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Original Article

Protective Effect of Aqueous Extract of *Terminalia arjuna* against Dehydrating Induced Oxidative Stress and Uremia in Male Rat

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Abstract

The present study has been designed to find out the protective effect of aqueus extract of *Terminalia arjuna* against dehydration induced oxidative stress and uremia, protection by plant extract in male Wister strain albino rats, and therefore to find out the scientific basis of local use of *Terminalia arjuna* bark extract by village ayurved doctors to protect the progressive kidney disorder (renal failure) relating to dehydration and other related problems. Water withdrawing for 15 days in male Wister strain albino rats resulted in a significant elevation in the level of blood nitrogenous products (i.e. urea and creatinine). On the other hand, it increased the levels of free radicals, melondialdehyde (MDA) and conjugated dienes (CD) along with a significant diminution in the activities of superoxide dismutse (SOD) and catalase in blood. All these water markers were significantly prevented after administration of aqueous extract of *Terminalia arjuna* bark. These results suggest that dehydration induced oxidative stress and uremia in male rats may be protected by using the above mentioned medicinal plants extract. This herbal extract showed no toxic effect on blood and kidney, based on the measurements of glutamic oxaloacetic transaminase and glutamic pyruvic transaminase activities (data not shown).

Keywords: Uremia; Oxidative stress; *Terminalia arjuna*; Dehydration; Renal failure; Reactive oxygen species; Ayurvedic.

Introduction

At present kidney diseases are a major problem across the globe. As per global and regional overview, 1783000 patients are suffering from End Stage Renal Disease (ESRD), of which dialysis has been given to 1371000 patients and kidney has been transplanted on 412000 patients suffering from renal failure whom live under particularly pro-oxidative conditions causing uremia (1, 2). Uremia is a potentially fatal condition that demands immediate treatment. Treatment option for uremia includes kidney transplantation and dialysis, which are very expensive and not free from side effects. Many scientists have tried to find out different phytomedicines to manage uremia (3). Typically, urea builds up in the patient's blood as the result of inefficiently operating kidneys, which usually results from either

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acute or chronic kidney failure (4). Oxidative stress is defined as an imbalance between formation of reactive oxygen species (ROS) and anti-oxidative defense mechanisms. Considering the profound biological effects of ROS, in recent years numerous clinical and experimental studies have been focused on detection of signs of oxidative stress in renal patients (5). There is good evidence indicating that uremia in general is associated with enhanced oxidative stress (5). Loss or deficiency of antioxidant activity (e.g. vitamin E deficiency) could also contribute to enhanced oxidative stress in uremia. Interstitial inflammation and oxidative stress may participate jointly in the development and reduction of the number of nephron units, which thereby limits sodium filtration (6). It is known that low density lipoprotein (LDL) from uremic patients, presents an elevated susceptibility to oxidation (7). Uremic oxidative stress is characterized from a biochemical point of view as a state of reactive aldehyde and oxidized thiol group accumulation, together with depletion of reduced thiol groups, which are particularly important as part of antioxidant defense (7). As a consequence of diminished renal catabolism and function, uremic oxidant mediators accumulate, favoring vascular cell dysfunction and progression to kidney failure and many other diseases (7). *Terminalia arjuna* (*T. arjuna*) is also an important medicinal plant widely used in the preparation of ayurvedic formulations for over three centuries, primarily as a cardiac tonic in India (8). Clinical evaluation of this plant indicates that it can be of benefit in the treatment of coronary artery diseases, heart failure and possibly hypercholesterolemia (9). However, most of the beneficial works on this plant have been carried out on hepatic or renal disorders (10). In this particular study, protective role of aqueous extract of *T. arjuna* bark was evaluated against dehydration induced oxidative stress and uremia in male rats. This work will also cover the basic physiological process for the management of uremia by loss of the antioxidant status in both blood and kidney and uremic profiles in blood. In contrast, this work has also a clinical dimension, because the results may be disseminated to the society, after proper investigation on the renal failure patients.

Experimental

Animal selection and care

This study was conducted on twenty four healthy adult male Wister strain rats, with a body weight of 108 ± 3 g. They were housed in cage (1 rats /cage) and acclimatized to laboratory conditions for 2 weeks prior to experimentation at constant temperature of 22 ± 3 ºC, with 12-12 h light-dark cycle (8.00-20.00h light: 20.00-8.00 h dark) at a humidity of 50±10%. They were supplied with an adequate dry food (pellet diet) and water *ad libitum*. The principle of laboratory animal care (NIH 1985) was followed duration the experiments and our University Ethics Committee approved the experimental protocol (11).

Plant materials

The bark of *T. arjuna* was collected from Gopali, Indian Institute of Technology, Kharagpur, Paschim Medinipur district of West Bengal, India. Taxonomist of Botany Department, Raja N. L. Khan Women's College, Midnapore identified the material and voucher specimen (number-BVS-7) was deposited in the Department of Botany, Raja N. L. Khan Women's College.

Preparation of aqueous extract of T. arjuna bark

At first, *T. arjuna* bark was dried at 40 ± 1 ºC in incubator and the dried parts were crushed using an electric grinder and the resulting powder was then separated. Next, 25 g of the fine powder was dissolves in 250 mL of distilled water and kept in an airtight glass jar. This mixture was incubated at 37 ± 1 ºC for 72 h in a Soxhlet extraction apparatus. The deep reddish brown extract of *T. arjuna* was collected. Then this extract was dried in a vacuum desiccator to obtain a dry mass, stored in a refrigerator at 4^{o}C and used for the next 7 days of our experiments. As per necessity, the extract was again prepared throughout the experimental period. When needed, the extract was suspended in de-ionized water and used in the study (12).

Experimental design

Twenty four healthy adult male Wistar strain rats were divided into four groups on the basis

of matching the body weights of the animals. The treatment schedule of each group was as follows:

(I) Group I or the control group

Animals were subjected to control groups feed dry food (pellet diet) and an adequate amount of water. Rats of this group received de-ionized water for 15 days prior to experimentation, followed by the next 15 days of experimental period through forceful oral route at 8.00 a.m. through gavage.

(II) Group II or the control plus the extract (T. arjuna) treated group

Animals were subjected to forceful oral administration of the aqueous extract of this plant parts at a dose of 400 mg/kg body weight / day/rat in 0.5 mL deionized water for 15 days prior to the commencement of experiment followed by the next 15 days of experimentation without dehydration. The plant extract was administered at 8.00 a.m. of each day by gavage.

(III) Group III or the dehydration group

Initially, rats were supplied with a normal diet and adequate amount of water for the first 15 days of experimentation. These rats were then induced to dehydration (according to the dehydration protocol) for the next 15 days of experimentation and 0.5 mL of de-ionized water was provided forcefully through oral route at 8.00 a.m. through gavage.

(IV) Group IV or the pretreatment followed by dehydration and extract administration group

Rats were subjected to preconditioning by oral administration of the aqueous extract of this plant parts for 15 days, prior to the induction of dehydration at the same dose as group II. From the 16^{th} day, animals were subjected to dehydration for the next 15 days and all the animals in this group were subjected to oral administration of the aqueous extract of these plant parts at the same dose as group II.

Animals sacrificed for plasma and organ collection

The whole experimental design was continued for 30 days and animals were sacrificed and then their blood and kidney collected from aorta and peritoneal cavity, respectively.

Dehydration protocol

Group I and group II animals were randomly placed as 1 rat/cage, with free access to dry food (pellet diet) and adequate water. The daily water intake/rat was measured. Groups III and IV animals were randomly placed as 1 rat/cage, with free access to dry food (pellet diet). Dehydration was achieved by withdrawing the drinking water bottle for 24 h and by providing 2 mL water to each rat after an interval of 24 h, throughout the 15 days dehydration period of experimentation (13).

Statistical analysis

Analysis of variance (ANOVA) followed by a multiple two-tail t-test with Bonferroni modification, was used for statistical analysis of the collected data. Difference were considered significant when $p < 0.05$.

Antioxidant enzymes

(I) Biochemical assay of catalase activity (CAT)

Catalase activity was measured biochemically. For the evaluation of CAT activity in plasma, collected blood was centrifuged and plasma fraction was separated. For kidney tissues, they were homogenized separately in 0.05 M tris hydrochloric acid (HCl) buffer solution (pH = 7.0) at a tissue concentration of 50 mg/mL. These homogenates were centrifuged separately at 10,000 g at 4 ^{o}C for 10 min. In a spectrophotometric cuvette, 0.5 mL of hydrogen peroxide (H_2O_2) and 2.5 mL of distilled water were mixed and absorbance was determined at 240 nm. Forty µL of tissue supernatant and plasma were separately added, and the subsequent six reading were noted at 30 sec intervals (14).

(II) Biochemical assay of superoxide dismutase (SOD)

Kidneys were homogenized in ice-cold 100mM tris-cocodylate buffer to give a tissue concentration of 50 mg/mL and blood centrifuged at 10,000 g for 20min at 4 ^{o}C. The SOD activity of these supernatants was estimated by measuring the percentage of inhibition of the pyragallol auto-oxidation by SOD. The buffer was 50mM

Table 1. Water intake (mL) /rat/day in the first 15 days, followed by the next 15 days of experimentation. Dehydration procedure followed by providing a little amount of drinking water to male albino rats for the next 15 days of experimental period. Data are expressed as mean ±SE (n=6). The statistical test used was ANOVA followed by multiple two-tail t-test. Data with the same superscripts (a) for a specific data did not differ from each other significantly ($p > 0.05$).

Group	Water intake (mL) /rat/day in the first 15 days of experimentation	Water intake (mL) /rat/day in the next 15 days of experimentation
I	16.4±0.6[a]	16.4±0.6[a]
II	16.8±0.3[a]	16.8±0.3[a]
III	16.9±0.4[a]	2.0
IV	16.2±0.4[a]	2.0

Group I: Control
Group II: Control + extract treated group
Group III: Dehydrating group
Group IV: Pretreatment followed by dehydration and extract coadministration

tris (pH = 8.2) containing 50 mM cocodylic acid (pH = 8.2), 1 mM ethylene diamine tetra acetic acid (EDTA) and 10mM hydrochloric acid (HCl). In a spectrophotometric cuvette, 2 mL of buffer, 100 µL of 2 mM pyragallol and 10 µL of supernatant were poured and the absorbance was noted at 420 nm for 3 min. One unit of SOD was defined as the enzyme activity that inhibited the auto-oxidation of pyragallol by 50 % (15).

Estimation of lipid peroxidation from the levels of malondialdehyde (MDA) and conjugated dienes (CD)

The kidneys were homogenized separately at a tissue concentration of 50 mg/mL in 0.1 M of ice-cold phosphate buffer (pH = 7.4) and the homogenates and blood samples were separately centrifuged at 10,000 g at 4 °C for 5 min. Supernatant and plasma were used for the estimation of MDA and CD. For the measurement of MDA, 0.5 mL homogenate and plasma were mixed separately with 0.5 mL normal saline and 2 mL of TBA-TCA mixture (0.392 g of TBA in 75 mL of 0.25 N HCl with 15 g of TCA, with the final volume of the mixture being made up to 100 mL with ethanol) and, then boiled at 100 °C for 10 min. The mixture was then cooled at room temperature and centrifuged at 4000 g for 10 min. The whole supernatant and plasma was transferred into a spectrophotometer cuvette and read at 535 nm. Calibration was performed by using the acid hydrolysis of 1, 1, 3, 3 tetra-methoxy propane, as a standard. The MDA present within the sample was calculated by using the extinction coefficient of 1.56×10^5 M/cm and expressed as the unit of nM/mg of tissue or nM/mL of plasma (16).

Quantification of CD was performed by a standard method. The lipids were extracted with the chloroform-methanol (2:1) mixture, followed by centrifugation at 10,000 g for 5 min. The chloroform layer was evaporated to dryness under a stream of nitrogen. The lipid residue was dissolved in 1.5 mL of cyclohexane and the absorbance was noted at 233 nm to measure the amount of hydrogen peroxide formed (16).

Table 2. Protective effect of pretreatment followed by coadministration of aqueous extract of *T. arjuna* bark on body growth and renal organo-somatic indices in dehydration induced oxidative stress in rat. Data are expressed as mean ± SE (n=6). ANOVA followed by multiple two-tail t-test and data with different superscripts (a, b) in a specific vertical column differed from each other significantly ($p < 0.05$).

Group	Initial body weight (g)	Final body weight (g)	Elevation/diminition in body growth (g%)	Renal-somatic index (g%)
I	110.2±5.5[a]	140.8±6.0[a]	30.6	0.80±0.02[a]
II	109.3±5.5[a]	143.7±6.0[a]	34.4	0.78±0.02[a]
III	108.4±2.3[a]	122.4±4.7b	14.0	0.69±0.04[b]
IV	109.8 ±4.2[a]	142.5 ±3.5[a]	32.7	0.82±0.02[a]

Group I: Control
Group II: Control + extract treated group
Group III: Dehydrating group
Group IV: Pretreatment followed by dehydration and extract coadministration

Table 3. Protective effect of pretreatment followed by coadministration of aqueous extract of *T. arjuna* bark on plasma and kidney SGOT and SGPT activities in dehydration induced oxidative stress and uremia in rat. Data are expressed as mean ± SE (n=6). The statistical test used was ANOVA followed by multiple two-tail t-test and data with the same superscripts (a) in a specific vertical column did not differ from each other significantly ($p > 0.05$).

	Plasma		Kidney	
Group	**SGOT (Unit/ L of plasma)**	**SGPT (Unit/ L of plasma)**	**SGOT (Unit/ mg of tissue)**	**SGPT (Unit/ mg of tissue)**
I	15.4±0.5[a]	23.4±0.2[a]	13.3±0.3[a]	24.4±0.3[a]
II	15.8±0.4[a]	23.6±0.2[a]	13.6±0.2[a]	23.6±0.2[a]
III	16.9±0.8[a]	22.1±0.1[a]	12.9±0.1[a]	25.1±0.5[a]
IV	16.1±0.7[a]	28.2±0.3[a]	13.2±0.5[a]	24.2±0.2[a]

Group I: Control
Group-II: Control + extract treated group
Group III: Dehydrating group
Group IV: Pretreatment followed by dehydration and extract coadministration

Blood uremia profile

(I) Biochemical estimation of blood urea

The collected blood was centrifuged and plasma fraction was separated. Urea level of the plasma measured by commercially available standard blood urea kit (Merck, Japan), using a semi-autoanalyser (Merck, Microlab-300, Japan) as per the standard protocol for phtotometric determination of urea according to the urease GLDH method (kinetic UV test). First, 10 µL of urea standard (50 mg/ 100mL) was mixed with 1000 µL of the monoreagent (composed of tris pH 7.8 120 mmol/l, 2-oxoglutarate-7 mmol/l, ADP 0.6 mmol/l, rease 6 ku/l, glutamate dehydrogenase 1ku/l and NADH 0.25 mmol/l) and incubated for around 60 sec at 25 °C, and absorbance was read at 37 °C for standardization. Then, 10 µL samples were used for the experimentation, as described before (17).

(II) Biochemical estimation of blood creatinine

The collected blood was centrifuged and plasma fraction was separated. Creatinine level of plasma was measured using commercially available standard blood urea kit (Merck, Japan) and a Semi-autoanalyser (Merck, Microlab-300, Japan) as per standard protocol for phtotometric determination of creatinine, based on Jaffe kinetic method without deproteinization. First, 100 µL of creatinine standard (1 mg/ 100mL) was mixed with 1000 µL of the monoreagent (buffer:NaOH 313 mmol/l and picric acid 8.73 mmol/l) and incubated for around 5 min at 25 °C and then absorbance was read at 37 °C for standardization. Then 100 µL samples were used

Table 4. Protective effect of pretreatment followed by coadministration of aqueous extract of *T. arjuna* bark on plasma and kidney catalase and SOD activities in dehydration induced oxidative stress in rat. Data are expressed as mean ± SE (n=6). The statistical test used was ANOVA followed by multiple two-tail t-test and data with different superscripts (a,b,c) in a specific vertical column differed from each other significantly ($p < 0.05$).

	Plasma		Kidney	
Groups	**Catalase (m mol of H_2O_2 consumption /dL of Plasma/min)**	**SOD (m mol of H_2O_2 consumption /dL of Plasma/min)**	**Catalase (m mol of H_2O_2 consumption/mg of tissue/min)**	**SOD (m mol of H_2O_2 consumption/mg of tissue/min)**
I	0.16±0.05[a]	0.83±0.05[a]	0.45±0.03[a]	1.33±0.08[a]
II	0.18±0.04[b]	0.90±0.06[b]	0.58±0.02[b]	1.40±0.08[b]
III	0.02±0.04[c]	0.49±0.04[c]	0.22±0.07[c]	.49±0.08[c]
IV	0.17±0.06[a]	0.88±0.06[b]	0.52±0.06[a]	1.38±0.05[b]

Group I: Control
Group II: Control + extract treated group
Group III: Dehydrating group
Group IV: Pretreatment followed by dehydration and extract coadministration

Table 5. Protective effect of pretreatment followed by coadministration of aqueous extract of *T. arjuna* bark on MDA and CD of plasma and kidney in dehydration induced oxidative stress in rat. Data are expressed as mean ± SE (n=6). The statistical test used was ANOVA followed by multiple two-tail t-test and data with different superscripts (a,b,c) in a specific vertical column differed from each other significantly ($p < 0.05$).

Group	Plasma		Kidney	
	MDA (n mol/ dL of plasma)	CD (n mol hydrogen peroxide/ dL of plasma)	MDA (n mol/ mg of tissue)	CD (n mol hydrogen peroxide / mg of tissue)
I	33.66±0.15[a]	399.56±0.14[a]	76.96±0.01[a]	327.26±0.06[a]
II	30.66±0.23[b]	379.56±0.55[b]	70.22±0.04[b]	290.48±0.05[b]
III	84.25±0.54[c]	435.49±0.84[c]	120.13±0.34[c]	419.25±0.54[c]
IV	32.56±0.26[a]	392.38±0.06[b]	86.04±0.06[a]	355.28±0.06[b]

Group I: Control
Group II: Control + extract treated group
Group III: Dehydrating group
Group IV: Pretreatment followed by dehydration and extract coadministration
MDA: Melondialdehyde
CD: Conjugated dienes

for analysis (18).

Toxicity study via biochemical estimation of glutamic oxaloacetic transaminase (GOT) and biochemical estimation of glutamic pyruvic transaminase(GPT)

For the assessment of toxicity in blood and kidney, GOT and GPT were measured, based on the method of Goel (19).

Results

Water intake and dehydration procedure

During the first 15 days of experimentation, all groups of rats were supplied with normal water. They were given a maximum of 16.4 ± 0.6 mL water/day/individual as intake. Then, during the next 15 days of experimentation, groups I and II were supplied with an adequate (normal) amount of water. However, groups III and IV were supplied with only 2 mL water/rat/24h (Table 1).

Table 6. Protective effect of pretreatment followed by coadministration of aqueous extract of *T. arjuna* bark on plasma urea and creatinine activities in dehydration induced oxidative stress and uremia condition in rat. Data are expressed as mean ± SE (n=8). The statistical test conducted was ANOVA followed by multiple two-tail t-test and data with different superscript (a,b,c) in a specific vertical column differed from each other significantly ($p < 0.05$).

Group	Plasma	
	Urea (mg/dL of blood plasma)	Creatinine (mg/dL of blood plasma)
I	30.2±0.7[a]	0.38±0.2[a]
II	25.4±0.7[b]	0.29±0.2[b]
III	75.2±0.8[c]	0.68±0.6[b]
IV	31.3±0.5[a]	0.33±0.4[a]

Group I: Control
Group II: Control + extract treated group
Group III: Dehydrating group
Group IV: Pretreatment followed by dehydration and extract coadministration

Body weight and somatic indices of kidneys

Body weight increased at the end of experiment in groups I, II and IV compared to their initial body weight (Table 2). In group III, the percentage of elevation in body growth was dramatically less than the other groups, due to the dehydration induced oxidative stress (Table 2). Renal somatic decreased significantly in group III, in comparison to groups I, II and IV. After administration of the *T. arjuna* extract in group IV, these indices were resettled towards the control level (Table 2).

Activities of SGOT and SGPT

The activities of SGOT and SGPT did not alter significantly in blood and kidney, among the test groups (Table 3).

Activities of catalase and SOD

In group II, CAT activities in blood and kidney were elevated, compared to group I, III and IV. After dehydration (group III), the activity of this enzyme in blood and kidney was decreased

significantly, compared with group I. There was a significant protection in catalase activity after pretreatment with the extract, followed by its coadministration (group IV) compared with the animals in the dehydrating group (group III) (Table 4).

Administration of the herbal mixture to non-dehydrating animals (group II) resulted in a significant elevation in the activity of SOD in blood and kidney, compared to the animals in groups I and IV. The activity of this enzyme was decreases significantly in blood and kidney in group III, in comparison with group I (Table 4). Pretreatment followed by coadministration of the extract to the animals in group IV resulted in a significant restoration of the SOD activity, compared with group III, and the values were resettled to the control level.

Quantification of MDA and CD

Quantities of MDA and CD were increased in blood and kidney of group III animals, compared to groups I, II and IV (Table 5). In the dehydrated rats (group III), 15 days of dehydration resulted in a significant elevation in the values of both parameters in blood and kidney. However, in group IV administration of the extract significantly decreased MDA and CD quantities.

Levels of blood urea and creatinine

Urea and creatinine levels were significantly increased in group III animals (the dehydration group), compared to groups I and II. But in group IV (pretreatment, dehydration and coadministration of extract) significantly low levels of urea and creatinine were observed, compared to group III, and the values resettled to the levels of control group.

Discussion

Dehydration is the risk factor at the point when urine production declines and finally results in no urine out put (anuria). Here, dehydration is only used for causing oxidative stress, elevation of blood urea and creatinine levels (3). Without urinary excretion of waste products, dangerous levels of urea accumulate in the blood (3). Decreased blood volume occurs with deficient fluid intake, which causes a reduced blood flow in kidney resulting in a decreased glomerular filtration rate (GFR). This can consequently lead to acute renal failure (ARF) (4). Hence, prolonged blood volume deficiency ultimately produces renal damage. Prolonged increase in urea and creatinine levels due to a decreased GFR, causes chronic renal failure (CRF) (4). When GFR decreases down to 90%, then end stage renal disease (ESRD) may occur.

Dehydration induced oxidative stress in blood and kidney has been established in this study to cause low activities of SOD and CAT. These are important antioxidant enzymes (20, 21). The decrease in the activity of antioxidant enzymes, as a result of dehydration, might be due to their use against the free radicals destruction and their inhibition by free radicals species (22). It is well established that SOD activity is inhibited by hydrogen peroxide, that reduces Cu^{2+} to Cu^{+} in SOD (23).The reduction of hydrogen peroxide is catalyzed by CAT, that protects tissues from highly reactive hydroxyl radicals (24).

Increase in the levels of oxidative stress products like MDA and CD in blood and kidney in the dehydrating group, again indicated the low level of antioxidant enzymes activities which causes progression of lipid peroxidation. Other possibilities for such elevation in MDA and CD could be the ischemia-reperfusion phenomenon (25, 26) or the high rate of catecholamine secretion that generates free radicals either through auto-oxidation or through metal ion or superoxide-catalyzed oxidation (12, 27). Recent studies have shown that oxidative stress is highly present in patients with renal disease (7). It is known that LDL from uremic patients presents an elevated susceptibility to oxidation. Uremic oxidative stress is characterized from a biochemical point of view as a state of reactive aldehyde and oxidized thiol group accumulation, together with depletion of reduced thiol groups, which are particularly important as part of antioxidant defense (28). As a consequence of diminished renal catabolism and function, uremic oxidant mediators accumulate urea and creatinine in blood (29). In this study, the *T.arjuna* extract was used due to its traditional

of this plant extract caused a significant elevation in the levels of antioxidant status. Moreover, this extract diminished the lipid peroxidation in blood cells and kidney of dehydrated animals.

Urea is metabolized by 'salvage pathway' and transfer of urea from plasma to colon, where it is broken down to ammonia by bacterial urease. Probably this process occurs also in the lower ileum (29). Oxidative stress appears to increase as CKD progresses, and correlates significantly with the level of renal function (30). In the present study, the levels of urea and creatinine were elevated due to the increase in oxidative stress in only the dehydrated animals. However, administration of plant extract decreased the uremic parameters near the levels of control animals. The extract had no general and metabolic toxic effect, as reflected from the insignificant variation in body growth and activities of SGOT and SGPT in blood and kidney tissues. This may be disseminated to the renal disease patients as a beneficial outcome.

Hence, we could conclude that correction and protection of the oxidative stress and uremia in the experimental animals have been found by applying the aqueous bark extract of *T. arjuna*. Further complex studies are needed to fully characterize the responsible active ingridents present in the plant and elucidate their possible mode of action and mechanism that is in progress.

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