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Modulatory effect of combination of green tea with various herbs on angiogenesis and on urolithasis

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Abstract
Green tea is one of the highly consumed drinks throughout the world after water and has gained popularity due to its various health benefits. The objective of this study was to investigate the regulatory effects of green tea on angiogenesis and on urolithasis. The methods used in this study are in-vitro nucleation assay, in-vitro Crystallization assay and Chorion allantois membrane (CAM) assay. To enhance the health benefits of green tea it was combined with herbs like *Ocimum gratissimum*, *Cymbopogon citratus* and *Cymbopogon flexuous*. The results demonstrated that The *Ocimum gratissimum* (tulsi), Cymbopogan cutratus and C. *citratus* (lemon grass) showed antilithiatic activity but they have less antilithiatic potential as compared to the standard drug cystone. The statistical data showed that there is no significant difference between C. *flexuosus* and O. *gratissium* plus green tea as compared to cystone. The antiangiogenic potential of these herbal teas could not be evaluated because of contamination in the chick embryos due to which they could not survive and the antiangiogenic potential of herbal teas could not came into conclusion.

Keywords: Angiogenesis, urolithasis, Green tea, *ocimum gratissimum*, *cymbopogon* citrates, cymbopogan *flexuosus*, C. *citratus*

Introduction
Urolithiasis can be defined as the clinical condition where the formation of crystal aggregates in the urinary tract results in kidney stones (Alelign & Petros 2018) [3]. The presence of a kidney stone in the human body can be externalized in very different ways, it can produce no symptoms but it can be associated with several pains caused for one or some of the following symptoms: gross or microscopic hematuria, obstruction of one or both kidneys, and urinary infections. Urolithiasis is considered one of the most common medical problems in the present society as it affects a high percentage of people but this illness exists since antique societies. In the last 10 years, the diagnosis of Urolithiasis was increased approximately by a 50%. The Urolithiasis is the formation of kidney or urinary tract stone that is most common and widespread disease known to man. Kidney stones are a common entity, affecting approximately 5% of women and 12% of men in general population. Prevalence of kidney stone disease is increasing and likewise the complications associated with stone disease. The most life threatening being sepsis and renal failure Urolithiasis is derived from a Greek word ouron (urine) and lithos (stone). Most common form of kidney stone in India is of calcium oxalate (80%) followed by calcium phosphate (15- 25%), then mixed stone (10-15%) and uric acid stone (2- 10%). In calcium oxalate stone, most common form is calcium oxalate monohydrate (COM) as compared to calcium oxalate dehydrate (COD) (Agarwal and Varma, 2014). Kidney stones are hard, solid particles that form in the urinary tract. In many cases, the stones are very small and can pass out of the body without any problems. However, if a stone (even a small one) blocks the flow of urine, excruciating pain may result, and prompt medical 3 treatment may be needed. Diet, excess body weight, some medical conditions, and certain supplements and medications are among the many causes of kidney stones. Kidney stones can affect any part of your urinary tract — from your kidneys to your bladder (Dyer R & Nordin BE 1967) [5]. Often, stones form when the urine becomes concentrated, allowing minerals to crystallize and stick together. Passing kidney stones can be quite painful, but the stones usually cause no permanent damage if they're recognized in a timely fashion. Depending on your situation, you may need nothing more than to take pain medication and drink lots of water to pass a kidney stone (Dhole *et al*., 2018) [4].

In other instances-for example, if stones become lodged in the urinary tract, are associated with a urinary infection or cause complications-recurrent stone formation is a common part of the medical care of patients with stone disease. A kidney stone usually will not cause symptoms until it moves around within your kidney or passes into your ureters — the tubes connecting the kidneys and the bladder. If it becomes lodged in the ureters, it may block the flow of urine and cause the kidney to swell and the ureter to spasm, which can be very painful. At that point, you may experience these signs and symptoms: Severe, sharp pain in the side and back, below the ribs. Kidney stones formation can take place due to several reasons such as cystinuria and hyperoxaluria. These two disorders are inherited metabolic disorder responsible for kidney stone formation. In cystinuria, cysteine, an amino acid which dos not dissolve in urine get accumulated leading to the formation of kidney stones, whereas in hyperoxaluria, body overproduce oxalae which settles down and forms stones. Besides these two inheritable metabolic disorder, kidney stone formation also occurs due to the over absorption of calcium from diet. The elevated calcium causes the formation of the crystals of calcium oxalate or phosphate elsewhere in the urinary tract (Johnson *et al*., 1979; Hoppe *et al*., 2003) [9, 8]. In-vivo studies showed that green tea can prevent kidney stone formation. The strategies for prevention of kidney stone may include fluid intake or diuretic agents which help in diluting urine. Recent studies suggested that kidney stones involve physiochemical proceedings such as super-saturations, nucleation, aggregation and accumulation within renal tubules. The health benefits of green tea for a wide variety of ailments, including different types of cancer, heart disease, and liver disease, were reported. Many of these beneficial effects of green tea are related to its catechin, particularly (-)-epigallocatechin- 3-gallate, content. There is evidence from *in vitro* and animal studies on the underlying mechanisms of green tea catechins and their biological actions (Rashidi *et al*.,2017) [13] There are also human studies on using green tea catechins to treat metabolic syndrome, such as obesity, type II diabetes, and cardiovascular risk factors (Higdon JV & Frei B). Long-term consumption of tea catechins could be beneficial against high-fat diet-induced obesity and type II diabetes and could reduce the risk of coronary disease. The aim of this study is to investigate the modulatory effect of combination of green tea on angiogenesis and on urolithasis.

Materials and Methods

Chemicals and reagents

Sodium chloride (NaCl), Sodium Phosphate (Na2HPO4), Sodium citrate (Na3C6H5O7), Magnesium sulphate (MgSO4), Sodium sulphate (Na2SO4), Potassium chloride (KCl), Sodium oxalate (Na2C2O4), Ammonium chloride (NH4Cl), Ammonium hydroxide (NH_4OH) and Calcium chloride dihydrate (CaCl2, $2H_2O$), heparin.

Preparation of tea

The plant parts used to prepare various infusions such as leaves of *Ocimum gratissimum* (tulsi), *Cymbopogon citratus* (lemon grass), *Cymbopogon flexuosus* (lemon grass) and moefeng green tea. Green tea was purchased in the form of 50g packet from the Bud white teas, Pvt. Ltd, New Delhi, and herbs were collected from the herbal garden, Rabindranath Tagore University, Bhopal, M.P. infusions were made by soaking 1% (combinations) and 2% (individual) infusions in 10 ml of hot distilled water (DW) maintained at at 90-95^{0}C for 5 minutes. After five min the aqueous infusions were filtered with whatman filter paper and stored for further use. Furthermore, cystone was used as a standard drug which was prepared by taking a fine powder of tablets (1 g) in 10 ml of distilled water (Farooq and Sehgal., 2019) [6].

In-vitro nucleation assay

Calcium oxalate were prepared to check the inhibition capacity of different tea combinations in- vitro (Kumar *et al*., 2013). In this method, calcium chloride solution (5mM) and sodium oxalate solution (7.5mM) were prepared in Tris-NaCl solution (0.5M and 0.15M respectively). The solution was kept at 37oC and pH was maintained at 6.5. After this, 950 µl of calcium chloride was taken in several test tubes in which tea (alone as well as combined) with different concentrations were added (25, 50, 100 34 and 200µg). This was performed along with the standard drug (cystone) at same concentrations. Addition of 950µl of sodium oxalate initiated nucleation which was measured spectrophotometrically at 620 nm after 2 hours of incubation period using the formula given below.

% Inhibition of nucleation = Absorbance of control – Absorbance of Test/ Absorbance of control X 100

In-vitro crystallization assay

In this assay, artificial urine (AU) was required which was prepared by the method given by the Beghalia *et al*., (2008). Sodium oxalate (Na2C2O4) solution 2mM and calcium chloride (CaCl2) solution 10mM were prepared separately. Also 0.9 g of sodium chloride (NaCl) was added in each solution to provide ionic indoor environment. Then the equal volume of both the solutions were mixed and stirred at 37oC and 6.5 pH was maintained to get the final urine concentration. Also, 0.01M sodium oxalate solution was prepared separately. Artificial urine (AU) 1ml was taken in different test tubes to which tea with different concentrations was added which is followed by the addition of 0.5ml of sodium oxalate solution that induced crystal formation. Cystone was used as standard drug with same concentration as that of teas.

% Inhibition of crystallization= Absorbance of control –Absorbance of Test/ Absorbance of control X100

Chorion allantois membrane (cam) assay

The CAM assay is the most common and simple test used to assess the inhibition or induction of angiogenesis. The complete procedure of CAM model is described below. Fertilized chicken eggs were marked with the pencil and were incubated at 37oC for four days (Jadhav *et al*., 2011) [15]. Eggs were rotated two times each day during the period of incubation. On 4th day, albumin was removed from the eggs with the syringe and windows were created (In-vivo) or yolk was spread into petry dishes to test the extracts (ex-vivo). Two eggs were used as a control, that was either devoid of herbal teas or given heparin (100µg/ml) which induced angiogenesis, while others were given different herbal infusions such as green tea in two eggs and green tea plus tulsi (O. *gratissium*) in another two eggs (100µg/ml) to check the inhibition of angiogenesis. This was done in

Laminar air flow. The extracts (herbal teas) and heparin was loaded on the small discs that was kept inside the egg shells on the desired sites and afterwards that eggs were incubated further for 3 days. After 24 hrs, the result was evaluated.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) by using Microsoft excel 2010 by used one way analysis of variance (ANOVA) followed by Tukey's test using SPSS software (version 18) for statistical analysis. At $p \leq 0.05$, values were considered statistically significant.

Results and Discussion

Nucleation assay in individual and binary infusions

Nucleation is the early stage of urolithasis where components of stone (salts) unite together and form clusters. The in-vitro nucleation process starts from the addition of sodium oxalate solution into calcium chloride solution which was estimated at 620nm. The different herbal infusions alone and in combination was evaluated for ant nucleation potential. A well-known polyherbal drug, cystone commonly used in treatment of kidney stone was taken as a standard drug. It was found that cystone inhibit the nucleation process strongly (43.69-78.97%) followed by C. *flexuosus* (FLEX) (35.43-76.62%), C. *citratus* (CTR) (27.89-75.83%) and O. *gratissium* (TUL) (27.20-73.59%) at 25-200µg/ml. The IC50 stands for inhibitory concentration and is defined as the concentration of a drug that is required for 50% inhibition *in vitro*. Lower the IC50 of the herbal extract higher is the antilithiatic potential. The IC50 was found in the following order: O. *gratissium* (43.09µg/ml), C. *citratus* (43.63µg/ml), C *flexuosus* (38.38µg/ml) and cystone (33.08µg/ml) that showed that cystone was more effective in inhibition of nucleation followed by C. *flexuosus*, O. *gratissium* and C. *citratus* **Fig. 1**

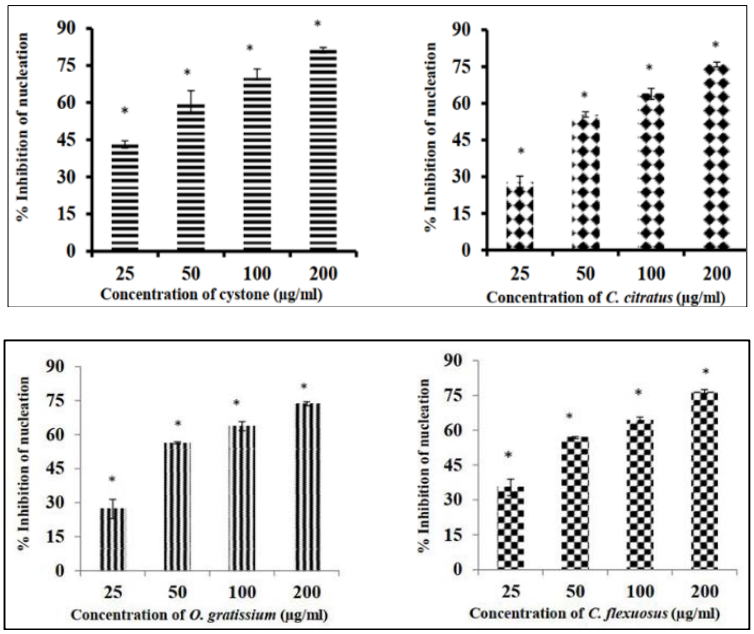

Fig 1: Inhibition of nucleation by (a) standard drug (cystone) (b) *C. citratus (c) O. gratissimum and C. flexuosus*. Values are expressed as mean ± standard deviation (n=3), p≤ 0.05 was considered statisticallysignificant. * represents significant difference between herbal teas as compared to cystone.

In case of green tea combination, result showed that cystone inhibit nucleation with the range of percent 43.69-78.97%followed by O. *gratissium* (tulsi) + green tea with the range of percent 41.20-79.13%, C. *citratus* (lemon grass) + green tea with 36.21- 72.79% and C *flexuosus* (lemongrass) + green tea with 34.49-70.54% inhibition at 25- 200µg/ml concentration. The IC50 of herbal teas with green tea combinations found was C. *citratus* + green tea (C+G) > C. *flexuosus* + green tea (F+G) > O. *gratissium* + green tea (T+G) > cystone was (45.21, 44.56, 39.89, 33.08µg/ml respectively) Hence, the % inhibition by green tea combinations were in following order: cystone > O. *gratissium* + green tea > C *flexuosus* + green tea + green tea > C. *citratus* + green tea. The % inhibition of nucleation of herbal teas with green tea combination and cystone are given Fig. 2.

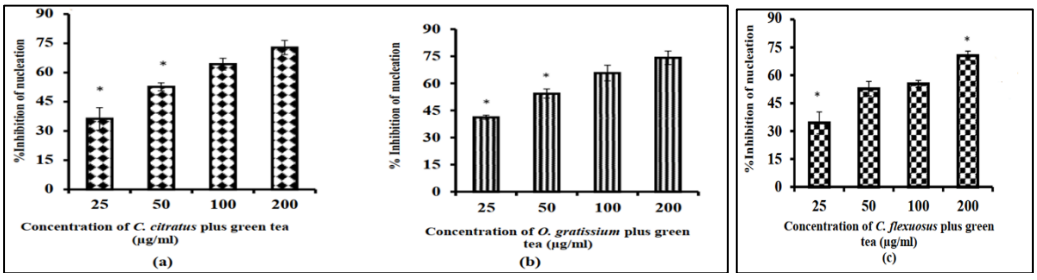

Fig 2: % Inhibition of nucleation by (a)*C. citratus*,(b) *O. gratissium* and (c) *C. flexuosus* with green tea combination. Values are expressed as mean ± standard deviation (n=3), p≤ 0.05 was considered statistically significant. * represents significant difference between herbal teas as compared to cystone**.**

Crystallization assay

Inhibition of crystallization with the different plant extracts was performed taking cystone as a standard drug at 25, 50, 100 and 200µg/ml. The % inhibition of crystallization with extracts and cystone was as follow: cystone (45.72-77.14%), C. *citratus* (36.41-73.88%), O. *gratissium* (43.95-73.23) and C. *flexuosus* (41.01- 69.27%). Also the IC50 of different extracts and cystone was of following order: Cystone (36.49µg/ml) followed by O. *gratissium* (37.93µg/ml), C. *flexuosus* 40.15µg/ml) and then C. *citratus* (44.42). Hence the % inhibition of different extract and cystone is of following order: cystone < O. *gratissium* < C. *flexuosus* < C. *citratus*. 45 The % inhibition of crystallization by herbal teas and standard drug (cystone) are given below Fig. 3

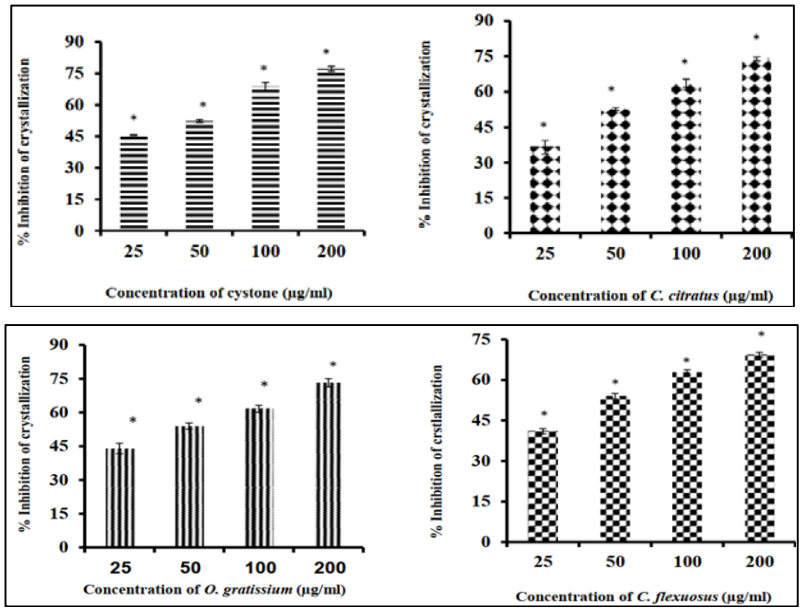

Fig 3: % Inhibition of crystallization by (a) Cystone (CYST) (b) *C. citratus* (CTR), *O. gratissium* (TUL) and *C. flexous* (FLX). Values are expressed as mean ± standard deviation (n=3), p≤ 0.05 was considered statistically significant. * represents significant difference between herbal teas as compared to cystone**.**

Crystallization assay

The crystallization assay evaluation with green tea combination showed that the IC50 of the cystone and herbal teas with green tea combination was in the following order: Cystone (27.35µg/ml) < C. *flexuosus* + green tea (F+G=27.96µg/ml) < C. *citratus* + green tea (C+G=30.21µg/ml) < O. *gratissium* + green tea (T+G=31.80µg/ml) with the % inhibition of crystallization: 48.83-78.59%, 47.11-73.69%, 47.78-72.69% and 46.24-71.62% respectively at 25, 50, 100 and 200µg/ml concentration each. This shows that the herbal teas are more effective in inhibition of crystallization with green tea combination as compared to alone Fig. 4

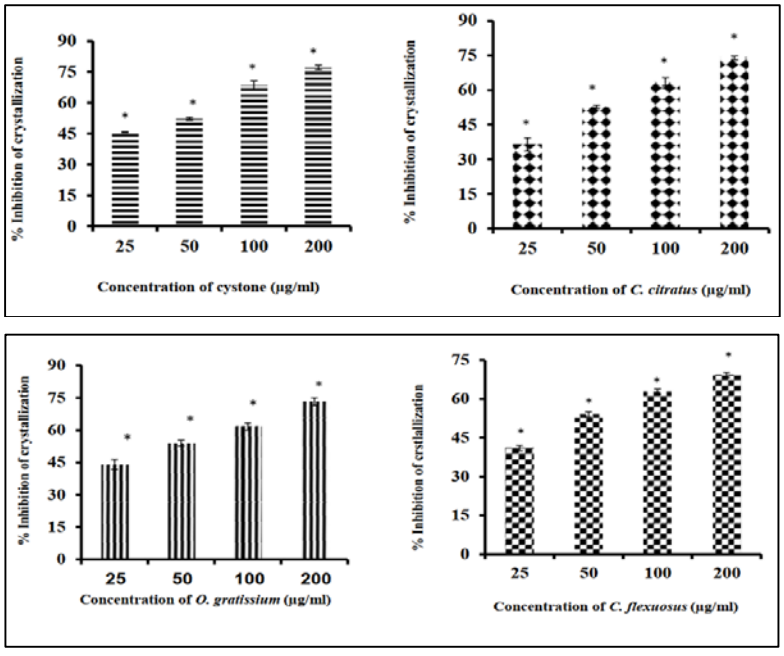

Fig 4:% Inhibition of crystallization by (a) Cystone (CYST), (b) *C. citratus* (CTR) *O. gratissium* (TUL) and *C. flexous* (FLX). Values are expressed as mean ± standard deviation (n=3), p≤ 0.05 was considered statistically significant. * represents significant difference between herbal teas as compared to cystone.

Anti-angiogenesis by using cam model

The angiogenesis was induced by heparin (100µg/ml) ex-ovo in petri dishes to check the inhibition of angiogenesis by herbal teas (O. *gratissium* and green tea plus O. *gratissium*) at 100µg/ml. These specific herbal tea were considered because of their sufficient antioxidant activity. The embryos loaded with heparin was considered as control and rest are considered as test sample. Following groups were made shown in Fig. 5

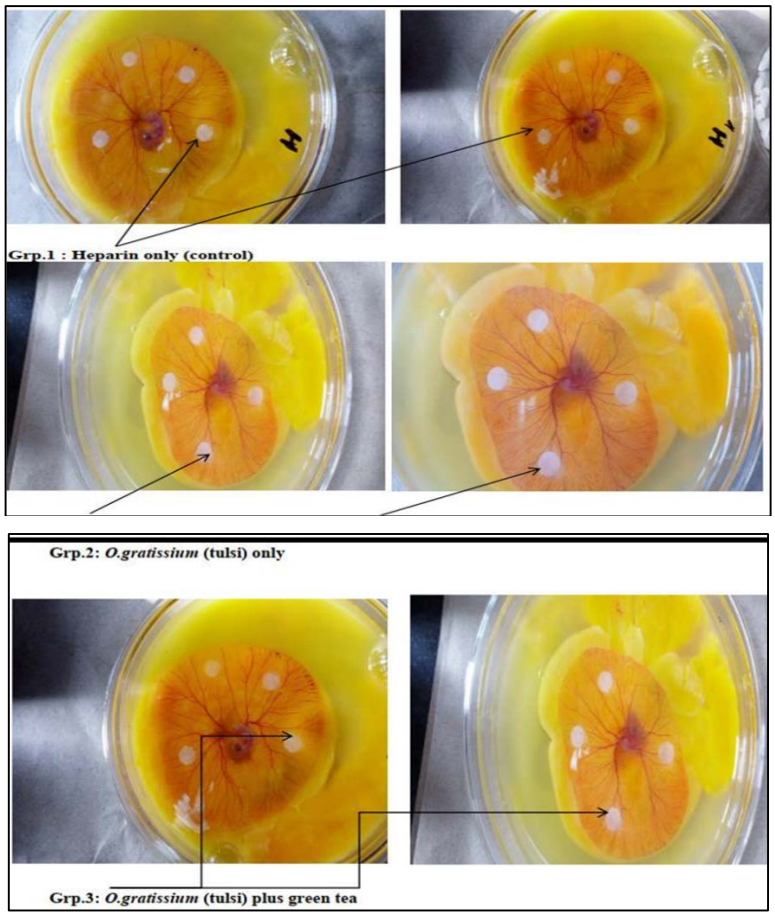

Fig 5: Photographic representation of angiogenesis in window method and in petridishes (in- vivo and ex-vivo).

Due to the contamination of these sample, the result could not came into the conclusion. I tried it more than 6 times but due to unfavourable conditions in laboratory, these samples could not survive ex-vivo and hence no antiangiogenic activity of herbal teas has been evaluated. Also in window methods, the embryos survived only for one day due to which the antiangiogenic potential of herbs could not be evaluated. In the comparative study of antilithiatic activity of Rivea hypocraterformis (ethanolic extract), Cyndon dactylone (methanolic extract) and Balanite aegypticae (water extract) in-vitro (Patel *et al*., 2010), the ethanolic extract of Rivea hypocraterformis showed equal potential of antilitiasis as cystone. Less activity was shown by methanolic extract of Cyndon dactyl one and water extract of Balanite aegypticae. It was reported that the Hibiscus rosa sinensis also have same antilithiatic properties as that of cystone at 150, 200 and 250 mg/kg body weight in albino rats (Vijayanarayana *et al*., 2007) [14]. *Ocimum sanctum* (tulsi) is a Holy plant and it has been used traditionally as a medicinal plants. Administration of O. *sanctum* (1g/kg body weight) in male albino rats for 60 days showed the decreasing level of serum calcium and inorganic phosphate level. It has also been reported to reduce number of tumor nodule formation in Lewis lung carcinoma (LLC)- injected mice. *Ocimum gratissimum* (tulsi) is also used as medicinal plant traditionally to treat various diseases including kidney stones. It 57 inhibited the 66.08% of nucleation of crystals at 1000mg/kg and 62.07% of crystallization at 100mg/kg. It is effective in inhibition of proliferation, migration, COX-2 protein induction, anchorage-independent growth and morphogenesis of breast cancer cells (Agarwal and varma, 2014) [1] Cymbopogan *citratus* have a significant antiangiogenic activity against the colorectal and breast carcinoma cell lines with the inhibition of 99 ± 0.8% at 100µg/ml.

Conclusion and Future

The *Ocimum gratissimum* (tulsi), Cymbopogan cutratus and C. *citratus* (lemon grass) showed antilithiatic activity but they have less antilithiatic potential as compared to the standard drug cystone. The statistical data showed that there is no significant difference between C. *flexuosus* and O. *gratissium* plus green tea as compared to cystone. The comparision of different herbal extract revealed that there is no significant difference among CYST, FLEX and (T+G) which have higher ant nucleation activity from CTR, TUL, C+G and F+G. It has been investigated that cystone, herbal teas (FLEX, CTR and TUL) and herbal tea combinations (C+G, T+G and F+G) showed similar inhibition of crystallization except CTR which demonstrated lesser anticrystallization activity.The antiangiogenic potential of these herbal teas could not be evaluated because of

contamination in the chick embryos due to which they could not survive and the antiangiogenic potential of herbal teas could not came into conclusion.
Further investigations are required to make a definitive conclusion.

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