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GC-MS Analysis and Antimicrobial Activity of an Iranian Traditional Medicinal Smoke (Anbarnasara)

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ABSTRACT

Introduction: In many countries, people use animal dung smoke to treat infections. Ancient physicians Avicenna and Zakaria Razi (Zakariyyā Rāzī) recommended these compounds to treat infections. In rural areas of Iran, people used female donkey dung (Anbarnasara) smoke to treat respiratory tract and burn wound infections. This study evaluates the antibacterial and antifungal properties of Anbarnasara smoke. **Methods**: The smoke from burning Anbarnasara was collected in a 50%-methanol solution. Following evaporation of methanol at 50ºC, the remaining compound was dissolved in DMSO, and various concentrations (3.1-100 mg/ml) were prepared. The antimicrobial effects of various concentrations (3.1-100 mg/ml) of Anbarnasara smoke solution (ASS) were investigated, using the agar well diffusion method on 15 different microorganisms, including eight standard microorganisms and seven bacteria species from clinical specimens. Also, GC-MS analysis was performed to identify the components in ASS. **Results**: Antifungal activity on *Candida albicans* was observed at 6.2- 100 mg/ml of ASS. Among Gram-positive and Gram-negative bacteria, the most significant inhibition zones belonged to *Staphylococcus epidermidis* (30.5± 0.70 mm) and *Proteus mirabilis* (25± 0.00 mm) at 100 mg/ml. GC-MS analysis showed 16 major peak areas, and of identified components, ~50% were phenolic compounds. **Conclusion**: Our results confirmed the ancient physicians' belief in the antibacterial and antifungal properties of Anbarnasara smoke.

INTRODUCTION

For about 70 years, people have been using antibiotics to control bacterial infections. Antimicrobial agents have saved many lives. However, irregular use of these compounds has resulted in the emergence of antibiotic resistance. Today, antimicrobial resistance is one of the significant public health concerns causing many deaths [1, 2]. In many countries, alongside industrial drugs, traditional medicines such as herbal medicines and traditional smokes are used for treating chronic infections [3]. In over 50 countries, people use medicinal smokes to treat infections and other diseases [4]. In Tanzania and South African, the smoke of burning elephant dung is used to treat convulsions in children and healing headaches [5, 6]. Famous ancient physicians Avicenna, Zakaria Razi, and Aghili Khorasani have described and recommended various medicinal smokes [7-9]. Medicinal smokes have impressive and fast pharmacological effects due to inhalation of therapeutic components [4].

In Iran, among traditional smokes, using the smoke from burning the Espand (*Peganum harmala*) and female donkey dung (Anbarnasara) is very common. Anbarnasara smoke is used to treat infectious wounds, especially burn wounds, localized skin abscesses, sinusitis, and vaginal infections [10, 11]. Anbarnasara smoke is known as a strong antibacterial and anti-allergic compound among people in rural areas. Some studies have recently shown the antibacterial, wound healing, and cytotoxic effects of the Anbarnasara smoke [3]. *Candida albicans* is one of the common causes of vagina fungal infections. *Staphylococcus aureus, S. epidermidis, E. coli, Enterococcus faecalis, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter aerogenes, Micrococcus luteus, Bacillus subtilis, Proteus mirabilis, Klebsiella pneumoniae,* and *Yersinia enterocolitica* are commonly detected in infectious wounds and chronic sinusitis [12-15].

Although using Anbarnasara smoke to treat infectious diseases is not common in medical and healthcare

systems, people in rural areas commonly use this medicinal smoke to treat infectious diseases.

The present research evaluated the antibacterial and antifungal activity of Anbarnasara smoke on standard bacterial and fungal agents, and seven bacteria species originated from wound and sinusitis infections. We also identified Anbarnasara chemical constituents by gas chromatography-mass spectrometry (GC-MS) analysis.

MATERIAL AND METHODS

Strains. Standard strains including *C. albicans* (PTCC No: 5027), *S. aureus* (PTCC No: 1112), *E. coli* (PTTC No:1330), *M. luteus* (PTCC No: 1110), *B. cereus* (PTTC No: 1053), *K.pneumoniae* (PTTC No:1015), and *Listeria monocytogenes* (PTCC No: 1306) were obtained from Persian Type Culture Collection (PTCC), Iranian Research Organization for Science and Technology (IROST), and *P. aeruginosa* (ATCC No: 27853) was obtained from a commercial company (Padtanteb Co, Iran). Also, pathogenic bacteria originated from clinical specimens, including *S. aureus* (No: 4310), *S. epidermidis* (No: 964111), *Staphylococcus haemolyticus* (No: 984211), *A. baumannii* (No: 964110), *E. aerogenes* (No: 974310), *Enterococcus* sp. (No: 984210), and *P. mirabilis* (No: 96421) were obtained from Shams Laboratory in Buin Zahra, Qazvin, Iran. Table 1 reflects the resistance profile of these bacteria.

Table1. Resistance profile of bacteria agents used in this study.

Organism \ Antimicrobial agent	trimethoprim	penicillin	ampicillin	amoxicillin	polymyxin B	chloramphenicol	vancomycin	tetracycline	fosfomycin	tigecycline
S. aureus (PTCC NO 1112)	R									
Enterococcus sp. (No:984210)							R			
E. aerogenes (No: 974310										
A. baumannii (No: 964110)	R		R	R		R			R	
P. mirabilis (No: 96421)					R			R		R
S. aureus (No: 4310)	R									
S. epidermidis (No: 964111)					R					
S. haemolyticus (No: 984211)					R					

Culture media and chemical reagents. Muller Hinton Agar, Nutrient Agar, methanol 99% were obtained from QUELAB, Dimethyl sulfoxide (DMSO) was obtained from DNAbiotech life science Co.

Extraction method. Donkey dungs (Anbarnasara) were collected from Buin Zahra, Qazvin, Iran, in June 2020. Dried Anbarnasara was powdered in a flask and heated. The generated smoke was collected into another flask on ice using a plastic interface and dissolved in methanol 50% (V/V) by frequent shaking. The solvent was allowed to evaporate in the oven at 50ºC [16], the remaining compound was dissolved in DMSO, and various concentrations (3.1-100 mg/ml) of Anbarnasara smoke solution (ASS) were prepared for antibacterial assay.

Antimicrobial activity. The antimicrobial activity of ASS was evaluated using the well-diffusion method on Muller Hinton agar (MHA) plates. Overnight bacteria cultures were used to prepare the microbial suspension in sterile physiological saline, with the 0.5 McFarland turbidity standard. A sterile cotton swab was dipped into the suspension, and MHA plates surface were inoculated by streaking the swab evenly over the entire agar surface. After inoculation of the MHA plats, 6-mm diameter wells were created and filled with 50 µl of various concentrations (3.1-100 mg/ml) of the ASS. MHA plates were incubated at 37°C, and after 24 hours, the inhibition zone for each concentration was measured in millimeters [17]. All experiments were repeated three times, and DMSO was used as a control in all experiments.

Statistical analysis. The data obtained from the antimicrobial assays were analyzed with Minitab software version 16 using the Kruskal-Wallis test analysis.

Gas Chromatography-Mass Spectrometry. The Anbarnasara smoke compounds in DMSO solvent were identified using GC-MS (Agilent, GC: 6890, MSD: 5973, UAS) with HP5- MS capillary column. The oven temperature was kept at 50˚C for 5 min initially, then raised to 280˚C for 10 min. The whole run time was 42 minutes. Injector and detector temperatures were set at 250˚C. Helium was used as the carrier gas at a flow rate of 1 mL/min. The injection volume was 1 µL. The retention time of every peak area was registered and

compared with Wiley the NIST Mass Spectrometry Data Center [18].

RESULTS

Antimicrobial activity. The antibacterial effect of ASS on Gram-positive bacteria was more than Gram-negative bacteria. Table 2 shows the inhibition zone diameter for all bacteria used in this study.

Table 2. The inhibition zone diameter (mm) for bacteria following treatment with various concentrations (3.1- 100 mg/ml) of ASS.

Concentration (mg/ml) / Strains	3.1	6.2	12.5	25	50	100
C. albicans (PTCC No: 5027)	----	12.5	15.5	18	20	22.5
S.aureus (PTCC No: 1112)	----	----	11.5	16.5	22	26.5
E. coli (PTTC No:1330)	----	----	----	10	12.5	15
M. luteus (PTCC No: 1110)	----	----	11	14	17.5	22
B. cereus (PTTC No: 1053)	----	----	----	----	5	12.5
K. pneumoniae (PTTC No:1015)	----	----	----	----	9.5	15
L.monocytogenes (PTCC No: 1306)	----	----	----	----	----	10
P. aeruginosa (ATCC No: 27853)	----	----	----	11.5	13.5	15.5
S. aureus (No: 4310)	----	3	10	15	18	22.5
S. epidermidis (No: 964111)	----	----	15	21	25.5	30.5
S. haemolyticus (No: 984211)	----	----	10.5	15.5	22.5	27.5
A. baumannii (No: 964110)	----	----	3	11	15	16
E. aerogenes (No: 974310)	----	----	----	----	8	14
Vancomycin-resistance Enterococcus sp. (No:984210)	----	----	----	11	20.5	23.5
P. mirabilis (No: 96421)	-----	-----	10.5	15.5	19.5	25

In the only fungi strain, *C. albicans*, the inhibition zone was not observed at the lowest concentration (3.1 mg/ml) of ASS (Fig. 1). The size of the inhibition zone was concentration-dependent, and a significant difference between various concentrations (*P*-value= 0.005)

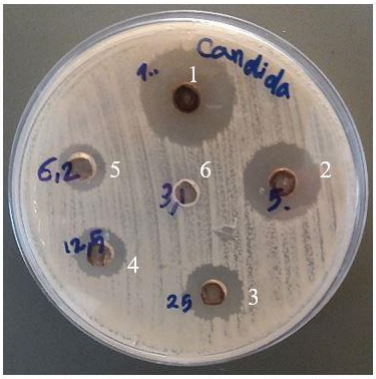

Fig. 1. The inhibition zone of various concentrations (3.1- 100 mg/ml) of ASS in *C. albicans*. 1, 100 mg/ml; 2, 50 mg/ml; 3, 25 mg/ml; 4, 12.5 mg/ml; 5, 6.2 mg/ml; 6, 3.1 mg/ml.

The inhibition zone was compared in Gram-positive and Gram-negative strains. The highest inhibition zone was observed at 100 mg/ml concentration in both Gram-positive and Gram-negative bacteria. The most significant inhibition zone at this concentration was observed in *S. epidermidis* with 30±0.7 mm. In *Staphylococcus* strains, no significant difference was observed in the highest concentration.

Antibacterial activity observed in vancomycin-resistance *Enterococcus* sp. and *M. luteus* strains showed no significant difference with most *Staphylococcus* strains (Fig. 2).

In two Gram-positive bacilli, the less inhibition zone observed was in *L. monocytogenes* with 10±0.0 mm at 100 mg/ml concentration while in *B. cereus* in addition to a more significant inhibition zone at this concentration (12.5± 3.5 mm), at the concentration of 50 mg/ml, 5 mm of inhibition zone was also observed (Fig. 3).

Chemical composition. GC-MS analysis showed 16 main peak areas (Fig. 6) with 49.38% of identified compounds belonging to phenolic compounds, i.e., Cyclohexanol, 2,3-dimethyl, Cyclohexanone, 2-(hydroxymethyl), 1,2-Benzenediol, 3-methoxy, Phenol, 2,6-dimethoxy (Syringol), Phenol, 4-methoxy-3-(methoxymethyl), 2,5-Dimethoxybenzyl alcohol, Ethanone, 1-(4-hydroxy-3-methoxyphenyl), 5-tert-Butylpyrogallol, 2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)-, (+)-s-2-Phenethanamine, 1-methyl-N-

vanilly, Phenol, 2,6-dimethoxy-4-(2-propenyl), Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl, Desaspidinol, 1-Butanone, 1-(2,4,6-trihydroxy-3-methylphenyl. Other main compounds were Cyclopropyl carbinol (11.95%), 1,2-Benzenedicarboxylic acid, diisooctyl ester (11,89%), and 3-Pyridinol (5.53%) (Table 3).

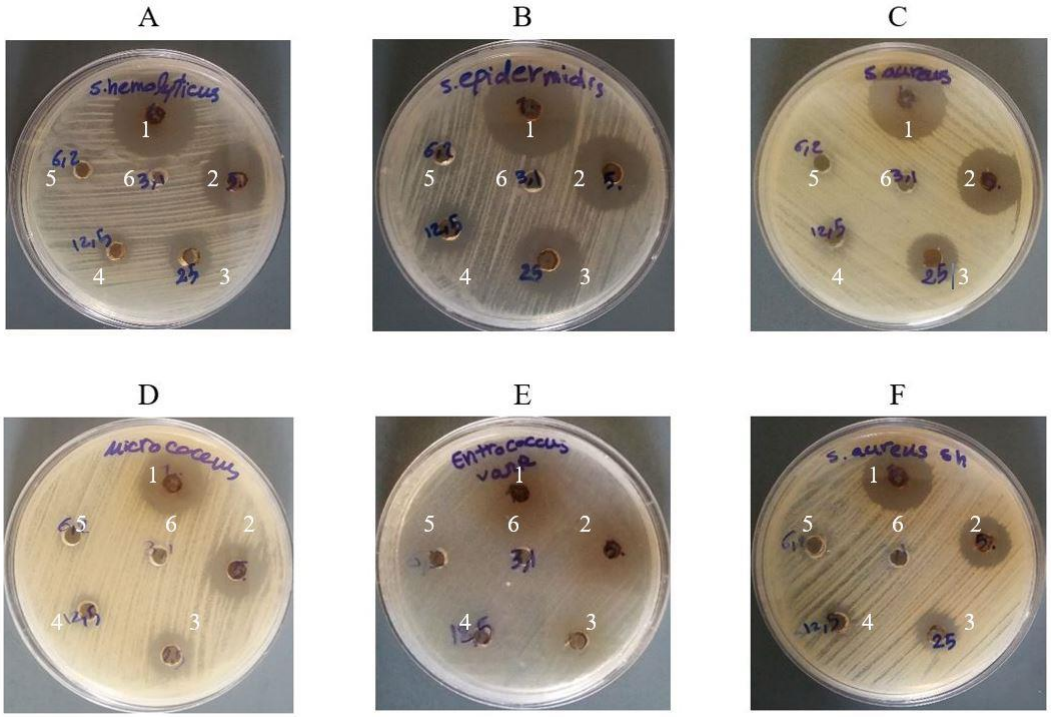

Fig. 2. The inhibition zone of the various concentrations (3.1- 100 mg/ml) of ASS in different strains. A, *S. haemolyticus*; B, *S. epidermidis*; C, *S. aureus* (standard strain), D, *M. luteus*, E, vancomycin-resistant *Enterococcus* sp.; F, *S. aureus* (clinical isolate). **1**, 100 mg/ml; **2**, 50 mg/ml; **3**, 25 mg/ml; **4**, 12.5 mg/ml; **5**, 6.2 mg/ml; **6**, 3.1 mg/ml.

Fig. 3. The inhibition zone of various concentrations (3.1- 100 mg/ml) of ASS. A, *L. monocytogenes*; B, *B. cereus.* **1**, 100 mg/ml; **2**, 50 mg/ml; **3**, 25 mg/ml; **4**, 12.5 mg/ml; **5**, 6.2 mg/ml; **6**, 3.1 mg/ml.

The highest antibacterial activity in Gram-negative bacteria was observed in *P. mirabilis* at 100 mg/ml with 25± 0.0 mm of the inhibition zone. In this strain, antibacterial activity was observed at other concentrations down to 6.2 mg/ml (Fig. 4).

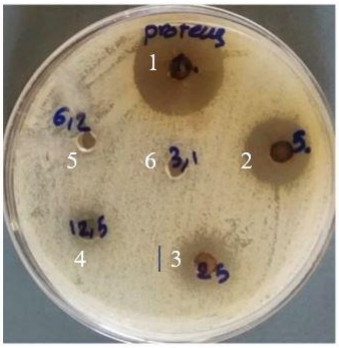

Fig. 4. The inhibition zone of various concentrations (3.1- 100 mg/ml) of ASS in *P. mirabilis*. **1**, 100 mg/ml; **2**, 50 mg/ml; **3**, 25 mg/ml; **4**, 12.5 mg/ml; **5**, 6.2 mg/ml; **6**, 3.1 mg/ml.

The lowest antibacterial activity in Gram-negative bacteria at the same concentration was in *E. aerogenes* with 14.5± 0.7 mm. There were no significant differences in other Gram-negative strains compared to the controls, *E. Coli*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, *E. aerogenes* (*P*-value = 0.161) (Fig. 5).

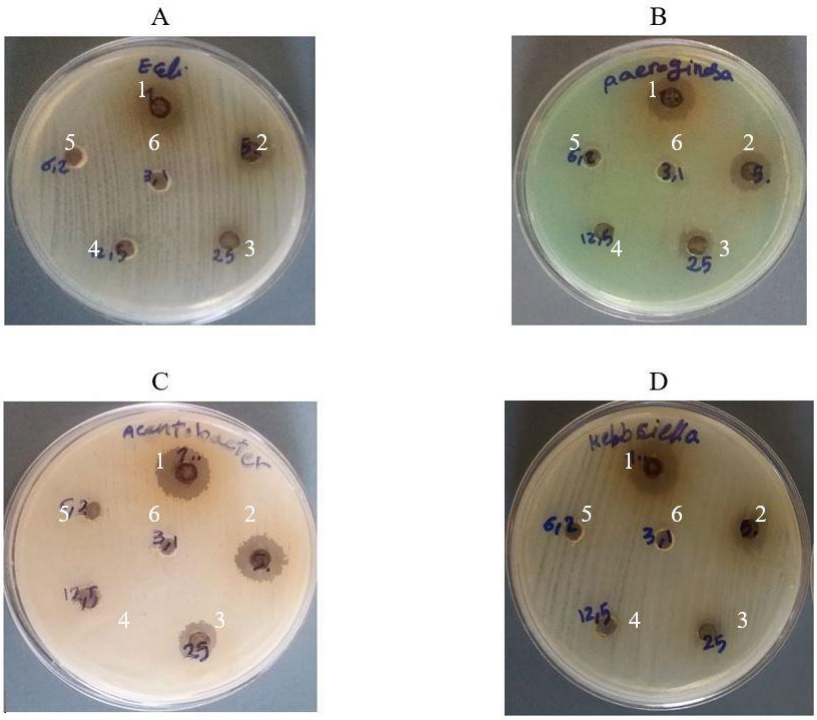

Fig. 5. The inhibition zone of various concentrations (3.1- 100 mg/ml) of ASS in Gram-negative bacteria. A: *E. coli*, B: *P. aeruginosa*, C: *A. baumannii*, D: *K. pneumoniae*. **1**, 100 mg/ml; **2**, 50 mg/ml; **3**, 25 mg/ml; **4**, 12.5 mg/ml; **5**, 6.2 mg/ml; **6**, 3.1 mg/ml.

DISCUSSION

In rural areas of Iran, Anbarnasara smoke is commonly used to treat infections. The healing effects of this traditional medicinal smoke have been reported in infected burn wounds, localized skin abscesses, sinusitis, and vaginal infections [11].

Candida albicans is one of the common causes of fungal vaginal infections [13]. Avicenna has recommended Anbarnasara smoke to treat vaginal infections [11]. In this study, our results showed the antifungal activity of ASS at low concentrations on *C. albicans*, corroborating Avicenna's recommendation on using Anbarnasara for vaginal infections.

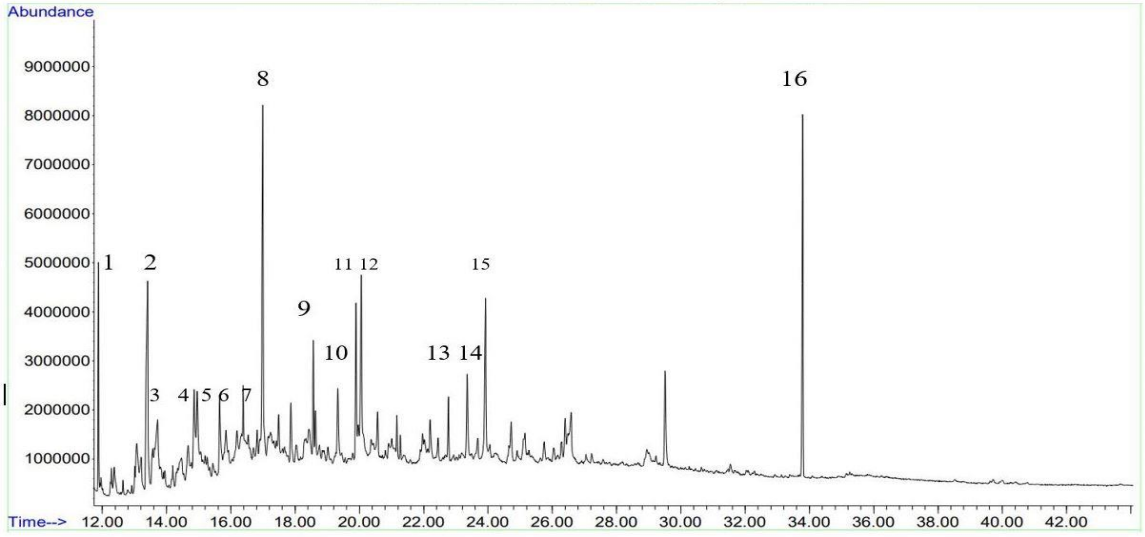

Fig. 6. GC-MS analysis of chemical constituent of the ASS.

Table 3. GC–MS peaks of identified compounds in ASS.

Peak	Retention Time (min)	Compound Name	% Total
1	11.88	Dimethyl sulfone	5.364
2	13.42	Cyclopropyl carbinol	11.590
3	13.71	3-Pyridinol	5.539
4	14.85	Cyclohexanol, 2,3-dimethyl Cyclohexanone, 2-(hydroxymethyl)	3.950
5	14.95	Toluic acid, 2-ethylcyclohexyl ester	3.452
6	15.64	1,2-Benzenediol, 3-methoxy	4.295
7	16.38	Cyclohexene, 2-ethenyl-1,3,3-trimethy	2.698
8	16.99	Phenol, 2,6-dimethoxy (Syringol)	14.914
9	18.56	Phenol, 4-methoxy-3-(methoxymethyl) 2,5-Dimethoxybenzyl alcohol	3.224
10	19.33	1,4-Dimethoxy-2,3-dimethylbenzene Ethanone, 1-(4-hydroxy-3-methoxyphenyl)	5.045
11	19.89	5-tert-Butylpyrogallol Trimethoxyamphetamine, 2,3,5	4.945
12	20.05	2-Propanone, 1-(4-hydroxy-3-methoxyphenyl) (+)-s-2-Phenethanamine, 1-methyl-N-vanilly	8.781
13	22.76	Phenol, 2,6-dimethoxy-4-(2-propenyl)	2.826
14	23.35	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)	3.922
15	23.92	Desaspidinol 1-Butanone, 1-(2,4,6-trihydroxy-3-methylphenyl)	7.560
16	33.78	1,2-Benzenedicarboxylic acid, diisooctyl ester	11.896

Previously, a study in Iran reported a 0.2% Mg/ml ASS effect on *S. sanguinis* and *E. faecalis* species. No significant difference was observed between the antibacterial effect of 0.2% ASS and the 0.2% chlorhexidine solution [10]. *Candida albicans* is the most common cause of oral candidiasis [19]. In our study, the antifungal effect of ASS was observed in all concentrations ranging from 6.2 mg/ml to 100 mg/ml, suggesting the ASS as a mouthwash. In Iranian traditional medicine, Anbarnasara smoke was used to treat the oral aphthous ulcer [10]. In the present study, in addition to the *C. albicans*, the ASS effect was evaluated on eight standard bacteria strains and seven clinical isolates often involved in sinusitis and infected wounds.

In over 50 countries, medicinal smokes are used to treat respiratory problems and wound infections [10]. In rural areas of Iran, people use Anbarnasara smoke to treat sinusitis and infected burn wounds [11]. The most common bacteria involved in sinus and wound infections are *Staphylococcus* spp., *E. coli*, *Enterococcus* spp., *Acinetobacter* spp., *P. aeruginosa*, *E. aerogenes*, *Micrococcus* spp., *Bacillus* spp., *P. mirabilis*, and *K. pneumonia* [14, 20]. In our study, the antibacterial activity of ASS on and Gram-positive strains showed to be more effective than Gram-negative strains. However, the inhibition zone at ASS 25 mg/ml in *P. aeruginosa*, *E. coli*, *Acinetobacter*, *k. pneumonia*, and *P. mirabilis* was significant. Burning Anbarnasara seems to produce microscopic scale particles that increase the absorption of bioactive components of this agent [4], which could be the reason for the effectiveness of Anbarnasara smoke on resistant bacteria such as *P. aeruginosa.*

Most of the clinical isolates used in this study were antibiotic-resistant strains (Table 1). *Proteus mirabilis*

and A. baumannii are two opportunistic bacteria contributing to chronic infectious wounds [12- 15]. In our study, low concentrations of ASS inhibited *P. mirabilis* and *A. baumannii* growth effectively.

Staphylococcus aureus and *P. aeruginosa* are the most common agents involved in burn wounds infections [12, 21], which provide the condition for colonization of yeast and fungal agents such as *Candida* spp., resulting in chronic infections [21]. Our study showed that the ASS was effective more on bacteria involved in wound infection, i.e., *S. aureus*, *C. albicans*, and *P. aeruginosa*. The few available reports on the antibacterial activity of Anbarnasara smoke used only standard bacteria [3, 4, 11], while in the present study, we used clinical isolates alongside standard strains.

Antibacterial activity of the methanolic extract and smoke of Anbarnasara on *S. aureus* and *E. coli* standard strains at 20 mg/ml revealed inhibition zones of 10 mm and 11 mm for *S. aureus* and 9 mm for *E. coli* in both methanolic extract and Anbarnasara smoke, lower than those with gentamicin, i.e., 19 mm for *S. aureus* and 17 mm for *E. coli* [3]. In our study, the inhibition zone in standard strain and clinical isolate of *S. aureus* were 16.5± 1.5 mm and 15 mm, respectively, at ASS 25 mg/ml. In the same concentration, the *E. coli* inhibition zone was 10 mm.

Our GC-MS analysis showed that most components were phenolic compounds. The prominent peak belongs to Phenol,2,6-dimethoxy (Syringol) with 14.91%, and the second prominent peak to 1,2-benzenedicarboxylic acid, diisooctyl ester (11.89%), followed by cyclopropyl carbinol (11.59%). In a similar study, a significant peak of Anbarnasara methanolic extract belonged to toluene in a mixture with tropylium ion, and the second prominent peak was dimethyl benzene (xylene). In their study, the molecular weight of isolated compounds was less than 100 atomic mass units (AMU) [3], while it was above 100 AMU in our study. The study also reported no cytotoxic activity in Anbarnasara smoke on L929 cells, i.e., normal cells, while decreased HeLa and KB cancerous cells viability [3], reflecting the safety of this compound for medical applications.

Several studies have reported antimicrobial activity, anti-inflammatory, antioxidant, and anti-carcinogenic properties of phenolic compounds [18, 22]. About 50% of identified components in our study belonged to phenolic compounds. The presence of phenols in this smoke can be one of the reasons for the antimicrobial activity of Anbarnasara smoke.

One of the significant compounds isolated in GC-MS analysis in this study was Cyclopropyl carbinol. Cyclopropyl carbinol derivatives are used to activate physiological substances and are antitumoral agents. Also, they are essential intermediate factors for enzyme inhibitors [23]. Antibacterial activity in ASS can be attributed to the presence of cyclopropyl derivates such as cyclopropyl carbinol (RT: 13.2). Some antibiotics belong to cyclopropyl derivates, such as 2-fluorocyclopropyl (fluoroquinolone derivatives) [24]. Another main component detected in our GC-MS analysis was 1,2-benzenedicarboxylic acid, diisooctyl ester (RT: 33.79). This compound has shown antifungal activity on six fungal species with MIC 10 µg/ml [25]. The amount of 1,2-benzenedicarboxylic acid, diisooctyl ester in our study was 11.89%, showing the possible role of this compound in the antifungal property of Anbarnasara smoke.

Desaspidinol was another identified compound in ASS (RT: 23.92 and 7.5%). This compound has shown an antibacterial effect on *S. aureus* and *B. subtilis* with inhibition zones diameter >10mm [26]. The antimicrobial activities and the antioxidant property of 5-tert butylpyrogallol identified in our GC- MS analysis have also been documented [27]. The antioxidant property of 1,2-benzene dicarboxylic acid and 1,2-benzenediol,3-methoxy has been confirmed [27]. Therefore, the antimicrobial activities of Anbarnasara smoke can be related to major phenolic compounds and other effective compounds.

Our analysis of the antimicrobial activity of Anbarnasara smoke solution followed by GC-MS-analysis that indicated the effective antibacterial and antifungal components corroborated the ancient physicians' belief in the therapeutic effect of this medicinal smoke

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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