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ORIGINAL ARTICLE

Inhibitory Effect of Piperine on Nucleation and Aggregation of Calcium Oxalate Crystals

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ABSTRACT
Piperine is a potent alkaloid obtained from Piper nigrum (Black pepper) and Piper longum (Long pepper) family Piperaceae. The study investigates the inhibitory potential of piperine on anti-nucleation and anti-aggregation of calcium oxalate crystals. Monohydrate and dihydrate crystals of calcium oxalate were synthesized. Piperine was isolated from Piper nigrum fruits by a single step method. The calcium oxalate crystals and piperine were characterized for their purity. Anti-nucleation and anti-aggregation assay of both the crystal types was studied separately. Piperine was found to exhibit significant inhibitory effect on nucleation and aggregation of monohydrate as well as dihydrate crystals of calcium oxalate. Piperine can be effectively employed in the management of urolithiasis and nephrolithiasis.
Keywords *Aggregation assay, Calcium oxalate, Nucleation assay, Piperine, Urolithiasis*

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INTRODUCTION

Piper spp. have been widely implemented in the treatment of several ailments by the ancient systems of medicine. Besides, piperine has been scientifically proven to exert antiviral, anti-inflammatory and bioavailability enhancing properties.

Formation of stone by the deposition of crystal in any part of the urinary system is known as urolithiasis. Majorly, calcium oxalate crystals as found; besides, these calcium phosphate, uric acid, creatinine and struvite can also deposit and lead to crystal formation [1].

In almost 80 % of urolithiasis cases, calcium oxalate monohydrate (COM) has been found to aggravate the condition by exhibiting greater affinity for renal tubular cells [2,3]. Although, calcium oxalate dihydrate (COD) are also the part of stone in many cases, hitherto it can even be observed in the urine of healthy subjects [4]. Current management of urolithiasis involves surgical procedures, medications and diets. Nonetheless, most are ineffective as far as relapses in patients is concerned [5,6,7,8].

The current study investigates the concentration dependent efficiency of piperine as an inhibitor for nucleation and aggregation of calcium oxalate crystals (monohydrate and dihydrate).

MATERIAL AND METHODS

Black pepper was Purchased from local market. Petroleum ether (b.p.40-60^{0}C) was purchased from (LobaChemiePvt Ltd). Calcium chloride, sodium oxalate were purchase from (Research-lab Mumbai, India),Thin layer chromatography plates were obtained from Merck, Germany.

Isolation of piperine

Black pepper corns were dried in shade & crushed with mechanical grinder to a coarse powder. The coarse powder was extracted using petroleum ether (b.p.40-60^{0}C) in a Soxhlet extractor for 5 hrs.The petroleum ether extract was cooled to precipitate crude piperine. The volatile oils and other impurities from the extract were separated using cold petroleum ether. Piperine was recrystallized from methanol. The pure piperine crystals were characterized using advanced analytical tools.

Synthesis of calcium oxalate monohydrate crystals

Crystals were synthesized as per earlier work with slight modification. About 0.735 g of calcium chloride dihydrate was dissolved in 50 ml distilled water. Simultaneously, 0.670 g of sodium oxalate was dissolved in 50 ml of 2N H_2SO_4. Both these solutions were mixed equally in a beaker to precipitate out calcium oxalate with stirring on a bath sonicator. The resulting calcium oxalate crystals were washed with liquid ammonia solution (25 % v/v) to remove traces of sulfuric acid. Finally, the crystals were rinsed with distilled water and dried at a temperature 60 °C for 2hours [9].

Synthesis of calcium oxalate dihydrate crystals

The calcium oxalate dihydrate crystals were prepared as per the protocol of Doherty et al.1994 with slight modification. Accurately, 32 ml of 0.2 M calcium chloride was added dropwise to 400 ml of 0.01 M sodium citrate that was kept continuously stirred on a magnetic stirrer for 10-15 min. To this solution 16 ml of 0.05 M sodium oxalate was added and stirred using probe sonicator (Biomedica BMI-599) for 10 min. The precipitate so obtained was separated by filtration through whatman filter paper. The residue was washed twice with distilled water and dried at a temperature of 60° C for 2 hours [10].

Characterization of Isolated Piperine and Calcium oxalate crystals (COM and COD)

The melting points of isolated piperinewas recorded using melting point apparatus (Veego Instrument).Thin layer chromatography was performed using Toluene: Ethyl Acetate (7:3) as solvent system. Further, quantitative analysis was recorded with high performance thin layer chromatography (CAMAG-Switzerland)) using n-Hexane: Ethyl acetate: glacial acetic acid (6:4:0.2) as mobile phase .UV-vis spectrophotometer (JASCO V-630) was used to determine the absorption spectrum in ethanol (95% v/v). Proton NMR of piperine was recorded using (Bruker AVANCE III HD). Microscopy of Piperine and Calcium Oxalate Crystals (COM & COD) were performed using Trinocular Microscope (Olympus & CH20i-IR). Piperine and Calcium Oxalate Crystals (COM &COD) having particle size and the stability were determined using a Nanoparticle Analyzer (Horiba Scientific & SZ-100). Surface conjugation and functional groups were determined by JASCO FT/IR-4100 Fourier-transform infrared spectroscopy (FTIR). Also, scanning electron microscopy was performed using Analytical Scanning Electron Microscope (JEOL JSM-6360 A).Powder XRD was performed using Bruker (D8ADVANCE).

Nucleation assay

Concentration dependent inhibitory effect of piperine on calcium oxalate (Calcium oxalate) crystal formation was evaluated. Calcium chloride ($CaCl_2$) (5 mmol/l) and sodium oxalate ($Na_2C_2O_4$) (7.5 mmol/l) solutions were prepared in Tris-HCl (0.05 mmol/l). Sodium chloride NaCl (0.15 mol/l) solution was prepared using buffer (pH 6.5). One ml each of different piperine concentration was mixed with 2 ml $CaCl_2$solution followed by the addition of 2 ml $Na_2C_2O_4$ solution. The optical density (OD) of the mixtures was then recorded at 620 nm wavelength. Percent inhibition of nucleation by piperine was calculated using the formula and compared to control [11].

$$\%\ inibition = 1 - \frac{(Turbidity\ Sample)}{(Turbidity\ Control)} X\ 100$$

Aggregation assayCalcium chloride $CaCl_2$ and Sodium oxalate $Na_2C_2O_4$ solutions (50 mmol/L each) were mixed together, heated to 60ºC in a water bath for 1 h and then incubated overnight at 37ºC to prepare seed Calcium oxalate crystals. After drying, Calcium oxalate crystal solution (0.8 mg/ml) was suspended in a 0.05 mol/l Tris-HCl and 0.15 mol/lNaCl buffer (pH 6.5). One ml of 0.005 mg/ml piperine was added to 2 ml Calcium oxalate solution, and then incubated at 37ºC for 30 min. OD of the final mixtures was then read at 620 nm wavelength and percent inhibition of aggregation was then calculated as described for nucleation assay [11].

RESULTS

Yield of the crude Piperine isolated from petroleum ether (40-60 0C) was found to be 1.23 % w/w. Upon recrystallisation, yield of pure Piperine was 0.56 % w/w.

Analysis of isolated Piperine Crystals

Melting point of the isolated piperine was found to be 132°C

Microscopy of Piperine and Calcium oxalate crystals

Crystal structure of piperine was observed by using trinocular microscope. Calcium oxalate monohydrate (COM) crystals were found to be acicular in nature. Whereas; Calcium oxalate dihydrate (COD) crystals were of tetrahedral shape (Fig.1). Moreover, scanning electron microscopy of these crystals confirmed these findings. (Fig 2)

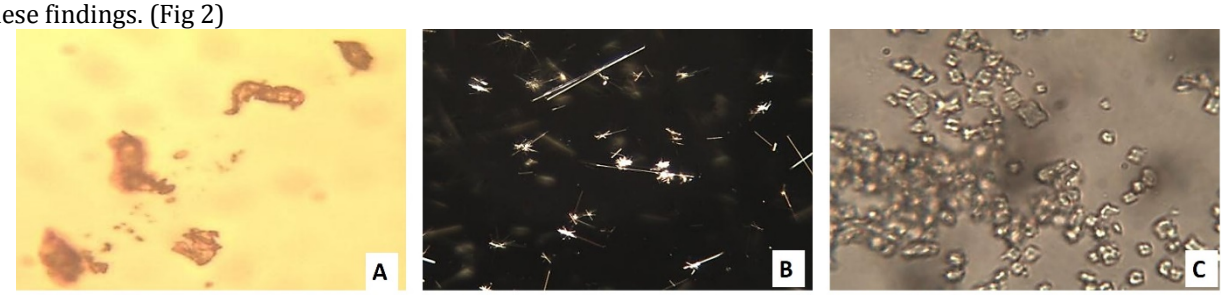

Fig 1: Microscopy of A: Piperine, B: Acicular crystals of COM; C: Tetrahedral crystals of COD (X450)

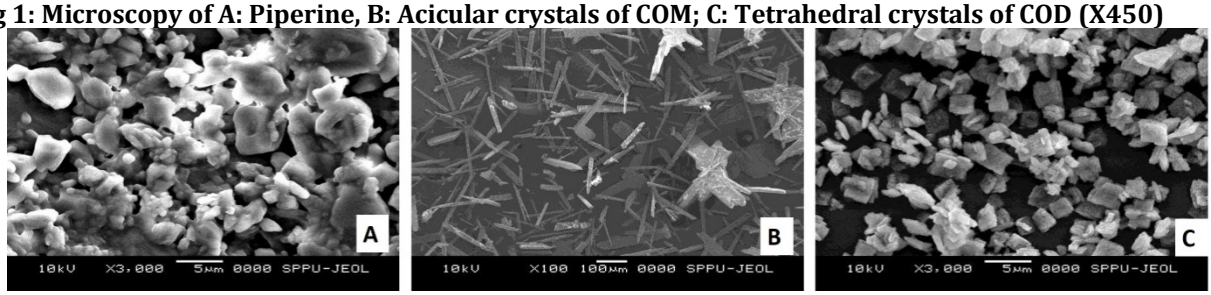

Fig 2 Scanning Electron Microscopy of A: Piperine (X 3000), B: COM (X 100) C: COD (X 600)

Particle size analysis of piperine, calcium oxalate crystal (COM & COD)

Particle size of piperine was 135 nm. Calcium oxalate monohydrate and calcium oxalate dehydrate having particle size was 84 nm and 318 nm respectively. The particle size was analyzed by particle size analyzer (Horiba)

UV-visible spectroscopy of piperine

Purity of compound was checked using UV-visible spectrophotometer. The λ max of piperine in ethanol was observed at 367 nm.

FT-IR of piperine, COM & COD

FT-IR spectra of piperine

FTIR exhibited 2940, 2803, 1630, 1585, 1449, 1366, 1032, 929, 847, 701 cm^{-1} (Fig 3)

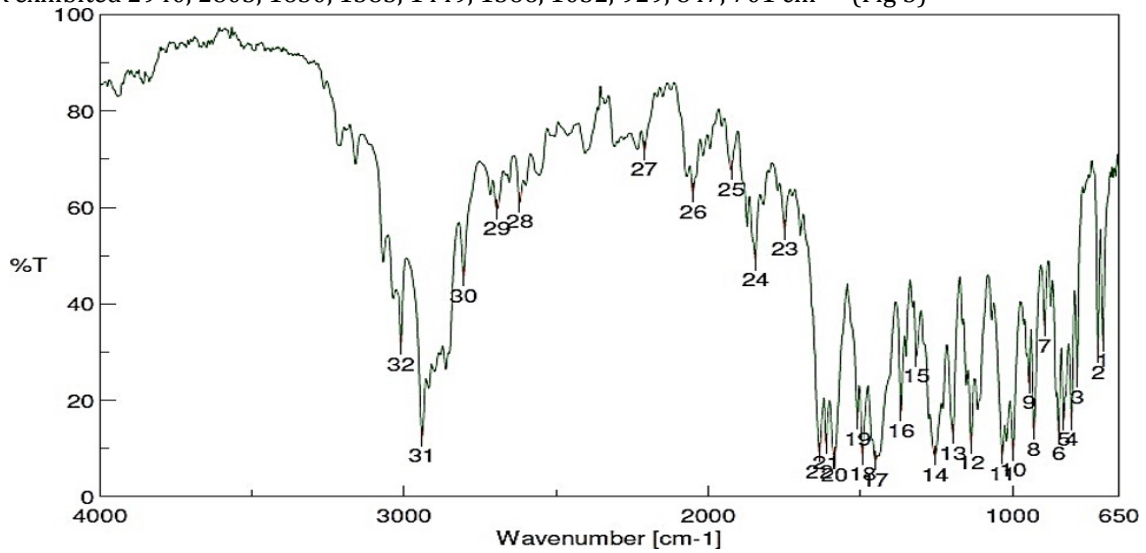

Fig 3: FTIR spectra of Piperine

FT-IR spectra of Calcium oxalate monohydrate crystals

The spectrum of COM exhibited several peaks *viz* 3733-3493, 1618, 1317, 1240-1092, 783, 754, 701-665 cm^{-1} (Fig 4)

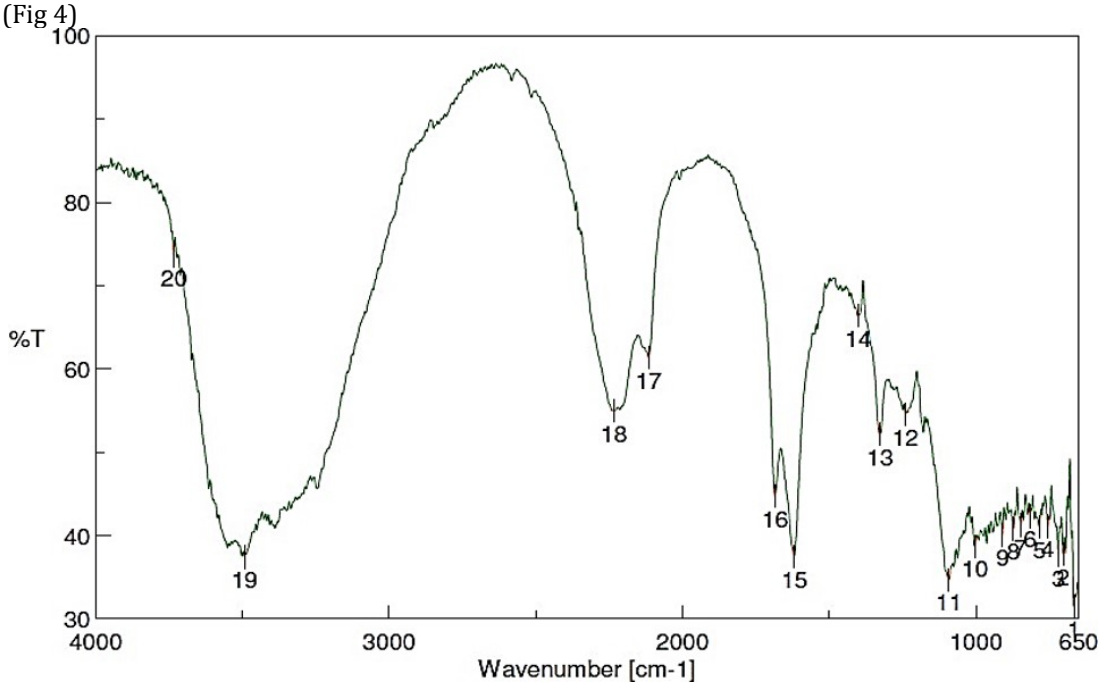

Fig 4: FT-IR of calcium oxalate monohydrate crystals.

FT-IR of calcium oxalate dihydrate crystals

Major peaks from the FTIR spectra of COD 3488-3057,1609, 1321, 953-883, 784-668 cm^{-1}. (Fig 5).

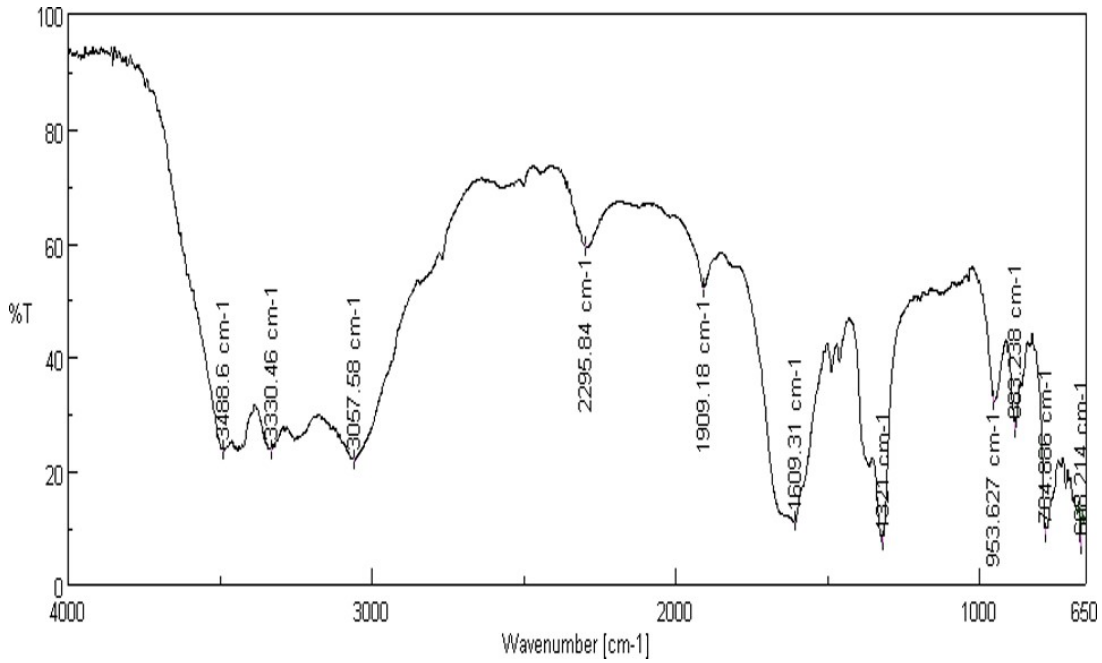

FIG 5 FT-IR OF COD

Proton NMR of piperine

Fig 6 shows the H1 NMR Spectra with peaks atδ1.567-1.675 ppm (6H, m, overlapping peaks from H- 19, H-20 and H-18), δ 3.527-3.639 ppm (4H, d, H-21 and H-17)δ 5.979 ppm (2H, s, H-2)δ 6.426-6.985 ppm(3H, d,overlapping peaks from H-11, H-10 and H-9) δ7.265-7.428 ppm (1H, dd, H-8) The chemical shifts are according to position of proton and carbon atoms of piperine.

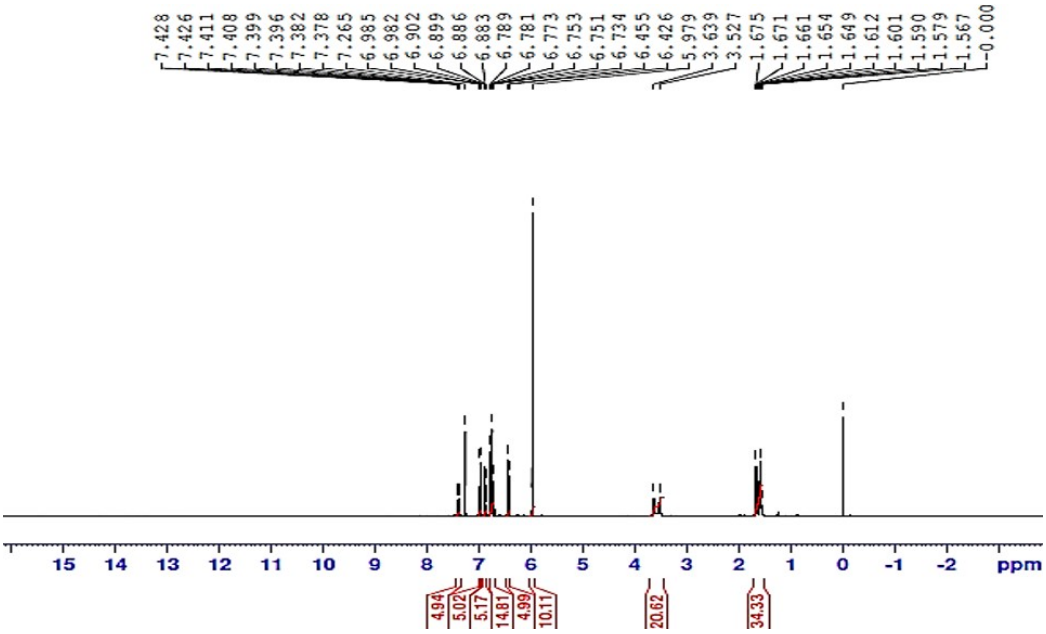

Fig 6 Proton NMR of Piperine

Powder X-Ray Diffraction

PXRD was performed to examine the crystalline state of piperine. Piperine exhibited characteristic diffraction peaks at $2\theta = 13^0$ & 22^0 respectively. Calcium oxalate crystals (COM and COD) exhibited characteristic diffraction peaks at $2\theta = 29^0, 23^0$ and $16^0, 25^0$Crespectively (fig 7).

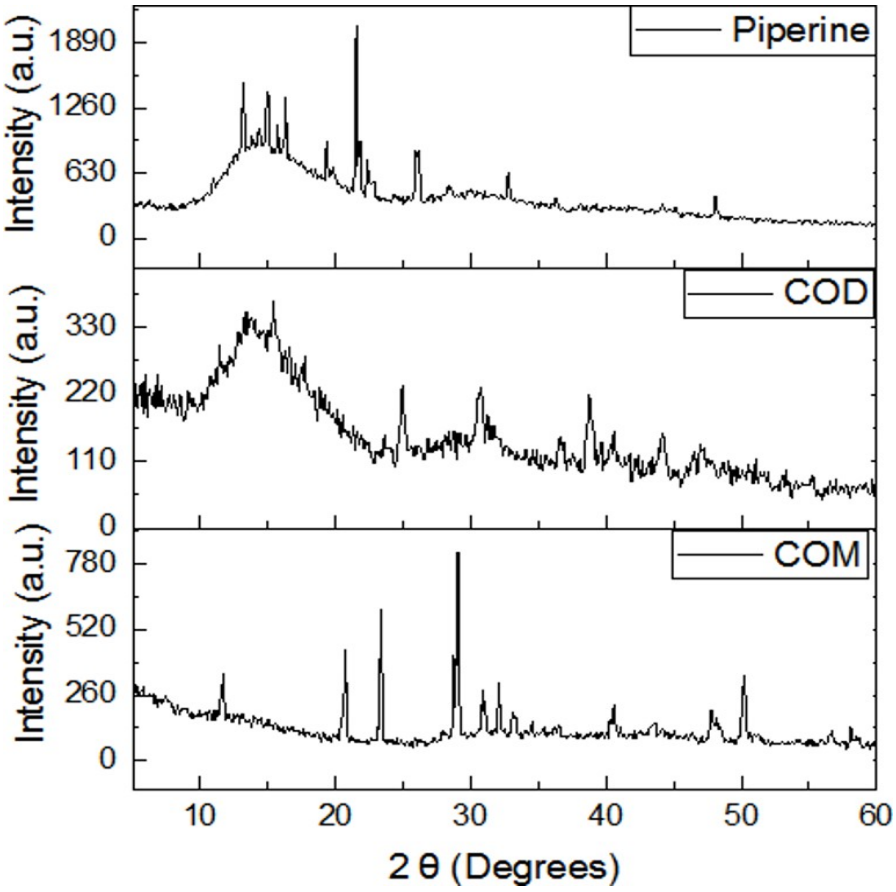

Fig7: PXRD pattern of Piperine, Calcium oxalate dihydrate crystals (COD) and calcium oxalate monohydrate crystals (COM).

Nucleation assay

Piperine was found to effectively inhibit the crystallization process by preventing nucleation of calcium oxalate through disintegration in smaller particles. From the results it is confirmed that the piperine exhibited significant nucleation-inhibition activity as compared to citric acid and 1 N HCl (Fig.8).

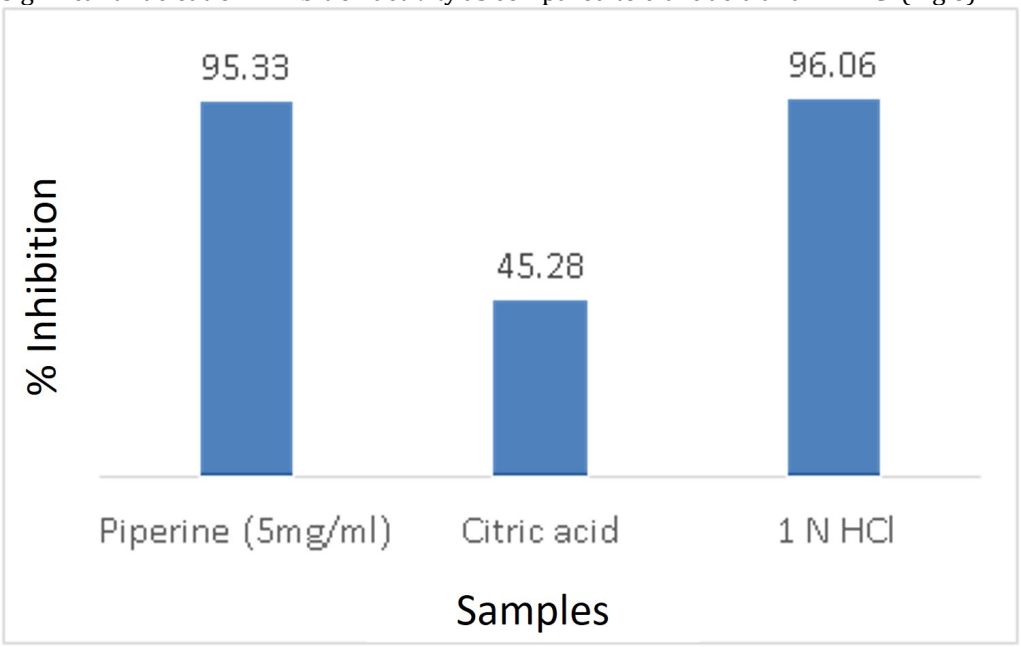

Fig 8Inhibition of Nucleation by piperine in comparison with citric acid and 1 N hydrochloric acid at 620nm.

Aggregation assay

Piperine compared to citric acid solution shows significant inhibition of aggregation of crystals and hence prevents the development of COD crystals. Fig9 shows the inhibition of aggregation of COD at 0, 30, 60 and 90 min time interval respectively against citric acid and 1 N hydrochloric acid, absorbance at 620 nm.

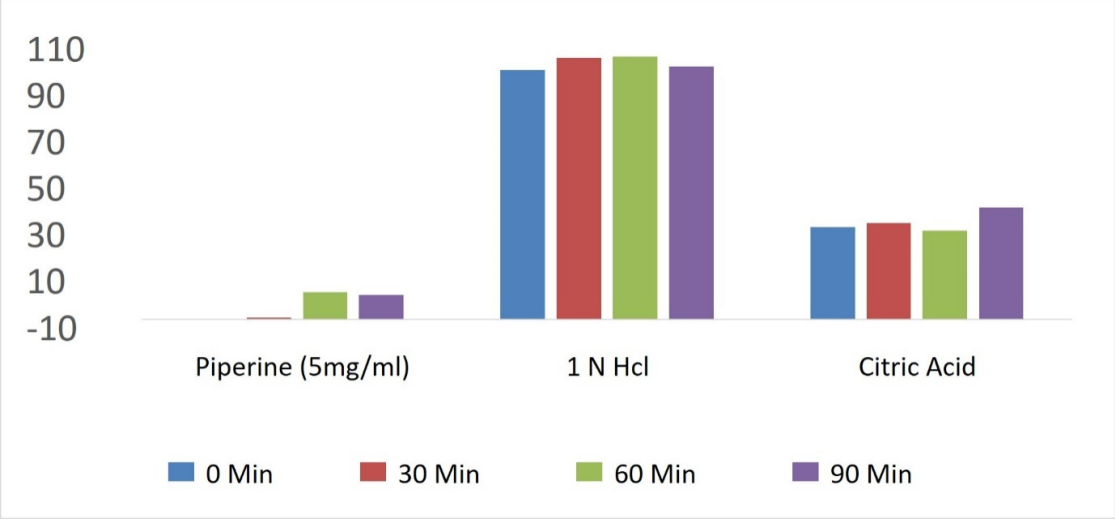

Fig. 9 Inhibitory effect of Piperine on aggregation assay at different time interval compared with citric acid and 1 N hydrochloric acid absorbance at 620nm.

DISCUSSION

Piper longum is one of the "Rasayana Drugs" in Ayurveda. Rasayana drugs enjoy several claims such as adaptogens and immunomodulator; anti-inflammatory treatment of mental diseases and is an important ingredient in "Panchkarma" [12]. Isolation of piperine using traditional method is cumbersome, and requires use of many solvents. The method developed in this study involves the use of petroleum ether for extraction and method for recrystallization. Piperine is sparingly soluble in petroleum ether at room temperature[13]. Increasing the temperature using continuous hot extraction method (soxhlet

apparatus) increases the solubility of piperine. The hot solution of piperine in petroleum ether on cooling, forces the piperine to crystallize out (by reducing its solubility in cold condition).Crude crystals of piperine may contain other alkaloids such as piperlongumine, and piperlonguminine The yield was around 1.23 % w/w. When the crude product was subjected to recrystallization in methanol, the yield of pure crystals was found to be 0.56 % w/w Piperine needles can be crystallize out from methanol [13].

The pure piperine crystals were observed under microscope wherein, they reveal the characteristic acicular shape. Thus, suggesting the presence of piperine. Further, particle size analysis carried out at particle size analyzer (Horiba, Japan) revealed the size range of 135 nm for piperine, whereas; 84 nm and 318 nm were the size range obtained for COM and COD respectively.

Instrumental characterization with UV-visible spectrophotometer exhibited typical absorbance of piperine at 367 nm. This is attributed to the six consecutive double bonds in the structure. Further, FT-IR spectra of the piperine confirmed the presence of major functional groups such as 3733-3493 cm^{-1}, ketonic group, alcohols, double bonds.

The H1NMR exhibited protons in the structure like phenolic and aliphatic protons. Moreover, the spectra obtained from powder X-ray diffractometer confirms the purity of isolated piperine.

Supersaturation of urine leads to crystallization of calcium oxalate particles within urinary tract. Citric acid and hydrochloric acid taken as standard compound. The prior step for the initiation of crystal in, in vitro crystallization is nucleation. The crystals are grown and form aggregates.

Nucleation is a primary stage in the development of crystals from its solution. rom its solution. Later, the crystal size grows as other molecules/particles deposits on the nucleated crystal. Subsequently, these nano or micro-crystals agglomerate to develop large crystal lattice. However, if the nucleation step is inhibited, it prevents crystal formation in a solution. Piperine (5 mg/ml) has significantly inhibited nucleation of calcium oxalate crystals. This could be attributed to the inhibition of juxta-position of molecules in formation of their crystal lattice [14].

Aggregation is the step in which, crystals of a solution attach to each other and form bulky particles. Reports reveal that crystal aggregation is a vital stage in the mechanism of stone formation in the urinary system [15]. Crystal aggregation is encouraged by viscosity of the surrounding medium. Viscosity in the urine can be raised by Tamm-Horsfall glycoprotein or other macromolecules [16].Reduction in crystal growth of calcium oxalate can be attributed to the inhibitory effect of Piperine in agglomeration of the crystals.

CONCLUSION

The present study confirms the anti-nucleation and anti-aggregatory property of piperine in inhibiting the crystallization process of calcium oxalate. Hence, it is advantageous in the treatment of urolithiasis. Thus, frequent consumption of *Piper nigrum* of *Piper longum* is beneficial in the prevention and/or management of urolithiasis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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STATEMENT ON THE WELFARE OF ANIMALS

This article does not contain any studies with human participants or animals performed by any of the authors.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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