

THE HORMESIS OF NAGAPARPAM IN EXPERIMENTALLY INDUCED PYELONEPHRITIC MICE

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Received: 20 Oct 2013, Revised and Accepted: 10 Nov 2013

ABSTRACT

Objective Nagarpam (NP), a herbo-mineral based Siddha medicine is widely used across the various parts of India for the treatment of various gastric ailments and other kidney related diseases. The lack of scientific evidence regarding the toxicity and efficacy of this drug upon prolonged usage by pyelonephritic patients probed us to initiate this study.

Methods The commercially available NP was analyzed for its solubility. The olive oil enriched emulsion was tested for the various phyto-compounds present in the drug. The anti-oxidant profile was evaluated followed by MIC and MBC of the emulsion with incremental concentration drug against Uropathogenic *E.coli* (UPEC) RRL – 02. Pyelonephritis was induced in experimental mice (n=18) for studying the efficacy and toxicity of NP by trans-urethral catheterization. Upon establishment of infection, the mice were separated into four groups (Group 1: Control, Group 2: Pyelonephritic model, Group 3: Amoxicillin (100 mg/ Kg body weight) and Group 4: NP (600 mg/Kg body)) of six animals each and treated with the NP emulsion for a period of 30 days. Urinary and tissue biochemical components such as protein, SOD, and catalase were monitored regularly and the histological examination were carried out on the formalin fixed kidney samples.

Results The NP emulsion contained various phyto-compounds such as tannins, glycosides and saponins. The emulsion had a bacteristatic effect on the pathogenic strain used in the study at 600 mg/Kg body weight, which was used as the concentration for the further *in vivo* studies. The administration of NP for a period of 10 days had a significant effect in restoring the reduced protein (p< 0.001) levels in the kidney and a decrease in the levels of SOD and catalase was observed relative to the control. However, the prolonged administration of the drug was found to be toxic to the mice at this concentration, which was reflected by the elevated levels of SOD and catalase with a significant decrease in the tissue protein levels (p< 0.01) at the end of the experimental period. This was also evidenced by the damage to the glomerular and tubular regions of the kidney in the NP treated animals.

Conclusion NP although effective in treating pyelonephritis at 600 mg/Kg bodyweight, was found to be toxic when administered for prolonged periods of time.

Keywords: Nagarpam, Urinary tract infections, Pyelonephritis, Toxicity.

INTRODUCTION

Complementary and alternative medicines have been routinely used for the treatment of various ailments across the globe, but have not gained popularity due to the lack of evidences gathered from scientific experimentation [1]. Siddha medication utilizes the herbo-mineral concoction system [2] for the treatment of various ailments across India. These herbal preparations are offered as medications as they are speculated to have a number of advantages such as stability and shelf-life over a period of time, storability and sustained availability upon administration and are known to contain metals and minerals as an integral part of their formulations [3]. The commonly supplemented metals in the formulations are zinc, mercury, lead and arsenic. Zinc, is an essential component of physiologically important enzymes such as carbonic anhydrase, superoxide dismutase and alkaline phosphatase and is essential for the proper functioning of the immune system, DNA synthesis, cell division, protection of proteins and lipids from oxidative damage [4].

A four-centre report by WHO report in 2009, where *E. coli*, the single major causative of urinary tract infections, was the organism under study, outlined the high levels of antibiotic resistance among pathogenic strains [5], thus raising the need for newer strategies that could help in combating and containing the issue [6]. Various medications are available in Siddha for the treatment of urinary tract infections; these medications are administered for a long period and lack scientific evidence to evaluate their potential toxicity with relation to humans. Therefore, an attempt was made to study the hormesis of the Siddha medication Nagarpam (NP) in treating uropathogenic *E.coli* induced pyelonephritis in mice to understand its anti-bacterial action and to study the toxicity on the kidneys upon prolonged usage at its anti-bacterial concentration.

MATERIALS AND METHODS

Chemicals

2, 2-Diphenyl- 1 - picrylhyrazyl (DPPH) was procured from Sigma Aldrich (Bangalore, India). All other chemicals, solvents and reagents were purchased from Sisco Research Laboratories (Mumbai, India) and the microbiological media from Himedia Pvt. Ltd (Mumbai, India).

Phytochemical analysis

The Siddha drug, NP was obtained from a registered drug store (Indian Medical Practitioners Co-operative pharmacy and Stores (IMCOPS) Ltd., Chennai, India). Ten grams of the drug was prepared as an emulsion in olive oil (gm /mL) and the phytochemical analysis was carried out using standard procedures to identify the compounds present [7].

Anti - oxidant profile

The anti-oxidant profile of the drug was determined (n = 9) in 96 well micro-titre plates (Tarsons, India) and absorbance's of the metabolites were measured in an automated microplate reader (Bio-Tek Instruments Inc. USA). The scavenging effects of the emulsion were analyzed by the DPPH radical scavenging activity [8], Deoxyribose radical scavenging activity [9], Superoxide anion radical scavenging [10], Lipid peroxidation inhibition activity. Also, the reducing power of the emulsion was determined as described by Oyaizu [11] *in vitro*.

Anti - bacterial analysis

Commensal *E. coli* 25922 strain (MTCC No. 443, IMTECH Chandigarh, India), a wild non-pathogenic strain of *E.coli* was utilized as the Standard strain for the study. The clinical isolate *Escherichia coli* RRL-

02 (Genbank accession number: JQ398845.1) was used as the Uropathogenic *E.coli* strain for the antimicrobial studies. The antibacterial activity of NP was carried out with varying concentrations (0 – 1000 mg/mL) by tube dilution method, for determining the minimum inhibitory concentration (MIC) and minimum bacteriostatic concentration (MBC). Growth curve analysis using commensal *E.coli* 25922 for relative comparison was carried out. For this study, amoxicillin, a broad-spectrum antibiotic at 100 mg/mL was chosen as the standard drug for the study.

Effect of drug on treating pyelonephritis

Female Swiss Albino mice (8 – 10 weeks) weighing 25 ± 2 g were housed in polypropylene cages (6 animals per cage) lined with paddy-husk bedding. An ambient temperature of $24 \pm 1^\circ\text{C}$, with a 12 h light/dark light cycle and $65 \pm 2\%$ relative humidity was maintained. The animals were fed with food and water *ad libitum* and the CPCSEA guidelines for Laboratory animal facility (ICMR, New Delhi, India) were followed. The Institutional Animal Ethical Committee (IAEC) of VIT University approved the study design and protocols (Approval number: VIT/ SBST/ IAEC/III/2011/19).

Eighteen (n= 18) female albino mice were used to generate the pyelonephritic model out of twenty four animals (n = 24) to test the effect of the NP emulsion. Each anesthetized mouse was infected with 2.5×10^8 cfu of ECRRL02/30 μL of sterile PBS, trans-urethraly using lubricated catheter delivering $\sim 10 \mu\text{L}^{-1}$ of inoculum into the bladder. After inoculation, the catheter was removed and the animal was monitored regularly for any discomfort, injury or inflammation due to the procedure [12]. Upon establishment of infection on the 7th day post inoculation, the animals were separated as six animals in each groups as follows: Control (Group 1), UPEC infected animals (Group 2) Amoxicillin treated animals (Group 3 treated with 100 mg/Kg body weight; concentration was derived from the anti-bacterial studies carried out using the pathogenic strain ECRRL02) and NP supplemented (Group 4 treated with 600 mg/Kg body weight of the NP emulsion as determined from the anti-bacterial studies using the ECRRL02). The animals were sacrificed on the 10th, 20th and 30th day after treatment, after anaesthetizing them by the intra-peritoneal administration of Ketamine. A portion of the kidney was processed for analyzing various biochemical parameters such as protein content [13], SOD [14], and catalase [15], the other portion was stored in 10% neutral buffered formalin solution for histo-pathological analysis.

Histo-pathological analysis

Kidneys fixed in 10% neutral buffered formalin were processed and embedded in paraffin. Four micron sections were cut and stained with Haematoxylin & Eosin (H&E). The sections were studied under 400X magnification (LabOMed, India) for evidence and degree of pyelonephritis and other morphological changes were observed.

RESULTS

Commercially available NP, a herbal medicine prepared with the pulp of *Aloe vera* extracts and enriched during drug preparation with zinc oxide was obtained and prepared into an emulsion using olive oil. Our studies due to the lacunae regarding the toxicity of the drug at higher concentrations upon prolonged usage, have explored the efficacy of NP as an alternative medication for pyelonephritis when compared to the regular antibiotics administered to the patients. The results obtained from our study are as follows:

The phytochemical analysis of the emulsified NP revealed the presence of a wide range of phyto-compounds such as tannins, saponins, flavanoids, glycosides, and terpenoids that could be responsible for its antioxidant and antimicrobial nature.

The antioxidant potential of the NP emulsion was carried out with incremental concentration starting from 0 – 500 $\mu\text{g}/\text{mL}$. It was observed that it has the potential to inhibit the generated superoxide radicals, the lipid peroxides, and the hydroxyl radicals. The NP emulsion also has a considerable reducing power activity. The antioxidant potential of the drug can be broadly mentioned as follows: superoxide inhibition ($90.3 \pm 0.2\%$ at 150 $\mu\text{g}/\text{mL}$ concentration) > hydroxyl radicals inhibition ($66.7 \pm 0.3\%$ at 50 $\mu\text{g}/\text{mL}$ concentration) > lipid peroxidation inhibition ($55.9 \pm 0.3\%$ at 250 $\mu\text{g}/\text{mL}$ concentration) > reducing power activity (32.6 ± 0.2 ascorbic acid equivalents at 50 $\mu\text{g}/\text{mL}$ concentration) > free radicals inhibition ($26.9 \pm 0.2\%$ at 100 $\mu\text{g}/\text{mL}$ concentration) as in the Table 1.

The antibacterial analysis of the NP emulsion using the non-pathogenic and pathogenic strain of *E.coli* indicated that NP has a bacteriostatic effect on the growth of the strains. The MIC of the drug was found to be 400 mg/Kg bodyweight while the MBC was found to be 600 mg/Kg bodyweight when compared to the standard drug amoxicillin, which inhibited the growth of the strains at 100 mg/Kg body weight.

Table 1: The above table shows the effect of Nagarpapam treatment on body weight and tissue biochemistry.

	Control	Pyeloneph Model	Amoxicillin treatment			Nagarpapam treatment			
			10 days	20 days	30 days	10 days	20 days	30 days	
Body wt (g)	25.2 ± 1.9	22 ± 2.3	22.3 ± 2.3	24.1 ± 2.2	24.9 ± 0.6	23.1 ± 0.6	22.6 ± 1.1	21.1 ± 1.5	
Kidney wt (g)	0.24 ± 0.01	0.27 ± 0.003	0.25 ± 0.001	0.24 ± 0.002	0.24 ± 0.001	0.27 ± 0.001	0.28 ± 0.007	0.24 ± 0.001	
Urinary protein content (mg/mL)	5.5 ± 0.3	8.5 ± 0.2	6.5 ± 0.1	5.7 ± 0.1	5.7 ± 0.2	7.8 ± 0.1	8.0 ± 0.1	8.6 ± 0.1	
Tissue	Protein content (mg/g of tissue)	2.5 ± 0.1	1.5 ± 0.2	2.2 ± 0.3	2.4 ± 0.3	$2.5 \pm 0.2^*$	$1.6 \pm 0.1^{***}$	$1.6 \pm 0.2^{**}$	$1.2 \pm 0.3^{**}$
	SOD ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	1.85 ± 0.2	2.2 ± 0.3	$2.0 \pm 0.2^{***}$	$1.9 \pm 0.05^{###/***}$	$1.89 \pm 0.01^{##/***}$	2.1 ± 0.3	$2.2 \pm 0.07^{***}$	2.4 ± 0.05
	Catalase (Units/mg protein)	0.9 ± 0.02	2.5 ± 0.08	$1.57 \pm 0.03^*$	1.1 ± 0.1	1.0 ± 0.1	2.1 ± 0.3	1.89 ± 0.04	2.1 ± 0.09

Values are expressed as mean \pm SD (n = 6). Significance was analyzed by one – way ANOVA is as follows Control vs. Treated: *: p<0.05; **: p<0.01; ***: p<0.001. Infected vs. Treated: #:p<0.05; ##: p<0.01; ###: p<0.001.

From the infected animals, it was evident on the seventh day post infection; pyelonephritis was established in the kidney of the mice. It was found that a significant decrease in the body weight of the infected animals (22 ± 2.3 g) when compared to the control (25 ± 1.9 g). This decrease in the body weight of the infected animals was brought back to normal in the case of the animals treated with Amoxicillin, whereas in the animals treated with NP, there was an increase in the body weight till 10th day, which started to decrease upon further treatment for 30 days. The total protein concentration in the kidneys of infected animals (1.5 ± 0.2 mg/g of Kidneys) were compared to control (2.5 ± 0.1 mg/ g of Kidney),

which indicated that there was a decrease in their concentration. This decrease was very minimally elevated on the 10th day. This was further found to decrease upon treatment with the drug for an extended time frame of 30 days (1.2 ± 0.3 mg/ g of Kidney; p < 0.01). Whereas in the case of the pyelonephritic mice treated with Amoxicillin, the decreased protein content in the kidneys were restored close to normal on the 10th day (2.2 ± 0.3 mg/ g of Kidney; p<0.05) and restored back to normal upon treatment with Amoxicillin for 30 days (2.5 ± 0.3 mg/ g of Kidney) when compared to the control animals. This difference in the protein content in the kidneys of the NP treated animals pave way to the

indication that there could be possible glomerular and tubular damage in the kidneys. This simultaneously poses the question whether this damage could have been due to the induced infection or drug administration for a prolonged time. This was clarified by analyzing the morphological changes in the tissue sections obtained from the mice after treatment with the drug for the various time intervals.

The histo-pathological analysis of the tissue samples obtained from the animals on the 7th day post infection with the *E. coli* RRL - 02 indicated that there was destruction of the normal glomerular architecture with inflammation in the tubular region (Fig. 1). This inflammation was accompanied by the infiltration of the neutrophil's and macrophages. When the sections obtained from the animals treated with Amoxicillin were analyzed it was seen that on the 10th day, there was a restoration of the near-normal architecture in the glomerular region. However, in the tissue sections obtained from the

animals treated with NP emulsion at 600 mg/Kg body weight it was noticed that on the 10th day, the damage to the glomerular architecture, and the infiltration of the neutrophil's although had considerably reduced; while the inflammation in the tubular region had not abated upon treatment. Also a decrease in the antioxidant enzymes, SOD and catalase were noticed. When the tissue sections obtained from the mice treated with NP emulsion for a period of 20 days and 30 days were examined after H&E staining, there was evidence of vacuolation in the tubular region. The degree of vacuolation in the tubular region of the mice treated with NP emulsion for 30 days were higher compared to the animals treated for 20 days, thus indicating that there could be possible damage to the kidneys due to the prolonged drug administration. This was also accompanied by the elevated levels of urinary protein, SOD and catalase, which had considerably reduced upon treatment with NP emulsion for a period of ten days.

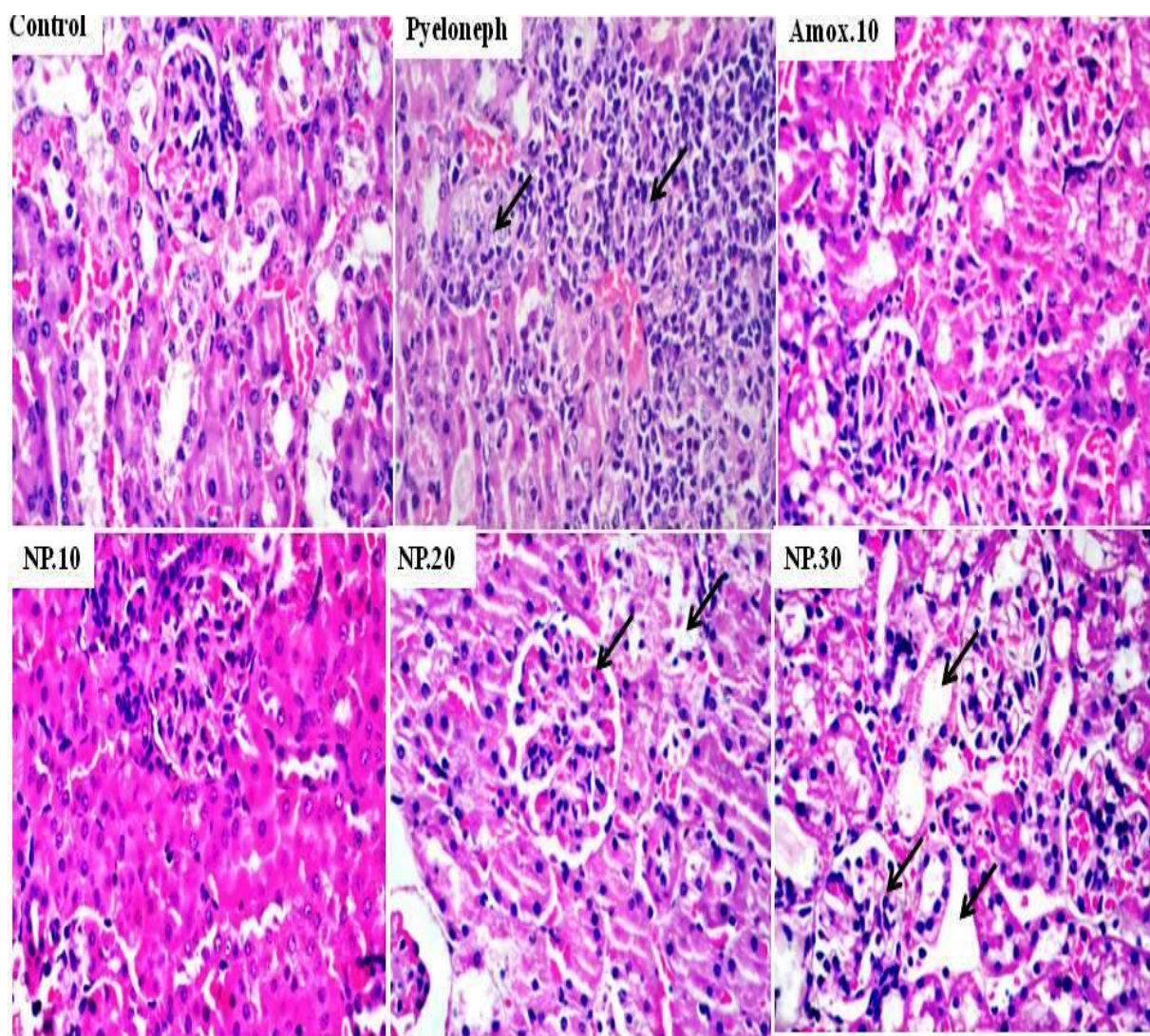


Fig. 1: The above figure represents the histo-pathological analysis of the kidney sections obtained from the control, amoxicillin and Nagarpam treated pyelonephritic mice.

All the sections shown here were taken at 400X magnification (Pyeloneph - kidney section obtained from mice with pyelonephritis. Amox.10 - pyelonephritic mice treated with 100 mg/Kg body weight of Amoxicillin for 10 days. NP.10, NP.20, NP.30 - mice treated with 600 mg/Kg body weight of Nagarpam for 10, 20 and 30 days respectively. The arrows represent the inflammation and destruction of glomerular architecture in the pyelonephritic

model. In the NP treated animals they represent the increased degree of vacuolation and destruction of the glomerulus.

DISCUSSION

Siddha drugs have been administered as a mode of treatment for several diseases across the states of India. These Siddha drugs are a mixture of herbal extracts and metal compounds, and therefore due

to their synergistic action have a less toxic effect and an increased bioavailability in the system, thereby resulting in increased potency of the drug [16]. Nagarpam is one such a drug that is prepared by the addition of incinerated Zinc oxide powders to the pulp of *Aloe vera*, a widely studied plant species for its anti-diabetic and other cosmetic applications [17]. This mineral enriched drug is widely used in the range of 100 – 200 mg/Kg body weight for the treatment of various skin [18], gastrointestinal and kidney related disorders and prepared by repeated incineration of the metals or their salts to eliminate their toxic effects [19 – 21].

The efficacy of this NP was investigated in treating UTI in the experimental animal model. Our *in vitro* anti-microbial analysis confirmed the bacteristatic activity of NP, which is primarily due to the presence of Zinc oxide in the formulation. The presence of Zinc could be the responsible factor for the bacteristatic effect of the drug against the *E.coli* strain used in the study. This could have been due to the electrostatic forces between the Zinc oxide particles and the negatively charged bacterial cell wall [22], due to which it has been commonly used as a component of fungicidal treatment and as a preservative to prevent bacterial and fungal growth [23].

The entry and re-colonization of the bacteria in the lipid rafts of the urothelium leads to the recurrence of UTI in the kidneys [24]. This also silently paves for an increased production of the ROS and RNS radicals in the body resulting in the subsequent damage to the slowly regenerating urothelium, inflammation and also increased protein loss from the kidneys, which is manifested as increased protein content in the urine of the infected mice [25 - 28]. This can be easily substantiated by the increased protein content in the urine from our study on the 7th day post infection, where due to prominent establishment of infection in the kidneys of the mice, there was damage to the glomerular region and this eventually resulted in the increased excretion of protein in the urine.

Zinc is an essential trace metal that is required for normal growth, DNA synthesis and wound healing in humans [6]. A wide-spread belief exists that Zn-deficiency can result in cancer, infection, skin diseases and wounds [29], due to which consumption of Zn at unknown or very high dosages commonly occurs [30]. This excessive consumption of Zinc as supplements poses a potential risk to human resulting in copper and iron deficiencies, poor growth and anaemia [31]. However, evidence relating to the use of this drug, which is reported to contain a high supplementation of Zinc (i.e., 388 mg/g) by the studies carried out by Sudha et al. [32], in treating urinary tract infection *in vivo* were lacking and therefore this was designed.

When the drug was administered to the mice for a period of 10 days, there was no toxic effects observed, but however when the drug was administered for a longer duration, toxic effects such as increased capsular space and vacuolation in the tubular region were noticed. Previous studies carried out by Ilango *et al.*[33], using NP up to 40 mg/Kg body weight for a period of 60 days have reported that the drug had caused no morphological changes on the kidney, thus confirming that this toxic effect could be the direct result of higher dosage. However, this higher dosage is essential as only at 600 mg/Kg body weight, the drug shows substantial anti-microbial property against the pathogenic strain used in the study. Also this toxic effect was reflected by the decrease in the body weight of the animals and by the variation in the levels of the various anti-oxidant enzymes analyzed. The drug treatment initially brought down the elevated levels of the anti-oxidant enzymes SOD and catalase, which were elevated due to infection. But this pattern of decrease in the levels of the anti-oxidant enzymes should have followed a similar pattern upon prolonged administration. But this pattern showed a deviation, whereby there was an increase in the levels of these enzymes, thus indicating that there could be a chance of toxicity due to the prolonged administration of the drug which contained large quantities of Zinc oxide particles.

Zinc when administered for a long period of time as an external supplement have been earlier reported to cause nephro-toxicity, where microscopic hematuria with or without renal failure have been reported in humans [34]. Also the results of this study coincide with the toxicity studies carried out Sharma and Paul [35], where they had reported NP to be slightly cytotoxic in the *in vitro* studies

they had carried out with Caco-2 cell lines. Nephro-toxicity and associated acute renal failure have also been reported in the case of other alternative treatment strategies involving high doses of metal supplementation [36]. In this study, the proximal tubules were affected and also evidence of tubulo-interstitial nephritis and glomerular damage due to the activity of the immune were reported.

Renal involvement during the ingestion of metal supplementation and its subsequent toxicity can present itself as renal failure, nephritic syndrome, tubule-interstitial nephritis with dysfunction or even lead to hypertensive encephalopathy, which at many times do not point towards metal accumulation and toxicity, thus eventually lead to the death of the individual [36, 37]. Continuous usage of Zinc at high doses for a long period of time can have adverse effect on the system, eventually leading to gastric distress and frequent food poisoning [38]. Also, it leads to a lower concentration of lipoproteins in the plasma and a decreased pattern in the absorption of copper, which could result in inhibiting iron transport and also by competing with its absorption in the body and eventually leading to anaemia [39].

CONCLUSION

Nagarpam, a herbal preparation enriched with Zinc oxide, is reported to be effective in treating pyelonephritis at 600 mg/Kg body weight. However, the continuous usage of this drug at high concentrations could lead to nephro-toxicity. Therefore more studies on optimising the dosage of this drug are warranted on the safe usage of this drug for longer periods of time.

ACKNOWLEDGEMENTS

The authors express their gratitude to Ms. Swetha A, M.Sc, who helped in generating the preliminary data for this study.

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