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Apple Juice Prevents Ethylene Glycol-Induced Urolithiasis in Wistar Rats (*Rattus norvegicus*)

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Abstract:
In this study the antiurolithiatic effect of the commercialized apple juice, Ceres™ and fresh apple juice in ethylene glycol-induced Wistar rats were tested. The calcium oxalate formation, body weight, kidney weight index, and biomarkers from blood and urine related to kidney function were evaluated. Twenty-one (21) male albino Wistar rats were divided into seven groups with three replicates each. Group I was not induced with ethylene glycol and untreated, while groups II, III, IV, V, VI, and VII were induced with ethylene glycol (0.75%) for 28 days. Group II was the negative control, it was untreated. Groups III and IV were the preventive group treated for 28 days with 200 µl of commercialized and fresh apple juice respectively. The group V and VI (curative group) was treated for 14 days with 200 µl of commercialized and fresh apple juice respectively. Group VII, treated with Rowatinex for 14 days. The results showed that the ethylene glycol successfully induced calcium oxalate crystals which cause urolithiasis. Both apple juice of the preventive group inhibits the formation of the calcium oxalate crystals in the histological section of the rat's kidney. It decreases the formation of calcium oxalate stones in the curative group. However, the apple juice has no effect in the body and kidney weight, BUN, creatinine, urine specific gravity and urine pH level. Data suggest that apple juice has no influence in restoring kidney function, however it show that it could prevent calcium oxalate stone formation, suggesting its antiurolithiatic potential.

Key words: Apple, Urolithiasis, Ethylene glycol, Calcium oxalate crystals, Antiurolithiatic

1. Introduction

The kidneys are one of the most important organs in the human body. It helps to detoxify and filter impurities from the blood, as well as the waste products from the urine (Sonali, 2013). Kidney stones are one of the common diseases in the urinary tract in our time. About one in seven men and one in 15 women will be diagnosed with kidney stones during their lifetime (Reyes, 2009). One Filipino every hour dies from kidney failure today, according to the Department of Health (DOH). It was once considered a disorder of the middle aged but today children, as young as 5 or 6 years old are showing symptoms of kidney disorder (Tarkan, 2008). Kidney stones (also called nephrolithiasis or urolithiasis) develop when a collection of minerals or other material form a small "stone." The stone can cause pain, block the flow of urine, and rarely cause long-term kidney problems if it is not recognized and treated promptly (Smith *et. al*, 2013). It is usually formed within the kidney, where urine collects before flowing into the ureter, the tube that leads to the bladder (Johnson, 2013). The chemical composition of stones depends on the chemical imbalance in the urine.

A kidney stone usually forms when substances that are normally found in the urine, such as calcium, oxalate, cystine, or uric acid, are at high levels. Calcium stones are the most common. They can be made of calcium oxalate (most common), phosphate, or maleate. Vitamin C and spinach contain oxalate. Calcium-based kidney stones are most commonly seen in young men between the ages of 20 and 30 (Krucik, 2012). However, in some children, stones can also form even if these substances are at normal levels. Knowing what the stone is made of is important in deciding which treatment to use. The substances form crystals, which become anchored in the kidney and gradually increase in size, forming a kidney stone. Stones that are very small (less than 5 millimeters [0.2 inches]) can usually pass on their own, while larger stones usually require treatment (Smith *et. al,* 2013).

Surgery, oral intake of medicine, good voiding habits and natural home remedies are types of the treatment to cure kidney stones. In patients with a solitary kidney, a kidney stone causing obstruction may lead to kidney failure and emergent referral to an urologist may be required to remove the stone or place a stent to bypass it. The type of procedure depends upon the location of the stone. If the stone is near the junction of the ureter or bladder, ureteroscopy may be an option. If the stone is high in the ureter near the bladder, an interventional radiologist may need to place a percutaneous nephrostomy or drain through the skin directly into the kidney to allow urine to drain. Patients with obstructing kidney stones who develop urinary tract infections may need to have a stent or nephrostomy placed to prevent progression of the infection. Large stones that are located in the kidney or the upper ureter may be unable to pass spontaneously. Lithotripsy, sometimes called extracorporeal shockwave lithotripsy (ESWL) uses ultrasound to deliver energy to break up the large stone into smaller stones and debris that then can be passed or captured by ureteroscopy. Patients with a large stone that fills the whole kidney (staghorn calculi) may require removal of the kidney. The patient may be recommended to take medication for pain, such as ibuprofen as an anti-inflammatory. Tamsulosin (Flomax) is a medication often prescribed to help promote stone passage (Stoppler, *et. al,* 2013). Good voiding habits, particularly frequent urination, are important. Therefore, of all the preventive recommendations, drinking enough fluids is the most important guideline for people with any type of kidney stones. Natural remedies are also an option to prevent surgery. Natural remedies like lemon juices, olive oil, raw apple cider vinegar, pomegranate juice and magnesium are used to cure kidney stones (Group, 2013). The lemon juice is an example of citrus fruit, which has a high amount of citrate, or citric acid. This citrate binds with calcium in the urine, thereby reducing the amount of calcium available to form calcium oxalate stones. It also prevents tiny calcium oxalate crystals from growing and massing together into larger stones. It makes the urine less acidic, which inhibits the development of both calcium oxalate and uric acid stones (Ebsco, 2011). Thus, patients with stones should avoid cola drinks with phosphoric acid, because they can severely reduce citrate levels in the urine (Vishalakshi, 2012).

Apple is one of the most popular fruits. It is widely used as a natural remedy for some diseases such as kidney stones. However, there has been no scientific study about its effect in the said disease. This wonderful fruit is packed with rich phyto-nutrients that are actually indispensable for optimal health. The antioxidants in apples have much health promoting and disease prevention properties (Rudrappa, 2014). Apples may help reduce the risk of developing cancer, hypertension, diabetes, and heart disease (Cool, 2013). It contains important nutrients such as vitamin C, B-complex, phytonutrients, and minerals like calcium, potassium and phosphorus (Nordqvist, 2013). Therefore, this fruit may be a cure for certain diseases like kidney stones.

This study generally aimed to evaluate the effect of apple juice on ethylene glycol-induced urolithiasis Wistar rats. Specifically, the effect of the apple juice on kidney in terms of calculi formation and kidney weight to body weight ratio, the effect of the apple juice in kidney function in terms of blood urea nitrogen (BUN) and serum creatinine, and the effect in the urine specific gravity and pH were determined.

2. Materials and Methods

Preparation of Apple Juice

A commercialized Ceres™ apple juice and fresh Fuji apple juice were used in this study. The commercialized apple juice was purchased from the market. The fresh apple was cut into small slices without removing the peels. The seeds were removed and the apple fruit was placed in the juicer to get its extract.

Experimental Animals

Nine week-old male albino rats of Wistar strain weighing between 100-150 g were used. The animals were fed with bow wow feed pellets and were given water *ad libitum*. Experimental animals were acclimatized for 14 days after purchasing them in Libertad, Pasay City to adjust to the new environment. The animals were housed in cages individually. The rats were under controlled conditions of 12:12 light dark cycle (Satish, 2010). Water bottles and feeders were cleaned regularly. All procedures regarding handling of the test animals were in accordance with the existing guidelines of the Philippine Association of Laboratory Animal Science (PALAS) for care and use of laboratory animals and with Administrative Order 40 of the Bureau of Animal Industry relative to Republic Act No. 8485 (PALAS CODE, 2002).

Induction of Urolithiasis

Ethylene glycol was used to induce urolithiasis. It was obtained from the instrumentation of Bicol University College of Science. The ethylene glycol (0.75%) was added to the drinking water of the experimental animals for 28 days, everyday. Six (6) groups were induced with ethylene glycol. The treatments was given once daily by gavage (Satishi, 2010).

Experimental Design

The experimental animals were divided into seven (7) groups. There were 3 replicates in each group. Group I served as the control group; it was not induced with ethylene glycol and untreated. Groups II, III, IV, V, VI, and VII were all induced with ethylene glycol for 28 days (Table 1). Group II served as the negative control; it was induced with ethylene glycol for 28 days but untreated. Groups III and IV served as the preventive group wherein, they were induced with 200 µl of commercialized apple juice and fresh apple juice, respectively for 28 days starting day 1 of induction of ethylene glycol. The groups V, VI, and VII were treated for 14 days, starting day 15 of induction of ethylene glycol. Groups V and VI are the curative group treated with 200 µl of the commercialized apple juice and fresh apple juice, respectively. The group VII, treated with one (1) capsule of Rowatinex.

Table 1. The experimental design

Treatments	Subgroups	Dose	Duration of Treatment
Control group	Untreated and Un-induced with EG (I)	N/A	N/A
	Untreated and Induced with EG (II) (Negative control)	N/A	N/A
Preventive group	Commercialized apple juice (III)	200 µl (1^{st}-28^{th} day)	28 days
	Fresh apple juice (IV)	200 µl (1^{st}-28^{th} day)	28 days
Curative group	Commercialized apple juice (V)	200 µl (15^{th}-28^{th} day)	14 days
	Fresh apple juice (VI)	200 µl (15^{th}-28^{th} day)	14 days
Positive group	Rowatinex (VII)	1 capsule (15^{th}-28^{th} day)	14 days

Body Weight and Kidney Weight

The weight of animals was recorded at the beginning of experiments and 24 h after the last treatment. Rats were dissected and the kidneys were removed and weighed. The kidney weight index was computed.

Urine Analysis

On day 28, animals of all the groups were kept in metabolic cages and urine samples were collected for 6 hours and were analyzed for the specific gravity, and pH using standard method. The urines collected were analyzed in Tanchuling Hospital laboratory.

Serum Analysis

The experimental animals were anesthetized by chloroform. The blood was collected through cardiac puncture of the rats. One mL of blood was placed in the vials and was sent to Tanchuling Hospital laboratory for the analysis of creatinine levels, and blood urea nitrogen. Blood chemical analysis related to kidney function were performed in Tanchuling Hospital laboratory.

Histological Evaluation

The right kidneys were excised and were placed in 10% formalin (Brzoska, et. al, 2002). These were sent to the Hi-Precision Diagnostic Laboratory for histological preparations. Histological evaluation of the kidney was done by examining the organ, comparing the histological morphology of untreated kidneys in varying extract concentrations to other treatment groups. The calculi/stone formation and the kidney weight were observed. The slides with the sample of the kidneys were examined under light microscope and CaC_2O_4 deposits were determined. Aggregations of CaC_2O_4 deposits (tubules containing CaC_2O_4 deposits) were counted in 10 microscopic fields and expressed as mean ± standard error for each group (Hadjzadeh *et. al*, 2007).

Statistical Analysis

The results were expressed as mean±SEM. Statistical analysis by using GraphPad InStat, ANOVA test was performed for multiple comparisons and was followed by Tukey's test and P<0.05 was considered as significant.

3. Results and Discussion

The study evaluated the amtiurolithiatic activity of apple juice in ethylene glycol-induced Wistar rats. Specifically, it determined the effect of commercialized apple juice (Ceres ™) and fresh apple juice on kidney in terms of calculi formation, body weight after 28 days, body weight difference, and kidney weight index; evaluated the effect of the apple juice in biomarkers related to kidney function in terms of blood urea nitrogen and serum creatinine; and determined the effect in the urine specific gravity, and pH.

Calcium Oxalate Crystal Formed in the Rat's Kidney

In this study, male Wistar rats were used because the urinary system of male rats resembles that of humans (Vyas *et. al*, 2011). Female Wistar rats as reported by Iguchi *et. al* (1999) and Lee *et. al* (1996), releases a female sex hormone which has an inhibitory effect on kidney stone formation wherein it showed significantly less in the amount of stone deposition in comparison with male albino Wistar rats (Karadi *et. al*, 2006).

In this study, the experimental rats were divided into seven groups. One group (group I) was untreated while the rest of the groups (group II-VII) were induced with E-glycol in their drinking water for twenty-eight days. The kidneys of the rats were subjected to histological analysis. The representative kidney sections of the experimental rats are shown in figure 2. Normal rats (not induced with EG) shows renal tubules with no dilation and inflammation of kidney (Fig. 2A). The normal histological section of the kidney shows that the cortex is characterized by spherical renal corpuscles and the oval or circular profiles of the convoluted tubules of the nephrons as described by Ozbek *et. al* (2003). However, the kidney of the untreated but induced with ethylene glycol Wistar rats showed presence of large-sized of calcium oxalate crystals in a renal tubule, tubules have dilation with interstitial inflammatory infiltrate due to the crystal deposits (Fig 2C and 2D). The crystals observed were large polygonal in shape, heterogeneous in distribution and pattern, which was the same observation viewed in the study of Shukla *et. al* (2013).

The present study successfully induced calcium oxalate formation in rat's kidney by adding the EG to drinking water just like in other studies (Hadjzadeh *et. al* (2008), Kalpeshin *et. al* (2011), Khalili *et.* a,l (2011)) (Fig. 2B and 2C). The studies mentioned developed calcium oxalate crystals in male Wistar rats following the same procedure.

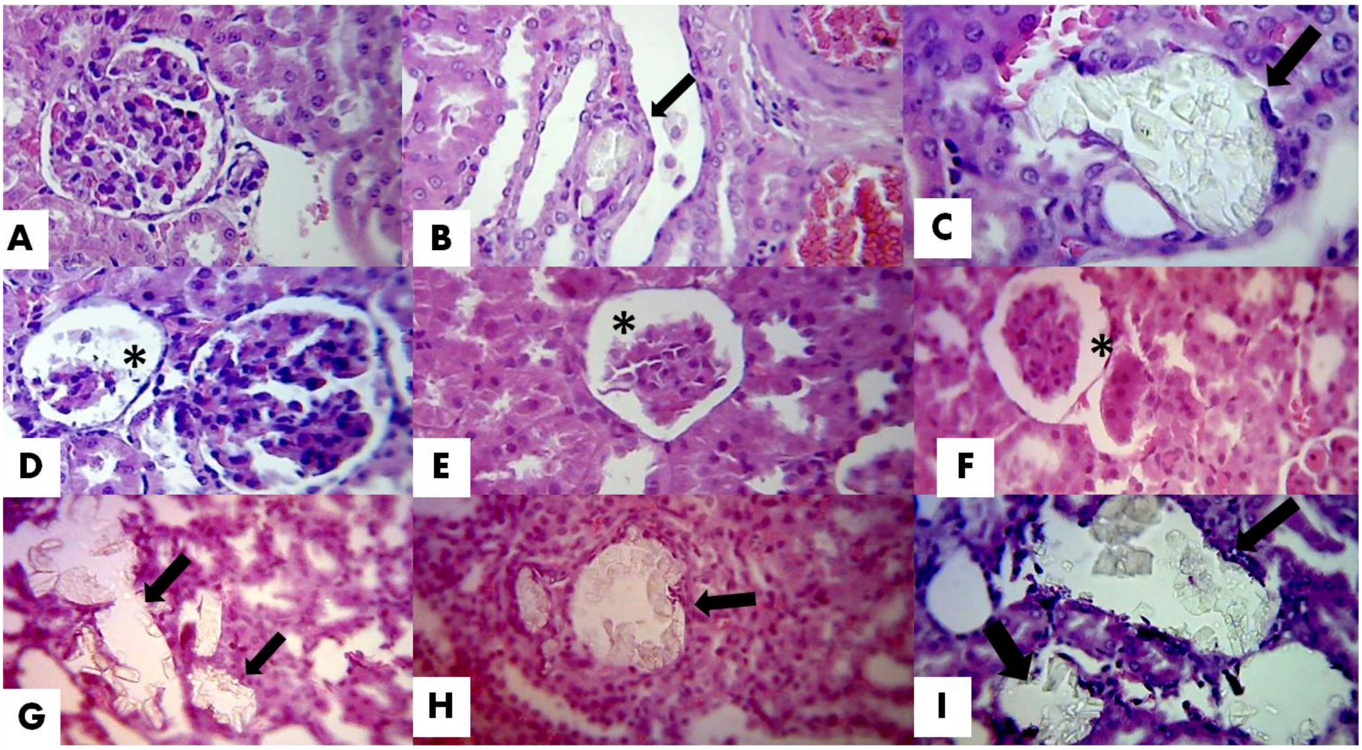

Figure 2. Representative microscopic images of glomerulus kidney sections from (A) Control group (B and C) Negative group, induced with E-glycol for 28 days (D and E) Commercialized apple juice for 28 days (F) Fresh apple juice for 28 days (G) Commercialized apple juice for 14 days (H) Fresh apple juice for 14 days (I) Rowatinex for 14 days; The arrow shows the presence of calcium oxalate crystals; The asterisks (*) indicate the renal tubular dilation. X=400x

In this study, groups III and IV are induced with EG and treated with apple juices, commercialized and fresh for twenty-eight days, respectively to serve as the preventive group. Histological sections show that after of both juices for twenty-eight days no calcium oxalate crystals were observed (Fig 2D and 2F). Kidney section of untreated rats showed abundant crystal deposition. In addition, renal epithelial cells had more tubular dilatation and damage as shown by large spaces in the tissue (Fig 2B and 2C). Also, the present study counted the number of calcium oxalate crystals formed. Results showed that that groups III and IV were statistically significant compared to the negative group ($P<0.05$) and comparable to the control group. This result suggests that apple juice prevents or inhibits calcium oxalate depositions, though histological sections show partial restoration of the kidney since renal tubular dilation with epithelial damage was still observed (Fig, 2D-2F). It was also shown that group III (commercial apple juice) was not statistically different from group IV (fresh apple group), suggesting that both juice have the same effect in protecting calcium oxalate deposition in the kidney (Table 2).

Table 2. Number of calcium oxalate crystals deposits in the kidney

Treatments	Subgroup	Counted number of calcium oxalate crystals
Control group	**Untreated (I)**	0 ± 0.00 [b]
	Negative control (II)	25 ± 8.15 [a]
Preventive group	**Commercialized apple juice (III)**	0 ± 0.00 [b]
	Fresh apple juice (IV)	0 ± 0.00 [b]
Curative group	**Commercialized apple juice (V)**	9.33 ± 7.42
	Fresh apple juice (VI)	3.33 ± 2.85
Positive group	**Rowatinex (VII)**	11 ± 5.86

Values expressed in Mean ± SEM; SEM= Standard Error of the Mean; a= Significant at P<0.05 compared to group I; b= Significant at P<0.05 compared to group II.

The present study aims to determine the curative potential of both apple juices. Two groups Group V and VI were induced with EG for 28 days and treated with apple juice for fourteen days. Histological sections of kidneys of rats treated with two apple juices show less tubules dilation with interstitial inflammatory infiltrate, still with calcium oxalate crystals but less in number (Table 2) and reduced in size (Fig 2G-2H). This effect is comparable with the positive control, Rowatinex is a commonly used as antiurolithiatic drug (Fig. 2I). The curative groups may not eliminate the calcium oxalate crystals but it decreases its formation. The same result was observed in the study of Thangarathinam *et. al* (2013) wherein less crystal deposits were observed compared to the untreated group.

Body and Kidney Weight

The body weight and kidney weights were also determined in this study. According to Kuo *et. al* (2003), kidney weight is increased in EG treated rats, it is due to renal papillary hypertrophy and crystal depositions. Due to the increase in promoter's level after ethylene glycol ingestion, it led to stone formation and aggregation in the kidney. Also, it was shown that crystal formation of kidney lead to weight loss. Results show, the initial body weight, the body weight difference, the body weight after 28 days of treatment and kidney weight index in all the groups does not have any significant difference compared to the other groups (Table 3).

Table 3. Body weight and kidney weight index

Treatments	Subgroup	Initial Body weight [ns]	Body Weight Difference [ns]	Body weight after 28 days [ns]	Kidney Weight Index [ns]
Control group	**Untreated (I)**	103.33 ± 3.33	10.53 ± 3.30	113.87 ± 6.47	0.44 ± 0.05
	Negative control (II)	120 ± 15.78	40.5 ± 11.23	160.5 ± 14.92	0.46 ± 0.04
Preventive group	**Commercialized apple juice (III)**	125 ± 7.64	45.07 ± 19.49	165.87 ± 18.96	0.42 ± 0.03
	Fresh apple juice (IV)	120 ± 15.28	36.03 ± 10.33	126.23 ± 18.75	0.46 ± 0.03
Curative group	**Commercialized apple juice (V)**	125 ± 2.89	6.23 ± 3.49	161.03 ± 10.15	0.58 ± 0.11
	Fresh apple juice (VI)	101.67 ± 1.67	15 ± 7.12	115.93 ± 8.52	0.38 ± 0.08
Positive group	**Rowatinex (VII)**	120 ± 0.00	27.93 ± 10.27	142.87 ± 15.31	0.54 ± 0.01

Values expressed in Mean ± SEM; SEM= Standard Error of the Mean; ns= not significant.

The deposition of stone in kidneys resulted to the increase in weight of the dry kidney and wet kidney weight in the study of Jie, *et. al* (1999). In terms of body weight there would be decrease in body weight because of the intense pain associated with stone formation which may lead to decrease in the food consumption (Ringold, *et. al*, 2005). However, these kinds of result were not well observed in the present study.

Effect on the Biomarkers related to Kidney Function

Several tests were also conducted in this study such as the serum analysis wherein the biomarkers related to kidney function like blood urea nitrogen (BUN) and serum creatinine were analyzed. These types of blood tests are biomarkers for kidney function.

Calcium oxalate stone deposition decreases glomerular filtration rate due to obstruction to the flow of urine. This leads to accumulation of waste products, particularly nitrogenous substances such as creatinine, uric acid and BUN in the blood (Ghodasara *et. al*, 2010). Among the 6 groups there was no significance in terms of the BUN (Figure 3). The apple juice has no effect in lowering the BUN.

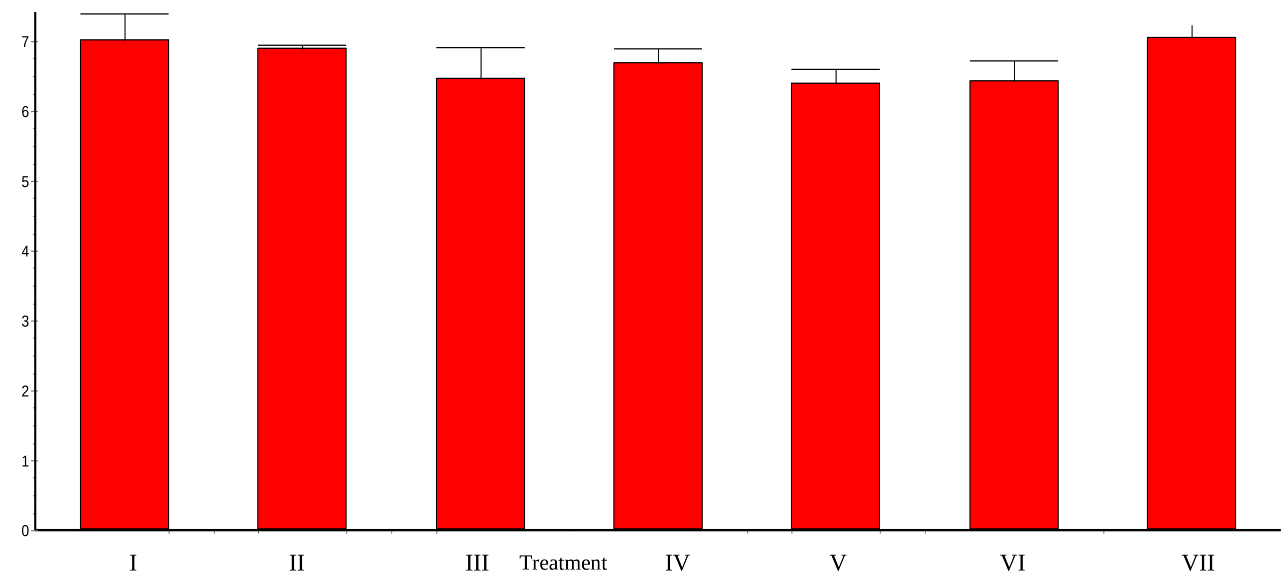

Figure 3. Effect of the natural and commercialized apple juice in the Blood Urea Nitrogen.

Values expressed in Mean ± SEM

In the serum creatinine, the preventive group (groups III and IV) showed highly significant levels (P< 0.05) in comparison to the control group (group I), however it is not statistically significant with the untreated group (Group II). Also, there was no significance in the curative group, groups V and VI. Creatinine is a waste product that comes from the normal wear and tear on muscles of the body (Pinney, 2014). The level of creatinine in the blood rises, if kidney disease progresses. This data suggests that the apple juice inhibited calcium oxalate formation, however, it has no effect in BUN or the lowering of creatinine, implying that it has no effect in restoring kidney function. In the study of Velpandian *et. al* (2012), using *Vediyuppu cheyaneer* treatment significantly (P<0.01) lowered the elevated serum levels of creatinine and BUN. It is also noteworthy that the positive group (Group VII) exhibited a high creatinine level among the treated (Figure 4), indicating that it has no effect in lowering creatinine.

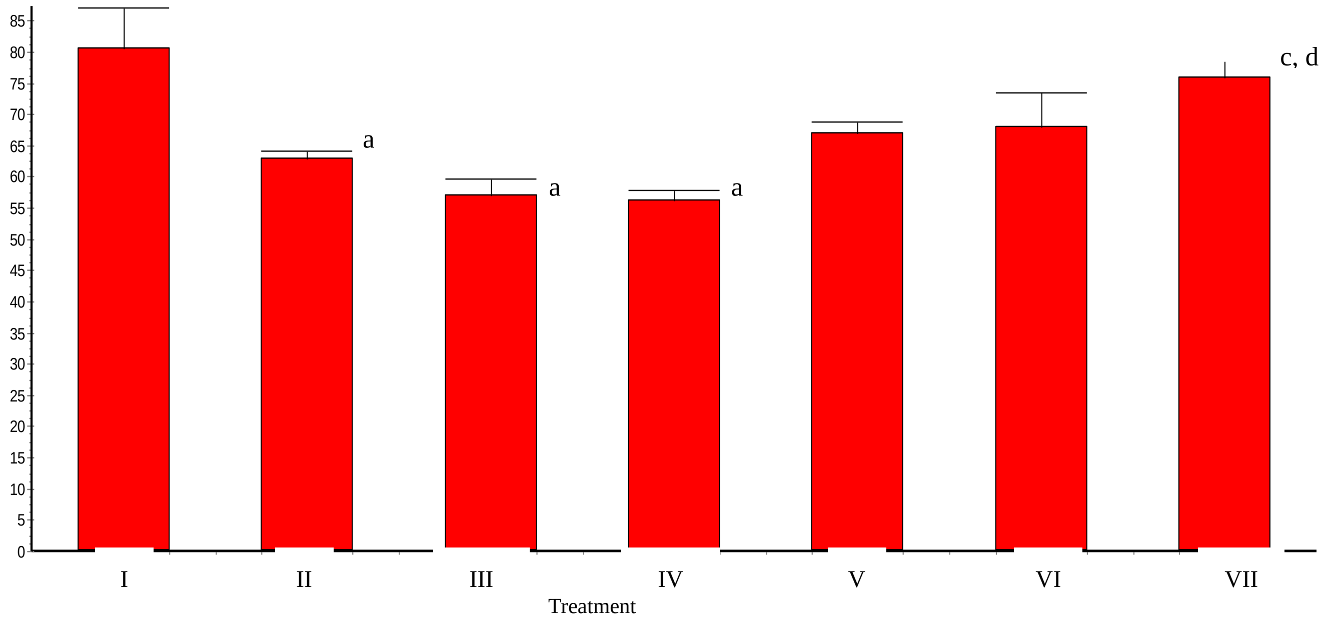

Figure 4. Effect of the natural and commercialized apple juice in the serum creatinine.
Values expressed in Mean ± SEM; BUN= Blood Urea Nitrogen; ns= not significant; a= Significant at P<0.05 compared to group I; b= Significant at P<0.05 compared to group II; c= Significant at P<0.05 compared to group III; d= Significant at P<0.05 compared to group IV.

Effect on Urine

Urinary chemistry is one of the important factors in determining the type of crystal formed and the nature of macromolecules included on the surface of the crystals. The urinary chemistry related to the calculi forming minerals provides good indication of the extent of stone formation (Makasana, *et. al*, 2014). Stone formation in kidney usually leads to polyuria (Velpandian, *et. al*, 2012). Specific gravity measures the urine density, or the ability of the kidney to concentrate or dilute the urine over that of plasma. Data in this study shows that there was no significant difference with the treated and untreated groups (Table 4), implying that the induction with EG has no influence with urine volume.

In the present study, pH of urine was also investigated. Urine of urolithiatic rats is known to become acidic. A lower pH would promote calcium oxalate formation (Dugdale, 2013). Results in this study showed that the pH level of the control group was neutral (table 4). The ethylene glycol induced rats have higher pH level compared to the control group. Moreover, all groups were considered significant (P<0.01) compared to Group I, the control group in terms of the pH level. However, there was no significant differences between the rest of the EG treated groups (table 4). On the other hand, it is also worth mentioning that pH becomes basic (Table 4), suggesting that condition is not conducive for calcium oxalate formation. As mentioned above a lower pH indicates kidney stone formation, specifically the calcium oxalate formation (Dugdale, 2013).

Table 4. Effect of the apple juices in the urine

Treatments	Subgroup	Specific Gravity [ns]	pH
Control group	**Untreated (I)**	1.00 ± 0.00	7 ± 0.00
	Negative control (II)	1 ± 0.00	9 ± 0.00 [a]
Preventive group	**Commercialized apple juice (III)**	1 ± 0.00	8.67 ± 0.33 [a]
	Fresh apple juice (IV)	1.00 ± 0.00	8.67 ± 0.33 [a]
Curative group	**Commercialized apple juice (V)**	1.00 ± 0.00	9 ± 0.00 [a]
	Fresh apple juice (VI)	1.00 ± 0.00	8.33 ± 0.33 [a]
Positive group	**Rowatinex (VII)**	1.00 ± 0.00	9 ± 0.00 [a]

Values expressed in Mean ± SEM; SEM= Standard Error of the Mean; ns= not significant; a= Significant at $P<0.05$ compared to group I.

In the study of Sayana *et. al* (2014), the rats treated with alcoholic extract of roots of *C. pareira* (velvet leaf) with different doses significantly reduced urinary calcium, uric acid and increased urinary magnesium levels, reduced serum calcium, creatinine and increased serum magnesium. Significant improvement renal function and kidney weight in prophylactic groups as compared to ethylene glycol controls. However, it did not show any effect on urinary oxalate, urine volume and any other serological parameters in the study of Mandavia *et. al* (2012).

The influence of apple juice against calcium oxalate stone formation observed in this study might be because of its flavonoids like quercetin and quercetin conjugates, antioxidants such as vitamins E and C, anti-microbial, and anti-inflammatory components (Alberto, *et. al*, 2006; Vinson, *et. al*, 2001; Sun, *et. al*, 2002; Hollman, 2000; Shaheen, *et. al*, 2001). Flavonoids as reported by several studies, especially the quercetin could prevent the formation of calcium oxalate calculi and their disaggregation through its anti-inflammatory and anti-oxidant mechanisms (Hadjzadeh, *et. al*, 2011). The presence of anti-microbial can also contribute to the antiurolithiatic effect (Edwin, *et. al*, 2008).

In the study of Touhami *et.* al (2007) which uses lemon juice, it was explained that the presence of a high antioxidant capacity was due to the presence of citrate, vitamin C, vitamin E and flavonoids such as eriocitrin, hesperetin and limonoids. Wherein, the vitamin E may prevent calcium oxalate crystal deposition in the kidney by preventing hyperoxaluria-induced peroxidative damage to the renal tubular membrane surface, (lipid peroxidation) (Huang, *et. al*, 2000 and Thamilselvan, *et. al,* 2005), which in turn can prevent calcium oxalate crystal attachment and subsequent development of kidney stones (Thamilselvan, *et. al*, 2005 and Santhosh, *et. al*, 2003).

4. Conclusion

The present study shows that the apple juices used, may have the capability to prevent the kidney stones formation. It might be used as an alternative medicine to decrease and prevent the formation of calcium oxalate crystals which causes urolithiasis.

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References

[1] A.B. Shukla, D.R. Mandavia, M.J. Barvaliya, S.N. Baxi, C.B. Tripathi. 2013. Anti-Urolithiatic Effect of Cow Urine Ark on Ethylene Glycol-Induced Renal Calculi. International Brazilian Journal of Urology.; 39: 565-71.

[2] C. Ebsco. August 2011. Kidney Stones. NAT Main Index.

[3] C.S. Kalpeshsinh, K.N. Fadadu, K.V. Patel, K.G. Patel, T.G. Gandhi, R. Tejal. 2011. Effect of flavonoid rich fraction of Citrus media Linn. On ethylene glycol induced urolithiasis in rats. Journal of Drug Delivery & Therapeutics.

[4] D.D. Dugdale, MD. 2013. Urine pH test. Medline Plus. Department of Medicine, University of Washington School of Medicine.

[5] D.R. Mandavia, M.K. Patel, J.C. Patel, A.P. Anovadiya, S.N. Baxi, C.R. Tripathi. 2012. Anti-Urolithiatic Effect of Ethanolic Extract of *Pedalium Murex* Linn. Fruits on Ethylene Glycol-Induced Renal Calculi. Enduorology and Stone Disease.

[6] E. Ozbek, and A. Ozbek. 2003. Kidney Damage by Dietary *Fusarium graminearum* in Rats: a Microscopic Study. The Journal of International Medical Research.

[7] E.F. Group III. 2013. 10 Natural Remedies for Kidney Stones. Global Healing Center.

[8] F. Jie, AG. Michael, SC. Paramjit. 1999. Impact of ammonium chloride administration on a rat ethylene glycol urolithiasis model. Scanning Microscopy.

[9] G. Krucik. August 15, 2012. Kidney Stones. Healthline.

[10] J. Edwin, E. Sheej, T. Vaibhav, G. Rajesh, T. Emmanuel. 2008. Antioxidant and Antimicrobial Activities of Cow Urine. Global Journal of Pharmacology 2: 20-2.

[11] J. Ghodasara, A. Pawar, C. Deshmukh, B. Kuchekar. 2010. Inhibitory effect of rutin and curcumin on experimentally-induced calcium oxalate urolithiasis in rats. Pharmacognosy Research ;2:388–92.

[12] J. Smith, and F.B. Stapleton. 2013. Patient information: Kidney stones in Children (Beyond the Basics). UpToDate.

[13] J. Sun, Y. Chu, X. Wu, RH. Liu. 2002. Antioxidant and antiproliferative activities of common fruits. Journal of Agriculture and Food Chemistry, 50:7449-7454.

[14] J. Vinson, X. Su, L. Zubik, P. Bose. 2001. Phenol antioxidant quantity and quality in foods: fruits. Journal of Agriculture and Food Chemistry, 49:5315-5321.

[15] J.R. Pinney. 2014. Kidney (renal) Disease.

[16] K. Johnson. 2013. Understanding Kidney Stones- The basics. WebMD.

[17] K.M. Santhosh, R. Selvam. 2003. Supplementation of vitamin E and Selenium prevents hyperoxaluria in experimental urolithic rats. Journal of Nutritional Biochemistry, 14:306-313.

[18] Khan, K. N. M., Hard, G.C., and Carl A., 2013. Kidney. In: Haschek and Rousseaux's Handbook of Toxicologic Pathology, Third Edition. Elsevier Inc. pp.1667.

[19] L. Tarkan. 2008. A Rise in Kidney Stones Is Seen in U.S. Children. The New York Times.

[20] L.C. Cool. 2013. The Fruit That Really Does Keep the Doctor Away. Healthline.

[21] M. Iguchi, C. Takamura, T. Umekawa, T. Kurita, K. Kohri. 1999. Inhibitory effects of female sex hormones on urinary stone formation in rats. Kidney International.

[22] M. Khalili, M.R. Jalali, M. Mirzaei-Azandaryani,. 2012. Effect of Hydroalcoholic Extract of *Hypericum Perforatum* L. Leaves on Ethylene Glycol-Induced Kidney Calculi in Rats. Urology Journal Vol. 9 No. 2.

[23] M. Touhami, A. Laroubi, K. Elhabazi, F. Loubna, I. Zrara, Y. Eljahiri, A. Oussama, F. Grases, A. Chait. 2007. Lemon juice has protective activity in a rat urolithiasis model. BMC Urology 5;7:18.

[24] MAR. Hadjzadeh, N. Mohammadian, Z. Rahmani, F. Rassouli. 2008. Effect of Thymoquinone on Ethylene Glycol-Induced Kidney Calculi in Rats. Urology Journal Endourology and Stone Disease.

[25] MC. Stoppler, MD. and W. Shiel. 2013. Kidney Stones. MedicineNet.com.

[26] MR. Alberto, MA. R. Canavosio, MC. M Nadra. 2006. Antimicrobial effect of polyphenols from apple skins on human bacterial pathogens. Electronic Journal of Biotechnology ISSN: 0717-3458.

[27] N. Thangarathinam, N. Jayshree, A. Metha, L. Ramanathan. 2013. Effect of polyherbal formulation on ethylene glycol induced urolithiasis. International Journal of Pharmacy and Pharmaceutical Sciences.

[28] P. Hollman, I. Arts. 2000. Flavonols, flavones, and flavanols-nature, occurrence and dietary burden. Journal of the Science and Food Agriculture, 80:1081-1093.

[29] P. Vishalakshi. 2012. A Study To Assess The Effectiveness Of Structured Teaching Programme Regarding Prevention Of Renal Calculi Among Pre-University College Students In A Selected Pre-University College, Mysore. Rajiv Gandhi University of Health Sciences Bangalore, Karnataka.

[30] R.V. Karadi, N.B Gadgeb, K.R. Alagawadi, R.V. Savadi. 2006. Effect of *Moringa oleifera* Lam. root-wood on ethylene glycol induced urolithiasis in rats. Journal of Ethnopharmacology.

[31] RL. Kuo, JE. Lingeman, AP. Evan, RF. Paterson, JH. Parks, SB. Bledsoe, et al. 2003. Urine calcium and volume predict coverage of renal papilla by Randall's plaque. Kidney International; 64: 2150-4.

[32] S. Ringold, JG. Tiffany, M. Richard. 2005. JAMA-Kidney stones.

[33] S. Shaheen, J. Sterne, R. Thompson, C. Songhurst, B. Margetts, P. Buerney. 2001. Dietary antioxidants and asthma in adults- population based case-control study. American Journal of Respiratory and Critical Care Medicine,164:1823-1828.

[34] S. Thamilselvan, M. Menon. 2005. Vitamin E therapy prevents hyperoxaluria-induced calcium oxalate crystal deposition in the kidney by improving renal tissue antioxidant status. BJU International, 96:117-126.

[35] SB. Sayana, C.C. Khanwelkar, V.R. Nimmagadda, R. Chavan, R. Vasant, N. Kumar. 2014. Evaluation of Antiurolithic Activity of Alcoholic Extract of Roots *of Cissampelos Pareira* in Albino Rats. Journal of Clinical and Diagnostic Research.

[36] T.R. Reyes. November 10, 2009. Surviving the (kidney) stone age. Philippine Council for Health Research and Development.

[37] U. Rudrappa. 2014. Apple fruit nutrition facts. Power your diet.

[38] V. Velpandian, S.S. Rajaprehidha, V. Banumathi, J. Anbu, and A. Anjana. 2012. Effect of *Vediyuppu cheyaneer* in ethylene glycol induced hyperoxaluria model in rats. International Journal of Life Science and Pharma Research.

[39] V. Vyas, R. Vyas, S. Joshi, D. Santani . 2011. Antiurolithiatic Activity of Whole-Plant Hydroalcoholic Extract of *Pergularia daemia* in Rats. Journal of Young Pharmacists.

[40] Y.H. Lee, W.C. Huang, J.K. Huang, L.S. Chang. 1996. Testosterone enhances whereas estrogen inhibits calcium oxalate stone formation in ethylene glycol treated rats. Journal of Urology.