Traditional medicine and herbal medications have gained attentions due to their valuable therapeutic potentials.[1-3] In Iranian traditional medicine, Anbarnesa smoke collected from burning female donkey’s dung obtained in spring has been employed for treating inflammatory oral ulcers (e.g., aphthous ulcers) and other inflammatory conditions such as infections of the middle and external ear with no significant side effects. In addition, it can be utilized to treat stomatitis and ear infections (otitis). In previous reports, Avicenna deployed Anbarnesa smoke for vaginal infection, decreasing the duration of the menstrual period and bleeding stanching.[3,4-10]

Anbarnesa smoke has a long history of antimicrobial and anti-infectious effects, but academic documents regarding its health-beneficial properties are lacking. For instance, Parvin et al.[11] reported that Anbarnesa smoke (in direct contact and without solvent) via at least 6 s of fumigation could create the growth inhibition zones on Pseudomonas aeruginosa and Staphylococcus aureus cultures. The antibacterial efficacy of Anbarnesa was believed to be due to the presence of some...
antibacterial agents in donkey’s dung produced through the process of fermentation and digestion of foods in the digestive system, as well as the presence of probiotics. However, further evaluations illustrated that Anbarnesa compositions did not have significant antibacterial effects against S. aureus and Escherichia coli. Sadeghi-Aliabadi et al. demonstrated that Anbarnesa smoke combined with water and n-hexane solvent had suitable cytotoxicity on Hela and KB cancerous cells. In addition, they investigated that this solution was nontoxic on L929 fibroblast cells when diluted with higher than 2 mg/mL concentrations. It was believed that the hydrolysis of dung lignin could produce different compounds with attractive antibacterial potentials. Another study assessed the antibacterial activity of Anbarnesa mouthwash 0.2% compared with chlorhexidine 0.2%, demonstrated that both of the mouth rinses had similar inhibitory growth zone, which was significantly better than in the case of examined control specimens for different bacterial species. Chlorhexidine 0.2% induced higher minimum inhibitory concentration (MIC) values than Anbarnesa smoke extracts included in mouthwash 0.2% for Streptococcus sanguis and Enterococcus faecalis species, while no significant differences were found between the two agents (or solutions or products) regarding MIC values against the other bacteria. Chlorhexidine 0.2% and mouthwash 0.2% produced with Anbarnesa smoke extracts showed higher growth inhibitory effect than control specimens against all bacteria except for E. faecalis. As a result, the prepared mouth rinse 0.2% had some suitable antibacterial potentials, but it is not as efficacious as chlorhexidine 0.2% on some selected species, with no significant effect on the E. faecalis species. Thus, Anbarnesa should be further evaluated and formulated as an alternative mouthwash with fewer side effects for plaque control/prevention of periodontal disease.

In this study, the chemical composition of Anbarnesa smoke was evaluated by gas chromatography–mass spectrometry (GC/MS), and its antiviral activity was analyzed based on 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay.

**Materials and Methods**

**Sample preparation**

Donkey droppings were collected from a farm in Flavarjan, Isfahan province, Iran, in spring 2019. They were burned, and the smock was cold trapped according to the Iranian traditional medicine method. A traditional and simple technique was used to collect Anbarnesa smoke. A copper cap was used on top of the container containing Anbarnesa sample and the steam from the Anbarnesa smoke was collected. Dark yellow oil was obtained and reserved in opaque glass for further analysis.

**Gas chromatography–mass spectrometry analysis**

GC-MS analysis was performed on a Hewlett Packard 5792A mass selective detector coupled with a Hewlett Packard 6890 gas chromatograph, equipped with a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm). The GC operating conditions were as follows: carrier gas, helium with a flow rate of 2 mL/min; column temperature, 60°C–280°C at 4°C/min; injector and detector temperatures, 280°C; volume injected, 0.1 mL of the oil; split ratio, 1:50. The MS operating parameters were as follows: ionization potential, 70 eV; ion source temperature, 250°C; resolution, 1000; ionization current 750 μA; mass range 35–425.

**Identification of Anbarnesa smoke components**

Identification of the constituents was performed based on computer matching using the library spectra (Library Database Wiley 275 L), and their retention indices with reference to an n-alkane series in a temperature-programmed run, interpreting their fragmentation pattern and comparison of the mass spectra with those reported in the literature.

**Antiviral activity**

**Cell toxicity assay**

The evaluation was based on the reduction of MTT. Accordingly, the MTT colorimetric assay was performed in 96-well plates. Hep2 (cervix adenocarcinoma) and Vero (African green monkey kidney) cells were seeded in 96-well plates at a concentration of 10³ cells per well and incubated for 24 h at 37°C in a 5% CO₂ enriched atmosphere. After treatment with various concentrations of each extract, cells were incubated for an additional 48 h at 37°C. After that medium was removed, the cells in each well were incubated with 200 mL of MTT solution (5 mg/mL) for 2 h at 37°C. Next, the MTT solution was discarded, and 200 mL insoluble formazan crystal was added. Optical density was measured at 570 nm. Data were obtained from triplicate wells. The 50% cytotoxic concentration (CC₅₀) was defined as the cytotoxic concentration of the compound by regression analysis.

**Virus samples**

Adenovirus (type 5) and herpes simplex type-1 (HSV-1) were grown on cells in DMEM medium until complete cytopathic effect (CPE). The titer viral was utilized at a final concentration of 100 TCID₅₀/mL.

**Antiviral activity assay**

A CPE reduction assay was employed to screen the obtained compounds’ antiviral activity. In brief, to confluent cell monolayers in a 96-well plate, 100 TCID₅₀ (50% tissue culture-infective dose) virus suspension and serial two-fold dilutions of the compound were added, simultaneously. The dilution mediums without samples and virus suspension were added to the cell cultures to serve as the cell and virus controls, respectively. The plates were incubated at 37°C in a humidified CO₂ atmosphere for 3 and 4 days (3 days for HSV1 and 4 days for adenovirus). The concentration that reduced 50% of CPE with respect to virus control was estimated from the plots of data plots and was defined as the 50% inhibitory concentration (IC₅₀). The selective index (SI) was calculated from the ratio CC₅₀/IC₅₀.
RESULTS AND DISCUSSION

Gas chromatography–mass spectrometry analysis

Twenty-two constituents, representing 97.1% of the Anbarnesa smoke, were identified [Table 1]. As a result, hexadecanoic acid (or palmitic acid, 29.4%) was the main constituent of the smoke. Notably, the main constituents were dominated by hexadecanoic acid (29.4%), cis-9-octadecenoic acid (17.7%), and octadecanoic acid (or stearic acid, 10.8%). In a previous study, Shafiee et al. [3] analyzed the Anbarnesa smoke using chromatography by GC mass device, and isolated its constituents as hexane, acetic acid, aconitane, beta carotene, and dimethyl amine. It was demonstrated that the compounds obtained from Anbarnesa smoke mainly have antibacterial, antifungal, anti-inflammatory, and antioxidant properties. These compounds were evaluated for treating neuralgia, rheumatism, capillary hemorrhage, and skin disorders. [3,5-10]

In our study, the antiviral effects of Anbarnesa smoke were evaluated for the first time.

Antiviral activity

Based on the results obtained from the MTT analysis, The CC_{50} value of the compounds on Hep2 and Verro cells was 2271.2 μg/mL and 5077.5 μg/mL, respectively. Furthermore, the IC_{50} value on adenovirus and HSV-1 was 802.55 μg/mL and >5077.5 μg/mL, respectively. In addition, the SI value of the compounds on adenovirus and HSV-1 was 2.82 and <1, respectively. In previous studies performed by Shafiee et al. [3], the cytotoxic effects of Anbarnesa on L929 fibroblast cell line were evaluated. The assessment of cytotoxicity was performed at 1, 24, and 72 hours. Cell viability was measured by an MTT test, and enzyme-linked immunosorbent assay reader machine was employed to read the results. The results of our study demonstrated that Anbarnesa was nontoxic in 1/64, 1/128, and 1/256 dilutions, while the toxicity was detected in 1/32 dilution after 72 h (of what?). In addition, in 1/8 and 1/16 dilutions, cell toxicity was identified in the first hour. Moreover, antibacterial, antifungal, anti-inflammatory, and antioxidant properties of Anbarnesa smoke were reported before. [4,5-11,13]

Our results illustrated that the compounds obtained from Anbarnesa smoke had no significant antiviral activity against adenovirus and HSV-1.

Conclusions

The chemical composition analysis of the Anbarnesa smoke was performed using GC/MS. Consequently, hexadecanoic acid (29.4%) could be detected as the major constituent of the smoke. The results obtained from antiviral evaluations demonstrated no significant toxicity against the viruses. Notably, the antiviral, anti-inflammatory, antibacterial, and wound healing potentials of Anbarnesa can be affected by environmental conditions and animal diets. Overall, future studies should be conducted to find the other biomedical and therapeutic potentials of Anbarnesa smoke, as well as innovative formulations from its compounds.

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Conflicts of interest

There are no conflicts of interest.

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