

THE EFFECT OF CALOTROPIS GIGANTEA LEAF EXTRACT ON GROWTH CONTROL OF STRUVITE CRYSTALS

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ABSTRACT: Throughout the world, a large number of people suffering from urinary stone problem and upto 15% people are affected by kidney stones at some point in their life. The stone comprise the composition of calcium phosphate, calcium oxalate, uric acid and magnesium ammonium phosphate (Struvite crystals). There are many attempts are under practice to reduce the growth of Struvite crystals by natural resources, and as a consequence, we planned to utilize *Calotropis gigantea*, commonly known as Yercum or Madar, belongs to the family Asclepiadaceae, for this purpose. *Calotropis gigantea* is widely distributed in eastern and southern parts of India, and it holds various biological applications in its credit. Based on the reports on various components from various parts of the plant, the present study focused on inhibitory effect of the methanolic extract of *Calotropis gigantea* leaves on the growth of Struvite crystals.

For this study, Struvite crystals were grown by single diffusion gel growth technique (confirmed by FTIR), and the inhibitory effect was evaluated. From the results, it is witnessed that the increase in the concentration (0.15% to 1.00%) of methanol extract of *Calotropis gigantea* leaves gradually reduced the growth of Struvite crystals significantly, from 2.49 g to 0.09 g, and reduce the nucleation rate of Struvite crystals as well. In addition, the responsible active compound is identified (Lupeol, a pharmacologically active triterpenoid) and isolated/characterized by high performance thin layer chromatography (HPTLC) / high performance liquid chromatography (HPLC) and NMR techniques.

Key words: *Calotropis gigantea*, Leaf extract, Methanol extract, Struvite crystals.

INTRODUCTION

A large number of people suffering from urinary stones problems [1]. Urinary stones have been found to contain calcium phosphate, calcium oxalate, uric acid and magnesium ammonium phosphate or Struvite crystals [2-4]. Among the magnesium phosphates, namely, ammonium magnesium phosphate hexahydrate (AMPH) commonly known as Struvite and magnesium hydrogen phosphate trihydrate have 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 urine 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 favors precipitation of Struvite crystals. Higher intake of phosphate (from Proteins) and magnesium based food and lower intake of water gives rise to the PO_4^{3-} and Mg^{2+} 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 a Struvite or branched calculus that involves the entire renal pelvis and calyces. Patients with infected Struvite calculi who receive no treatment have about a 50% chance of losing the kidney [18, 19]. Urinary stones are characterized by high reoccurrence rate therefore requiring a preventive treatment by using the medicinal plants [20, 21].

Calotropis gigantea commonly known as Madar or Yercum belongs to the family Asclepiadaceous and is a shrub about 6 m high which is widely distributed in eastern and southern parts of India [22]. Studies by various researchers have proved that plants are one of the major sources for drug discovery and development [23]. Plants are reported to have antimicrobial, haemolytic, anticancer, anti-inflammatory, antidiabetic, antioxidant properties etc [24]. The chemical constituent shows that the presence of Root: cardiac glycosides, seven oxypregnane-oligo glycosides, calotroposides A-G. Rootbark: β -amyirin, two isomeric crystalline alcohols, giganteol, isogiganteol and cardenolides. Latex: akundarin, latex contains 0.45% uscharin, 0.15% cal toxin, 0.15% calactin, latex also contains α -calatropeol, β - calatropeol, β - amyirin and calcium oxalate, it also yields a nitrogen and sulphur containing fish and cardiac poison, gigantol. Latex also contains traces of glutathione and a proteoclastic enzyme similar to papain. Leaves: alkaloids, glycosides, mudarine. Stembark: β -calatropeol, -amyirin, giganteol. Flower: α -calatropeol, β -calatropeol, amyirin, cardioactive glycosides, mudarine, asclepin, bitter resins akundarin, calotropin [25-30]. In the present investigation, the effects of methanol extract of *Calotropis gigantea* leaves are used as additives to induce the nucleation and growth of Struvite crystals by single diffusion gel growth technique and are reported for the first time. Lupeol

is the active compound that is identified and isolated by High performance thin layer chromatography (HPTLC) technique and column chromatography. The isolated compounds were confirmed by Fourier transform infrared (FTIR) and ^{13}C NMR spectra. The purity was checked by High performance liquid chromatography (HPLC) technique. This study incorporated a multidisciplinary approach in characterizing Struvite crystals grown *in vitro* to help formulate prevention or dissolution strategies in controlling urinary stone growth.

MATERIALS AND METHODS

Chemicals and reagents

Analytical grade anhydrous methanol, calcium chloride, magnesium acetate, oxalic acid, sodium metasilicate, ammonium dihydrogen phosphate were purchased from Sigma-Aldrich. Lupeol (purity 99%), was also purchased from Sigma-Aldrich. Ethyl acetate, toluene, glacial acetic acid and methanol used in the present research work were of HPLC grade and were procured from E. Merck.

Collection of plant material

The leaves of *Calotropis gigantea* were collected in the month of June from the bishop Heber college, Trichy, Tamil Nadu, India. The plant was identified and confirmed by Dr. S. John Britto, Director, Rapinat herbarium, St. Joseph College, Tiruchirapalli, Tamil Nadu.

Preparation of methanol extracts

The leaves of *Calotropis gigantea* were washed in running water, cut into small pieces and then shade dried for a week at 35-40°C, after that it was grinded to a uniform powder of 40 mesh size [31]. The methanol extracts were prepared by soaking 100 g of the dried powder plant materials in 1 L of methanol by using a soxhlet extractor continuously for 10 hr. The extracts were filtered through whatmann filter paper No. 42 (125mm). The filtered extract was concentrated and dried by using a rotary evaporator under reduced pressure. The obtained residue 1.2 g (leaves) was used to prepare the series (0.15, 0.25, 0.50, 0.75 and 1.0%) of methanol supernatant concentrations for *in vitro* studies (Table 1).

Growth and characterization of Struvite crystals

Glass test tubes were used as a crystallization apparatus and the single diffusion reaction technique was employed [32,33]. One of the reactants, 0.5 M ammonium dihydrogen phosphate (ADP), was mixed with sodium metasilicate solution, the density of 1.04g/cm³ 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 gel in 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). 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 [34].

The nomenclature of different additive solution on the growth of Struvite crystals

An attempt was made to study the effect of the methanol extract of *Calotropis gigantea* leaves on the growth of Struvite crystals generated 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 aqueous extract of five medicinal plants on the growth of Struvite crystals. A series of five different concentrations of 0.15, 0.25, 0.50, 0.75 and 1.00% of these each plant extracts was added in equal amounts in supernatant solution and the average weight of the grown crystal were measured.

Table 1: Supernatant solutions added to the set gels for Struvite crystals.

Supernatant Solutions (SS) (Groups and Treatments)	Compositions
I (Control)	10 ml of 1 M magnesium acetate
II (Distilled water)	5 ml of 1 M magnesium acetate +5 ml of distilled water
III (0.15% methanol extract)	4ml of 1 M magnesium acetate +5 ml of 0.15% of methanol extract of leaves of

	<i>Calotropis gigantea</i> separately
IV (0.25% methanol extract)	5 ml of 1 M magnesium acetate +5 ml of 0.25% of methanol extract of leaves of <i>Calotropis gigantea</i> separately
V(0.50% methanol extract)	5 ml of 1 M magnesium acetate +5 ml of 0.50% of methanol extract of leaves of <i>Calotropis gigantea</i> separately
VI(0.75% methanol extract)	5 ml of 1 M magnesium acetate +5 ml of 0.75% of methanol extract of leaves of <i>Calotropis gigantea</i> separately
VII(1.00% methanol extract)	5 ml of 1 M magnesium acetate +5 ml of 1.00% of methanol extract of leaves of <i>Calotropis gigantea</i> separately

Isolation and identification of Lupeol from *Calotropis gigantea* by HPTLC

The HPTLC plates Si 60F₂₅₄ (20cmX10cm) were purchased from E. Merck (India). 100mg/ml of methanol extracts of leaves of *Calotropis gigantea* was taken for analysis. The extracts were filtered and vacuum dried at 45°C. The dried extracts was separately redissolved in 1ml of methanol and sample of varying concentration (5-30 µl) for Lupeol were spotted for quantification. 1mg of standard Lupeol was prepared in 1ml of chloroform, and different amounts of (50-250 ng) Lupeol were loaded onto a TLC plate to get the calibration curve [35-38].

A Camag HPTLC system equipped with an automatic TLC sampler ATS₄, TLC scanner 3 and integrated software Win CATS version 3, was used for the analysis. Samples were washed on a pre-coated silica gel HPTLC plates Si 60F₂₅₄ (20cm x 10cm) plate of 200µm-layer thickness, for quantification of Lupeol in leaves of *Calotropis gigantea*. The samples and standards were applied on the plate as 8mm wide bands with a constant application rate of 150Nl s⁻¹, with an automatic TLC sampler (ATS₄) under a flow of N₂ gas, 15mm from the bottom, 15mm from the side, and the space between two spots was 6mm in the plate.

Detection and estimation of Lupeol

The linear ascending development was carried out in a Camag twin through chamber (20cm x 10cm), which was pre-saturated with a 25ml mobile phase, Toluene: Ethyl acetate (8.0: 2.0 v/v) for Lupeol for 30 minutes, at room temperature (25°C±2°C) and 50±5% relative humidity. The length of the chromatogram run was up to 90 mm. Subsequent to the development; the TLC plate was dried in a current of air, with the help of air dryer, in a wooden chamber with adequate ventilation. The dried plate was dipped into freshly prepared Libermann Burchard reagent. Quantitative estimation of the plate was performed in the absorption-reflection mode at 538nm, using a slit width 6.00 x 0.45 mm, with data resolution 100µm/step and scanning speed 20mm/sec. The source of radiation utilized was a tungsten lamp emitting continues visible spectra of 366 nm. Determination of Lupeol in methanolic extracts of leaves of *Calotropis gigantea* was performed by the external standard method, using pure standards. Each was carried out in triplicate.

Calibration curve and linearity

The calibration performed by analysis of working standard solutions of Lupeol (1000 to 6000 ng for *Calotropis gigantea*) was spotted on recoated TLC plate, using semiautomatic spotter under nitrogen stream. The TLC plates were developed, dried by hot air and photo metrically analyzed as described earlier. The calibration curves were prepared by plotting peak area versus concentration (ng/spot) corresponding to each spot.

Recovery

To determine the recovery, known concentrations of standards were added to a reanalyzed sample of *Calotropis gigantea* leaves. The spiked samples were then analyzed by the proposed HPTLC method and the analysis was carried out in triplicate.

Isolation of Lupeol by column chromatography

The condensed methanol extract of leaves powder (150 g) of *Calotropis gigantea* was subjected to column chromatography over TLC grade silica gel. Elution of the column first with n-hexane, increasing amount of ethyl acetate in n-hexane and finally with methanol yielded a number of fractions. The preparation of solvent systems used to obtain Lupeol (32 mg/90 g) were n-hexane-ethyl acetate (90:10) from fraction 5 and 6. The compounds were detected on TLC plates by spraying with Libermann Burchard reagent and heated at 100°C for 10 minutes.

Purification of isolated compounds by High performance liquid chromatography

The analytical HPLC system (Shimadzu) was equipped with a diode array detector, a 20 μ l loop, 200 x 4.6 mm C18 column, methanol (HPLC grade, 0.2mm filtered) used as a mobile phase. The isolated Lupeol compounds were separated using a mobile phase of methanol: water (75:25 v/v) at a flow rate of 1.0 ml/min, column temperature 30 °C. Injection volume was 40 μ l and detection was carried out at 346 nm.

Characterization of isolated compounds:

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. ^{13}C NMR spectrum was acquired on Bruker AM 200 SY instruments (^{13}C , 50.32 MHz) using TMS as internal standard and CDCl_3 as solvent.

Statistical analysis

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

RESULTS AND DISCUSSIONS

Effect of *Calotropis gigantea* on Struvite crystals

The effect of *Calotropis gigantea* 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 $\text{Mg CH}_3\text{COO}_2 \cdot 4\text{H}_2\text{O}$ was led to the maximum nucleation of crystals growth within 24 hr of adding the supernatant solutions Fig. 1 (1a). In the presence of *Calotropis gigantea*, nucleation was delayed and reduced masses of the crystals were observed 96 hr after adding the supernatant solutions Fig. 1 (1b-g). Morphology of the harvested crystals after addition of *Calotropis gigantea* 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 1.7 cm to 0.2 cm at 1% concentration of extracts was observed in (Fig. 3c-g). With an increase in the concentration of *Calotropis gigantea* from 0.15% to 1% (v/v), the weight of the formed crystals was gradually reduced from 2.49 g to 0.09 g 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 Struvite crystals.

Crystals	Group	Treatments	Mean (gm) \pm sd
Struvite	A	Control	2.43 \pm 0.014
	B	Control+ Distilled water	2.39 \pm 0.057
	C	Control+0.15% extracts	1.22 \pm 0.014 ^{a,b}
	D	Control+0.25% extracts	0.46 \pm 0.014 ^{a,b,c}
	E	Control+0.50% extracts	0.24 \pm 0.014 ^{a,b,c,d}
	F	Control+0.75% extracts	0.16 \pm 0.014 ^{a,b,c,d,e}
	G	Control+1.00% extracts	0.08 \pm 0.014 ^{a,b,c,d,e,f,ns}

Values represent mean (gm) \pm 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 $p < 0.05$.

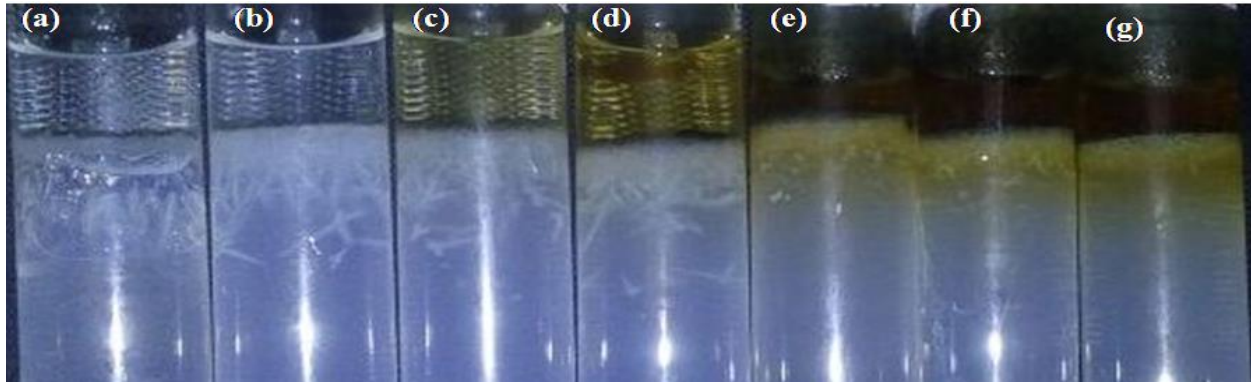


Figure 1: The effect of *Calotropis gigantea* on Struvite crystals in the gel method (a) without any additive (b) with the distilled water (c) with the 0.15% extract (d) with the 0.25% extract (e) with the 0.50% extract (f) with the 0.75% extract (g) with the 1.00% extract after 7 days.

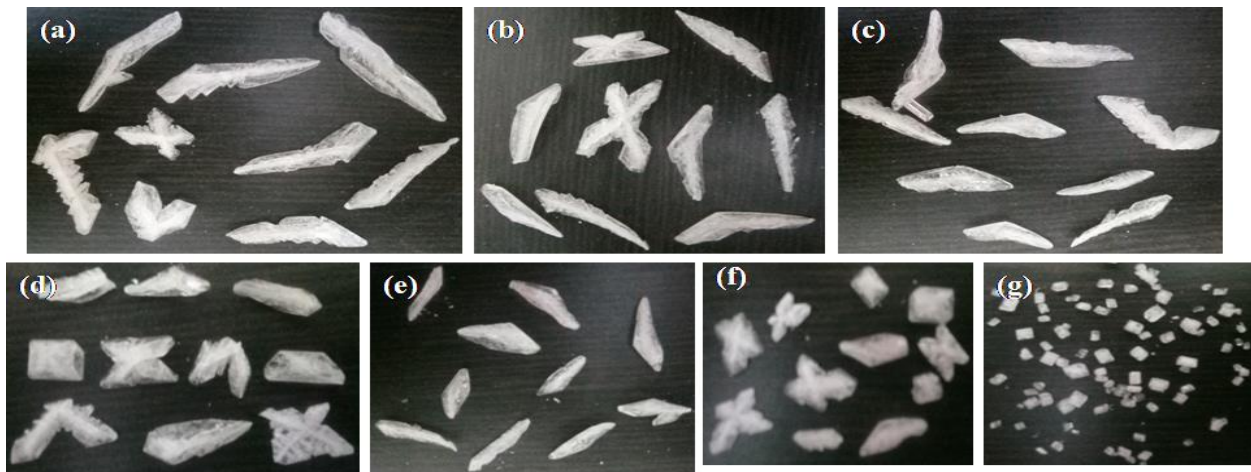


Figure 2: The harvested crystals of Struvite obtained from *Calotropis gigantea* in the gel method (a) without any additive (b) with distilled water (c) with 0.15% extract (d) with 0.25% extract (e) with 0.50% extract (f) with 0.75% extract (g) with 1.00% extract after 7 days.

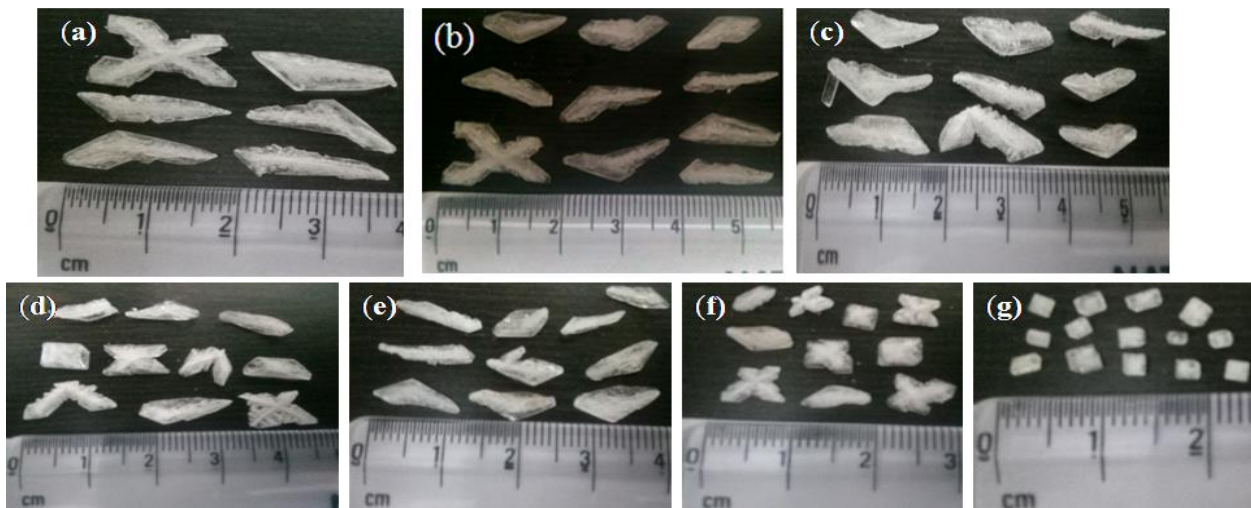


Figure 3: The measurement of Struvite crystals obtained from *Calotropis gigantea* in the gel method (a) without any additive (b) with distilled water (c) with 0.15% extract (d) with 0.25% extract (e) with 0.50% extract (f) with 0.75% extract (g) with 1.00% extract after 7 days.

Characterization of Struvite crystals

The FTIR spectra of Struvite crystals obtained in the presence and absence of the *Calotropis gigantea* is shown in Fig. 4. In fig. 4 (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_3 antisymmetric stretching vibration and the peak at 757 cm^{-1} is due to the water liberation 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. 4(f), a band at and 2374 cm^{-1} is due to the antisymmetric and symmetric stretching vibration of NH_4 units. The peak at 1600 cm^{-1} is due to HOH deformation of water and the peak at 1439 cm^{-1} is due to the HNH deformation modes of NH_4 units. The band at 1003 cm^{-1} is due to V_3 antisymmetric stretching vibration and the peak at 757 cm^{-1} is due to the water liberation and NH_4 rocking modes.

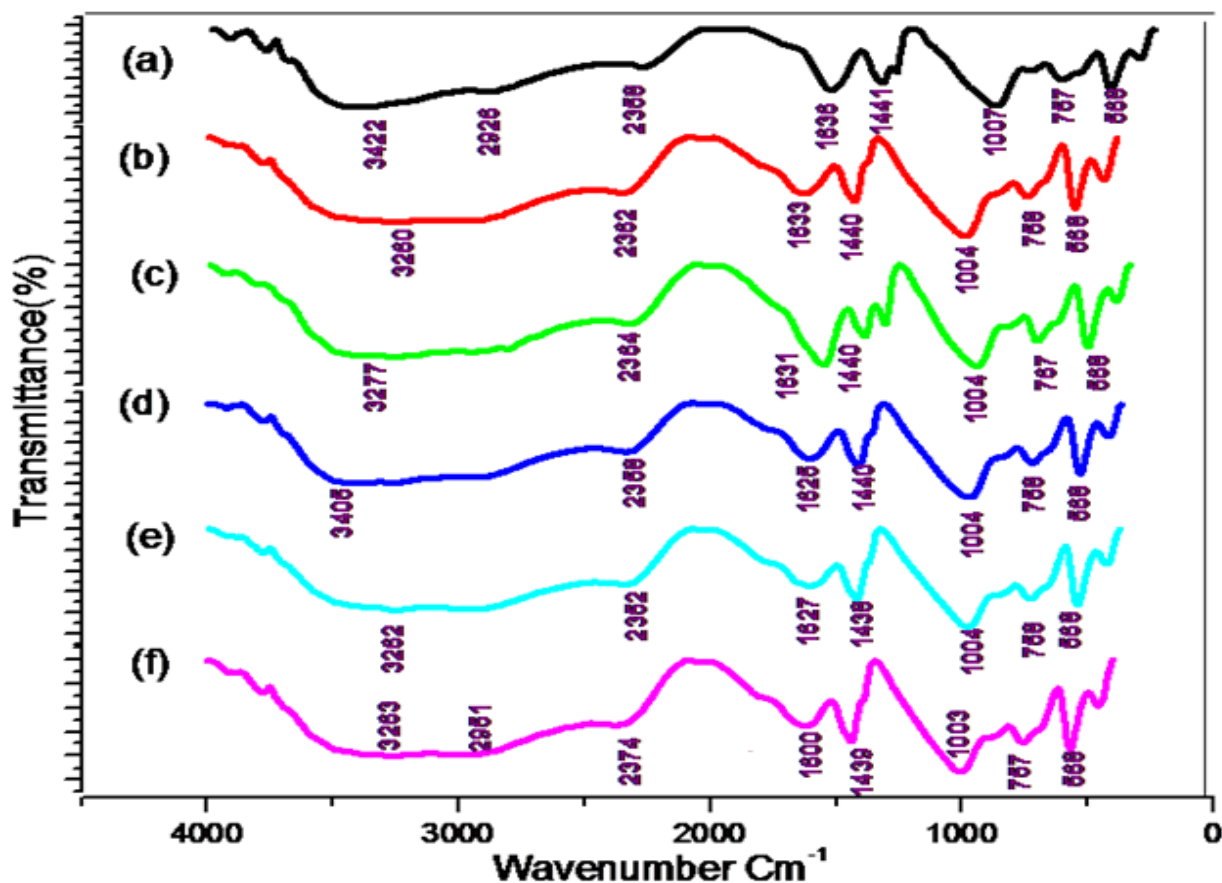


Figure 4: The FTIR spectra of Struvite crystals obtained from *Calotropis gigantea* in the gel method (a) without any additive (b) with 0.15% extract (c) with 0.25% extract (d) with 0.50% extract (e) with 0.75% extract (f) with 1.00% extract after 7 days.

Quantitative determination of Lupeol in *Calotropis gigantea* leaves by HPTLC technique

HPTLC fingerprint patterns have been therefore evolved for extracts of *Calotropis gigantea*. Lupeol standard was quantitated accurately using silica gel F254 HPTLC pre-coated plates with the mobile phase for n- Hexane : Ethyl acetate (80:20v/v), the R_f value for Lupeol was about 0.55. The chromatograms of Lupeol and methanol extract of *Calotropis gigantea* are shown in Fig. 5. The R_f value of standard Lupeol was matched with the R_f value of *Calotropis gigantea* extract (0.55). The amount of Lupeol isolated is 0.473 mg per 100 mg leaves of *Calotropis gigantea*.

Calibration curve and Linearity

The calibration curve was prepared by plotting peak area versus concentration (ng/spot) corresponding to each spot (Fig. 6). The regression equation and correlation curves for Lupeol in *Calotropis gigantea* were, regression via height $y= 6.232+0.101X$ and $r= 0.99215$ $sdv= 6.53$ (Fig. 6a) and regression via area $y=130.296+2.085 X$ and $r=0.99976$ $sdv=1.12$ (Fig. 6b).

Accuracy and recovery

The results showed that the percentage recoveries after sample processing and application were in the range of 99.77 % to 100.11 % (Lupeol) (Table 3). The percentage of Lupeol in *Calotropis gigantea* leaves (Table 4).

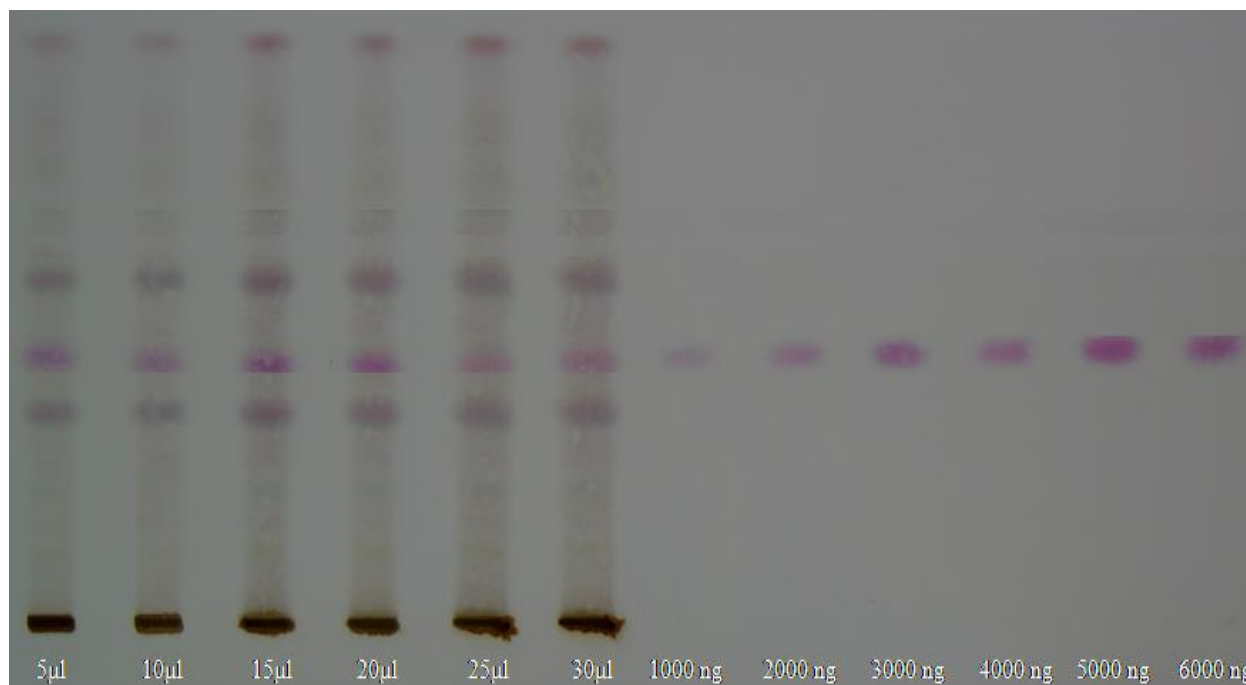
Table 3: Recovery study of Lupeol by HPTLC ($n=3$).

Compound	Amount of compound present in the plant material (mean, $\mu\text{g}/100 \text{ mg}$)	Amount of standard added (μg)	of Amount of standard found in mixture (μg)	Recovery (%)
Lupeol	257	257	620.00	100.10 ± 1.14
		620	1110.10	99.80 ± 0.93

Table 4: Amount of Lupeol in *Calotropis gigantea* leaves.

Compound	Quantity (mean) (mg/100 mg)	Mean \pm SE	CV (%)
Lupeol	0.257	0.257 ± 0.004	0.72

“n” is number of determination, “SE” is standard error, “CV” is cumulative value



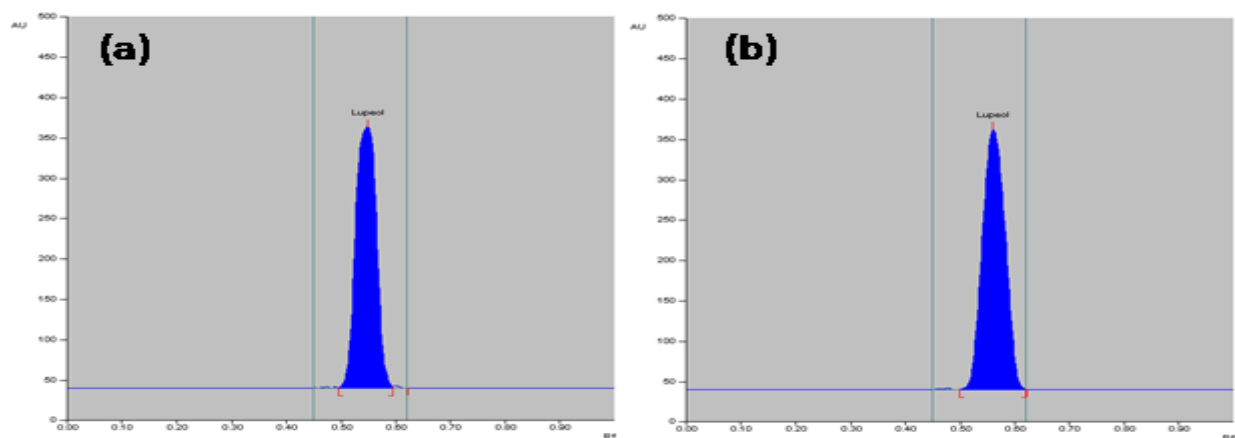


Figure 5: HPTLC chromatogram of Lupeol in *Calotropis gigantea* leaf extract. (a) HPTLC chromatogram of standard Lupeol, (b) HPTLC chromatogram of Lupeol in *Calotropis gigantea* leaves

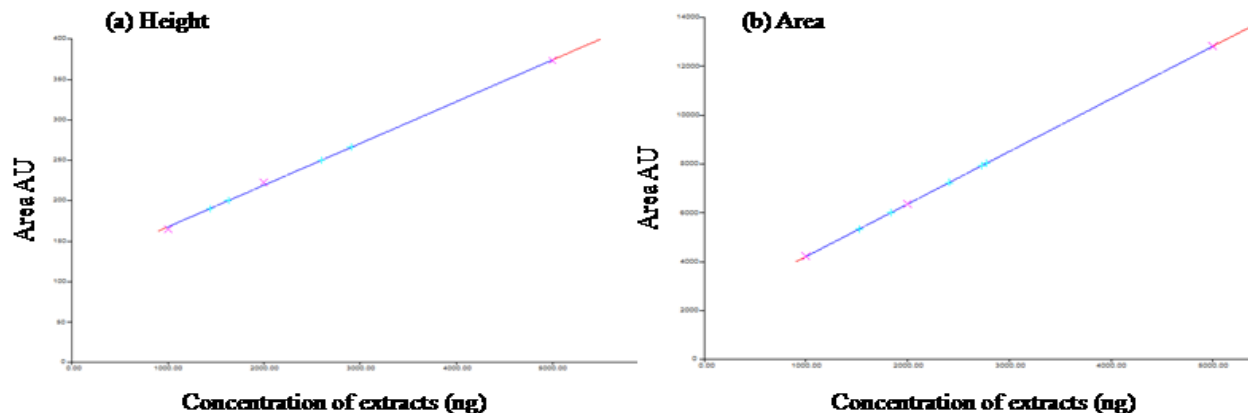


Figure 6: Linear graph for Lupeol in *Calotropis gigantea* in all tracks (concentration vs. area)

Structural elucidation of isolated compound Lupeol

Lupeol was isolated by column chromatography of fractions 5-6 from *n*-hexane-ethyl acetate (9:1) from leaves of *Calotropis gigantea*. Lupeol melting point is 213°C, which corresponds to the molecular formulae $C_{30}H_{50}O$, IR: (KBr) ν_{max} : 3434.62 cm^{-1} (Hydrogen bonded OH Stretch), 2947.33 cm^{-1} and 2354.07 cm^{-1} (C-H Stretch in CH_2 and CH_3), 2108.94 cm^{-1} ($\text{C}\equiv\text{C}$ Stretch), 1640.99 cm^{-1} (C=C Symmetric Stretch), 1563.14 (C=C Asymmetric stretch), 1417.55 cm^{-1} (C-H deformation in CH_2 and CH_3), 1032.72 cm^{-1} (C-O Stretch of secondary alcohol), 880.49 cm^{-1} (=C-H bending exocyclic CH_2) (Fig. 6). In the ^{13}C NMR spectrum of Lupeol δ : δ 37.90 (C-1), δ 20.08 (C-2), δ 79.45 (C-3), δ 38.26 (C-4), δ 55.82 (C-5), δ 18.26 (C-6), δ 27.37 (C-7), δ 38.68 (C-8), δ 50.87 (C-9), δ 34.93 (C-10), δ 19.41 (C-11), δ 20.08 (C-12), δ 35.66 (C-13), δ 40.51 (C-14), δ 25.10 (C-15), δ 29.42 (C-16), δ 40.51 (C-17), δ 48.95 (C-18), δ 48.42 (C-19), δ 151.03 (C-20), δ 27.37 (C-21), δ 38.52 (C-22), δ 25.10 (C-23), δ 15.01 (C-24), δ 15.13 (C-25), δ 15.13 (C-26), δ 14.9 (C-27), δ 16.05 (C-28), δ 109.33 (C-29) and δ 18.26 (C-30) (Fig. 7). The structure of Lupeol was confirmed by comparison with spectral data reported in literature [45-47]. The IR and ^{13}C NMR spectra of Lupeol isolated from the methanol extract in this study is reproduced in Figs. 7 and 8, respectively.

Identification of isolated compound by HPLC

The chromatograms of standard Lupeol and the Lupeol isolated by methanol extract of the leaves of *Calotropis gigantea* are shown in Fig. 9. The Retention time of Lupeol standard (3.073) match with the retention time of Lupeol isolated from the *Calotropis gigantea* extract (3.065) as shown in Figs. 9(a) and 9(b).

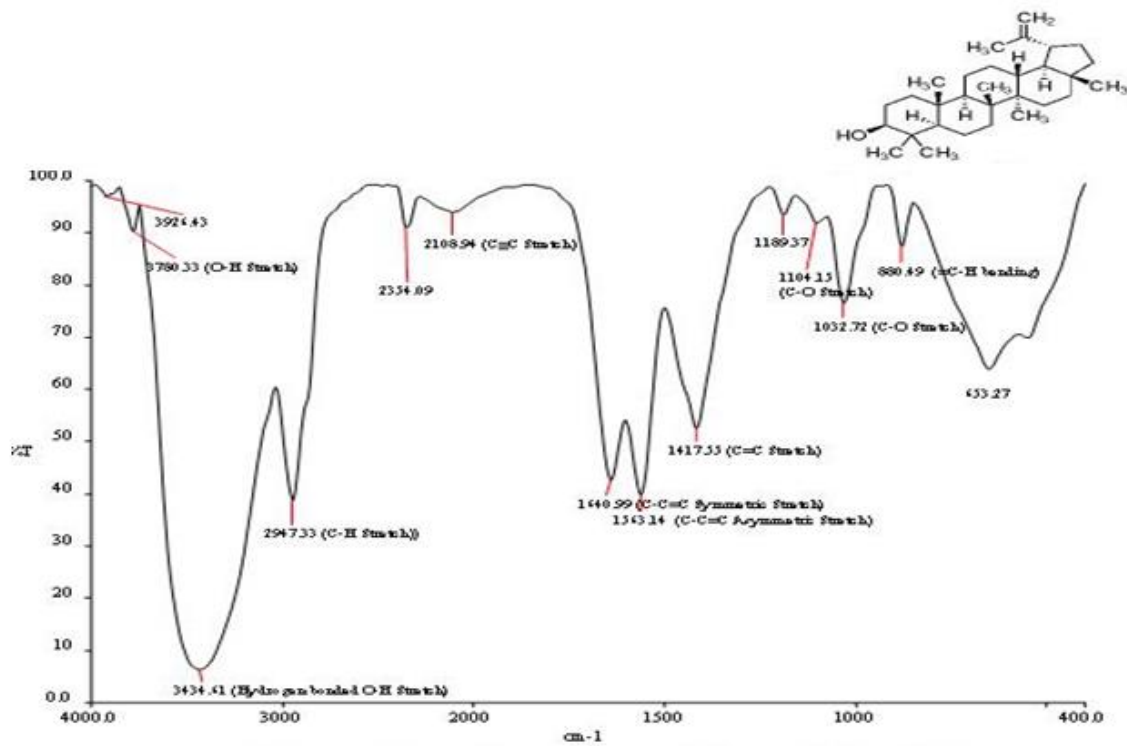


Figure 7: FTIR spectrum of Lupeol isolated from the leaves of *Calotropis gigantea*

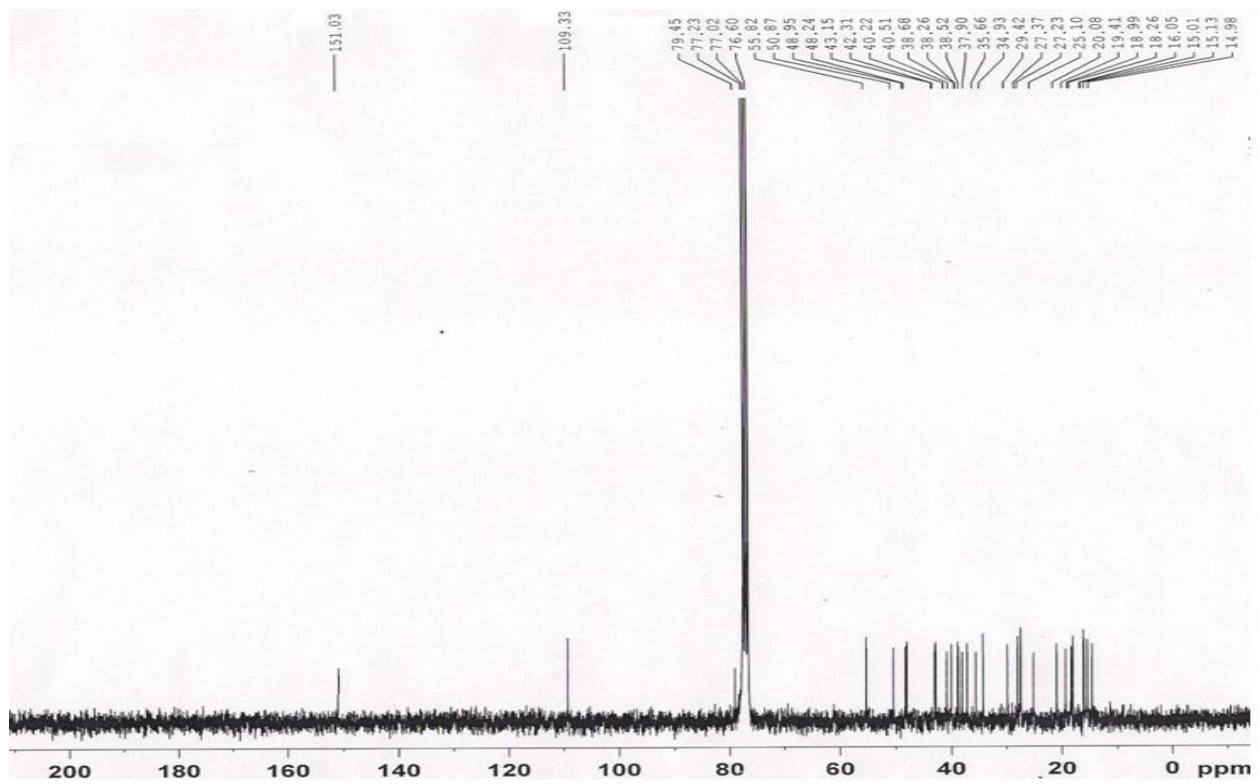


Figure 8: ¹³C NMR spectra of Lupeol isolated from the leaves of *Calotropis gigantea*

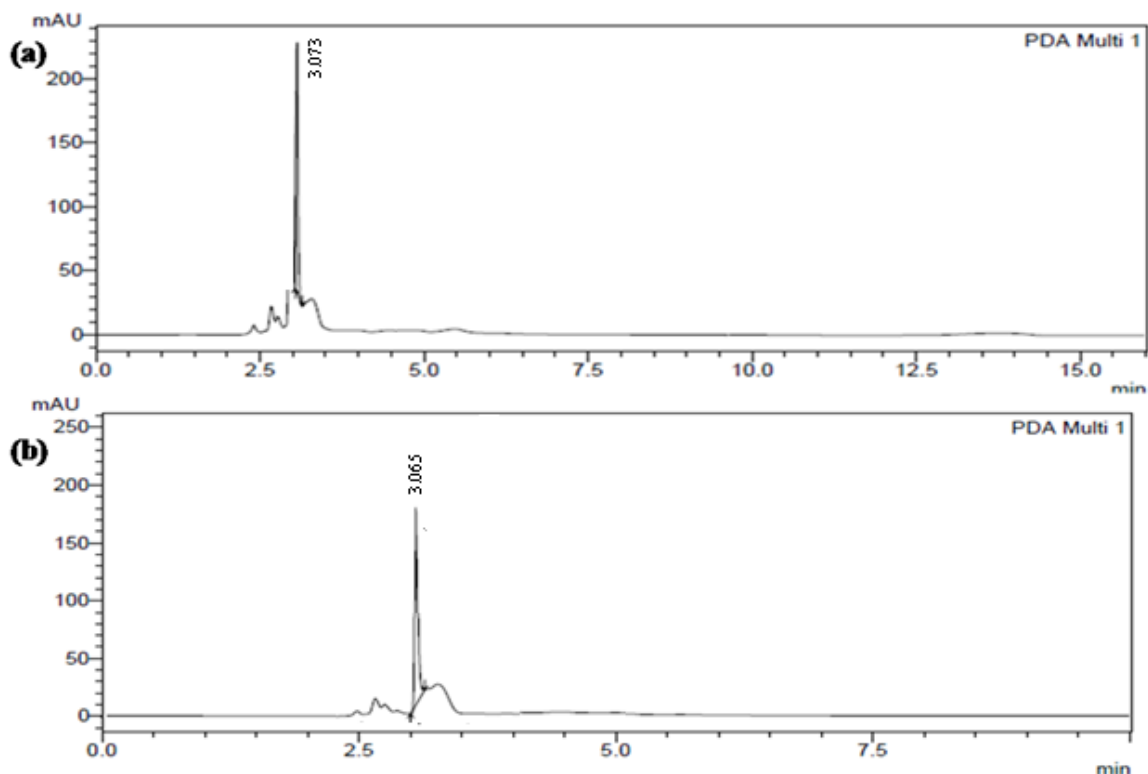


Figure 9: (a) HPLC chromatogram of standard Lupeol (b) HPLC chromatogram of Lupeol isolated from the leaves of *Calotropis gigantea*

CONCLUSION:

- Methanol extract of the leaves of the medicinal plant, *Calotropis gigantea*, has been used for this present investigation.
- From the spectral (IR and NMR) and chromatographic (HPTLC and HPLC) interpretation, the isolated compound of the methanol extract was identified as Lupeol, a pharmacologically active triterpenoid.
- Struvite crystals grown by single diffusion gel growth technique and their growth inhibitory effect was examined using the methanolic extract.
- From the observation of various results in this study, it is evident that the *Calotropis gigantea* can reduce the formation of ammonium magnesium phosphate hexahydrate (Struvite crystals, a major component of urinary stone) by suppressing the nucleation rate.
- In addition, the methanolic extract disintegrate the existing crystals as follows: Struvite crystals weight ogradually reduced from 2.49 g to 0.09 g by gradual addition of the methanolic extract from 0.15% to 1.00%.
- In conclusion, the results reveal that the methanolic extract of *Calotropis gigantea* leaves may be considered as a better agent (Lupeol component) to control and reduce the growth of Struvite crystals.

ACKNOWLEDGEMENT

Reena Loice acknowledges Dr. Beatrice valdaris of Bishop Herber College, Trichy for constant support for this research and K. Manjula, Managing Director, Bio Techno Solutions Training and Research Institute, Tiruchirapalli for providing the infrastructure to carry out the proposed research work.

CONFLICTS OF INTERESTS

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

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