ORIGINAL RESEARCH PAPER
Volume - 10 | Issue - 09 | September - 2021 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

"*IN VITRO* STUDY OF HOMOEOPATHIC MEDICINE SARSAPARILLA Q, 6C, 12C, 30C, 200C, 1M AS AN INHIBITOR OF CALCIUM OXALATE AND CALCIUM PHOSPHATE CRYSTALLISATION."

Homeopathic

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ABSTRACT

Background: Around 12% of world population has been infected by renal stone disease which has multiphase etiological factors with high recurrence rate. Thus, in order to reduce the recurrence rate, use of homoeopathic intervention can be the most effective alternative option and Sarsaparilla is one of the frequently prescribed medicines by homoeopath in renal stones.

Aim: To find out the inhibitory action of homoeopathic medicine Sarsaparilla Q, 6C, 12C, 30C, 200C, 1M on calcium oxalate and calcium phosphate crystallization by *in vitro* study.

Methodology: *In vitro* crystallisation of calcium oxalate and calcium phosphate was carried out to evaluate effectiveness of Homoeopathic Medicine Sarsaparilla as an inhibitor. Calcium Oxalate crystallisation assay was experimented by using JASCO-UV/VISIBLE-630 Spectrophotometer. Slope of Nucleation and Aggregation phases is calculated using linear regression analysis and percentage of inhibition was calculated. Calcium phosphate crystallisation inhibition by Sarsaparilla was calculated by measuring concentration of calcium and phosphate ions, using Digital Photo colorimeter by Trinder and Gomeri method respectively. Antioxidant activity of Sarsaparilla Mother Tincture and potencies was measured by DPPH free radical scavenging assay by spectrophotometer.

Result: Maximum inhibition of calcium oxalate crystallisation has been noticed in Sarsaparilla Mother Tincture and 1M potency. While Sarsaparilla 30C showing maximum inhibition of Calcium Phosphate crystallisation. Sarsaparilla mother tincture was able to decolorize during DPPH assay in which Inhibition % was decreased as potency increased (Q>30C>1M).

Conclusion: The present in vitro study has shown potential role of Homoeopathic medicine Sarsaparilla as an inhibitor of calcium oxalate and calcium phosphate crystallisation.

KEYWORDS

Renal calculi; Sarsaparilla; Homoeopathy; Crystallisation; *in vitro*

INTRODUCTION-

According to NIDDK (National Institute of Diabetes and Digestive and Kidney disease, part of National Institute of Health, U.S.A), kidney stone is a solid pebble like piece of material that can form in one or both of kidneys when level of certain minerals in urine is high. Renal calculus is stone like body composed of nucleus surrounded by urinary salts bound together by colloid matrix of organic materials [1]. Sometimes calcium salts deposited outside of the renal parenchyma is termed as nephrocalcinosis which may be associated with urolithiasis. [2] It is documented in history that bladder stone was the commonest in old age but from last many years kidney stones are more prevalent.[3][4] The highest risks have been reported in some Asian countries. The frequency of recurrence rate is high (21% to 53%) in adults. [5] Epidemiological studies reported that calcium oxalate accounts for 60% to 90% of stones in children, followed by calcium phosphate (10–20%), struvite (1–14%), uric acid (5–10%), cystine (1– 5%), and mixed or miscellaneous (4%)[4]. Different risk factors and etiology affect prevalence of the disease like gender, genetic factors, climate, temperature, diet, etc.[5] 12% of the Indian population is suffering from urinary stone diseases and 50% of them may loss their kidney function. [6] Due to urolithiasis, number of other urological disorders is increasing which has been associated with an increased risk of end stage of renal failure. [6] Some known etiological factors like hyper excretion of relatively insoluble urinary constituents, physical changes in urine i.e. pH of urine, altered urinary crystalloids and colloids, vitamin A deficiency, hyperthyroidism, etc. cause damage to renal tissues and lead to the formation of renal stone[1]. The mechanism of renal stone formation is a complex biological process that involves physicochemical changes. However, it is noted that stone formation is usually dependent on the level of imbalance between urinary inhibitors and promoters of crystallization. [6] Crystallization of calcium based stones occurs in supersaturated urine if it is with low concentration of inhibitors. [6] The events of stone formation include crystal nucleation, crystal growth, crystal aggregation, and retention of crystals within the kidneys. [6] Cluster is formed by free atoms, ions and molecules such as calcium oxalate, octocalcium phosphate, hydroxyapatite and brushites and which made urine supersaturated. [7] It has been suggested that oxalate increases the availability of free radicals by inhibiting enzymes responsible for their degradation.[7] Reactive oxygen species is thought to be one of the factors involved in renal cell injury. Thus, reduction of renal oxidative stress could be an effective treatment option. [7] Stones causing obstruction and gives severe renal colic should be removed surgically such as ESWL and PCNL. [2] Limitations of surgeries and with increase rate of urolithiasis recognize the use of alternative therapies. There is none of the conventional therapies are 100% effective which are available for management but also it may have complications. Medicinal plants had been used as an alternative therapy since Vedic era for both prevention and treatment of urinary stone diseases.[8] Homoeopathy as a system of therapeutics has shown remarkable results in many diseases like renal stone. Homoeopathy is the most holistic mode of treatment for kidney stones and it cures the underlying causes of the disease through its law of similar. The earlier studies have shown antilithiac properties of Homoeopathic dilutions in various potencies. Sarsaparilla is one of those medicines mentioned in Homoeopathic Materia Medica and Homoeopathic Repertory used for treatment of kidney stones. Sarsaparilla (Smilax), a member of the liliaceae family, which has action on kidney as a diuretic. [9]Multiple trials were carried out to evaluate the action of Sarsaparilla in dilutions which has shown effective result and can be used as preventive medicine.[10] Though, the different action of homoeopathic medicine on crystallisation and its mechanism still remained unknown. Therefore, the following *in vitro study* is undertaken to find out the inhibitory action of sarsaparilla on calcium oxalate and calcium phosphate crystallisation.

MATERIALS AND METHODS-

Preparation and application of potentised Sarsaparilla:

Homoeopathic Medicine Sarsaparilla Mother Tincture and potencies like 6C, 12C, 30C, 200C and 1M are procured from standard Homoeopathic Pharmacy as per norms of Homoeopathic

Pharmacopoeia and SBL.

Calcium Oxalate crystallization assay [11,12]

Calcium Oxalate crystallization assay was carried out using *Hess et al* method with few modifications. Stock solutions were prepared of $CaCl_2$ (8.5 mM) and $K_2C_2O_4$ (1.0 mM), containing 200 mM NaCl and 10 mM sodium acetate, pH was adjusted to 5.7. Solutions were filtered and warmed up to 37ºC in a circulating water bath. Now, 1.0 ml of the $CaCl_2$ solution is transferred into a light path quartz cuvette. 0.1ml of Sarsaparilla containing Q, 6C, 12C, 30C, 200C, 1M and ethanol as a control were added to each solution of $CaCl_2$ respectively. 1.0 ml of the $K_2C_2O_4$ solution was added to light path quartz cuvette and was placed in a spectrophotometer. After addition of the oxalate-containing solution, automated time course of the optical density (OD) at 620 nm was measured by JASCO-UV/VISIBLE-630 Spectrophotometer for 20 minutes each over 60 seconds. The maximum increase value of OD is calculated as slope of nucleation by linear regression analysis. After few seconds values of OD start decreasing is measured as a slope of aggregation by linear regression analysis.

Process was performed in triplicates. Percentage inhibition by different dilutions is calculated as:

Percentage Inhibition = (Sc-St/Sc) x 100

Where, Sc is slope of nucleation/ Aggregation for control
St is slope of nucleation/ Aggregation for test

Calcium Phosphate Crystallization assay [13,14,15,16]

To determine the extent of Calcium Phosphate precipitation, homogenous Crystallization system was prepared to study the *in vitro* crystallisation (Chaudhary et al 2010). To perform crystallization assay 5.0 ml system was prepared containing 2.5ml of Tris Buffer (pH 7.4), 0.5ml of 50 mM $CaCl_2$ and 0.5ml of 50 mM KH_2PO_4 with 0.1ml Sarsaparilla Q, 6C, 12C, 30C, 200C 1M and ethanol as control. After incubating this assay system at 37°C, precipitates obtained were centrifuged at 4500 rpm for 15minutes and precipitates were dissolved in 5ml of 0.1 N HCl. The calcium ions (Ca^{2+}) and phosphate ions (HPO_4^{2-}) concentration in the precipitate represented the extent of precipitation (crystallization) of these ions and the sample containing inhibitory biomolecules minimized the extent of their precipitation. The calcium and Phosphate ions were estimated by Trinder, 1960 and Gomeri, 1941 method respectively. Percentage inhibition of calcium phosphate crystal in the presence of Sarsaparilla Q, 6C, 12C, 30C, 200C, 1M and control system as follow (process were performed in triplicates) :

% inhibition = ((C—T)/C) × 100,

Where, T is the concentration of Ca^{2+} or $HPO4^{-2}$ in presence of Sarsaparilla
C is the concentration of Ca^{2+} or $HPO4^{-2}$ in presence of control

DPPH free radical scavenging assay [17]

The scavenging activity of homoeopathic dilution of Sarsaparilla for the potency having maximum inhibition was checked with DPPH radical. A spectrophotometric assay of antioxidant determination was used with some modifications. A volume of 0.1 mM solution of DPPH was prepared by adding 25 mg of DPPH in 100 ml of methanol. Solutions of 90 µl of DPPH solution and different 10 µl Mother Tincture and dilutions of Sarsaparilla were prepared and kept for 30 minutes in cool place. These solutions were placed in light path quartz cuvette of Spectrophotometer one by one. The DPPH solution was used as experimental control. The process was done in triplicate. The absorbance was measured at 517 nm in spectrophotometer. The absorbance was taken as follows:

Scavenging activity % = ([Ac − At)/Ac] x100

Where, Ac is absorbance value of control
At is absorbance value of test

RESULTS-

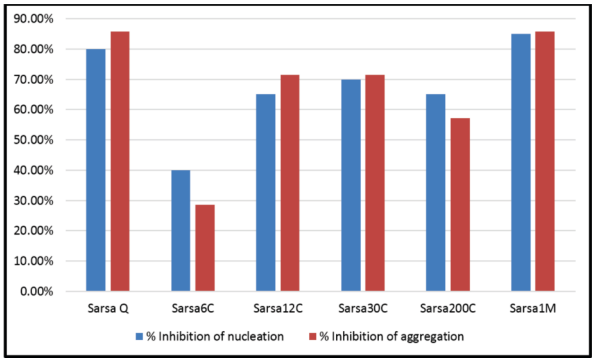
Calcium Oxalate crystallisation

In present study, we have assessed the mechanism of calcium oxalate crystallisation and its inhibition by Homoeopathic Medicine Sarsaparilla Q, 6C, 12C, 30C, 200C and 1M. In the table 1 the effect of different potencies and Mother Tincture of Sarsaparilla on nucleation and aggregation phase of calcium oxalate crystal formation. Maximum inhibition during nucleation phase is observed in Mother Tincture and 1M potency of Sarsaparilla (0.1ml) which is 80% and 85% respectively. Where, maximum inhibition in aggregation phase was observed in MT and 1M which is 85.71%. The minimum inhibition is shown in 6C potency.

Table 1 - Effect of Sarsaparilla on Nucleation and aggregation of calcium oxalate crystallisation

Sr. No	Sample	Absorbance Mean ± SD	% Inhibition	
			Nucleation	Aggregation
1	control	0.1524±0.004	-	-
2	Sarsaparilla MT	0.1256±0.001	80%	85.71%
3	Sarsaparilla 6C	0.1191±0.003	40%	28.57%
4	Sarsaparilla 12C	0.1289±0.002	65%	71.42
5	Sarsaparilla 30C	0.1319±0.001	70%	71.42%
6	Sarsaparilla 200C	0.0754±0.001	65%	65%
7	Sarsaparilla 1M	0.0501±0.0005	85%	85.71%

(Absorbance values are Mean ± Standard deviation of triplicates)



Graph 1- Inhibition of Calcium Oxalate Crystallisation

Calcium phosphate crystallisation assay:

During mineralization phase concentration of calcium ions and phosphate ions with different potencies and Mother Tincture of Sarsaparilla was measured by colorimeter with respect to Ethanol (control).

Table 2 showed concentration of calcium ion and inhibition percentage by sarsaparilla with respect to ethanol. Maximum inhibition has been seen in 30C potency which is 31.14% and minimum inhibition has seen in mother tincture and 1M potency.

Table 3 showed concentration of phosphate ion and inhibition percentage by sarsaparilla with respect to ethanol. Maximum inhibition has been seen in 30C potency which is 37.87% and minimum inhibition in mother tincture and 1M potency.

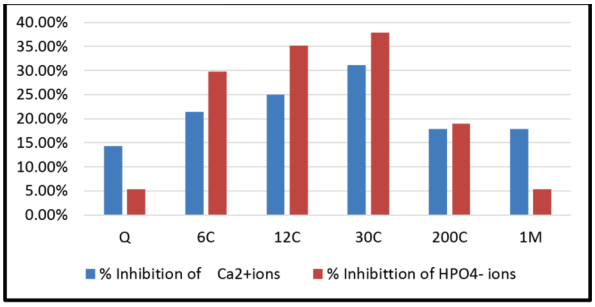
Table 2- concentration of calcium ion and inhibition percentage (Absorbance is mean values of triplicates)

	Sample	Absorbance Mean ± SD	Conc.	%inhibition
1	Blank	0	-	-
2	working standard	0.32	-	-
3	Ethanol	0.28±0.01	17.5	-
4	Sarsaparilla Q	0.24±0.005	15	14.28%
5	Sarsaparilla 6c	0.21±0.005	13.75	21.42%
6	Sarsaparilla 12C	0.21±0.000	13.12	25.02%
7	Sarsaparilla 30C	0.2±0.005	12.05	31.14%
8	Sarsaparilla 200C	0.23±0.005	14.37	17.88%
9	Sarsaparilla 1M	0.23±0.000	14.37	17.88%

Table 3- concentration of phosphate ion and inhibition percentage (Absorbance is mean values of triplicates)

	Sample	Absorbance Mean±SD	Conc.	%inhibition
1	Blank	0	-	-
2	working standard	0.31	-	-
3	Ethanol	0.37±0.005	23.87	-
4	Sarsaparilla Q	0.35±0.000	22.58	5.40%
5	Sarsaparilla 6c	0.26±0.005	16.77	29.70%

6	Sarsaparilla 12C	0.24±0.01	15.48	35.14%
7	Sarsaparilla 30C	0.23±0.000	14.83	37.87%
8	Sarsaparilla 200C	0.3±0.005	19.35	18.90%
9	Sarsaparilla 1M	0.35±0.000	22.58	5.40%

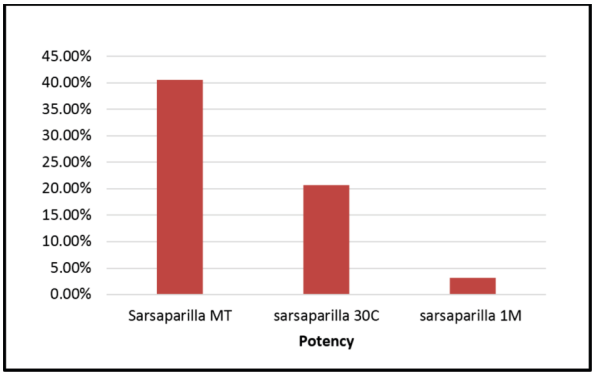


Graph 2 - Inhibition of Calcium Phosphate Q, 30C, 1M

Antioxidant activity (DPPH free radical scavenging activity):
The antioxidant activity was checked by DPPH scavenging activity. The Potency showing highest inhibition was considered. Sarsaparilla Mother Tincture and 1M has shown maximum inhibition in calcium oxalate crystallisation where as in calcium phosphate crystallisation 30C potency has shown the maximum inhibition. Triplicates were performed. Sarsaparilla Mother Tincture containing solution showed major changes. It was highly decolorized from purple to yellow and showed 40.58% DPPH inhibition.

Table 4- Antioxidant activity of Sarsaparilla

	Sample	Abs1	Abs2	Abs3	Mean±SD	% scavenging Activity
1	Blank	0	0	0	0	-
2	Experimental control	0.7754	0.7757	0.7787	0.7746±0.001	-
3	Sarsaparilla MT	0.4585	0.4616	0.464	0.4614±0.002	40.58%
4	Sarsaparilla 30C	0.613	0.6162	0.6193	0.6161±0.003	20.66%
5	Sarsaparilla 1M	0.7521	0.7516	0.7512	0.7516±0.004	3.20%



Graph 3 - % scavenging activity of Sarsaparilla Q, 30C, 1M

DISCUSSION-

A great German physician Samuel Hahnemann has discovered the new therapeutic system of medicine in 17th century which was known as "Homoeopathy" later on. This new approach was to cure the disease by administering drug of similar in kind which became cardinal principle of homoeopathy: "similia similibus curentur" or "likes cure like". Gradually this new system got its pick after publishing his work "The theory of chronic disease" and "Organon of medicine" and he became the father of homoeopathy. In his initial days of practice, he used to prescribe crude drug substances which had started showing some side effects. On his realization he started diluting crude drug substances and made new method of preparing homoeopathic drugs known as "High dilution". Nowadays, Homoeopaths are using high dilutions to treat diseases and getting tremendous results. Over many years non believer of homoeopathy has questioned its ability to cure the disease with high dilutions even they have called its effect as "placebo". However, despite of every challenges homoeopathy has managed to preserve its originality and survived through all thick and thins because of efforts were made by its believers. Recently, new scientific approaches have been made to prove its medicinal effects such as RCT (Randomized control trials*), in vitro, in vivo* studies. Though, understanding of molecular mechanism of action of homoeopathic medicines still remains enigmatic.

Mankinds as well as animals are suffering from renal stone disease for ages. The history of urolithiasis dates back to the dawn of civilization [4] Sarsaparilla is used as diuretic in traditional medicine. In Homoeopathy, Sarsaparilla is commonly prescribed in Mother Tincture form. But, recently in many studies it has been noticed that the dilutions of Sarsaparilla has been showing great result [11, 13, 17-20]. Though, Antiurolithiac action in high dilution is not evaluated yet. Therefore with the objective to assess the effective alternative treatment of kidney stone by Homoeopathic Medicine Sarsaparilla, which is procured from GMP (Good Manufacturing Practice) approved Companies of India and to know its mechanism of action in different dilutions, the *in vitro* study has been undertaken on Calcium Oxalate and Calcium Phosphate crystallisation.

The methods used in assays were already mentioned above. In calcium oxalate crystallisation assay, the absorbance values after adding different dilutions in system solution for 20 minutes were taken using spectrophotometer. In nucleation phase, absorbance values were increasing slowly which shows the inhibition of stone formation. Maximum inhibition was seen in medium and higher dilutions like 30C, 200C and 1M. In calcium phosphate crystallisation assay, firstly concentration of calcium and phosphate ions in prepared stock solution were calculated using photo colorimeter and then percentage inhibition were measured, in which only 30C potency has shown the effective inhibition. Present finding suggest that Sarsaparilla can be effective if it is given in proper dose. Further investigations on doses for Sarsaparilla in renal stone disease needs to be done. Considering oxidative stress to be possible etiological factor [21] for renal stone formation, Antioxidant activity was performed for Mother Tincture and potencies showing maximum inhibition of crystallisation by DPPH free radical scavenging assay. When, DPPH solution were added in samples and kept for 30 minutes some changes were observed. Solution containing mother tincture showed highly decolorized from purple to yellow color. In this assay Inhibition % was decreased as potency increased (Q>30C>1M). This shows need of further investigation in order to find out the relation between Antioxidant and Homoeopathic dilutions.

CONCLUSION-

Present experimental investigation has shown efficacy of Homoeopathic Medicine Sarsaparilla Q, 6c, 12c, 30c, 200c as an inhibitor of calcium oxalate and calcium phosphate crystallisation. After reviewing the result (table 1, 2 and 3) Sarsaparilla is more effective in calcium oxalate stone disease than the calcium phosphate. In this study Antioxidant activity assay was also performed which showed higher Antioxidant potential of Sarsaparilla Mother Tincture than the dilutions selected here. We may link Antioxidant activity with antiurolithiac action. But further investigation is needed.

ACKNOWLEDGEMENT-

Author would like to acknowledge the Bharati Vidyapeeth (Deemed to be University), Homeopathic Medical College& Hospital, Pune and Bharati Vidyapeeth (Deemed to be University), Poona college of Pharmacy, Pune, to provide opportunity and required resources to conduct the present study. I am also grateful to my colleague Dr. Sharada Gowda and Dr. Ankita Rabediya for helping me to complete my work. Lastly, I would like to thank my family members for being with me in every step and showing their immense support.

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