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Curative and nephrotoxic potential of methanolic leaf extract of *Plukenetia conophora* Mull arg. in rats challenged with *Pseudomonas aeruginosa* urinary tract infection

SS Enitan, KA Digban, M Olley, OG Otuneme, TG Faloye and IO Adediji

Abstract
The curative and nephrotoxic potential of methanolic leaf extract of *Plukenetia conophora* (*MLEPC*) was assessed in rats challenged with experimental *Pseudomonas aeruginosa* urinary tract infection. Rats with culture-proven *Pseudomonas aeruginosa* UTI were treated with different doses of *MLEPC* (250, 500, 750 and 1000 mg/Kg/bid), as well as equal volume (50:50) of 500 mg/Kg/bid *MLEPC* and 500 mg/Kg/bid Ciprofloxacin suspension combined, administered orally twice daily for 7days. The animals were sacrificed on day 2, 4, 8 and 16 to determine bacterial burden in urine and urinary organs homogenates, as well as urine leucocytes count using standard methods. Urinary levels of acid phosphatase (ACP), alkaline phosphatase (ALP), Creatinine and Urea were also evaluated spectrophotometrically using kits to determine the nephrotoxic potential of the extract. The treatment outcome of our experimental *P. aeruginosa* UTI in animal model revealed that the *MLEPC* produce a progressive significant reduction ($P<0.001$) in the bacterial load in urine, bladder and kidneys homogenates, as well as urine leucocytes count in a dose-dependent manner comparable to that of the positive control (Ciprofloxacin). Combination of *MLEPC* with Ciprofloxacin resulted in a partial synergistic effect. All the extract-treated groups, had sterile pyluria by day 4 of therapy, as well as sterile bladder and kidney culture by day 8, except for 250mg/Kg/bid with a mean urine, bladder and kidneys bacterial count of 3.7 log CFU/ml, 1.4 log CFU/bladder and 1.7 log CFU/kidneys, respectively and mean urine leucocytes count of 9 cells/mm^3. By day 16, all the animals have fully recovered, no bacterial growth was observed except for the negative control. Nephrotoxicity studies showed no evidence of nephrotoxicity in even the most challenging experiment. The result of this study revealed the UTI-curative activity of Methanolic extract of *P. conophora*, with no potential risk of nephrotoxicity.

Keywords: *Plukenetia conophora,* Urinary tract infection, *Pseudomonas aeruginosa*, curative potential, Nephrotoxicity

1. Introduction
The urinary tract of humans has been documented to be a common site of *Pseudomonas aeruginosa* infection, often following catheterization, cystoscopy, and gynaecological surgery [1, 2]. Urinary tract infection (UTI) caused by *P. aeruginosa* is a significant cause of morbidity and mortality worldwide and require effective prompt treatment as delay can be fatal [3]. Ciprofloxacin is the preferred oral agent for UTIs caused by susceptible bacteria such as *P. aeruginosa* because of its high renal excretion rate (>60%) and non-toxic effect on the nephrons [4, 5]. However, it is well recognized that *P. aeruginosa* UTI is difficult to treat and manage because of increasing prevalence of antibiotic resistant strain [6, 7]. These have triggered an interest in the application of alternative, novel, non-antibiotic based methods for preventing and treating UTI. As a result, advocacy on continuous search for alternative medicine and herbal remedies is been intensified [8, 9].
Plukenetia conophora Mull arg. is one of such medicinal plants, gaining fast recognition in traditional medicine [10]. The plant *Plukenetia conophora*, formerly known as *Tetracarpidium conophorum*, commonly called African Walnut belongs to the family Euphorbiaceae. It is a climbing shrub 10-20ft long found mostly in the Southern and Western regions of Nigeria and other West African countries like Cameroun and Gabon [11, 12]. Almost all the parts of *P. conophora* are considered to be medicinal. For instance, the leaves are used to treat indigestion, constipation, diarrhoea, dysentery, syphilis, thrush, intestinal worm infection, hiccups, eczema and parasitic skin conditions mostly among children, the elderly and immunosuppressed [13-15].

The bark in particular is chewed for toothache and to prevent and control high blood pressure while the root is used for healing of haemorrhoids, frost bite and varicose ulcers [16, 17].

In our previous *in vitro* susceptibility studies [18], we demonstrated the antibacterial activity of the methanolic leaf extract of *Plukenetia conophora* on selected urinary isolates. *P. aeruginosa* in particular showed the highest zone of inhibition (18 mm) at the highest concentration tested (200 mg/ml). Our preliminary phytochemical screening of the extract, revealed the presence of tannins, anthraquinones, saponins, flavonoids, resins and alkaloids, while cardiac glycosides and steroids were absent. Given these findings, this present study is therefore aim at evaluating the curative potential of the methanolic leaf extract of *P. conophora* against experimental *P. aeruginosa* UTI in animal model in order to determine if the previous observed *in vitro* activity will translate into significant activity *in vivo*. And owing to the fact that plausible evidence exist that some herbal remedies may either interact or interfere with the action of some standard drugs in modulating therapeutic activities [19], we therefore see the need to investigate the combined effects of *P. conophora* and Ciprofloxacin in the experimental animals, with a view to establish if the anticipated effects are elicited either singly or combined.

On the other hand, to our knowledge, no study has shown that regular intake of *P. conophora* can cause nephrotoxicity in animal or man. We therefore see the need to determine if this extract is nephrotoxic at the varied concentrations tested following oral administration. It is hoped that the outcome of this investigation will provide adequate scientific justification on the use of *P. conophora* as alternative treatment option for UTI with little or no nehprotoxic potential.

2. Materials and Methods

2.1 Plant materials

A bulk of pesticide free leaves of *P. conophorum* (Figure 1) were collected from a private farmland in Igueben Local Government Area of Edo State, coordinates: *6º 30, N 6º00E* in central southern Nigeria in the evening in the month of August, 2014. The leaves were identified at the Department of Botany, College of Natural and Applied Science, Igbinedion University, Okada, Edo State. The leaves were washed with distilled water and dried at room temperature (25±2 ˚C) for 14 consecutive days. The air-dried leaves were pulverized using pestle and mortal in the laboratory. 50 g of the coarse powder was then soaked in 500ml of aqueous methanol (20:80) in a homogenizer and left for 24 h with frequent agitations according to the method described by [20]. Afterwards, the homogenate was filtered three times through sterile cheese cloth and centrifuged at 200 xg for 10 minutes and the supernatant was collected and then filtered into separate sterile container using a funnel containing sterile Whatman No.1 filter paper for clarification. The filtrate was concentrated under reduced pressure and after removal of the solvent, yield of extract was obtained and the extract stored in the dessicator.

Fig 1: Leaves of *Plukenetia conophora* Mull arg. (African walnut)

2.2 Preparation of various concentrations of extract

The extract was reconstituted daily by shaking 10 g of the extract in 10 mL of distilled water to obtain a 1000 mg/mL extract solution. Additional lower concentrations (750, 500 and 250 mg/mL) were made from the stock (1000 mg/mL) with sterile distilled water. All these were done aseptically.

2.3 Antibiotic

Ciprofloxacin® (Geltec Pvt Ltd, India) was purchased from the Department of Pharmacy, Igbinedoin University Teaching Hospital Okada, Edo state 20 mL of Ciprofloxacin® suspension was prepared daily by dissolving 20 tablets of 500mg Ciprofloxacin® in 20 mL of sterile distilled water to obtain a concentration of 500 mg/mL.

2.4 Experimental animals

Animal use protocol was in accordance with international standard on the care and use of experimental animals [21]. Female Wistar albino rats weighing 200±30g (mean±SD) were purchased from the Animal Production and Health

Department, University of Benin, Benin City (Edo State) and were clinically examined upon arrival and any that showed signs of abnormality or disease were excluded from the study. Only symptom-free animals were used. They were housed in the Experimental Animal House, College of Health Sciences, Igbinedion University Okada (Edo state, Nigeria) separately in well ventilated wire-bottom steel cages, under hygienic conditions, with proper aeration at 25±2 ºC, and a relative humidity of 45-50%. The rats were randomly assigned into 8 groups of 10 rats each and fed on standard pellet diet (10g/100g body weight) twice daily and tap water ad libitum. Prior to experimentation, the rats were allowed to stabilize in the Animal House with standard 12-hour light-dark cycle, for a period of 10 days. The Animal Care and Ethics Committee of Igbinedion University, Okada approved the experimental protocols.

2.5 Pre-infection phase

To test for any pre-existing infection with *Pseudomonas aeruginosa*, about 0.3 mL urine sample (from 3-4 pools every 2 h) was aseptically collected from the external urethral meatus of each rat with the aid of a micropipette into ice-cooled sterile Eppendorf tubes after gentle compression of the bladder through the external abdominal wall. One portion of each urine sample (5-µL) was inoculated onto MacConkey agar (MCA) and Cystein Lactose Electrolyte Deficient (CLED) Agar and sub-cultured onto Cetrimide agar medium selective for Pseudomonas. From the overnight cultures of the viable colonies on selective media, colonies were subsequently sub-cultured onto Nutrient agar plates and incubated at 37 ºC for 18-24hrs. Isolates were characterized by their colony morphology, Gram staining characters, pigment production, motility and biochemical tests as described by [22]. Identification of isolates was by Bergey's Manual for Determinative Bacteriology [23].

The second portion of urine was centrifuged in a cold centrifuge for 10 min at 500-700g. Smears were made from urine sediment and slides were air dried and fixed in methanol. Presence of leucocytes in urine sediment was detected by microscopic examination of Gram stained smear as described by [2] and quantified by hemocytometer- count procedure as described by [24]. Only rats that had negative urine bacteriological culture for Pseudomonas were used for the study. Any rat with urine culture which tested positive for Pseudomonas with significant leucocyturia (> 5 cells/mm^3) present was considered to be unsuitable and was replaced before the experimental infection, and no animal was replaced thereafter.

2.6 Infection phase

2.6.1 Test isolate

Stock culture of *P. aeruginosa* was obtained from the Microbiology Laboratory of the Igbinedion University Teaching Hospital (IUTH), Okada. The test organism was previously isolated from a female out-patient with symptomatic UTI, having bacteruria of ≥ 10^5cfu/ml.

2.6.2 Preparation of test isolate

The isolate were sub-cultured from preserved agar slant onto selective and differential solid media and re-identified biochemically using standard methods as described by [22].

2.6.3 Standardization of inoculum

Colonies from the pure culture of the isolate was inoculated into nutrient broth (NB) and incubated at 37 ºC for 18 to 24 h. Surface viable count was carried out as described by [25]. The bacteria was washed once with saline and then adjusted to the desired concentration.

2.6.4 Rat model of urinary tract infection

The UTI model used was adapted from previous studies by [26] and [27] with some modifications. Briefly, following water restriction for 4 h prior to and after infection, respectively, rats were anesthetized intraperitoneally with ether using a hypodermic needle (25 x 0.5 mm) and were induced to void urine by gentle compression of the bladder through the external abdominal wall. By using a 23-gauge blunt, slightly bent needle, 0. 5 ml of the bacterial suspension with a concentration of 1.2 X 10^7 CFU/ml in phosphate buffered saline (PBS) was trans-urethrally inoculated into the bladder of each rat in Group 1-7 (except, Group 8 which served as the zero control), for about a 5-second period. Immediately after the inoculation, the urethral needle was removed and the external urethral meatus was clamped for 4 h. After the indicated interval, the clamp was removed and urine sample was collected from each inoculated rat as earlier described. Specimens were treated as infectious and handled according to standard precautions. Post-infection, bacteria burden in urine (bacteriuria) was determined and the presence of leucocytes in urine sample was detected by microscopic examination of Gram stained smear as described by [2], while leucocyturia was quantified by hemocytometer count [24]. Bacteria burden in urinary organs homogenates was also determined. Briefly, two rats from each infected group were sacrificed by cervical dislocation as described by [28]. Bladder and kidneys were harvested under sterile conditions and cut open. The cut surface of each organ was imprinted onto CLED agar plates. The organs were rinsed with 100 ml of sterile physiological saline solution, followed by absorption of that adhering to the organ tissue by sterile filter paper. The bladder and a pair of kidneys were homogenized in 2 and 4 ml of distilled water, respectively. Serial 10-fold dilutions of the tissue homogenates were inoculated onto agar (0.1 ml/9-cm-diameter plates) and incubated overnight at 37 °C, and the colonies were counted. Bacterial counts were expressed as the number of colony-forming units (CFU) per ml of urine, bladder or kidneys.

Isolation and identification of *P. aeruginosa* in urine, bladder and kidneys culture from infected rats 18 h post-inoculation was used to confirm the development of urinary tract infection in the infected rats before commencement of extract intervention. Meanwhile, no bacteria were isolated from the un-infected rats (Zero control).

2.7. Treatment phase

Extract was assessed for its curative ability to eradicate *P. aeruginosa* from the urinary tract of rats. Therapy was initiated 18 h post-bacterial challenge and lasted for 7 days. All experimentally urinary tract infected rats were treated as shown in Table 1. Group 1, 2, 3 and 4 received 250, 500, 750 and 1000 mg/Kg/bid of *MLEPC,* respectively. While Group 5 was treated with equal mixture (50:50) of 500 mg/Kg/bid *MLEPC* and Ciprofloxacin combined. Group 6 and 7 which served as the positive and negative control received a standard oral dose of Ciprofloxacin (500mg/Kg/bid) and sterile Phosphate Buffer Saline (PBS), respectively. While Group 8 which was uninfected, served as the zero control and received no treatment. The volume of extract (10 ml/Kg body weight) administered to individual rat using intragastric tube was calculated, recorded and adjusted daily with changes in body

weight throughout the treatment phase. They were observed daily for any observable change. Study animals were treated every 12 h (during the period 7.00-7.30 AM/PM) for 7 consecutive days with an appropriate volume of suspension

Table 1: Experimental pharmacological protocol

Groups	N	Treatments
G1	10	Received 250 mg/Kg/bid *MLEPC*
G2	10	Received 500 mg/Kg/bid *MLEPC*
G3	10	Received 750 mg/Kg/bid *MLEPC*
G4	10	Received 1000 mg/Kg/bid *MLEPC*
G5	10	Received equal mixture (50:50) of 500 mg/Kg/bid (*MLEPC* + *Ciprofloxacin*)
G6	10	Received 500 mg/Kg/bid of *Ciprofloxacin* only (Positive control)
G7	10	Received Sterile PBS (Negative control)
G8	10	Uninfected and received no treatment (Zero control)

MLEPC = Methanolic leaf extract of Plukenetia conophora; N = Number of rats per group; PBS = Phosphate Buffer Saline

(10 ml/kg), which was shaken gently immediately prior to administration and delivered by oral gavage. At day 2, 4, 8, and 16, two rats were sacrificed from each group and the total CFU recovered from urine, whole-bladder and kidneys homogenates was determined, as well as urine leucocytes count. Response to therapy was assessed based on microbiologic and immunologic criteria.

2.8 Nephrotoxicity studies

2.8.1 Urine collection for evaluation of nephrotoxicity indicator parameters

With gentle compression of the bladder through the external abdominal wall, urine was collected from the external urethral meatus of each rat with the aid of a micropipette into a small bijour bottle containing 20 µL of 20% sodium azide as a preservative. The urine was frozen at -20 ºC and used for the determination of urinary acid phosphatase (ACP), alkaline phosphatase (ALP), creatinine and urea.

2.8.2 Urinary acid phosphatase (ACP)

Spectrophotometric method using ACP kit supplied by BioSystem Reagents and Instruments (Spain) was used to assess the level of ACP in urine and tissue homogenates as described by [29].

2.8.3 Urinary alkaline phosphatase (ALP)

Spectrophotometric method using ALP kit supplied by Teco Diagnostic (Anaheim, USA) was used to assess the level of ALP in urine and tissue homogenates as described by [30].

2.8.4 Urinary Creatinine

Urinary Creatinine Assay Kit (Cell Biolabs, Inc., USA) was used to measure the levels of creatinine in urine according to manufacturer's instructions and as described by [31].

2.8.5 Urinary Urea

Urea in urine sample was measured by urease-berthelot colormetric method using Urease kit (Randox Laboratories Ltd, United Kingdom) according to manufacturer's instructions and as described by [32].

2.9 Statistical Analyses

Data for the bacterial colony forming unit found in the urine, bladder and kidneys specimens, urine leucocytes count and renal parameters (ACP, ALP, Creatinine and Urea) were presented as means of 2 rats using line charts. Data were analyzed using one way analysis of variance (ANOVA) and Tukey-Kramer Multiple Comparisons Test using SPSS-18.0 (Statistical packages for social Scientists – version 18.0) statistical program. P values<0.05 were considered significant [33].

3. Results and Discussions

The mean urine bacterial counts of the 250 mg/Kg/bid extract treated group decreased significantly (P<0.001) from 6.9 to 3.7 log CFU/ml on day 4 of therapy, while sterile pyluria was obtained by day 8. There was significant increase (P<0.001) in the mean urine leucocytes count, from 16 to 21 cells/mm^3 by day 2, which however declined significantly (P<0.001) to 14 cells/mm^3, 9 cells/mm^3 and 3 cells/mm^3 by day 4, 8 and 16, respectively. While mean bacterial counts in bladder and kidneys culture reduced significantly (P<0.001) from 5.3 to 1.4 log CFU/bladder and from 4.2 to 1.7 log CFU/kidneys, respectively, by day 8. However, negative urinary organ homogenates culture was obtained by day 16.

For the 500 mg/Kg/bid and 750 mg/Kg/bid extract treated groups, mean urine bacterial counts decreased significantly (P<0.001) from 6.6 to 3.9 log CFU/ml, and from 6.8 to 4.0 log CFU/ml, respectively, while the mean urine leucocytes counts increased non-significantly (P>0.05) from 14cells/mm^3 to 18 cells/mm^3 and significantly (P<0.01) from 17 cells/mm^3 to 22 cells/mm^3, respectively, by day 2 of therapy. However, a non-significant (P>0.05) decline was observed by day 4 of therapy when sterile pyluria was obtained for the two groups, 13 cells/mm^3 and 19 cells/mm^3, respectively. It further declined significantly (P<0.001) to 5 cells/mm^3 and 7 cells/mm^3, respectively by day 16. On the other hand, mean bacterial counts in bladder and kidneys from 500 mg/Kg/bid extract treated group reduced significantly (P<0.001) from 4.6 to 1.5 log CFU/bladder and from 3.5 to 1.7 log CFU/kidneys, respectively, also from 4.8 to 1.3 log CFU/bladder and from 3.7 to 1.6 log CFU/kidneys in the 750 mg/Kg/bid extract treated group on day 4 of therapy, while negative urinary organ homogenates culture was obtained by day 8 for both groups.

In the 1000 mg/Kg/bid extract and combined 500 mg/Kg/bid extract + Ciprofloxacin treated groups, sterile pyluria was obtained by day 2 of therapy, with a significant rise (P<0.001) in mean urine leucocytes counts, from 18 cells/mm^3 to 25 cells/mm^3 and from 12 cells/mm^3 to 23 cells/mm^3, respectively. However, by day 4, 8 and 16, the mean urine leucocytes counts of 1000 mg/Kg/bid extract treated group declined to 22 cells/mm^3 (P>0.05), 17 cells/mm^3 (P<0.01) and 10 cells/mm^3, (P<0.001) respectively, while that of combined 500 mg/Kg/bid extract + Ciprofloxacin treated group declined to 18 cells/mm^3 (P<0.01), 15 cells/mm^3 (P>0.05) and 8 cells/mm^3 (P<0.001), respectively. On the other hand, there was significant reduction (P<0.001) in the mean bacterial counts in bladder and kidneys from 1000 mg/Kg/bid extract (from 4.4 to 3.7 log CFU/bladder and from 4.3 to 2.0 log CFU/kidneys) and combined 500 mg/Kg/bid extract + Ciprofloxacin (from 5.1 to 4.6 log CFU/bladder and from 4.0 to 1.8 log CFU/kidneys) treated groups, respectively, by day 2 of therapy. While negative bladder and kidneys cultures were obtained by day 4 of therapy. Both 1000 mg E and Combined 500 mg E+C proved to be more effective than the 250 mg E, 500 mg E and 750 mg E as evident by sterile pyluria on day 2 of therapy, although no significant difference (P>0.05) was observed between the two said groups.

As was expected, the microbiologic outcome of the extract was found to be comparable with that of the positive control group in which mean urine bacterial count decreased significantly (P<0.001) from 6.9 to 2.5 log CFU/ml, while the

mean urine leucocytes count increased non-significantly (P>0.05) from 13 to17 cells/mm^3 by day 2 of therapy. Sterile pyluria was obtained by day 4 of therapy. Moreover, the mean urine leucocytes count further declined to 15 cells/mm^3 (P>0.05), 10 cells/mm^3 (P<0.01) and 6 cells/mm^3 (P>0.05) by day 4, 8 and 16, respectively. The mean bacterial counts in the bladder and kidneys homogenates decreased significantly (P<0.001) from 4.3 to 1.6 log CFU/bladder and from 3.7 to 1.3 log CFU/kidneys, respectively by day 4 of therapy. However, negative urinary organ homogenates cultures were obtained by day 8.

But, on the other hand, mean bacterial counts in urine, bladder and kidneys from the negative control group increased significantly (P<0.001) from 7.5 to 9.3 log CFU/ml, from 4.5 to 4.8 log CFU/bladder and from 3.4 to 4.7 log CFU/kidneys, respectively, by day 8. However, bacteria burden recovered from urine, bladder and kidneys of the said group declined significantly (P<0.001) to 5.4 log CFU/ml, 4.3 log CFU/bladder and 4.2 log CFU/kidneys, respectively, by day 16. While, the number of urine leucocytes increased significantly (P<0.001) from 15 to 26 cells/mm^3 (day 0-8) which later declined to 22 cells/mm^3 by day 16 (Fig. 2-4 & 5).

Furthermore, the outcome of our nephrotoxicity studies revealed that the mean urine levels of acid phosphatase, alkaline phosphatase, creatinine and urea were significantly reduced (P<0.001) in a dose-dependent manner by day 8 in the 250mg/Kg/bid (from 97 to 81 nKat/L; from 53 to 40 IU/L; from 302 to 280 µmol/L, from 208 to 192 mmol/L, respectively), 500mg/Kg/bid (from 92 to73 nKat/L, from 55 to 36 IU/L, from 297 to 270 µmol/L; from 220 to 187 mmol/L, respectively), 750mg/Kg/bid (from 95 to 68 nKat/L; from 52 to 37 IU/L; from 303 to 253 µmol/L; from 212 to 162 mmol/L, respectively), 1000 mg/Kg/bid (from 110 to 60 nKat/L; from 51 to 30 IU/L; from 301 to 230 µmol/L; from 217 to 163 mmol/L, respectively) *MLEPC*, as well as the combined 500 mg/Kg/bid *MLEPC* + Ciprofloxacin (from 102 to 48 nKat/L, from 54 to 39 IU/L, from 302 to 212 µmol/L and from 223 to 151 mmol/L, respectively) and the positive control (from 95 to 71 nKat/L, from 53 to 37 IU/L, from 304 to 263 µmol/L and from 215 to 163 mmol/L, respectively), except the zero control; when compared with the negative control group in which the mean urine levels of the nephrotoxicity indicator parameters were significantly in-

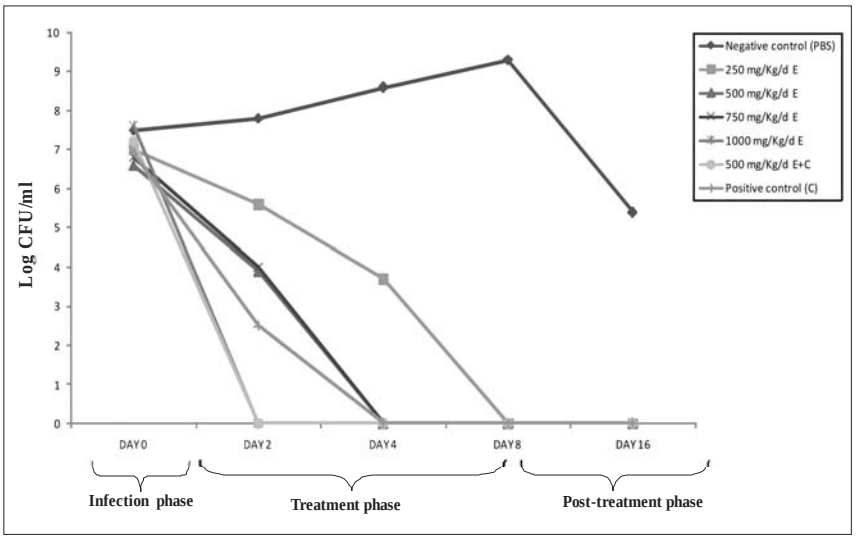

Fig 2: Changes in urine bacterial counts of treated and control rats. Each symbol represents the average urine bacterial counts of all animals in each group at each time point (Day 0 -16).

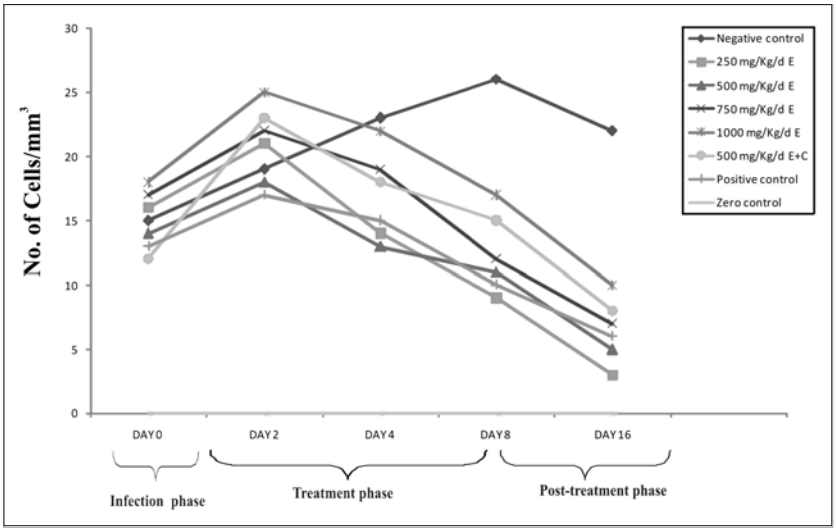

Fig 3: Changes in urine leucocytes counts of treated and control rats. Each symbol represents the average urine leucocytes counts of all animals in each group at each time point (Day 0 -16).

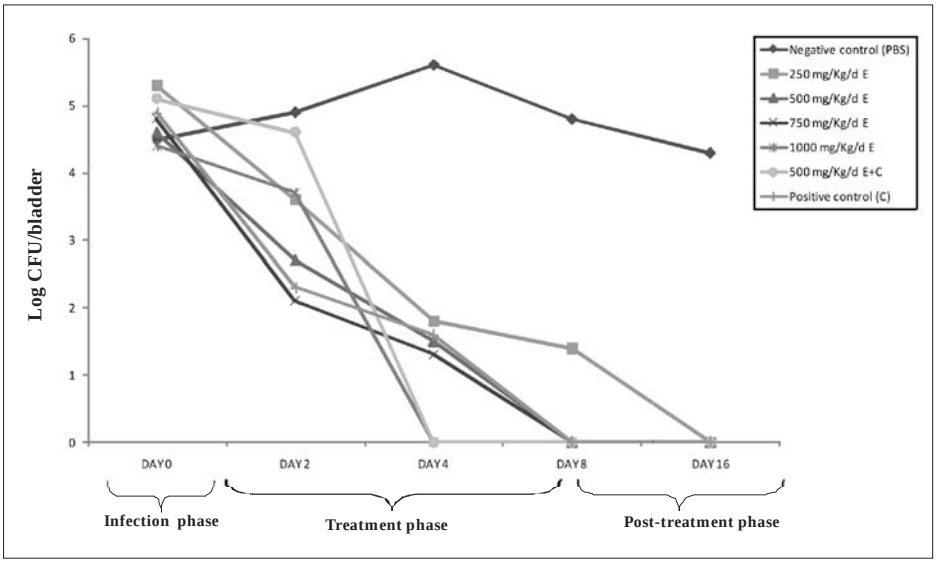

Fig 4: Changes in bladder bacterial counts of treated and control rats. Each symbol represents the average bladder bacterial counts of all animals in each group at each time point (Day 0 – 16).

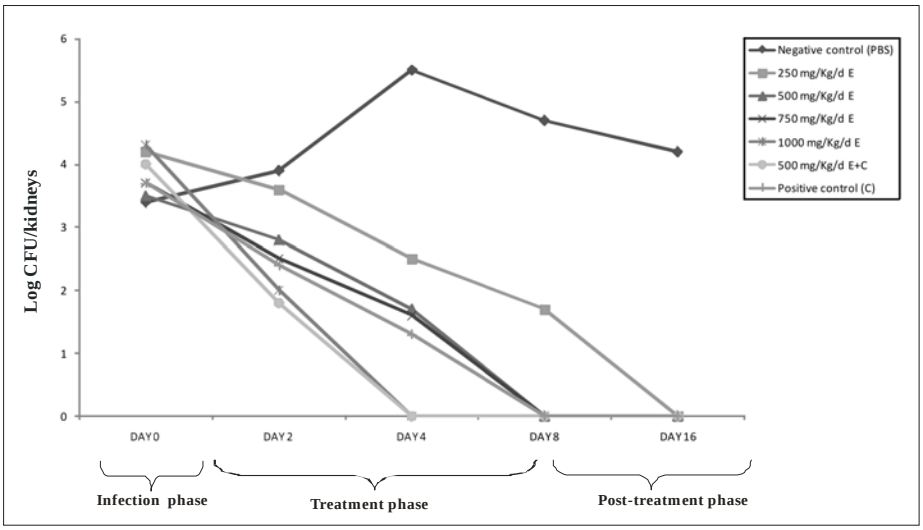

Fig. 5: Changes in kidneys bacterial counts of treated and control rats. Each symbol represents the average kidneys bacterial counts of all animals in each group at each time point (Day 0 -16).

-creased (P<0.001): acid phosphatase (from 93 to 120 nKat/L), alkaline phosphatase (from 50 to 78 IU/L), creatinine (290 to 336 µmol/L) and urea (from 213 to 277 mmol/L). However, by day 16, the nephrotoxicity indicator parameters returned close to the pre-treatment levels (baseline status) in all the treated groups, except the negative control (Fig. 6, 7, 8 & 9).

Most often, microbiological, immunological or histopathological studies are carried out to assess host's response to treatment of an infection. In this study, we assessed microbiological and immunological responses of infected experimental animals to extract therapy by comparing bacterial load and leukocytes count in the various treated groups with that of the controls, before therapy (day 0), during therapy (day 2 and 4) and after therapy (day 8 and 16). We defined microbiological success as negative post-treatment urine and urinary organ homogenate cultures, while microbiological failure as positive post-treatment urine and urinary organ homogenate cultures.

The present culture data indicate occurrence of microbiologic success in all the various extract treated groups, as evident by sterile urine and urinary organ homogenate cultures post-treatment. Treatment outcome shows that methanolic extract of *P. conophora* exhibited a high curative activity *in vivo*. Bacteria burden in urine, bladder and kidneys were significantly reduced (P<0.001) in a dose-dependent manner during and after therapy was completed in all the extract treated rats with sterile leucocyturia by day 4 of therapy and sterile urinary organ homogenate culture by day 8, except for the 250mg/Kg/bid treated group in which bacteria persisted in the urinary organs beyond day 8, but not up to day 16 in the absence of bacteruria. The extract proved to be more effective than the positive control treated with ciprofloxacin (P<0.001), especially at the highest concentration tested (1000mg/Kg/bid), except for the negative control group with positive urine and urinary organ homogenate cultures by day 16.

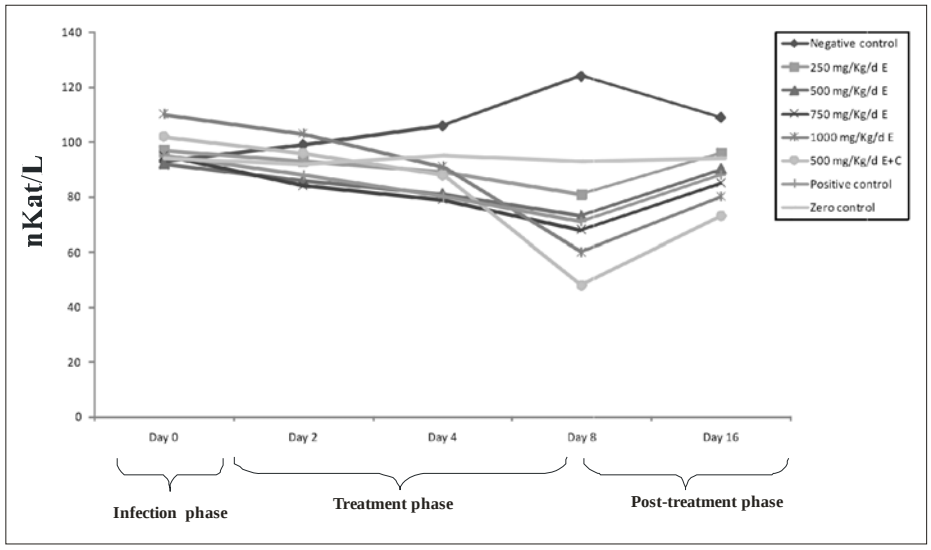

Fig. 6: Changes in urine acid phosphatase levels of treated and control rats. Each symbol represents the average acid phosphatise levels of all animals in each group at each time point (Day 0 -16).

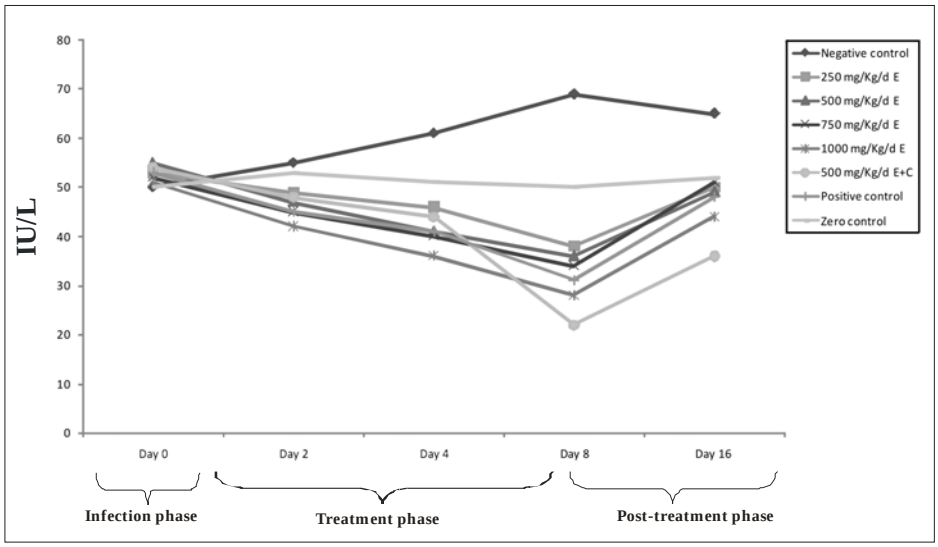

Fig 7: Changes in urine alkaline phosphatase levels of treated and control rats. Each symbol represents the average alkaline phosphatise levels of all animals in each group at each time point (Day 0 -16).

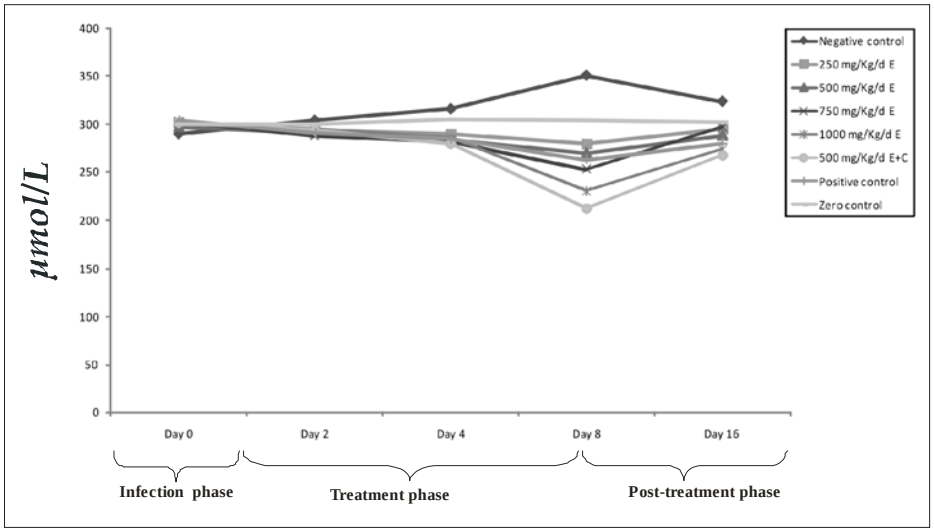

Fig. 8: Changes in urine creatinine levels of treated and control rats. Each symbol represents the average creatinine levels of all animals in each group at each time point (Day 0 -16).

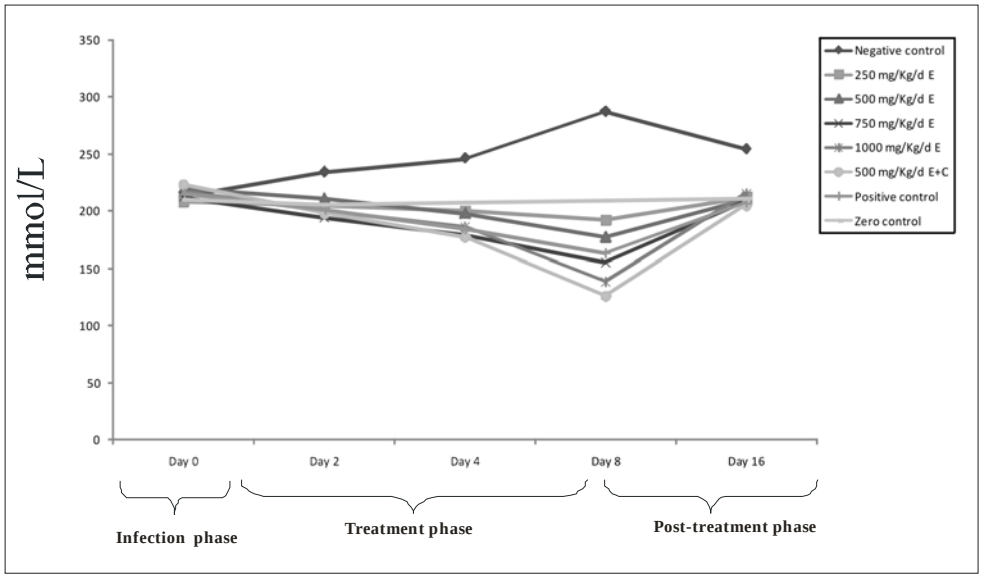

Fig 9: Changes in urine urea levels of treated and control rats. Each symbol represents the average urea levels of all animals in each group at each time point (Day 0 -16).

The combined 500mg/Kg/bid extract + Ciprofloxacin seem to possess a synergistic effect. Its curative activity was comparable with the extract, only at the highest concentration tested (1000 mg/kg/bid). It proved to be more effective than the 500 mg/Kg/bid extract or 500 mg/Kg/bid Ciprofloxacin administered singly, as evident by sterile leucocyturia by day 2 of therapy (P<0.001). The inherent curative potential of the extract appears to be enhanced when combined with Ciprofloxacin as its potency was observed to have increased when used in combination with the preferred drug for treatment of UTI. The anticipated antagonistic effect did not emerge. This further underscores the reason why several plants are now being used in part or as a whole or in combination with other plants or standard antibiotics to either prevent or treat urinary tract infections. The combination of different antimicrobial agents has been shown to behave synergistically in preventing the adhesion and growth of uropathogens [5, 34].

Furthermore, the outcome of this current study revealed a significant rise and fall in leucocyturia post-infection and post-therapy, respectively. In health, leucocytes are hard to come by in normal urine. The presence of leucocytes in urine is therefore, a good indicator of an immunological response of the uroepithelium to microbial assaults. According to [2] and [35], the appearance of leucocytes in urine is a hallmark of active bacterial infection, while its disappearance following therapy suggests resolution of the infection.

In this current study, we observed sterile leucocyturia which persisted during and after therapy was completed. In the absence of conditions such as polycystic kidney disease, interstitial nephritis and cystinuria, it is not uncommon to have sterile leucocyturia during and after treatment of urinary tract infection with antibiotics, which may persist for up to a week following discontinuation of the antibiotics [36].

Since, bacteruria and leucocyturia were significantly reduced in this current animal study post-therapy, this shows that methanolic leaf extract of *P. conophora* possesses curative efficacy *in vivo* at all dose levels tested. The curative activity observed in the extract-treated rats in this present study mirrored our previously established *in vitro* antibacterial activity data [22] and also supports the works of [37] and [38], who showed that plant extracts that can cause significant reduction in bacteruria and leucocyturia could serve as alternative treatment-option for UTI. On the other hand, with regard to the renal profile of the experimental animals, we found out that the urinary levels of ACP, ALP, creatinine and urea were significantly reduced (P<0.001) in the extract, ciprofloxacin and combined extract + ciprofloxacin treated rats following 7 days of therapy, when compared to the negative control in which there was significant elevation (P<0.001). Here, a rise in the urinary enzymes, creatinine and urea as was initially anticipated was absent, it can therefore be inferred that the extract did not cause nephrotoxicity at all the concentrations tested. Meanwhile, no significant difference (P>0.05) was observed in the zero control rats throughout the observation period. Besides, a much more significant reduction in the levels of renal parameters was observed when extract was co-administered with ciprofloxacin than when administered singly. This in a way suggests that combination therapy permit the usage of lower doses without risk of renal toxicity.

Biochemically speaking, significant elevation in the levels of renal parameters is indicative of renal alterations. Though these parameters were elevated in the negative control group, the elevation observed is thought to be a direct outcome of microbiologic failure, as animals in this group were treated with sterile PBS with no antimicrobial property and as a result, they failed to achieve microbiologic cure. It is normal to have these parameters elevated in UTI, as presence of bacteria and leucocytes in urine have been documented to cause an elevation in the levels of urinary enzymes [39, 40].

Insignificant changes in the renal parameters of the zero control rats confirm no occurrence of renal alternations in this group of animals since they were uninfected at the first instance. While significant reduction (P<0.001) in the extract and ciprofloxacin treated rats proved that the treatments (at the concentrations tested) were non-nephrotoxic. Significant decrease in bacteruria and leucocyturia observed in the extract and ciprofloxacin treated rats is also thought to be responsible for the reduced levels of urinary enzymes. Reduced or complete absence of bacteruria and leucocyturia, which is characteristic of a successfully treated UTI, has been correlated with reduced levels of urinary enzymes [41, 42].

4. Conclusion

The previously reported excellent *in vitro* antibacterial activity of methanolic leaf extract of *P. conophora* against urinary tract pathogens has been confirmed in animal model. The extract proved to be effective in the treatment of *P. aeruginosa* UTI, without risk of nephrotoxicity even at the highest concentration tested. If these findings are extrapolated to humans, they further underscore the curative potential of *MLEPC* and may likely produce clinical benefit in humans.

Competing Interests

Authors have declared that no competing interests exist.

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