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In Vitro Investigation on Antimicrobial and Antifungal Effects of Medicinal Smoke Anbar Nesara

Sedigheh Mousaei [1], Mohamad Mahdi Ejtehadi [2], Zahra Esmaeili [3], Fatemeh Mahjoub [4], Maliheh Motavasselian [5], Lida Jarahi [6], Zahra Mortezaei [7]*

1 Student Research Committee, Department of Persian Medicine, School of Persian and Complementary Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

2 Ejtehadi Medical Laboratory, Sciences of Laboratory, Mashhad University Medical Sciences, Mashhad, Iran.

3 Department of Microbiology and Virology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

4 Department of Persian medicine, School of Persian and complementary medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

5 Department of Persian Medicine, School of Persian and Complementary Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

6 Department of Community Medicine, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

7 Department of Microbiology and Virology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

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*Corresponding Authors: Zahra Mortezaei: Department of Microbiology and Virology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. *Tel*: +98-51-35240481, *E-mail:* z.yeganeh592000@gmail.com.

ABSTRACT

Background: Bacterial antibiotic resistance is increasing, and using natural alternatives is very important. Medical smoke has been prevalent in the treatment of various diseases for many years. This study investigates the antimicrobial effect of Anbar Nesara (AN) smoke on *Enterococcus faecalis*, *Streptococcus pyogenes*, *Escherichia coli*, and *Candida albicans*.

Methods: In this study, AN smoke is considered as a case, and antibiotic and antifungal groups as controls. Standard and clinical strains of *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli*, and *Streptococcus pyogenes* were prepared. The antibacterial effect of the extract was determined by diffusion in agar and micro-broth dilution.

Results: This research showed that AN smoke can effectively affect *Enterococcus faecalis*, *Escherichia coli*, and *Streptococcus pyogenes* at concentrations above 100 mg/ml and on *Candida albicans* at concentrations above 15.62 mg/ml. The most significant effects were related to the concentration above 500 mg/dL, significantly different from antibiotic discs. The most sensitive microorganism to AN smoke is *Candida albicans*, and the most resistant bacterium to it is *Escherichia coli*.

Conclusion: AN smoke has antimicrobial properties and can be considered as complementary treatment.

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Introduction

During the last two decades, due to the widespread use of antibiotics, steroids, and other immunosuppressive drugs, the frequency and severity of infectious diseases have increased significantly. The antimicrobial resistance crisis (AMR) has increased the global prevalence of infectious diseases affecting the human population that cannot be treated with known antibiotic agents. This crisis will have heavy costs on human society as debilitating and fatal diseases increase. One of the factors that caused this crisis was the increasing frequency of AMR phenotypes among microbes. This issue is an evolutionary response to humans' widespread and often unnecessary use of antibiotic drugs (1, 2).

Vaginitis is one of the most common diseases of women and is responsible for 10 million doctor's visits per year (3). *Candida vulvovaginitis* is a fungal infection caused by various species of *Candida*, mainly *Candida albicans*. *Candida vaginitis* is the second most common cause of vaginal infections in women after bacterial vaginosis. According to studies, at least 75% of women of reproductive age experience candidal vulvovaginitis once in their life. Its symptoms include itching, burning, pain during sexual intercourse, inflammation, and redness of the vulva and vagina, and it may be considered one of the most common non-fatal and extremely annoying diseases (4).

The typical treatment of *Candida vulvovaginitis* is azole compounds, associated with a gradual increase in drug resistance during treatment. In addition, during the last two decades, due to the widespread use of antibiotics, steroids, and other immune-suppressive drugs, the prevalence and severity of these infections have increased significantly. Resistance to azoles is considered one of the causes of recurrence and chronicity of vulvovaginitis (5, 6). Today, with the increasing antibiotic resistance, there is a need to discover or develop novel and effective drugs to treat these infections (7, 8).

One alternative method can be complementary medicine. Natural medicines and their replacement with new drugs are more important than before. As one of the systematic traditional medicines, Iranian medicine investigates, diagnoses, and treats many diseases and has a special place among complementary medicines worldwide. In Iranian medicine, local methods of using drugs to deliver the effective substance to the organ better have been common. One of the local forms of drug use in the past was "smoking."

In Iranian medicine, medicinal smokes are widely used for the treatment of diseases, among which the following can be mentioned: Qast smoke in the treatment of uterine mood disorders, Aklil-ul-Mulek smoke in the treatment of uterine myositis, Javashir and Sekbinj smoke in the treatment Amenorrhoea, turpentine smoke and maqal in multiple miscarriages. In more than 50 countries of the world, the use of medical smoke is common and even accepted among ordinary people (9). The smoke from burning female donkey dung (Anbar Nesara) is one of the medicinal smokes used in Iranian folk medicine. Among other things, it is used in treating chickenpox blisters, inflammatory mouth lesions such as canker sores or inflammations such as otitis media and externa, and urinary and genital infections of women and bleeding (10). Many studies have investigated the antibiotic, anti-inflammatory, wound healing, and anti-cancer effects of Anbar Nesara (AN) smoke (11-15). The method of smoke collection has been different in different studies. Plants and medicinal compounds have particular substances called effective or active substances, which have physiological effects on living organisms. These substances are made in tiny amounts during special and complex biochemical processes and are known as secondary metabolites (16). Different extraction methods can affect the active ingredients (17, 18). Various ways can also extract medicinal smoke, but so far, no study has been done to compare the different methods of extracting smoke and its effects.

According to the above, this study was conducted to compare the antibacterial and

antifungal effects of different methods of extracting Anbar Nesara (AN) smoke on standard and clinical strains of *Enterococcus faecalis*, *Escherichia coli*, *Streptococcus pyogenes*, and *Candida albicans* in a laboratory environment (in vitro).

Materials and Methods

The dung was obtained from a female donkey in Chenaran villages near Mashhad city, whose feeding was under control. In this study, standard bacteria strains, *Escherichia coli* ATCC25922, *Enterococcus faecalis* ATCC29212, *Streptococcus pyogenes* ATCC19615, and *Candida albicans* ATCC10231 were purchased from Tehran Pasteur Institute in lyophilized form, and the desired samples were cultured in nutrient broth medium. Clinical samples were also prepared from Dr. Ejtihadi Mashhad's medical diagnosis laboratory to investigate and compare the effect of dung smoke on both standard and clinical groups.

Preparation of Anbar Nesara (AN) smoke

Carbon black was prepared with three different methods described in the articles, and the researcher's innovative method is as follows:

A (Preparation of soot in the glass container: According to Parvin et al.'s instructions, the raw sterile disks were placed in sterile plates and placed inside the container, and according to the instructions, each 30 pieces of dung with an approximate weight of 7 grams were burned. It took up to 8 hours. A thin layer of soot formed on the wall of the glass chamber and was collected by a sterile method (15).

B (Preparation of soot by condensation method: According to the method of Talebi and colleagues, soot was burned, and the resulting smoke turned into a liquid in a Becher with ice placed behind it. Then, it was collected by a sterile syringe (19).

C (Collection of soot in n-hexane solution: soot was prepared according to the instructions of Sadeghi et al. with the design of a similar device (20).

D (Innovative method: disposable plates with specific weights were placed in sterile glass containers, and 100 grams of dung were burned in the container. Soot was placed on the plates, and burning continued until the weight of the soot inside the plates reached 500 mg.

Antibiotic sensitivity tests

Based on the instructions mentioned in the articles, discs were prepared in different concentrations. According to the last method (method d), 500 mg of soot was dissolved in 1 ml of DMSO, and a solution of 500 mg/ml of Anbar Nesara (AN) soot extract was obtained. Then, by successive dilution, concentrations of 250, 125, 62.5, 31.25, 15.625, and 7.81 mg/ml were prepared from Anbar Nesara (AN) soot extract.

The antimicrobial properties of different concentrations of Anbar Nesara (AN) soot extract were determined by diffusion method in agar according to CLSI standard clinical-laboratory rules. For this purpose, discs (with a diameter of 6 mm) were treated with Anbar Nesara (AN) soot extract in the concentration range of 7.81 to 500 mg/ml (20 microliters per disc) and kept in an incubator at 37 °C for 24 hours. Then, the discs were placed on Mueller Hinton culture medium and blood agar (Quelab, Canadian product), which were inoculated with bacterial suspensions according to McFarland's half (with a concentration of 1.5×10^8 cfu/ml). Also, discs of Ceftriaxone, Ciprofloxacin, Cefotaxime, Ceftazidime, Gentamicin, Cotrimoxazole, Tobramycin, Amikacin, Imipenem, Cefixime, Clindamycin, Nystatin antifungal disc and DMSO disc were used as control. Then, the plates were incubated overnight at 37 °C. Antibacterial property was obtained by measuring the halo of non-growth. This experiment was repeated more than three times.

Mueller Hinton broth medium (Merck, Germany) and a 96-well microplate in a sterile container were used in the micro broth dilution method. The desired concentrations of Anbar Nesara (AN) extract (from 3.12 to 400 mg/ml) were prepared in sterile tubes, and 100 µl were poured into each well. Ninety microliters of Mueller Hinton broth and 10 µl of bacteria were added to all wells. It should be noted that the bacterial suspensions were prepared according to the McFarland method (with a concentration of 1.5×10^8 cfu/ml) before the experiment.

In addition, DMSO was also used as a control and nutrient broth medium for positive control (Figure No. 1). At zero time, optical absorbance was measured using an ELISA reader (BioTec 800TS, made in the United States) at a wavelength of 630 nm. The microplate was placed in an incubator at 37 °C for 24 hours; after that, it was read again with an optical absorption ELISA reader. This experiment was repeated twice.

Statistical analysis

After three repetitions, the results of this study were compared using a t-test, One-way ANOVA, and the Least significant difference test (LSD) in SPSS statistical software version 27.0, and $p< 0.05$ was considered significant.

Results

In extracting soot using methods A, B, and C, the discs did not have much effect on pathogens. Only discs prepared by the fourth method were effective against bacteria. In this study, due to the two-phase mixture of Anbar Nesara (AN) extract and Nutrient Broth medium, it was impossible to measure MIC and MBC after 24 hours, and the numbers read by the ELISA reader were less than the zero time. They could not be accepted and cited (Figure No. 1).

The average halo of non-growth of Escherichia coli in the clinical group was higher in all antibiotic discs except cotrimoxazole than in the AN disc, and there was a significant difference. Compared to cotrimoxazole, the soot disk had better efficacy (Table 1).

In the comparison of the average halo of non-growth of *Escherichia coli* in the standard group, the AN disc was more effective than the discs of clindamycin, ceftriaxone, cefotaxime, and tobramycin, and this difference was significant. The other discs, cefixime, imipenem, gentamicin, clindamycin, cefotaxime, ciprofloxacin, cotrimoxazole, and amikacin, had better effects on controlling the growth of standard *Escherichia coli*, and this difference was significant (Table 2).

When the average non-growth halo of *Enterococcus faecalis* was compared in the clinical and standard groups, the AN disk was more effective than the cefixime, ceftriaxone, cefotaxime, imipenem, tobramycin, amikacin, and ciprofloxacin disks, and this difference was significant. In the rest of the cotrimoxazole antibiotic discs, gentamicin was significantly better than the AN disc (Tables 1 and 2).

The average non-growth halo of *Streptococcus pyogenes* in the clinical and standard groups in the soot disk (AN) was 16 and 17 mm, respectively, which was higher than all antibiotics, and this difference was significant (Tables 1 and 2).

Candida albicans non-growth halo in the clinical and standard group: The mean non-growth halo in the nystatin disk was higher than in the soot disk (AN), and there was a significant difference (tables no. 1 and 2).

The LSD test, used to compare non-growth halo between different pathogens in the clinical and standard groups, showed that AN disc was effective in all four groups and that there was a significant difference in all four groups. *Candida albicans* had the highest diameter of non-growth halo, and *Escherichia coli* had the lowest.

Table 1. Comparison of diameter of non-growth halo (mm) for antibiotic discs and AN disc in clinical microorganisms.

Pathogen	Antibiotic	Antibiotic discs		*AN Disc*		P_Value
		Average halo diameter	standard deviation	Average halo diameter	standard deviation	
Escherichia coli	Gentamicin	20.0000	.30000	10.0000	.20000	<0.001
	Clindamycin	30.0000	.40000			<0.001
	Imipenem	15.0000	.50000			<0.001
	Amikacin	23.0000	.10000			<0.001
	Cefixme	11.0000	.20000			<0.004
	Ciprofloxacin	22.0000	.70000			<0.001
	Ceftriaxone	15.0000	.10000			<0.001
	Cefotaxime	15.0000	.40000			<0.001
	Tobramycin	22.0000	.80000			<0.001
	Co-trimoxazole	.0000	.00000			<0.001
Entrococcus faecalis	Gentamicin	22.0000	.10000	13.0000	.60000	<0.001
	Clindamycin	30.0000	.20000			<0.001
	Imipenem	.0000	.00000			<0.001
	Amikacin	.0000	.00000			<0.001
	Cefixme	.0000	.00000			<0.001
	Ciprofloxacin	.0000	.00000			<0.001
	Ceftriaxone	.0000	.00000			<0.001
	Cefotaxime	.0000	.00000			<0.001
	Tobramycin	.0000	.00000			<0.001
	Co-trimoxazole	23.0000	.30000			<0.001
Streptococcus pyogenesa	Gentamicin	.0000	.00000	16.0000	.10000	<0.001
	Clindamycin	.0000	.00000			<0.001
	Imipenem	.0000	.00000			<0.001
	Amikacin	.0000	.00000			<0.001
	Cefixme	.0000	.00000			<0.001
	Ciprofloxacin	.0000	.00000			<0.001
	Ceftriaxone	.0000	.00000			<0.001
	Cefotaxime	.0000	.00000			<0.001
	Tobramycin	.0000	.00000			<0.001
	Co-trimoxazole	.0000	.00000			<0.001
Candida albicans	Nystatin	30.0000	.50000	20.0000	.50000	<0.001

Table 2. Comparison of diameter of non-growth halo (mm) for antibiotic discs and AN disc in standard microorganisms (.0000 in the table means: "No inhibition observed").

Pathogen	Antibiotic	Antibiotic discs		*AN Disc*		P_Value
		Average halo diameter	standard deviation	Average halo diameter	standard deviation	
Escherichia coli	Gentamicin	25.0000	.50000	12.0000	.20000	<0.001
	Clindamycin	.0000	.00000			<0.001
	Imipenem	33.0000	.20000			<0.001
	Amikacin	23.0000	.30000			<0.001

	Cefixme	24.0000	.40000			<0.001
	Ciprofloxacin	19.0000	.50000			<0.001
	Ceftriaxone	.0000	.00000			<0.001
	Cefotaxime	.0000	.00000			<0.001
	Tobramycin	.0000	.00000			<0.001
	Co-trimoxazole	31.0000	.70000			<0.001
Enterococcus faecalis	Gentamicin	20.0000	.50000	15.0000	.70000	<0.001
	Clindamycin	28.0000	1.00000			<0.001
	Imipenem	.0000	.00000			<0.001
	Amikacin	.0000	.00000			<0.001
	Cefixme	.0000	.00000			<0.001
	Ciprofloxacin	.0000	.00000			<0.001
	Ceftriaxone	.0000	.00000			<0.001
	Cefotaxime	.0000	.00000			<0.001
	Tobramycin	.0000	.00000			<0.001
	Co-trimoxazole	22.0000	.50000			<0.001
Streptococcus pyogenes	Gentamicin	.0000	.00000	17.0000	.60000	<0.001
	Clindamycin	.0000	.00000			<0.001
	Imipenem	.0000	.00000			<0.001
	Amikacin	.0000	.00000			. <0.001
	Cefixme	.0000	.00000			<0.001
	Ciprofloxacin	.0000	.00000			<0.001
	Ceftriaxone	.0000	.00000			<0.001
	Cefotaxime	.0000	.00000			<0.001
	Tobramycin	.0000	.00000			<0.001
	Co-trimoxazole	.0000	.00000			<0.001
Candida albicans	Nystatin	30.0000	.40000	20.0000	.10000	<0.001

Discussion

The results of this study showed that among the bacteria, the most resistant to Anbar Nesara (AN) extract is Escherichia coli (12 mm), and the largest diameter of the non-growth halo is related to *Streptococcus pyogenes* (17 mm). The largest diameter of the non-growth halo in all groups of bacteria and fungi (20 mm) is associated with Candida albicans yeast (Figure no. 2).

Compared to the antibiotics that were used, the effect of Anbar Nesara (AN) on *Escherichia coli* was lower than all antibiotics except cefixime. In cefixime, the diameter of the non-growth halo was 11 mm, while in Anbar Nesara (AN) extract, this diameter was reported to be 12 mm. The non-growth halo of other antibiotics on *Escherichia coli* was above 15 mm. Regarding *Enterococcus faecalis*, it should be demonstrated that only the non-growth halo of gentamicin, clindamycin, and cotrimoxazole antibiotics was in the range of 20 mm, and other antibiotics had no effect on the growth of *Enterococcus faecalis*. Also, antibiotics were not effective on the growth of *Streptococcus pyogenes*, which is remarkable compared to the non-growth halo of Anbar Nesara (AN) smoke, which was 16 mm. This research showed that Anbar Nesara (AN) smoke can be effective on *Enterococcus faecalis*, *Escherichia coli*, and *Streptococcus* at concentrations above 100 mg/ml and *Candida albicans* at concentrations above 15 mg/ml. The most effective part is related to the 500 mg/ml concentration. Also, the results of this research showed that the method of soot extraction is effective, so the greatest effect was related to the

soot extracted using the researchers' innovative method.

In their study, Parvin et al. concluded that *Staphylococcus aureus* and *Pseudomonas aeruginosa* are sensitive to dung smoke, and the diameter of the non-growth halo rises with the increase in smoking time (21). In another similar study, the effect of Anbar Nesara (AN) on the pathogens *Staphylococcus aureus* and *Pseudomonas* has been investigated by the method of sooting the disk, and it has been considered more effective on the pathogens (22). In this study, no results were obtained from the disc fumigation method; maybe the examined type of microbe required a higher concentration of Anbar Nesara (AN) extract to have an antimicrobial effect, and in this method, the concentration of the extract was not acceptable to eliminate them.

Talebi et al. also showed that the diameter of the non-growth halo of *Staphylococcus aureus* is significantly greater than that of *Bacillus cereus*. However, in this research, the substance extracted could have been more effective on the samples (19). Salari et al., who used Sadeghi et al.'s method (method C) to collect soot, found it to be effective against *Acinetobacter*, *Enterobacter*, and *Klebsiella pneumoniae* bacteria (23), which, of course, is due to the lack of common bacteria species in these studies. It is not logical to compare the results with the current research.

Akbari Zare, who used a method similar to Sadeghi et al.'s (Method C) to collect soot, has mentioned the effect of Anbar Nesara (AN) on *Escherichia coli* and *Enterococcus* (24). Also, in the study of Akbari Zare, about 6.2 ml g/ml of Anbar Nesara (AN) extract on *Candida albicans*, the diameter of the non-growth halo was reported to be 12.5 mm (22). Still, this method could have been more effective in our study. This lack of effectiveness can be due to the difference in the type of Anbar Nesara (AN) used in the research. According to the fourth method, smoke was effective on bacterial and fungal pathogens.

The results of the present study showed that, as mentioned in the articles, the smoke extraction method can affect the quantity and quality of secondary metabolites (17, 18). The active ingredients of natural compounds can be affected by genetic and physiological changes and changes (16). The difference in the results obtained in various studies can be due to the type of pathogen and its resistance, the difference in environmental conditions, the sampling season of the livestock diet, the kind of pasture fodder (25), the physiological conditions of the animal (21) and different methods of soot extraction (18).

Despite the strengths of this study, there are some limitations, including: the use of limited microbial strains, the failure to examine its effects in cells and mice, and the failure to determine its toxicity, which could affect the validity of the results of this study.

Since this substance (dung) is available and relatively economical, it is suggested that future studies investigate its effect with the drugs used in the treatment of *Candida albicans* infection and also on more pathogenic strains such as *Trichomonas* and *Gardenella*. Unfortunately, due to the unavailability of antifungal discs such as clotrimazole and fluconazole in this study, there is a possibility that the antifungal effect of Anbar Nesara (AN) amber extract is greater than that of common drugs such as clotrimazole or fluconazole; However, it should be noted that the sensitivity of *Candida albicans* to nystatin is higher than other antifungal drugs. Limited studies have investigated the active substances of Anbar Nesara (AN) (14, 26). Still, considering the effect of the soot extraction method on the active substances, more research is suggested to investigate the active substances in Anbar Nesara's (AN) smoke. In addition, since in the sources of traditional Iranian medicine, the effectiveness of the smoke itself on some women's infections is emphasized, and not the extract obtained from the smoke, it is possible that the reason for the response in the high dose was due to the form of

the drug used because the active smoke contains hot and smoky components and penetrates more into the target organ. With a lower dose, this need can be met. The use of Anbar Nesara smoke, especially for vaginal infections, has been recommended since the time of Avicenna to reduce this infection. However, future studies may focus on the effects of its components separately (23). In addition, its simultaneous use with other antimicrobial drugs and investigation of their synergistic effects can be considered in future studies.

Conclusion

As a coclusion, the greatest inhibitory effect of Anbar Nesara against *Streptococcus pyogenes* was obtained. However, despite the importance of laboratory studies in evaluating the performance of antimicrobial substances, the generalizability of the results of these studies in clinical settings requires more clinical research.

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Ethics approval and consent to participate

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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