

Full Length Research Paper

Spirulina protects against tacrolimus-induced hepatic and renal toxicity in rats: A biochemical and histological study

Zakaria A. Elzawahry¹, Marwa A. Abass¹, Manal R. Abd El-Haleem², Reda A. Abdel Hamid³ and Hebatallah H. Atteia^{4*}

¹Departments of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Zagazig University, Egypt.

²Department of Histology and Cell Biology, Faculty of Medicine, Zagazig University, Egypt.

³Department of Anatomy, Faculty of Medicine, Zagazig University, Egypt.

⁴Department of Biochemistry, Faculty of Pharmacy, Zagazig University, Egypt.

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Tacrolimus is a powerful immunosuppressive agent with hepatotoxic and nephrotoxic effects. It has a protective role against many toxicants. This study was conducted to evaluate the possible protective role of spirulina against tacrolimus induced hepatotoxicity and nephrotoxicity. Forty adult male albino rats divided into 4 groups. Group I, control group, Group II, spirulina group (received spirulina 500 mg/Kg body weight (bw)/day orally), Group III, tacrolimus group (received tacrolimus 12 mg/kg bw/day orally); and Group VI, prophylactic group (orally administered spirulina for 3 days before and 28 days concurrently with tacrolimus in the same previous doses). Tacrolimus induced adverse effects on both liver and kidney functions and structure that was manifested by elevated hepatic transaminases, total and direct bilirubin, albumin, blood urea nitrogen, serum creatinine and creatinine clearance. There was a significant decrease in serum total antioxidant capacity (TAC) and hepatic and renal total thiol molecules (TTM), with a significant increase in serum malondialdehyde in tacrolimus group. Histopathologically, tacrolimus induced swelling and granulation of hepatocytes, congestion of blood sinusoids and degeneration of bile ductules, glomerular hypertrophy and segmentation, swelling, degeneration and hyalinosis of renal tubules. Spirulina pre- and co-treatment significantly improved these deleterious effects. This was accompanied by partial restoration of the expression of PCNA near to the normal level observed in control rats. Moreover, spirulina treatment did not alter the trough blood tacrolimus levels or tacrolimus-induced immunosuppression. Further studies are warranted to evaluate whether transplant patients on tacrolimus treatment may benefit from the protective effects of spirulina.

Key words: Antioxidant, malondialdehyde (MDA), total antioxidant capacity (TAC), tacrolimus, total thiol molecules (TTM), proliferating cell nuclear antigen (PCNA), spirulina.

INTRODUCTION

Tacrolimus is an immunosuppressant macrolide produced by *Streptomyces tsukubaensis*. It is used to prevent rejection of transplanted organs by inhibiting

calcineurin enzyme that is crucial for the multiplication of T-lymphocytes which are vital to the immune process (Tanaka et al., 1987; Fruman et al., 1992). Protocols that

do not include calcineurin inhibitors often is associated with limited graft survival that makes tacrolimus considered as the backbone of most immunosuppressive regimens (Jantz et al., 2013). Despite its high clinical efficiency, tacrolimus has been well known for its adverse reactions. In particular, patients receiving tacrolimus chronically are at high risk to develop cholestasis and renal damage (Yadav et al., 2013; Banhara et al., 2015). Nephrotoxicity was reported in approximately 52% of kidney transplantation patients, 40% of liver transplantation patients receiving tacrolimus and in 59% of heart transplantation patients in US randomized trial (Boudjema et al., 2011). Moreover, tacrolimus toxicity clearly showed that induced lipid peroxidation can be partially reversed with antioxidants in children (Grunot et al., 2002). Histopathologic examination revealed that tacrolimus induces renal necrosis and apoptosis. It also increases reactive oxygen species production and decreases antioxidant status (Piao et al., 2014). Therefore, a big need arises to alleviate tacrolimus induced oxidative stress or to reduce its dose to a safer level. Conceivably, reducing tacrolimus dose can impair its therapeutic efficacy.

Spirulina is a great source of natural protein with all amino acids, phyto-nutrients, antioxidants, carbohydrate, mucopolysaccharides, vitamins and trace minerals. Many people use it as an effective natural appetite suppressant. It is known to have important beneficial effects on cellular metabolism and homeostasis (Abou Gabal et al., 2015). Spirulina was reported to have antioxidant, antimutagenic and antineoplastic effects (Premkumar et al., 2004; Khan et al., 2006; Abdel-Daim et al., 2016). The antioxidant and cytoprotective effects of spirulina can be attributed to its antioxidant active constituents including C-phyco-cyanins, β -carotene, vitamins, and minerals (Upasani and Balaraman, 2003; Abdel-Daim et al., 2013; Abdel-Daim, 2014; EL-Sabagh et al., 2014). Moreover, it was previously demonstrated that spirulina can protect against end organ toxicities induced by different chemotherapeutic agents as well as lead acetate-induced hepatotoxicity by ablating oxidative stress and lipid peroxidation (Khan et al., 2006; Hemalatha et al., 2012). Spirulina has also a cardioprotective effect against tilmicosin-induced cardiac toxicity in mice (Ibrahim and Abdel-Daim, 2015). Abdelkhalek et al. (2015) and Abdel-Daim et al. (2016) have reported the hepatorenal protective effects of spirulina platensis against deltamethrin-induced toxicity by minimizing lipid peroxidation and improving antioxidant capacity. Spirulina platensis also exerted antioxidant, anti-inflammatory and immunomodulatory effects in acetic acid-induced experimental ulcerative colitis (Abdel-

Daim et al., 2015). As far as immunosuppressive effects of tacrolimus are concerned; spirulina, was previously proved to have a remarkable immunosuppressive effect both *in-vivo* and *in-vitro*. Therefore, spirulina gains more and more attention from medical scientists as a natural treatment for allergic, autoimmune and transplant-related diseases (Hayashi et al., 1994; Kim et al., 1998; Ramirez et al., 2002; Rasool and Sabina, 2009; Kumar et al., 2010). Accordingly, this study aimed to investigate whether, and how, spirulina may alleviate tacrolimus induced hepatotoxicity and nephrotoxicity by assessment of liver and kidney function tests, oxidative stress markers as well as hepatic and renal histopathologic examination. Lastly, to verify any role for spirulina interaction with tacrolimus, we measured tacrolimus trough levels and lymphocytic proliferation assay in the presence and absence of spirulina.

MATERIALS AND METHODS

Spirulina tablets 500 mg were obtained from DXN Co., Malaysia. Tacrolimus 1 mg capsules were from Hikma Pharmaceutical Co., Jordan. Alanine amino transferase (ALT) and aspartate amino transferase (AST) kits were purchased from Diamond diagnostics (Cairo, Egypt). Alkaline phosphatase (ALP), total and direct bilirubin kits were from Biodiagnostic (Dokki, Giza, Egypt). Albumin kit was obtained from spectrum-diagnostics albumin-BCG kit (Egyptian Company for Biotechnology "S.A.E", Obour city Industrial area, Cairo, Egypt). Blood urea nitrogen (BUN) and creatinine colorimetric kits were purchased from Biomerieux (Lyon, France).

Experimental design

The present study was carried on 40 adult male albino rats, weighing about 180 to 200 g. Rats were caged under standardized environmental conditions. They were housed in a spate well ventilated cages, under standard conditions, with free access to standard diet and water *ad libitum*, throughout the whole period of the experiment (28 days). The experiment was performed in accordance with the guidelines for the Care and Use of Laboratory Animals (Institute of Laboratory Animals Resources, 1996). Rats were classified into four groups received the following for 4 weeks. Group I (control group) included 10 animals which did not received any medications. Group II (spirulina group) included 10 animals that were treated with spirulina dissolved in distilled water in a dose of 500 mg/kg body weight orally *via* orogastric tube (Khan et al., 2006; Abdel-Daim et al., 2013). Group III (tacrolimus group) included 10 animals. The animals received orally tacrolimus (6.7 mg/kg body weight) once daily by orogastric tube. Tacrolimus was dissolved in distilled water. This dose was equivalent to 1/20 of LD50; 134 mg/kg (NIIRDN, 1994; Lewis, 2004). Group IV (prophylactic group: Tacrolimus + Spirulina) included 10 animals that were treated with spirulina and tacrolimus. Spirulina was given in a dose of 500 mg/kg body weight orally 3 days before and 28 days concomitantly with tacrolimus according to Khan et al. (2006) and Abdel-Daim et

*Corresponding author. E-mail: hebahosany@yahoo.com. Tel: 002-01016483450.

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al. (2013).

At the end of the experiment, the animals were weighed, then subjected to light ether anesthesia. Blood was collected through microcapillary tube from retro-orbital plexus and used for biochemical analysis. Rats were then sacrificed by decapitation. The obtained specimens from liver and kidney were divided into two parts. One part was frozen in liquid nitrogen (-170°C) and kept at -80°C for the determination of total thiol molecules (TTM). The other part was fixed immediately in 10% neutral buffered formalin and processed to get paraffin blocks for light microscopy examination. Five micrometers were stained with Haematoxylin and Eosin (H&E), and proliferating cell nuclear antigen (PCNA) immunostaining.

Biochemical study

Liver function tests

The activities of ALT and AST enzymes in serum were determined as described by Reitman and Frankel (1957). ALP activity was assayed according to the method of Belfield and Goldberg (1971). Total bilirubin and direct bilirubin were measured by the method of Walter and Gerade (1970). Serum albumin was determined colorimetrically according to the modified bromocresol green binding assay (BCG) (Tietz, 1990).

Kidney function tests

BUN and serum creatinine levels (mg/dl) have been measured according to the methods of Kaplan (1965) and Bjurosson (1979), respectively. Creatinine clearance (ml/min) as an index of glomerular filtration rate was calculated from serum creatinine and a 24 h urine sample creatinine levels using the formula: Creatinine clearance = (Urine creatinine (mg/dl)/Serum creatinine (mg/dl)) × (Urine volume (ml)/Urine collection time (h) × 60).

Oxidative stress markers

Serum total antioxidant capacity (mmol/l): The determination of the anti-oxidative capacity is performed by the reaction of antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide. The antioxidants in the sample eliminate a certain amount of the provided hydrogen peroxide. The residual hydrogen peroxide is assayed colorimetrically by enzymatic reaction which involves the conversion of 3,5-dichloro-2-hydroxyl benzensulphonate to a colored product (Koracevic et al., 2001).

Serum malondialdehyde (MDA, μmol/l): MDA was determined by measuring thiobarbituric reactive species using the method of Yagi (1998) in which the thiobarbituric acid-reactive substances react with thiobarbituric acid to produce a red colored complex with peak absorbance at 532 nm.

Total thiol molecules (TTM): TTM were measured in hepatic and renal tissues according to Sedlak and Lindsay's method (1968). Briefly, 0.2 ml Tris-HCl, 0.02 M EDTA buffer and 5,5'-Dithiobis-2-nitrobenzoic acid (in pure methanol) were added to test tubes containing tissue homogenate. The tubes were mixed and incubated for 15 min at room temperature, the samples were centrifuged at 3000 g for 10 min and ultimately the absorbance of the supernatant was measured at 412 nm. The TTM capacity was expressed as nmol per mg of protein in samples. Biodiagnostic kit (Dokki, Giza, Egypt) was used for the colorimetric determination of total protein in tissue homogenate.

Therapeutic drug monitoring: Tacrolimus trough levels (ng/ml)

were evaluated in blood at the end of the experiment 8 h after the last injection of tacrolimus by double antibody radioimmunoassay method (Winkler et al., 1995).

The lymphocyte proliferation assay (*in vitro*): The lymphocyte proliferation assay was done *in vitro* parallel to the experiment to investigate the influence of spirulina on the immunosuppressive effect of tacrolimus. It was done by isolation of peripheral blood lymphocytes by Histopaque density gradient centrifugation technique, the mononuclear cell layer was collected and washed three times with Hank's Balanced Salt Solution (300 ×g, 10 min) and resuspended in RPMI-1640 (Lonza, Germany). Isolated lymphocytes were incubated with tacrolimus at a concentration of 35 μg/L and combined tacrolimus and spirulina in a concentration of 35 and 250 μg/L, respectively for 2 h. The lymphocyte proliferation was measured by using XTT cell proliferation assay kit (ATCC) cat. no. 30-1011K according to the instruction manual and measuring the absorbance of the assay by ELISA BrdU (Colorimetric) kit (Roche Diagnostics, Penzberg, Germany).

Histological study

Specimens from the liver and kidney for light microscopy examination were fixed in 10% saline formalin and processed to prepare serial sections of 5-μm-thickness paraffin sections for (1) Haematoxylin and Eosin (H&E) stain (Wilson and Gamble, 2002), (2) immunohistochemically staining for localization of proliferating cell nuclear antigen (PCNA) reactivity (Ramos-Vara et al., 2008). PCNA was carried out by means of the avidin biotin-peroxidase complex method (Dako ARK™, Peroxidase, Code No. M0879, Dako, Glostrup, Denmark) following the manufacturer's instructions. Paraffin sections (5 μm) were dewaxed, hydrated and microwave-treated (0.01 M Trisodium citrate). Endogenous peroxidase was eliminated by incubation in 10% H₂O₂ in phosphate-buffered saline (PBS), pH=7 and 4. Sections were incubated with the specific primary antibody mouse monoclonal anti-PCNA antibody PC 10 (Dako, Santa Barbara, CA) at 1:20 dilution for 1 h. After 3 PBS washes, sections were incubated for 30 min with biotinylated rabbit anti-mouse immunoglobulin. After repeated washes with PBS, slides were incubated with avidin and biotinylated horseradish peroxidase (1:200) for 30 min. Diaminobenzidine tetrahydrochloride (DAB) was used as chromogen substrate-chromogen that resulted in a brown-colored precipitate at the antigen site. After repeated PBS washes, slides were counterstained in diluted hematoxylin and rehydrated. Sections of human lymph node with germinal centers served as positive control slides. All steps of immunohistochemistry were performed at room temperature in a humidity chamber. Negative control slides were made using the same previous steps except the primary antibody was replaced by buffer.

E-Morphometric analysis

Using image analyzer at Faculty of Dentistry, Ain shams University, the mean number of PCNA positive cells were measured. It was measured in randomly chosen five fields/section in five sections in all rats in each group at magnification of 400.

F-Statistical analysis

Data were represented as means ± standard deviation (SD). The differences were compared for statistical significance by analysis of variance (ANOVA) and student's t-test. Difference was considered significant at p < 0.05. The statistical analysis was performed using Epi-Info version 6.1 (Dean et al., 2000).

Table 1. Changes in the liver and kidney function tests in the studied groups.

Variable	Groups				ANOVA
	Control group (N= 10)	Spirulina group (N= 10)	Tacrolimus group (N= 10)	Tacrolimus + Spirulina group (N=10)	F-value
ALT (IU/l)	60.33 ± 6	58.19 ± 5.06 ^{NS}	128.28 ± 1.33 [#]	74.33 ± 1.76* ^{\$}	646.98
AST (IU/l)	119.17 ± 1.15	118.50 ± 1.22 ^{NS}	220.88 ± 1.35 [#]	130.85 ± 1.35* ^{\$}	15087.69
ALP (IU/l)	75.70 ± 1.67	74.51 ± 1.89 ^{NS}	175.18 ± 1.40 [#]	90.00 ± 0.52* ^{\$}	10759.67
Total bilirubin (mg/dl)	0.61 ± 0.03	0.59 ± 0.02 ^{NS}	1.63 ± 0.05 [#]	0.77 ± 0.01* ^{\$}	2495.73
Direct bilirubin (mg/dl)	0.31 ± 0.02	0.30 ± 0.01 ^{NS}	0.75 ± 0.04 [#]	0.35 ± 0.03* ^{\$}	622.56
Serum albumin (gm/dl)	5.6 ± 0.30	5.5 ± 0.20 ^{NS}	2.0 ± 0.04 [#]	5.2 ± 0.10* ^{\$}	840.63
Blood urea nitrogen (mg/dl)	41.40 ± 3.35	40.52 ± 2.55 ^{NS}	94.20 ± 5.89 [#]	58.40 ± 4.09* ^{\$}	364.47
Serum creatinine (mg/dl)	0.98 ± 0.09	0.99 ± 0.01 ^{NS}	1.80 ± 0.08 [#]	1.03 ± 0.03* ^{\$}	414.11
Creatinine clearance (ml/min)	42.1 ± 0.3	42.0 ± 0.5 ^{NS}	22.4 ± 0.1 [#]	37.8 ± 0.32* ^{\$}	2416.23

Data are expressed as mean ± standard deviation (SD). ^{NS}Non-Significantly different from control group. [#]Significantly different from the control group P < 0.001. *Significantly different from tacrolimus group P < 0.001. ^{\$}Significantly different from Spirulina group P < 0.001.

Table 2. Comparison of tacrolimus trough level between rats of tacrolimus group and protected group (spirulina + tacrolimus).

Variable	Groups		t-test	P-value
	Tacrolimus (N= 10)	Tacrolimus + Spirulina (N=10)	Student t-test value	
Tacrolimus trough level (ng/ml)	31 ± 4	28 ± 3 ^{NS}	1.9	> 0.05

Data is expressed as mean ± SD. ^{NS}Non-Significantly different from tacrolimus group.

RESULTS

Biochemical changes

General observation and body weight gain

During the whole period of the study, rats treated with tacrolimus showed decreased food intake as compared to other studied groups. There was a significant decrease (P < 0.05) in body weight gain (BWG%) for tacrolimus group as compared to the control group (8.7 g ± 2.1 vs. 36.5 g ± 3.5). On the other hand, there was a significant increase in BWG% in prophylactic group compared to the tacrolimus group (27.1 g ± 2.5 vs. 8.7 g ± 2.1, respectively).

Liver and kidney function tests

There was no statistical significant difference between control group and spirulina group regarding liver and kidney function tests as shown in Table 1. Rats treated by tacrolimus showed a significant increase in serum ALT, AST, ALP, total and direct bilirubin, as well as BUN, serum creatinine and a significant decrease in albumin and creatinine clearance compared to control rats. Pre- and co-treatment with Spirulina showed a significant improvement in these functional parameters in

comparison with tacrolimus-treated rats (Table 1).

Oxidative stress markers

There was a significant decrease in serum MDA and an increase in TAC as well as hepatic and renal TTM in spirulina-treated rats as compared with control group as shown in Table 2. Rats treated by tacrolimus showed a significant increase in serum MDA and a significant decrease in serum TAC as well as hepatic and renal TTM compared to control rats. Prophylactic group (spirulina + tacrolimus) showed a significant decrease in serum MDA and an increase in TAC as well as hepatic and renal TTM in comparison with tacrolimus-treated rats (Figure 1).

Therapeutic drug monitoring

As shown in Table 2, tacrolimus trough level did not differ in rats treated by tacrolimus either alone or in combination with spirulina.

Lymphocyte proliferation assay

There was a non-statistical significant difference in

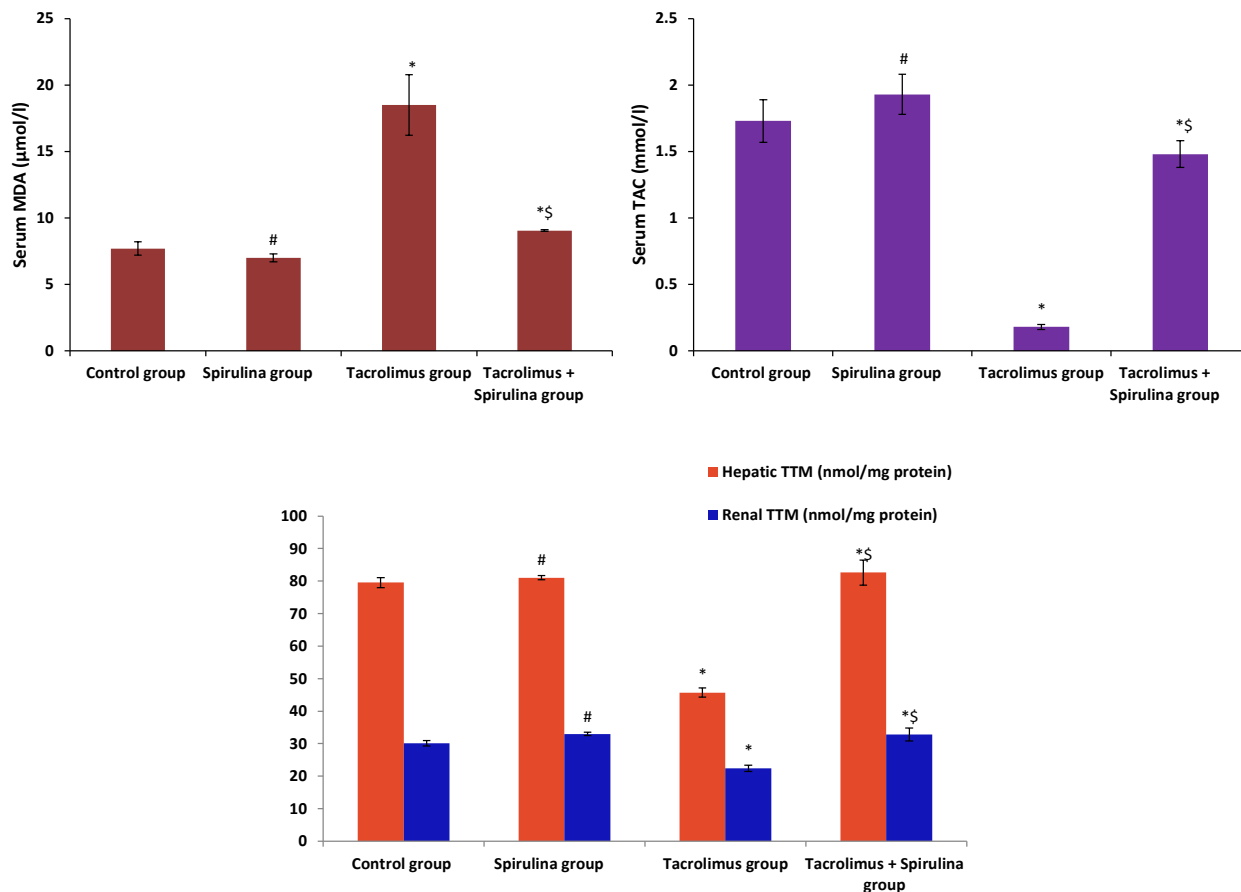


Figure 1. Changes of the oxidative stress markers in the studied groups. Data are expressed as mean \pm SD. #Significantly different from the control group $P < 0.05$. *Significantly different from tacrolimus group $P < 0.001$. §Significantly different from Spirulina group $P < 0.001$.

Table 3. Statistical comparison of lymphocyte proliferation assay between adult male albino rats of the treated group (tacrolimus) and the protected group (spirulina + tacrolimus).

Variable	Groups		t-test	P-value
	Tacrolimus	Tacrolimus + Spirulina	Student t-test value	
Lymphocyte proliferation assay	0.31 \pm 0.02	0.33 \pm 0.06 ^{NS}	1	> 0.05

Data is expressed as mean \pm SD. ^{NS}Non-Significantly different from tacrolimus group.

lymphocyte proliferation assay between tacrolimus group and protected group (spirulina + tacrolimus) (Table 3).

Histopathological changes

Histopathological changes in H&E stained sections

Groups I and II (Control and Spirulina groups): Light microscope examination of the liver of the control rats and spirulina treated rats showed hepatic lobules with

cords of hepatocytes with central vesicular nuclei radiating from the central vein and separated by blood sinusoids (Figure 2a). Examination of the renal cortex of the control and spirulina treated rats under light microscope showed normal renal corpuscles with glomeruli, Bowman's capsules lined by simple squamous epithelium. Proximal convoluted tubules (PCT) had eosinophilic cuboidal epithelium and narrow lumen, whereas distal convoluted tubules (DCT) had wide lumen (Figure 3a).

Group III (Tacrolimus group): Light microscope

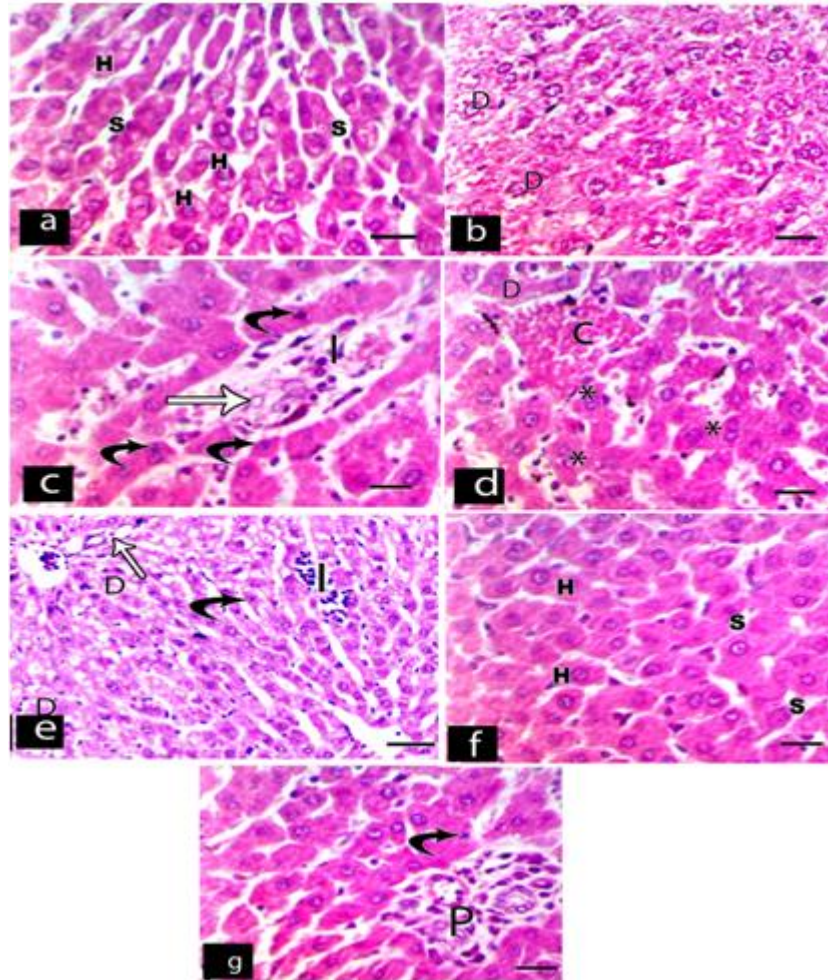


Figure 2. A photomicrograph of sections in the liver of adult male rats of different studied groups: a: group I (untreated control group); hepatocytes (H) well arranged in radiating cords, separated by blood sinusoids (S). b, c, d, e: group II (Tacrolimus group); degenerated, swollen hepatocytes (D) with granulated cytoplasm, multiple apoptotic figures (curved arrows), severe sinusoidal congestion (C) and inflammatory cellular infiltrates (I), and degenerated bile ductules (white arrow). f, g: group III (tacrolimus and Spirulina group); normal hepatocytes (H) well arranged in radiating cords, separated by blood sinusoids (S), and few apoptotic figures (curved arrows), and portal tracts showing bile ductules proliferation (P). H&E stained sections X 400 scale bar= 50 μ m.

examination of the liver revealed different changes in the hepatic lobule. Some hepatocytes showed swelling, degeneration and granulation of cytoplasm. Many degenerated bile ductile and sinusoidal congestion were also seen. There are also inflammatory cellular infiltrates and multiple apoptotic figures (Figure 2b to d). The renal cortex revealed different changes. Most of glomeruli are distorted. Some glomeruli are hypertrophied with enlarged malpighian corpuscles with congestion of glomerular capillaries. Others have widening of the capsular space or segmentation of the glomeruli. The glomeruli showed vacuolation. Proximal convoluted tubules lined with exfoliated degenerated cells and

presence of hyaline casts, some cells showing pyknotic nuclei were also observed. DCT showed vacuolation of cytoplasm and hylanosis. There was also inflammatory cellular infiltrates (Figure 3b to d). There was also inflammatory cellular infiltrates. Peritubular hemorrhage, capillary and vascular congestion were also seen (Figure 2b to d).

Group IV (Tacrolimus + spirulina group): Light microscope examination of liver sections of group IV revealed that hepatocytes preserved normal appearance and normal liver architectural, some cells showed mild degeneration with few apoptotic figures (Figure 2f and g).

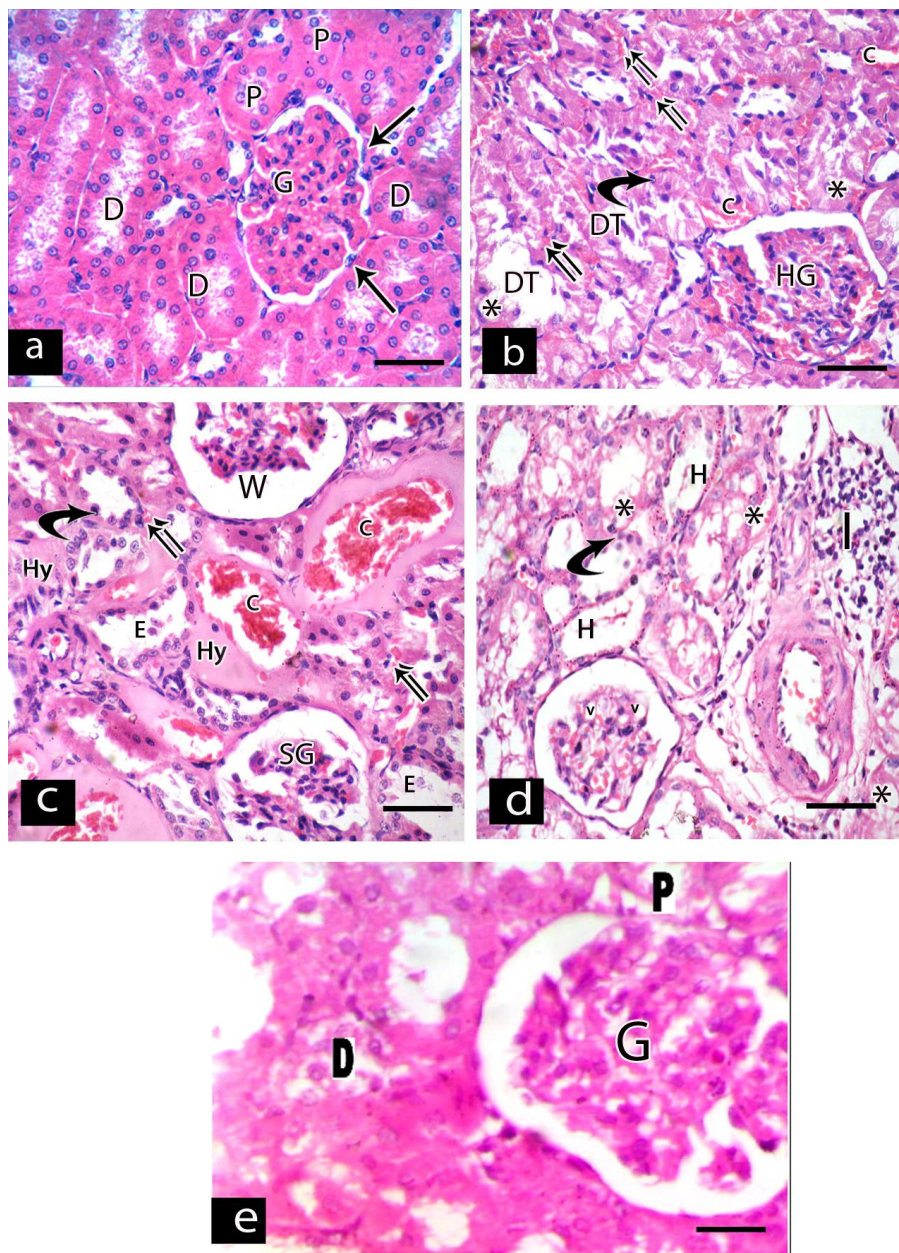


Figure 3. A photomicrograph of sections in the renal cortex of adult male rats of different studied groups. a: group I (untreated control group); showing normal glomeruli (G), with its capillary tufts surrounded by Bowman's capsule that are lined by simple squamous cells (arrows). Note the proximal convoluted tubules (P) with narrow lumen & intense acidophilic cells and distal convoluted tubules (D) with wide lumen & the pale acidophilic cells. b, c, d : group II (Tacrolimus group); showing that most of glomeruli are distorted . Some glomeruli are hypertrophied (HG) with enlarged malpighian corpuscles with congestion of glomerular capillaries. Others have widening of the capsular space (w) or segmentation of the glomeruli (SG). Glomerular cytoplasmic vacuolations (v) is seen. Most of renal tubules are distorted (DT). Exfoliated degenerated tubular cells (E), hyaline casts in the lumen, dark-stained pyknotic nuclei (curved arrow), numerous intracellular vacuoles in the tubular cells of proximal convoluted tubules & distal convoluted tubules (asterisk), and hyalinosis of the convoluted tubules (H) are observed. A focal areas of dense interstitial mononuclear cellular inflammatory cellular infiltrations (I), peritubular hemorrhage (double arrow) and peritubular capillary & vascular congestion (c) are seen. e: group III (Tacrolimus and Spirulina group); nearly normal glomeruli (G), mild degeneration in proximal convoluted tubules (P) and distal convoluted tubules (D). H&E stained sections X400; scale bar=50 μ m.

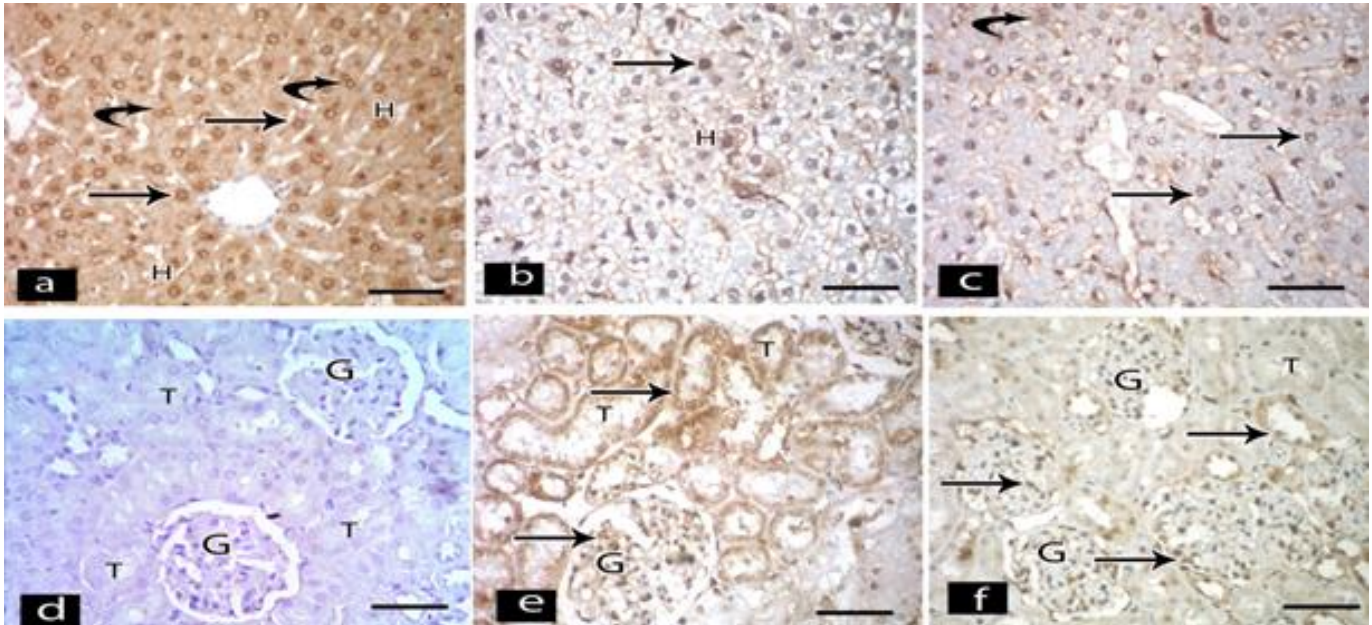


Figure 4. PCNA immunohistochemically stained sections in the liver (a-c) and in the renal cortex (d-f) of albino rats of different groups. (a & d) Control group; (b & e) tacrolimus group; (c & f) Tacrolimus + Spirulina group. PCNA stained sections in the liver (a-c); hepatocytes (H), multiple mitotic figures (curved arrows), positive nuclear immune reaction (arrows). a: showing strong expression of PCNA in most of nuclei & cytoplasm of hepatocytes (H) and mitotic figure was seen. b: showing disturbed hepatic structure, low expression of PCNA (arrows) in the irregular nuclei of hepatocytes (H). c: showing moderate expression of PCNA (arrows) in normal hepatocytes nuclei. Note multiple mitotic figures (curved arrows). PCNA stained sections in the renal cortex (d-f); Glomerulus (G), proximal & distal convoluted tubules (T), positive nuclear immune reaction for PCNA (arrows). d: showing negative immune reaction of PCNA in glomerular and in cells of the proximal & distal convoluted tubules in the control group e: showing strong positive nuclear & cytoplasmic reaction in many glomerular cells and tubular cells. f: showing positive nuclear reaction in few glomerular cells and few distal convoluted tubules cells. PCNA immunoperoxidase X 400, Scale bar: 50 µm.

Renal cortex of prophylactic group showed early mild hydropic degeneration and a few lesions (Figure 3e).

Histopathological changes of PCNA immunostained sections

The hepatic sections stained for proliferating cell nuclear antigen (PCNA) antibodies showed strong immune reaction in hepatocytes in the control and spirulina groups (Figure 4a). Tacrolimus group sections showed mild immune reaction in disrupted hepatocytes with irregular intended nuclei separated by irregular dilated hepatocytes (Figure 4b). Spirulina protected group revealed moderate nuclear reaction in most of hepatocytes with multiple mitotic figures (Figure 4c). The kidney sections stained for PCNA antibodies showed negative immune reaction in glomerular, PCT and DCT cells in the control and spirulina groups (Figure 4d). Tacrolimus group sections showed strong positive nuclear reaction in many glomerular cells and some tubular cells (Figure 4e). Spirulina protected group revealed nuclear reaction in few glomerular cells and positive immunoreaction in few PCT and DCT cells (Figure 4f).

Morphometric results

The mean number of PCNA immunostained cells/high power field (HPFs) showed a non-significant difference between control group and spirulina group in both liver and kidney specimens. Regarding the mean number of PCNA immunostained hepatocytes/HPFs in tacrolimus group compared with the control, there was a highly significant decrease, but tacrolimus plus spirulina group showed a highly significant increase compared with tacrolimus group that was non-significant compared with control (Table 4). However, there was a highly significant increase in the mean number of PCNA immunostained renal tubular cells/HPFs in tacrolimus group compared with the control, but tacrolimus + spirulina group showed a highly significant decrease compared with tacrolimus group and a non-significant increase compared with control (Table 4).

DISCUSSION

Tacrolimus is an immunosuppressive drug that binds to protein and inhibits the phosphatase activity of calcineurin in T lymphocytes to reduce the activity of the

Table 4. The mean number of PCNA positive cells/high power field (HPFs) in different groups.

Group	Control	Spirulina	Tacrolimus	Tacrolimus + Spirulina
Area percentage of PCNA stained hepatocytes/HPFs	10.09±1.03	10.28±1.00	2.04±0.47 [#]	6.10±0.03 ^{\$}
Area percentage of PCNA stained renal tubular cells/HPFs	1.4±0.13	1.73±0.14	19.28±1.27 [#]	4.52±0.66 ^{\$}

Data Values are expressed as mean± SD of n=6 animals. [#]Significantly different from the control group P < 0.001. ^{*}Significantly different from tacrolimus group P < 0.001. ^{\$}Significantly different from Spirulina group P < 0.001.

patient's immune system and so lower the risk of organ rejection (Naesens et al., 2009). It is a potent immunosuppressive agent that is used to treat solid organ transplant recipients, and it has played a large role in the improvement of graft survival rates. However, especially in high doses, it can induce renal toxicity and cholestatic hepatitis (Taniai et al., 2008). Therefore, the objective of the present work was to demonstrate the possible protective role of spirulina against the hepatic and renal damage induced by tacrolimus.

In the present study, tacrolimus treatment induced variable toxic effects, evidenced with a marked reduction in the BWG%; more than 75% decrease compared to control; there was also significant impairment in liver and kidney function tests. Tacrolimus administration induced significant elevations in AST, ALT, ALP, total and direct bilirubin which reach 1.5 or more times the upper limit of control group. These results were in agreement with studies of Taniai et al. (2008) who reported that tacrolimus produced increase in ALT, AST activities and total bilirubin level. Singh and Watt (2012) found also that many patients taking tacrolimus had a long term mild increase in liver enzymes. Elevated serum level of hepatic enzymes indicate liver damage, cellular leakage and loss of functional integrity of hepatocytes (Mishra et al., 2015). Supporting these notions, we found that tacrolimus induced histopathological changes including swelling of hepatocytes, granulation of cytoplasm, liver congestion, degenerated bile ductules, inflammatory cellular infiltrates and inflammatory cellular infiltrates. Similar findings were detected by Yadav et al. (2013) who found that tacrolimus induced hepatotoxicity in the form of cholestatic hepatitis and liver congestion.

Pre- and concomitant administration of spirulina with tacrolimus here significantly reversed tacrolimus induced changes in liver function tests. Thus, this reduction in the hepatic enzymes activities clearly pointed to the membrane stabilizing activity of spirulina. Reduction in the levels of AST, ALT, ALP and bilirubin towards the control values is an indicator of the protective effects of spirulina. The histological examination of the liver sections confirmed the aforementioned results where spirulina pre- and co-administration along with tacrolimus can restore the normal cellular architecture of the liver and reverse tacrolimus induced histopathological effects. In line with this, previous studies showed that spirulina returned the elevated serum levels of hepatic enzymes

near to normal levels in deltamethrin-intoxicated rats and other models of toxicity through its potent antioxidant and free radical-scavenging activities (Abdel-Daim et al., 2013; Abdel-Daim, 2014; Abdel-Daim et al., 2016).

Regarding tacrolimus induced nephrotoxicity in the current study, there were also significant elevations in BUN, serum creatinine and a significant reduction of creatinine clearance in tacrolimus treated group, in agreement with Abdel-Daim et al. (2013, 2016). Similar results were also reported by Di Benedetto et al. (2009) who found a significant increase of serum creatinine (>1.8 mg/dl) in patients developing renal dysfunction following liver transplantation due to calcineurin inhibitors. In concordance, the results obtained from the present study showed that microscopical examination of the kidney of adult albino rats treated with tacrolimus showed vacuolation of glomeruli and distal tubule. Banhara et al. (2015) reported that distal tubular dysfunction is prevalent among kidney transplant patients using tacrolimus. Moreover, Boudjema et al. (2011) suggested that tacrolimus induced nephrotoxicity is dose-dependent in transplant patients. Nephrotoxicity is a major clinical obstacle related to tacrolimus and is usually responsible for the discontinuation of treatment (Porayko et al., 1994). This is in agreement with Gaston (2006), where tacrolimus induced nephrotoxicity as manifested by severe interstitial fibrosis, peritubular calcification, and focal glomerulosclerosis; these changes may result in irreversible chronic renal failure in patients undergoing renal transplantation patients. Furthermore, other changes were observed in the kidney including swelling of proximal tubules, hyalanosis and presences of hyaline casts in proximal and distal tubules. These changes were similar to the results of Randhawa et al. (1997).

Although spirulina has demonstrated protection against multiple drug and toxin-induced systemic toxicity (Khan et al., 2006; Alam et al., 2013; Abdel-Daim et al., 2016; Bashandy et al., 2016) its protective effect on tacrolimus-induced toxic injury has never been investigated. This prompted us to evaluate whether and how, spirulina may ameliorate tacrolimus-induced hepato and nephrotoxicity. Accordingly, when rats administered spirulina concomitantly with tacrolimus, liver and kidney function tests returned near to control values, suggesting the cytoprotective ability of spirulina in liver and kidney cellular integrity, restoring their normal functions.

Spirulina was previously proven to have potent

antioxidant activities (Romay et al., 1998; Lissi et al., 2000; Chu et al., 2010). These activities were largely related to phycocyanin protein of spirulina. This protein contains a tetrapyrrole phycocyanobilin, which has been reported to have a significant antioxidant and radical scavenging properties, offering protection against oxidative stress (Bashandy et al., 2016). Similarly, in the current study, spirulina treatment had significantly improved the antioxidant parameters (serum TAC, hepatic and renal TTMs) compared to the control group. In confirm, a recent study has indicated that spirulina shows free radical scavenging and potent antioxidant activity during deltamethrin intoxication (Abdel-Daim et al., 2015; Abdelkhalek et al., 2015; Abdel-Daim et al., 2016). Furthermore, spirulina contains superoxide dismutase that exerts indirect action by retarding oxygen radical generating reactions rate (Belay, 2002; EL-Sabagh et al., 2014).

Supportive data were provided from the present histologic and immunohistochemistry studies, where spirulina co-administration ameliorated tacrolimus induced hepatocellular and renal cellular regeneration and proliferation in H&E stained section that were further supported by PCNA immunostaining. Spirulina protected group showed partial restoration of immunoreaction to PCNA in most of the hepatocytes and renal cells comparable to control rats. Ozaki et al. (2001) studied the role of spirulina in reducing nephrotoxicity, cellular hyperplasia and PCNA overexpression in peroxisome proliferators. Moreover, Makhoulf and Makhoulf (2012), tested the hepatoprotective effect of spirulina against ionizing radiation induced liver injury; they found spirulina could significantly increase hepatocytes DNA content and proliferation, the authors explained these effects by abundant content of spirulina of beta carotene and superoxide dismutase.

An additional objective in this study was to evaluate the possibilities of interaction between tacrolimus and spirulina that can reduce therapeutic efficacy of tacrolimus. Both the trough level of tacrolimus and lymphocyte proliferation assay did not change significantly in absence and presence of spirulina.

Conclusively, it was shown that orally administered spirulina may be associated with a decrease in tacrolimus induced hepatotoxicity and nephrotoxicity in adult male albino rats. Further studies are warranted to evaluate whether transplant patients on tacrolimus treatment may benefit from the protective effects of spirulina.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Abdel-Daim MM, Abuzead SM, Halawa SM (2013). Protective role of

- Spirulina platensis against acute deltamethrin-induced toxicity in rats. PLoS One 8:e72991.
- Abdel-Daim MM (2014). Pharmacodynamic interaction of Spirulina platensis with erythromycin in Egyptian Baladi bucks (*Capra hircus*). Small Ruminant Res. 120:234-241.
- Abdel-Daim MM, Farouk SM, Madkour FF, Azab SS (2015). Anti-inflammatory and immunomodulatory effects of Spirulina platensis in comparison to Dunaliella salina in acetic acid-induced rat experimental colitis. Immunopharmacol. Immunotoxicol. 37:126-139.
- Abdel-Daim M, El-Bialy BE, Rahman HG, Hefny HA, Hassan AM (2016). Antagonistic effects of Spirulina platensis against sub-acute deltamethrin toxicity in mice: Biochemical and histopathological studies. Biomed. Pharmacother. 77:79-85.
- Abdelkhalek NK, Ghazy EW, Abdel-Daim MM (2015). Pharmacodynamic interaction of Spirulina platensis and deltamethrin in freshwater fish Nile tilapia, *Oreochromis niloticus*: impact on lipid peroxidation and oxidative stress. Environ. Sci. Pollut. Res. Int. 22: 3023-3031.
- Abou Gabal AA, Aboul-Ela HM, Ali EM, Khaled AEM, Shalaby OK (2015). Hepatoprotection, DNA damage prevention and antioxidant potential of spirulina platensis on CCl₄- induced hepatotoxicity in mice. Am. J. Biomed. Res. 3(2):29-34.
- Alam MA, Haider N, Ahmed S, Alam MT, Aziz A, Perveen A (2013). Tahlab (Spirulina) and few other medicinal plants having anti-oxidant & immunomodulatory properties described in Unani medicine- A review. Int. J. Pharmaceut. Sci. Res. 4: 4158-4164.
- Banhara PB, Gonçalves RT, Rocha PT, Delgado AG, Leite M Jr, Gomes CP (2015). Tubular dysfunction in renal transplant patients using sirolimus or tacrolimus. Clin. Nephrol. 83(6):331-337.
- Bashandy SA, El Awdan SA, Ebaid H, Alhazza IM (2016). Antioxidant potential of spirulina platensis mitigates oxidative stress and reprotoxicity induced by sodium arsenite in male rats. Oxid. Med. Cell. Longev. 7174351.
- Belay A (2002). The potential application of Spirulina (Arthrospira) as a nutritional and therapeutic supplement. in health management, Review. J. Am. Nutraceut. Assoc. 5:27-48.
- Belfield A, Goldberg DM (1971). Revised assay for serum phenyl phosphatase activity using 4-amino-antipyrine. Enzyme 12(5):561-573.
- Bjuorsson TD (1979). Use of serum creatinine concentration to determine renal function. Clin. Pharmacol. 28:477-481.
- Boudjema K, Camus C, Saliba F, Calmus Y, Salamé E, Pageaux G, Ducerf C, Duvoux C, Mouchel C, Renault A, Compagnon P, Lorho R, Bellissant E (2011). Reduced-dose tacrolimus with mycophenolatemofetil vs. standard-dose tacrolimus in liver transplantation: A randomized study. Am. J. Transplant. 11:965-976.
- Chu WL, Lim YW, Radhakrishnan AK, Lim PE (2010). Protective effect of aqueous extract from spirulina platensis against cell death induced by free radicals. BMC Complement. Altern. Med. 10:53.
- Dean AG, Dean JA, Coulombier D, Brendel KA, Smith DC, Burton AH, Dicker RC (2000). Epi-Info (6.1): A word processing data base and statistical program for epidemiology and microcomputer office, Center for disease control, Atlanta Georgia, USA.
- Di Benedetto F, Di Sandro S, DeRervo H, Spaggiari M, Montalti R, Ballarin R, Cappelli G, Gerunda GE (2009). Sirolimus monotherapy effectiveness in liver transplant recipients with renal dysfunction due calcineurin inhibitors. J. Clin. Gastroenterol. 73(3):280-286.
- EL-Sabagh MR, Eldaim MAA, Mahboub D, Abdel-Daim M (2014). Effects of Spirulina platensis algae on growth performance, antioxidative status and blood metabolites in fattening lambs. J. Agric. Sci. 6:92.
- Fruman DA, Klee CB, Bierer BE, Burakoff SJ (1992). Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A. Proc. Natl. Acad. Sci. U S A. 89(9):3686-3690.
- Gaston RS (2006). Current and evolving immunosuppressive regimens in kidney transplantation. Am. J. Kidney Dis. 47(4):S3-21.
- Grunot E, Elinuv H, Kohen R (2002). Markers of oxidative stress in cyclosporine-treated and tacrolimus-treated children after liver transplantation. Liver Transpl. 8(5):469-476.
- Hayashi O, Katoh T, Okuwaki Y (1994). Enhancement of antibody production in mice by dietary *Spirulina platensis*. J. Nutr. Sci. Vitaminol. 40:431-441.

- Hemalatha K, Pugazhendy K, Jayachandran K, Jayanthi C, Meenambal M (2012). Studies on the Protective Efficacy of Spirulina against Lead Acetate Induced Hepatotoxicity in *Rattus norvegicus*. *Int. J. Chem. Anal. Sci.* 3:1509-1512.
- Ibrahim AE, Abdel-Daim MM (2015) Modulating Effects of Spirulina platensis against Tilmicosin-Induced Cardiotoxicity in Mice. *Cell J.* 17:137-144.
- Institute of Laboratory Animal Resources (ILAR) (1996). Guide for the Care and Use of Laboratory Animals, 7th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington DC.
- Jantz AS, Patel SJ, Suki WN, Knight RJ, Bhimaraj A, Gaber AO (2013). Treatment of acute tacrolimus toxicity with phenytoin in solid organ transplant recipients. *Case Rep. Transplant.* 2013.
- Kaplan A (1965). Urea Nitrogen and Urinary Ammonia. In: *Satndard Methods of Clinical Chemistry*. Meites S. (Ed.). pp. 245-256. Academic Press, New York.
- Khan M, Shobha JC, Mohan IK, Rao Naidu MU, Prayag A, Kutala VK (2006). Spirulina attenuates cyclosporine-induced nephrotoxicity in rats. *J. Appl. Toxicol.* 26(5):444-451.
- Kim H, Lee E, Cho H, Moon Y (1998). Inhibitory effect of mast cell-mediated immediate-type allergic reactions in rats by spirulina. *Biochem. Pharmacol.* 55:1071-1076.
- Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V (2001). Method for the measurement of antioxidant activity in human fluids. *J. Clin. Pathol.* 54:356-417.
- Kumar N, Singh S, Patro N, Patro I (2010). Evaluation of protective efficacy of Spirulina platensis against collagen-induced arthritis in rats. *Inflammopharmacology* 17(3):181-190.
- Levis RJ Sr (2004). *Sax's Dangerous Properties of Industrial Materials*. 11th Edition. (2004). Wiley-Interscience, Wiley & Sons, Inc. Hoboken, NJ. P 3344.
- Lissi EA, Pizarro M, Aspee A, Romay C (2000). Kinetics of phycocyaninebilin groups destruction by peroxy radicals. *Free Radic. Biol. Med.* 28(7): 1051-1055.
- Makhlouf R, Makhlouf I (2012). Evaluation of the effect of spirulina against gamma irradiation induced oxidative stress and tissue injury in rats. *Int. J. Appl. Sci. Eng. Res.* 1:152-164.
- Mishra G, Khosa RL, Singh P, Jha KK (2015). Hepatoprotective potential of ethanolic extract of Pandanus odoratissimus root against paracetamol-induced hepatotoxicity in rats. *J. Pharm. Bioallied Sci.* 7(1):45-48.
- Naesens M, Kuypers DR, Sarwal M (2009). Calcineurin inhibitor nephrotoxicity. *Am. Soc. Nephrol.* 4(2):481-508.
- NIIRDN Drugs in Japan (Ethical Drugs). (1995). (Yakugyo Jiho Co., Ltd., Tokyo, Japan) – P 7200.
- Ozaki K, Joel FM, Joseph KH, Cindy RM, Matthew LN, Nyska A (2001). Unique Renal Tubule Changes Induced in Rats and Mice by the Peroxisome Proliferators 2,4-Dichlorophenoxyacetic Acid (2,4-D) and WY-14643. *Toxicol. Pathol.* 29(4):440-450.
- Piao SG, Lim SW, Doh KC, Jin L, Heo SB, Zheng YF, Bae SK, Chung BH, Li C, Yang CW (2014). Combined treatment of tacrolimus and everolimus increases oxidative stress by pharmacological interactions. *Transplantation* 98(1):22-28.
- Porayko MK, Textor SC, Krom RA, Hay JE, Gores GJ, Richards TM, Crotty PH, Beaver SJ, Steers JL, Wiesner RH (1994). Nephrotoxic effects of primary immunosuppression with FK-506 and cyclosporine regimens after liver transplantation. *Mayo Clin. Proc.* 69(2):105-111.
- Premkumar K, Abraham SK, Santhiya ST, Ramesh A (2004). Protective effect of spirulina fusiform on chemical induced genotoxicity in mice. *Fitoterapia* 75(1):24-31.
- Ramos-Vara JA, Kiupel M, Baszier T, Bliven L, Brodersen B, Chelack B, Czub S, Del Piero F, Dial S, Ehrhart EJ, Graham T, Manning L, Paulsen D, Valli VE, West K (2008). American Association of Veterinary Laboratory Diagnosticians Subcommittee on Standardization of Immunohistochemistry Suggested guidelines for immunohistochemical techniques in veterinary diagnostic laboratories. *J. Vet. Diagn. Invest.* 20:393-413.
- Randhawa PS, Starzl TE, Demetris AJ (1997). Tacrolimus (FK 506) associated renal pathology. *Adv. Anat. Pathol.* 4 (4):265-276.
- Rasool M, Sabina EP (2009). Appraisal of immunomodulatory potential of *Spirulina fusiformis*: An *in vivo* and *in vitro* study. *J. Nat. Med.* 63(2):169-175.
- Reitman S, Frankle S (1957). Glutamic oxaloacetic transaminase colorimetric method. *Am. J. Clin. Pathol.* 28:56.
- Remirez D, Gonzalez R, Merino N, Rodriguez S, Ancheta O (2002). Inhibitory effects of Spirulina in zymosan-induced arthritis in mice. *Mediators Inflamm.* 11:75-79.
- Romay C, Armesto J, Remirez D, González R, Ledon N, García I (1998). Antioxidant and anti-inflammatory properties of C-phycocyanin from blue-green algae. *Inflamm. Res.* 47(1):36-41.
- Sedlak J, Lindsay RH (1968). Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* 25(1):192-205.
- Singh S, Watt KD (2012). Long term medical management of liver transplant recipient. *Mayo Clin. Proc.* 87(8):779-790.
- Tanaka H, Kuroda A, Marusawa H, Hashimoto M, Hatanaka H, Kino T, Goto T, Okuhara M (1987). Physicochemical properties of FK506, a novel immunosuppressant isolated from *Streptomyces tsukubaensis*. *Transplant. Proc.* 19(5 Suppl 6):11-16.
- Taniai N, Akimaru K, Ishikawa Y, Kanada T, Taakinuma D, Mizenguchi Y, Mamada Y, Yoshida H, Takiri T (2008). Hepatotoxicity caused by tacrolimus and cyclosporine after living donor liver transplantation. *J. Nippon. Med. Sch.* 75 (3):187-191.
- Tietz NW (1990). *Clinical guide to laboratory tests*. 2. Philadelphia: WB Saunders. P 566.
- Upasani CD, Balaraman R (2003). Protective effect of Spirulina on lead induced deleterious changes in the lipid peroxidation and endogenous antioxidants in rats. *Phytother. Res.* 17:330-334.
- Walter M, Gerade H (1970). Bilirubin assay. *Microchem. J.* 15:231-236.
- Wilson I, Gamble M (2002). The hematoxylin and eosins, In: *Theory and practice of histological techniques*, Bancroft JD, Gamble M (Eds.). 5th ed., Churchill Livingstone, Elsevier Science Limited, London, UK, pp. 125-138.
- Winkler M, Wonigeit K, Undre N, Ringe B, Oldhafer K, Christians U, Pichlmayr R (1995). Comparison of plasma vs whole blood as matrix for FK 506 drug level monitoring. *Transplant. Proc.* 27(1):822-825.
- Yadav DK, Gera DN, Gumber MR, Kute VB, Patel MP, Vanikar AV, Trivedi HL (2013). Tacrolimus induced severe cholestasis complicating renal transplantation. *Ren. Fail.* 35(5):735-737.
- Yagi K (1998). Simple assay for the level of total lipid peroxides in serum or plasma. *Methods Mol. Biol.* 108:101-106.