IN VITRO STUDY ON NIGELLA SATIVA AS INHIBITOR OF CALCIUM OXALATE CRYSTALLIZATION

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ABSTRACT

Objective: *This study aims to test the efficacy of Nigella Sativa plant as an inhibitor of calcium oxalate crystallization.* ***Material & Methods:*** *Nigella Sativa alcohol extract was made by maceration of 100 grams Nigella seeds which were dried and powdered for 72 hours and then filtered, so as to produce 100 percent Nigella alcohol extract. Then make dilutions of 10 and 50 percent. The precipitation of calcium oxalate at 37 0C and pH 5.7 was studied by measurement of turbidity at 620 nm with and without inhibitor which is Nigella Sativa.* ***Results:*** *Tmax, the slope of nucleation (SN) and slope of aggregation (SA) was measured with control and with inhibitor. With increasing concentration of inhibitor, Tmax progressively increased, whereas SN and SA decreased. The nucleation percentage inhibition ratio was 86.0% and inhibition of crystal aggregation took place by 66.6% with 100% extract. Crystallization was inhibited (growth and aggregation) in a concentration dependent manner.* ***Conclusion:*** *Nigella Sativa is strong inhibitor of calcium oxalate crystallization. However, comprehensive work is needed to establish the results.*

Keywords: *Nigella Sativa, inhibitor, calcium oxalate.*

ABSTRAK

Tujuan: *Penelitian ini bertujuan untuk menguji efikasi tanaman Nigella Sativa sebagai penghambat kristalisasi kalsium oksalat.* ***Bahan & Cara:*** *Ekstrak alkohol Nigella Sativa dibuat dengan cara maserasi 100 gram biji Nigella yang dikeringkan dan dijadikan bubuk selama 72 jam dan selanjutnya disaring, sehingga dihasilkan sebanyak 100 persen ekstrak alkohol Nigella. Kemudian dibuat pengenceran sebanyak 10 dan 50 persen. Pengendapan kalsium oksalat pada 370C dan pH 5.7 dipelajari dengan pengukuran kekeruhan pada 620 nm dengan dan tanpa inhibitor Nigella Sativa.* ***Hasil:*** *Tmax, kemiringan nukleasi (SN) dan kemiringan agregasi (SA) diukur dengan kontrol dan inhibitor. Meningkatnya konsentrasi inhibitor, tmax semakin meningkat, sedangkan SN dan SA menurun. Rasio penghambatan persentase nukleasi adalah 86.0% dan penghambatan agregasi kristal terjadi sebesar 66.6% dengan ekstrak 100%. Kristalisasi dihambat (pertumbuhan dan agregasi) dan bergantung pada konsentrasi.* ***Simpulan:*** *Nigella Sativa adalah penghambat kuat kristalisasi kalsium oksalat. Namun, diperlukan pekerjaan yang komprehensif untuk menetapkan hasil.*

Kata Kunci: *Nigella Sativa, inhibitor, kalsium oksalat.*

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INTRODUCTION

Urolithiasis is a recurrent renal disease. It affects 4-8% in the UK, 15% in US, 20% in Gulf countries and 11% population in India. Stone formation tends to recur at a very high rate; without preventive measures after a first stone. After 3 years this is about 40%, by 10 years up to 75% and by 25 years virtually every patient has formed at least one more stone.[1] Pakistan is located in the stone belt region. It stretches from Egypt, through the Middle East, Indian subcontinent, Thailand and Philippines.

There is a high prevalence of renal tract stones in this country.[2] Although the incidence and prevalence of stone disease are not known in Pakistan due to lack of centralized epidemiological data, it roughly constitutes 40-50% of the urological workload in major hospitals.[3] In Pakistan, the predominant stone is Calcium Oxalate found in 60-65% patients with stone disease.[4] It is true for countries like India, Bangladesh and Saudi Arabia.

Although much is understood about the physical chemistry involved in nephrolithiasis, the inciting factor and sequence of events that lead to the

formation of a kidney stone remains elusive. Significant research has been performed using animal models and cell culture experiments; however, it is unclear whether these outcomes are applicable to human kidney stone formers. Recent efforts to understand the pathogenesis of nephrolithiasis in humans have led to a delineation of the human surgical anatomy, histopathology, and metabolic factors in a variety of kidney stone formers.[5]

Medicinal plants contain chemical compounds like glycosaminoglycans (GAGS) which are inhibitors of calcium oxalate crystallization. Macromolecules of higher molecular weight are obtained from the plant extracts. They exert their effect similar to natural urinary inhibitors and slow down crystal nucleation, growth and aggregation.[6] Grapefruit and lemon juice[7] reported to increase urinary citrate excretion thus exerting their crystallization inhibition effect in vivo as well in vitro probably through the formation of calcium citrate, which is more soluble than CaOx. B, ligulata, A, indica and H. Hirsute also exerts their antilithogenic effect through crystal growth inhibition.

Investigations on the effect of Ammi visnaga seeds on kidney stones revealed that the antilithiatic effect is mainly because of highly potent diuretic activity and amelioration of uraemia and hyperbilirubinemia by seeds of Ammi visnaga.[8] In an experiment, the ethanolic extract of Asparagus racemosus Wild has been found to inhibit renal stone formation. Renal stone formation was induced in adult albino wistar rats by oral administration of 0.75% ethylene glycolated water for 28 days. The extract markedly reduced the concentration of ions responsible for formation of stone in the urine and increased the concentration of magnesium. Magnesium is considered as inhibitor of crystallization.[9]

Besides inhibitory action, herbal medicines have several phytoconstituent and exert their beneficial effects on urolithiasis by multiple mechanisms like: helps in spontaneous passage of calculi by increasing urine volume, pH and anti-calcifying activity (Diuretic activity), balance the Inhibitor and promoter of the crystallization in urine and affects the crystal nucleation, aggregation and growth (Crystallization inhibition activity) relieves the binding mucin of calculi (lithotriptic activity), improve renal function , regulate oxalate metabolism, regulates the crystalloid colloid imbalance and improve renal function, thus prevents recurrence of urinary calculi, improve renal tissue antioxidant status and cell membrane integrity and prevent recurrence (Antioxidant activity), ACE and Phospholipase A2 Inhibition ,exerts significant anti-infective action against the major causative organisms (Antimicrobial activity), reveals marked improvement in symptoms of urinary calculi like pain, burning micturition and haematuria.[10]

Nigella Sativa is also called Kalonji or black seed. In Indian and Middle Eastern cookings, it is used as a seasoning. The seeds of Nigella are dried, roasted and added to the dishes.[11] Nigella Sativa exhibits strong antimicrobial, hepatoprotective, antidiabetic activity, anti-inflammatory activity, antifertility activity, antioxytocic activity, cytotoxic activity, anthelmintic activity, analgesic activity etc. It has been reported that renal calculi is broken down to pieces and eliminated from the body if treated with Nigella mixed with honey.[12] The formation of calcium oxalate renal calculi can be prevented significantly if an extract of Nigella Sativa seeds in the dose of 250 mg/kg is given.[13]

Hadjzadeh et al.[14] investigated the effects of thymoquinone as a major component of NS seeds on EG-induced CaOx kidney calculi in rats and discovered that Number and size of crystals of calcium oxalate decreased in great number when a dose of 5 mg/kg of thymoquinone was given to rats. These crystals were present in different parts of renal tubules. A higher dose of thymoquinone had also preventive and therapeutic effects on CaOx kidney calculi, although the therapeutic effect of thymoquinone with a dose of 5 mg/kg was more potent.

OBJECTIVE

This study aims to test the efficacy of Nigella Sativa plant as an inhibitor of calcium oxalate crystallization.

MATERIAL & METHODS

Dried seeds of the Nigella were obtained from the local herbalist and were authenticated by the botanist. The voucher specimen is available with the herbalist. The black seeds were powdered and 100 g of powder were macerated in a 50% aqueous–ethanol solvent and kept at 40°C in an

incubator for 72 hours. It was mixed occasionally during the incubation time. The mixture was filtered through a filter paper and the solvent was removed this is 100% alcoholic extract of Nigella Sativa.[5] Dilutions of 10 % and 50 % were made.

The precipitation of calcium oxalate at 37^0C and pH 5.7 was studied by the measurement of turbidity at 620 nm. A spectrophotometer UV/VIS (Helios, Unicam, UK) was employed to measure the turbidity of the formation of calcium oxalate. Pure Chemicals, Analar grade, including calcium chloride anhydrous, sodium oxalate, sodium chloride, and sodium acetate were used for this study.[6] The work was carried out at Bahauddin Zakariya University, Multan, Pakistan.

The calcium oxalate monohydrate crystallization was obtained by the mixture of calcium chloride (8 mmol/l) and sodium oxalate (1 mmol/l), containing 200 mmol/l sodium chloride and 10 mmol/l sodium acetate. These concentrations were chosen because they are close to physiological urinary concentrations. pH value was adjusted to 5.7 (pH meter, Bante, china). A pH of 5.7 was selected because it is a pH value frequently observed in the first morning urines of calcium stone formers.[7]

A volume of 1.5 ml of the calcium chloride solution was transferred into a 10-mm lightpath cuvette in a cell holder and blank reading was taken. 1.5 ml of sodium oxalate was added to the previous volume and the measurement was immediately taken.

A mixture of 1 ml of calcium chloride (8 mM) and 1ml of inhibiting solution was mixed in the cell. Blank reading was taken and then volume of 1 ml of sodium oxalate (1mM) was added to the previous volume and measurement was immediately started for 30 minutes. The maximum increase of optic density with time, termed S N, mainly reflects the maximum rate of formation of new particles and thus crystal nucleation. Maximum time, namely tmax, corresponds to the time between the addition of oxalate and the moment at which maximum absorbance (equilibrium) is measurable.

After equilibrium has been reached, crystals can neither nucleate nor grow. A progressive decrease of optic density with time was observed. The maximum decrease of optic density at 620 nm with time, therefore, reflects the rate of decrease in particle number due to crystal aggregation. As optic density decreases crystal aggregation increases. The rate of aggregation, SA, is derived from the maximum decrease in optic density.[8]

The experiment was repeated with different concentrations of the alcoholic extract of Nigella Sativa. Three replicates of all the experiments were taken. Concentrated solutions of the modifier were pipetted into the calcium containing solution before oxalate was added. The percentage inhibition ratio was calculated as (1-[SNm/SNc]) × 100 for rate of nucleation and (1 [SAm/SAc]) × 100 for the rate of aggregation where 'm' stands for modifier and 'c' for control.

RESULTS

Alcoholic extract of Nigella Sativa on CaOx crystallization kinetics has been evaluated in this study. Table 1 summarizes maximum values of t max, SN and SA in control experiments and with inhibitor (10, 50, and 100%). With increasing concentration of inhibitor, t max progressively increased, whereas SN and SA decreased.

In the presence of Nigella seeds, tmax increased and the values of SN and SA reduced. When the concentration of Alcoholic extract of Nigella Sativa seeds was increased (100%), the nucleation percentage inhibition ratio was 86.0% and inhibition of crystal aggregation took place by 66.6 %. Crystallization was inhibited (growth and aggregation) in a concentration dependent manner as shown in the Table 1.

Table 1. Values of turbidity slopes, percent inhibition, and tmax without and with inhibitor at various concentrations.

Concentration of inhibitor (%)	Turbidity slopes		Percent inhibition (growth)	Percent inhibition (aggregation)	t_{max} (Min)
	S_N	S_A			
0	0.380	0.120			8
10	0.077	.057	79.7	52.5	12
50	0.058	0.044	84.7	63.33	14
100	0.053	0.040	86.0	66.66	18

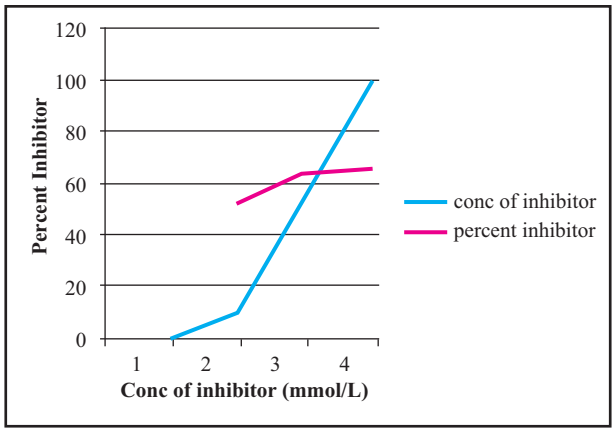

Figure 1. Plot showing effect of Nigella Sativa on crystallization (growth and agregation).

DISCUSSION

The highest increase in optic density at 620 nm with time has direct relation with crystal growth.[8] Certainly optic density is an accurate measure of particle concentration per unit volume.[9] After attaining the maximum value and becoming stable, absorbance decreases gradually. It shows a decrease in particle number because of crystal aggregation.[10]

Hennequin et al.[8] described an induction time in nucleation inhibition studies. The induction time is the beginning of nucleation of particles, which are detectable by turbidimetry. Delay in the ti shows inhibition of nucleation. However, it is difficult to detect as CaOx nuclei are too small to be detected by turbidimetry (1000A).

Hence, maximum time, tmax, is used instead of ti. Maximum time tmax is time between the addition of oxalate and the time at which maximum absorbance is measurable. Delay in the beginning of nucleation affects the time at which maximum absorbance is reached (tmax). Nigella Sativa prolonged tmax in concentration dependant manner.

In the control experiment tmax was 8 minutes and in the experiment with inhibitor, it increased to 12, 14, and 18 respectively with increasing the concentration from 10, 50, and 100% respectively. Prolongation of tmax in the presence of Nigella shows that Nigella Sativa has inhibited the nucleation. Values of SN and SA also decreased in the presence of the alcoholic extract of Nigella Sativa as compared to control.

It appears that if crystal growth and aggregation interfere somehow, the renal stone recurrence may be prevented.[11] The inhibiting capacity of Nigella Sativa shows the presence of some elements capable of inhibiting calcium oxalate crystallization. Yadav et al.[12] describe that plants possess many constituents.

Beneficial effects of these constituents are exerted on urolithiasis by numerous mechanisms. They increase the urine volume, pH, and anti-calcifying activity and thus help spontaneous passage of calculi; involved in crystallization inhibition activity by bringing the inhibitors and promoters of crystallization in equilibrium in urine and thus steps in the crystallization process i.e., nucleation, aggregation, and growth are inhibited; play role in lithotriptic activity by relieving the binding of mucin of calculi, make the renal function better, regulate crystalloid and colloid imbalance consequently preventing recurrence of urinary calculi, etc.

The mechanism of inhibition of Nigella Sativa is yet to be discovered. Probable mechanisms through which plants inhibit crystallization are described. Medicinal plants contain chemical compounds like Glycosaminoglycans (GAGS) which themselves possess an inhibitory effect in the crystallization of calcium oxalate. Macromolecules of higher molecular weight of plant extract exert their action similar to natural urinary inhibitors and inhibit crystal nucleation, growth, and aggregation.[13]

Farook et al.[14] studied the inhibition of mineralization of urinary stone forming minerals by medicinal plants and reported that fruit juice sequester the insoluble calcium salts and hydroxyl acids present in the fruit juice and chelate single or mixed ligands. It is expected that hydroxyl acids form metal ion complexes with calcium. The hydroxyl acids present in the urine may decrease the amount of ionized calcium available for calcium oxalate precipitate.

Seaweed sargassum graminifolium (Turn.) (SGP) has many negatively charged –OSO3, –COO–, and –OH groups, and these anions strongly coordinate with Ca2+ ions.[15] The numerous negatively charged groups of SGP were able to chelate Ca2+ ions, as a result, concentration of Ca2+ ions on the surface of the SGP molecules is rapidly increased. This resulted in a higher energy interface on the surface of SGP molecules.

The adsorption of Ca2+ ions would result in a simultaneous decrease in free Ca2+ ions and an increase in the energy state of Ca2+ ions. Both the high-energy interface and high energy state Ca2+ ions would then promote the formation of

thermodynamically metastable COD.16 Nigella might have acted in the same manner. The results that Nigella seeds have antiurolithiatic activity are corroborated by Hadjzadeh et al.[17] who showed that ethanolic extract of seeds of Nigella Sativa had a protective result on renal stone formation in kidneys of rats.

The extract also decreased the number of calcium oxalate renal calculi formed in the treated groups by 57%. Hadjzadeh et al.[18] also discovered that Thymoquinone in Nigella Sativa has a preventive effect on CaOx calculi formation in the kidneys of rats. Similarly, thymoquinone (a phytochemical compound found in the plant Nigella Sativa) has a disruptive effect on CaOx crystals formed by EG (ethylene glycol), demonstrating a therapeutic effect on kidney calculi in rats.

Besides, it has been proved that by mixing calcium chloride and potassium oxalate, calcium oxalate crystals were produced due to high supersaturation. When inhibitor Nigella Sativa was added, the free ionic activity of calcium was reduced and therefore supersaturation was reduced. This inhibited the process of crystallization. Another mechanism of inhibition by the inhibitor under study may be that it is bound to calcium oxalate crystal surfaces, blocked growing sites, and modified attractive or repulsive forces between crystals so that growth and especially crystal aggregation was inhibited. Nigella Sativa might have acted on both the growth and aggregation stages of the crystallization as is evident from the result.

CONCLUSION

Nigella Sativa is strong inhibitor of calcium oxalate crystallization. However, comprehensive work is needed to establish the results.

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