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ORIGINAL ARTICLE

In vitro Assessment of Antioxidant and Antiurolithic Activities of Ethanol Extract of Whole Plant *Biophytum sensitivum* (Linn.) DC

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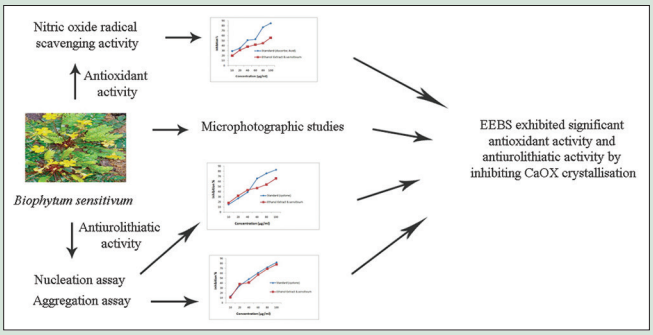
ABSTRACT

Background: Previous research studies have demonstrated that the formation of urinary stones leads to oxidative stress in patients; hence, search for antiurolithic drugs possessing antioxidant activities from natural sources has gained great potential. **Objectives:** The present study was undertaken to investigate *in vitro* antioxidant and antiurolithic potency of ethanol extract of whole plant *Biophytum sensitivum* Linn. DC (EEBS). **Materials and Methods:** The antioxidant potential of EEBS was determined by nitric oxide radical scavenging assay. Inhibition capacity of EEBS on calcium oxalate (CaOx) crystallization was evaluated by nucleation assay, aggregation assay, and microscopic assay (image analysis of CaOx crystal morphology). **Results:** Half-maximal inhibitory concentration (IC_{50}) value of nitric oxide radical scavenging activity of EEBS was found to be 90.12 µg/mL and that of ascorbic acid (standard) was 37.23 µg/mL. In nucleation assay, IC_{50} of EEBS was found to be 68.82 mg/mL, compared with 52. 41 mg/mL for cystone (standard). In aggregation assay, IC_{50} value was indicated as 52.39 mg/mL and for cystone, it was found to be 41.62 mg/mL. Addition of various concentration of EEBS (20, 40, 80, and 160 mg/mL) resulted in change in structure of CaOx crystals. EEBS a concentration of 160 mg/mL reduced the size of CaOx crystals to 812.68 µm whereas size of CaOx crystal treated with the control was 1398.05 µm. The size reduction of CaOx crystals was found to be dose-dependent. **Conclusion:** This plant can be used alone or in combination with other herbal drugs, as EEBS showed significant antioxidant and antiurolithic activities.

Key words: Aggregation assay, antioxidant, antiurolithic, *Biophytum sensitivum,* microscopic assay, nucleation assay

SUMMARY

- Ethanol extract of *Biophytum sensitivum* exhibited significant scavenging effect on nitric oxide radicals compared to the standard (ascorbic acid)
- Ethanol extract of *B. sensitivum* showed an inhibitory effect on nucleation and aggregation of calcium oxalate crystals in a concentration-dependent manner
- In microphotographic studies, Ethanol extract of *B. sensitivum* at concentration of 160 mg/mL reduced the size of calcium oxalate crystals to 812.68 µm whereas calcium oxalate crystal treated with control was 1398.05 µm.



Abbreviations Used: EEBS: Ethanol extract of *Biophytum sensitivum*; CaOx: Calcium oxalate; IC_{50}: Half-maximal inhibitory concentration; ESWL: Extracorporeal shockwave lithotripsy; Tris-HCl: Tris hydrochloride; NaCl: Sodium chloride; $CaCl_2$: Calcium chloride; $Na_2C_2O_4$: Sodium oxalate; COM: Calcium oxalate monohydrate; COD: Calcium oxalate dihydrate; Abs: Absorbance

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INTRODUCTION

Urolithiasis is the third most common affliction of the urinary tract which is exceeded by the urinary tract infections and prostate diseases.[1] Kidney stone formation is a worldwide problem and is estimated that 12% of world population experiences renal stone disease with a recurrence rate of 70–80% in male and 47–60% in female.[2] Crystallisation of calcium oxalate (CaOx) begins with increased urinary supersaturation with subsequent formation of the solid crystalline particles within the urinary tract, followed by nucleation, growth, aggregation, and retention within the kidneys.[3] Calcium-containing stones, especially CaOx monohydrate, CaOx dehydrate, and basic calcium phosphate, are the most commonly occurring stones. CaOx stones are generally found in two forms: CaOx monohydrate (Whewellite) and CaOx dihydrate (Weddellite). CaOx monohydrate is thermodynamically most stable and common form. It has greater affinity for renal tubular cells and is responsible for the formation of stones in the kidney than CaOx dihydrate.[4] Other types of stone include uric acid stone, struvite stone, cystine stone, silicate stone, protease-related stone, and dihydroxyadenine crystals.[5]

At present, there were no satisfactory drugs available in the market for the treatment, prevention, or recurrence of stones.[6] Synthetic drugs used for the treatment of kidney diseases are associated with higher incidence of adverse drug reactions. Invasive procedures such as extracorporeal shockwave lithotripsy, ureteroscopy, and nephrolithotomy are considered to be effective, but they are costly, may reduce renal functions, increase possibility of acute renal injury, infections, and recurrence of kidney stone formation.[7]

Urolithiasis is a complex process that occurs due to imbalance between promoters and inhibitors of stone formation in the kidneys.[8] This

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natural crystallization inhibition capacity is in deficit in stone formers.[9] Recent clinical and preclinical studies have reported that CaOx crystals directly induce renal epithelial cell injury mediated through lipid peroxidation and oxygen free radical generation.[10,11] Various etiological factors are involved in stone formation[12] so that treatment aimed at multiple targets, such as antispasmodic, anti-inflammatory, diuretics, antioxidant, antibiotics, muscle relaxants, and analgesics. Multiple chemical constituents present in medicinal plants may offer effective, inexpensive, and safe remedy for the treatment of urolithiasis. The various marketed antiurolithic herbal formulations have been used worldwide. Ureteric calculus disappeared within 55 days of treatment with "Cystone" a herbomineral composition by relaxing the detrusor muscles and increasing diuresis.[13]

Biophytum sensitivum Linn. DC (*B. sensitivum*; Common names: *Nilaccurunki*, *Tintaanaalee* in Tamil; *Mukkutti* in Malayalam; and *Lajalu*, *Lajjaalu*, and *Lakshmana* in Hindi) belongs to family Oxalidaceae.[14] Phytochemical investigation of *B. sensitivum* had revealed the presence of large amount of phenolic and polyphenolic compounds, saponins, polysaccharides, pectin, and essential oils. Main bioactive constituents are bioflavonoids such as amentoflavone with trace amounts of cupressoflavone, luteolin, isoorientin, and isovitexin.[15,16] *B. sensitivum* has been used as a traditional folk medicine for various ailments. Grounded leaves of *B. sensitivum* has been used for diuretic effect and powdered form for urolithiasis.[17] Recent pharmacological studies showed that it has antioxidant,[18] antibacterial,[19] antidiabetic,[20] antitumor,[21,22] cardioprotective,[23] immunomodulation, radioprotective, anti-inflammatory activities,[24] and many more. Hence, the search for antiurolithic drugs possessing significant antioxidant activities from natural sources has gained great potential; the present study was aimed to investigate *in vitro* antiurolithic and antioxidant activity of ethanol extract of whole plant *B. sensitivum*.

MATERIALS AND METHODS

Chemicals

Cystone was procured from Himalaya health care, Bangalore, India. Other reagents used in this study including ascorbic acid, sodium nitroprusside, naphthyl ethylenediamine dihydrochloride, glacial acetic acid, sulfanilic acid reagent, calcium chloride ($CaCl_2$) dihydrate, tris buffer, and sodium oxalate ($Na_2C_2O_4$) were of analytical grade and obtained from Himedia laboratories, Mumbai, India.

Plant source and identification

The whole plant, *B. sensitivum,* was collected from Shevaroy Hills, Salem District, Tamil Nadu and was taxonomically identified and authenticated by Dr. A. Balasubramanian, Executive Director, ABS Botanical conservation, Research and Training Centre, Kaaripatti, Salem (Dt) T.N.(Ref. No.-AUT/JKK/095).

Preparation of extracts

The whole plant was washed and dried in the shade for about 3 weeks. Dried plant was coarsely powdered, sieved (mesh size = 40), and stored in airtight container at room temperature. Powdered plant material (500 g) was sequentially extracted with petroleum ether (60°C–80°C) for defatting the drug and then with 70% ethanol using Soxhlation method. The obtained solvent extract was filtered and evaporated to dryness at 45°C under reduced pressure using a rotary evaporator. The dried extract was stored in the airtight container.[25]

Phytochemical investigation

The ethanol extract of *B. sensitivum* (EEBS) was tested for the presence of carbohydrate, alkaloids, flavonoids, tannins, glycosides, saponins, terpenes, steroids, protein, and phenolic compounds using the standard procedures.[26]

*In vitro a*ntioxidant/free-radical scavenging activity assay

Nitric oxide radical scavenging assay

This assay was done according to the method of Garat *et al*.[27] Griess ILosvay reagent was modified using naphthyl ethylenediamine dihydrochloride (0.1%w/v) instead of the use of 1-naphthylamine (5%). A volume of 2 mL of 10 mM sodium nitroprusside prepared in 0.5 mM phosphate buffer saline was added to 0.5 mL of various concentration of EEBS or standard (10, 20, 30, 40 60, 80, and 100 µg/mL). The mixture was incubated at 25°C for 2.5 h. After incubation, 1.5 mL of reaction mixture was mixed with 1.5 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% naphthyl ethylenediamine dihydrochloride) and incubated at room temperature for 5 min. The absorbance (Abs) was read at 546 nm and ascorbic acid was taken as standard. Percentage inhibition was calculated using the formula:

Percentage Inhibition (%) = Abs of control – Abs of test/Abs of control × 100

Determination of inhibition capacity of extract on calcium oxalate crystallization

Nucleation assay

Inhibition capacity of plant extract on CaOx crystallization was determined according to the method described by Hennequin *et al*.[28] This assay was conducted with EEBS or standard compound cystone at 10, 20, 40, 60, 80, and 100 mg/mL concentrations. For each sample testing, 1 mL of 0.025 M $CaCl_2$, 2 mL of 0.05 mol/L Tris–buffer, 1mL of plant extract or standard at different concentrations were added to test tube initially, then 1 mL of 0.025 M $Na_2C_2O_4$ was added at room temperature (37°C) to study the percentage of inhibition. Procedure was repeated for six duplicates for each sample. The rate of nucleation was determined by comparing appearance of crystals that reached critical or optically detectable size in the presence of extract and that of control with no extract. The Abs was recorded at 620 nm and the percentage inhibition was calculated using the formula:

Percentage Inhibition (%) = Abs of test/Abs of control × 100

Aggregation assay

Rate of aggregation of CaOx crystals was determined by following the method of Atmani *et al*.[29] The CaOx crystals were prepared by mixing 1 mL of 0.025 M $CaCl_2$ and 1 mL 0.025 M $Na_2C_2O_4$. Both solutions were then equilibrated at 60°C in a water bath for 1 h. The solutions were then cooled overnight at 37°C. The crystals formed were centrifuged for 5 min and harvested crystals were evaporated for 5 min at 37°C. The crystals were used at concentration of 0.8 mg/mL, buffered with tris hydrochloride 0.05 mol/L, and sodium chloride 0.15 mol/L at pH 6.5. Experiments were conducted at 37°C with 1 mL of EEBS or cystone (standard) at various concentrations (10, 20, 40, 60, 80, and 100 mg/mL). Stirred well and then the rate of aggregation was estimated by comparing the turbidity in the presence of EEBS or cystone (standard) with that of control. The Abs at 620 nm was recorded. The rate of aggregation or percentage inhibition rate (Ir) was estimated using the following formula:

Percentage Inhibition (Ir) =1 - Turbidity of test/Turbidity of control × 100

Microscopic assay (image analysis of calcium oxalate crystal morphology)

Incubation of metastable solutions of $CaCl_2$ and $Na_2C_2O_4$ resulted in the formation of CaOx crystals. The harvested crystals were centrifuged and placed on a Petri plate glass slide and various concentration of EEBS (20, 40, 80, and 160 mg/mL) and control were then applied directly to the crystal. Change in structure of CaOx crystals was compared with the control by observing under microscope after 30 min to determine how crystals were dissolved by extract. Crystal size was observed under Leica stereo zoom dissecting microscope with digital imaging system at ×4 and the photographs were taken.[30]

Statistical analysis

Results were expressed as mean value ± standard deviation. Student's *t*-test was used for comparison between values of samples and standards. Differences were considered statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

Percentage yield of petroleum ether and ethanol extract of *B. sensitivum* were 4.92% w/w and 12.54% w/w, respectively. Preliminary phytochemical investigation indicated that EEBS showed the presence of phytochemicals such as carbohydrates, steroids, flavonoids, alkaloids, fixed oils, tannins, saponins, protein, amino acids, and phenolic compounds.

Inhibition of nitric oxide radical

Generated nitric oxide radical from sodium nitroprusside is measured by Greiss reduction. Sodium nitroprusside at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions. Figure 1 of this study illustrated that the half-maximal inhibitory concentration (IC_{50}) value of EEBS was found to be 90.12 µg/mL and that of ascorbic acid (standard) was 37.23 µg/mL. The result indicated that EEBS had significant scavenging effect on nitric oxide radicals in a concentration-dependent manner ($P < 0.05$). Standard ascorbic acid at a concentration of 10–100 µg/mL inhibited production of nitric oxide radical by 29%–86% whereas EEBS inhibited nitric oxide radical generation by 20%–56%, thereby exhibited radical scavenging activity. The inhibitory potentials of EEBS against this highly reactive compound may be attributed to their ability to compete with oxygen for nitric oxide leading to reduced production of nitrite ions.

Nucleation assay

The *in vitro* inhibitory effect of various concentration of EEBS (10, 20, 40, 60, 80, and 100 mg/mL) on different phases of CaOx crystallization was determined. The EEBS at various concentrations exhibited inhibitory effect on nucleation and was comparable with that of cystone. There was a steep decrease in the Abs with increasing concentration of the extract. Figure 2 depicts that the percentage inhibition of extract on nucleation of CaOx crystals was found to be 18–66%, whereas with cystone, it was 15–83%. IC_{50} of the EEBS was 68.82 mg/mL, compared with 52.41 mg/mL for cystone. The extract might contain some phytochemicals that inhibit the growth of crystals and thereby reducing the possibility of tubular injury. A similar inhibition of CaOx monohydrate stones was also reported for *Adiantum capillus*[31] and *Terminalia arjuna*.[32]

Aggregation assay

Figure 3 illustrated that EEBS extract showed a significant dose-dependent inhibition on aggregation of CaOx crystals. The percentage inhibition of the EEBS on CaOx aggregation was found to be 11%–78%, whereas with cystone, it was 13%–82%. IC_{50} of the plant extract was 52.39 mg/mL, and for cystone, it was found to be 41.62 mg/mL. Higher concentrations of EEBS showed lower turbidity (aggregation). Hence, this result indicated that EEBS possess phytoconstituents that inhibit the aggregation of CaOx crystals. Rapid crystal formation is the most critical step in urolithiasis.

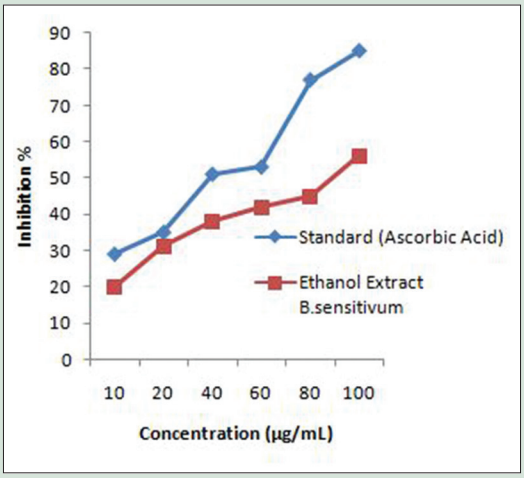

Figure 1: Nitric oxide radical scavenging activity

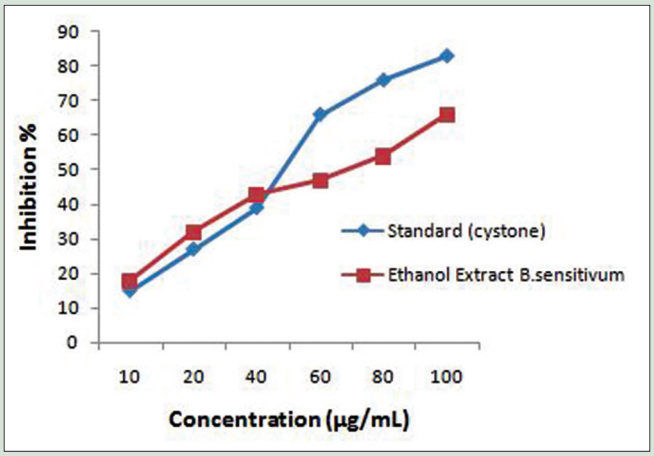

Figure 2: Nucleation assay

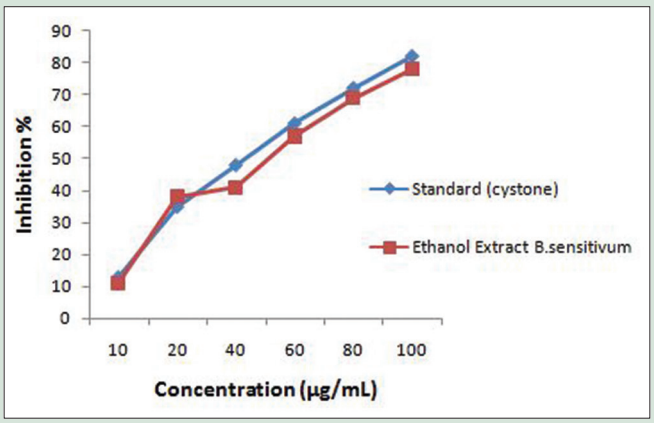

Figure 3: Agreggation assay

Microscopic studies

In microphotographic study, incubation of metastable solutions of $CaCl_2$ and $Na_2C_2O_4$ resulted in the formation of CaOx crystals. The corresponding size of CaOx crystals treated with control and various concentration of EEBS was illustrated in Figures 4-8. Addition of EEBS at various concentration resulted in size reduction of CaOx crystals in dose-dependent manner. Crystal size was compared with the control by observing under microscope. Higher concentrations of EEBS (160 mg/mL) reduced the size of CaOx crystals to (812.68μm), whereas CaOx crystal size treated with control was 1398.05 µm. Our results suggest that phytochemicals from the plant exert their action directly on the crystals.[33]

Crystal size is a limiting factor in stone formers as large particle occludes, less likely to pass through urinary tract and subsequently induce injury on urinary tract. Therefore, antiurolithic activity is mainly associated with the dissolution of stone forming constituents in urine which further prevent its crystallization and recurrence. Extract of B. ciliata promoted the formation of calcium oxalate dehydrates crystals rather than calcium oxalate monohydrate crystals.[34] Previous research studies mentioned the significance of polyphenols and flavonoids in the antioxidant and antiurolithic activities of different plant extracts.[32,35]

CONCLUSION

Phytochemical investigation of EEBS revealed that it contained large amounts of phenolic and polyphenolic compounds, saponin, polysaccharides, pectin, and essential oil. Preclinical or clinical data confirmed that the formation of urinary stones leads to oxidative stress in patients. Significant antioxidant property of EEBS is due to the presence of bioactive phytoconstituents such as amentoflavone, a bioflavonoid with trace amounts of cupressoflavone, luteolin, isoorientin, and isovitexin. Saponins are known to have anti-crystallization properties by disaggregating the suspension of mucoproteins, the promoters of crystallization.[36] Antiurolithic activity is attributed mainly due to the presence of saponins. A saponin-rich fraction of Herniaria hirsuta was also found to be a potent inhibitor of CaOx stone formation.[37] Hence, this plant, used alone or in combination with other herbal drugs, may exhibit excellent antiurolithic and antioxidant activities.

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Figure 4: CaOx crystal treated with control

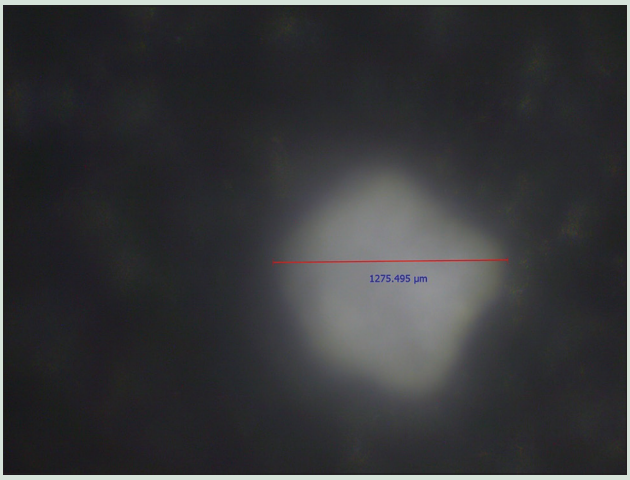

Figure 5: CaOx crystal treated with EEBS (20 mg/mL)

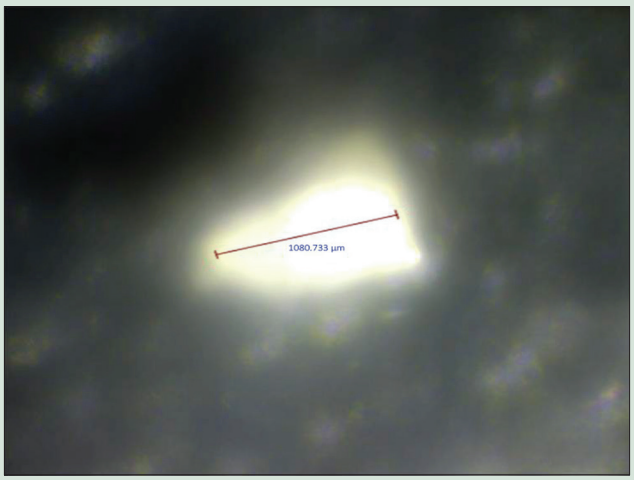

Figure 6: CaOx crystal treated with EEBS (40 mg/mL)

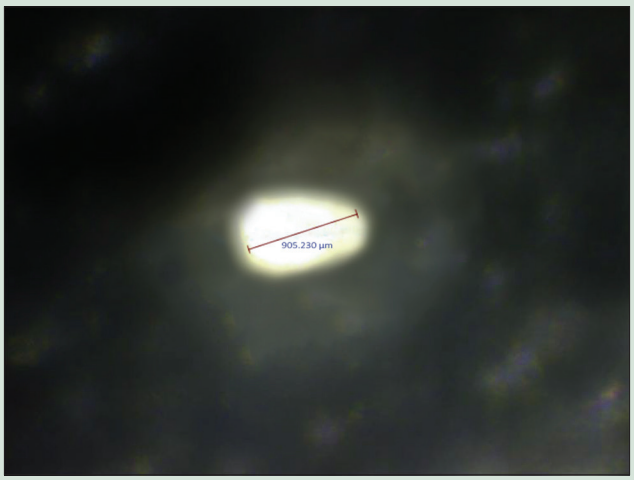

Figure 7: CaOx crystal treated with EEBS (80 mg/mL)

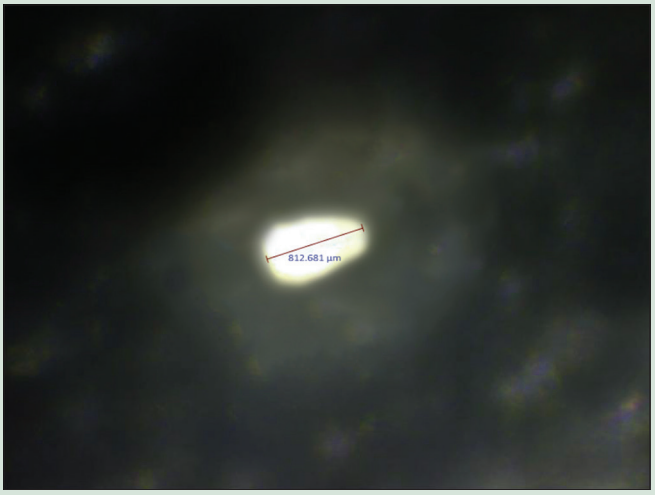

Figure 8: CaOx crystal treated with EEBS (160 mg/mL)

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Nill.

Conflicts of interest

There are no conflicts of interest.

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