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# Detection of some types of bacteria in patients with kidney stones and the use of corn silk extracts to effectively inhibit the urease enzyme in *Klebsiella* Species

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**Abstract** The objective of the present study was to investigate the physicochemical and phytochemical properties of the gum exudates of *Mangifera indica*. The proximate composition was determined by using AOAC methods. Antioxidant capacity was determined using the free radical 2,2- diphenyl- 1-picrylhydrazyl (DPPH) method, The total phenolic contents were measured using Folin-Ciocalteu reagent assay according to the method described by [1] with some modifications. The gum was observed under Scanning electron microscope. Moisture, dry matter, crude fiber, crude fat, total ash, minerals and carbohydrates percentage of gum exudates of *Mangifera indica* are 9.48 ± 0.61, 90.51 ± 0.61, 0.86 ± 0.22, 51.43 ± 1.28, 0.2 ± 0.10, 4.32 ± 0.54, 33.68 ± 0.55 respectively. Antioxidant capacity; DPPH radical scavenging assay: $IC_{50}$ value for gum exudates of *Mangifera indica* was 2.36 ± 0.63 mg of Gallic acid equivalents per mL. Total Phenol Content was 243.59 ± 0.27 ppm.

**Keywords** Antioxidant capacity, DPPH, *Mangifera indica*, Total phenol content

## Introduction

Renal stones are a common clinical problem with a subsequent burden for the health system [1]. It is one of the most common painful diseases [2]. The symptoms related to the renal stones are highly dependent on the stones locations, the stones located within calyces are considered to be asymptomatic. While the initial symptoms of stone existing in the ureter are a cute onset of severe flank pain probably associated with nausea, vomiting and hematuria [3]. There are four main types of kidney stones; calcium stones, uric acid stones, struvite stones, and cysteine stones [4].

Struvite kidney stones or another name "infection stones" which have a proportion about (10-15%) of all kidney stones. are known to occur more frequently in women than in men (at a 2:1 ratio) a finding that might be attributable to the higher incidence of urinary tract infection (UTI) in women [5]. Struvite calculi are important clinically because they can lead to sepsis and renal failure [6]. In fact these stones are mainly composed of magnesium ammonium phosphate $MgNH_4PO_4.6H_2O$ (struvite) and calcium carbonate apatite $Ca_{10}(PO_4)_6.CO_3$ [7]. The urine composition does not seem to be a factor in the spontaneous formation of struvite crystals; therefore, stones containing struvite are considered associated with (UTI) [8]. They are primarily caused by urea splitting bacteria such as *proteus, pseudomonas, Klebsiella*, and *staphylococcus* [9].

## Corn Silk

Today, researchers have focused on the drug discovery from medicinal plants Medicinal plants are regarded as an acceptable, cheap, easily available and safe source of active compounds for pharmaceutical [10]. Corn silk is a long, weak, and shiny fibers at the top of corn's ear [11]. Traditionally, it is used for making tea as a healthy and medical drink in Asian communities especially in China [12]. However, corn silk becomes very important in drugs

development, because of its bioactive constituents which include oxidant prevention agent limits, anti-diabetic activity anti-proliferative effects diuretic activity anticoagulant activity, antifungal, anti-fatigue, and treating obesity [13].

## Materials and Methods

### Patients

The present study comprised of 100 individuals patient with kidney stones (group1) (60 male and 40 female). Patients aged from 20 to 75 years old, were attending to the urologic department in Ghazi Al-Hariri Hospital for the period from December 2016 to February 2017, Patients were diagnosed by urologist in the hospital. Patients didn't Suffers from any other disease. The urine samples were collected to done general urine examination and urine cultures

### The Bacteriology

### Culture of urine for 100 patients with kidney stone (group 1)

Performed on MacConkey agars, inoculating 0.001 mL of urine and streaking the surface to quantitative colony counts .The plates were aerobically incubated for 24 hr. at 35°C.

### Identification of bacteria in urine of 100 patients with kidney stone (group 1)

Identification of suspected isolates was done according to the colony morphology and biochemical tests.

**Colony morphology** All isolates were primarily identified according to the general culture characteristic (color, shape, texture and size) of the colony onto MaCconky agar and eosin methylene blue EMB agar after incubated overnight at 37ºC. Other characteristics were observed like lactose fermentation.

### Biochemical tests to identify bacteria in urine of patients with kidney stone

Following tests were used to identify bacteria.

### Citrate utilization test

Simmon citrate agar slant was stabbed with fresh bacterial isolates and incubated at 37 ºC for 24 hrs. Changing the color from green to blue is indicating a positive result. This test used to detect the bacterial ability to utilize sodium citrate as a carbon source.

### Oxidase test reagent

A filter paper was moistened with several drops of freshly prepared 1% oxidase reagent, and then a small portion of the tested colony was picked up by a wooden stick and placed on moistened filter paper. The color conversion to blue or purple color within 30 second this indicated to a positive reaction.

### Motility test

Tube containing motility media was stabbed once in the center of media with an inoculating needle, then incubated at 37°C for 24hrs. The motile bacteria spread out from the injected line of inoculation.

### Urease test

Urea agar slant was inoculated heavily over the entire slant surface and incubated at 37°C for 24hrs. Urease test is positive if the indicator was changed to purple-pink color, while keeping the media its yellow-orange color indicates a negative result. This test used to detect bacterial capacity to produce urease enzyme which hydrolyzes urea to ammonia and carbon dioxide.

### Indole test

Peptone broth was inoculated with a new culture of each suspected isolate and incubated at 37°C for 24hrs. Avolume of 0.5 ml from Kovac's reagent, was added directly to the bacterial culture tube and if the culture produces tryptophanse which hydrolyze tryptophan to indole, pyruvic acid and ammonia, red ring will appear at the top of the broth and this indicated a positive result.

### Storage of bacteria

The bacteria storage in Brain heart infusion.

**Extraction of Corn Silk Part**

**Plant Materials**

The samples of corn plant were collected at harvesting time where their materials are fully maturated and developed. Firstly, the corn silk flowers were gathered from corn fields of the faculty of Agriculture's farm of Baghdad University in February 2017. Secondly, they were dried in a shaded well-ventilated place. Thirdly, Cuts (0.4 mm) them using a knife mills then keeping them stored in glass containers at room temperature for further processing [14].

**Preparation of the Crude Extract** [14]

1. Three Erlenmeyer flask labelled with 99.9% ethanol extract, 80% v/v ethanol/water extract and water extract contain 1L of 99% ethanol, 80% v/v ethanol/water and water respectively
2. one hundred g of chopped corn silk were added to each flask and exposed to a hot continuous extraction in a Ultrasonic at steady temperature of (50 ± 1.0 °C) for 5 hours (cycle 1)
3. decanted the solvents from each flask , added 1L of each solvents and exposed to a hot continuous extraction in a Ultrasonic at steady temperature of (50 ± 1.0 °C) for 5 hours (cycle 2)
4. Repeat step 3 (cycles3)
5. Each of three previous extracts was filtered through Whatman No. 1 filter paper to remove the debris.
6. Then, each filtered sample was condensed by a rotary flash evaporator under vacuum at 50°C.
7. Lyophilizing each condensed samples in a freeze-dryer to obtain a crude 99.9% ethanol Extract (99.9%EE), 80% ethanolic extract (80%EE), and water extract (AE).
8. Lastly, all extracts were stored at 4 °C for subsequent analysis

The same experiment was returned to the one hundred g of the corn silk powder Which was crushed by the electric mill with 80% ethanol.

- **Calculation**

The below equation used to determine the yield as percentage of the quantity of the initial material of (100g).

$$\text{Yield } \% = \frac{\text{yield} * 100}{100 \text{ g}}$$

**Urease inhibition part**

**Preparation concentration**

1. Prepared the stock solutions of organic solutions by dissolve organic extracts dried and aqueous extract dried by phosphate-buffer (pH=7)

2. Prepared from it various concentration (1000, 500, 250, 125, 62.5) mg\L diluted with phosphate-buffer (pH=7) for organic extract and aqueous extract.

3. Then filter it with micro filter 0.45μm and used or stored at 4 °C until further use.

***Klebsiella* Species urease inhibition assay**

**Activation of microorganisms**

The specimen of the colonies was taken by a loop that contains 5 ml of sterilized Brain heart agar. The loop has been shaken well and incubated in the incubator for 24 hours at 37 °C. The loop was sterilized via flame before using it to ensure that the planted bacteria are not contaminated

- **Principle of urease inhibition assay**

The inhibition of urease examination was performed spectrophotometrically in 96-well Microplate, Urease activity was
continuously measured with the rate of ammonia generation .

- **Procedure of urease inhibition assay**

1. Dissolve 38.71 g of urea broth powder in 1000 ml distilled water.
2. Then, thoroughly mix to dissolve the medium completely then sterilize the results by Autoclave.

3. After that, 40% urea was sterilized by filtration and added to the medium.
4. After activation, under a sterile tube and aseptic ambience, the desired colony was taken by a loop to the test tube that contains 5 ml of the sterilized urea broth
5. The solution: (100µl) of bacteria diluted of *Klebsiella* species was incubated with 100µl of extracts (99.9% EE), (80% EE), and (AE) dissolved in phosphate puffer in concentrations of (1000, 500, 250, 125, 62.5, and 31.25 mg/L) at 30 °C for 24 hour.
6. Change in absorbance (optical density) was measured at 630 nm on ELISA plate reader in compare with standard urease inhibitor i.e. thiourea (TU) [15].

- **Calculation**

The percentage of inhibition was calculated by using the formula given below.

$$\% \text{ Inhibition} = 100 - \left\{\frac{\text{Absorbance of Test Compound}}{\text{Absorbance of Control}} \times 100\right\}$$

Measuring the effects of different concentrations of inhibitors on production of ammonia was used to evaluate the $IC_{50}$ of the active compounds are calculated by plotting the relation between % Inhibition and concentration of inhibitors. The $IC_{50}$ values were determined using Graphpad Prism7 software.

## Results and Discussion

### Result of urine culture

The urine culture was performed for 100 patients with kidney stones, making sure that the patients did not take any anti - bacterial medication. The results showed that 71% of the patients have no growth in their urine culture and 29% have positive urine culture (13%, 10%, 5% and 1% of *Klebsiella, E. coli, Proteus,* and *Pseudomonas*) respectively. The results of urine culture are shown in Table 1. Ureolytic infection- induced stones are estimated to constitute 15–20% of all urinary stones. *Proteus, Pseudomonas,* and *Klebsiella* are the most common bacterium responsible for struvite stone [16]. *Escherichia coli* causes the majority of asymptomatic bacteriuria, cystitis, pyelonephritis, and catheter-associated urinary tract infection (UTI)

**Table 1:** Bacteria types and Percentage in urine culture

| Bacteria types | Percentage |
|---|---|
| *Klebsiella* | 13% |
| *Proteus* | 5% |
| *E-coli* | 10% |
| *Pseudomonas* | 1% |
| No growth | 71% |

### Diagnosis of bacteria

Primary bacteria identification with nonspecific media MacConkey agar show in Table 2, is a selective and differential culture medium for bacteria designed to selectively isolate Gram-negative. *Klebsiella, Proteus, E. coli, and Pseudomonas* they have growth on MacConkey agar. MacConkey agar distinguishes those Gram-negative bacteria that can ferment the sugar lactose (Lac+) from those that cannot (Lac-) fermentation. *Klebsiella* and *E. coli* are lactose fermenting. The bile salts precipitate in the immediate neighbourhood of the colony, causing the medium surrounding the colony to become hazy. *Proteus* and *Pseudomonas* are Non-lactose fermenting. Table 3: shows bacteria identification in Citrate utilization test, indole test, Oxidase test, motility test and Urease test.

**Table 2:** The Primary bacteria identification with MacConkey agar

| Bacteria species | MacConKey Agar | Ferment the sugar lactosue |
|---|---|---|
| *Klebsiella* | Positive(+) | Lac+ |
| *Proteus* | Positive(+) | Lac- |
| *E-coli* | positive(+) | Lac+ |
| *Pseudomonas* | Positive(+) | Lac- |

**Table 3:** Bacteria identification with, Citrate utilization test indole test, Oxidase test, motility test and Urease test

| | **Citrate utilization test** | **Indole test** | **Oxidase test** | **Motility test** | **Urease test** |
|---|---|---|---|---|---|
| ***Klebsiella*** | Positive(+) (blue color) | Positive(+) or Negative(-) | Negative(-) | Negative(-) | Positive(+) |
| ***Protuse*** | Negative(-) (green color) | Positive(+) or Negative(-) | Negative(-) | Positive(+) | Positive(+) |
| ***E. coli*** | Negative(-) (green color) | positive(+) | Negative(-) | Positive(+) | Negative(-) |
| ***Pseudomonas*** | Positive(+) (blue color) | Negative(-) | Positive(+) | Negative(-) | Negative(-) |

**Plant Extract**

**Extraction of corn silk**

Table 4 shows yield of extracts of corn silk with respect to solvents. The percentage yields were calculated against 100g of corn silk material subjected to each extraction method .The percentage yield of aqueous extract (AE) (was high yield than others 9.1%. The next was **80%** ethanol extract (80%EE) with (2.4%). The percentage was (0.93) for 99.9% ethanol extract (99.9%EE). These results disagree with those reported by Nurhanan, et al. [17] who mention that the yield of extracts are found to be in ethanol higher than water. However, more polar aglycones or flavonoid glycosides are extracted with pure alcohols or with water−alcohol mixtures, and for less polar flavonoids (isoflavones, flavanones, methylated flavones and flavonols) [18].

According to the results shown in Table 4, it is noticed that the water solvent gives higher yield than others which can considered as factor in solvent cost reduction.

When we returned the experiment to the powder of the corn silk which was crushed by the electric mill with 80% ethanol the yield of extracts was only 0.84 g, however, the yield of extracts of powder of the corn silk less than yield of extracts of cut corn silk (0.4mm) because when the particle sizes are too small, unhomogeneous extractions can form and the analyte re-adsorption on the matrix surfaces, which hinders the extraction. In our study, because of the re-adsorption of the extracted solutes, a lower flavonoid yield was found when the particle size was smaller than 0.4mm. Hence, a particle size of 0.4 mm was selected for subsequent tests [19].

**Table 4:** Comparison analysis of extraction yield, in 100g of corn silk, fractions obtained using different solvents

| **Type of solvents used in extraction** | **Yield(g)** | **Color of extract** | **yield%** |
|---|---|---|---|
| 99.9% ethanol | 0.93 | yellow | 0.93% |
| 80% ethanol | 2.4 | light brown | 2.4% |
| Water | 9.13 | brown | 9.1% |

**Study urease inhibition by corn silk extract.**

A study on enzyme inhibition is an important area of pharmaceutical science. This study in the past have led to the discoveries of several successful drugs, useful against a variety of pathophysiological conditions. Natural products have played an important role in the development of new therapeutic agents against urease enzyme, such as flavonoids, that exhibited excellent urease inhibitory activity. Specific inhibitors interact with enzymes and block their activity towards their corresponding natural and synthetic substrates [14].

**Urease inhibition in *Klebsiella species***

Urea Broth medium was developed by Rustigian and Stuart. This medium is especially recommended by Indian Pharmacopoeia. The pink color is given as a positive result of the bacteria produced urease Klebsiella aerogenes.

Because Urea Broth Medium convert to alkaline as the use of urea by the organisms release ammonia through the incubation, showed by pink color [20]. The inhibitory activity of Extracts (99.9%EE), (80%EE), and (AE) to Klebsiella Species are shown in the table 5. It is noticed that all three extracts (99.9%EE), (80%EE), and (AE) show a potent urease inhibitory activity and the $IC_{50}$ values are shown in Figure (1), (2), (3). ($IC_{50}$ = 235.9, 305.5, and 247.3 mg/L) respectively as compared with thio urea as shown in Figure (4), which shows inhibitory of ($IC_{50}$ =138.1mg/L). The extract (99.9%EE) have less $IC_{50}$ so it's the best urease inhibitor from the other extracts.

**Table 5:** Klebsiella species urease inhibitory activity of Extracts (99.9%EE) (80%EE), (AE) as compared with standard urease inhibitor thiourea

| Conc. species | Inhibition % of thiourea | Inhibition % of 99.9E.E. | Inhibition % of 80% E.E. | Inhibition % of A.E. |
|---|---|---|---|---|
| 31.25 | 29.78 | 23.72 | 9.927 | 28.81 |
| 62.5 | 34.62 | 29.53 | 23.97 | 34.62 |
| 125 | 38.25 | 33.89 | 31.23 | 37.77 |
| 250 | 39.70 | 38.98 | 32.62 | 39.22 |
| 500 | 45.76 | 41.64 | 36.25 | 40.67 |
| 1000 | 52.30 | 44.30 | 42.42 | 44.3099 |
| $IC_{50}$ | 138.1 | 235.9 | 305.5 | 247.3 |

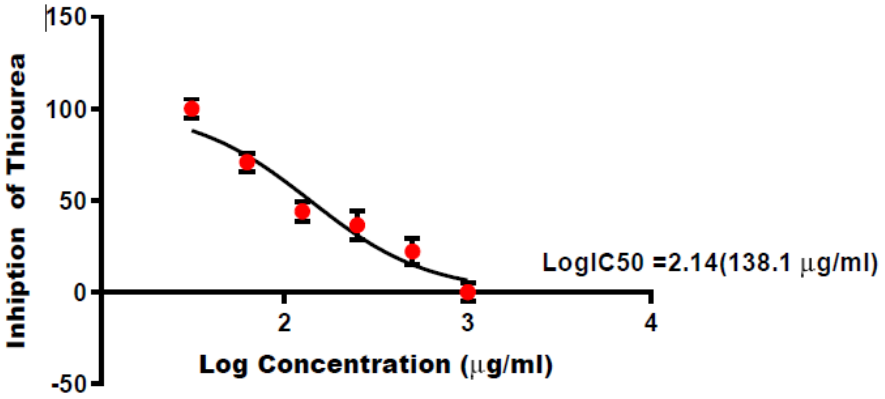


*Figure 1: The $IC_{50}$ of Klebsiella species urease inhibition by thiourea*

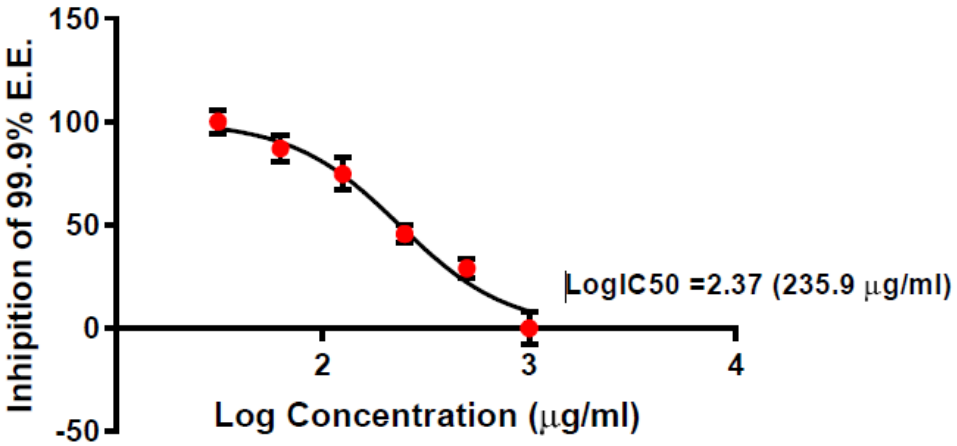


*Figure 2: $IC_{50}$ of Klebsiella species urease inhibition by Different concentrations of 99.9% ethanolic extract (99.9%EE)*

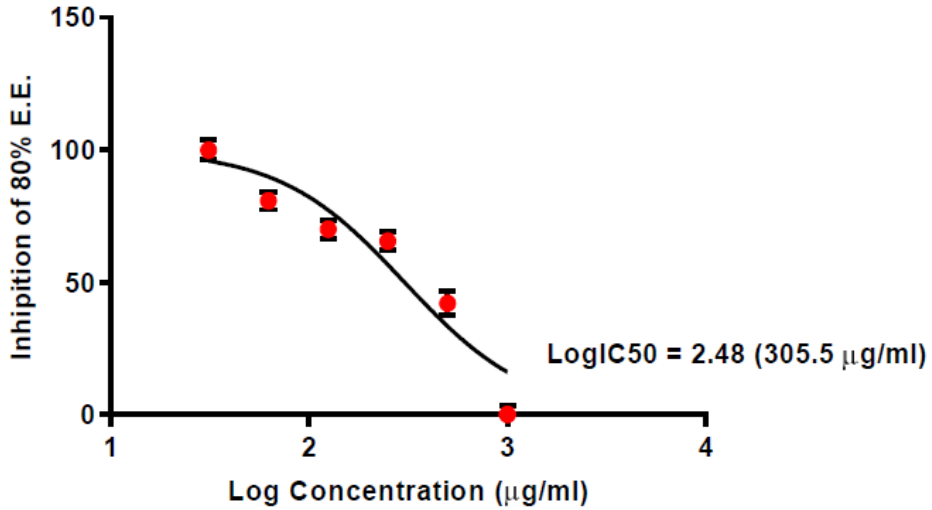


*Figure 3: $IC_{50}$ of Klebsiella species urease inhibition by different concentrations of 80% ethanolic extract (80%EE)*

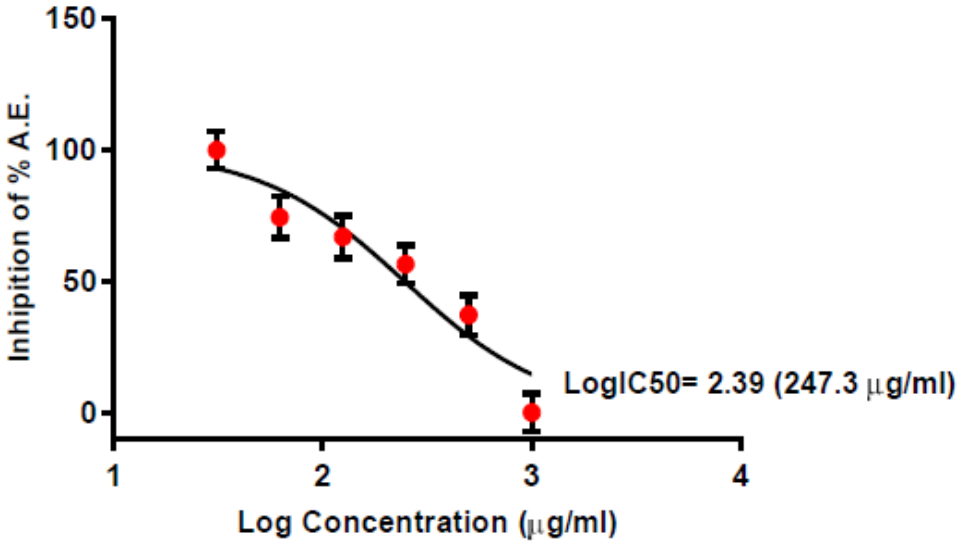


*Figure 4: $IC_{50}$ of Klebsiella species urease inhibition by different concentrations of aqueous extract (AE)*

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