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ORIGINAL ARTICLE

Mitochondrial Nephrotoxicity induced by Tacrolimus (FK-506) and Modulatory Effects of *Bacopa monnieri* (Farafakh) of Tabuk Region

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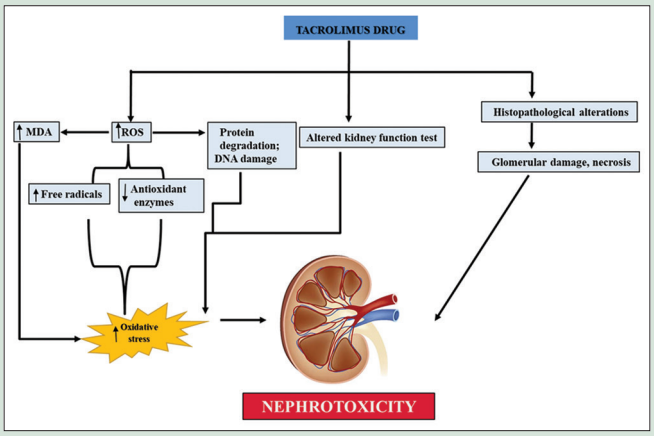
ABSTRACT

Background: Tacrolimus is a known immunosuppressive drug used widely for organ transplantation, but its nephrotoxicity mechanism is still unclear. **Objectives:** The present study investigates the protective efficacy of *Bacopa monnieri* (BM), against tacrolimus-induced nephrotoxicity in rats. **Materials and Methods:** Group 1 (control group); administered orally with normal saline for 30 days; Group 2 (BM extract treated group); Group 3 (tacrolimus-treated group); and Group 4; (tacrolimus plus BM extract treated group). Tacrolimus-treated rats received 1 mg/kg body weight of tacrolimus intraperitoneally for 30 days, and BM-pretreated rats were administered with the dose of 200 mg/kg orally by gavage once a day for 30 days. **Results:** Tacrolimus-induced nephrotoxicity was assessed biochemically and histopathologically. Pretreatment with BM has shown to possess a significant protective effect against tacrolimus-induced kidney functions regarding urea, creatinine, and albumin levels, respectively. The creatinine, mitochondrial lipid peroxidation (thiobarbituric acid reactive substances), and protein carbonyl levels were significantly increased dramatically, and however, the total proteins, albumin, glutathione, superoxide dismutase, and glutathione peroxidase were decreased when pretreated with tacrolimus. The nephroprotective efficacy of the BM extract was further evident by histopathological analysis and DNA fragmentation. **Conclusion:** The outcome of this study indicates that BM extracts exerted protection against tacrolimus-induced kidney toxicity.

Key words: Antioxidant activity, *Bacopa monnieri*, DNA fragmentation, nephrotoxicity, tacrolimus

SUMMARY

- Nephroprotective properties of the ethanol extract of *Bacopa monnieri* and its antioxidant activity was evaluated. It showed anti-oxidative and protective efficacy against tacrolimus by significant attenuation of oxidative stress parameters in mitochondria isolated from the kidney, augmentation in renal function biochemistry as well as the restoration of renal structures.



Abbreviations Used: ANOVA: Analysis of variance, BM: *Bacopa monnieri*, BUN: Blood urea nitrogen, DNPH: Dinitrophenylhydrazine, DPPH: 2,2-diphenyl-1-picrylhydrazyl, DSR: Deanship of Scientific Research, EOBPV: Egyptian Organization for Biological Products and Vaccines, GPx: Glutathione peroxidase, GSH: Glutathione, H and E: Hematoxylin and eosin, H_2O_2: Hydrogenperoxide, IAEC: Institutional Animals Ethics Committee, IC: Inhibitory concentration, Ip: Intraperitoneal, mLPO: Mitochondrial lipid peroxidation, Mn-SOD: Mn-superoxide dismutase, PC: Protein carbonyl, ROS: Reactive oxygen species.

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INTRODUCTION

Humans are incessantly exposed to exogenous chemicals, drugs, and environmental pollutants deliberately and inadvertently that harm the kidney. Kidneys are extremely susceptible to damage caused by reactive oxygen species (ROS) due to oxidative stress. Tacrolimus is a known immunosuppressive drug used in organ transplantation and has both short-term and long-term advantages over conventional drugs. After transplantation, toxicity of tacrolimus is an important determinant of morbidity and mortality. This drug has higher nephrotoxicity, neurotoxicity, and diabetogenicity.[1] Tacrolimus is absorbed in the duodenum and jejunum after oral administration and have a great erraticism of pharmacokinetics.[2]

Herbal remedies have been used for a long time for the management and detoxification from drugs of addiction. Because of the efficiency, fewer side effects and comparatively low cost, medicinal plants, and herbal drugs are prescribed widely. *Bacopa monnieri* (Linn.) Pennell, from family Scrophulariaceae, has been studied widely for its hepatoprotective and nephroprotective properties.[3,4] Numerous clinical studies inveterate the valuable actions of BM.[5] These pharmacological actions are mainly

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attributed to the saponin compounds present in the alcoholic extract of the plant. The primary chemical constituents isolated from BM are triterpenoids, saponins with jujubogenin and pseudojujubogenin as the aglycones including bacosides A1–A3, bacopasaponins A-G, and bacopasides I-V.[6,7] It has also been shown to possess potent antioxidant activity.[8]

However, the efficacy of the BM against an immunosuppressive drug is scarce. Therefore, the present study was aimed to reconnoiter the protective efficacy of BM against tacrolimus-induced nephrotoxicity in rats.

MATERIALS AND METHODS

Plant collection and extract preparation

BM was collected from the local market, authenticated by the departmental botanist and was shade dried, blended to form the coarse powder. Briefly, 70 g of dry powder was taken, and to it, 70% ethanolic (v/v) was added with intermittent shaking and kept overnight. The process was repeated three times, pooled, filtered and extracted in soxhlet apparatus at 60°C.

Animals

A total of 40 male adult Sprague Dawley rats weighing 150–180 g were used for the experiments and purchased from the breeding unit of Egyptian Organization for Biological Products and Vaccines, Abbassia, Cairo. They were housed in steel mesh cages and maintained for 1 week acclimatization period on commercial standard and pellet diet and drinking water *ad libitum*. The housing cycle was 12:12 h light-dark cycle under controlled temperature (20°C–22°C). The animal use protocol had been approved by the Institutional Animals Ethics Committee of Tanta University.

Phytochemical screening of the plant

BM extracts were subjected to phytochemical testing for the identification of chemical constituents, including, tannins, saponins, phenols, total antioxidant capacity, and flavonoids as reported by Saggu *et al.*[9,10]

Animal treatments

Randomly animals were divided into four groups of ten rats each. Group I animals served as control. Group II animals were given BM extract at a dose of 200 mg/kg body weight, Group III animals were injected 1 mg/kg body weight of tacrolimus intraperitoneally, daily for 30 days, and Group IV received tacrolimus + BM extract, respectively, for 30 days. Oral administration of the BM extract was given orally by gavage before the administration of tacrolimus. After 24 h, the animals were sacrificed by cervical dislocation, and their kidneys were removed immediately. One kidney of each animal was processed for the preparation of mitochondria, and the other kidney was fixed in 10% buffered formalin (chilled) for histopathological studies.

Sample preparation

Preparation of homogenate and determination of antioxidant markers

Samples of blood were taken from the orbital sinus, and centrifugation separated the serum at 3000 g for 10 min using capillary tubes, and the Auto-analyzer estimated renal function parameters.

The kidneys were removed and dissected free from the surrounding fat and connective tissue. Subsequently, renal tissue was homogenized, and mitochondria from tissues were isolated from the fasted animal by the differential centrifugation method as illustrated.[11,12] The protein concentration in the stock suspension was determined by using the Lowry method.[13]

Quantitative determination of glutathione, superoxide dismutase, and mitochondrial glutathione peroxidase activity

The reduced glutathione (GSH) content was determined by the method Beutler *et al.*[14] The activity of Mn-superoxide dismutase (MnSOD) was established by the way as described by Flohe and Otting[15] The change in absorbance was measured at 438 nm and expressed as U/mg of mitochondrial protein.

The activity of GSH peroxidase was assayed by the method described by Flohe.[16] Results were expressed as U/mg of mitochondrial protein.

Estimation of mitochondrial lipid peroxidation and protein carbonyl

The mitochondrial lipid peroxidation was quantitated by monitoring the formation of thiobarbituric acid reactive substances (TBARS) according to the method by Waseem and Parvez.[12] The rate was determined as n moles of TBARS formed/mg of protein using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$. The protein carbonyl (PC) content was measured using dinitrophenylhydrazine as described by Sohal *et al.*[17]

Histopathological examination

The histopathology was carried out according to Scheuer and Chalk.[18] Briefly, after excision of kidneys, instantly they were washed using chilled saline solution and treated for further bioassays. A small section was straightaway fixed in 10% formalin and later embedded in paraffin, 5 μm sectioned were cut and stained with hematoxylin and eosin, and examined under a light microscope.

Estimation of fragmentation of DNA.

Samples from the kidney of the ten animals/group were mechanically dissociated in hypotonic lysis buffer and centrifuged at 13,800 g for 15 min. Colorimetric determination by Diphenylamine assay was performed, and small DNA fragments from the supernatant were immediately separated, and the pellet containing large DNA pieces were used for the estimation. The developed blue color was colorimetrically quantified spectrophotometrically at 578 nm.[19] The formula expressed percentage of DNA fragmentation in each sample: % DNA fragmentation = (O. D supernatant/O. D supernatant + O. D pellet) × 100.(O. D. optical density).

Statistical analysis

All the data are presented as means ± standard deviation for at least three replications for each prepared sample. One-way analysis of variance followed by Student–Newman–Keul's test was done to assess the significant differences among the groups, using SigmaPlot, Systat Software program version 11.

RESULTS

Initial phytochemical screening of *Bacopa monnieri* extract

Phytochemical analysis of the BM extract showed the presence of tannins, saponins, phenols, and flavonoids. Total phenol content present in BM extract was found to be 40.8 mg/g GAE and the total flavonoid is 16.2 mg/g of CE equivalent, respectively [Table 1].

Effect of tacrolimus and *Bacopa monnieri* on kidney function test

In the present study, treatment with tacrolimus induced a significant elevation in serum blood urea nitrogen (BUN) and creatinine. However, the levels of total protein and albumin were significantly decreased ($P < 0.001$) when compared to healthy animals (Group 1).

These alterations were attenuated considerably in rats treated with the BM extract ($P < 0.05$) [Table 2].

Effect of *Bacopa monnieri* extract on tacrolimus induced perturbations on isolated mitochondrial enzymatic and nonenzymatic antioxidants

Tacrolimus induces oxidative stress and produces a generation of free radicals and peroxidative damage to the tissues. The activities of antioxidant enzymes, namely, GSH, Mn-superoxide dismutase, GPx were significantly reduced in tacrolimus-induced rats when compared to control animals. Figure 1 reflects the reduced mitochondrial GSH level was significantly decreased in the Group III as compared to the normal control ($P < 0.001$) (Group I) but increased dramatically in the Group IV as compared to the tacrolimus treated group ($P < 0.05$). A similar trend of the decrease was also observed in the activities of GPx and MnSOD.

The TBARS values increased significantly in the tacrolimus-treated group when compared with the control, and BM-treated animals ($P < 0.001$) [Table 3]. The PC values were significantly reduced in comparison with controls. However, BM oral extract treatment in rats resulted in significant increases in values ($P < 0.05$) [Table 3]. The significant elevation of plasma PCs indicates the presence of oxidative stress and upregulation of NAPQI production, which stimulates protein oxidations.

Histopathological alterations

The histopathological changes in the kidney sections in the different groups are shown in Figure 2a-f. Kidney sections in control group and BM revealed normal structure of glomeruli, proximal convoluted tubules, and distal convoluted tubules in both cortex and medulla [Figure 2a and b], on the other hand, kidney sections in groups treated with tacrolimus showed some histopathological lesions in glomeruli and some parts of the urinary tubules such as marked atrophied glomeruli, leukocytic infiltrations, congestion in blood vessels and marked degeneration in proximal and distal convoluted tubules and vacuolization in tubular cells focal necrosis [Figure 2c and d] at the concentration 1 mg/kg body weight for 30 days. In contrast; kidney sections in rats treated with TAC + BM revealed a better improvement in glomerular damage with minimal vacuolization and degeneration in tubular cells and the Malpighian corpuscles appeared as normal structure [Figure 2e and f].

Table 1: Total flavonoids; total phenolics contents in *Bacopa monnieri* extract

Parameter	BM leaves extracts
Total phenolic compound (mg/g gallic acid)	40.8
TF (mg/g dry weight)	16.2

The value represents mean±SD of three determinations. TF: Total flavonoids; SD: Standard deviation; BM: *Bacopa monnieri*

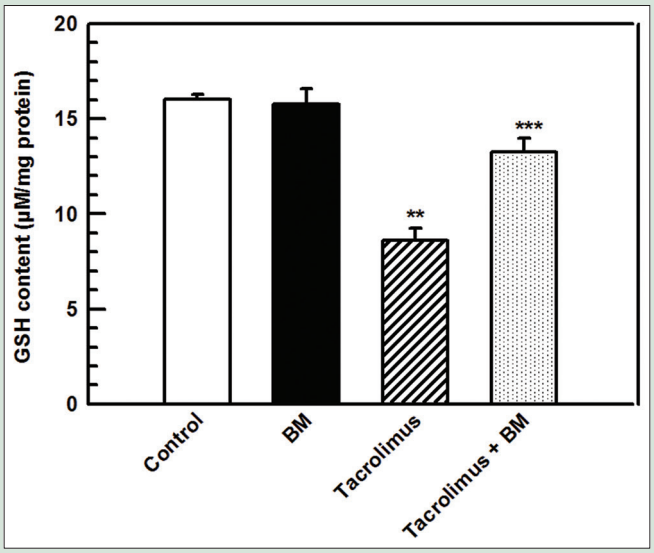

Figure 1: Effect of *Bacopa monnieri* extracts administration (200.0 mg/kg) on GSH activity in tacrolimus induced nephrotoxicity in experimental rats. Values are mean ± standard deviation, $N = 10$ animals in each group. ***Statistically significance at $P < 0.001$, in comparison to the normal control group; **Statistically significance at $P < 0.01$, compared with tacrolimus treated group

Table 2: Effect of *Bacopa monnieri* leaves extract administration on kidney function test: Creatinine, blood urea nitrogen, total protein and albumin levels in tacrolimus-induced nephrotoxicity in rats

Group	Parameter			
	BUN (IU/L)	Creatinine (IU/L)	Albumin (g/dL)	Total protein (g/dL)
Control	17.41±0.84	1.33±0.26	0.33±0.02	8.41±0.43
BM extract	18.41±0.43	2.13±0.12	0.37±0.03	7.54±0.56
Tacrolimus	38.41±0.35**	4.78±0.28**	0.74±0.05**	3.17±0.18**
Tacrolimus + BM extract	28.0±1.85*	2.14±0.15*	0.52±0.02*	6.04±0.52*

Bar represen ts mean±SE. n=10 animals per group. Statistical significances: **P<0.001, compared with normal control group; *P<0.05, compared with tacrolimus treated group. BUN: Blood urea nitrogen; SE: Standard error; BM: *Bacopa monnieri*

Table 3: Effect of *Bacopa monnieri* extracts on Mn-superoxide dismutase and glutathione peroxidase activities in total antioxidant capacity-treated rats

Group	Parameter			
	Mn-SOD	GPx	m-LPO (µmoles of TBARS formed/h/g of tissue)	PC (nmoles of carbonyl/mg protein)
Control	4.12±0.41	0.43±0.17	0.68±0.02	1.43±0.32
BM extract	5.02±0.23	0.37±0.29	0.72±0.03	1.54±0.36
Tacrolimus	1.21±0.12**	0.16±0.10**	2.72±0.65**	3.04±0.29**
Tacrolimus + BM extract	3.02±0.62*	3.04±0.09*	1.32±0.57*	1.85±0.21*

Values are means±SE. n=6 animals per group. Mn-SOD and GPx are expressed as U/mg mitochondrial protein. Statistical significances are indicated by *P<0.01 when compared with normal control group; **P<0.05, compared with tacrolimus treated groups. SE: Standard error; BM: *Bacopa monnieri*; Mn-SOD: Mn-superoxide dismutase; GPx: Glutathione peroxidase; TAC: Total antioxidant capacity; TBARS: Thiobarbituric acid reactive substances; PC: Protein carbonyl; m-LPO: Mitochondrial lipid peroxidation

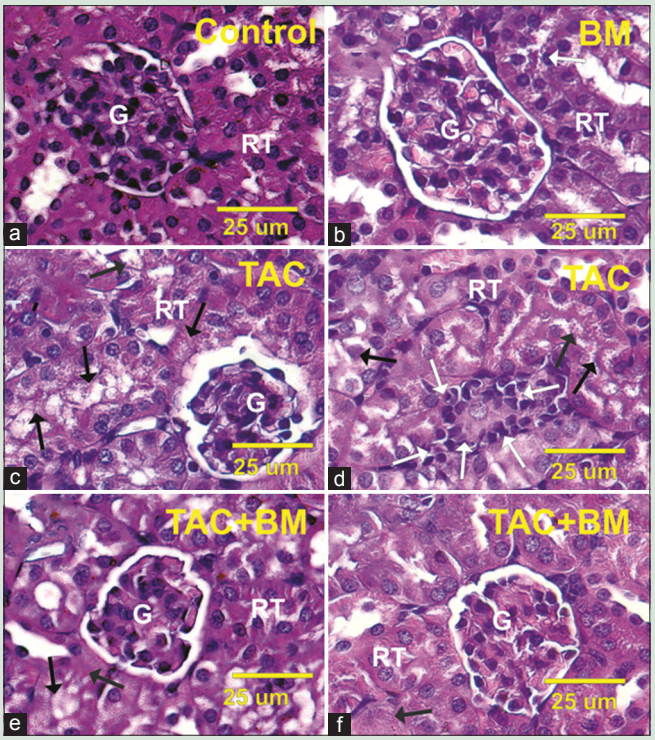

Figure 2: (a-f) Photomicrographs of kidney sections in the different groups under study stained with hematoxylin and Eosin. (a and b) Kidney sections in control and *Bacopa monnieri* showed normal structure of renal cortex which comprised renal corpuscles (G), proximal and distal convoluted tubules. (c and d) Kidney sections in treated group with TAC showed atrophied glomeruli (G), leukocytic infiltrations (Black arrows), and congestion in blood vessels and marked degeneration in proximal and distal convoluted tubules (White arrows). (e and f) Kidney sections in groups treated with TAC + *Bacopa monnieri* revealed minimal vacuolization and degeneration in tubular cells (White arrows) and the malpighian corpuscles appeared as normal structure

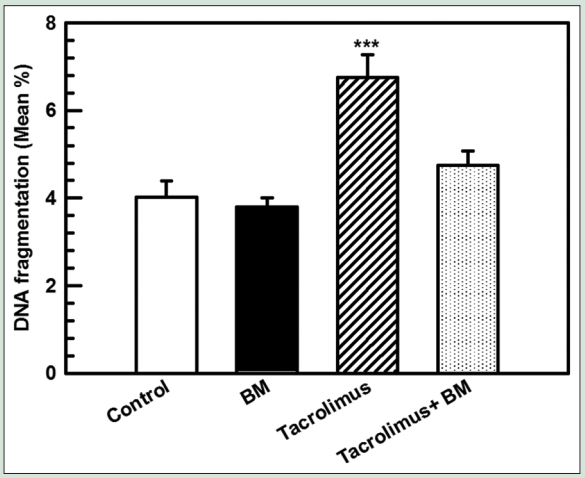

Figure 3: DNA fragmentation of rats treated with tacrolimus, *Bacopa monnieri* extract, and combination of both. Statistical significances are indicated by ***$P < 0.001$ when compared with normal control groups

As compared with the normal control group, the mean percentage of DNA fragmentation when treated with 1 mg/kg of Tacrolimus for 30 days is significantly increased ($P < 0.001$). Treatment with 200 mg/kg of BM extracts prevents the cellular damage substantially [Figure 3].

DISCUSSION

Tacrolimus is a known immunosuppressive drug which binds to FKBP12 and works by inhibiting calcineurin, a calcium-calmodulin-dependent phosphatase which ultimately inhibits T-cell activation.[20]

The current study aimed to analyze the potential impact of ethanolic extract of BM on tacrolimus-induced nephrotoxicity. We have examined the antioxidant profile, renal markers, and renal morphology in tacrolimus-treated rats together with the phytochemical screening of the plant. We have found that the extract possessed tannins, saponins, phenols, flavonoids and showed radical scavenging activity [Table 1].

Mitochondria play an essential role in the cytoxicities of several drugs and chemicals.[21,22] In the present work, the observed nephrotoxicity due to immunosuppressive tacrolimus treatment was manifested by marked increases in serum creatinine, BUN clearance, and a decrease in total protein and albumin.

During stressful situations increase in energy demand necessitates a multifold upsurge in oxygen supply to active tissues to maximize energy yield. The augmented metabolic rate results in the generation of ROS free radicals in massive amounts, causing an imbalance between ROS production and antioxidant defense. PC is a universally accepted biomarker of PC accumulation and oxidation of protein. Tacrolimus pretreatment aggravated the increase in protein oxidation, and BM pretreatment restored the levels in insolated kidney mitochondria. It has been found that BM contains saponins, alkaloids, phenols, and antioxidants which might be the reason for the protective efficacy of the extract against the ROS-induced oxidative stress.

In this study, it has been found that the antioxidant and nonantioxidant enzymes were substantially suppressed in tacrolimus-treated rats. However, treatment with BM substantially up-regulated the levels of antioxidant and non-antioxidants when compared with the tacrolimus-treated animals. Commonly, increased levels of MDA are used as an index of oxidative injury due to oxygen free radicals. Some of the authors reported the protective efficacy of juniper oil against tacrolimus induced nephrotoxicity in rats and also showed the anti-inflammatory and antinociceptive influences of some juniper species in Swiss male albino mice.[23,24] In this study in tacrolimus-treated rats, there was a significant increase of MDA levels along with decreased levels of GPx. It is a well-known fact that ROS generation plays a vital role in peroxidation of lipid membranes of the tissue, resulting in subcellular damage as evident in the histopathological examination [Figure 2]. In our study, the kidney of tacrolimus-treated rats has shown characteristic morphological findings such as glomerular atrophy, degeneration in renal tubules, necrosis, and vacuolation. Similarly, Oyouni *et al.*[25] reported the nephroprotective efficacy of *Ocimum basilicum* in mice treated with tacrolimus at the dose of 3 mg/kg b. wt. Nephroprotective effects of BM extracts were further confirmed by measuring the DNA fragmentation in rat kidney tissue. It prevents the cellular DNA damage and safe to use.

CONCLUSION

Taken together, BM pretreatment in rats protected against tacrolimus-induced nephrotoxicity may be through free radical scavenging activity due to the presence of antioxidants and flavonoids in the extract. Protective efficacy was accompaniment by a significant attenuation of oxidative stress parameters in mitochondria isolated from kidney, augmentation in renal function biochemistry as well as the restoration of renal structures evident by histopathological, DNA fragmentation findings.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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