

The Evaluation of Lithiasis/Antilithiatic Activity of Oxalate-Degrading Bacteria and Routine Antibiotics

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ABSTRACT

Background: Nephrolithiasis is a common urinary tract disease, in addition to the pain and treatment costs, there might be significant complications resulting from the stones. This study aimed to investigate the lithiasis/antilithiatic activity of oxalate-degrading bacteria and routine antibiotics in rats. Male Wistar rats were divided into 3 groups. The rats in the group I received normal rat chow and drinking water (Positive control group). Groups II (Negative control group), and III rats received 1%Ethylene glycol containing diet for 30 days. Also, groups III rats received probiotic. In this study, 6strains of *Lactobacillus* and 2*Bifidobacterium* were used. Also, urinary and serum samples were collected and analyzed on 0 and 30th day. Then, Biochemistry and histopathological studies of the kidney, antibiogram, and oxalate degrading ability tests were carried out. The treatment group showed a significant reduction of hyperoxaluria, also, serum creatinine and calcium were decreased. In the treatment with probiotics, much improvement in the histopathological derangement was observed and showed a remarkable effect as histopathological features of the kidney's tissue reached up to the normal level which was almost similar to the positive control group. Based on the above results it can be concluded that oxalate-degrading probiotics have significant inhibitory effects on calcium oxalate urolithiasis.

Keywords: Calcium Oxalate; Ethylene Glycol; Nephrolithiasis; Oxalate-degrading probiotics.

INTRODUCTION

Urinary stone formation is one of the most common diseases of the genitourinary system, that despite its long history in the medical community, it is still considered to be a stable disease for which there is no definite cure (Hatch, 2013). The formation of kidney stones is mostly due to an imbalance in the consumption of oxalate rich-foods, on the other hand, hereditary background and lack of expression of several essential bacteria are effective in the process of decomposition of oxalate (Miller, 2013). Usually, the stones formed in the upper and lower parts of the kidney, and calyx separate from their place and enter the pelvis, and bladder. Larger stones require more advanced methods than ESWL, PCNL, and Laser to remove stones from different parts of the urinary tract, but these treatments will bear a huge cost for the patient and the community, while it is still not a definite treatment (Khan, 2017; Abratt,2010; McFarland,2018). And also, the presence of kidney stones in people's bodies is the cause of many kidney and liver diseases. Oxalate- or calcium-rich foods are usually involved in the formation of kidney stones. Oxalate increases in the

gut ,so the intestinal absorption of oxalate can increase serum, and urinary oxalate levels and therefore will cause the formation of calcium oxalate crystals (Turroni, 2007; Fijan,2014). Recently, it is claimed that there are several oxalate-degrading bacteria in the gastrointestinal tract that can reduce the oxalate content entered the digestive tract. However, the decrease in the density of these bacteria would increase the formation of oxalate stones and subsequently, the frequent formation of calcium oxalate crystals in the kidneys (Hill,2014). On the other hand, the reduction of these bacteria can be attributed to gastrointestinal diseases, oxalate-rich foods, and high antibiotic consumption. Studies show that oxalate-degrading bacteria, such as *Oxalobacter formigenes*, *Lactobacillus acidophilus*, *Bifidobacterium*, and *Enterobacteriaceae* can degrade oxalate during enzymatic pathways and convert it into a format and carbon dioxide (Venugopalan, 2010). The enzymes oxalyl coenzyme A decarboxylase (oxc) and formyl coenzyme A transferase (frc) play a key role in oxalate-degrading bacteria to degrade oxalate (La Fata,2018; Dimidi,2017). For the first time, Alison et al. (Allison,1977; Sidhu 1997; Ettinger,2014) identified oxalate-degrading bacteria called *Oxalobacter formigenes*, which could specifically decompose and convert oxalate into carbon dioxide and format. Subsequently, other researchers, including Hatch in a study of CF patients with kidney stones, reported that the colonization of *Oxalobacter formigenes* was reduced in the gastrointestinal tract due to overuse of antibiotics and that the risk of kidney stones was increased in these patients (Hatch, 2008). Neuhaus evaluated people with the kidney stones and reported that the number of oxalate-degrading bacteria, including *Oxalobacter formigenes* was significantly lower in the colon of patients than in the normal people, which in turn could increase urinary oxalate levels (Neuhaus,2000). Venugopalan and Amini studied the spinal cord injured patients with hyperoxaluria and found that these individuals were negative for oxalate-degrading bacteria. These individuals received an oral suspension of *Oxalobacter formigenes* and lactobacilli within two months (Amini2016; Venugopalan ,2010). The results showed that the oxalate content in a 24-hour urine sample was decreased from 0.6 to 0.41 mmol; and Of the 36 patients at the end of the study course, 22 (61%) were positive for the colonization of oxalate-degrading bacteria (Afkari, 2019; Liebman,2011). Also, other studies demonstrated that several strains of *Lactobacillus*, including *acidophilus*, *gasseri*, *plantarium*, *Streptococcus thermophilus*, and *Bifidobacterium infants*, had reduced the level of urinary oxalate. Between these strains, *acidophilus* and *gasseri* were tended to be more degrading of oxalate (Goldfarb,2004;Goldfarb,2019; Hatch 2008). On the other hand, studies showed that a high intake of antibiotics would increase kidney stone formation which may be caused by reducing the number of oxalate-degrading bacteria, this study aims to investigate lithiasis/antilithiatic activity of oxalate-degrading bacteria and routine antibiotics in rats.

MATERIALS AND METHODS

Experimental Animals & Dividing of Male Wistar Rats

Male Wistar rats weighing between 200-220g, were used in the study. Animals were housed in a laboratory, kept in a 12-hour light-dark cycle, temperature-controlled room (23±2°C), and relative humidity (50±10%). Animals were given a standard diet and drinking water. Also, Ethylene glycol was used to induce kidney stones in rats. Male Wistar rats were divided into three groups (n=6). The rats in the group I received normal rat chow and drinking water (Positive control group) Groups II (Negative control group), and III rats received 1% Ethylene glycol containing diet. Also, groups III rats received probiotic for 30days.

Preparation of probiotic culture

In the present study, *Lactobacillus acidophilus* PTCC 1643, *Lactobacillus Delbruki* PTCC1737, *Lactobacillus. Planetarium* PTCC 1745, *Lactobacillus.Casei* PTCC1608 , *Bifidobacterium Bifidum* PTCC 1644, *Bifidobacterium animalis subsp. Lactis* PTCC 1736, *Streptococcus salivarius subsp. Thermophilus* PTCC 1738 and also strains *L. paracasei* AKPL-IR <<http://www.ncbi.nlm.nih.gov> (JF461540.1), *L. Paracasei* AKKL-IR <<http://www.ncbi.nlm.nih.gov> (JF461539.1) were used showing the high power in oxalate degrading in culture media.

Media and determining the oxalate-degrading capability

The procedure for determining the oxalate-degrading capacity of probiotic isolates was based on the method previously described by Campieri. Briefly, 5ml of filtered sterilized ammonium oxalate solution [20 mM/l ammonium oxalate and 40 g/l dextrose (Germany)] was added to 5 ml of the base media (Protease peptone 20 g/l, the yeast extract 10 g/l, Tween 80 2ml/l, KH₂PO₄ 4g/l, NA acetate 10g/l, di-ammonium-hydrogen-citrate 4g/l, MgSO₄.7H₂O 0.1 g/l and MnSO₄ 0.1 g/l). Culture broths inoculated at 2% into the base media containing 20 mM ammonium oxalate. The base media supplemented with 0.05%(v/v) L-cysteine hydrochloride when inoculating probiotic species and all cultures incubated anaerobically at 37C for 48 hours. A media control (ammonium oxalate base media) prepared as above, but without inoculating bacteria. Optical density (600 nm) and plate counts (Colony Forming Units/ml) performed to determine the growth of each strain in Ammonium oxalate based media, which compared with growth in basal media. Ammonium oxalate based media cultures and the media control centrifuged at 3000 RPM for 10 mins and the supernatants filter sterilized using 0.45 µm filters (Sartorius AG, Göttingen, Germany). The culture filtrates stored at 4C until plate counts recorded and the analysis performed on strains that grew in 20mM ammonium oxalate based media, but all strains cultured in an ammonium oxalate plate (20mM, 40 mM and 60 Mm), to test the oxalate degradation power in the bacteria studied Campieri (Campieri,2001; Okombo,2010).

Antibiotic susceptibility test for oxalate-degrading probiotics

Routine antibiotics were used to investigate the antibiotic susceptibility of the bacteria used in this study. To perform the test, the pure colonies were first inoculated in M.R.S broth medium (under anaerobic conditions at 37-38°C for 24h) and then cultured onto a plate containing Tomato Juice Agar medium with a sterilized swab using lawn culture method. With the aid of sterile forceps, the antibiotic discs including vancomycin (30mcg), novobiocin (30mcg), polymixin B (300units), doxycycline (30mcg), rifampin (30mcg), erythromycin (15mcg), ampicillin (30mcg), tetracycline (30mcg), chloramphenicol (30mcg), amoxicillin (25cmg), kanamycin, amikacin, gentamicin, cephalexin, clindamycin, penicillin (10units), oxacillin (1mcg) were seeded onto the medium with certain distances of 15mm from the edge of the plate and 24mm between the two discs. The plates were then placed in an anaerobic jar at 37°C for 37-48 hours. The antibiotic susceptibility of oxalate-degrading bacteria was assessed by measuring the diameter of the zone of inhibition (ZOI).

In vivo urinary oxalate levels using selected probiotic

In this study, for the Intervention group twice suspensions of probiotic with distilled water were gavaged to the rats (doses of $\sim 10^{11}$ CFU) for 30days, they were weighed weekly and their urine & serum samples were gained in the 0, 2nd, and 4th weeks by placing the animals in metabolic cages for a 24h period.

Collection of urine and serum

On the day 0,15 and 30, the rats were placed in metabolic cages and 24-hour urine was collected with 0.02 percent sodium azide as a preservative to prevent bacterial growth. After determining urinary volume and pH, urine was aliquoted for various tests. Urinary Oxalate, Calcium, and Creatinine were determined using the commercial kit in semiautomatic photometer according to the manufacturer's protocol. Each week one-hour urine samples were collected before the start of 24hr urine sample collection and examined by the light microscope to analyze CaOx-crystalluria (24). The rats anesthetized with Xylasein/Ketamine by Intramuscular (IM) injection (Xylasein 0.55 and Ketamine 10mg/kg Bodyweight) and the blood was taken from the orbital sinus into the centrifuge tube without anticoagulant, allowing to clot at room's temperature and then centrifuged to collect serum. After collecting the serum it was subjected to analysis of serum Creatinine, urea and Calcium.

Analysis of histopathology and CaOx crystal in kidney

The kidney tissue from each group was fixed at 10% neutral buffered formalin, trimmed, processed, and set in paraffin. Sections from each kidney were stained with hematoxylin and eosin and examined under the light microscope for pathological analysis as well as the polarized light

microscope for visualizing CaOx crystal. The pathological analysis was examined with the help of a qualified pathologist. The statistical analysis of the antibody level among the different groups was performed based on a one-way and two-way ANOVA using SPSS version 16. where $P_{\text{-value}} < 0.05$ was considered a significant difference.

RESULTS

Serum & Urine analysis

After administration of ethylene glycol (1%) and ammonium chloride (1%) for 3 days, a significant difference was observed in a way that calcium oxalate crystals in urine of each group of animals was observed when compared to the positive control group. On the administration of probiotics for 30 days (Intervention group), also a significant reduction of calcium oxalate crystals in urine was noted when compared to the negative control group. Ethylene glycol (EG) and ammonium chloride (AC) treated groups (the negative control group), calcium, creatinine and oxalate were found to be increased significantly in urine, while, in Intervention group, probiotics significantly reduced the levels of creatinine, oxalate and calcium in urine comparing to that of the negative control group. On treatment with probiotics, serum calcium, urea and creatinine were found to be significantly reduced when compared to the negative control group (Table 1).

Evaluation of oxalate-degrading activity in probiotic bacteria studied

In this study, oxalate kit and ammonium oxalate used in culture medium were used to evaluate and confirm the oxalate-degrading capacity of bacteria, and different concentrations of ammonium oxalate (20 mM, 40 mM and 60 mM), were used in the MRS culture medium containing bacteria. After incubation, it was observed that all strains could grow at a concentration of 20 mmol of ammonium oxalate, and the strains of *Lactobacillus planetarium* and *Lactobacillus casei* were able to grow at the concentration of 40 mmol of ammonium oxalate, while *L. paracasei* AKPL-IR and *L. Paracasei* AKKL-IR managed to grow and decompose ammonium oxalate at the concentration of 60 mmol, which were recorded on the NCBI site as potent oxalate-degrading bacteria.

Antibiogram test for oxalate-degrading probiotics

In this study, the resistance pattern of oxalate-degrading bacteria to common antibiotics was evaluated by the NCCLS method (Diagram 1), using the antibiotic discs. All bacteria studied showed 100% sensitivity to tetracycline, oxacillin, novobiocin, vancomycin, rifampin, gentamicin, clindamycin, and polymyxin B, as well as intermediate sensitivity and resistance to other antibiotics. In other words, the oxalate-degrading bacteria had an intermediate pattern to ampicillin, amoxicillin, penicillin, doxycycline, cephalixin, and were completely sensitive to most of antibiotics, but none showed 100% resistance.

Analysis of histopathology and CaOx crystal in kidney

In kidney histopathology analysis, CaOx crystal was found to be significantly increased in the animals of the negative control group. In the Intervention group, after treatment with probiotics, the CaOx crystal level was significantly reduced when compared to the negative control group. Histopathological reporting revealed normal construction of kidney tissue in the positive control group. In the urolithiasis group, tubules have shown irregular material and structure, while in the treatment with probiotics, much improvement in histopathological derangement was observed, which was almost similar to the positive control group (Fig. 1-3).

DISCUSSION

Dietary oxalate plays an important role in the excretion of urinary oxalate (Uox) and calcium oxalate crystal is one of the most essential factors in kidney stone formation. Today, so many research were conducted and showed that the identification of intestinal oxalate degrading bacteria provided a new direction for the reduction of Uox (Pirbaglou, 2016; Behnsen, 2013). The present study seeks to examine the efficacy of using oxalate-degrading probiotics to degrade the intestinal oxalate thereby preventing hyperoxaluria and CaOx urolithiasis in rats (Heeney, 2018; Rossi 2016;

Talei&soleimani., 2021). In this study, ethylene glycol (EG) ingestion to rats has been widely used as an experimental model for the study of nephrolithiasis. When EG is used, kidney crystal deposition can be quite incompatible. To achieve a uniformly high rate of kidney's crystal deposition, ammonium chloride (AC) has been used in combination with ethylene glycol. When male Wistar rats were treated by 1% Ethylene glycol with 1% or 2% ammonium chloride, almost all of them showed deposition of the calcium oxalate (CaOx) crystals in the kidney within 3 days. After administration of the lithogenic agent in the negative control group, large and plentiful crystals were observed in the urine and kidneys, symptoms such as blood excretion of the urine, shrinking and degeneration of the kidneys, a severe decrease in a 24-hour urine volume, the presence of calcium oxalate crystals in the kidneys, severe weight loss in the rats, as well as wounds and hemorrhages in the hands, and urinary and fecal excretion in the rats was accompanied by pain, anal bleeding, and severe physical contraction were observed. As well, in macroscopic and microscopic examination of renal tissue, impaired renal filtration, renal atrophy, and hematuria were observed.

Creatinine and urea are the indicators of kidney and tubular damage. Glomerular filtration rate (GFR) decreases in kidney's tissue injury; it may be due to the presence of stone in the urinary tract, which obstructs urine flow and the waste products (Shimizu, 2015; Kim, 2018). Urinary creatinine is found to be increased in the negative control group, which may be due to a decrease in GFR.

According to the results of the present study, the Intervention group experienced a decrease in the sedimentation of oxalate and calcium in the urinary tract, which would demonstrate the lack of formation of renal stones. In fact, after treatment with *oxalate-degrading probiotics* for 30 days, urinary crystals were reduced significantly. This effect could help preventing the stone formation by the excretion of small particles and reducing their chances of retention in the urinary tract. Reduction of calcium level in urine provides less calcium to bind with oxalate which will result in the reduction of calcium oxalate crystals. in the intervention group, there seems to be a sharp decrease in urinary oxalate levels and calcium oxalate crystals in their kidneys, so that the healing of inflammation of the hands is observed in all rats of the group consuming oxalate-degrading bacteria, as well as rats' weights. And the 24-hour urine volume is similar to the positive control group; also, at the end of the treatment period, there was no significant difference between the weight of the intervention rats and the healthy group. It is to say that, in the intervention group, parenchymal components of the renal tissue, were histologically normal and lacked any pathological lesions and calcium oxalate sedimentation.

Histologically, no renal glomerulus atrophy, kidney bruise, and the presence of calcium oxalate deposits or crystals were seen at the end of the course of treatment with oxalate-degrading probiotics. Also, Kwak et al. investigated the role of lactobacilli in reducing calcium oxalate crystals. After isolating and identifying lactobacilli, the oxalate degrading activity was evaluated using oxalate kits (Kwak, 2001). The oxalate-degrading lactobacilli was then added to the diet of rats with calcium oxalate stone-forming kidneys and high levels of urinary oxalate. These rats were treated with lactobacilli, after 30 days, no calcium oxalate crystals were observed and the regeneration was seen in the renal tissue of the rats. In two similar studies, Hoppe and Hatch examined oxalate-degrading bacteria in patients with hyperoxaluria or those with calcium oxalate stones in the urinary tract and kidneys. These bacteria were present in very small numbers, and most patients were negative for colonization of *Oxalobacter formigenes*, indicating that after treatment with oxalate-degrading lactobacilli, the urinary oxalate levels were decreased by 92% due to the stable establishment and colonization of oxalate-degrading bacteria (Whittamore,2017; Hatch, 2003). By prescribing oxadrop (containing oxalate-degrading bacteria), Lieske et al. were able to significantly reduce the urinary oxalate levels in patients with hyperoxaluria and calcium oxalate stones (Lieske, 2005). Taking this drug for three months reduced the urinary oxalate excretion by more than 90%. The results of our study are consistent and similar to their study.

On the other hand, In vivo, and in vitro studies have shown that taking Clarithromycin and Doxycycline eliminated the strains of *Oxalobacter formigenes* and lactobacilli. In the presence of

ampicillin and clavulanic acid, these bacteria are killed much faster. Gastrointestinal microbiota, including *Oxalobacter formigenes* and *Lactobacilli*, are highly sensitive to streptomycin and penicillin, causing the loss of 90% of the bacteria in the gastrointestinal tract (Perez, 2002). Susana researched *Lactobacilli* and their susceptibility to antibiotics and evaluated the antibiotic susceptibility pattern in 22 strains of *Bifidobacteria* and *Lactobacillus* species to 12 antibiotics. All strains were resistant to chloramphenicol, imipenem, and most strains were resistant to metronidazole; four were sensitive to tetracycline, and half of them were sensitive to cefixime, and the other half were resistant, but all were sensitive to vancomycin (Susanne, 2003). In the current study, oxalate-degrading *Lactobacilli* showed 100% sensitivity to tetracycline, oxacillin, novobiocin, vancomycin, rifampicin, gentamicin, clindamycin, polymyxin B, and also showed intermediate sensitivity and intermediate resistance to other antibiotics, but none of the antibiotics had 100% resistance. Therefore, characterization and isolation of oxalate-degrading Probiotics from the human colon are required and could be analyzed for future studies.

CONCLUSION

based on the above discussion it is concluded that the oxalate-degrading Probiotics demonstrated a significant lithotriptic effect on experimentally induced calcium oxalate urolithiasis. The present study was an agreement with the studies reporting that the presence of Probiotics is responsible for the lithotriptic activity of probiotic drugs.

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Table1: Effect of oxalate-degrading bacteria on serum & urinary parameters in rats

Sample	parameters	unit	Positive control group M±SD	Negative control group M±SD	treated group M±SD	S/NS
Urine/24h	Oxalate	mmol/d	5.2±3.2	8.6±0.25	4.1±2.19	<i>p</i> <0.05
	Calcium	mg/d	1.57±1.1	5.8±0.19	1.83±0.22	<i>p</i> <0.05
	Creatinine	mg/d	0.88±1.22	1.1±0.15	0.71±0.01	<i>p</i> <0.05
	Urine volume	ml/d	8.30±0.12	4.32±1.24	9.35±5.13	<i>p</i> <0.05
Serum	Calcium	mg/dl	7.82±1.21	9.12±1.08	7.14±2.02	<i>p</i> <0.05
	Creatinine	mg/dl	0.82±1.02	2.02±1.52	0.78±0.42	<i>p</i> <0.05
	Urea	ml/dl	38.42±3.21	69.15±4.32	40.36±2.53	<i>p</i> <0.05
Wight		g	220±10	120±5	230±5	<i>p</i> <0.05

Data are presented as mean± standard deviation ; S: Significant; NS:Non Significant.

Diagram.1: The result of antibiogram test:

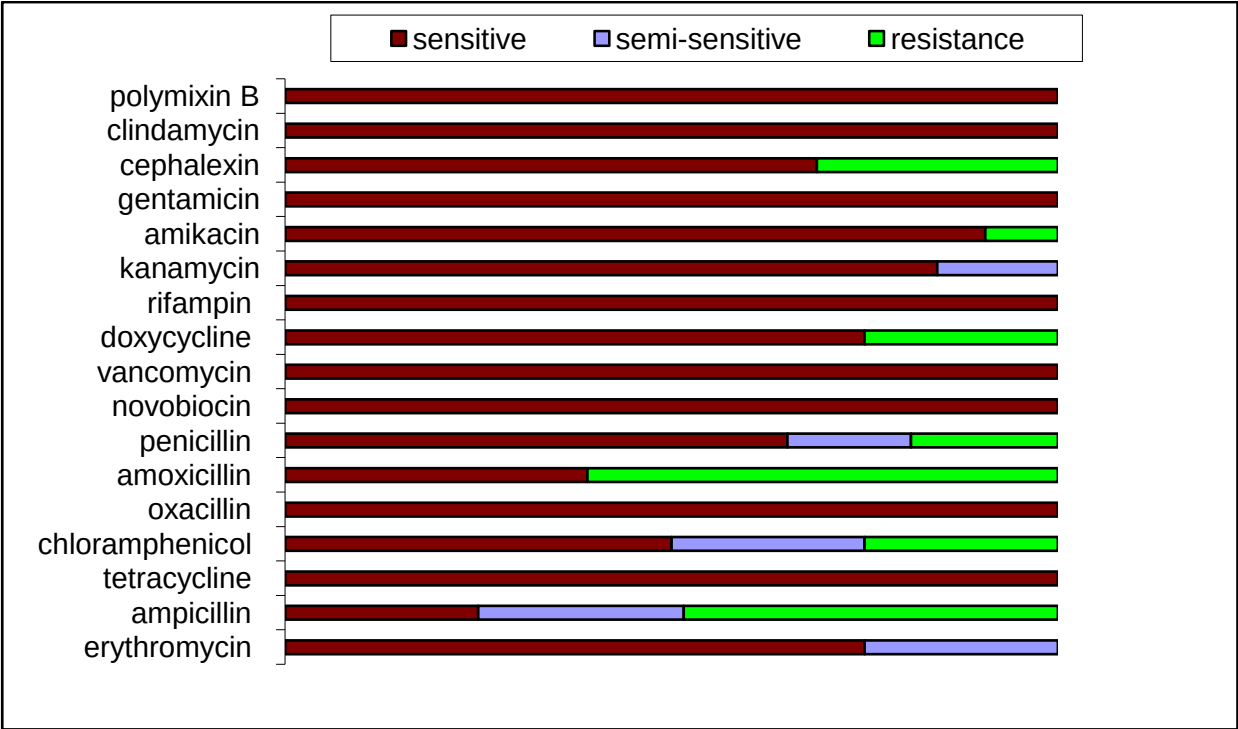
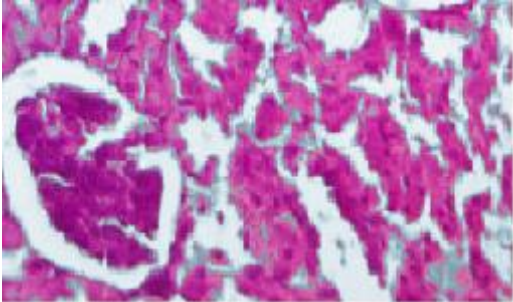
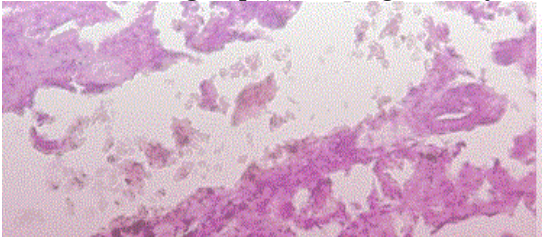


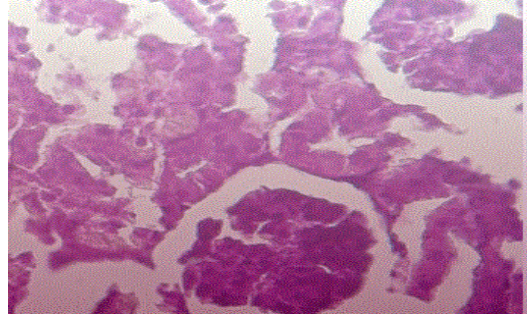
Fig.1–3:Microscopic images by light microscopy of kidney sections after Haematoxylin / Eosin staining.



Positive control group (A) showing a healthy structure of glomerulus,tubules, and blood vessels.



The **negative control group (B)** Shows a distorted and abnormal structure with calcium oxalate deposits.



In the **probiotic-treatment group (C)** indicates an intact tissue structure of the kidney, normal blood vessels, similar to the positive control group.