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"*In vitro* Studies on Indigenous Medicine for Urolithiasis: Efficacy of Aqueous Extract of *Aerva lanata* (Linn.) Juss. Ex Schult on Growth Inhibition of Calcium Hydrogen Phosphate Dihydrate"

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Aerva lanata (L.) Juss. ex Schult., is one of the medicinal plants widely used by traditional healers for the treatment of urolithiasis. A perusal of literature revealed that no studies have so far been undertaken to evaluate its efficacy against Calcium Hydrogen Phosphate Dihydrate (CHPD) or brushite type urinary crystals. Hence, an attempt is made in this study to assess the validity of this indigenous knowledge by using *in vitro* single diffusion gel growth technique. The morphology of CHPD crystals was studied by microscopy. The structural and chemical changes of the treated crystals were assessed by FTIR, XRD, TGA/DTA and SEM/ EDX analysis. The study revealed that aqueous extract of *Aerva lanata* is very effective to make structural changes and reduction in size of the brushite type urinary crystals.

Keyword: Urolithiasis, *Aerva lanata,* Calcium Hydrogen Phosphate Dihydrate, SEM, EDX, FT-IR, TGA/DTA and XRD.

1. Introduction

Urolithiasis is a common clinical condition referred to as kidney stone and it affects 12% of the world population [1]. The disease frequency is on the rise due to imbalanced life style, mental stress, lack of physical exercise, unnatural dietary habits and dilution of life sustaining natural processes. In modern medicine, drugs without side effects are not available to disintegrate/dissolve urinary stone [2]. In the indigenous system of medicine, there are many reports regarding the efficacy of phyto-medicines for the treatment of urinary stones. *Aerva lanata* (L.) Juss. ex Schult., is one such medicinal plant widely recommended by traditional healers for the treatment of urolithiasis [3]. Either whole plant or shoots /roots are used as a single drug in traditional medicine [4]. In the present investigation, we aim to assess the validity of this indigenous knowledge by conducting *in vitro* crystallization experiments based on reverse pharmacological approach [5]. The study aims to explore the synergetic action of phyto- chemicals in the aqueous shoot extract of *Aerva lanata* on *in vitro* growth inhibition of CHPD (brushite type) urinary crystals.

2. Materials and Methods

An ethnobotanical survey was conducted among the traditional healers and herbal collectors of Kottayam district to document their traditional knowledge on antiurolithiatic medicinal plants. 60% of the respondents suggested that **Cherula** [*Aerva lanata* (L.) Juss. ex Schult] is as effective as **Kalloorvanchi** [*Rotula aquatica* Lour.] to disintegrate urinary stones [3]. Hence, Cherula [*Aerva lanata* (L.) Juss. ex Schult] was selected for the *in vitro* validation of its traditional antiurolithiatic property. The plant specimens were collected from CMS college campus and authenticated in the Department of Botany, CMS College, Kottayam. The voucher specimen (accession no: 3402) is deposited in the CMS Herbarium, C.M.S. College, Kottayam. The aerial parts of the plant were cleaned in tap water, shade dried and powdered. The aqueous extract (40 mg/ml) with appropriate dilution was used for further *in vitro* inhibition studies on CHPD/brushite type urinary crystals. The proportions of additive [*Aerva lanata* shoot extract (Als)], with respect to $CaCl_2$ and double distilled water used in different treatments are as follows:

- Treatment 1 **(Control)** - 5 ml $CaCl_2$: 5 ml water
- Treatment 2 **(ALS1)** - 5 ml $CaCl_2$: 1 ml Als : 4 ml water
- Treatment 3 **(ALS2)** - 5 ml $CaCl_2$: 2 ml Als : 3 ml water
- Treatment 4 **(ALS3)** - 5 ml $CaCl_2$: 3 ml Als : 2 ml water
- Treatment 5 **(ALS4)** - 5 ml $CaCl_2$: 4 ml Als : 1 ml water

2.1 Single diffusion gel growth technique:

The antiurolithiatic property of *Aerva lanata* was verified by the growth dissolution studies of Calcium Hydrogen Phosphate Dihydrate (CHPD), employing hydrogel method developed by Henisch *et al.* (1998) [6], with appropriate modifications as suggested by Rajendran and Dale (2010) [7] and Parekh *et al.* (2007) [8]. The hydrogel was prepared from Sodium Metasilicate ($Na_2SiO_3.9H_2O$, m.wt.284 g/mol) and specific gravity of the gel was adjusted to 1.06 using deionized water. Freshly prepared $CaCl_2$ solutions were used for growing CHPD crystals. The pH of the gel solution was adjusted to 6.5 by adding one molar Orthophosphoric acid. The gel thus prepared was used as a medium for growing crystals employing sterile Petri- plates and Test tubes.

The initial stages of crystal growth were studied by microscopic observation of the CHPD crystals grown in the micro-slides. A drop of solution prepared for gel was put at the middle of a sterile micro slide and it was kept inside a sterile Petri dish. The gel is then covered with cover slip and allowed to set slowly. Thereafter, suitable concentration of the calcium chloride solution was poured up to the level of the cover slip. The poured solution was allowed to diffuse slowly through the gel to initiate nucleation, aggregation and growth of micro-crystals. Different concentrations of the **Als** extract were then added to calcium chloride to study the growth inhibition and dissolution of CHPD crystals. The slides were observed after 5 minutes using a compound microscope (Olympus); the photographs were taken and were subjected to morphometric analysis using image analyzer software.

CHPD crystal growth inhibition studies were also conducted in sterile test tubes. For that, 10ml of the gel solution was poured in and was allowed to set slowly at room temperature. Equal volume of Calcium chloride solution was added into each tube. Different concentrations of the **Als** extract (I% to 4%) were then added through the sides of the test tubes and the inhibitory effects were analyzed based on the changes noted with respect to number, size and morphology of crystals. After 3 weeks, the crystals were harvested from the gel, washed in double distilled water, filtered and then air dried. The dried crystals were characterized by SEM/EDX, FTIR, TG/DTA and XRD techniques.

3. Results and Discussion

The results of the present study revealed the synergetic action of bioactive compounds of *Aerva lanata* on *in vitro* growth inhibition of calcium phosphate urinary crystals (Fig. 1 and Fig. 2). With increasing concentration of the

shoot extract (Als1-Als4) a gradual reduction in the size with alteration in the morphology of crystals were observed (Table 1). The morphology of the crystal formed in the control (star & sword) gets altered into spindle, round or oval in the treatments ALS2 to ALS4.The interference with crystal growth and aggregation is a possible therapeutic strategy for prevention of recurrent stone diseases. It is suggested that, macro molecules of higher molecular weight of plant extracts exert their action similar to natural urinary inhibitors and inhibit crystal nucleation, growth and aggregation [9]. Moreover, the frequency of the sharp edged and larger crystals or crystal aggregates were much reduced in treatments (ALS1, ALS2, ALS3 & ALS4). This finding is in agreement with the results of earlier studies based on a glycoprotein inhibitor of calcium oxalate crystal growth [10, 11]. As pointed out by Parekh *et al.,* (2007) [8] change in morphology of crystal is an important phenomenon because, if the painful star type or spiky, needle, irregular stones were converted into smooth spherical or oval grain like ones, then, their passage through the urethra is less painful.

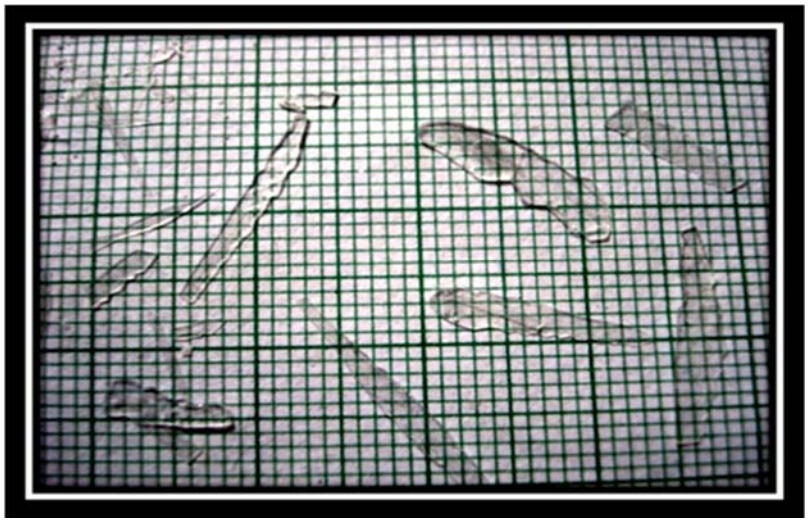

Fig 1: Harvested CHPD crystals

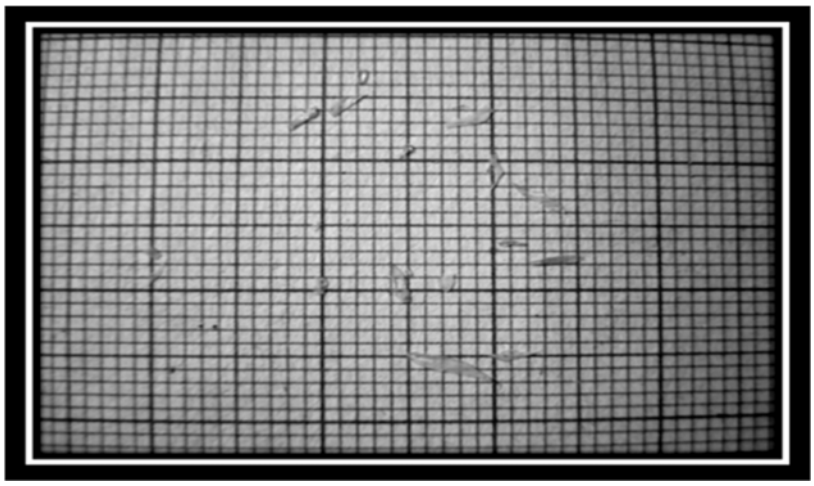

Fig 2: Harvested ALS4 crystals

Table 1: Average apparent dimensions of the grown crystals in control and Als extract treatments

S. No.	Treatments	Shape	Area range (µm^2)	Average number of crystals
1	C (CHPD)	Irregular Star Sword Leaf like	38-4125	4
2	ALS1	Dumbbell Spindle Round Irregular	3-358	9
3	ALS2	Spindle Oval Dumbbell	2-289	19
4	ALS3	Spindle Dumbbell Oval	1- 203	38
5	ALS4	Round Dumbbell Spindle	0.6– 117	43

FT-IR spectra of the gel grown crystals (control-CHPD and treatment-ALS4) are shown in Fig. 3. In the control (CHPD), the presence of water of crystallization was referenced from absorptions at 3543.16, 3487.57 and 3282.79 cm^{-1}, which are due to intermolecular and weakly H bonded OH. The absorption at 1650.29 cm^{-1} is due to H-O-H symmetric bending vibrations and P=O associated stretching vibrations were observed at wave numbers 1211.2, 1129.96 and 1064.34 cm^{-1}. Likewise, the P-O-P asymmetric stretching vibrations were observed at 873.10 and 790 cm^{-1}. The absorption at 665 cm^{-1} is due to (H-O-) P=O and the strong absorption at 578 and 525 cm^{-1}are due to acid phosphates. The comparison of the FTIR spectra of ALS4 with that of control (CHPD) revealed similarity with most of the bands along with two additional bands at 2827 cm^{-1} and 2884 cm^{-1}, which indicate C-H stretch (alkanes). Besides, it shows two shifts. The peak at 3282 cm^{-1} was shifted to a lower value at 3276 cm^{-1}; another peak at 1211 cm^{-1} was shifted to a higher value at 1214 cm^{-1}. A shift from 3282 cm^{-1} to 3276 cm^{-1} indicates new hydrogen bond formation between H-OH. The shift and appearance of two new peaks as mentioned above clearly indicate the incorporation of bioactive organic moiety of the **Als** (*Aerva lanata* shoot) extract within the crystal framework of CHPD.

The X-ray diffractogram of the gel grown CHPD crystal (Fig. 4) matches with the JCPDS data (72-0713). As it is evident from the Fig. 5, the XRD pattern of **Als** extract treated crystals (ALS4), shows shift in the peak positions, change in peak intensity and appearance of new peeks which shows the effectiveness of the **Als** extract to inhibit/reduce the growth and modify the crystal structure of CHPD crystals.

The TG-DTA curve of CHPD shows weight loss in two stages (Fig. 6). The major weight loss of about 20% occurs between 123 ºC and 191 ºC which indicates the loss of water of hydration. The endothermic peak in DTA around 123 ºC with the associated shoulders indicates the stepwise removal of water during this temperature range. Subsequently in the high temperature range of 191-441 ºC, two molecules of $CaHPO_4$ combine and result in the elimination of a water molecule leading to the formation of calcium pyrophosphate and nearly 74% of the sample was stable. The observed mass loss corresponds well with the DSC curve. The following chemical reactions are expected to occur during the dehydration and decomposition stages [12].

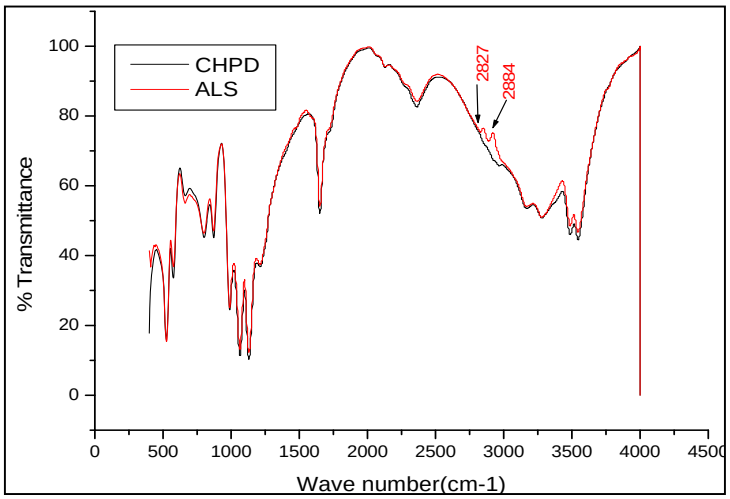

Fig 3: FTIR of control-CHPD (C) and treatment (ALS) crystals

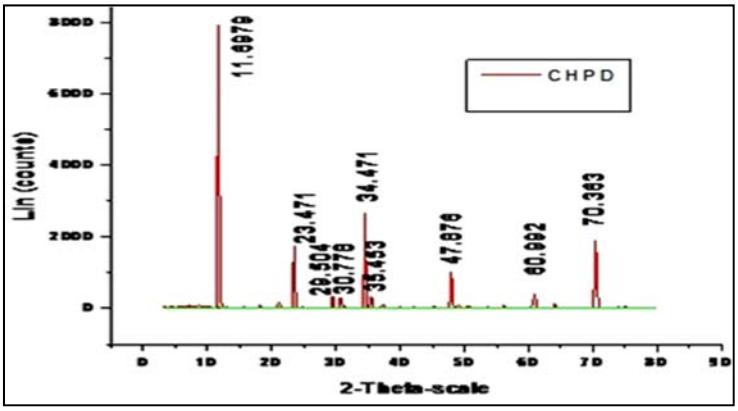

Fig 4: XRD pattern of CHPD

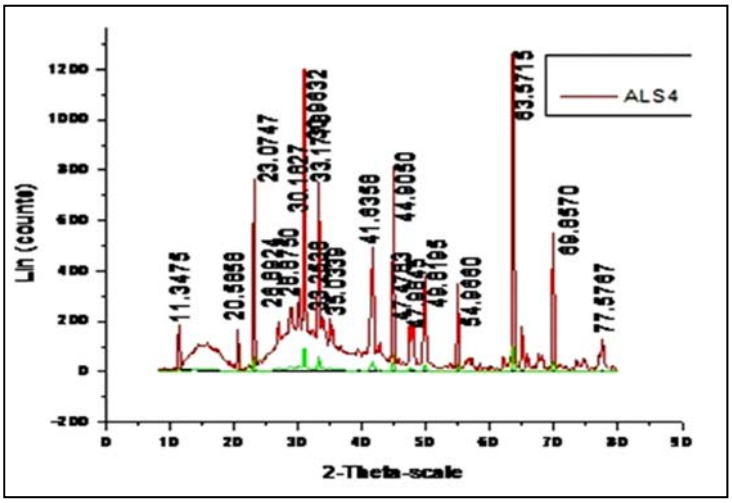

Fig 5: XRD pattern of ALS4

$2CaHPO_4.2H_2O \rightarrow 2CaHPO_4 + 4\ H_2O\uparrow$ --- (1)

$2CaHPO_4 \rightarrow Ca_2P_2O_7 + H_2O\uparrow$ ---------- (2)

The thermal degradation pattern of ALS4 crystals is not identical with that of CHPD (Fig. 7). In ALS4 crystals, the major weight loss of about 21.5% occurs between 110 ^{0}C and 192 ^{0}C. The first stage shows 2 shoulders along with a prominent peak at 189 ^{0}C. Onset of the second stage is at 370 ^{0}C and it shows nearly 3.5% weight loss up to 480 ^{0}C and the rest of the sample (75%) was stable up to the temperature 819 ^{0}C. This altered pattern of TG/DTA curve of *Aerva lanata* shoot treated (ALS4) crystals suggests incorporation of bioactive compounds from the aqueous shoot extract of *Aerva lanata*.

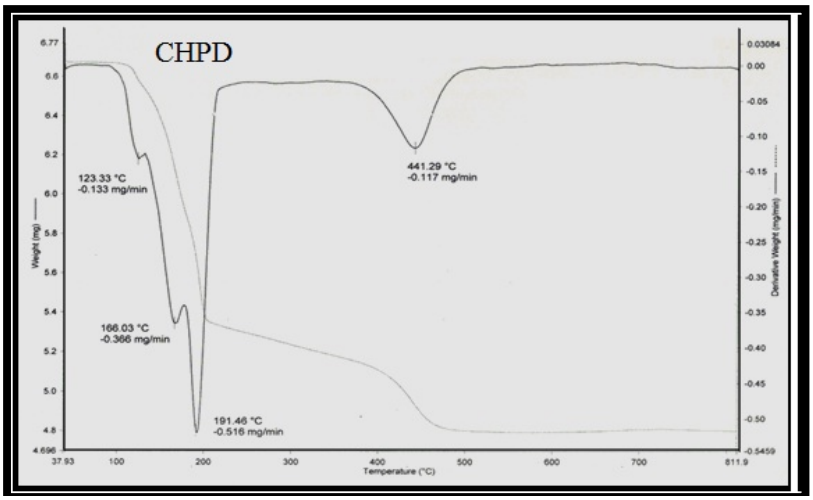

Fig 6: Thermogram of CHPD

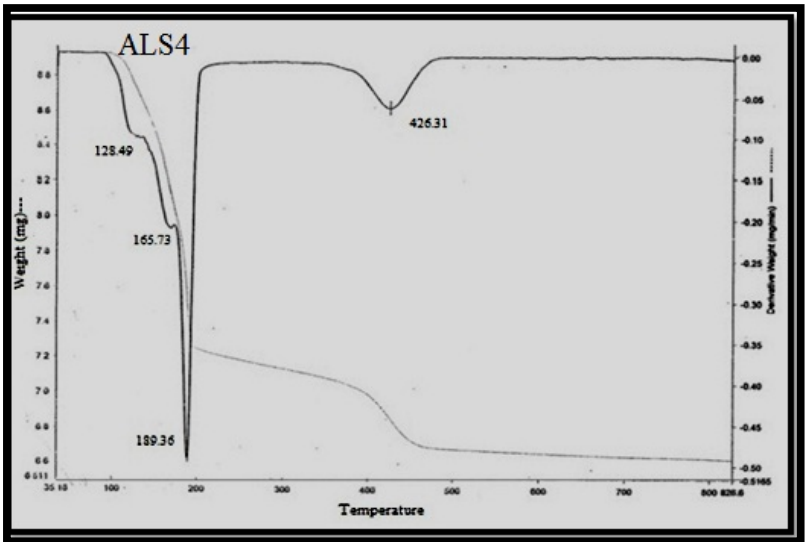

Fig 7: Thermogram of ALS4

The results of the SEM analysis also revealed the basic structural changes of the **Als** treated crystals (Fig. 9) from pure CHPD crystals (Fig. 8).The SEM of CHPD crystals shows a continuous sheet like formation without any intermediate space whereas the SEM of ALS4 shows a network structure with lots of space in between.

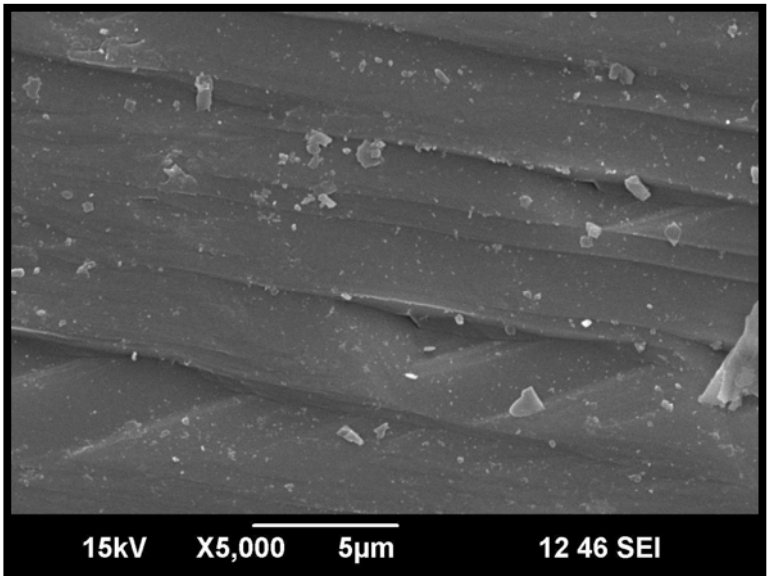

Fig 8: SEM of CHPD crystals

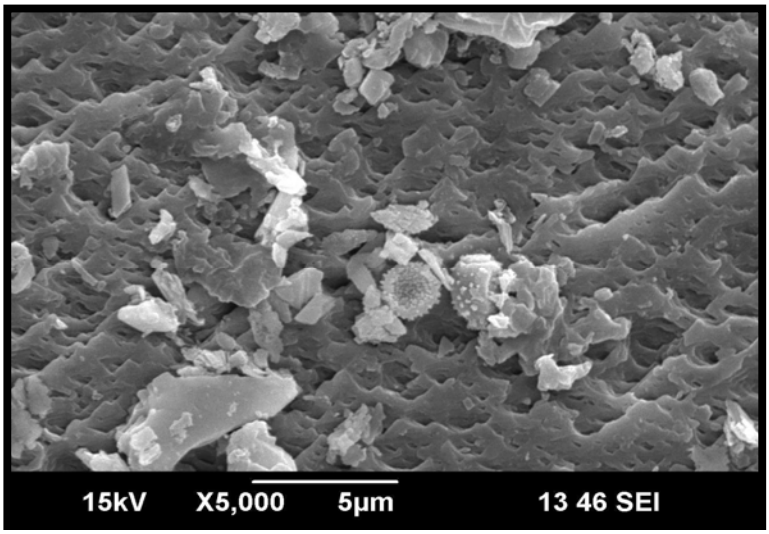

Fig 9: SEM of ALS4 crystals

The Energy Dispersive X-ray Spectroscopy (EDX) analysis of Als treated crystals (ALS4) revealed the presence of 3.72% of Carbon atoms along with expected Oxygen (36.57%), Phosphorous (32.65%) and Calcium (27.06%) atoms (Fig. 10). The above data give additional evidence to support the incorporation of organic moiety from the Als extract (Table: 2).

Table 2: Elemental analyses of CHPD & ALS4

Element	Pure CHPD	ALS4
	(Atom %)	(Atom %)
C	------	**3.72**
O	20.84	36.57
P	39.11	32.65
Ca	40.05	27.06
Total	100	100
Ca/P	1.02	0.83

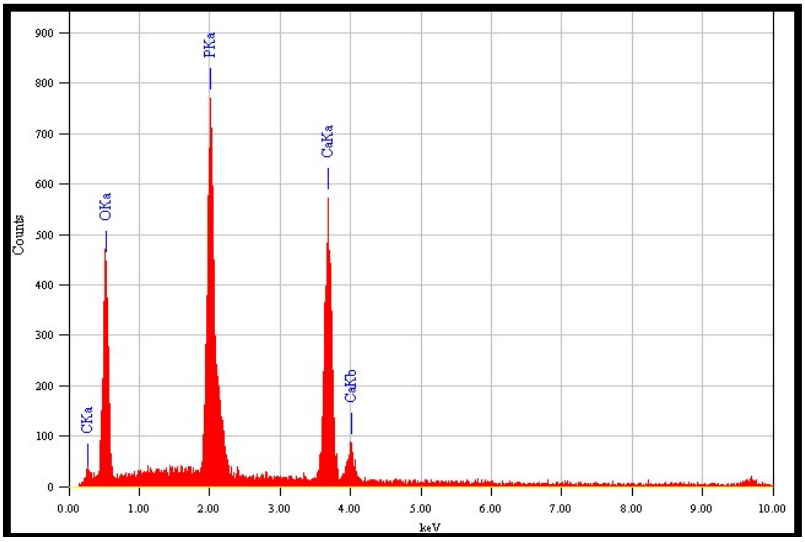

Fig 10: EDX of ALS4

The CHPD/Brushite crystal has a plate-like morphology dominated by (010) faces and the structure within the (010) plane is composed of two corrugated rows of Ca^{2+} and HPO_4^{2-} that are offset in the <010> direction. Between these calcium and phosphate containing sheets, are layers of water molecules bound to the calcium ions above and below the (010) plane [13]. It is assumed that when the interconnecting layers of water molecules, that bound to the calcium ions above and below the (010) plane, is unavailable for bonding due to incorporation of bioactive compounds in **Als** extract, further growth of CHPD crystals may get arrested. This explains one of the possible mechanisms for the efficacy of **Als** extract on growth inhibition of CHPD crystals under *in vitro* conditions. The FTIR and TGA/DTA data provide additional evidence that organic moiety from **Als** extract might have formed a bond with -OH on the surface of (010) face of CHPD. The role of phytoactive compounds of *Aerva lanata* in reducing the aggregation of CHPD/ brushite type crystals is substantiated in the present investigation. It is assumed that incorporation of the bioactive compounds within the framework of CHPD might have blocked further growth and aggregation ALS4 crystals. *In vivo* studies conducted by Soundararajan *et al*. (2006) [14] and Selvam *et al* (2011) [15] further revealed that bioactive substances of *Aerva lanata* end up in the urine and exert their action to disintegrate calcium oxalate type of crystals too. In short, the findings of the present investigation along with earlier *in vivo* studies substantiate the traditional botanical knowledge of *Aerva lanata* as an excellent single drug for the treatment of Urolithiasis.

4. Summary

The shoot extract of *Aerva lanata* was evaluated for its antiurolithiatic activity employing single diffusion gel growth technique. The morphological and structural examination of the treated CHPD crystals with optical/ scanning electron microscopy, FTIR, XRD and TGA/DTA analysis forms an evidence for the dissolution property of the bioactive compounds in shoot extract of *Aerva lanata*. The results of the present study provide conclusive evidence to corroborate the traditional botanical knowledge of the *Aerva lanata* as an effective antiurolithiatic agent.

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