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Research Article

RADICAL SCAVENGING ACTIVITY OF *NEOLAMARCKIA CADAMBA* (ROXB) BOSSER IN NEPHROLITHIASIS RELATED OXIDATIVE STRESS

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ABSTRACT

Objective: To investigate the role of *Neolamarckia cadamba* (Roxb) Bosser in reducing nephrolithiatic oxidative stress in wistar rats. Methods: Nephrolithiasis induced in rats by supplementing ethylene glycol (EG) in drinking water for 28 days. Animals were divided into six groups, each containing six viz. vehicle control, nephrolithiatic control and cystone (750mg/kg, p.o) served as antilithiatic control. Methanol fruit extract of *N. cadamba* fruit extract (MFNC) in doses of 200, 400 mg/kg, p.o. were administered to rats after receiving EG in both curative and preventive regimes. DPPH activity was assessed to evaluate the antioxidant activity of the extract. Oxidative stress reducing property of MFNC in nephrolithiasis was estimated for both enzymatic and non enzymatic markers of oxidative stress like Thiobarbaturic acid relative substances (TBARs), conjugated diene (CD), Lipid hydroperoxides (HP), reduced glutathione (GSH), SGOT and SGPT. Estimation of Antioxidant enzymes, Superoxide dismutase (SOD), Catalase (CAT) and Glutathione Reductase (GST) were evaluated. Results: Lithiatic groups elevated the level of oxidative stress markers like TBARs, CD, HP, SGOT, SGPT whereas the MFNC extract lowered these values towards control. Antioxidant enzymes (SOD, CAT and GST) concentrations elevated by the MFNC administration by lowering the oxidative stress. Conclusion: the results suggest the renoprotection of MFNC by preventing the oxidative stress induced by nephrolithiasis.

Keywords: *Neolamarckia cadamba*, nephrolithiasis, oxidative stress, antioxidant enzymes.

INTRODUCTION

Patients with kidney diseases are found to have significant oxidative stress due to their declined renal functions[1]. Oxidative stress (OS) is produced by the imbalance between free radical production and antioxidant defence. Oxidative stress has both positive and negative effects on the body, however, they are effective in several host defence mechanisms like hydrolysis of pathogens and denaturation of antigens that invading into the body and is considered to be harmful when the OS damages the body cells and tissues by peroxidation of lipid membrane, oxidation of protein or DNA and disruption of cytokines, development of various diseases, cell injury and apoptosis [2, 3].

Oxidative stress includes classical oxidative stress, chlorinated oxidative stress, nitrosative oxidative stress and carbonyl stress. Antioxidants are the radical scavengers, able to inhibit the process of oxidation and protect the body against free radicals that cause many physiological conditions[4]. Antioxidant activity in human body is carried out by the action of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, glutathione reductase (GST) and non enzymatic antioxidants include hydrophilic and lipophilic antioxidants. Ascorbic acid, uric acid, bilirubin, albumin and flavonoids are the hydrophilic non enzymatic antioxidants. Alpha tocopherol, ubiquinol and carotenoids are classified as lipophilic non enzymatic antioxidants[5, 6]. Many antioxidant compounds exert its effect by interfering in the oxidative reaction by reacting with ROS and produce non reactive complex or weak radical type compounds. Mitochondria and mitochondrial cytochrome oxidase enzyme (cyto P450) are mainly account for the production of 90% of oxidant production. NADPH oxidase generates ROS in phagocytes and endothelial cells.

Urolithiasis, the common recurrent renal diseases formed by the mineral deposits in kidney and associated structures. Epidemiological studies proved that various factors like age, nutrition, climate, fluid intake etc. are the most critical ones contribute the development of renal stone. Oxidative stress involves in many kidney diseases. The organ which is highly vulnerable to ROS attack and damage is kidney. In the present scenario, oxidative stresses are the recent area of research in molecular mechanism of many renal diseases[7]. *In vivo* and *in vitro* experiments have revealed that raised oxidative stress is involving in nephrolithiasis. Antioxidants retard this oxidative stress induced by oxalate metabolism. Ethylene glycol (EG) administration to experimental animals induces oxalate lithiasis. Renal cells produce free radical mediated lipid peroxidation when exposed to oxalate stress[8]. The impaired antioxidant protection is responsible for the deposition and retaining of oxalate crystals in renal cells. Weak antioxidant defense and high oxidative load have been reported in stone formers. Antioxidant balance and free radical production are considered as major factors leading to the process of crystal aggregation and deposition in renal tissues[9]

Both hepatic and renal androgen receptors (AR) signalling plays a major role in the process of oxidative stress and nephrolithiasis[10]. Androgen receptors enhance oxalate biosynthesis and CaOx crystal formation by way of up regulating the glycolate oxidase and NADPH oxidase subunit at the transcriptional level. This up regulation increases the production of oxalate and oxidative stress, which results in renal tubular

injury. Mitochondria are the major site of ROS generation. They are the crucial site for ATP production by cytochrome enzymes. The level of ROS is 5-10 folds higher in mitochondria than other cytosolic and nuclear compartments[11]. The generated free radicals affect ATP synthesis by interfering in the energy transduction process and results in decreased membrane potential, damage the membrane and DNA and production of abnormal protein[12]. Other sites for ROS generation are endoplasmic reticulum, peroxisomes, lysosomes etc. About 1-3% of inspired molecular oxygen is converted to most powerful precursor of H_2O_2 i.e. O_2-. In the presence of reduced metal ion, $Fe2^+$, H_2 interact with variety of substrate and produce cellular damage. Breakdown of H_2O_2 leads to the generation of most reactive and damaging ROS, OH-. ROS is considered as damaging molecule even though they are produced in the respiratory process. The level of ROS is known to increase the level of natural antioxidants during the diseased condition and in ageing. The function of kidney is relies on aerobic metabolism for the production of ATP by oxidative phosphorylation[13]. The role of lipid peroxidation and oxidative function was studied on depleted antioxidant vitamin and enhanced oxidative stress in nephrolithiasis[14]. This paper provides a brief overview of clinical trial on wistar albino rats to check the therapeutic property of methanol fruit extract of *N. cadamba* (MFNC) against CaOx induced oxidative stress.

MATERIALS AND METHODS

Plant Material

The fruits of *N. cadamba* were collected from University of Kerala, Karyavattom ($8^0$37'36N, 76^o50'14E), Thiruvananthapuram, India. The plant material was authenticated by the Department of Botany, University of Kerala (voucher number: KUBH 5811) and powdered and subjected to methanol extraction.

Experimental Animal

Adult male albino rats of wistar strain (150- 250g) were used for the antiurolithiatic activity and the animals were acclimatized to standard laboratory conditions (12-hr light and 12-hr dark cycle) and provided with regular rat chow and drinking water at *ad libitum* (IAEC -KU-23/2011-12-ZOOL -GP (3)).

Study Protocol

Calcium oxalate nephrolithiasis in rats was induced by free access to drinking water containing 0.75% ethylene glycol (EG) for (28 days) and 2% ammonium chloride (AC) was supplemented for the first few days to enhance the stone formation in renal tissues. Animals were divided into nine groups containing four rats in each group. Group 1 served as normal control and received regular standard rat food and drinking water at *ad libitum*. EG and AC in drinking water was fed to group II – IX for the induction of renal calculi till the 28th day. Group II were treated with standard anti urolithiatic drug, cystone (750mg/kg body weight). Dose for the methanol fruit extract was selected as 200 and 400mg/kg body weight. Group III were considered as lithiatic control treated with EG/AC whereas group IV and V received the MFNC at a dose of 400mg/kg and 200mg/kg body weight respectively after treatment with EG/AC in drinking water. They were served as post treatment regimens (PR). Groups VI-IX was taken as co treatment regimens supplied with EG/AC for 28 days (CR). Among the CR, group VI and VIII received MFNC (400mg/kg and 200mg/kg body weight) from 15th day to 28th day. Group VII and IX received MFNC (200mg/kg and 400mg/kg body weight) from 1st day to 28th day. All drugs were given once daily by oral route using gastric tube.

Total *In Vitro* Antioxidant Capacity

DPPH Photometric Radical Scavenging Assay

DPPH (2, 2, diphenyl 1-picryl hydrazyl) assay was based on the measurement of loss of DPPH colour after reaction with test compounds[15]. The extracts were mixed with 150µL DPPH solution in methanol making up to a final volume of 3.0mL. The mixtures were shaken vigorously and stored in dark at room temperature. The decrease in absorbance of the reaction mixture was monitored at 517nm in UV-visible spectrophotometer. The percentage radical scavenging capacity (RSC) was calculated as follows.

$$\text{RSC (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\ \%$$

Assessment of Oxidative Stress

The lithiasis induced oxidative stress was assessed by measuring the antioxidant levels in kidneys of albino rats. After the experimental period, animals were sacrificed by cervical decapitation and blood was collected under anesthetic condition and both kidneys were taken out. One kidney was used for the antioxidant assay. Serum was separated and analysed for serum marker enzymes like serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT). Kidney was used for the estimation of antioxidant activity by estimating lipid peroxidative markers (TBARs, CD and hydroperoxides), non enzymatic antioxidant (GSH) and antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GST)).

Kidney Marker Enzymes

Determination of Serum SGOT and SGPT

In vitro determination of aspartate aminotransferase was carried out with method using 2, 4-Dinitrophenylhydrazine (DNPH) and the quantitative determination of alanine aminotransferase in serum was assessed with modified UV (IFCC) kinetic assay method[16].

Lipid Peroxidative Markers

Estimation Of Thiobarbaturic Acid Relative Substances (TBARS)

The concentration of TBARS in the tissue was estimated by the method of Nichans and Samuelson (1968). In this method Malonedialdehyde (MDA) and other thiobarbituric acid reactive substances (TBARS) react with thiobarbituric acid in an acidic condition to generate pink MDA-TBA complex which was read at 535nm.

Estimation Of Conjugated Diene (CD)

Conjugated diene was estimated according to the method of Recknagel and Ghoshal (1966). The tissue homogenate was mixed with 5ml chloroform/methanol mixture and centrifuged. Three ml of lower layer was taken out and dried at 45˚C and mixed with cyclohexane (1.5 ml) and absorbance was measured at 233nm.

Estimation of Lipid Hydroperoxides (HP)

Lipid hydroperoxides in the tissue was estimated by the method of Jiang *et al*. (1992). Oxidation of ferrous ion (Fe^{2+}) under acidic condition in the presence of xylenol orange leads to the formation of a chromatophore with an absorbance maximum at 560nm.

Reduced Glutathione (GSH)

Reduced glutathione was estimated by the method of Ellman (1959) based on the development of the yellow colour when 5, 5-dithiobis (2 nitrobenzoic acid) (DTNB) was added to the extract and was read in spectrophotometer at 412nm.

Antioxidant Enzymes

Estimation of Superoxide Dismutase (SOD)

The enzyme assay of SOD was based on the inhibition of NADH phenazine methosulphate tetrazolium formation. The reaction was inhibited by the addition of NADH. After incubation period, the reaction was stopped by the addition of the glacial acetic acid and the colour formed at the end the reaction was extracted in butanol layer and measured at 560 nm using butanol as blank and the system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme reaction which gave 50% inhibition of Nitro blue tetrazolium (NBT) reduction in 1 minute under the assay condition and expressed as specific activity in unit[21].

Estimation of Catalase (CAT)

The UV light absorption of hydrogen peroxide can be measured between 230 and 250nm. On decomposition of hydrogen peroxide by catalase, the absorption decrease with time. The enzyme activity can be arrived from this decrease. Calculated the decomposition of H_2O_2 using the extinction co-efficient 0.036 per µ mole/ml[22].

Estimation of Glutathione Reductase (GST)

The enzyme glutathione reductase catalyzed the reduction of oxidized glutathione to reduced glutathione. The decreased NADPH concentration which is proportional to the enzyme activity was measured. The reduction of the absorbance (oxidation) of NADPH was followed for 10 minutes at 37°C. The sample with FAD and free from FAD were measured simultaneously. The coefficient was determined by dividing the amount of reduction of absorbance of the assay with added FAD/10 minutes[23].

Statistical Analysis

The results were expressed as mean ± SEM and analyzed using one way ANOVA followed by Tukey test. Differences between data sets were considered significant at P≤0.05.

RESULTS

DPPH Photometric Radical Scavenging Assay

In the present study, antioxidant activity of the fruit extracts was investigated by DPPH scavenging assay and reducing power of the extract. The DPPH radical scavenging activity of the fruit extract is based on the ability of DPPH to decolourise solution in the presence of antioxidants. DPPH free radicals contain odd electron, which is highly reactive and responsible for the absorption at 517 nm and develop purple colour. Profound antioxidant activity of MFNC was noticed from DPPH assay (Fig: 1).

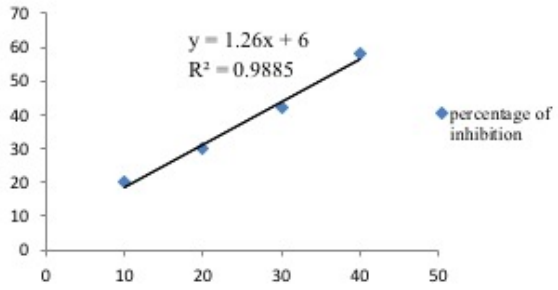

Fig 1. DPPH scavenging activity of MFNC

Lipid Peroxidative Markers Assay

The present study focuses on the oxidative stress induced by calcium oxalate (CaOx) by checking the activity of antioxidant enzymes in kidneys. All the MFNC administered rats have reduced the level of TBARs that has been increased in the lithiatic control rats. The co treatment regimes, group VII (14.36±0.68), group VIII (15.31±0.54) and group IX (19.14±0.52) showed significant reduction in TBARs value (Table 1). Significant ($p<0.01$) reduction in CD has been observed from all the extract administered groups. Group VI have showed a decreased CD value of 28.37±0.48 among the co treated rats. The observed HP value in the control rat was 7.59±0.27. MFNC administration in both preventive and curative regimes reduced the value of HP when compared with group III. A highly significant ($p<0.01$) decrease in HP was observed from group V (9.36±0.61) and group IX (9.32±0.54). Both treatment regimes has increased the GSH values. However, the increased GSH value was observed in group VIII (63.57±0.63) of the co treatment groups.

Kidney Marker Enzymes Assay

Both SGOT and SGPT are increased significantly ($p<0.01$) in group III, but supplementation with the fruit extract in groups IV to IX, were observed with lowered SGOT level (Table 2).

Estimation of Antioxidant Enzymes

The SOD level in group III was significantly ($P<0.01$) decreased when compared with normal control rats that received standard rat feed and water. However, significant increase in superoxide enzyme concentration HAS reported in antilithiatic control rats. The activity of SOD was significantly ($p<0.01$) elevated in the group IV, post treatment. The value is highly significant ($p<0.01$) when compared to Group I and III ($p<0.01$). Group VII and group IX of the co treatment regimens exhibited elevated SOD activity and the values are in the range of cystone administered groups of rats. The catalase activity has decreased significantly ($p<0.01$) in lithigenic rats (3.18±0.45) when compared to group I. All the methanol fruit extract treated group of animals increased the concentration of CAT activity. Group IX was reported with the catalase activity of 6.4±1.83 and is highly significant ($p<0.01$) when compared with lithiatic and antilithiatic control groups. The significant decrease in antioxidant level of GST was observed from the lithiatic control rats. Co treatment groups reported with elevated GST and the values suggest that the MFNC can reduce the oxidative stress generated by renal calculi in experimental rats (Table 3).

Table 1: Effect of MFNC on antioxidant activity in urolithiatic rats

Treatment groups	Thiobarbaturic acid reactive substances (TBARs)	Conjugated diene (CD)	Hydroperoxides (HP)	Reduced glutathione (GSH)
Group I	12.09±0.35	25.26±0.39	7.59±0.27	69.23±0.39
Group II	15.84±0.29 c**	79.80±0.95	15.76±0.50	61.48±0.53
Group III	61.48±0.72 c**	198.41±0.69	16.60±0.63a**	40.44±0.54a**
Group IV	29.46±0.54 c**	44.29±0.74b**c*	10.46±0.67 c**	62.22±0.63 c**
Group V	21.48±0.57 c**	45.31±0.68b**c**	9.36±0.61 c**	68.43±0.69 c**
Group VI	25.00±0.57 c**	28.37±0.48 b**c**	12.43±0.71 c**	62.15±0.55 c**
Group VII	14.36±0.68 c**	38.19±0.32 b**c**	11.34±0.69 c**	38.31±0.62
Group VIII	15.31±0.54 c**	55.51±0.39 b**c**	11.00±0.57 c**	63.57±0.63 c**
Group IX	19.14±0.52 c**	41.15±0.58 b**c**	9.32±0.54 c**	55.00±0.57 c**

Each value is the mean ± SEM for 4 animals, a-indicates significant difference with normal control groups, b- indicates significant difference with antilithiatic groups, c-indicates significant difference with lithiatic groups, d- indicates significant difference with CR, e- indicates significant difference with PR, *- P<0.05, **-P<0.01

Table 2: Effect of MFNC on SGOT and SGPT in urolithiatic rats

Treatment groups	SGOT	SGPT
Group I	54.72±15.84	129.93±8.05
Group II	113.94±5.91	152.10±3.06
Group III	346.83±24.24a*	504.86±43.57a**b**
Group IV	110.77±11.52c**e**	31.51±2.61b**c**e**
Group V	126.18±3.06c**	244.79±7.98c**
Group VI	121.55±20.32c**e**	69.48±5.13c**e**
Group VII	148.68±16.41 c**e**	55.66±13.53c**e**
Group VIII	160.80±3.39 c**e**	121.98±15.41c**e**
Group IX	101.42±7.93 c**e**	29.46±7.16c**e**

Each value is the mean ± SEM for 4 animals, a-indicates significant difference with normal control groups, b- indicates significant difference with antilithiatic groups, c-indicates significant difference with lithiatic groups, d- indicates significant difference with CR, e- indicates significant difference with PR, *- P<0.05, **-P<0.01.

Table 3: Effect of MFNC on antioxidant enzyme activity in urolithiatic rats

Treatment	SOD activity	CAT activity	GST activity	Kidney protein
Group I	1.80±0.10	7.18±0.36	0.04±0.001	5.003±0.39
Group II	2.57±0.14	4.3±0.10	0.03 ±0.003	5.373±0.037
Group III	0.56±0.16a*b*	3.18±0.45a**	0.01±0.003	4.733±0.29
Group IV	7.22±0.36 a**b**c**	5.10±1.22c**	0.01±0.001	0.322±0.014 a**c**
Group V	0.33±0.06 a*b**	4.32±0.46	0.02±0.003	0.338±0.009 a**c**
Group VI	0.48±0.12 a*b**	4.31±1.50	0.03±0.014	0.277±0.015 a**c**
Group VII	8.92±0.62 a**c**	5.07±1.34c**	0.03±0.009	0.257±0.019 a**c**
Group VIII	2.50±0.02 c**	4.55±1.72	0.02±0.001	0.275±0.026 a**c**
Group IX	5.57±0.73 a**b**c**	6.4±1.83b**c**	0.03±0.005	0.323±0.022 a**c**

Each value is the mean ± SEM for 4 animals, a-indicates significant difference with normal control groups, b- indicates significant difference with antilithiatic groups, c-indicates significant difference with lithiatic groups, d- indicates significant difference with CR, e- indicates significant difference with PR, *- P<0.05, **-P<0.01.

DISCUSSION

DPPH is stable nitrogen centered free radical and accept an electron or hydrogen radical to become stable diamagnetic molecule. When DPPH radical react with reducing agents, corresponding hydrazine will form and thus the solution loses colour. When the DPPH radical receive an electron or hydrogen radical from an antioxidant, the purple colour of the DPPH solution turned yellow[24]. Here, the study revealed the ability of MFNC to reduce free radicals. The earlier studies had showed that the antioxidant activity of herbal extract reduced the incidence of calcium oxalate calculi formation. Stone formation in EG treated animals results in hyperoxalauria and related renal oxalate retention and oxalate excretion[25]. Free radical elevation is the major risk factor in the course of EG induced urolithiasis. In the early stage, systemic circulation brings toxic substances to kidneys and these substances produce free radicals. In later stage, progressive accumulation of leukocytes and defective antioxidant enzymes keep kidneys under huge oxidative stress[26], which could be the reason for the significant ($p<0.01$) decrease in antioxidant enzymes in lithiatic control rats. In the present study, increased oxidative stress could be ascribed to significant ($p<0.01$) reduction in antioxidant enzymes in kidneys. OS is one of the causes of nephrolithiasis which decrease the protective antioxidant enzymes such as SOD, CAT and GST. The role of

increased oxidative stress in kidneys and protective role of ROS scavengers and antioxidants are reported from several studies.

The lipid peroxidation (LPO) produced by hydrogen peroxide, superoxide anion and hydroxyl radicals which result in altered structural integrity of cell membrane, cell organelle and loss of essential fatty acids by the formation of peroxide products[27]. The increased kidney oxalate concentration in EG treated groups of animals shown to inhibit the catalase activity[28]. It may be ascribed to the cause of significant ($p<0.01$) decrease in catalase activity in group III (lithiatic control rats). It is revealed that MFNC elevated the catalase activity by decreasing stone depositions in kidney. Earlier studies have reported that agents who decrease the oxidative stress are able to protect the cells from calcium oxalate induced toxic effect. Today natural antioxidants are used as complementary and alternative therapy against urolithiasis. GSH act as metabolic regulator for detoxification and metabolism. The decreased level of GSH in lithiatic control rats is attributed to the increased oxidative stress by generation of free radicals[29]. GSH is an important free radical scavenger and involved in the repair of free radical mediated tissue damage[30] and which could be the reason for the improved antioxidant status in MFNC administered rats.

Mitochondria are the major contributors of free radical mediated oxalate toxicity[31]and studies have reported that oxalate disrupts the electron transport chain in mitochondria and induce the leak of free radicals and reduced the antioxidant enzymes and resulted in mitochondrial dysfunction. In the present study, the antioxidant activity of the fruit extract has protected the kidneys of experimental rats by decreasing the lipid peroxidative markers and increased the antioxidant enzymes. Thus it is suggested that MFNC administration can be effective against free radical induced lipid peroxidation in oxalate induced experimental nephrolithiasis. In tissues, H_2O_2 detoxification involves two mechanisms. The enzyme catalase converts H_2O_2 to oxygen and water and GSH reduces the H_2O_2 by the enzyme GPx[32]. The enzyme catalase has inhibited by the increased oxalate concentration. The elevated levels of oxalate are considered as the reason for the decreased activities of catalase in lithiatic control rats (group III).

The significant ($p<0.01$) decrease in the concentration of antioxidant enzymes, SOD, CAT and GST in methanolic fruit extract of *N. cadamba* may be due to the property of the extract in lowering oxidative stress by decreasing the production of hydroxyl radical. Significant ($p<0.01$) restoration of antioxidant enzymes in group IV (400mg/kg b. wt.) of post treatment group is an indication of recovery of antioxidant enzymes due to the radical scavenging property of the MFNC. The high SOD in 400mg/kg received groups revealed that the fruit extract increased the superoxide radical scavenging and enhances cytoprotection against free radical attack. The kidney stone inhibitory property of fruit extract can be due to the increased antioxidant activity of MFNC in wistar rats. The leading cause of crystal depositions in renal tissue is due to the imbalance between oxidant and antioxidant level. The significant ($p<0.01$) increase in antioxidant enzyme activity in the fruit extract of methanol received groups suggest the effectiveness of methanol fruit extract for reducing oxidant production. The low concentration of renal glutathione level is positively correlated to LPO. This is the reason behind the decreased glutathione reductase in calcium oxalate induced rats[33]. Free radical scavengers and metabolic inhibition of LPO are necessary for protecting the renal cells against oxalate induced renal cell injury. The MFNC administration in experimental rats have decreased the concentration of intracellular calcium which might be due to the activity of the extract in increasing the cGMP activation and was reported to control the increase in intracellular calcium concentration[34]. The polyphenolic contents of fruits and vegetables are reported to have antioxidant activities and the free radical scavenging activity of the plant extract is attributed to the flavonoid phytochemicals in it [35]. The selected plant material was reported with the phenols and flavonoids[36] and can be ascribed to the oxidative stress reducing property of *N. cadamba.*

CONCLUSION

Thus the possible mechanism of antilithiatic activity of fruit extract may be attributed to the antioxidant and free radical scavenging property of the methanolic fruit extract. It is suggested that MFNC administration is more effective against oxalate stress induced by nephrolithiasis and protect the renal tissues against free radicals.

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