Table 1. The effect of the essential oil was different due to the types of microorganisms. Figure 3 shows the results of the disk diffusion agar antimicrobial method. According to the results, the highest (14.30

mm) and lowest (8.70 mm) diameter of the inhibition zones were related to *B. subtilis* and *E. aerogenes*, respectively ($p < 0.05$). In this regard, *B. subtilis* and *E. aerogenes* were the most sensitive and most resistant microbial strains against the essential oil, respectively. The diameters of the inhibition zone for the bacteria *S. pyogenes*, *S. aureus*, *Sh. dysenteriae*, and *S. typhi* were 12.90, 13.80, 10.80, and 9.10 mm, respectively.

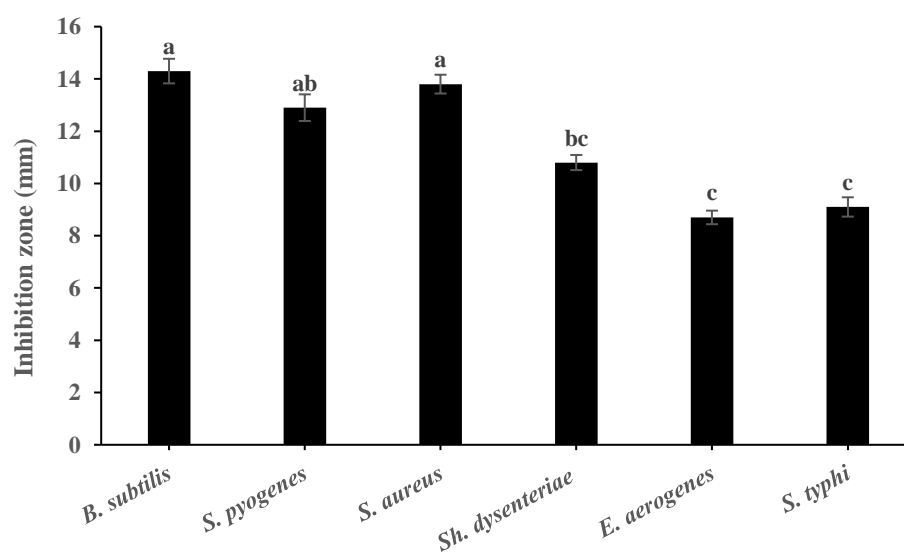


Figure 3. Antibacterial effect of *P. fraxinifolia* essential oil based on disc diffusion agar method.

Figure 4 shows the results of the antimicrobial essential oil based on the well diffusion agar method. *B. subtilis* and *E. aerogenes* were the most sensitive (with an inhibition zone diameter of 15.80 mm) and the most resistant (with an inhibition zone diameter of 9 mm) microbial strains against the essential oil, respectively. The diameter of the inhibition zones for the bacteria *S. pyogenes*, *S. aureus*, *Sh. dysenteriae*, and *S. typhi* were 13, 15.60,

11.60, and 10.60 mm, respectively. It is worth mentioning that the essential oil showed more antimicrobial activity in the well diffusion agar method compared to the disk diffusion agar method, which may be due to the direct contact of the essential oil with the microorganisms in this method. While in the disk diffusion agar antimicrobial method, the essential oil must penetrate from the surfaces of the disk into the medium to show its inhibitory effect [3, 21, 22].

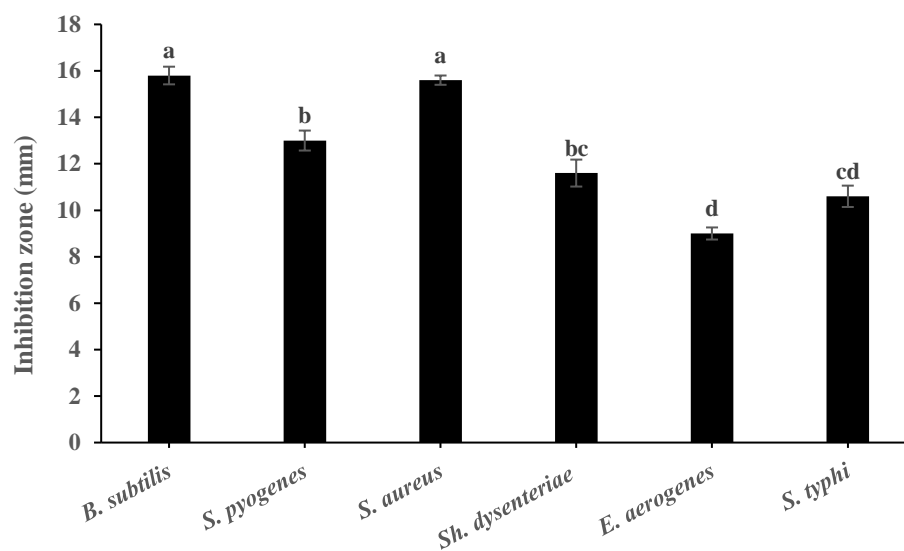


Figure 4. Antibacterial effect of *P. fraxinifolia* essential oil based on well diffusion agar method.

The results of the minimum inhibitory concentration and bactericidal concentration of the essential oil are shown in Table 1. As can be seen, the minimum concentration of the

essential oil that was able to inhibit microbial growth or kill microorganisms was lower for the bacterium *B. subtilis*, which is in line with the results of the disk diffusion and well diffusion agar methods.

Table 1. Antibacterial effect of *P. fraxinifolia* essential oil based on minimum inhibitory concentration and minimum bactericidal concentration methods.

Bacterial type	Minimum inhibitory concentration (mg/mL)	Minimum bactericidal concentration (mg/mL)
<i>B. subtilis</i>	2	64
<i>S. pyogenes</i>	4	128
<i>S. aureus</i>	2	128
<i>Sh. dysenteriae</i>	16	512

<i>E. aerogenes</i>	32	> 512
<i>S. typhi</i>	32	512

In addition, it is important to note that Gram-negative bacteria showed higher resistance to the essential oil compared to Gram-positive bacteria, probably due to the more complex cell membrane structure based on lipopolysaccharide in Gram-negative bacteria compared to the single-layer mucopeptide in Gram-positive bacteria. In this way, the lipopolysaccharide layer limits the speed of propagation of hydrophobic compounds of the essential oil in the cell membrane of Gram-negative bacteria [4, 5, 12, 19, 21, 23-33]. There are very limited studies on the antimicrobial effect of *P. fraxinifolia* tree essential oil. In the study of Akhbari and colleagues [17], the antimicrobial activity of the essential oil and methanolic extracts of *P. fraxinifolia* against 11 microorganisms was investigated and their power was evaluated both qualitatively and quantitatively in terms of the presence or absence of inhibitory zones, the diameter of the inhibitory zone, and the minimum inhibitory concentration values. The essential oil showed antimicrobial activity against *Staphylococcus epidermidis*, *Escherichia coli*, and *B. subtilis* tested. The maximum inhibitory zones and the minimum inhibitory concentration values were 22 mm and 250 µg/mL, respectively, indicating a high sensitivity for the microbial strain *S. epidermidis* to the leaf essential oil. Although the methanolic extract of the plant leaf also showed antimicrobial activity against *Aspergillus brasiliensis* and *Sh. dysenteriae* in both disk diffusion and microdilution tests, and the stem extract showed a stronger and wider spectrum of antimicrobial activity [17]. The antimicrobial effect of the essential oil is due to its phenolic compounds. These active

biological components play an inhibitory role in microbial growth through enzyme inhibition or reaction with sulfhydryl groups of proteins through non-specific states and modification of protein function [34].

4. Conclusion

The results of this study showed that the essential oil of *P. fraxinifolia* contains significant amounts of phenolic and flavonoid compounds and has a significant antioxidant effect. The results of microbial tests showed that the minimum growth inhibitory concentration for *B. subtilis* and *E. aerogenes* was 2 and 64 mg/mL, respectively, and the minimum bactericidal concentration was 32 and greater than 512 mg/mL, respectively. The largest diameter of the inhibition zone of the essential oil in the disk diffusion agar and well diffusion agar tests was related to the *B. subtilis* species and the smallest diameter was related to the *E. aerogenes* species. However, in order to benefit from the antimicrobial and antioxidant potential of *P. fraxinifolia* essential oil as a natural preservative in the food industry, it is suggested that more research be conducted on the effect of *P. fraxinifolia* essential oil in reducing microbial growth and lipid oxidation and ultimately increasing the preservation of various foodstuffs.

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ارزیابی پتانسیل آنتی‌اکسیدانی، فنل و فلاونوئید کل و فعالیت ضد میکروبی اسانس لرگ بر باکتری های

بیماری زا: مطالعه در شرایط آزمایشگاهی

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اطلاعات مقاله	چکیده
تاریخ های مقاله :	افزودن نگهدارنده های شیمیایی باعث افزایش ماندگاری محصولات غذایی می شود، اما استفاده طولانی مدت و بی رویه از نگهدارنده های شیمیایی باعث افزایش مقاومت میکروارگانیسم ها و خطرات سلامتی مرتبط با جذب آنها می شود. گیاهان دارویی هم در دنیا و هم در ایران تنوع زیادی داشته و قابلیت استفاده بعنوان جایگزین ترکیبات شیمیایی را دارا می باشند. در این مطالعه، اثر ضد میکروبی اسانس لرگ (<i>Pterocarya fraxinifolia</i>) بر علیه باسیلوس سوبتیلیس، استرپتوکوکوس پیورنز، استافیلوکوکوس اورئوس، شیگلا دیسانتری، انتروباکتر ائروژنز و سالمونلا تیغی توسط دیسک دیفیوژن آگار، چاهک آگار، حداقل غلظت مهارکنندگی و حداقل غلظت کشندگی بررسی شد. محتوای فنول کل و فلاونوئید اسانس به ترتیب با استفاده از روش های فولین سیوکالتو و کلرید آلومینیوم تعیین گردید. برای ارزیابی فعالیت آنتی اکسیدانی اسانس از دو روش مهار رادیکال های آزاد DPPH و ABTS استفاده شد. محتوای فنول و فلاونوئید کل اسانس ۳۸/۶۳ میلی گرم گالیک اسید در گرم و محتوای فلاونوئید آن ۱۹/۲۰ میلی گرم کوئرستین در گرم بود. اسانس لرگ قادر به مهار رادیکال های آزاد DPPH (۵۸/۶۰ درصد) و ABTS (۵۹/۸۰ درصد) بود. نتایج فعالیت ضد میکروبی اسانس به روش دیسک دیفیوژن آگار و چاهک آگار به خوبی نشان داد که باسیلوس سوبتیلیس و انتروباکتر ائروژنز به ترتیب حساس ترین و مقاوم ترین سویه های میکروبی نسبت به اسانس می باشند. حداقل غلظت مهارکنندگی رشد برای این باکتری ها به ترتیب ۲ و ۶۴ میلی گرم در میلی لیتر و حداقل غلظت کشندگی به ترتیب ۳۲ و بزرگ تر از ۵۱۲ میلی گرم در میلی لیتر به دست آمد.
کلمات کلیدی:	
اسانس لرگ، نگهدارنده طبیعی، ضد میکروب، آنتی اکسیدان، ترکیبات زیست فعال.	
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