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Nephroprotective effects of some essential oils against anobacteria-induced infection in mice

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ABSTRACT

The purpose of this study was to evaluate the nephroprotective effects of nine essential oils against nanobacteria-isolated from human kidney stones in Swiss mice. We found evidenceof their inflammatory infiltration and accumulation in the renal tubules indicating that the kidney is a preferred site for mineralization by nanobacteria. Among all tested essential oils, dill, almond, cinnamon, sesame and olive were found to possess highly nephro-protective effect against nanobacteria-induced infection in mice. They prevented the nanobacterial-nephro-toxicity as evidenced by a significantly reduced ($p \leq 0.01$) level of serum urea and creatinine. Moreover, the nephroprotective effects of the prior oils were confirmed by a reduced intensity of renal cellular damage, as evidenced by histological findings. Although, rocket, mint, clove and lettuce oils had no renal protective effect as the biochemical and pathological findings were significantly altered. The oils-treated sub-cultures from kidneys were negative for nanobacterial growth from dill, almond, cinnamon, sesame and olive treatments while the growth were positive for the rest of the tested oils.

In conclusion, nanobacteria may be involved in the pathogenesis of nephrolithiasis and dill, almond, cinnamon, sesame and olive oils have a protective role against nanobacteria-induced nephrotoxicity in mice.

KEYWORDS

Nanobacteria;
Essential oils;
Kidneys;
Urolithiasis.

INTRODUCTION

Urolithiasis is identified to be an affliction to mankind from early eras and remains a main concern regarding health and well being today. Stone disease influences 10 to 12% of the population in developed countries with a peak incidence between 20 and 40 years of age[1].

There are many theories that explain the pathogenesis of stone formation, for example, the supersaturation theory and the inhibitors theory. Epidemiological studies reveal that about 80% of all kidney stones are composed of calcium salts (75% calcium oxalate), while about 5% are pure uric acid[2]. Nanobacteria are carbonate apatite forming, cytotoxic bacteria recently discovered in human and bovine blood and were pub-

lished as an infectious cause for pathological calcification[3]. Ten years ago, the claim that nanobacteria promote the nucleation of kidney stones provoked much controversy and in polycystic kidney disease[4].

In our previous study we have recently isolated nanobacteria from Egyptian patients with urolithiasis[5]. Cuerpo et al[6] showed that when these bacteria were injected intravenously, they accumulate in kidney and produced apatite. These bacteria have been shown to accumulate in the kidney[7]; however, studies on *in vivo* effects of these bacteria on the kidneys are lacking. In this study we investigated the effects of intra-peritoneal inoculation of nanobacteria on kidneys of Swiss mice. Furthermore, we determined the potential protective effects of nine essential oils against nanobacteria-induced nephrotoxicity in mice by evaluating the levels of serum urea and creatinine (indicators of renal function) and by histopathological changes in kidney tissues.

MATERIALS AND METHODS

Animals

Seven-week-old male Swiss Albino mice (*Mus musculus*) weighing 15-30 g were obtained from the Center of Laboratory Animal Research, Giza, Egypt. All mice were kept in a specific pathogen-free animal room under the controlled condition of temperature (23°C) and lighting (12 h dark-light cycle) and were provided with standard laboratory diet and tap water. Mouse experiments were conducted under ethics approval from Cairo University. Animal Ethics Committee in accordance with the guidelines of the Council on Animal Care.

The animals were allowed to acclimate to the environment for 1 week before the experiment.

Plant material

Plant target species are summarized in TABLE 1. The plant materials were identified at the Herbarium of the department of Botany, Faculty of Science, Cairo University. A voucher sample was deposited in the department of Pharmacology, Faculty of Veterinary Medicine, Cairo University, Egypt.

Extraction of essential oils

Essential oils were obtained by Clevenger hydrodistillation method. The dried plant material (200 g), was cut into small pieces, and placed in a flask (4 l) together with doubly distilled water (1.5 l). The mixture was boiled for 3 h; collected essential oils were dried with anhydrous sodium sulphate and kept at –18 °C until its use.

TABLE 1: List of tested essential oils and their species

Plant species	Family	Common name	Plant part
1. Lactuca sativa	Asteraceae	Lettuce	Leaves
2. Eruca sativa	Brassicaceae	Rocket (Salad)	Leaves
3. Sesamum indicum	Pedaliaceae	Sesame	Seeds
4. Cinnamomum zeylanicum	Lauraceae	Cinnamon	Bark
5. Prunus dulcis	Rosaceae	Almond	Drupe
6. Anethum graveolens	Apiaceae	Dill	Leaves
7. Olea europaea	Oleaceae	Olive	Fruits
8. Mentha longifolia	Lamiaceae	Mint	Leaves
9. Syzygium aromaticum	Myrtaceae	Clove	Buds

These oils were dissolved in tween 80 1:2 (v/v) to give stock solutions after which they were mixed for total solubility at 1800 rpm for 10 minutes.

Nanobacterial culture

Urinary tract stones were collected from male and female patients hospitalized in the *Kasr El Aini*, Cairo University, Egypt. Stones were demineralized in 1M HCl and then neutralized[8], centrifuged at 14,000 X *g* for 15 min, and the pellets used for immuno-fluorescence staining (IIFS) and transmission electronic microscopy (TEM). Part of the pellets were suspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK), sterile- filtered, and supplemented with gamma fetal bovine serum (γ-FBS) (Sera-Lab, Crawley Down, Sussex, UK) under nanobacterial culture conditions.

The nanobacterial cultures were produced according to the method of Ciftcioglu et al.[9]. Subcultures were carried out in serum free RPMI-1640 (Gibco, Paisley, UK) after 4 weeks of initial inoculation and kept under tissue culture conditions (37°C, 5% CO2, and 95% air). The cultures were harvested by centrifugation at 20,000 g for 30 min at 4°C, washed with phosphate buffered saline (PBS, pH 7.2), and freshly used for experimental work.

The cultures were prepared using strict aseptic techniques in a cell culture facility. Nanobacterial samples

were filtered through 0.2-mm filters before culturing. Subcultures were made using γ-FBS as a culture supplement. FBS and nanobacteria were γ-irradiated, when indicated; at a minimum dose of 30 kGy given at room temperature during about 16 h. Subculturing of nanobacteria in serum-free DMEM was performed with monthly passages. Serum-free nanobacteria attach firmly to the bottom of the culture vessel. These cultures were passaged or harvested with a rubber scraper. Cultures were established on Loeffler medium supplemented with 10% conditioned medium from nanobacterial culture, and DMEM replaced water in the formula[10]. The incubation period was 6 weeks under cell culture conditions. Only pure nanobacterial cultures were used. Control experiments were performed to determine whether spontaneous crystallization could occur in a culture medium. The samples were viewed under Light microscopy with differential interference contrast (DIC) optics. The presence of nanoparticles in stones was confirmed by morphological evidence with scanning electron microscopy (SEM) and transmission electronic microscopy (TEM) of inoculated 3T6 cell monolayers.

Experimental design

Mice were divided into eleven groups of 10 mice each were treated by oral gavage:

First group were kept as negative control without nanobacteria inoculation and administered normal saline in tween 80 dissolved in 1:2 (v/v).

2nd group were kept as control positive inoculated by intra-peritoneallly (I/P) with nanobacteria (0.19 mg of bacterial protein/0.4 ml in DMEM) by 2 doses with 2 weeks interval.

Animals from groups III to XI were administrated orally with 0.1 ml oils of rocket, dill, almond, cinnamon, sesame, olive, mint, clove and lettuce oils, respectively, daily for six weeks. All animals were inoculated after one week from the onset of treatment with nanobacteria (0.19 mg of bacterial protein/0.4 ml in DMEM) by 2 doses with 2 weeks interval.

Blood samples

Three weeks after the 2nd bacterial dose, blood samples were collected from the orbital plexus of all mice. The collected blood samples were separated at 2500 rpm for 15 minutes after been completely become clotted. Serum samples were separated and used for determination of urea and creatinine measured using commercial available diagnostic kits (Alkan Company, Cairo, Egypt). After collection of the blood samples, all mice were sacrificed and the kidneys were removed and processed for re-isolation of nanobacteria and histopathological examination. Animals were handled according to the local rules and regulation of Experimental Animals, Cairo University.

Re-isolation of nanobacteria from kidneys of experimentally infected mice

Under sterile condition pieces of kidney from all groups were taken and grinding well then filtered by a 0.22μm filter and was prepared subculture in DMEM and the growth of nanobacteria from positive growth control wells was confirmed by Loeffler media. In an attempt to quantify indirectly the influences of the essential oils on the slowly multiplying nanobacteria, an absorbance of 15 mAbs for the positive growth control on day 4 was used as the reference point for establishing growth or no growth for each test compound. The growth was monitored weekly by measuring the absorbance at 650 nm for one month for all groups.

Histo-pathological examinations

Kidney tissue specimens were collected from all groups immediately after sacrificing of mice and fixed in 10% formol saline. Paraffin sections of 5μ thickness were prepared, stained by Hematoxylene and Eosin (H & E) and examined microscopically.

Statistical analysis

The experimental results obtained are expressed as the mean ± standard deviation (SD). The data was subjected to one way analysis of variance (ANOVA) followed by student t-test using the statistical analysis software (SPSS) Ver. 15, under windows XP. Statistical significance was considered at P values as $p \leq 0.05$ and $p \leq 0.01$.

RESULTS

In all experimental groups, no mortality was observed during the period of the study. In our study we investigated the effects of intra-peritoneal administra-

tion of nanobacteria on kidney in Swiss mice. The result of TEM of inoculated 3T6 cell monolayers has shown intracytoplasmic vacuolar formations containing 200 to 300 nm nanoparticles in cell cultures (Figure 1). Culture of nanobacteria on solid media take about 6 to 8 weeks indicating low rate of multiplication and appear at the bottom of the plate as pin point stony like appearance which due to its ability for mineral precipitation or biomineralization which lead to renal stone formation, while under the microscope appear yellowish or grayish whit stony like shape (Figure 2).

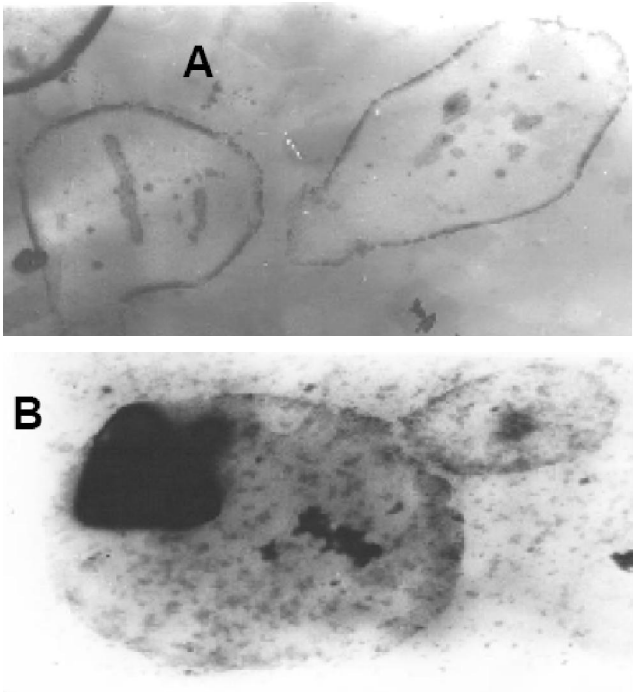

Figure 1 : TEM analysis of renal stone-inoculated 3T6 monolayers. (A) Free 3T6 cells. (B) Nanoparticles inside vesicles in 3T6 cells

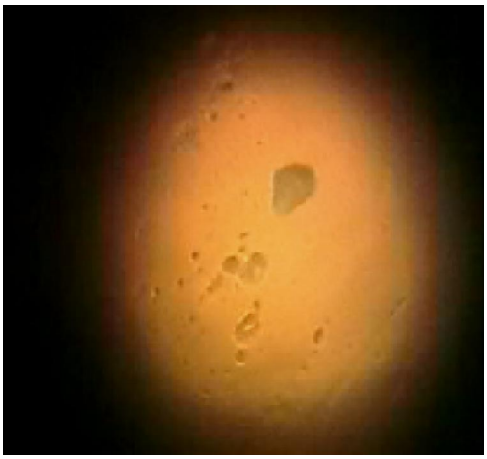

Figure 2 : Culture of nanobacteria on Loeffler media showing stony like appearance at the bottom of the plate

A significantly increased ($p \leq 0.01$) level of serum urea and creatinine was observed in mice groups received dill, almond, cinnamon, sesame and olive oils. Although, rocket, mint, clove and lettuce oils had no renal protective effect as the biochemical findings were significantly altered (TABLE 2).

TABLE 2 : The effect of tested oils on kidney function parameters in nanobacteria- induced infection in mice (n=10)

Group Number	Treatment	Urea mg/dl	Creatinine mg/dl
I	Normal (control negative)	44.50 ±2.45	0.85 ± 0.08
II	Nanobacteria-Infected (control positive)	72 ± 3.43*	2.55 ± 0.20*
III	Nanobacteria-Infected Rocket (Salad)	69 ± 2.16	2.81 ± 0.18
IV	Nanobacteria-Infected Dill	47.5 ± 1.71†	0.92 ± 0.13†
V	Nanobacteria-Infected Almond	45 ± 1.42†	0.95 ± 0.11†
VI	Nanobacteria-Infected Cinnamon	43 ± 1.65†	0.88 ± 0.09†
VII	Nanobacteria-Infected Sesame oil	44 ± 1.50†	0.90 ± 0.11†
VIII	Nanobacteria-Infected Olive	42.5 ± 1.85†	0.97 ± 0.14†
IX	Nanobacteria-Infected Mint	66 ± 2.20	1.90 ± 0.34
X	Nanobacteria-Infected Clove	63 ± 2.66	2.05 ± 0.21
XI	Nanobacteria-Infected Lettuce	65 ± 2.70	2.45 ± 0.33

The histopathological features of the kidneys from various treatments groups are as presented in Figure 3. Renal sections from the non infected control and groups of dill, almond, cinnamon, sesame and olive treatments showed no histological changes, as evidenced by the normal appearance of glomeruli and tubules (Figures 3A and 3C). In contrast, kidney sections from the infected control and groups of rocket, mint, clove and lettuce oils treatments exhibited altered renal architecture with extensive focal interstitial nephritis, atrophy of glomerular tuft and distention of Bowman's space and tubular structures (Figures 3B and 3D). These histopathological observations were in line with biochemical findings and all these results support each other.

The oils-treated sub-cultures from kidneys were negative for nanobacterial growth from dill, almond, cinnamon, sesame and olive treatments while the growth were positive for the rest of the tested oils.

DISCUSSION

The phyto-preparations of medicinal plants have

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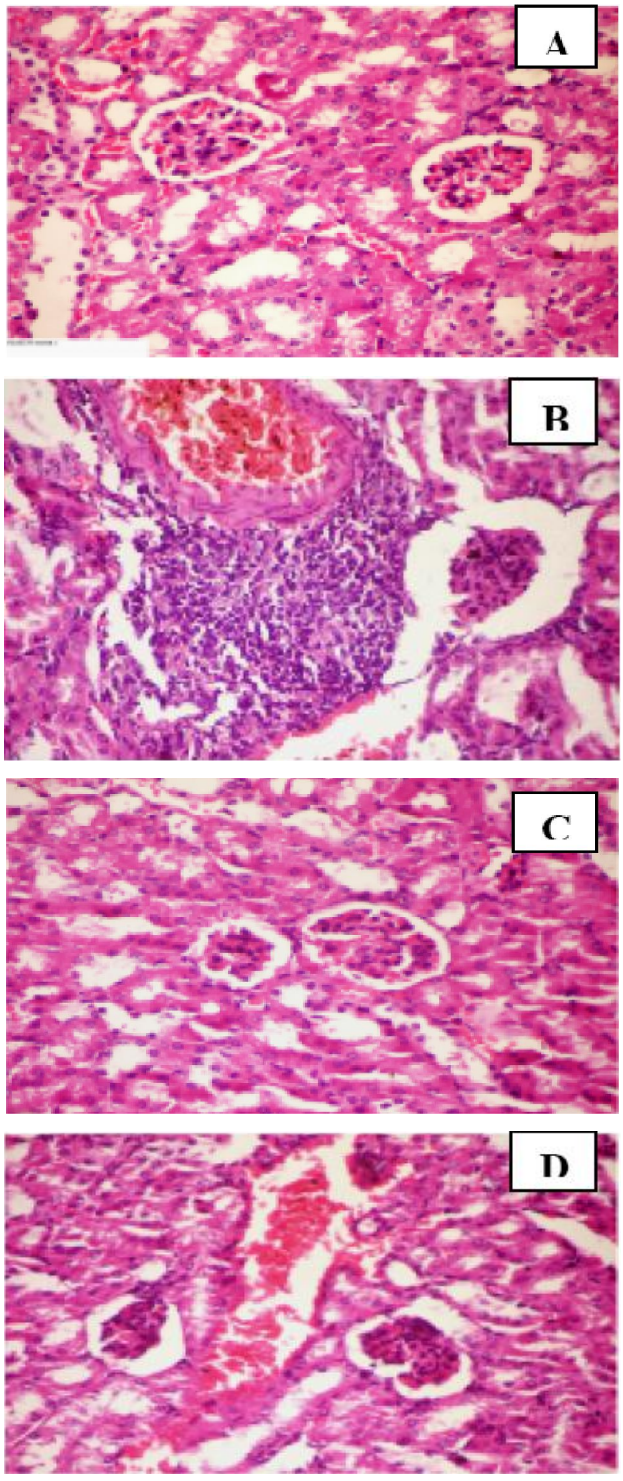

Figure 3 : Kidney sections of each group (H and E stained X 400). (A) Control group showing normal appearance of glomeruli and tubules; (B) Nanobacteria-infected non treated group exhibiting focal interstitial nephritis, atrophy of glomerular tuft and distention of Bowman's space and tubules; (C) Cinnamon treated group showing almost normal appearance of glomeruli and tubules; (D) Rocket treated group showing congestion of blood vessels and distention of Bowman's space.

gained special interest in recent decades as alternative products that could solve problems associated with the appearance of strains of microorganisms with reduced susceptibility to traditional antibiotics due to the diversity of mechanisms of action.

We found evidence of their inflammatory infiltration and accumulation in the cortex. It could be that the kidney is a preferred site for mineralization by these tiny bacteria. It is also plausible that other body calcifications could have a nanobacterial element. The effect of essential oils in protective kidney stones formed by nanobacteria, appeared evident in this study. Biomineralization in cell culture medium yielded the formation of biofilms and mineral aggregates very similar to those found in tissue calcifications and renal pathologies, suggesting that nanobacteria could be efficient nuclei of mineralization, which start the formation of kidney stones[11].

Among all tested essential oils, dill, almond, cinnamon, sesame and olive were found to possess highly nephro-protective effect against nanobacteria-induced infection in mice. They revented the nanobacterial-nephrotoxicity as evidenced by a significantly reduced ($p \leq 0.01$)

level of serum urea and creatinine. Urea is an excreting substance that is produced in protein metabolism. Creatinine is an excreting substance that is produced in creatine metabolism in a nonenzematic pathway. Increasing in urea and creatinine may be due to renal infections and disorders[12]. Also, no pathological changes were observed in kidneys from those groups, as evidenced by the normal appearance of glomeruli and tubules. In this respect, Dahiya and Purkayastha[13] found that dill oil had broad antibacterial activity against both Gram positive bacteria such as *Staphylococcus aureus, S. aureus* MRSA, *Enterococcus* sp. and Gram-negative bacteria *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*.

The antibacterial activities exhibited by dill oil might be attributed to these major components. the major components detected in dill oil are carvone (41.5%), limonene (32.6%), and apiol (16.8%)[14]. Lazarou et al[15] The probable mechanism of sesame oil action include preventing the bioactivation of the mutagens (through inhibition of metabolic enzymes such as CYP450 family), scavenging free radicals[16], modulat-

ing the antioxidant defense system[17] and may, therefore, protect the macromolecules like nucleic acids, proteins and lipids against oxidative damage and confer protection at cellular level[18].

Maria-Neto et al[19] isolated a novel antimicrobial protein belonging to the 2S albumin family was isolated from *Sesamum indicum* kernels against several bacteria and fungi. The histopathological examination showed grossly enlargment and inflamation of the kidney of infected mice. Microscopical examination revealed sever interstitial nephritis and thickening in parietal layer of Bowman's capsule. These finding confirmed that nanobacteria isolate is renal pathogenic isolate. These results are in accordance with that obtained by Sohshang et al[20] who reported those bacteria or other agents producing such nidi, if present in blood and in urine accelerate pathologic calcification *in vivo*. Also this is clinically important because blood contains phosphate near its saturation level. Also, Driessens et al[21] showed that nanobacteria may thus participate in activation-inhibition processes regulating a large number of responses inside and outside cells. Thus, nanobacteria could have multiple pathologic actions in the body. Giachelli et al[22] reported that when apatite is found in soft tissue considered being pathological calcification. Among various hypotheses proposed for pathological tissue calcification, recent evidence supports the possibility that self-replicating calcifying nanoparticles (CNPs) can contribute to such calcification. These CNPs have been detected and isolated from calcified human tissues, including blood vessels and kidney stones, and are referred to as nanobacteria**.** Also apatite on nanobacteria produces carbonate on their cell walls which may initiate kidney stone formation[9]. In conclusion, nanobacteria may be involved in the pathogenesis of nephrolithiasis and dill, almond, cinnamon, sesame and olive oils have a protective role against nanobacteria-induced nephrotoxicity in mice and the process is probably mediated through their antioxidant properties.

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