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In Vitro Effect of Lemon and Orange juice on Struvite Crystallization

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Abstract: ***To investigate the inhibitory effect of lemon and orange juices on the growth of struvite crystals. Struvite crystals were grown by the single diffusion gel growth technique and the inhibitory effect of lemon and orange juices on the growth of struvitecrystals has been studied. With an increase in the concentration of lemon and orange juices, the weight of the formed crystals was gradually reduced from 2.49 g to 0.09 g (lemon juice) and 2.49 g to 0.21 g (orange juice) for the struvite crystals, respectively. The crystals harvested from the treated lemon and orange juices were characterized by Fourier Transform Infrared Spectroscopy (FTIR) to confirm the functional groups. Results obtained indicated that lemon juice has the potential to inhibit the formation of struvitecrystals as compared to orange juice. This study confirms that using lemon juice can promote the formation of ammonium magnesium phosphate hexahydratecrystals and reduce the nucleation rate of struvitecrystals, a major component of urinary stone.***

Key words: Lemon juice, Orange juice, Magnesium acetate, Ammonium dihydrogen phosphate, Fourier Transform Infrared Spectroscopy (FTIR).

I. INTRODUCTION

A large number of people are suffering from urinary stone problem all over the globe. Not only the humans but animals and birds also suffer from the urinary stone problem. The occurrence in some areas is so alarming that they are known as 'Stone Belts'. The area of high incidence of urinary calculi include British islands, Scandinavian countries, Central Europe, Northern Australia, Northern India, Pakistan, Mediterranean countries [1, 2]. The financial costs of the disease are staggering; in the United States, for instance, the health bill for treatment of kidney stones runs to billions of dollars annually[3]. More recent studies suggest that there has been a gradual increase in the annual incidence and a decrease in the age of onset of the disease perhaps the result of change in lifestyle and diet[4]. Majority of the calculi are composed of calcium salts, oxalates and phosphates. Among the phosphates, magnesium phosphates, namely, Ammonium Magnesium Phosphate Hexahydrate (AMPH) commonly known as Struvite and Magnesium Hydrogen Phosphate Trihydratehave also been reported to occur as constituents in renal calculi [5–8] not only in adults but also in children [9, 10]. Struvite calculi, found in 15–20% of urinary calculi [11, 12], are mostly related to urinary tract infections with ureolithic microorganisms in humans and animals [5, 13, 14]. Struvite is also known as triple phosphate stone, infection stone or urase stone. They are found more frequently in women and in persons older than 50 years [15,16]. An elevated urinary pH reduces the solubility of magnesium ammonium phosphate and favor sprecipitation of Struvite crystals. Higher intake of phosphate(from Proteins) and magnesium based food and lower intake of water gives rise to the $PO4^{3-}$ and Mg2 ions in the supersaturated urine, which leads to the conditions of formation of Struvite[17].Struvite stones may grow rapidly over a period of weeks to months and, if not adequately treated, can develop into aStaghorn or branched calculus that involves the entire renal pelvis and calyces. Patients with infected Staghorn calculi who receive no treatment have about a 50% chance of losing the kidney [18, 19].

Gel growth (in vitro) of a few urinary stone constituents and the inhibitory role played by some extracts or juices of natural products in crystal growth were studied earlier [20-22]. This technique has been successfully used to study the growth inhibition of calcium oxalate crystals [23] and calcium hydrogen phosphate dihydrate (CHPD), i.e., Brushite crystals [24] using herbal extracts of Tribulusterrestris Linn. And Bergenia Ligulata Linn. Growth inhibition studies of Struvite in the presence of some of the herbal extracts of Boerhaaviadiffusa Linn. [25], Rotula aquatic Lour[26] and Commiphorawightii[17] were successfully carried out.

Previously, two studies demonstrated that citrate behaves like an inhibitor of calcium oxalate crystal growth [27,28]. It is able to fix at the surface of the crystals to reduce their size. According to the study, the lemon juice is being the richest in this element. The

impact of lemon juice as an inhibitor is higher than that of orange juice. Citrate is 6 times concentrated in the lemon juice compared to the orange juice[26]. This is in agreement with our observations that orange juice increased inhibition of crystal growth, but it did not alter the rate of nucleation and aggregation significantly. And also in this study, lemon juice was found to be the most potent inhibitor of struvite crystals. Therefore, it is very much necessary to study the growth-inhibition of Struvite crystals. In the present investigation, Struvite crystals were grown by single diffusion gel growth technique and the growth inhibition study of the Struvite crystals in the presence of the different concentration of lemon and orange juice separately are reported for the first time.

II. MATERIALS AND METHODS

A. Materials and instruments

Analytical grade of anhydrous magnesium acetate, ammonium dihydrogen phosphate, sodium metasilicate, were all purchased from sigma-aldrich, New Delhi, India. Fourier Transform Infrared (FTIR) spectra were recorded with a nominal resolution of 4 cm^{-1} and a wave number range from 400 to 4000 cm^{-1} using the KBr pellet technique.

B. Preparation of fresh citrus fruit juice

The fresh citrus fruit lemon and orange were purchased from the local Gandhi market, Trichy. The procured fruits were washed and dried at room temperature. The outer portion of these fruits was wiped with 70% alcohol and fresh juice was collected by using sterile syringe for further in vitro study.

C. In-vitro growth of Struvite crystals

Glass test tubes were used as a crystallization apparatus and the single diffusion reaction technique was employed [17,25]. One of the reactants, 0.5 M ammonium dihydrogen phosphate (ADP), was mixed with sodium metasilicate solution the density of 1.04g/cm^3 at pH9.4, so that the pH of the mixture was maintained at 6 and left undisturbed for 2-3 days. After gelation took place, the supernatant solution of 1 M Magnesium acetate was gently poured onto the set gel in various test tubes. After pouring on each supernatant solution, the test tubes were capped with airtight stopples. The experiments were conducted at room temperature (37°C).

D. The nomenclature of different additive solution on the growth of struvitecrystals

An attempt was made to investigate the putative activity of the fruit of lemon and orange juice separately as inhibitors of Struvite crystal formation in gel method. The supernatant solutions as given in (Table 1) were added to the set gels and the results were noted. The experiments were repeated four times. To study the effect of the fruits of lemon and orange juice on the growth of Struvite crystals, a series of five different concentrations of 1% to 5% (v/v) of these each lemon and orange juice were added in equal amounts in supernatant solution and the average weight of the grown crystal were measured separately.

E. Characterization of the grown crystal

The grown Struvite crystals were characterized using FTIR to verify the compound and structure of the grown crystal. FTIR was performed by Hitachi 570 FT-IR spectrophotometer technique to verify the proper formation of crystal and their purity [24,26].

F. Statistical analysis

The masses of the crystals (gm) are presented as the mean ±Standard deviation for the control and treatment samples. One-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparison were made between groups. Values of $p<0.05$ was considered to be significant.

Table- 1: Supernatant solutions added to the set gels for struvite crystals

CRYSTALS	Supernatant Solutions (Groups and Treatments)	Compositions
Struvite	A	10 ml of Magnesium acetate
	B	5 ml of Magnesium acetate ±5 ml of Distilled water
	C	5 ml of Magnesium acetate ±5 ml of 1% of aqueous lemon and orange juice separately
	D	5 ml of Magnesium acetate ±5 ml of 2% of aqueous lemon and orange juice separately

	E	5 ml of Magnesium acetate ±5 ml of 3% of aqueous lemon and orange juice separately
	F	5 ml of Magnesium acetate ±5 ml of 4% of aqueous lemon and orange juice separately
	G	5 ml of Magnesium acetate ±5 ml of 5% of aqueous lemon and orange juice separately

III. RESULTS AND DISCUSSIONS

A. Effect of lemon juice on Struvite crystals

The effect of lemon juice on nucleation and crystallization characteristics of struvite crystals is determined by measuring the weight of the formed crystals. In the gel method, the control using pure Mg CH_3COO_2•$4H_2O$ led to the maximum nucleation of crystals growth within 24 h of adding the supernatant solutions Fig. 1 (1a). In the presence of lemon juice, nucleation was delayed and reduced masses of the crystals were observed 96 h after adding the supernatant solutions Fig. 1 (1b-g). Morphology of the harvested crystals after addition of lemon juice as shown in Fig. 2.The largest single struvite crystals having dimensions of 3.2 cm as observed in (Fig. 3a). The sizes of the struvite crystals were reduced from 3.2 cm to 0.2 cm at 1% lemon juice was observed in (Fig. 3c-g).With an increase in the concentration of lemon juice from 1% to 5% (v/v), the weight of the formed crystals was gradually reduced from 2.47 g to 0.09 g at 5% concentration of lemon juice respectively. The ANOVA statistical analysis was performed and different parameters have been evaluated, and $p<0.05$ has suggested that the correlation is significant as shown in (Table 2).

Table- 2: ANOVA statistical analysis for harvested lemon struvite crystals

CRYSTALS	GROUP	TREATENTS	Mean (gm)±SD
Struvite	A	Control	2.49±0.014
	B	Control+Distilled water	2.46±0.057
	C	Control+1% orange juice	$1.28±0.014^{a,b}$
	D	Control+2% orange juice	$0.48±0.014^{a,b,c}$
	E	Control+3% orange juice	$0.29±0.014^{a,b,c,d}$
	F	Control+4% orange juice	$0.17±0.014^{a,b,c,d,e}$
	G	Control+5% orange juice	$0.09±0.014^{a,b,c,d,e,f\text{-ns}}$

Values represent mean (gm) ± S.D (n=4) Comparisons between means are as follows. a: A *vs* B-G, b: B *vs* C-G, c: C *vs*D-G, d: D *vs* E-G, e: E *vs* F-G, f: F *vs* G. Statistical significance were considered to be $^{a}p<0.05$,$^{b}p<0.05$,$^{c}p<0.05$,$^{d}p<0.05$,$^{e}p<0.05$.

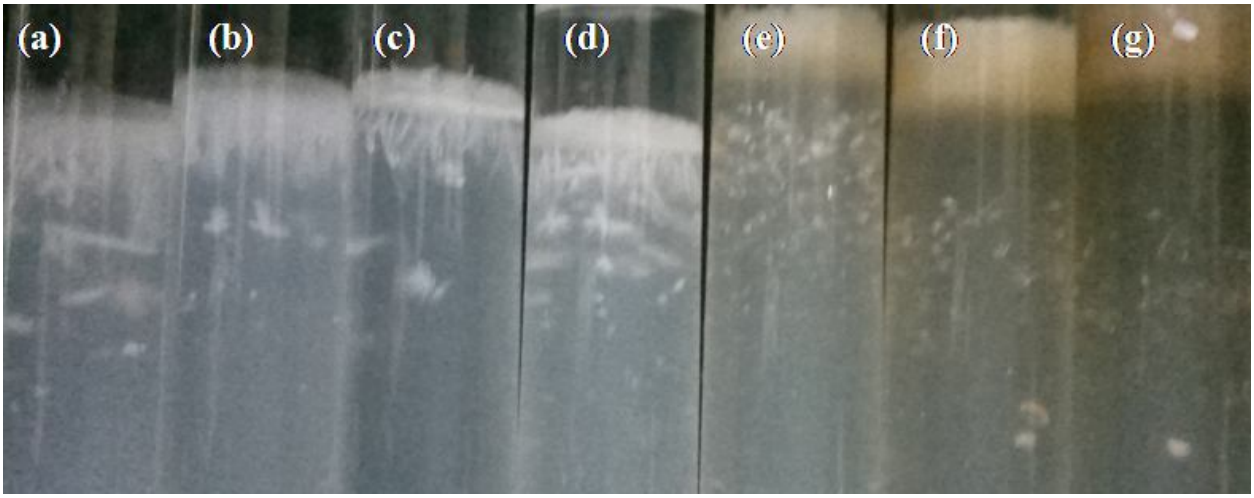

Fig. 1**:** The effect of lemon juice on struvite crystals in the gel method (a) without any additive (b) with the distilled water (c) with the 1% lemon juice(d) with the 2% lemon juice (e) with the 3% lemon juice (f) with the 4% lemon juice (g) with the 5% lemon juice after 7 days.

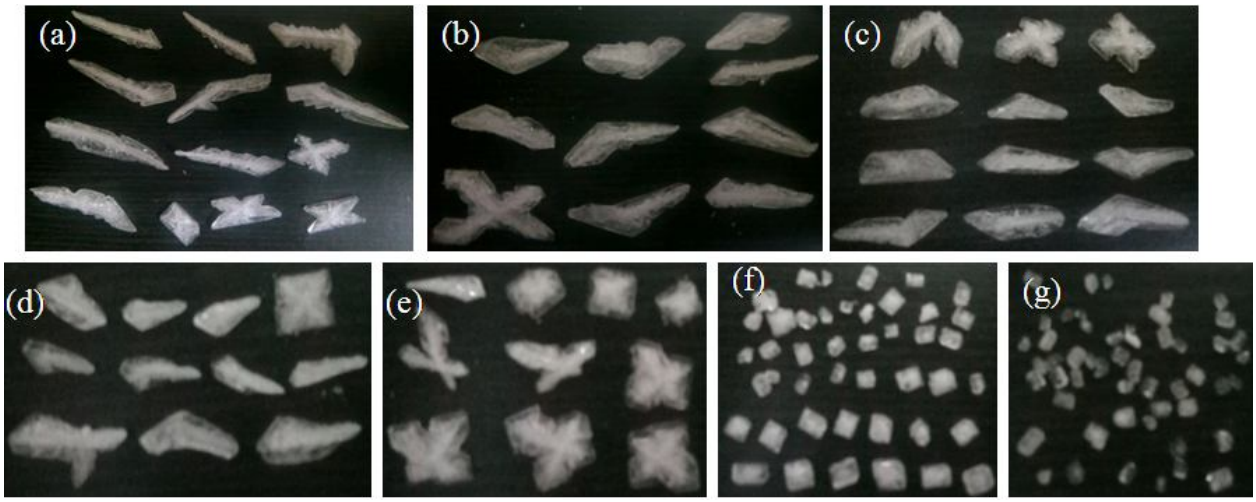

Fig. 2: The harvested crystals of struvite obtained from lemon juice in the gel method (a) without any additive (b) with the distilled water (c) with the 1% lemon juice(d) with the 2% lemon juice (e) with the 3% lemon juice (f) with the 4% lemon juice (g) with the 5% lemon juice after 7 days.

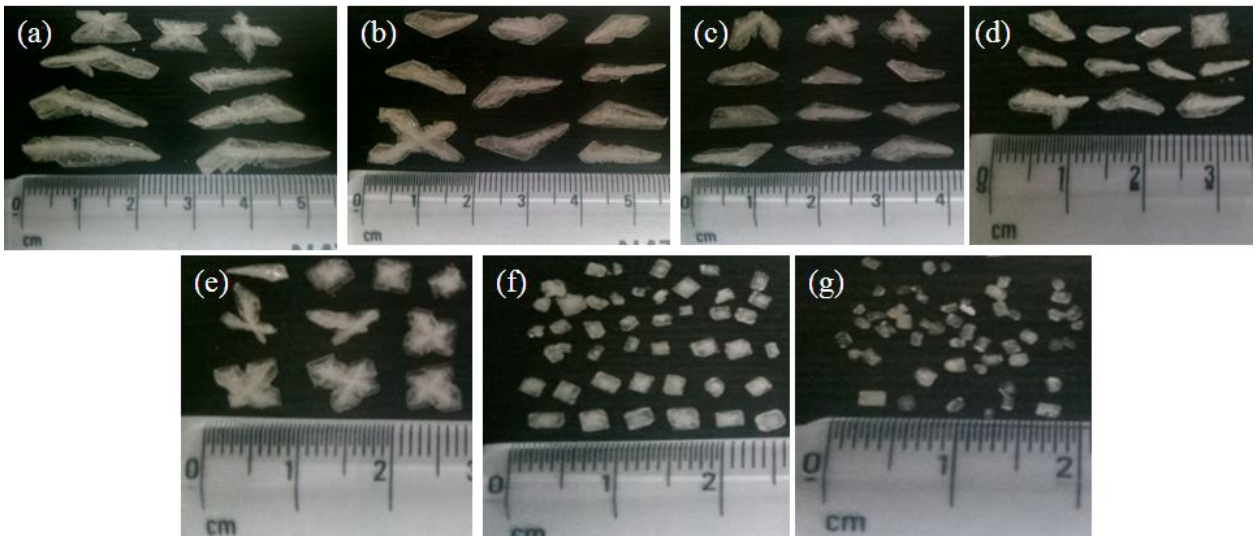

Fig. 3: The measurement of struvite crystals obtained from lemon juice in the gel method (a) without any additive (b) with the distilled water (c) with the 1% lemon juice(d) with the 2% lemon juice (e) with the 3% lemon juice (f) with the 4% lemon juice (g) with the 5% lemon juice after 7 days.

B. Effect of orange juice on Struvite crystals

The effect of orange juice on nucleation and crystallization characteristics of struvite crystals is determined by measuring the weight of the formed crystals. In the gel method, the control using pure Mg CH_3COO_2•$4H_2O$ led to the maximum nucleation of crystals growth within 24 h of adding the supernatant solutions Fig. 4 (4a). In the presence of orange juice, nucleation was delayed and reduced masses of the crystals were observed 96 h after adding the supernatant solutions Fig. 4 (4b-g). Morphology of the harvested crystals after addition of orange juice as shown in Fig. 5.The largest single struvite crystals having dimensions of 3.2 cm as observed in (Fig. 6a). The sizes of the struvite crystals were reduced from 3.2 cm to 0.5 cm at 1% orange juice was observed in (Fig. 6c-g).With an increase in the concentration of orange juice from 1% to 5% (v/v), the weight of the formed crystals was gradually reduced from 2.47 g to 0.21 g at 5% concentration of orange juice respectively. The ANOVA statistical analysis was performed and different parameters have been evaluated, and $p<0.05$ has suggested that the correlation is significant as shown in (Table 3).

Recently, growth inhibition studies of Struvite crystals in the presence of some of the herbal extracts [17,18,25]were attempted in literature. In the present work, Struvite crystals growth was reduced due to the inhibitory effect of fruit of lemon juice as compared

to the orange juice under *in vitro* conditions. As lemon juice contain citric acid, this further supports the earlier theories on citrate inhibition and indicate that, consumption of lemon juice may help to inhibit the growth of struvite crystals containing urinary calculi in body.

Table- 3: ANOVA statistical analysis for harvested orange struvite crystals

CRYSTALS	GROUP	TREATENTS	Mean (gm)±SD
Struvite	A	Control	2.49±0.014
	B	Control+Distilled water	2.46±0.057
	C	Control+1% Lemon juice	$1.47\pm0.021^{a,b}$
	D	Control+2% Lemon juice	$0.68\pm0.014^{a,b,c}$
	E	Control+3% Lemon juice	$0.43\pm0.014^{a,b,c,d}$
	F	Control+4% Lemon juice	$0.32\pm0.014^{a,b,c,d,e}$
	G	Control+5% Lemon juice	$0.21\pm0.014^{a,b,c,d,e,f\text{-ns}}$

Values represent mean (gm) ± S.D (n=4) Comparisons between means are as follows. a: A *vs* B-G, b: B *vs* C-G, c: C *vs*D-G, d: D *vs* E-G, e: E *vs* F-G, f: F *vs* G. Statistical significance were considered to be $^{a}p<0.05$,$^{b}p<0.05$,$^{c}p<0.05$,$^{d}p<0.05$,$^{e}p<0.05$.

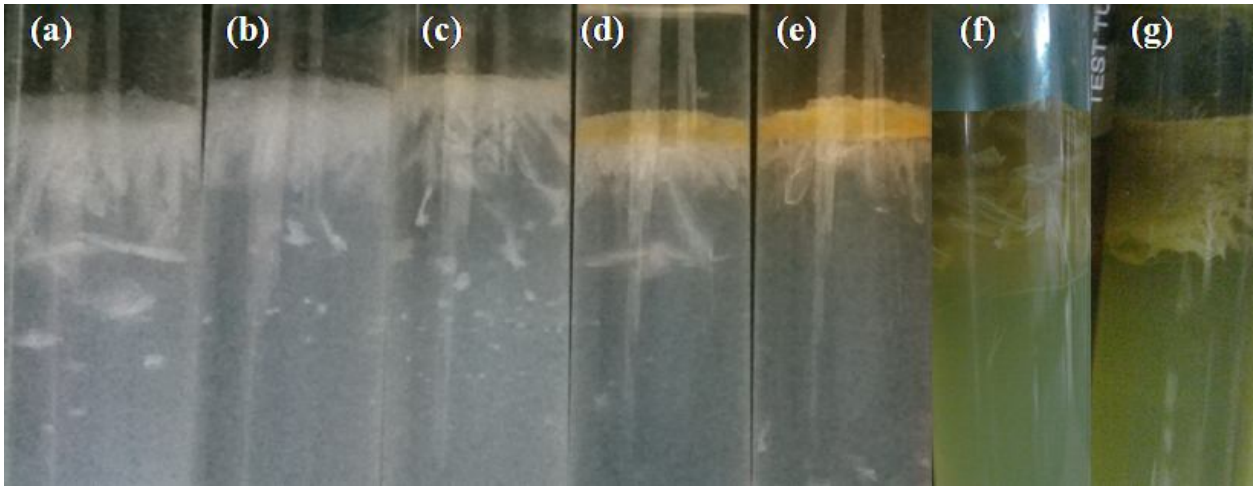

Fig. 4: The effect of orange juiceon struvite crystals in the gel method (a) without any additive (b) with the distilled water (c) with the 1% lemon juice(d) with the 2% lemon juice (e) with the 3% lemon juice (f) with the 4% lemon juice (g) with the 5% lemon juice after 7 days.

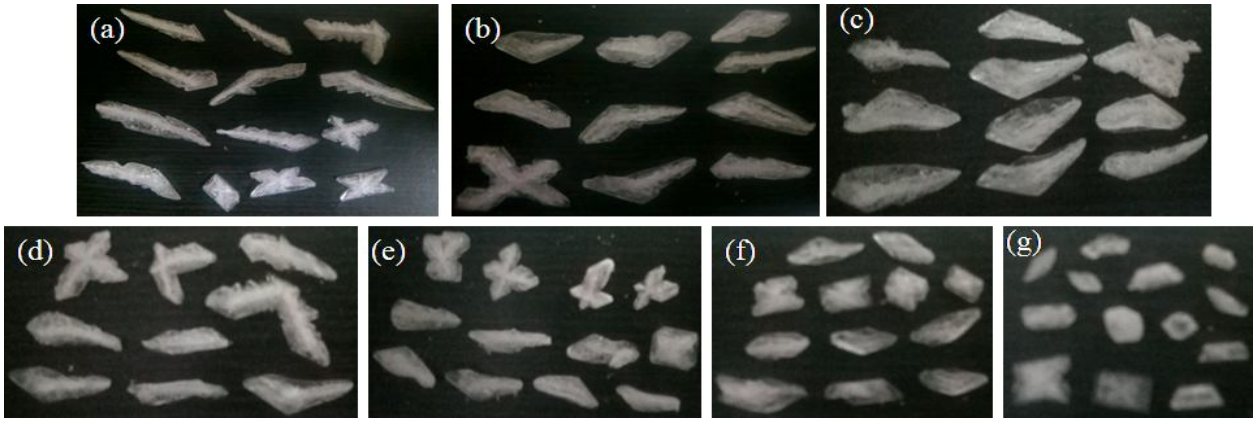

Fig. 5: The harvested crystals of struvite obtained from orange juice in the gel method (a) without any additive (b) with the distilled water (c) with the 1% lemon juice(d) with the 2% lemon juice (e) with the 3% lemon juice (f) with the 4% lemon juice (g) with the 5% lemon juice after 7 days.

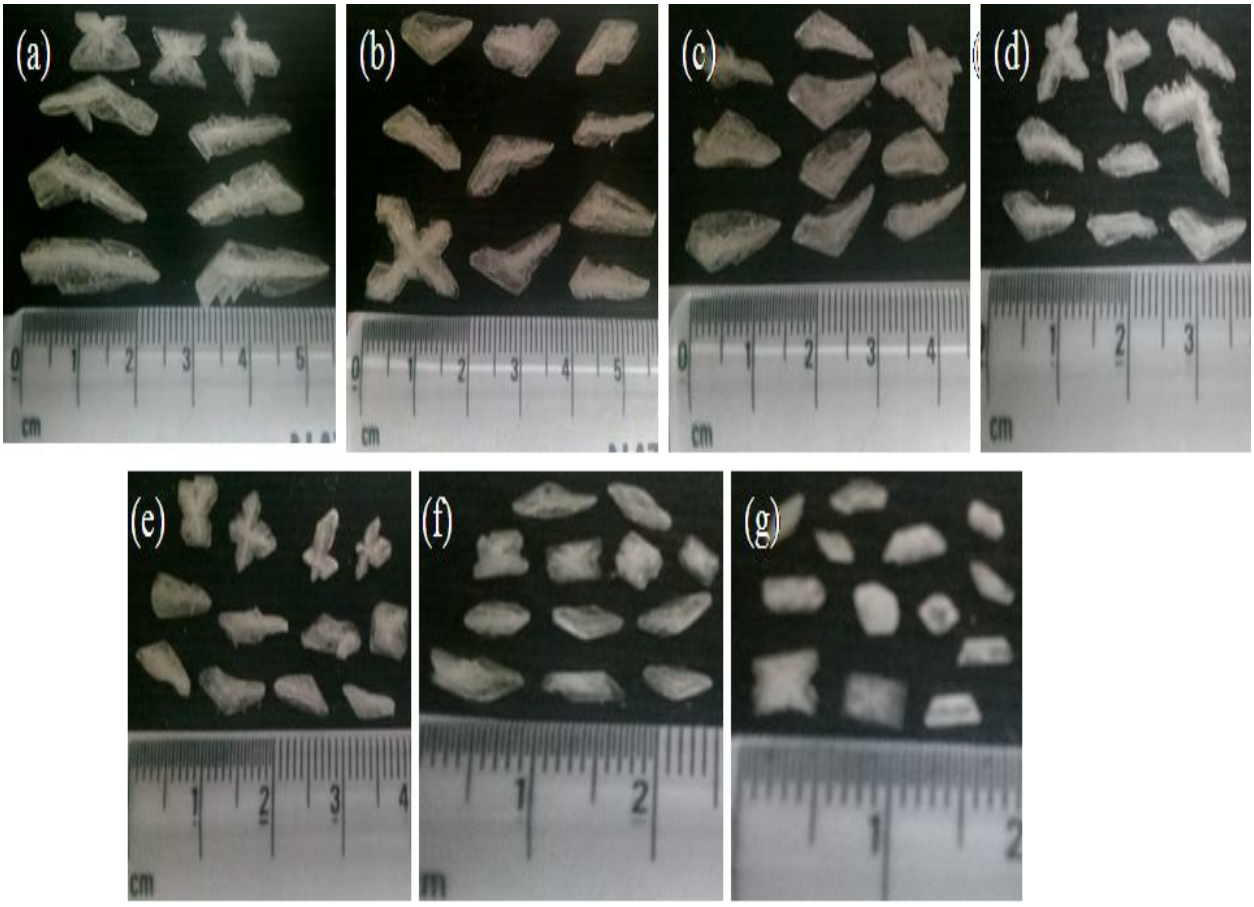

Fig. 6: The measurement of struvite crystals obtained from orange juice in the gel method (a) without any additive (b) with the distilled water (c) with the 1% lemon juice(d) with the 2% lemon juice (e) with the 3% lemon juice (f) with the 4% lemon juice (g) with the 5% lemon juice after 7 days.

C. Characterization of Struvite crystals

The FTIR spectra of Struvite crystals obtained in the presence and absence of the lemon and orange fruit juice are shown in Fig. 7and 8. In fig. 7 and 8(a) without any additive, a strong band at 2358 cm^{-1} are due to the antisymmetric and symmetric stretching vibration of NH_4 units. The peak at 1636 cm^{-1} is due to HOH deformation of water and the peak at 1441 cm^{-1} is due to the HNH deformation modes of NH_4 units. The band at 1007 cm^{-1} is due to V_3antisymmetric stretching vibration and the peak at 757 cm^{-1} is due to the water liberational and NH_4 rocking modes. The peak at 568 cm^{-1} is due to the V_4 bending modes of the PO_4 units. In the presence of 5% lemon juice Fig. 7(f), a band at and 2374 cm^{-1} is due to the antisymmetric and symmetric stretching vibration of NH_4 units. The peak at 1625 cm^{-1} is due to HOH deformation of water and the peak at 1438 cm^{-1} is due to the HNH deformation modes of NH_4 units. The band at 1004 cm^{-1} is due to V_3antisymmetric stretching vibration and the peak at 758 cm^{-1} is due to the water liberational and NH_4 rocking modes. The peak at 568 cm^{-1} is due to the V_4 bending modes of the PO_4 units. In the presence of 5% orange juice Fig. 8(f), a band at and 2365 cm^{-1} is due to the antisymmetric and symmetric stretching vibration of NH_4 units. The peak at 1637 cm^{-1} is due to HOH deformation of water and the peak at 1441 cm^{-1} is due to the HNH deformation modes of NH_4 units.. The band at 1005 cm^{-1} is due to V_3antisymmetric stretching vibration and the peak at 760 cm^{-1} is due to the water liberational and NH_4 rocking modes. The peak at 577 cm^{-1} is due to the V_4 bending modes of the PO_4 units.

Several researcher have reported crystallization characterization of Struvite crystals using FTIR techniques[17,25]. The peaks shift from 2358 to 1674 cm^{-1} and from 1441 to 1438 cm^{-1} for HNH deformation modes of NH_4 units previously reported. The shifting further supports that the lemon juice can promote the formation of ammonium magnesium phosphate hexahydrate crystals and reduce the nucleation rate of struvite crystalsas compared with orange juice.

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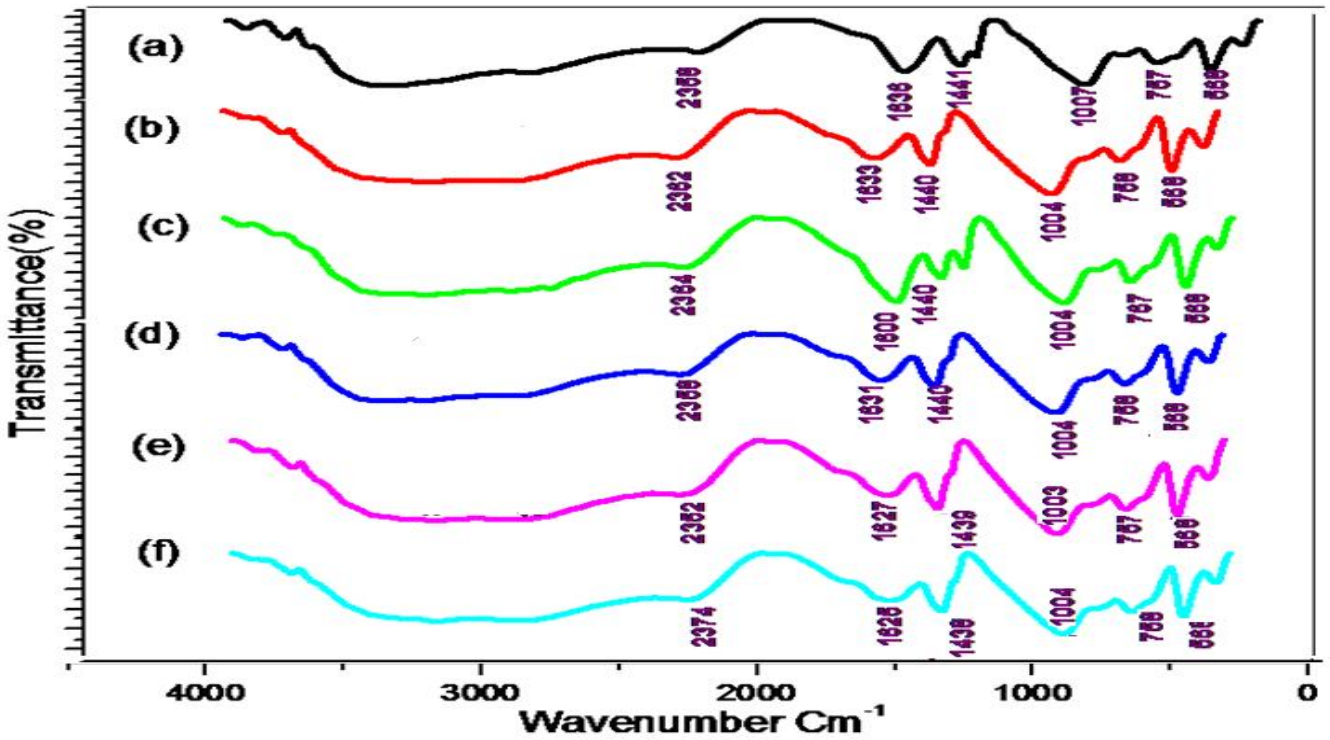

Fig. 7**:** The FTIR spectra of struvite crystals obtained from lemon juice in the gel method (a) without any additive (b) with the 1% lemon juice (c) with the 2% lemon juice (d) with the 3% lemon juice (e) with the 4% lemon juice (f) with the 5% lemon juice after 7 days.

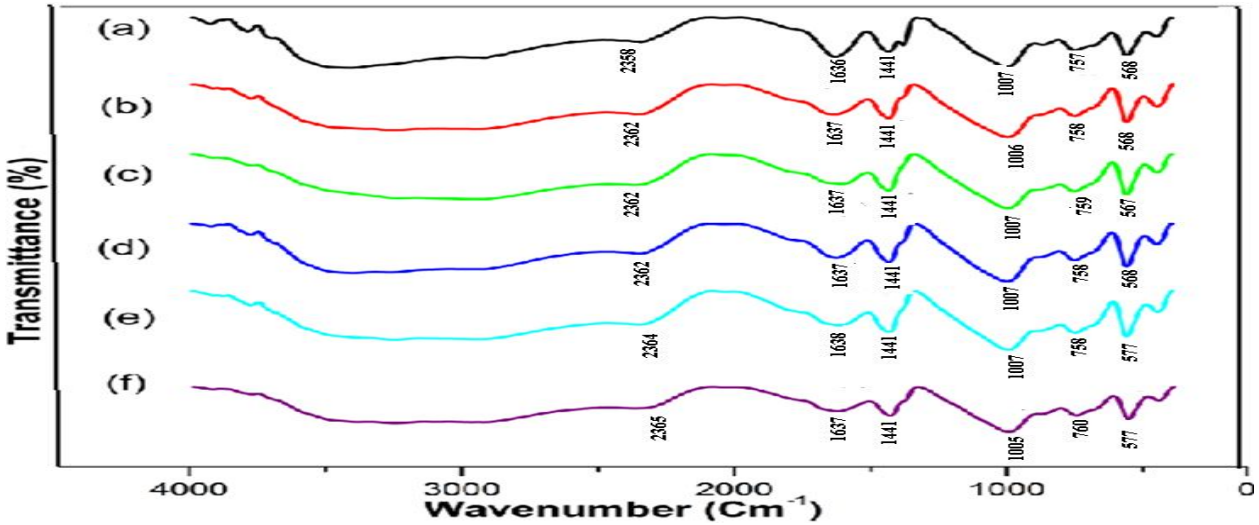

Fig. 8: The FTIR spectra of struvite crystals obtained from orange juice in the gel method (a) without any additive (b) with the 1% lemon juice (c) with the 2% lemon juice (d) with the 3% lemon juice (e) with the 4% lemon juice (f) with the 5% lemon juice after 7 days.

IV. CONCLUSION

Struvitecrystals were grown by single diffusion gel growth techniques and were characterized by FTIR technique for the experimental confirmations of the grown crystal. With an increase in the concentration of lemon and orange juices, the weight of the formed crystals was gradually reduced from 2.49 g to 0.09 g (lemon juice) and 2.49 g to 0.21 g (orange juice) for the struvite crystals, respectively. FTIR techniques confirmed its functional groups of Struvite crystals. One way ANOVA performed with treated and untreated crystal growth data obtained from Struvite crystals showed significant differences ($p<0.05$). This study

confirms that using lemon juice can promote the formation of ammonium magnesium phosphate hexahydrate crystals and reduce the nucleation rate of struvite crystals, a major component of urinary stone. This study is focused to find new natural product for the treatment of urinary stone.

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A. Conflicts Of Interests

The authors declare that they have no conflict of interest. It has not been published elsewhere. That it has not been simultaneously submitted for publication elsewhere. All authors agree to the submission to the journal.

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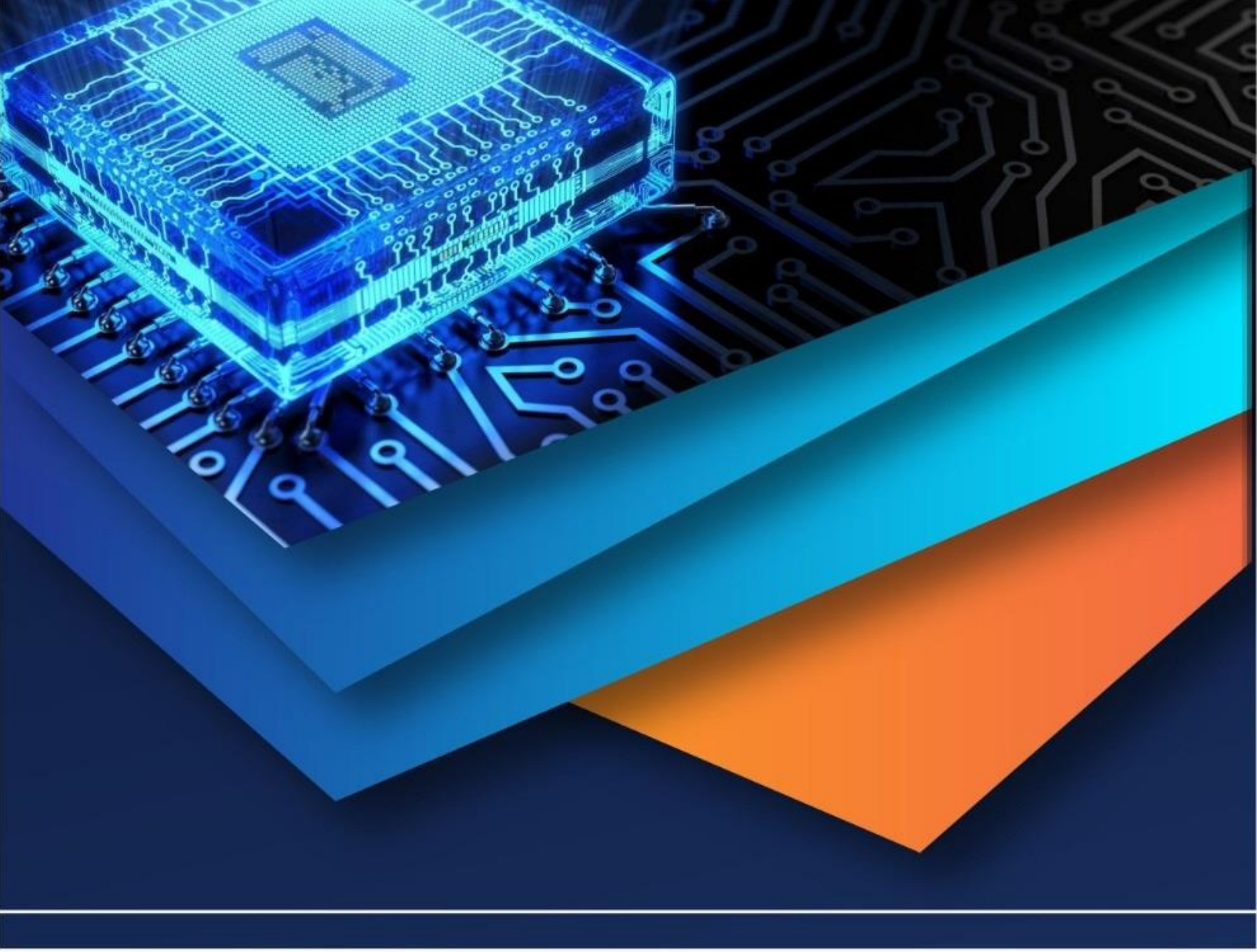

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