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INHIBITION OF GROWTH OF *BRUSHITE* CRYSTALS USING KULITTH-*YUSH* WITH AND WITHOUT CITRIC ACID

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Urolithiasis, horsegram, *Macrotyloma uniflorum/ Dolichos biflorus*, *Piper longum*, *Piper nigrum*, *Zingiber officinale*

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ABSTRACT: The present study examined the potential of horsegram *yush/* broth on preventing growth of calcium hydrogen phosphate dihydrate (*Brushite*) crystals *in vitro*. Crystal length was measured daily over a period of 11 days Two types of *yush* were tested (a) *akruth yush* made with only salt or (b) *kruth yush* with *Trikatu* consisting of equal proportions of long pepper (*Piper longum)*, black pepper (*Piper nigrum)*, and dry ginger (*Zingiber officinale)*. These *yush* were tested also with addition of different concentrations of citric acid or lemon juice. The *yush* were prepared as prescribed in Ayurvedic texts. *Kruth yush* significantly inhibited crystal growth and was more effective than *akruth yush*. Addition of citric acid to the *kruth yush*, reduced crystal size from Day 2 and continued until Day 11. Percent reduction was more with higher citric acid concentration (302% with 0.001 M, 35.5% with 0.0055 M and 50.2% with 0.01M citric acid). However, citric acid alone with horsegram *yush* did not reduce crystal size significantly. Lemon juice was effective in reducing crystal size from Day 2 itself. Percent reduction was 31.4% with 1.04% lemon juice, 33.5% with 1.56%, 43.9% with 2.08% and 52.7% with 3.13% lemon juice. Reduction in crystal length was 0.017cm with 1.04% lemon juice and 0.088 cm with 3.13% lemon juice was comparable to 0.0415 cm length obtained with 0.001 M citric acid. The study showed that use of *kruth* horsegram (*yush+trikatu*) with addition of lemon juice was effective in reducing size of *brushite* crystals.

INTRODUCTION: Urolithiasis is widely prevalent in Asia as approximately one-fifth of the Asian population is estimated to suffer from it [1]. In India, the incidence has increased over the years; from less than 40 per 100,000 in the 1960's to 442.7-930/100000 three decades later.

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Prevention and treatment of urolithiasis relies on increased use of fluids and drugs like diuretics to decrease calciuria, alkalis to increase urinary citrate excretion, and allopurinols to reduce uric acid synthesis and uric acid excretion in patients having hyperuricemia and hyperuricosuria.

Other drugs may be prescribed to induce hypermagnesiuria. However, the effectiveness of these drugs is not guaranteed and recurrence is not uncommon [2]. Lithotripsy and ureteroscopic extraction also do not guarantee prevention of recurrence of the stones that can be as high as 50-80% [3].

The treatment itself is associated with side effects like haemorrhage, hypertension, tubular necrosis, subsequent kidney fibrosis leading to cell injury, and recurrence of renal stone formation [4]. Also, these methods may not be affordable for many patients. Ayurveda recommends several plants, each to be prepared using distinct procedures, for treatment of *ashmari* i.e. urinary/kidney stones [4, 5].

It recommends the use of horsegram/*kulith* (*Macrotyloma uniflorum*) and its preparations as pathyas for the treatment of *asmari*. One of the pathyas made using horsegram is *kulith yush*, a soup-like preparation.

The Ayurvedic text, *Sharangadhara Samhita*, describes two types of *kulitth-yush*, namely *akruth yush* that consists of only horsegram cooked in water and *kruth yush* that contains salt, and *katu* (pungent) substances like pepper and ginger that are cooked along with the horsegram in water [6]. In the present investigation, we added *trikatu*, that consists of pepper (*P. nigrum*), long pepper (*P. longum*), and dry ginger (*Z. officinale*), to *kruth yush*. Disturbance of tridoshas is said to be a factor in the genesis of *ashmari*, and *trikatu* is used to restore the balance between the three *doshas*. Therefore, we studied the inhibitory effect of *akruth yush* and *kruth kulith yush* made with *trikatu* on the growth of *brushite* crystals, using an *in-vitro* model.

Citrate has been reported to prevent stone formation [7], and hypocitruria has been reported to be a metabolic disturbance in a large proportion of patients with recurrence of renal calculi [8]. A Cochrane review by Phillips *et al.,* [9], noted that citrate therapy significantly reduced the stone size and new stone formation was significantly lower. However, there are few studies, if any, in the literature on the use of citric acid or lemon juice with *kulith yush*. Therefore, for the *kruth yush*, in addition to salt and *trikatu*, we studied the effects of added citric acid or lemon juice (*Citrus limon*), on the *brushite c*rystals *in-vitro*.

METHODOLOGY: This study was carried out in 3 steps.

Step 1: Standardization of the preparation of *kulith yush* with and without *trikatu* and with the souring agents.

Step 2: Sensory evaluation of *kulith yush* to determine the organoleptic acceptability of the *yush* and to identify the acceptable concentration of the souring agent added.

Step 3: Study of the effect of *kulith yush* on calcium hydrogen phosphate *dihydrate* or *brushite* crystals grown *in-vitro* using single-diffusion gel growth technique.

Procurement of Horsegram: Two types of horsegram (red and brown) were studied. Red horsegram samples were obtained from Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra and a local market in Rajasthan. The brown horsegram samples were collected from seven regions namely, local markets in Anantpur and Puttaparthi in Andhra Pradesh, Bengaluru in Karnataka, Pune and Mumbai in Maharashtra, and Bharade and Vidale varieties from local farmers in the Konkan regions of Maharashtra.

Sampling: A sample of 100g was drawn from each of the samples of the red and brown horsegram varieties by quartering method. The 100g portions were then hand-mixed thoroughly to obtain a composite sample of 700g of the brown *kulith* and 200 g sample of the red *kulith* (as the amounts obtained were relatively less). From the composite sample of brown *kulith*, 200g was drawn for the preparation of *yush* and for the red *kulith*, a 50 g portion was drawn for the same purpose.

Step 1: Preparation of *kulith yush*: The *akruth* and *kruth yush* were prepared according to the procedure described by Tripathi [6]. First, 40g of *kulith* was accurately weighed and then ground into a smooth paste with 80 ml of deionized water. The paste was then divided into two portions for the *akruth* and *kruth yush*. For the *akruth yush*, 15g of the paste was weighed accurately, 240ml of water was added, and the mixture was boiled for 15 minutes. For the *kruth yush*, salt, ginger and pepper are usually added. However, in the present study instead of using pepper and ginger, *trikatu* was added to 15g of *kulith* paste along with 240ml of water. The *trikatu* contained *Piper longum*, *Piper nigrum* and *Zingiber officinale*, in the ratio of 1:1:1, w/w as recommended in the texts. Initially, 2.25g of *trikatu* was added, but the *yush* had a very strong and unpalatable flavour. After several trials, 0.5g of

trikatu was found to be the most acceptable. Therefore, 0.5g of *trikatu* was added along with 0.6 g of salt to the *kulith* paste and water mixture, that was then boiled for 15 minutes.

Use of the souring agent: Lemon juice and citric acid were used separately to prepare the *kruth kulith yush* to determine its effect on the growth of *brushite* crystals, and to compare the effect of the pure acid in the *kruth yush* with that of lemon juice. Lemon juice or citric acid in different concentrations **Table 1** were added to the mixture after the *yush* had cooled, and the *yush* was mixed well.

TABLE 1: CONCENTRATIONS OF SOURING AGENTS USED IN *KRUTH KULITH YUSH*

Acid/ Souring agent	Quantity	Concentration of the souring agent
Acid		
Citric acid	0.03g	0.001M
	0.165g	0.0055M
	0.3g	0.01M
Souring agent		
Lemon juice	2.5ml	1.04% (v/v)
	3.75ml	1.56% (v/v)
	5ml	2.08% (v/v)
	7.5ml	3.13% (v/v)

*Concentration of souring agent calculated on the basis of amount of fruit taken.

Step 2 - Sensory Evaluation of *kulith yush*: Thirty semi-trained panellists from the Department of Food Science and Nutrition, SNDT Women's University, aged 20-23 years were asked to rate the *yush* samples on a 7- point hedonic rating scale for taste, flavour, sourness and overall acceptability. Scores ranged from 1 = Dislike extremely to 7 = Like extremely. Sourness of the *yush* was also scored as follows: 1 - No perception; 2 - Inadequate perception; 3 -Palatable, 4 -More sour than 3 but tolerable; 5 - Unpalatably sour. Aftertaste was evaluated, using the following scores: 1-No aftertaste; 2, Mild aftertaste; 3 –Moderate aftertaste; 4 –Strong aftertaste.

In addition, panelists were also asked to indicate the concentration of the souring agent at which the *yush* was most palatable. The *yush* samples were presented to the panel members in glass bowls completely covered with aluminum foil to mask the color in order to minimize bias in evaluation. A straw was inserted through the aluminum foil to allow the panel members to taste the samples. Members were instructed to rinse their mouths with water after tasting each sample. Mean scores were calculated for each of the five sensory attributes.

Step 3 - Growth-inhibition Study of *kulith yush* on Calcium Hydrogen Phosphate Dihydrate (CHPD, *Brushite*) Crystals: This was carried out at the Crystal Growth Laboratory, Department of Physics, Saurashtra University, Rajkot, Gujarat. CHPD or *brushite* crystals were grown *in-vitro* by the single diffusion gel growth method. *Brushite* crystals were chosen for the study because these crystals are considered to be precursors for other thermodynamically more stable calcium phosphates. They provide sites for the heterogenous nucleation of calcium oxalate monohydrate crystals, promote their aggregation, and act as the nidus for further phosphate and oxalate stone formation.

Brushite crystals generally precipitate in the descending thin limb of the loop of Henle itself, because this segment has high levels of supersaturation for calcium phosphates from the tubular fluids. Therefore, by inhibiting the growth of *brushite* crystals, it may be possible to overcome the growth and aggregation of stable phosphate and oxalate stones [10].

Gel Preparation: Silica hydrogel was used as the medium for crystal growth. Stock solution of sodium meta-silicate was prepared by dissolving 11g of sodium meta-silicate in 100ml double distilled water. Double distilled water was added to make the appropriate volume of the stock solution, and its specific gravity was adjusted to 1.05. Monosalicylic acid is formed that polymerizes to form a three-dimensional gel network composed of Si-O links.

The chemically inert gel network acts like a three-dimensional matrix in which crystal nuclei are delicately held and supplied with nutrients for growth. Diffusing a reactant into the gel leads to a critical concentration at some regions in the gel network leading to the formation of nuclei. Further supply of nutrients to the nuclei leads to aggregation and crystal formation [11].

Macro Size CHPD Crystal Growth: Glass test tubes of 2.5 cm diameter and 15 cm length were used for growing the crystals. Sodium meta-silicate solution (specific gravity 1.05) was adjusted to pH 4.0 using 1M orthophosphoric acid. Into each test-tube, 20ml of this solution was transferred. The tubes were kept for gelation for 48 hours. After completion of gel formation, 20 ml of 1M aqueous solution of calcium chloride (reactant) was carefully poured on the set gels. Elongated, platelet type and star shaped crystals grew very rapidly within two days after pouring calcium chloride solutions (Plate 1).

PLATE 1: CALCIUM HYDROGEN PHOSPHATE DIHYDRATE CRYSTALS IN SODIUM-META SILICATE GEL

Growth Inhibition Study: For this, *kulith-yush* was filtered through Whatman no.1 paper, followed by Whatman no. 42, to obtain a clear filtrate. After crystals grew to a measurable size and could be seen with the naked eye (after 8-10 days), 10ml of the reactant was replaced with 10ml of the filtered *kulith yush*. Growth inhibition was assessed by measuring the apparent length of crystals with a traveling microscope of 0.001cm least count, after every 24 hours for 11 days. Length of 5 crystals that grew just below the interface was measured to maintain uniformity. Growth inhibition by the *yush* was compared with a control (this contained only the reactant i.e. 20ml of 1M calcium chloride solution) and with a tube containing 10ml of deionized water along with 10ml of the reactant.

Statistical Analysis: Statistical analysis was carried out using SPSS package version 16.0. Means and standard deviations were calculated. Analysis of variance was applied to determine whether the mean scores for each of the sensory attributes significantly differed between the souring agents added. Student's t-test was used to determine whether the mean scores for sensory attributes between *yush* made with citric acid and lemon juice differed significantly. Paired difference t-test was applied to the data on growth inhibition study to compare the size of crystals grown in control and deionized water, and to determine whether the inhibitory effect of akruth and *kruth yush* with *trikatu* and salt differed significantly. Paired difference t-test was also applied to determine whether the inhibitory effect differed significantly between a*kruth yush* and *kruth yush* with souring agent as well as between *kruth yush* with *trikatu* and salt alone and the *yush* made with the souring agent.

RESULTS:

Sensory Evaluation of *Yush:*

***Yush* Preparation with Citric Acid:** Three concentrations of citric acid were added to the kruth *kulith yush*, i.e., 0.001M (0.013% acid, w/v), 0.0055M (0.07% acid, w/v) and 0.01M (0.13% acid, w/v). Perception of sourness and aftertaste of the *yush* increased significantly, with an increase in the concentration of citric acid (F= 45.56, p= 0.000). Sourness was perceived to be 'inadequate' in the *yush* prepared with 0.001M citric acid, whereas the *yush* with 0.0055M citric acid was considered palatable. The *yush* with 0.01M citric acid was described as 'more sour but tolerable' and received the highest scores for overall acceptability, taste and flavor **Table 2.**

***Yush* Preparation with Lemon Juice:** Four concentrations of lemon juice were added to kulith-*yush* - 1.04% (v/v, 0.05% citric acid), 1.56% (v/v, 0.08% citric acid), 2.08% (v/v, 0.10% citric acid) and 3.13% (v/v, 0.16% citric acid). Scores for sourness (F= 14.54, p= 0.000) and aftertaste (F= 5.30, p= 0.002) differed significantly between the various concentrations of lemon juice added to the *yush*. The *yush* with 2.08% lemon juice received the highest score for taste and flavor, although, its overall acceptability was marginally lower than for the *yush* with 1.56% lemon juice **Table 2**.

Lower concentrations of lemon juice i.e. 1.04% and 1.56%, were described by the panelists as 'palatable' but 'mild' in terms of sourness and aftertaste. *Yush* with 3.13% lemon juice was found to have a strong aftertaste and had a lower overall acceptability score **Table 2**. Based on the sensory attribute scores, the most acceptable concentrations of lemon juice was found to be 2.08%, and for Citric acid- 0.13% (0.01M).

TABLE 2: MEAN SCORES FOR SENSORY CHARACTERISTICS OF *KULITH YUSH* PREPARED WITH CITRIC ACID AND LEMON JUICE

Souring agentconcentration	Overall Acceptability	Taste	Flavour	Sourness	Aftertaste
Citric acid					
0.001M	3.77± 1.28	4.17 ± 1.23	3.90± 1.30	2.30± 0.89	1.83± 0.75
0.0055M	4.10± 1.24	4.30± 1.42	4.07± 1.28	2.63± 0.93	2.37± 0.85
0.01M	4.50± 1.33	4.50± 1.38	4.27± 1.26	3.63± 0.89	2.57± 1.04
Lemon juice					
1.04% (v/v)[a]	3.93± 1.44	4.17± 1.42	4.00± 1.29	2.57± 0.90	2.00± 0.83
1.56% (v/v)	4.23± 1.19	4.37± 1.00	4.10± 1.12	2.97± 1.10	2.00± 1.08
2.08% (v/v)	4.20± 1.13	4.50± 1.28	4.20± 1.13	3.60± 1.07	2.37± 1.13
3.13% (v/v)	4.13± 1.55	4.37± 1.61	4.10± 1.54	3.93± 1.08	2.70± 1.09

Inhibition of Crystal Growth:

Comparison of the Effect of *Kruth Yush* (with *Trikatu* and Sodium Chloride) with Control and Deionized Water: Addition of kruth kulitth *yush* prepared with brown (t= 6.49, p= 0.000) and red varieties of horsegram (t= 2.26, p= 0.001) had a significantly greater inhibitory effect on *brushite* crystal growth as compared to that in control **Fig. 1** and the tube containing deionized water (brown variety t= 2.26, p= 0.005 and red variety (t= 2.26, p= 0.02).

Percent decrease in crystal size as compared to that in control, on the 11th day was 58.2% and 46.2%, in the presence of *kruth yush* prepared with brown and red varieties of horsegram, respectively. As compared to deionized water, the percent decrease in crystal size on the 11th day was 48.7% and 34.0% in the presence of *kruth yush* prepared with brown and red varieties of horsegram.

In the tube containing deionized water, *brushite* crystals grew rapidly until day 3, whereas in the control tube, crystals continued to grow rapidly for the first 7 days. Maximum length of crystal size in both control and deionized tubes was seen on Day 8, after which there was a plateau in the crystal size until day 11. Crystals grown in deionized water were significantly smaller as compared to those in the control tube (t=6.0, p=0.000). In contrast, decrease in crystal size in the presence of *kruth yush* began from the second day itself. There was no significant difference in the effect of the brown and red varieties, particularly after the 3rd day.

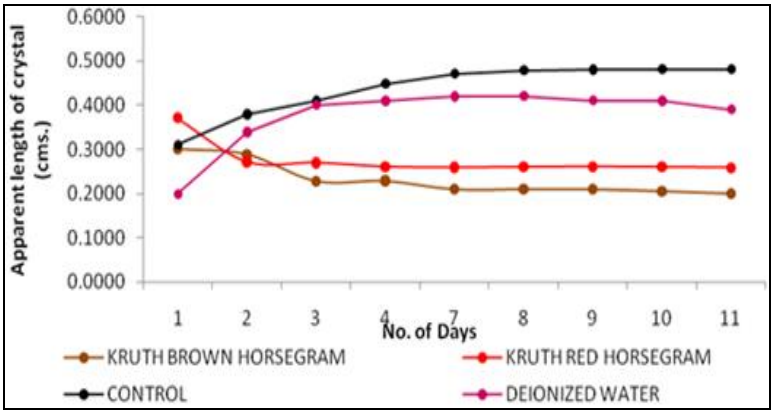

FIG. 1: GROWTH OF CRYSTALS OVER 11 DAYS IN THE PRESENCE OF *KRUTH YUSH* PREPARED FROM BROWN AND RED VARIETIES OF HORSEGRAM

Comparison of Effect of Kruth and A*kruth Yush* from Brown and Red Varieties of Horsegram: Crystal size reduced more rapidly within the first three days with *kruth yush* prepared with brown and red varieties of horsegram than with the a*kruth yush* **Fig. 2**. Over eleven days of the study, crystal

size decreased by 33.3% and 30.2% in the presence of *kruth yush* prepared with brown and red horsegram, respectively. In comparison, reduction in crystal size over 11 days in the presence of a*kruth yush* prepared with brown horsegram was 30.6% and with red horsegram, the reduction was 21.1%. There was no statistically significant difference between the kruth and akruth *yush*.

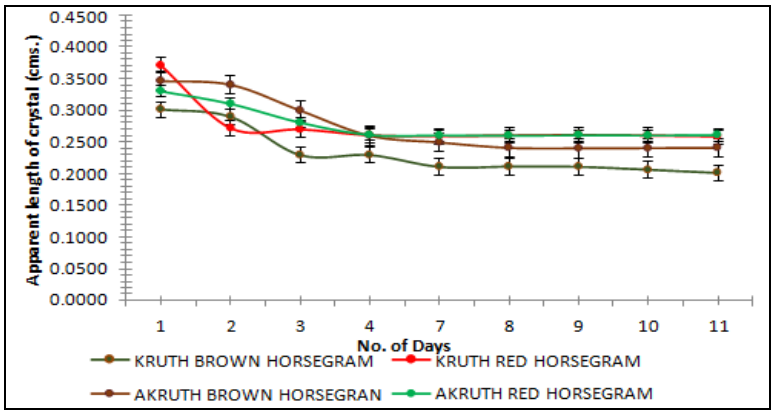

FIG. 2: GROWTH OF CRYSTALS OVER 11 DAYS IN THE PRESENCE OF ARUTH AND KRUTH *YUSH*

Effect of *Kruth Yush* with Citric Acid and Lemon Juice: The effect of citric acid and lemon juice added at different concentrations to *kruth yush* on the growth of *brushite* crystals was studied. Also, crystal growth inhibition in the presence of citric acid and lemon juice was compared with that in the control tube and in the presence of deionized water.

Effect of *Kruth Yush* with Citric Acid: As seen in **Fig. 3**, in the presence of all four concentrations of citric acid, reduction in crystal size was evident on Day 2 itself and continued until the 11th day. In contrast, in the presence of *kruth yush (*salt and *trikatu*), *and* in the presence of *akruth yush*, decrease in the crystal size showed a plateau after the 7th day **Fig. 3.**

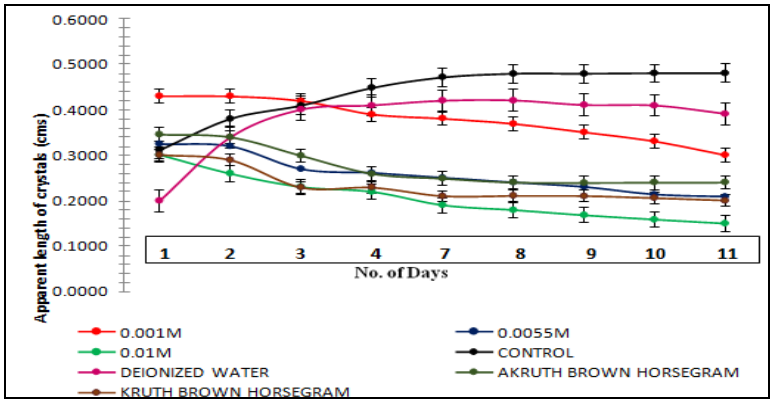

FIG. 3: GROWTH OF CRYSTALS OVER 11 DAYS IN THE PRESENCE OF *KRUTH YUSH* WITH CITRIC ACID COMPARED TO CONTROL, DEIONIZED WATER, AKRUTH AND *KRUTH YUSH* (SALT AND *TRIKATU* ONLY)

Significant reduction in crystal size was observed with the concentrations of citric acid added **Table 3**, as compared to in the control tube, with deionized water and with both *akruth* and *kruth yush* (salt and *trikatu* only) made with brown horsegram.

TABLE 3: RESULTS OF PAIRED T-TEST COMPARING THE EFFECTS OF *KRUTH YUSH* WITH CITRIC ACID, *AKRUTH* AND *KRUTHYUSH* (SALT AND *TRIKATU*) ON MEAN CRYSTAL SIZE

	t value (p value)		
Concentration of citric acid	**0.001M**	**0.0055M**	**0.01M**
Control	1.80 (0.044)	5.43 (0.0003)	6.36 (0.0001)
Deionized water	1.65 (0.04)	3.40 (0.005)	4.45 (0.001)
Akruthyush	12.51 (0.000)	3.22 (0.000)	11.74 (0.000)
Kruthyush	16.09 (0.000)	6.47 (0.0001)	3.86 (0.002)

Growth inhibition was more at higher concentrations of citric acid. In the presence of *yush* with 0.001M citric acid, crystal size reduced gradually until the 3rd day, *Yush* with 0.0055M citric acid showed a sharp decrease on the 3rd day, after which there was a small and gradual decrease.

In the *yush* with 0.01M citric acid, the crystal size reduced from Day 2 itself, and continued until the 11th day **Fig. 3.**

Percentage reduction in the crystal size in *kruth yush* was 30.2% with 0.001M citric acid, 35.5% at 0.0055M and was 50.2% with 0.01M citric acid.

With all three concentrations of citric acid, the reduction in crystal size was significantly more as compared to that in control, with deionized water and in both the akruth and *kruth yush* (salt and *trikatu* only) made with brown horsegram.

Effect of Citric Acid Solution: Addition of citric acid solutions alone at the same concentrations did not significantly reduce the crystal size. With 0.001M standard citric acid solution, crystals continued to grow up to the 11th day indicating a lack of inhibitory effect.

With 0.0055M citric acid, reduction in crystal size was observed only after the 5th day, whereas with 0.01M citric acid, only a slight reduction of 17.64% was seen on day 11 **Fig. 4.**

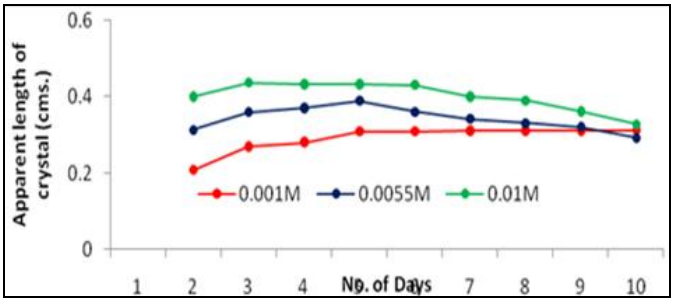

FIG. 3: GROWTH OF CRYSTALS OVER 11 DAYS IN THE PRESENCE OF 3 CONCENTRATIONS OF CITRIC ACID

Effect of *Kruth Yush* with Lemon Juice: Since lemon juice is commonly used as a souring agent in Indian cookery, it was added to *kruth yush* at four different concentrations to study its crystal growth inhibitory effect. *Kruth yush* containing lemon juice showed a decrease in crystal size from day 2 itself, with a steep decrease particularly until day 4. In contrast, the crystal size with akruth and *kruth yush* (salt and *trikatu* only) showed a plateau after day 7 **Fig. 4.** The rate of growth inhibition was higher at higher concentrations of lemon juice. In *yush* with 1.04% lemon juice, crystal size reduced gradually until day 8, with a sharper decrease thereafter. *Yush* with 1.56% and 2.08% lemon juice showed a sharp decrease within the first three days. However, with 1.56% lemon juice, a plateau was observed thereafter. On the other hand, with 2.08% and 3.13% lemon juice, gradual reduction continued until day 11 and day 9, respectively.

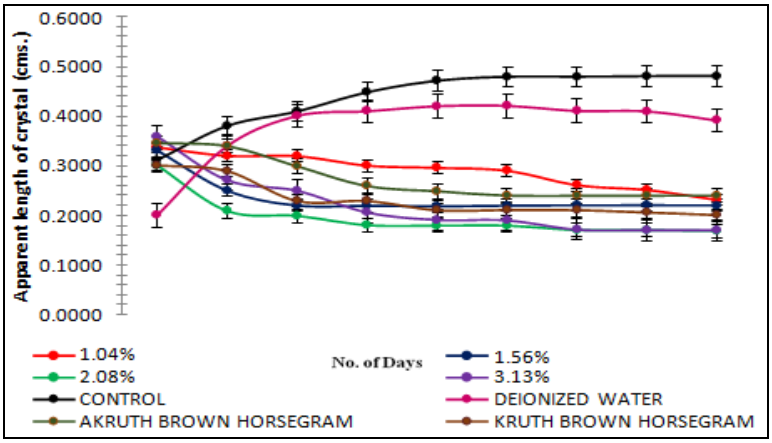

FIG. 4: GROWTH OF CRYSTALS OVER 11 DAYS IN THE PRESENCE OF *KRUTH YUSH* WITH LEMON JUICE COMPARED TO CONTROL, DEIONIZED WATER, AKRUTH AND *KRUTH YUSH* (SALT AND *TRIKATU* ONLY)

Percent reductions in crystal size with lemon juice were 31.4% with 1.04% lemon juice, 33.5% with 1.56% juice, 43.9% with 2.08% juice and 52.7% with 3.13% lemon juice. The reductions in crystal size was significantly greater for the *yush* with lemon juice as compared to that in control, and with deionized water. The results of paired t-test comparing the effects of a*kruth yush* with *kruth yush* with lemon juice at four different concentrations were – 1.04% (t= 1.98, p=0.04),

1.56% (t=3.96, p=0.002); 2.08% (t=9.33, p=0.000) and 3.13% (t=6.05, p=0.000). Similarly, the results of paired t-test comparing the effect of *kruth yush* (salt and *trikatu*) with *kruth yush* with lemon juice at four different concentrations were – 1.04% (t= 7.19, p=0.000), 1.56% (t=5.43, p=0.000); 2.08% (t= 5.22, p=0.000) and 3.13% (t=5.15, p=0.000).

Comparison between Effect of *Yush* with Citric Acid and *Yush* with Lemon Juice: Citric acid is the primary acid in lemon juice [12]. Hence, growth inhibitory effect of *yush* with lemon juice, that contains citric acid, was compared with that of *yush* containing synthetic citric acid.

Fig. 5 shows that in *yush* with lemon juice there was a steeper decrease during the first 2 days as compared to *yush* with citric acid. Even at the lowest concentrations, *yush* with lemon juice (1.04%) showed a 0.017cm reduction in crystal size in the first 2 days whereas with *yush* with citric acid (0.001M), the reduction was only 0.0004cm. At the highest concentrations, *yush* with 3.13% lemon juice showed a 0.088cm reduction in crystal size in the first 2 days whereas the reduction in *yush* with citric acid (0.001M) was only 0.0415cm.

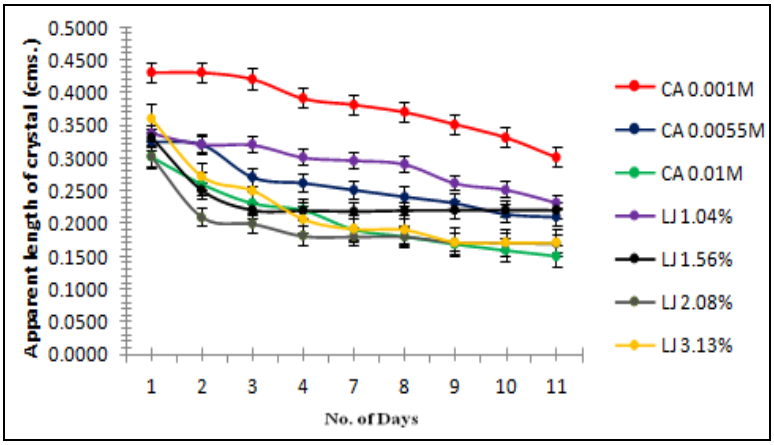

FIG. 5: GROWTH OF CRYSTALS OVER 11 DAYS IN THE PRESENCE OF *KRUTHYUSH: COMPARISON BETWEEN* CITRIC ACID AND LEMON JUICE

Crystal size using the Acceptable Concentrations of Citric Acid and Lemon Juice: Sensory evaluation of the *yush* preparations had shown that the acceptable concentrations were 0.01M for citric acid and 2.08% lemon juice. With the *yush* containing 0.01M citric acid, there was a 50.2% decrease in crystal length, whereas *yush* with 2.08% lemon juice led to 43.9% reduction. Thus, at the acceptable concentration, citric acid had a greater inhibitory effect than did the lemon juice. **Fig. 6** shows the growth of *brushite* crystals in the control tube, in the presence of deionized water and in *yush* with citric acid and lemon juice at the organoleptically acceptable concentrations.

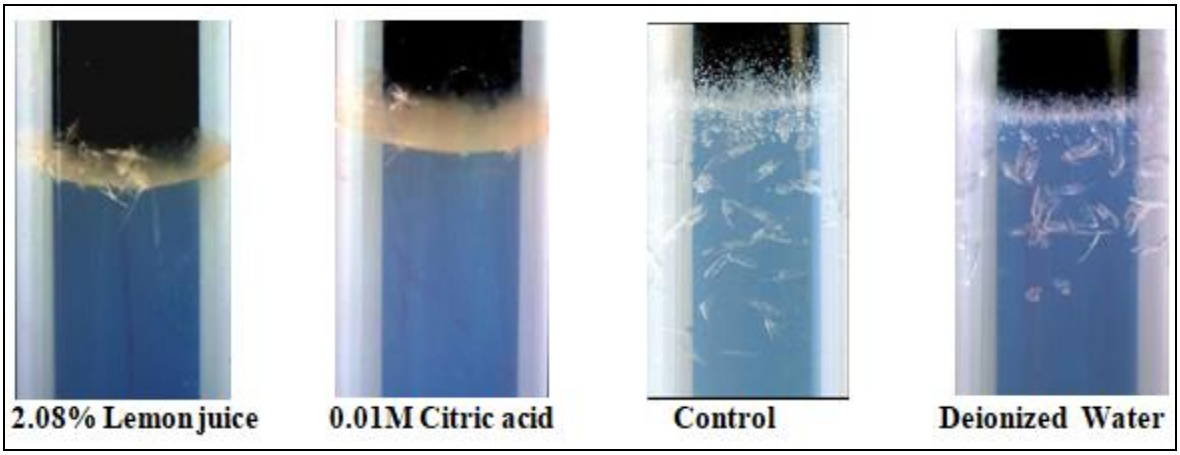

FIG. 6: COMPARISON OF CRYSTAL GROWTH WITH CONTROL, DEIONIZED WATER, LEMON JUICE AND CITRIC ACID

DISCUSSION: The results of the present study confirmed the Ayurvedic benefits of using horsegram (kulith) *yush* in Ayurvedic literature. Addition of *trikatu* to the *yush* helped in preventing the growth of *brushite* crystals. The use of either citric acid or lemon juice also helped in reducing the growth of crystals, indicating that *kruth yush* prepared with these agents would be helpful in prevention and treatment of renal stone.

Horse gram has been studied by several investigators for its beneficial properties. Kaundal and co-workers [13] have reviewed the pleiotropic effect of horse gram. Earlier reports in the literature indicate that extracts and/or preparations of horse gram alone or in combination with other substances prevented or dissolved urinary crystal formation both *in-vitro* [14, 15] and *in-vivo*. The beneficial effect of horsegram extract could be due to several mechanisms.

One mechanism may be a diuretic effect. Patel & Acharya *et al.,* [16] observed that the aqueous extract had a diuretic effect and the levels of calculus inhibitors like magnesium, and citrate as well as decreasing levels of promoters like calcium, uric acid and oxalate. They also observed pathological changes in the kidney along with an increase in glomerular activity. In [17] cases of nephrou-reterolithiasis patients given 1g of Swetaparpati (an Ayurvedic formulation) with 50ml kulattha kwatha (decoction) three times/d for 1 month, expulsion of stones improved, particularly from the ureter. The effect was attributed to the diuretic effects of Swetaparpati and Kulattha [17]. The diuretic effect could be due to a dipeptide, pyroglutamylglutamine (PN), and in rats, it was seen that the natural PN was 2-3 times more potent as a diuretic than the synthetic compound [18].

Another mechanism could be due to the binding protein(s) present in horsegram [19]. This is likely to be a dimeric protein containing an abundant amount of acidic amino acids and having a sequence similar to a calcium-binding protein, calnexin in *Pisum sativum* [20], as well as polar compound [21]. The dimeric protein, DB58, from horsegram has been found to have greater conformational flexiblility than other horsegram proteins that allows it to spread easily on the crystal surface thereby preventing aggregation [22].

A third mechanism could be attributed to the antioxidants in horsegram, that might confer protective effects against peroxidative damage to the renal epithelial cells and inhibit the fixation of crystals and subsequent growth [23]. Antioxidants would be useful in reducing oxidative stress. Horsegram decreased malondialdehyde production, increased antioxidant enzymes, and inactivated NF-κB [24].

The antioxidant activity of horsegram samples used in the present study ranged from 77.6 to 91.8 % inhibition [25]. Antioxidant effect of horsegram could be attributed to its saponin, lectin, and polyphenol present content [26].

Saponins: The saponin content of horsegram samples used in the present study varied from 46.4 mg/100g to 84.3 mg/100g, with brown horsegram containing more saponins [25] (Venkateswaran, 2010). Due to their amphiphilic nature, saponins may coat the crystals, prevent mucoprotein aggregation in renal tubular cells, and prevent the crystals from adhering to the epithelial cells [27, 28].

Lectins: Lectins could block crystal binding sites and prevent stone formation by modulating alkaline phosphatase (ALP) activity and preventing bacterial colonization in the kidneys [25], which has been associated with increased calcification in the renal cortext. The horsegram seed lectin, DBL has been shown to bind to N-acetyl-galactasomine [29], the precursor for the formation of chondroitin sulfate, a mucoprotein. By binding to the precursor, DBL would prevent formation of the mucoprotein, which acts as a binding site for crystals [27]. Lectins prevented the adhesion of calcium oxalate crystals to BSC-1, MDCK cells and fibroblasts 3T3 cells by blocking the cell sites for crystal binding [30]. Also, lectins agglutinate some resident bacteria of the digestive tract and selectively remove these from the lumen [22]. Thus, in kidney infections, lectins may prevent the formation of bacterial colonies and stop the latter from acting as a nidus for crystal growth.

Venkateshwaran [25] reported that lectin activity in *yush* was 16HU/g *yush*, whereas the activity in raw horsegram was approximately 3 times lower (5.33HU/g sample). A very small fraction (1-5%) of dietary lectins are absorbed from the gut. Hence,

increase in lectin activity after 15min. of boiling and the fact that up to 5% is absorbed, might be of significance in terms of the role it could play in reducing the risk of stone formation [25].

Polyphenols: Polyphenols in horsegram may also contribute to the protective effects. Epigallocatechin gallate (EGCG) from green tea decreased the formation of calcium oxalate stones and increased SOD activity in both rat kidney tissues and MDCK cell lines [31].

In the present study, addition of *trikatu* to the *yush* enhanced its growth inhibitory activity. *Trikatu* has pleitropic properties and can regulate multiple signaling molecules. It contains flavonoids, alkaloids, phenols, tannins, quinine, cardiac glycosides, sterols [32]. Piperine might enhance the activity of protein unfolding enzymes such as AAA+ protease thereby accelerating proteolytic mechanisms [32]. *Trikatu* has also been shown to have antibacterial effects against four test organisms strains namely *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *V. harveyi* [33]. The salt added to the *yush* might have also contributed to the growth-inhibitory effect of *brushite* crystals.

Citrate is a strong inhibitor of crystallization of calcium salts. In the present study, addition of acid in the form of citric acid, or lemon juice to the *yush* inhibited crystal growth. At the lowest concentrations, *yush* with lemon juice showed a decrease of 31.4% in crystal size over 11 days as compared to a 30.2% decrease in *yush* with citric acid. At the highest concentrations, a slightly higher percentage decrease over 11 days was seen in *yush* with lemon juice (52.7%) than in *yush* with citric acid (50.2%). Lemon juice contains citric acid and malic acid [12] and the latter might have acted synergistically with the citric acid, resulting in the slightly greater crystal growth inhibitory effect.

However, at the organoleptically acceptable concentrations *yush* with citric acid (0.01M) had a greater inhibitory effect than that with lemon juice (2.08%). Percentage acid present was 0.13% and 0.10% respectively. It could be postulated that lower percentage reduction observed in *yush* with lemon juice could be due to the slightly lower percentage of citric acid. In a review Palermo *et al.*, [34] noted that citrate therapy significantly reduced stone size in four studies and new stone formation in seven studies. Stone size stability was observed in four studies and the need for treatment for removal of renal stones was much lower in participants who were given citrate.

Hence, it is worthwhile to explore dietary modifications, particularly citrus fruits like lemon for their potential. Growth inhibition by citrate was suggested to be due to synergistic activity of antioxidant compounds in lemon juice [35, 36]. Bargouthy and Somani [7] reported that citrus fruit juices including lemon juice/lemonade had favorable effect. Joshi and Joshi [37] reported that 100% lemon juice altered the morphology of *brushite* crystals from needle and platelet shape to snowflake shape along with a reduction in crystal length *in-vitro*. Citric acid also has been demonstrated to inhibit the growth of urinary crystals both *in-vitro* and *in-vivo*.

Joshi and Joshi [37] and Parekh and Joshi [38] demonstrated in vitro that citric acid at 0.1- 1.0M concentrations reduced the length of *brushite* crystals. Complete growth inhibition of crystals was observed at 0.8, 0.9 and 1.0M concentrations of citric acid. Citrate has an alkalinizing effect on urine, possibly because it reduces renal reabsorption of citrate and allows more citrate to be excreted in urine. Citrate also inhibits the spontaneous nucleation and agglomerate of calcium oxalate crystals. Also, it sequesters calcium ions through formation of citrate calcium complexes that are more soluble in urine at physiological urine pH levels. Thus binding of citrate to form soluble calcium citrate complexes renders calcium unavailable for *brushite* formation. Citric acid was also postulated to reduce ionic calcium concentration and thus inhibit both spontaneous and heterogenous nucleation [37] (Joshi and Joshi, 2003).

It has been suggested citric acid and conditions above pH 6 favor *brushite* crystal growth [39]. Citric acid and/or lemon juice would neutralize the excess alkali and favor crystal growth inhibition. Thus, a combination of an aqueous extract of horsegram combined with *trikatu* i.e. *kruth yush* along with citric acid / lemon juice may prove useful.

This study demonstrated the benefits in an *in-vitro* system and should be studied with patients for its efficacy.

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