



The Study of Antibacterial Properties of Anbarnasara Smoke on Multi-Drug Resistant Bacteria Isolated From Urinary Infection in Pregnant Women

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Published Online December 30,
2020

Keywords: Donkey dung
smoke, Antibacterial effect,
Urinary infection, MDR isolates,
Anbarnasara



Abstract

Background: Using smoke from burning donkey dung has been popular in the treatment of many diseases in Iran.

Objective: This study aimed to investigating the antimicrobial properties of donkey dung smoke on multi-drug resistant (MDR) bacteria isolated from urinary infection.

Materials and Methods: First, 300 and 200 urine samples were collected from pregnant and non-pregnant women in Isfahan, Iran. Then in each group, 100 bacterial isolates including *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Staphylococcus saprophyticus* were isolated. Antibiotic resistant protocol was determined by antibiogram test. Donkey dung was sterilized, disintegrated, and heated. The smokes were concentrated in n-hexane solvent (65%) and were collected after evaporation of the solvent. Finally, the antibacterial activities of the concentrations of 0.25, 0.5 and 1 mg/mL of the smokes were detected using disk diffusion and macrodilution methods.

Results: The most abundant MDR isolates causing urinary infections in pregnant and non-pregnant women was *Escherichia coli*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of donkey dung smoke on MDR isolates from pregnant women were 0.25 mg/mL and 0.5 mg/mL, respectively. In the case of MDR isolates in non-pregnant women, the MIC of the smoke on *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* was 0.25 mg/mL, and the MBC on these isolates was 0.5 mg/mL.

Conclusion: The smokes from donkey dung investigated in the present study have suitable potentials for controlling the infections after *In vivo* analysis.

Received June 3, 2020; Revised September 15, 2020; Accepted September 22, 2020

Background

Urinary tract infections are among the most common infections in outpatients and inpatients, which can cause significant medical complications. These infections account for huge treatment costs, and produce adverse effects not only on individuals but on society. Moreover, the resistance of bacteria against antibiotics has increased the tendency to use drugs and plant compounds in recent years. Therefore, using natural drugs has received particular attention and importance.¹ The use of medicinal plants in treatment of diseases has a long history. Using plant compounds to treat infections is a global traditional method, especially in developed countries. Today, despite the large proportion of the drugs manufactured artificially in pharmaceutical industry, it has been estimated that at least one-third of all pharmaceutical products are either

directly obtained from plants or chemically changed after extraction from the medicinal plant. In most parts of the world, plants are known as the most important medical resources of human life.²

Urinary tract naturally lacks any microorganisms. Urinary tract infections occur when a microorganism of any types invades the tissues of kidney, bladder, or urethra. The risk of infection is higher in women than men. Bladder infections are just painful; but if they reach the kidneys, they may cause serious problems.^{3,4} Urinary tract infections are considered as serious threats to the health of the community due to the lack of proper diagnosis and treatment which may lead to severe complications such as urinary tract disorders, high blood pressure, hypertension, septicemia, chronic kidney damage (particularly in children), and high risk of birth

weight loss or premature birth (5).

Infecting agents usually enter the urinary tract through blood circulation or from fistula. Sometimes, patients catch the infection from hospital environment so what infection is not present when the patient is admitted; and is established within 72 h after a bed or up to 6 weeks after the release.⁴ Urinary tract infections are generally treated arbitrarily, occupying most antibiotics consumption in the world. Physicians should be aware of the contributing factors affecting urinary tract infections and the pattern of antibiotic resistance in order for administration of proper treatment and prevention of drug resistance.^{6,7} These infections can be caused by gram-negative bacteria such as *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Citrobacter* spp., and *Pseudomonas aeruginosa*. In addition, gram-positives such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Enterococcus* spp. contribute to urinary tract infections.⁵

Although natural health promoting compounds are mainly present in various medicinal plants, some other compounds may also be found in non-plant products such as animal products.⁸ Excretory products of animals such as elephant, camel, cow, and donkey stool and urine are prime examples of these natural products.⁹ Large number of researches have been done into the smokes of these products. Some researches, for instance, have shown that the smoke from elephant dung has been traditionally used to treat headaches, toothache, sinus infections, and nose bleeding.¹⁰ Donkey dung has also been the focus of some researches, and it has been found that the dung is collected in spring for healing purposes. The donkey feeds on the best medicinal plants such as *Foeniculum vulgare*, *Coriandrum sativum*, *Descurainia Sophia*, *Cichorium intybus*, and *Matricaria chamomilla* in spring and, therefore, its smoke contains the components of different pharmaceutical plants. Moreover, no side effects on the users have been reported for this smoke. In some other studies, the components of donkey dung have been separated using chromatography method, and their antioxidant, antibacterial, and anti-inflammatory properties have been approved.¹¹ Hydrolysis of donkey dung components such as Lignin produces some anti-microbial compounds including weak acids and phenol compounds.^{12,13} This study aimed to investigate the effect of donkey dung smoke on antibiotic resistant bacteria causing urinary tract infections in pregnant and non-pregnant women.

Materials and Methods

Specimens

The clinical urine specimens were taken from 300 pregnant and 200 non-pregnant women in several medical laboratories in Isfahan, Iran during 9 months from January to August 2017. The isolated bacteria were identified by biochemical testing, and their antibiotic resistance pattern was detected by Kirby-Bauer disk

diffusion test protocol.¹⁴ Fresh donkey dung specimens (Figure 1) were collected from just parturient donkeys in villages around Isfahan in the spring, 2017 and deposited in aluminum foils in order to sterile at 121°C for 15 minutes in autoclave. Then the dung was disintegrated and 100 g of it was placed in a lab balloon and heated at 100°C on an electrical heater. A heat stable plastic tube was attached to the balloon nozzle and the other side of it was placed in the shacked cooled n-hexane solvent (65%). An air flow was entered into the balloon every 5 minutes for better smoke production. The schematic view of the apparatus is shown in Figure 2. Next, the smokes were dried by rotary (Figure 3) and the concentrations of 0.25, 0.5, and 1 mg/mL of them were prepared in dimethyl sulfoxide 99.9%.¹⁵



Figure 1. Fresh Donkey Dung Specimens Used in the Present Study.

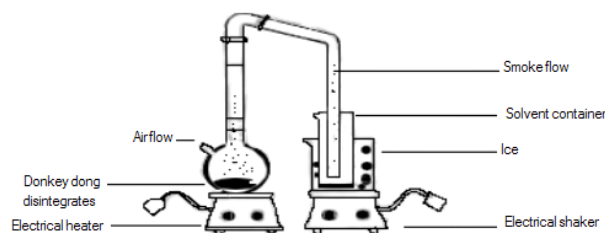


Figure 2. The View of Designed Apparatus for Donkey Dung Smoke Production and Concentration.



Figure 3. The Donkey Dung Smokes Prepared in the Present Study.

Evaluation of Antimicrobial Effects of Donkey Dung Smoke

To carry out the evaluation using disk diffusion method, first microbial suspensions equivalent to McFarland standard no.0.5 were prepared from antibiotic resistant clinical isolates and inoculated to Müller Hinton agar (MHA) plates. Then the concentrations of 0.25, 0.5 and 1 mg/mL were prepared from smokes, and 30 µL of each dilution was added to standard blank paper disks and put on a side of the bacterial culture. Disks containing 30 µL gentamicin and 30 µL dimethyl sulfoxide (DMSO) were employed as positive and negative controls, respectively. Finally, the inoculated media were incubated at 37°C for 24 hours, and the diameters of bacterial growth inhibition zones were detected. The assessments were done in triplicate.¹⁶

Macrodilution method was applied to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). To this end, first, the concentrations of 0.25, 0.5 and 1 mg/mL of smokes were prepared. Then 1 µL of each bacterial isolate suspension equivalent to McFarland standard no.0.5 in Müller Hinton broth was added to 1 ml of each concentration and incubated at 37°C for 24 h. The lowest concentration with no growth turbidity was considered as MIC. The MBC was determined by transferring limited growth media to solid medium (MHA). The concentration with no growth in MHA was considered as MBC.¹⁷

Statistical Analysis

Data were analyzed by independent *t* test and the analysis of variance (ANOVA).

Results

Isolates

One hundred multi-drug resistant (MDR) bacteria were isolated from urine specimens of pregnant and non-pregnant women. Results obtained from the abundance of clinical specimens are presented in Table 1.

Antibacterial Effects

The results from growth inhibition zones by 3 concentrations of donkey dung smoke on MDR isolates from urine specimens of pregnant and non-pregnant

women in disk diffusion test are shown in Table 2. The maximum diameter of growth inhibition zone on the MDR bacterial isolates was associated with the positive control (gentamicin), which had significant difference with other groups (*P*<0.001). The second most significant inhibition on the growth of MDR isolates belonged to the concentration of 1 mg/mL, and the lowest effect was associated with the concentration of 0.25 mg/mL of donkey dung smoke. The important point was that – just about *Pseudomonas aeruginosa*, the maximum effect of donkey dung smoke was observed by the concentration of 0.5 mg/mL with significant difference with other concentrations.

The results from macrodilution test for detection of MICs and MBCs are shown in Table 3. The MIC of donkey dung smoke on all MDR isolates from pregnant women urine specimens was 0.25 mg/mL, and the MBC on these isolates was 0.5 mg/mL. The MIC of donkey dung smoke on the MDR isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* from non-pregnant women urine specimens was shown by the concentration of 0.25 mg/mL, while the MBC on the mentioned isolates was 0.5 mg/mL. In the case of *Klebsiella pneumoniae* and *Proteus vulgaris* isolates, these concentrations were 0.5 and 1 mg/mL, respectively.

Discussion

In the present study, multiple drug resistant bacteria were isolated with different abundance from the urine specimens of pregnant and non-pregnant women, and

Table 1. The Abundance of MDR Isolates Responsible for the Urinary Tract Infections in Pregnant and Non-Pregnant Women Referred to Several Medical Laboratories in Isfahan, Iran

Isolate	Non-pregnant Women	Pregnant Women
<i>Escherichia coli</i>	55 (55%)	65 (65%)
<i>Klebsiella pneumoniae</i>	21 (21%)	26 (26%)
<i>Proteus vulgaris</i>	11 (11%)	5 (5%)
<i>Staphylococcus epidermidis</i>	7 (7%)	0 (0%)
<i>Staphylococcus aureus</i>	3 (3%)	2 (2%)
<i>Pseudomonas aeruginosa</i>	0 (0%)	2 (2%)
<i>Staphylococcus saprophyticus</i>	3 (3%)	0 (0%)

Table 2. The Mean and Standard Deviations of Growth Inhibition Zones by Donkey Dung Smoke on 6 MDR Isolates From Pregnant Women

Isolate	Growth inhibition zone (mm) by different concentrations				
	0.25 (mg/mL)	0.5 (mg/mL)	1 (mg/mL)	Positive Control (Gentamicin)	Negative control (DMSO)
<i>Escherichia coli</i>	7.58±1.70	11.73±2.00	13.22±1.71	20.35±1.49	0.00±0.00
<i>Klebsiella pneumoniae</i>	7.20±1.13	12.11±2.05	13.39±1.50	20.44±1.20	0.00±0.00
<i>Proteus vulgaris</i>	7.18±1.02	10.96±1.57	11.90±1.04	21.15±1.52	0.00±0.00
<i>Staphylococcus aureus</i>	7.00±1.73	12.33±1.52	12.66±0.57	22.00±2.00	0.00±0.00
<i>Pseudomonas aeruginosa</i>	7.57±0.97	12.12±1.51	12.33±1.81	21.16±1.13	0.00±0.00
<i>Staphylococcus saprophyticus</i>	7.10±1.01	11.33±1.52	12.00±1.00	21.33±0.57	0.00±0.00

Table 3. The Mean and Standard Deviations of Growth Inhibition Zones by Donkey Dung Smoke on 5 MDR Isolates From Non-Pregnant Women

Isolate	Growth inhibition zone (mm) by different concentrations				
	0.25 (mg/mL)	0.5 (mg/mL)	1 (mg/mL)	Positive Control (Gentamicin)	Negative control (DMSO)
<i>Escherichia coli</i>	7.39±2.06	12.55±1.91	13.26±1.54	20.30±1.35	0.00±0.00
<i>Klebsiella pneumoniae</i>	6.23±1.06	11.02±2.31	11.38±1.47	11.66±0.57	0.00±0.00
<i>Proteus vulgaris</i>	6.60±0.54	10.20±0.83	12.00±0.70	20.86±1.19	0.00±0.00
<i>Staphylococcus aureus</i>	10.50±0.70	14.50±0.70	13.00±1.41	21.50±0.70	0.00±0.00
<i>Pseudomonas aeruginosa</i>	6.50±0.70	11.50±0.70	13.30±0.42	23.50±0.70	0.00±0.00
<i>Staphylococcus saprophyticus</i>	7.39±2.06	12.55±1.91	13.26±1.54	20.30±1.35	0.00±0.00

were forwarded to Isfahan (Iran) medical laboratories. The most abundant MDR isolates in pregnant women was *Escherichia coli* (55%); the least abundant infection was caused by *Staphylococcus aureus* and *Staphylococcus saprophyticus* (30%). The most abundant MDR infections in non-pregnant women were associated with *Escherichia coli* (65%), and the least abundant was related to *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In two studies conducted by Zeeman et al on the prevalence of the bacterial urinary tract infections in more than 70 000 pregnant women¹⁸ and Salvatore et al on the relationship between prevalence of the urinary tract infections and pregnancy,¹⁹ the most abundant isolates were detected to be *Escherichia coli*. Their study results were in agreement with our findings in this study.

Donkey dung smoke with the concentration of 1 mg/mL showed the most significant antibacterial effect on *Pseudomonas aeruginosa* isolates from non-pregnant women with 14.50 mm growth inhibition zone. This effect was seen on *Klebsiella pneumoniae* isolates from pregnant women with 13.39 mm growth inhibition zone ($P < 0.001$). In macrodilution test, the concentration of 0.25-0.5 mg/mL of donkey dung smoke showed the most growth inhibition and bactericidal effects on MDR isolates from pregnant and non-pregnant women. Khadri et al detected the inhibitory effect of *Cymbopogon schoenanthus* L. Spreng essential oil on bacteria causing urinary infections, and showed that the essential oil had greatest effects on *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*.²⁰ The results of that study were consistent with the findings in our study in the case of *Escherichia coli* and *Staphylococcus aureus*. The results could be partly attributed to the effective compounds of *Cymbopogon schoenanthus* and donkey dung, both of which have eugenol (2-methoxy-4-allyl phenol) acetate and acetic acid. Donkey dung also contains hexane, beta-carotene and dimethylamine which have anti-bacterial properties. In another study by Talebi et al,²¹ the effect of donkey dung smoke on a number of gram-positive bacteria was investigated. The mean diameter of growth inhibition zone on *Staphylococcus aureus* was significantly higher than the mean diameter produced by vancomycin.²¹ Similarly, in the present study donkey dung smoke had significant effect on the *Staphylococcus*

aureus isolates from urine specimens of pregnant and non-pregnant women.

Biological smokes of plant tissues and animal wastes have been studied for controlling different infectious bacteria. In a study by Dehkordi et al to evaluate the effects of Guajol® ointment synthesized from smoke condensate of jennet feces, it was concluded that the treatment of wounds by 5% Guajol® ointment resulted in more collagen fibers and fibroblast cells formation on 7th day, as well as in the recovery of epithelial and epidermal tissues and hair follicles on 21st day, which could supplement the results of this study for treatment of genital wounds by removing urinary bacteria causing secondary infections from the given wounds.²² Najafi and Fazel Jafari reported that Espand seeds smoking for 8 days was only effective on Gram-positive bacteria, but had no effect on Gram-negative bacteria.²³ Shahverdi et al studied the antimicrobial and cytotoxic effects of Espand seeds smoke on *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Escherichia coli*, *Staphylococcus aureus*, and *Aspergillus niger*. They found that the smoke was effective on all tested microorganisms, and the differences in the degrees of effectiveness might have been due to the nature of the investigated microbes resulting in the differences in anti-microbial material permeability into them. They further suggested that this smoke could be included in the category of strong antibiotics like gentamicin.²⁴ In another study conducted by Parvin et al on the impact of medicinal smokes of Espand seeds and donkey dung on some hospital acquired pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, it was revealed that by increasing the smoking time duration for sensitive bacteria, the anti-bacterial effects of smokes were increased based on the diameter of growth inhibition zones.¹² The findings from the present study also revealed that the donkey dung smoke had satisfactory anti-bacterial properties against antibiotics resistant bacteria isolated from the pregnant and non-pregnant women and, therefore, may be used as a traditional/industrial treatment after *in vivo* evaluation.

Conclusion

Urinary tract infections are among the most common infections in outpatients and inpatients, which can cause

significant medical complications. These infections account for huge treatment costs, and produce adverse effects not only on individuals but on society. Also, the resistance of bacteria against antibiotics has increased the tendency to use drugs and plant compounds in recent years. Therefore, using natural drugs has received particular attention and importance. In this study, it was concluded that the donkey dung smoke had satisfactory anti-bacterial properties against antibiotics resistant bacteria isolated from pregnant and non-pregnant women, and could be used as a traditional/industrial treatment after *in vivo* evaluation. Therefore, the smokes from donkey dung examined in the present study have suitable potentials for controlling the infections after *in vivo* analysis.

Authors' Contributions

All authors participated in conducting the project and approval of the final manuscript.

Ethical Approval

The study was approved by the ethics committees of the Islamic Azad University of Shahrekord branch, Iran, IR.IAU.SHK.REC.1397.046

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

Financial Support

This study was extracted from MSc thesis conducted in Falavarjan Branch, Islamic Azad University, Isfahan, Iran.

Acknowledgement

This study was extracted from MSc thesis conducted in Falavarjan Branch, Islamic Azad University, Isfahan, Iran. Authors express their thanks to the laboratory personnel of medical laboratories in Isfahan, Iran, for collecting the urine specimens from the patients.

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