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Short Communication

Consideration antimicrobial and antioxidant properties of *anbarnesa smoke* ointment

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ABSTRACT

Anbarnesa or donkey dung is one of the most effective smokes used for treatment of many infectious and viral diseases. It is also used as disinfectants. The purpose of this study was to evaluate the antimicrobial and anti-radical activity of *Anbarnesa* smoke and to provide ointment. In this research study, the donkey dung was burned by the designed machine and the smoke was collected in a diethyl ether solvent. The solvent evaporated at environment temperature and was used to test the dried specimens. To determine the MIC and MBC of the sample at the presence of bacteria was used Macro broth dilution method. The MIC and MBC of the smoke was shown on Staphylococcus aureus 1.87, 3.75 mg/mL and on Escherichia coli 3.75, 7.5 mg/mL, respectively. The average percentage of the inhibitation of free radicals in concentration of 9, 6, and 3% of smoke, was found to be 28.37, 18.86 and 8.86, respectively.

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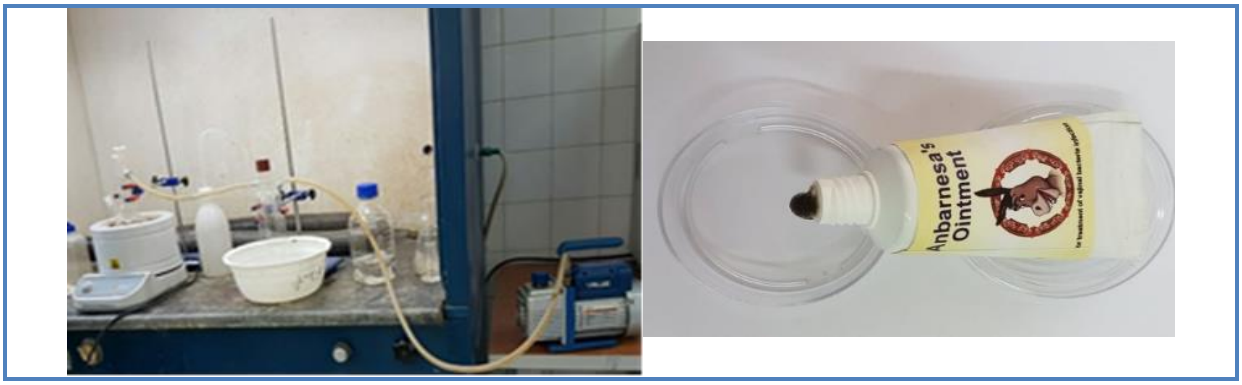
Graphical Abstract



Introduction

In recent years, the use of medicinal plants has increased due to the cost and cost less, as well as the compatibility of patients with these drugs and side effects of chemical drugs. The use of herbal and non-herbal medicines as a smoke has been found in over 50 countries all over the world. Consumption in traditional medicine and in general is relatively acceptable [1]. The routes of smoke entering the body are by inhalation and topically, which is used in medical science for treatment of pulmonary diseases, nervous system disorders, skin problems, and reproductive system. The advantages of using medicine smokes is the rapid transfer to the brain, effective absorption by the body and low cost of these materials. One of these types of smokes is the smoke from the burning of *Anbarnsara*. By hearing this name, the unconscious person will imagine a sweet and savory drug in his mind; however, Anbarnesara is a donkey dung. In many parts of Iran, especially in thecentral provinces the donkey dung and the smoke from its incomplete combustion are used to treat respiratory diseases. In fact, the use of donkey dung has been developed as an antimicrobial and antiseptic agent. On the other hand, the benefits of this material is easily available and affordable [2-3]**.** Inhaling of Anbarnesa smoke is one of the best ways to treat the chronic and acute sinusitis. It also has a great impact on the rapid restoration of various types of ulcers, especially burn wounds. Pouring the mixture of anbarnesa and vinegar on the forehead is beneficial in the treatment of Nosebleed. Smoke the Anbarnesais useful for reducing the bleeding and shortening of the women's menstruation period and treatment of vaginal infections [4]. Mainl, natural smoke of animal is used in the treatment of some viral and microbial diseases, allergic diseases, relief of colds and the treatment of some benign tumors and cysts, especially cysts in women's genitals[5]. Microorganisms play an important role in development of human diseases. By increasing the number of strains resistant to various antibiotics, the emergence of resistant strains between the gram negative bacteria and gram positive coccias such as Staphylococcus and complications such as nausea, vomiting, diarrhea, kidney damage and allergic reactions, many efforts have been made to use the potential of antimicrobial compounds such as plants [6-7]. Plants and their compounds include essential oils and plant extracts are have Potential Ability for substitution with chemical drugs While the side effects of these compounds are lower compared to chemical drugs [8]. Considering the increased antibiotic resistance due to the use of antimicrobial drugs for the prevention and treatment of infections, as well as side

effects and their harmful effects, investigating the medicinal plants is required to discover new sources of drug against bacterial infections [9]. Free radicals can damage the molecules of the biological systems of the body and cause many diseases in animals, and especially human [10]. The harmful effects of free radicals can be reduced by antioxidants, as these substances cause trapping and inhibiting the production of free radicals, so they will prevent the possible illnesses caused by their existence and activity. Nowadays, several synthetic antioxidants, such as butylatedhydroxytoluene, butylatedhydroxyanisole and some other synthetic materials made in the industry are undesirable due to carcinogenic effect. For this reason, using natural antioxidant has been considered by researchers[11-12]. The DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a lipophilic radical that has a maximum absorption of Wavelength of 517 nm. In the DDPH test, the hydroxyl groups of the antioxidant compounds by giving H to DPPH free radicals, reduce DPPH molecules which is accompanied by a change in the color of the reaction solution from dark violet to light yellow. As a result, the absorption at a wavelength of 517 nm was reduced. Absorption at a wavelength of 517 nm indicated the remaining DPPH [13]. Ointment is a semi solid product in which the drug is placed on a suitable base that has hydrophilic or hydrophobic properties and is used for external use such as betamethasone ointment [14]. Wound healing is one of the most basic issues human beings have encountered since the beginning of creation. Several medications and ointments are used to repair the open wounds, each of which has deficiencies, limitations and multiple side effects [15-17]. In traditional medicine, various herbs and natural substances are used to heal the wounds [18-20].

Material and Method

In this study, pure diethyl ether and Dimethyl sulfoxide and Methanolwas were purchased from the Merck Company. Anbarnesa (Donky Dung) was collected from the Alborz mountain chain from the north of Iran. Also a digital balance with 0.001 g accuracy (Kern Company, Germany) was used for measuring the weight of samples. As well, the magnetic stirrer (IKA، C-MAG HS7), vacuum pump, autoclave (RT. 1), incubator (BINDER), spectrophotometer (Jenway), inoculationloop, microbiological culture, sampler, pipette tip, DPPH reagent, and laboratory Glassware are employed in this research.

Extraction of smoke from burning anbarnesa

In the practical process, Anbarnesa dried powdere (30 g) burnedin a balloon and stirred at 150-200 °C. Then balloon was connected to a trap containing diethyl ether (Et_2O, 60 mL) and placed in the ice and water container and end of the trap was connected to the vacuum pump (Figure 1). The smoke from the sample burning was collected in a solvent. The solvent was evaporated in the vicinity of the air and sample dried for antimicrobial and antioxidant activity test.

Figure 1. Burning *Anbarnesara* powder.

Antimicrobial test

To evaluate the antimicrobial properties of *Anbarnesa* smoke, its MBC and MIC properties

was determined using the tube dilution method. For evaluation of the antimicrobial activity, we used two sample gram positive bacteria (Staphylococcus aureus) and gram negative bacteria (Escherichiacolias) [21-22].

The studied bacterias

The studied bacteria were Escherichia coli with No .ATCC 25922 and Staphylococcus aureus with number ATCC 9144, which was Prepared from the Institute of Scientific Researches of Iran.

Preparation of bacterial suspension

The bacterial lyophilized ampoules (Staphylococcus aureus and Escherichia coli) was first opened in sterile conditions and transferred to Trypticase Soy Broth (TSB) liquid medium, and incubated for 24 h at 37 °C. Then, to ensure that the bacteria were pure from the Trypticase Soy Broth mediumwas streaking on agar medium, and incubated for 48 h at 37 °C. To obtain the amount of bacterial inoculation from the first culture, a re-culture was conducted in the Trypticase Soy Broth medium, and incubated at 37 °C for 24 h. From fresh and 24-hour culture of bacteria, different amounts were transferred to the sterile Trypticase Soy Broth culture medium until an optical absorption of 0.1 at 600 nm wavelength was achieved. Then by transferring 1 mL of bacterial suspension into a tube containing 9 mL of peptone water water, 0.01% Sequential dilutions were prepared up to 10-7. The 100 µL from each dilution was cultured on plates containing the nutrient agar medium**.** Plates were incubated at 37 °C for 24 h and the average number of bacteria in a cuvette with optical absorption of 0.1 was calculated for staphylococcus aureus bacteria equivalent to 3.4×10^{10} and Escherichia coli equal to 4.2×10^{11}. All the culture mediumwere prepared according to the merck companyinstructions, and sterilized using an autoclave [23-24].

Preparation of concentration of extract

To prepare the extract, it required a solvent to solve the smoke without having an antimicrobial effect. So, DMSO was used as a solvent. From completely dried sample, a concentration of 15 mg/mL in a DMSO solvent was prepared for antibacterial studies.

Minimum inhibitory concentration (MIC) by macro broth dilution method

Using the dilution method in tube, minimum growth inhibitory concentration (MIC) and minimum bactericidal concentration of antimicrobial substance was determined. for investigation of the MIC properties was prepared ten sterile tubes containing tryptic SoyBroth culture medium (1 mL) that 7 tubes was used for testing different dilutions of the extract.two tubes were used a positive control (Containing diluted extract plus culture medium) and a negative control (Contains microbial suspension plus culture medium).Also, a tube containing microbial suspension and culture medium was employed to studying the growth of bacteria in an environment containing DMSO for extraction. The sample (1000 µL)was added by sampler to the first tube of each series. The initial concentration of the extract was 15 mg/mL which was obtained by adding 1 mL of extract from the first tube containing 1 mL of culture medium concentration of 7.5 mg/mL. After being mixed the sample solution and the culture medium by sampler, it was removed from the tube 1 mL and entered the second tube. In this way, dilution of 1.2 was obtained with an effective amount of 3.75 mg/mL. Then, 1 mL was transferred from the second tube to the third and dilution of 1.4 was achieved with an effective amount of 1.87 mg/mL. It was

done until the tube number 8, and from the last tube 1 milliliter was lifted and thrown out. Thus, all the tubes contained 1 mL of liquid, with the difference that a concentration gradient was created from the first tube until the tube No. 8. That sample dilution is reduced in them and each tube contains half the sample dilution in the previous tube. To All tubes except tubes 8 (to control the sterility of the sample in the final dilution) was transferred 100 µL of microbial suspensions. Diligenceof extracts for Staphylococcus aureus and Escherichia coli were performed separately. All the tubes were incubated at 37 °C for 24 h (as seen in Figure 2). After incubation time, the concentration was compared with the control sample and the lowest concentration was determined as MIC concentration.

Figure 2. MIC test steps.

Minimum bactericidal concentration (MBC)

From all the tubes which the bacteria did not grow samples are taken. In order to determine the minimum bactericidal concentration of the extract, was employed the surface method. For this purpose, 100 µL of tubes showing the non-growth of bacteria in the MIC were poured onto nutrient agar culture medium and spread on the medium through player (Figure 3). After incubation for 24, the cultured plates were controlled for microbial growth. In the tube containing the lowest concentration of the extract, the bacteria did not grow, and considered as the MBC of the extract.

Figure 3. Bacterial culture on agar medium.

Determine antioxidant activity by DPPH

To conduct the test, 3.9 mL of DPPH stokes made (0.004 g of DPPH In 100 mL methanol) was poured into the test tube. Then 0.1 mL of each extract was added to it and placed in dark for 30 min and its absorbance was read at the wavelength of v517 nm. Percentage of DPPH radical containment was calculated using the following Equation 1.

$$I(\%)= 100\times (A0 - As)/A0 \quad (1)$$

Where A0 was absorption control (contains all the reactive components without a sample) and as was the sample absorption. The results were displayed IC50 that show the amount of antioxidants that is needed for reaching the DPPH concentration to 50% of the initial value)[25].

Preparation ointment

1 g of sample with 5 mL of alcohol was completely mixed to obtain a colloidal homogeneous compound with Eucerin. 20

drops of oleic acid (emulsifier) were added to the composition to produce a completely homogeneous and stable product. 10 g of Eucerin was added to the compound and mixed up to be completely homogeneous. The solution volume with vaseline reached 50 gr and mixed again to get homogeneous (Figure 4).

Figure 4.*Anbarnesa's*ointment.

Statistical analysis of data

All the trials were repeated three times. Statistical analysis was performed using the SAS software version 9.3. Comparison of the mean of the data was analyzed using the protected least significant difference (PLSD) test at 5% probability level.

Results

In the dilution experiments in the tube, the minimum inhibitory concentration and the minimum bactericidal concentration of the extract were determined. The results revealed that the minimum concentration of the Anbarnesa smoke inhibitor by Macro Dilution Method was 3.75 mg/mL for E. coli and 1.87 for Staphylococcus aureus bacteria while the minimum bactericidal concentration on these bacteria was found to be 7.5 and 3.75, respectively (Table 1).

Table 1. Results of the MIC, MBC test.

Bacteria	MIC[a]	MBC[b]
Escherichia coli	7.5	**3.75**
Staphylococcus aureus	3.75	**1.87**

[a, b]The results for this experiment are from three replicates.

Results of antiradical activity evaluation

The results of the analysis of variance (ANOVA) test revealed a significant difference in the concentrations different of Anbarnesa, in terms of anti-radical activity at a probability level of 5%, statistically (Table 2).

Table 2. Analysis of variance of antioxidant activity effect of different concentrations of smoke from Anbarnesa

Sources of changes	Degrees of freedom	Antioxidant activity
Treatment	2	**285.54
fault	6	0.38
Coefficient of changes (percent)	-	31.32

** Indicator Significant in probability level of 5% based on the Least Significant Difference test (LSD).

Based on the comparison of the averages, the rate of changes of the activity of anti-radical was in the range between 8.86 and 28.38%, that most of this amount was dedicated to the concentration of 9%. In contrast, the smallest amount was related to a concentration of 3% (Table 3).

Table 3.Comparison of mean of the effects of different quantities of Anbarnesa smoke.

Antioxidant activity	Concentrations(percent)
8.86	3
18.86	6
28.37	9
1.24	LSD5%

Different letters in the columnIndicator Significant in probability level of 5% based on the Least Significant Difference test (PLSD)**.**

Discussion

In Macro broth dilution method, the minimum inhibitory concentration of the extract was determined. The dilution method is an accurate and highly sensitive method to find outthe antimicrobial properties and antimicrobial properties of herbal extracts [26].The results of this study demonstrated that, the Anbarnesa smoke had antimicrobial effects on the resistant microbes such as Staphylococcus aureus, which was in consistent with the study conducted by Parvin *et al*. [27-28]. In the study of Salar and Mohamadisani, MIC and MBC of smoke on Staphylococcus aureus bacteria showed a same number of 6.25. The results of the current study demonstrated a better performance compared to this study [29]. Secondary metabolites, especially plant phenols, form a large group of compounds that act as initial antioxidants [30].Among the plant's antioxidant compounds, phenolic compounds are widely distributed in many plants. The antioxidant properties of phenolic compounds are mainly due to their regenerative capacity and their chemical structure, enabling them to neutralize free radicals and formation of a complex with metal ions and shutdown of single and triple oxygen molecules. The phenolic compounds, by electron transport to free radicals, inhibit fat oxidation reactions [31]. Generally, the mechanism of phenolic compounds for antioxidant activity is to neutralize free lipid radicals and prevent the breakdown of hydrogen peroxides into free radicals [32]. Research has shown that many plant compounds can act as free radical neutralizers [33]. DPPH measurements are widely considered as valid parameters in the evaluation of the antioxidant activity of pure compounds as well as plant extracts in laboratory conditions [34].In this study, with increasing the concentration of the Anbarnesa extract, its anti-radical activity increased, which was consistent with the study of Taha Nejad *et al.*, which showed that with increasing concentrations of the extract of the Malva, its anti-radical effect enhanced [35].

Conclusions

Currently, investigation of antimicrobial activity has become an important clinical subject that is mainly due to development of the microbial resistance. In this regard, the present study demonstrated that, the antibacterial effect of smoke collected from *Anbarnesa* on the lack of growth of gram positive and negative microorganisms. Also, the study of anti-radical activity of *Anbarnesa* in concentrations of 3, 6 and 9% showed that, the Anbarnesa has the most antioxidant activity in higher doses. In addition, with the production of a suitable drug with herbal source and less pharmacological complications can be hope to treat the infections caused by Staphylococcus aureus and Escherichia coli bacteria. For this purpose, *Anbarnesa* smoke was used to prepare the ointment.

Appreciation

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Conflict of interest

We have no conflicts of interest to disclose.

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