RESEARCH ARTICLE

ASSESSMENT OF ANTIUROLITHIATIC PROPERTY OF PHYTIC ACID AND EXTRACTS OF *PRUNUS DULCIS, MUSA ACUMINATE, PISUM SATIVUM* BY THE INHIBITION OF FORMATION OF CALCIUM OXALATE CRYSTALS

Preeti Sirohi, Shadma Afzal, Nidhi Chaudhary and N.K. Singh*

Department of Biotechnology, Motilal Nehru National Institute of Technology Allahabad, Prayagraj (U.P.), India
Email: nksingh@mnnit.ac.in

Received-04.01.2021, Revised-18.01.2021, Accepted-29.01.2021

Abstract: The problem of kidney stone formation among the people is growing at a distressing frequency. Reason behind this is the non-favourable food habits, infections in the renal organs and low water consumption and retention by the body. Thus the best way to address the situation of kidney stone formation is through their inhibition at early or transient stage through the use of infusions of plant molecules which form part of our daily diet. The study was concentrated around the inhibition and modulation of calcium oxalate monohydrate crystalsand its visualization and characterization when they are under the influence of prepared plant infusions and solutions. The modulations were achieved by using phytic acid, extracts of *Prunus dulcis* (Almond), *Musaacuminata*(Banana) and *Pisum sativum* (Peas). The inhibition was planned via phytic acid solution of 1mg/mL concentration and infusions of plant extracts of 20%(w/v) concentration and the crystals were prepared using double displacement reaction between calcium chloride and oxalic acid. XRD and FTIR were used to characterize the formed crystals whereas visualization and nephelometry were used to study inhibition. All the infusions and solution have shown significant inhibitory effect with maximum inhibition of 51.38% shown by phytic acid and followed by 41.58%, 26.86%, 28.14% inhibitions by banana, pea and almond respectively. The study concluded that phytic acid have maximum inhibitory effect on calcium oxalate crystals among the used components and can be used to prevent the formation of urinary calculi.

Keywords: Calcium oxalate crystals, Kidney stone, Nephlometry, Phytic acid

INTRODUCTION

The stone disease usually originates in hot, arid, or dry climates such as northern Australia, central Europe, northern India and Pakistan and Mediterranean countries. Between 1% and 15% of people globally suffer from kidney stones at least once in their lifetime(Morgan et al. 2016). In 2015, 22.1 million cases of kidney stones were reported, subsequently leading to 16,100 deaths(Vos et al. 2016; Joshi et al. 2005).

The stone development is often connected with an increased consumption of fast foods and foods containing high fructose corn syrup(Knight et al. 2010). Other dietary productswhich contribute in the stone formation include high consumption of starchy foods, animal proteins, and oxalates, as well as low consumption of fluid and calcium.Calcium stone is the most common type of kidney stones worldwide. Calcium-containing stones denote about 80% of all kidney stone cases which typically contain calcium oxalate (80%) either alone or in combination with calcium phosphate (5-10%)(Coe et al., 2005).The stone development initiates from the occurrence of cores and the formation of these coresis from supersaturated urine. Super-saturation hangs on the factors like urinary pH, ionic strength, and solute concentration of certain glycoproteins, complexations and the pathogenic factors.

Various experiments have been performed to understand the exact mechanisms of urolithiasis. A sort of success has been achieved in it how the different physiological condition of kidney like renal injury are associated with super-saturation which help in nucleation and further crystal growth(Khan 2006).The pathophysiological mechanisms through which kidney stone formation take place and the factors affecting it(Khan 2018).Many potential inhibitors had been searched to retard the process of urolithiasis. Plants extracts have always been proved to natural cures of physiological anomalies such as*Phyllanthus niruri*had used to inhibit kidney stone formation(Freitas et al. 2002).In a similar work, inhibitory effects of herbal extracts of *Tribulus terrestris* and *Bergenia ligulata* was proved using *in vitro* study of crystal growth(Joshi et al. 2005).

Many inhibitors have been studied for retarding the kidney stone formation(Joshi et al. 2005; Ahmed et al. 2016). For the formation and inhibition study double diffusion method is widely employed for its obvious reason of easy visualization and characterization. However this method has not been used because of its time consuming nature. In the present study COM were prepared and its growth, visualization and characterization were observed under normal conditions and under the action of plant infusions of *Prunus dulcis* (Almond), *Musaacuminata* (Banana), *Pisum sativum* (Peas) and phytic acid solution.

*Corresponding Author

MATERIALS AND METHODS

Reagents

Phytic acid was purchased from Sigma Aldrich. All the chemicals and chemicals used in this study of laboratory grade.

Preparation of Stock Solution

0.5M, 100mLof calcium chloride and oxalic acid stock solutions were prepared. In addition to this phytic acid solution of concentration 1mg/mL was prepared.

Preparation Of Plant Infusions

50g each of *Prunus dulcis* (Almond), *Pisum sativum* (Peas) were weighed and dried at 60°C in an incubator for 24 hours. After 24 hours, 20g of each were weighed and crushed using mixer grinder and added to 100mL of distilled water to make a solution. For preparing infusion of *Musa* (Banana), 50g of banana was weighed and crushed using mixer grinder and added to 100mL of distilled water to make a solution. These solutions were incubated at 4°C for 24 hours. After 24 hours the solutions were filtered 3 times until clear plant infusions were obtained.

Crystal Growth

Double displacement between calcium chloride and oxalic acid was used to grow COM crystals. The nucleation of COM crystals occurred due to the reaction between calcium chloride and oxalic acid which is, as follows- $CaCl_2 + H_2C_2O_4 = CaC_2O_4 + 2HCl$. The COM crystals were obtained as precipitate.

Crystal Characterization

X-ray diffraction (XRD)

Crystals of calcium oxalate were formed as stated above and the obtained crystals were dried in an incubator for 24 hours at 60°C. After 24 hours the dried crystals was collected and subjected to XRD. X-ray diffraction (XRD) patterns were recorded at 40 kV and 30 mA with Cu Kα radiation (Wavelength= 0.15406 nm) in the 2θ range from 10 to 90° with 2°/min. The results were recorded.

Fourier Transform Infra-red (FTIR) Spectroscopy

FTIR was used to characterize the above grown crystals as calcium oxalate.The Fourier transform infrared radiation transmittance (FTIR) analysis of calcium oxalate crystals was recorded at room temperature (25 ±2 ºC) at spectral range of 4000 – 400 cm^{-1}.

Inhibition Characterizations

Visualization

Took 5 different clean and clear glass slides and to one of them added 30µL of 0.5M $H_2C_2O_4$, 30µL of 0.5M $CaCl_2$and 80µL of deionized water. On the other four slides added 80µL each of infusions of *Prunus dulcis* (Almond), *Musaacuminata* (Banana), *Pisum sativum* (Peas) and phytic acid solutionrespectively, followed by adding 30µL of 0.5M $CaCl_2$ and 30µL of 0.5M $H_2C_2O_4$using pipette. Then the slides were allowed to rest still for 10 minutes and then the visualized observations were recorded.

3 different infusions and phytic acid stock solution were prepared (Table 1).

Table 1. Different solutions prepared using plant infusions

Solution Name	Component
A	deionised water
B	20 %(w/v) of *Prunus dulcis* (Almond)
C	20 %(w/v) of *Pisum sativum* (Peas)
D	20 %(w/v) of *Musa* (Banana)
E	1mg/mL of Phytic Acid

All the solutions were taken on clear glass slides and 30 µL of 0.5 M calcium chloride solution was added on each slide. After that 30 µL of oxalic acid was added slowly on each mixture of $CaCl_2$ and infusions leading to the formations of calcium oxalate crystals seen as white turbidity.

Turbidity Measurement

For measuring turbidity the reference solution was prepared by mixing 5mL each of 1%(w/v) hydrazine sulphate and 10%(w/v) of hexamethylene tetramine. The solution was allowed to stand still for 24 hours. After 24 hours the solution was added with 90mL distilled water to obtain a reference solution of 400NTU (Nephlometric Turbidity Unit).

In clean and fresh 5 test tubes, out of 5 tubes one tube have10mL of deionized water and in rest 4 tubes 10mL of infusions of *Prunus dulcis* (Almond), *Musaacuminata* (Banana), *Pisum sativum* (Pea), phytic acid solution were added respectively. To each tube then added 10mL of 0.5M $CaCl_2$ followed by adding 10mL of 0.5M $H_2C_2O_4$ to each tube. To measure turbidity first zero was set by using 30mL distilled water. After setting the meter to zero using 30mL distilled water and calibrating the device to 400NTU for the reference solution, prepared by method already stated, the turbidity meter was ready for use. The turbidity of solutions of each tube was measured by properly shaking the tubes to prevent any settlement of the crystals and to ensure the error free reading.The below stated formula was used to quantitatively describe the inhibitory effects of plant infusions and phytic acid solution.

$$\text{Inhibition (\%)} = \frac{(\text{Turbidity of reference solution}) - (\text{Turbidity of solution with infusion})}{(\text{Turbidity of solution with deionised water})} \times 100$$

RESULTS

Characterization of crystals

X-Ray Diffraction (XRD)

The characterization of the crystals was performed in order to validate the grown crystals as COM. Therefore a powder XRD pattern of the grown crystals were recorded and is shown in the figure. Thecharacteristics peaks analogous to the diffraction planes 020, 101, 112, 130 and 202confirms the wurtzite structure of the calcium oxalate crystals. The prominent peaks in XRD pattern of calcium oxalate crystals corresponds to the diffraction planes 020, 101, 112, 130 and 202confirms thecrystalline nature. Sharp and narrow peaks depicted the crystallinity and purity of the synthesized Calcium oxalate crystals (Wahab et al., 2009; Jamdagni et al., 2018).

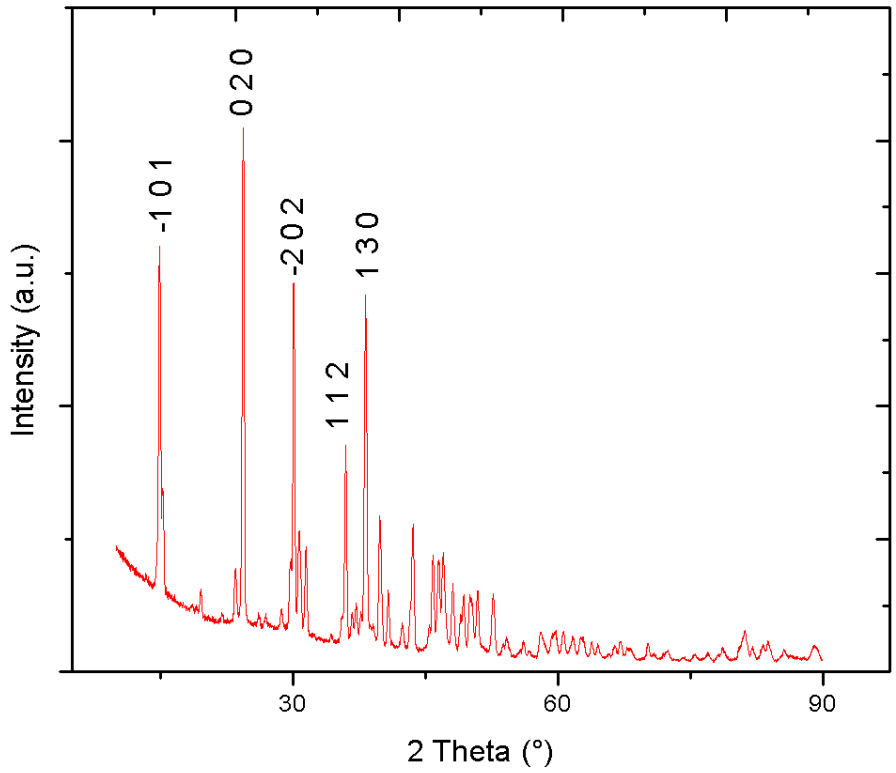

Fig. 1: XRD pattern of the synthesized calcium oxalate crystals showing narrow and sharp peaks.

FTIR (Fourier-transform infrared spectroscopy)

A PerkinElmer Spectrum Version 10.4.00 FTIR spectrophotometer was used to record the IR spectrum using calcium oxalate pellets and the spectrum is shown in figure. Typical bands for calcium oxalate stones were the strong bands around 778.53 (C=O asymmetrical stretching), 1314.93 (C–C symmetrical stretching), and 1604.64 (OC≡O asymmetrical stretching) (Channa et al. 2007, Silverstein et al. 2005). In the present study, the typical bands of calcium oxalate stones are appeared. The peak at 519 cm^{-1} arises due to O-C-O in-plane bending. The metal-carboxylate stretch appears at 1316 cm^{-1}(C–C symmetrical stretching). A strong band around 760.23 cm^{-1} (C=O asymmetrical stretching) was appeared. The water molecules coordinated with the calcium oxalate molecules produce characteristic peaks corresponding to the bending modes at 1621 cm^{-1}(OC≡O asymmetrical stretching). The peaks for asymmetric and symmetric stretch of the coordinated water molecule are shown by the broad spectrum of peaks above 3000 cm^{-1}(Girija et al. 1998). The five peaks in between 3000 cm^{-1} and 3500 cm^{-1} correspond to the asymmetric and symmetric stretch of the water molecules coordinated with the calcium oxalate molecules(Ali et al. 2006).

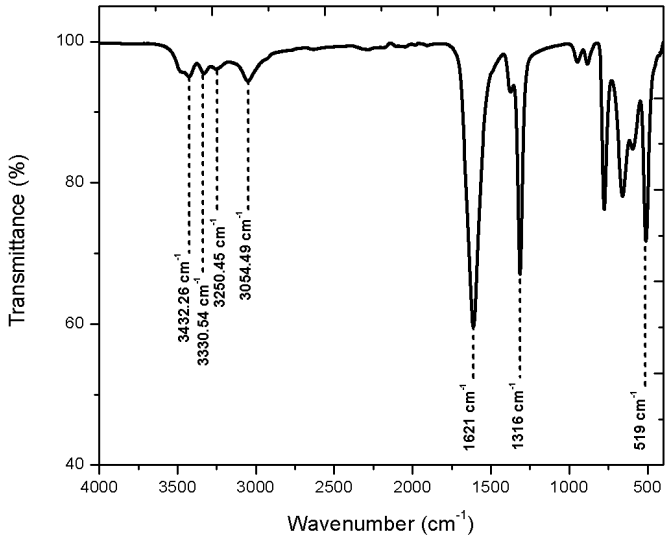

Fig. 2: FTIR spectrum of the synthesized Calcium oxalate crystals. According to the obtained FTIR spectra, the synthesized calcium oxalate crystals showed all the typical bands.

Microscopic observations and Turbidity measurement

Our study concentrated on the inhibition of the COM crystals growth by the use of infusions of the plants and fruits part of daily diet of common individuals. *Musa acuminata*(Banana) has cooling effect on the body and its richness in potassium helps in maintain kidney health(Kumar et al. 2012).*Prunus dulcis* (Almond) contains magnesium which controls blood sugar levels and therefore restricts the formation of kidney stone. *Pisum sativum* (Peas) has immense health benefits including preventing heart diseases, regulating blood sugar level, fighting nervous system and improves digestion and kidney health. Phytic acid acts as potential chelator of calcium and therefore may be protective against kidney stone(Nouvenne et al. 2014).

From the visualization of the slide, the presence of white turbidity was directly proportional to crystal formed. As the results observed under microscope revealed thatcrystals formed in the glass slide containing phytic acid were least turbid because of minimal formation of crystals (Fig.1). Phytic acid was followed by banana. Least inhibition of calcium oxalate crystals was observed in the case of almond and pea. Deionised water was used during crystal formation on the glass slide without infusion to maintain constant volume of mixture so that more turbid appearance don't arise in the reference casedue to concentration difference. To quantitatively examine the inhibitory effect of above stated infusions on calcium oxalate crystal growth, a different experimental procedure was employed using turbidity meter.

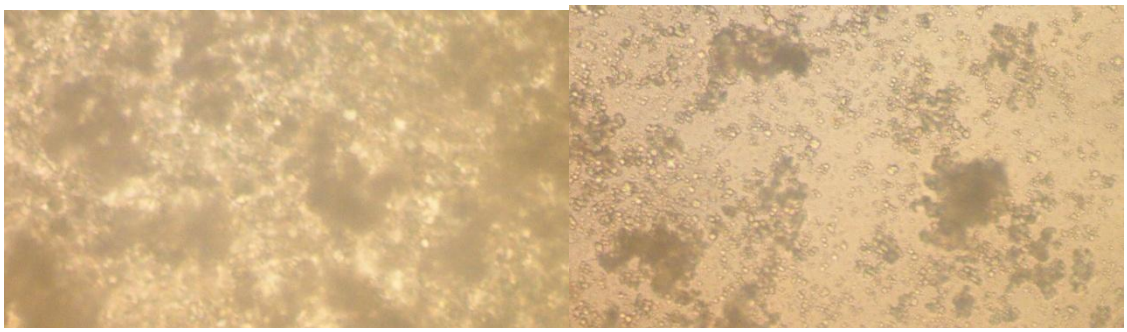

Fig. 3: Microscopic observations of calcium oxalate crystals without phytic acid and with phytic acid

In Turbidity meter, artificial light source emits a known intensity of light through a sample. The suspended $Ca(C_2O_4)$ crystals scatter or absorb the light. The scattered light is then measured at an angle of 90° in the device showing its turbidity. So, the turbidity is directly proportional to the amount of crystals formed.

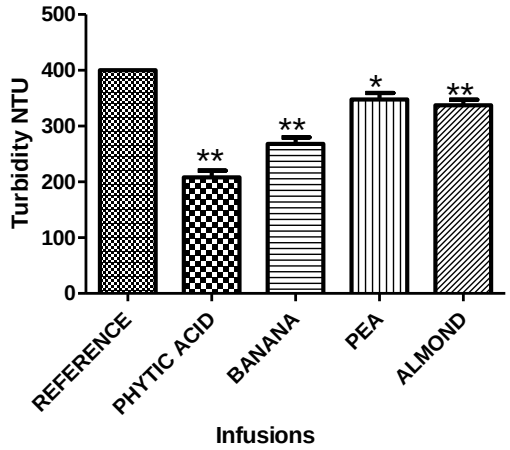

Fig. 4: Turbidity measurement of different food compounds after treating the calcium oxalate crystals.

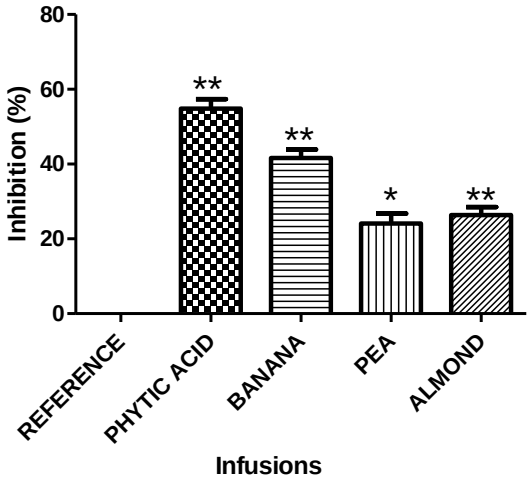

Fig. 5: Calcium oxalate crystals inhibition (%) of different food compounds

Through the graph it is clearly understood that the inhibition of COM was observed using the phytic acid solution and plant infusions. However the use of phytic acid solution significantly decreased the turbidity of the solution and therefore showing the inhibition of crystal formation by 51.38%. Out of 3 plant infusions studied banana was most successfully able to inhibit growth of crystals with the turbidity and inhibition being 274NTU and 41.58% respectively. Pea and almond showed almost similar inhibitory effect with 26.86% and 28.14% inhibition respectively.

DISCUSSION

The inhibitory effect of phytic acid may be attributed due to its chelating effect lead to less availability of calcium result in less formation of COM crystals(Cheryan et al. 1980). *Musa acuminata* contains about 20-30mg of magnesium per 100gm(Wall 2006). Studies have shown inhibitory effect of magnesium inthe growth of COM crystals. The inhibitory effect of magnesium on the growth of COM crystals is mainly due to formation of ion complexes with oxalate and magnesium oxalate is more soluble than calcium oxalate. Due to its oxalate chelating property, magnesium comes under the consideration of inhibitor of calcium oxalate crystallization(Ryall 1997). In case of almond and pea, the percentage composition of phytic acid is 0.35% - 9.42% and 0.22% to 1.2% respectively(Schlemmer et al. 2009).According to USDA Food Composition Databases the magnesium content in almond and pea are 255mg per 95g and 47.9mg per 145g respectively, thus proving their inhibitory action on the growth of COM. However other constituents of both almond and pea can also prove to be inhibitory to COM growth. It can be noted that lower phytic acid and magnesium content of pea maybe responsible for its lesser inhibition of the growth in comparison to almond.

Therefore it can be seen that phytic acid, *Prunus dulcis* (Almond), *Musaacuminata* (Banana), *Pisum sativum* (Peas) prove to be inhibitory to the growth of COM. However this was an*in vitro* study under controlled conditions. But in human body, process of urolithiasis is complex mechanisms that occur in the body under dynamic conditions of supersaturated

urine. But nevertheless, this study helps in screening and isolation of the potent inhibitors of the kidney stone formation.

Therefore it is clearly visible through the study that the use of banana, pea and almond and phytic acid can inhibit the growth of COM to a significant extent. The advantages of use of banana, pea and almond are that they form part of daily diet of every common household and are cheaply available and accessible.

CONCLUSION

Calcium oxalate crystals were grown both in absence and presence of plant infusions and phytic acid, and the grown crystals were characterised usingXRD and FTIR. The inhibitory effect of phytic acid, *Prunus dulcis* (Almond), *Musaacuminata*(Banana) and *Pisum sativum* (Peas) were qualitatively studied visually and quantitatively using nephelometry. In *in vitro* conditions, the solution of phytic acid had shown maximum inhibition of growth of COM crystalfollowed by the extracts of *Musa accuminata* and almost same extend of inhibition shown by*Prunus dulcis* (Almond) and *Pisum sativum* (Pea). This study is useful to formulate the necessary dosages in order to prevent formationof urinary calculi.

ACKNOWLEDGEMENT

Authors are thankful to the Director, Motilal Nehru National Institute of Technology Allahabad, Allahabad-211004, UP, India for providing the Lab facility and financial support for performing this experiment. The author is also thankful to the Ministry of Human Resource and Development, Government of India, New Delhi, India for providing scholarship during the research work.

REFERENCES

Ahmed, S., Hasan, M.M. and Mahmood, Z.A. (2016). Inhibition of calcium oxalate crystals growth by *Macrotyloma uniflorum* (Lam.) Verdc, *Phaseolus lunatus* Linn, and *Phaseolus vulgaris* Linn: An *in vitro* study. Journal of Pharmacognosy and Phytochemistry,5(1):124-130.

Ali, A.M., Raj, N.A.N., Kalainathan, S. and Palanichamy, P. (2008). Microhardness and acoustic behavior of calcium oxalate monohydrate urinary stone. Materials Letters, 62:2351–2354.

Channa, N.A., Ghangro, A.B., Soomro, A.M. and Noorani, L. (2007). Analysis of kidney stones by FTIR spectroscopy. J. Liaquat Uni. Med. Health Sci., 6:66–73.

Cheryan, M. and Rackis, J.J. (1980). Phytic acid interactions in food systems. CRC Critical Reviews in Food Science and Nutrition,13(4):297-335. https://doi.org/10.1080/10408398009527293

Coe, F.L., Evan, A. and Worcester, E. (2005). Kidney stone disease. J. Clin. Invest., 115(10):2598–2608. https://doi.org/10.1172/JCI26662

Frietas, A.M., Schor, N. and Boim, M.A. (2002). The effect of *Phyllanthus niruri* on urinary inhibitors of calcium oxalate crystallization and other factors associated with renal stone formation. BJU International,89:829–834

Girija, E.K., Latha, S.C., Kalkura, S.N., Subramanian, C. and Ramasamy, P. (1998). Crystallization and microhardness of calcium oxalate monohydrate. Materials Chemistry and Physics, 52:253-257.

Jamdagni, P., Khatri, P. and Rana, J.S. (2018). Green synthesis of zinc oxide nanoparticles using flower extract of *Nyctanthesarbor-tristis* and their antifungal activity. Journal of King Saud University-Science,30(2):168-175.

Joshi, V.S., Parekh, B.B., Joshi, M.J. and Vaidya, A.B. (2005). Herbal extracts of *Tribulus terrestris* and *Bergenia ligulata* inhibit growth of calcium oxalate monohydrate crystals in vitro. Journal of Crystal Growth,275:e1403–e1408.

Khan, A. (2018). Prevalence, pathophysiological mechanisms and factors affecting urolithiasis. Int. Urol. Nephrol., 50(5):799-806. https://doi.org/10.1007/s11255-018-1849-2

Khan, S.R. (2006). Renal tubular damage/ dysfunction: key to the formation of kidney stones. Urol. Res., 34:86–91. https://doi.org/10.1007/s00240-005-0016-2

Knight, J., Assimos, D.G., Easter, L. and Holmes, R.P. (2010). Metabolism of fructose to oxalate and glycolate. Horm.Metab. Res., 42(12):868–873. https://doi.org/10.1055/s-0030-1265145

Kumar, K.P.S., Bhowmik, D., Duraivel, S. and Umadevi, M. (2012). Traditional and Medicinal Uses of Banana.Journal of Pharmacognosy and Phytochemistry,1(3):51-63.

Morgan, M.S.C. and Pearle, M.S. (2016). Medical management of renal stones. BMJ., 352: i52. https://doi.org/10.1136/bmj.i52

Nouvenne, A., Ticinesi, A., Morelli, I., Guida, L. and Borghi, L. (2014). Fad diets and their effect on urinary stone formation. Transl.Androl. Urol., 3(3): 303–312. https://doi.org/10.3978/j.issn.2223-4683.2014.06.01

Ryall, R.L. (1997). Urinary inhibitors of calcium oxalate crystallization. World J. Urol.,15:155-164.

Schlemmer, U., Frolich, W., Prieto, R.M. and Grases, F. (2009). Phytate in foods and significance for humans:Food sources, intake, processing, bioavailability, protective role and analysis. Mol.Nutr. Food Res.,53:S330 –S375. https://doi.org/10.1002/mnfr.200900099

Silverstein, R.M., Webster, F.X. and Kiemle, D.J. (2005). Infrared spectroscopy. In: Bennon D, Yee J (eds.) Spectrometric Identification of Organic Compounds, 7th ed. New York, John Wiley and Sons, Inc., pp. 72–126.

Vos, T., Allen, C., Arora, M., Barber, R.M., Bhutta, Z.A., Brown, A., Carter, A., Casey, D.C., Charlson, F.J., Chen, A.Z. and Coggeshall, M. (2016). Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet, 388:1545–1602.

Wahab, R., Ansari, S.G., Seo, H.K., Kim, Y.S., Suh, E.K. and Shin, H.S. (2009). Low temperature synthesis and characterization of rosette-like nanostructures of ZnOusing solution process. Solid State Sciences, 11(2):439-443.

Wall, M.M. (2006). Ascorbic acid, vitamin A, and mineral composition of banana (Musa sp.) and papaya (*Carica papaya*) cultivars grown in Hawaii. Journal of Food Composition and Analysis, 19:434–445.