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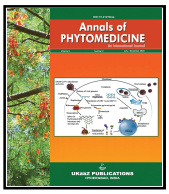

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Protective effect of different parts of *Terminalia catappa* L. in the hematological and biochemical profiles of immunosuppressed mice

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Abstract

Desi badam botanically equated as *Terminalia catappa* L. belongs to the family Combretaceae. This tropical tree has gained significance because of the therapeutic potentials of leaves since time immemorial. Existing evidences to validate the medicinal properties of other parts such as bark, fruits, wood are limited. In our previous studies, we have standardized and validated the aqueous extract of bark, fruits and wood of *T. catappa* using botanical, chemical and analytical protocols. *In vitro* experiments were also conducted to evaluate the antioxidant, anti-inflammatory, anticancer and antimicrobial properties. In the present study, animal experiments are carried out to evaluate the protective effect of aqueous extract of different parts of *T. catappa* on cyclophosphamide induced immune-suppressive mice. Low, medium and high doses (125, 250, 500 mg/kg) of the selected plant parts were administered to Swiss albino mice. The effect of test extracts on hematological profile, biochemical profile and histopathological profile was compared with cyclophosphamide (immunosuppressant) treated and levamisole (immunostimulant) treated mice. From the results obtained, it could be concluded that bark, fruit and wood of *T. catappa* ameliorated cyclophosphamide induced cytotoxicity in mice. Among the different doses, medium dose of the test extracts was more efficacious than the low and high doses.

1. Introduction

Cyclophosphamide is a well-known cancer chemotherapeutic agent and widely used in the treatment of various types of cancers such as lymphoma, myeloma and chronic lymphocytic leukaemia (Pass *et al*., 2005; Sultana *et al*., 2011). Unfortunately, immunosuppression induced by cyclophosphamide *via* DNA cross linking of actively dividing cells (Oger, 2007) increases incidence of secondary infections and mortality (Kajaria *et al*., 2013; Hamrita *et al*., 2012), which is a major limiting factor in clinical chemotherapy (Maschmeyer *et al*., 2007). Therefore, many attempts are being made to obtain immunomodulatory agents from plant kingdom that can reduce the cytotoxic side effects of cyclophosphamide and enhance immunity in chemotherapy-treated patients (Balekar *et al*., 2014; Yadav *et al*., 2015; Sibi and Varghese, 2014; Shruthi *et al*., 2018).

Terminalia catappa L. (TC), a large spreading tree belonging to the family Combretaceae, is distributed throughout the tropics in coastal environments. Various extracts of leaves and bark of TC have been reported to exhibit antibacterial (Neelavathi *et al*., 2013; Sangavi *et al*., 2015), anti-fungal (Parimala Gandhi *et al*., 2015), anti-inflammatory (Sivaranjani *et al*., 2015; Venkatalakshmi *et al*., 2015), antioxidant and antitumour (Venkatalakshmi *et al*., 2014) activities. LC-ESI-MS/MS analysis revealed the presence of Isorhamnetin in bark, fruit and wood, Rottlerin and Limocitrin in bark and wood, and Iristectorin-A in fruit and wood. The same study revealed that the aqueous extract of fruit is more effective in controlling the growth of EAC cell lines (84.97%) when compared to bark (67.89%) and wood (37.34%) of TC (Venkatalakshmi *et al*., 2016a).

A detailed literature survey was carried out to learn the phytopharmacological significance of different parts of TC (Venkatalakshmi *et al*., 2016b). Safety profiles of the aqueous extracts of bark, fruits and wood of TC were evaluated through acute oral toxicity studies. Oral administration of test substances at a dose level of 2000 mg/kg body weight caused no adverse toxic effects and suggested that the test substances are safe to consume (Venkatalakshmi and Brindha, 2020). In the present study, protective effect of the aqueous extract of different parts of TC was evaluated in altered hematological and biochemical profiles of cyclophosphamide induced immune-suppressive mice model.

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2. Materials and Methods

2.1 Collection and authentication of plant materials

Plant parts such as bark, fruits and wood of TC were collected from farm houses and Gardens in and around Mannargudi, Tamil Nadu, India. The identity of the plant specimens was confirmed using Flora of Presidency of Madras (Gamble, 1997). The botanical identity was authenticated by comparing with the herbarium specimen deposited at RAPINAT Herbarium, St. Joseph's College, Tiruchirappalli, Tamil Nadu, India (Voucher specimen number P.N.001/2012). Medicinally useful parts of the plants were studied in both fresh and dried conditions as per the standard textual methods (Trease and Evans, 1983).

2.2 Experimental animals

The study was conducted at the Central Animal Facility registered (No.817/04/ac/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India. The study was conducted after the approval by the Institutional Animal Ethical Committee, SASTRA University (IAEC Approval Number: 166/SASTRA/IAEC/RPP). Swiss albino mice of either sex (72 numbers) were used to evaluate the protective effect of TC on cyclophosphamide induced immuno suppressed mice. Among the different parts available, we have chosen bark, fruit and wood of the plant for the present study. Three doses low (125 mg/kg), medium (250 mg/kg) and high (500 mg/kg) of aqueous extracts of bark, fruits and wood of TC were prepared as per standard protocol (Brindha *et al*., 1981). Levamisole was used as a standard drug.

2.3 Experimental design

Animals were divided into twelve groups, each comprising of six mice. Treatments given to each group and work plan were as follows. Study period was 14 days. Mice from all groups were immunized by injecting 0.1 ml of 20% sheep erythrocytes (SRBCs) intraperitoneally (*i.p.*) on day 7.

2.4 Determination of haematological parameters

The blood samples were collected from individual animals of all the groups by retro orbital bleeding. To estimate haematological parameters, blood was mixed with ethylene diamine tetra acetate (EDTA) and fed to the autoanalyzer (GENESIS™ Veterinary Haematology Analyzer) and parameters such as total WBC count, differential WBC count, RBC profile and platelet profile were evaluated (Prabhu *et al*., 2013).

2.5 Estimation of blood glucose (Folin and Wu, 1919)

A volume of 0.1 ml of the plasma was added to 3.4 ml of water, 0.2 ml of 10% sodium tungstate and 0.2 ml of 2/3 N sulphuric acid in order to precipitate the protein. The solution was mixed well and centrifuged (3000 rpm,10 min). To 1 ml of the filtrate, 2 ml of alkaline copper sulphate solution was added and placed in a boiling water bath for 8 min, cooled and added 2 ml of phosphomolybdic acid.

Group I Immunized control	Vehicle only (Distilled water)
Group II Negative control	Cyclophosphamide (100 mg/kg/p.o.) 3rd, 6th, 9th days
Group III Negative control + Standard drug	Cyclophosphamide (100 mg/kg/p.o.) 3rd, 6th, 9th days + Levamisole (50 mg/kg/p.o.)
Groups IV, V and VI	Cyclophosphamide (100 mg/kg/p.o.) 3rd, 6th, 9th days + Aqueous extract of TC bark (125, 250, 500 mg/kg., p.o., respectively)
Groups VII, VIII and IX	Cyclophosphamide (100 mg/kg/p.o.) 3rd, 6th, 9th days + Aqueous extract of TC fruit (125, 250, 500 mg/kg., p.o., respectively)
Groups X, XI and XII	Cyclophosphamide (100 mg/kg/p.o.) 3rd, 6th, 9th days + Aqueous extract of TC wood (125, 250, 500 mg/kg., p.o., respectively)

Various concentrations of glucose standard solution were prepared and made up to 1 ml with water. After adding 2 ml of alkaline copper sulphate solution, the mixture was placed in a boiling water bath for 8 min, cooled and added 2 ml of phosphomolybdic acid. A blank was also maintained. Glucose reduces the cupric ions present in the alkaline copper reagent to cuprous ions, which reduces the phosphomolybdic acid to phosphomolybdous acid, a blue coloured compound which was read at 620 nm in a Systronics 119 UV/VIS Spectrophotometer. The amount of glucose present in the sample was estimated using a standard graph of glucose. The glucose content was expressed as mg/dl.

2.6 Estimation of serum cholesterol (Zak *et al.*, 1953)

A volume of 0.1 ml of sample was added to 4.9 ml of ferric chloride precipitating reagent, mixed well and centrifuged (2000 rpm). From this, 2.5 ml of filtrate was added to a mixture of 2.5 ml diluting reagent and 4 ml concentrated sulphuric acid and mixed thoroughly. Various concentrations of cholesterol standard solutions were prepared and made up to 5 ml with diluting reagent. A volume of 4 ml of sulphuric acid was added to all the tubes. A blank was also maintained and the colour developed was read at 560 nm.

2.7 Estimation of protein (Lowry *et al*., 1951)

A volume of 0.1 ml of diluted sample (serum/tissue) was made upto 1.0 ml with water and 4.5 ml of alkaline copper reagent was added to all the tubes including blank, containing 1.0 ml of water. The tubes were incubated for 10 min at room temperature. Then 0.5 ml of Folin's Phenol reagent was added to all the tubes and further incubated for 20 min at room temperature. The colour developed was read at 620 nm using blank. Aliquots of protein standard were also treated as above. The protein content was expressed as g/dl.

2.8 Estimation of albumin (Dandekar, 2002)

A volume of 0.2 ml serum was pipetted in-to the test tube. To this, 5.8 ml of sodium sulphate and 2 ml of ether were added and mixed by inversion. The mixture was allowed to stand for 30 min, before centrifuging (rpm) for 10 min. The formed globulin was carefully detected at the bottom, at the junction of water and ether layer, and removed using capillary chopper, the clear filtrate was used for estimation of albumin content. Four test tubes were prepared for colour development. To the first tube, 3 ml of water was added. A volume of 3 ml standard protein solution was added to second tube. 0.1 ml of serum and 2.9 ml of sodium chloride added to third tube, and 3 ml of above filtrate was added to the fourth tube. To all the tubes, 3 ml of biuret reagent was added and mixed and after 10 min the colour developed was read at 520 nm.

2.9 Estimation of aspartate transaminase [EC 2.6.1.57] (King, 1965)

The assay mixture containing 1 ml of substrate (Aspartate) and 0.2 ml of serum was incubated for 1 h at 37°C. To this, 1 ml of dinitro phenyl hydrazine (DNPH) was added and kept at room temperature for 20 min. Various concentrations of pyruvate was used as standard. Serum was added to the control tubes after the reaction was arrested by the addition of 1 ml of DNPH. To this, 5 ml of NaOH was added and the colour developed was read at 520 nm. The level of AST was expressed as U/L. The oxaloacetate formed will react with aniline citrate converting it to pyruvate. Pyruvate reacts with DNPH to give 2,4, dinitro phenyl hydrazine of pyruvate (brown colour). The intensity of the colour is read at 520 nm

2.10 Estimation of alanine transaminase [EC 2.6.1.2] (King, 1965)

The assay mixture containing 1 ml of substrate (Alanine) and 0.2 ml of serum was incubated for 1 h at 37°C. Then 1 ml of DNPH was added and kept at room temperature for 20 min. Various concentrations of pyruvate were used as standard. Serum was added to control tubes after the reaction was arrested by the addition of 1 ml of DNPH. Then 5 ml of NaOH was added. The keto acid pyruvate resulted from ALT activity coupled with 2,4-DNPH formed hydrazone, which gave a brown colour in alkaline medium, and was read at 540 nm using Systronics 119 UV/VIS Spectrophotometer. The level of ALT was expressed as Units/l.

2.11 Estimation of serum alkaline phosphatase [EC 3.1.3.1] (King, 1965)

The reaction mixture containing 1.5 ml carbonate buffer, 1 ml di-sodium phenyl phosphate, 0.1 ml magnesium chloride and 0.1 ml of serum was incubated at 37°C for 15 min. The reaction was arrested by the addition of Folin's Ciocalteau phenol reagent. Control tubes were also treated similarly but serum was added after the reaction was arrested with Folin's phenol reagent and then added 1 ml of sodium carbonate. Various concentrations of phenol were used as standard. ALP converts phenyl phosphate to inorganic phosphate and phenol at alkaline pH. Phenol so formed reacts with Folin's Ciocalteau reagent. The colour developed was read after 10 min at 640 nm in Systronics 119 UV/VIS Spectrophotometer. The activity of ALP was expressed as Units/l.

2.12 Estimation of blood urea (Natelson *et al*., 1951)

To 0.1 ml of blood, 3.3 ml distilled water, 0.3 ml 10% sodium tungstate, and 3.3 ml 2/3 N H_2SO_4 were added and centrifuged for 10 min at 3000 rpm. After centrifugation, 2 ml distilled water, 0.4 ml DAM (in full) reagent and 1.6 ml H_2SO_4-H_3PO_4 reagent were added to 2 ml of the supernatant in a test tube and incubated in a boiling water bath for 30 min. After incubation, the test tube was cooled and the color developed was read at 480 nm. Different volumes of working standards (0.2, 0.4, 0.6, 0.8 and 1.0 ml) were taken and made up to 2 ml with distilled water. Then 0.4 ml of DAM reagent and 1.6 ml of sulphuric acid, phosphoric acid reagent was added to all the test tubes and heated in a boiling water bath for 30 min. A containing 2 ml of distilled water was treated the same way. Then test tubes were cooled and intensity of the colour was read at 480 nm. From the standard optical density values, the amount of urea in the sample was calculated and expressed as mg/dl.

2.13 Estimation of creatinine (Bonsness and Taussky, 1945)

A mixture of 0.1 ml serum, 2 ml 5% sodium tungstate and 2 ml 2/3 N H_2SO_4 was centrifuged at 3000 rpm for 10 min. A volume of 2 ml of supernatant was taken in reaction tube and to this, 1 ml picric acid and 1 ml sodium hydroxide were added and incubated at 37°C for 15 min. The intensity of colour was measured at 550 nm. For standard, different volumes of working standard (0.2, 0.4, 0.6, 0.8 and 1.0 ml) were taken. The volume was made upto 2 ml with distilled water. Then 1 ml of picric acid and 1 ml of sodium hydroxide were added and incubated at 37°C for 15 min. A blank containing 2 ml of distilled water was treated the same way. The intensity of colour was measured at 550 nm. From the standard optical density values, the amount of creatinine in the sample was calculated and expressed as mg/dL.

2.14 Statistical analysis

All the results were expressed as mean ± SEM The data were statistically analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons. Statistical presentations were organized using Graph Pad Prism Software, Windows version 6.0. Inter group comparison were carried out and p values < 0.05, $p < 0.01$ and $p < 0.001$ were considered significant.

3. Results

The animals treated with cyclophosphamide, a synthetic immunosuppressant, were presented with 6% decrease in their body weight on day 14, when compared with initial weight. Animals treated with synthetic immunostimulant levamisole had shown 4% increase in body weight. Among the other groups, in which animals received three different doses of test extracts along with cyclophosphamide, medium dose treated animals exhibited 3-3.6% increase in body weight which was comparable to immunized control (Table 1). No significant changes were observed in the weight of the organs such as brain, heart, liver, spleen, thymus and kidneys of the experimental animals (Table 2).

Table 1: Effect of test extracts on changes in body weight

Groups	Day 0	Day 7	Day 14	Change in weight %
I	38.24 ± 2.6	38.65 ± 2.6	39.32 ± 2.9	↑ 3%
II	42.5 ± 2.01	41.68 ± 1.37	39.97 ± 2.48	↓ 6%
III	39.53 ± 1.35	39.61 ± 0.82	40.95 ± 0.18	↑ 4%
IV	41.99 ± 3.44	41.88 ± 2.79	42.08 ± 2.91	↑ 0.21%
V	35.69 ± 3.51	36.15 ± 3.65	36.86 ± 3.46	↑ 3.3%
VI	40.96 ± 1.89	41.22 ± 2.34	41.35 ± 2.94	↑ 1%
VII	38.24 ± 3.34	37.85 ± 2.74	37.86 ± 2.28	↓ 1%
VIII	40.63 ± 0.69	39.69 ± 0.92	41.80 ± 0.94	↑ 3%
IX	38.92 ± 3.78	38.97 ± 3.20	39.30 ± 2.87	↑ 1%
X	40.49 ± 2.94	41.53 ± 2.89	40.99 ± 3.89	↑ 1.2%
XI	38.58 ± 2.84	38.98 ± 2.58	39.96 ± 2.75	↑ 3.6%
XII	34.58 ± 6.08	34.18 ± 5.56	35.23 ± 4.66	↑ 1.87%

Values are Mean ± SD (n=6), values are significant when

I - Immunized control
II - Negative control (CYP - Cyclophosphamide)
III - CYP + Levamisole
IV - CYP + LDAETCB (Low dose of aqueous extract of *T. catappa* bark)
V - CYP + MDAETCB (Medium dose of aqueous extract of *T. catappa* bark)
VI - CYP + HDAETCB (High dose of aqueous extract of *T. catappa* bark)
VII - CYP + LDAETCF (Low dose of aqueous extract of *T. catappa* fruit)
VIII - CYP + MDAETCF (Medium dose of aqueous extract of *T. catappa* fruit)
IX - CYP + HDAETCF (High dose of aqueous extract of *T. catappa* fruit)
X - CYP + LDAETCW (Low dose of aqueous extract of *T. catappa* wood)
XI - CYP + MDAETCW (Medium dose of aqueous extract of *T. catappa* wood)
XII - CYP + HDAETCW (High dose of aqueous extract of *T. catappa* wood)

Table 2: Effect of test drugs on changes in organ weight

Groups	Brain (g)	Heart (g)	Liver (g)	Spleen (g)	Thymus (g)	Kidney (g)
I	0.48 ± 0.01	0.19 ± 0.01	1.76 ± 0.01	0.12 ± 0.00	0.05 ± 0.00	0.31 ± 0.01
II	0.44 ± 0.02	0.20 ± 0.01	1.95 ± 0.06	0.10 ± 0.01	0.05 ± 0.00	0.33 ± 0.01
III	0.46 ± 0.01	0.20 ± 0.01	1.87 ± 0.00	0.11 ± 0.00	0.05 ± 0.00	0.29 ± 0.01
IV	0.47 ± 0.01	0.18 ± 0.01	1.74 ± 0.08	0.13 ± 0.00	0.05 ± 0.00	0.27 ± 0.01
V	0.51 ± 0.01 ^{c}	0.26 ± 0.03$^{\bullet c}$	2.04 ± 0.08	0.17 ± 0.00	0.06 ± 0.01	0.33 ± 0.03
VI	0.48 ± 0.01	0.21 ± 0.00	2.18 ± 0.08	0.25 ± 0.06^{*c}	0.06 ± 0.00	0.32 ± 0.03
VII	0.49 ± 0.01	0.19 ± 0.01	1.83 ± 0.12	0.13 ± 0.00	0.04 ± 0.00	0.28 ± 0.01
VIII	0.46 ± 0.01	0.21 ± 0.01	1.89 ± 0.03	0.12 ± 0.00	0.05 ± 0.00	0.30 ± 0.01
IX	0.47 ± 0.01	0.20 ± 0.01	1.62 ± 0.08	0.16 ± 0.01	0.05 ± 0.01	0.27 ± 0.01
X	0.46 ± 0.00	0.21 ± 0.02	1.80 ± 0.14	0.17 ± 0.02	0.06 ± 0.00	0.28 ± 0.01
XI	0.46 ± 0.02	0.21 ± 0.01	2.37 ± 0.21$^{\bullet}$	0.20 ± 0.00$^{\bullet}$	0.06 ± 0.01	0.33 ± 0.01
XII	0.44 ± 0.02	0.18 ± 0.01	1.78 ± 0.06	0.14 ± 0.00	0.04 ± 0.01	0.26 ± 0.01^{c}

Values are Mean ± SEM (n=6), values are significant when
$^{*}p < 0.001$, $^{\#}p < 0.01$, $^{\bullet}p < 0.05$ vs Immunized control
$^{a}p < 0.001$, $^{b}p < 0.01$, $^{c}p < 0.05$ vs Negative control

I - Immunized control
II - Negative control (CYP - Cyclophosphamide)
III - CYP + Levamisole
IV - CYP + LDAETCB (Low dose of aqueous extract of *T. catappa* bark)
V - CYP + MDAETCB (Medium dose of aqueous extract of *T. catappa* bark)
VI - CYP + HDAETCB (High dose of aqueous extract of *T. catappa* bark)
VII - CYP + LDAETCF (Low dose of aqueous extract of *T. catappa* fruit)
VIII - CYP + MDAETCF (Medium dose of aqueous extract of *T. catappa* fruit)
IX - CYP + HDAETCF (High dose of aqueous extract of *T. catappa* fruit)
X - CYP + LDAETCW (Low dose of aqueous extract of *T. catappa* wood)
XI - CYP + MDAETCW (Medium dose of aqueous extract of *T. catappa* wood)
XII - CYP + HDAETCW (High dose of aqueous extract of T. *catappa* wood)

Table 3: Effect of test extracts on WBC profile

Groups	WBC(10^9/l)	NEU(10^9/l)	LYM(10^9/l)	MO(10^9/l)	EO(10^9/l)	BA(10^9/l)
I	7.77 ± 0.23	1.5 ± 0.02	5.8 ± 0.23	0.5 ± 0.02	0.005 ± 0.00	0.003 ± 0.00
II	4.76 ± 0.45*	0.86 ± 0.12	3.5 ± 0.28*	0.31 ± 0.07	0.003 ± 0.00	0.00 ± 0.00
III	8.1 ± 0.31^{a}	1.4 ± 0.05	6.1 ± 0.32^{a}	0.5 ± 0.04^{c}	0.005 ± 0.00	0.005 ± 0.00
IV	4.8 ± 0.45$^{\#}$	0.87 ± 0.06	3.5 ± 0.34*	0.4 ± 0.04	0.01 ± 0.00	0.00 ± 0.00
V	7.9 ± 0.33^{c}	2.25 ± 0.28^{b}	4.8 ± 0.17	0.6 ± 0.04^{a}	0.03 ± 0.01$^{\bullet}$	0.00 ± 0.00
VI	7.1 ± 0.35^{a}	1.39 ± 0.04	5.2 ± 0.39^{b}	0.5 ± 0.02	0.01 ± 0.00	0.00 ± 0.00
VII	5.8 ± 0.68	1.49 ± 0.40	3.7 ± 0.21*	0.6 ± 0.06^{a}	0.02 ± 0.00	0.00 ± 0.00
VIII	7.3 ± 0.58^{b}	2.04 ± 0.28^{c}	4.7 ± 0.29	0.53 ± 0.01^{b}	0.02 ± 0.00	0.006 ± 002^{c}
IX	6 ± 0.07	1.41 ± 0.01	4.2 ± 0.06^{a}	0.4 ± 0.02	0.02 ± 0.00	0.00 ± 0.00
X	5.6 ± 0.31	1.6 ± 0.6	3.4 ± 0.07*	0.6 ± 0.01^{a}	0.02 ± 0.00	0.006 ± 0.00^{c}
XI	7.7 ± 0.54^{c}	2.6 ± 0.1^{a}	4.7 ± 0.43	0.4 ± 0.03	0.03 ± 0.00$^{\bullet}$	0.003 ± 0.00
XII	6.9 ± 0.71^{b}	1.6 ± 0.8	4.5 ± 0.32$^{\bullet}$	0.9 ± 0.05*^{a}	0.02 ± 0.00	0.00 ± 0.00

Values are Mean ± SEM (n=6), values are significant when
$^{*}p < 0.001$, $^{\#}p < 0.01$, $^{\bullet}p < 0.05$ vs Immunized control
$^{a}p < 0.001$, $^{b}p < 0.01$, $^{c}p < 0.05$ vs Negative control

NEU - Neutrophils, LYM - Lymphocytes, MO - Monocytes, EO - Eosinophils, BA - Basophils
I - Immunized control, II - Negative control, III - CYP + Levamisole, IV - CYP + LDAETCB, V - CYP + MDAETCB, VI - CYP + HDAETCB, VII - CYP + LDAETCF, VIII - CYP + MDAETCF, IX - CYP + HDAETCF, X - CYP + LDAETCW, XI - CYP + MDAETCW, XII - CYP + HDAETCW

Table 4: Effect of test extracts on RBC profile

Groups	HCT(%)	RBC(10^{12}/l)	HB(g/dl)	MCV(fl)	MCH(pg)	MCHC(g/dl)	RDW(%)	RSD(fl)
I	45.6 ± 0.59	9.5 ± 0.06	11.1 ± 0.09	47.9 ± 0.41	11.6 ± 0.05	24.3 ± 0.11	13.5 ± 0.17	6.5 ± 0.10
II	48.2 ± 0.13	10.1 ± 0.08	11.3 ± 0.09	47.7 ± 0.27	11.2 ± 0.20	23.6 ± 0.24•	13.7 ± 0.15	6.6 ± 0.11
III	45.8 ± 0.49	9.5 ± 0.07	11.1 ± 0.11	48.2 ± 0.15	11.7 ± 0.02	24.3 ± 0.06	13.4 ± 0.21	6.5 ± 0.11
IV	45.9 ± 0.95	9.8 ± 0.16	11.3 ± 0.25	46.4 ± 0.21•	11.4 ± 0.08	24.6 ± 0.04[b]	14.2 ± 0.15•	6.6 ± 0.02
V	48.6 ± 0.53	10.4 ± 0.13	11.7 ± 0.15	46.9 ± 0.06	11.3 ± 0.00	24.2 ± 0.04	13.8 ± 0.11	6.5 ± 0.04
VI	47.8 ± 1.47	9.8 ± 0.43	11.2 ± 0.23	48.8 ± 0.59	11.4 ± 0.25	23.4 ± 0.21[#]	14.5 ± 0.13[*a]	7.1 ± 0.02[*a]
VII	47.5 ± 1.26	9.9 ± 0.24	11.9 ± 0.33	47.8 ± 0.10	12.1 ± 0.04[a]	25.2 ± 0.00[*a]	13.6 ± 0.07	6.5 ± 0.06
VIII	46.7 ± 0.06	9.7 ± 0.05	11.9 ± 0.15	48.3 ± 0.23	12.3 ± 0.21[a]	25.4 ± 0.32[#a]	13.8 ± 0.06	6.7 ± 0.00
IX	47.6 ± 0.15	10.0 ± 0.01	11.9 ± 0.08	47.5 ± 0.18	11.8 ± 0.10	24.9 ± 0.12[a]	14 ± 0.06	6.6 ± 0.02
X	53.1 ± 1.43	11.1 ± 0.17	13.1 ± 0.42•	47.8 ± 0.57	11.8 ± 0.22[c]	24.8 ± 0.17[a]	14.4 ± 0.03[*b]	6.9 ± 0.08[*c]
XI	38.8 ± 5.02[b]	8.3 ± 1.06[c]	9.4 ± 1.20[c]	46.5 ± 0.15	11.3 ± 0.02	24.2 ± 0.04	14.2 ± 0.06[#]	6.6 ± 0.07
XII	47.4 ± 0.06	10.1 ± 0.06	11.7 ± 0.06	47.4 ± 0.06	11.8 ± 0.04	24.8 ± 0.13[a]	14.7 ± 0.06[*a]	6.9 ± 0.04[*c]

Values are Mean ± SEM (n=6), values are significant when
*$p < 0.001$, #$p < 0.01$, •$p < 0.05$ vs Immunized control
[a]$p < 0.001$, [b]$p < 0.01$, [c]$p < 0.05$ vs Negative control

I - Immunized control, II - Negative control, III - CYP + Levamisole, IV - CYP + LDAETCB, V - CYP + MDAETCB, VI - CYP + HDAETCB, VII - CYP + LDAETCF, VIII - CYP + MDAETCF, IX - CYP + HDAETCF, X - CYP + LDAETCW, XI- CYP + MDAETCW, XII - CYP + HDAETCW

Table 5: Effect of aqueous extracts of test substances on platelet profile

Groups	PLT	PCT	MPV	PDW
I	599 ± 4.33	0.38 ± 0.03	6.45 ± 0.02	41.9 ± 0.19
II	595 ± 14.91	0.38 ± 0.01	6.5 ± 0.00	43.5 ± 0.39
III	592 ± 32.22	0.38 ± 0.02	6.42 ± 0.05	42 ± 0.25
IV	645 ± 20.29	0.42 ± 0.01	6.45 ± 0.26	42.1 ± 0.35
V	606 ± 12.91	0.38 ± 0.01	6.4 ± 0.26	42.3 ± 0.18
VI	1026 ± 244.33•[c]	3.59 ± 0.03•[c]	6.15 ± 0.11	41.8 ± 0.88
VII	813 ± 12.24	0.54 ± 0.01	6.6 ± 0.08	42.6 ± 0.19
VIII	835 ± 24.15	0.55 ± 0.02	6.45 ± 0.07	43.7 ± 0.44
IX	649 ± 3.24	0.42 ± 0.03	6.4 ± 0.26	42.9 ± 0.28
X	490 ± 58.66	0.31 ± 0.04	6.4 ± 0.02	43.5 ± 0.61
XI	669 ± 115.16	0.43 ± 0.07	6.46 ± 0.05	43.7 ± 1.56
XII	705 ± 12.04	0.46 ± 0.01	6.4 ± 0.05	42.2 ± 0.31

Values are Mean ± SEM (n=6), values are significant when
*$p < 0.001$, #$p < 0.01$, •$p < 0.05$ vs Immunized control
[a]$p < 0.001$, [b]$p < 0.01$, [c]$p < 0.05$ vs Negative control

I - Immunized control, II - Negative control, III - CYP + Levamisole, IV - CYP + LDAETCB, V - CYP + MDAETCB, VI - CYP + HDAETCB, VII - CYP + LDAETCF, VIII - CYP + MDAETCF, IX - CYP + HDAETCF, X - CYP + LDAETCW, XI - CYP + MDAETCW, XII - CYP + HDAETCW

3.1 Effect of test extracts on WBC profile

In the present study, cyclophosphamide treated mice were presented with highly significant (p<0.001) reduction in the number of total WBC and lymphocytes count when compared with immunized control mice. Highly significant increase in the total WBC count was observed in the mice co-treated with levamisole and test extracts (medium and high dose) along with cyclophosphamide. Among the three doses selected, near normal values were obtained in the mice treated with medium dose (250 mg) of the test extracts. Significant increase in neutrophil and monocyte count was observed in the mice treated with test extracts (250 mg) along with cyclophosphamide when compared with negative control group (Table 3).

3.2 Effect of test extracts on RBC and platelet profile

No significant changes were observed in the HCT, RBC, HB and MCV. Slight variations were observed in MCHC, RDW and RSD. In platelet profile, except group X, in all other groups there was an increase in platelet count, but it was statistically not significant. Significant increase in platelets was observed in group VI. There was a very small decrease in the number of platelets in group II when compared to group I, but not significant (Tables 4 and 5).

3.3 Effect of test extracts on glucose and cholesterol

There was no significant change in the level of glucose in all groups except in group IV and X where marked elevation in glucose was observed compared to control ($p < 0.05$). No significant changes were observed in cholesterol level. In cyclophosphamide treated group, a slight increase in cholesterol was observed but statistically insignificant. Among the treatment groups, medium dose of aqueous extract of fruits has shown results comparable to control (Figue1).

3.4 Effect of test extracts on total protein and albumin

Highly significant reduction in total protein and albumin was observed in cyclophosphamide treated group II when compared to group I. This shows the toxic effect of cyclophosphamide. In all the treatment groups, significant restoration of protein and albumin was observed when compared with group II (Figure2).

3.5 Effect of test drugs on liver marker enzymes

Highly significant elevations in the levels of aspartate transaminase, alanine transaminase and alkaline phosphatase indicated hepatotoxicity induced by cyclophosphamide in group II. In all other groups, the altered liver marker profile was brought back to near normal significantly as compared to group II (Table 6).

Table 6: Effect of test extracts on liver marker enzymes

Groups	AST (Units/l)	ALT (Units/l)	ALP (Units/l)
I	69.9 ± 2.436	20 ± 0.6325	176.4 ± 16.42
II	103.2 ± 5.062*	32.3 ± 1.726*	234 ± 9.925#
III	72.6 ± 1.618a	23.3 ± 0.7601a	162 ± 7.887a
IV	61.2 ± 2.187a	20 ± 1.673a	165.6 ± 2.277a
V	67.1 ± 1.441a	24.6 ± 1.282a	151.2 ± 6.831a
VI	66.6 ± 1.004a	21 ± 0.9661a	162 ± 6.831a
VII	71.9 ± 1.909a	21 ± 0.3651a	136.8 ± 4.554a
VIII	68.4 ± 1.386a	21.6 ± 0.2108a	151.2 ± 6.831a
IX	66.8 ± 0.8414a	25.6 ± 0.5578•b	165.6 ± 9.107a
X	67.4 ± 0.6580a	20.6 ± 0.9189a	147.6 ± 8.209a
XI	65.8 ± 0.8844a	23.6 ± 0.7601a	165.6 ± 16.42a
XII	63.7 ± 1.294a	21 ± 0.6325a	151.2 ± 7.887a

Values are Mean ± SEM (n=6), values are significant when
*$p < 0.001$, #$p < 0.01$, •$p < 0.05$ vs Immunized control
a$p < 0.001$, b$p < 0.01$, c$p < 0.05$ vs Negative control

I - Immunized control, II - Negative control, III - CYP + Levamisole, IV - CYP + LDAETCB, V - CYP + MDAETCB, VI - CYP + HDAETCB, VII - CYP + LDAETCF, VIII - CYP + MDAETCF, IX - CYP + HDAETCF, X - CYP + LDAETCW, XI - CYP + MDAETCW, XII - CYP + HDAETCW

Table 7: Effect of test extracts on blood urea and creatinine

Groups	Urea (mg/dl)	Creatinine (mg/dl)
I	27 ± 2.280	0.6 ± 0.04216
II	46.6 ± 4.104*	1.3 ± 0.1932*
III	29 ± 1.592a	0.7 ± 0.07303a
IV	24 ± 0.3651a	0.6 ± 0.06325a
V	24 ± 1.265a	0.88 ± 0.0211a
VI	28 ± 1.265a	0.7 ± 0.03801a
VII	26.6 ± 1.687a	0.6 ± 0.08433a
VIII	28 ± 1.673a	0.7 ± 0.03651a
IX	27.6 ± 1.476a	0.6 ± 0.05578a
X	27 ± 0.9661a	0.7 ± 0.07303a
XI	26 ± 0.3651a	0.8 ± 0.00843a
XII	28 ± 1.282a	0.7 ± 0.05578a

Values are mean ± SEM (n=6), values are significant when
*$p < 0.001$, #$p < 0.01$, •$p < 0.05$ vs Immunized control
a$p < 0.001$, b$p < 0.01$, c$p < 0.05$ vs Negative control

I - Immunized control, II - Negative control, III - CYP + Levamisole, IV - CYP + LDAETCB, V - CYP + MDAETCB, VI - CYP + HDAETCB, VII - CYP + LDAETCF, VIII - CYP + MDAETCF, IX - CYP + HDAETCF, X - CYP + LDAETCW, XI - CYP + MDAETCW, XII- CYP + HDAETCW

3.6 Effect of test extracts on renal markers

Toxic metabolites of cyclophosphamide induce free radical mediated damage to nephrons, thereby producing nephrotoxicity. In the present study, elevated levels of serum urea and creatinine in group II animals clearly depicted cyclophosphamide induced nephrotoxicity. The values were significant when compared to group I. Altered values were brought back to near normal in the animals treated with test extracts along with cyclophosphamide.

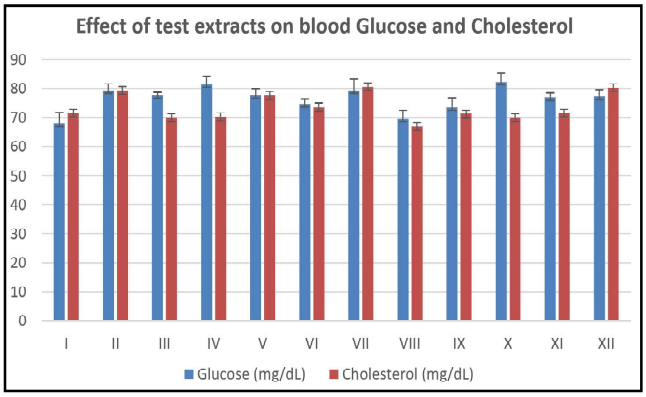

Figure 1: Effect of test extracts on glucose and cholesterol

Values are Mean ± SEM (n=6)
*$p < 0.001$, #$p < 0.01$, @$p < 0.05$ vs Immunized control
a$p < 0.001$, b$p < 0.01$, c$p < 0.05$ vs Negative control

I - Immunized control, II - Negative control, III - CYP + Levamisole, IV - CYP + LDAETCB, V - CYP + MDAETCB, VI - CYP + HDAETCB, VII - CYP + LDAETCF, VIII - CYP + MDAETCF, IX - CYP + HDAETCF, X - CYP + LDAETCW, XI - CYP + MDAETCW, XII - CYP + HDAETCW

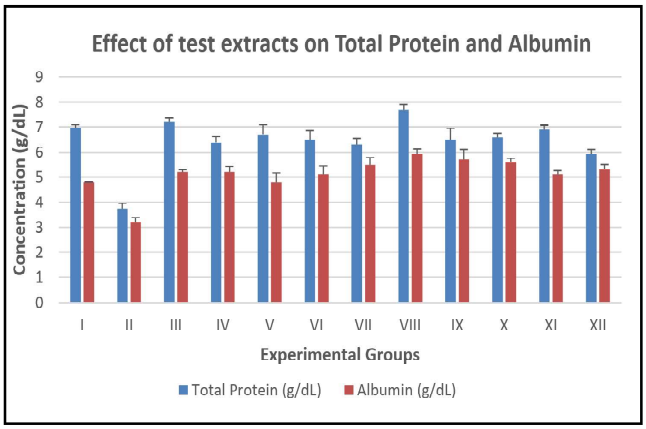

Figure 2: Effect of test extracts on total protein and albumin

Values are Mean ± SEM (n=6)
*$p < 0.001$, #$p < 0.01$, •$p < 0.05$ vs Immunized control
a$p < 0.001$, b$p < 0.01$, c$p < 0.05$ vs Negative control

I - Immunized control, II - Negative control, III - CYP + Levamisole, IV - CYP + LDAETCB, V - CYP + MDAETCB, VI - CYP + HDAETCB, VII - CYP + LDAETCF, VIII - CYP + MDAETCF, IX - CYP + HDAETCF, X - CYP + LDAETCW, XI - CYP + MDAETCW, XII - CYP + HDAETCW

3.7 Histopathological studies

Histopathological observations of liver, kidney and thymus sections of animals treated with medium dose of the test drugs did not show any significant changes. The observed changes were within normal limits.

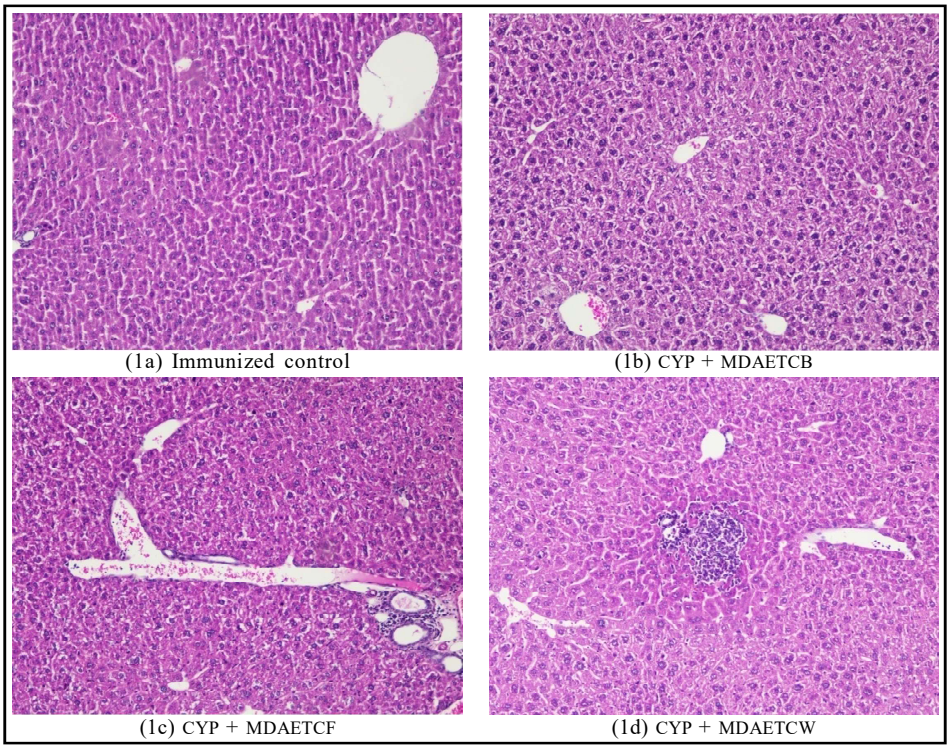

Plate 1: Photomicrographs of histopathological studies - Liver

MDAETCB - Medium dose of aqueous extract of *T. catappa* bark
MDAETCF - Medium dose of aqueous extract of *T. catappa* fruit
MDAETCW - Medium dose of aqueous extract of *T. catappa* wood

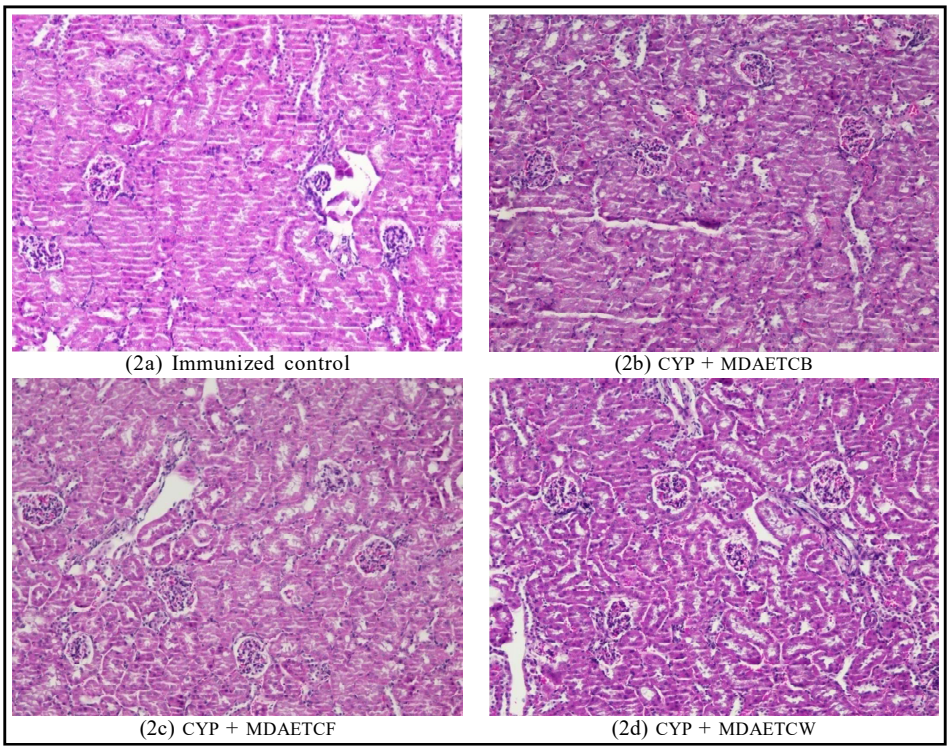

Plate 2: Photomicrographs of histopathological studies - Kidney

MDAETCB - Medium dose of aqueous extract of *T. catappa* bark
MDAETCF - Medium dose of aqueous extract of *T. catappa* fruit
MDAETCW - Medium dose of aqueous extract of *T. catappa* wood

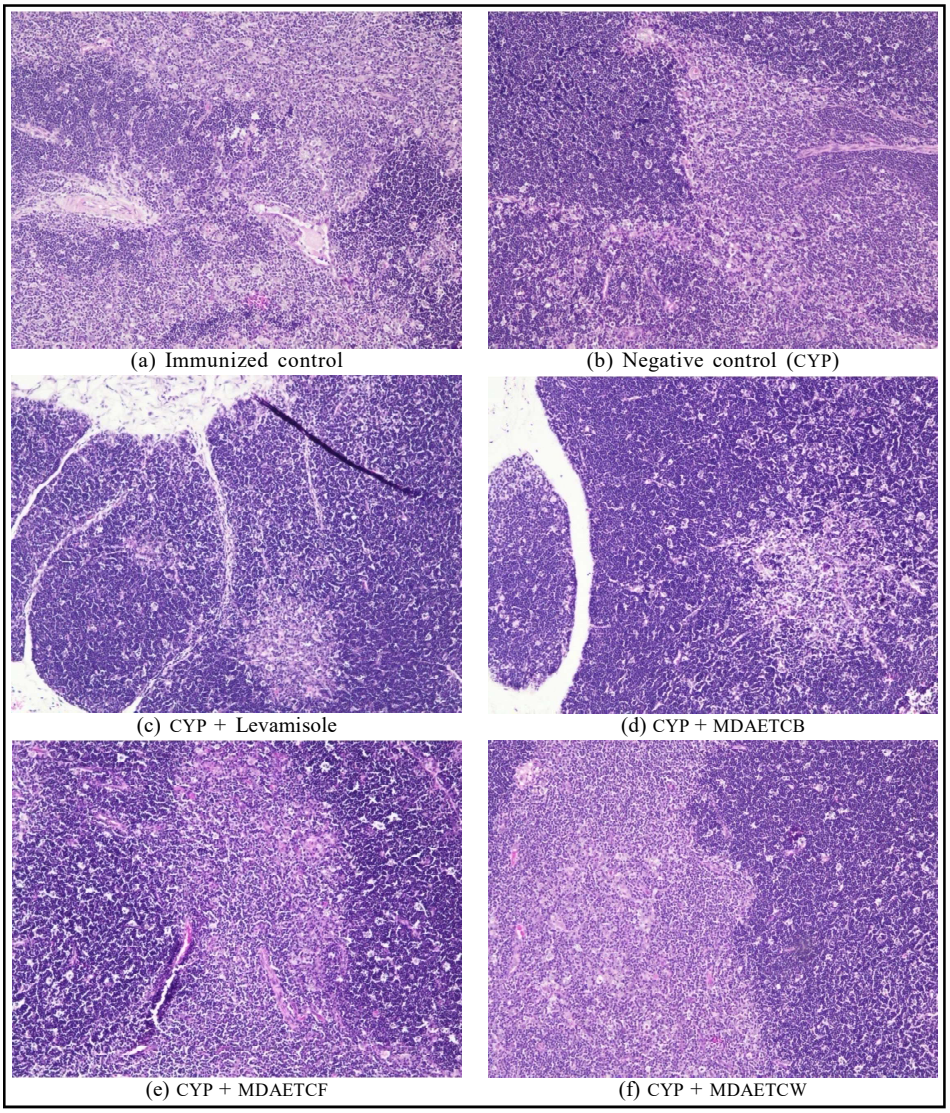

Plate 3: Photomicrographs of histopathological studies - Thymus

CYP - Cyclophosphamide
MDAETCB - Medium dose of aqueous extract of *T. catappa* bark
MDAETCF - Medium dose of aqueous extract of *T. catappa* fruit
MDAETCW - Medium dose of aqueous extract of *T. catappa* wood

4. Discussion

Cyclophosphamide is an oxazaphospharine class of alkylating agent widely used in cancer chemotherapy and used to stimulate immunosuppression with a very narrow therapeutic index and undergoes a complicated process of metabolic activation and inactivation (Ekhart *et al*., 2008). The suppressive effects of cyclophosphamide on the lymphoid organs, white blood cell counts and other immune functions have been well documented (Zaidi *et al*., 1990; Jena *et al*., 2003; Ramadan *et al*., 2012). Bone marrow is the site of continuous proliferation and turnover of blood cells and also a source of cells involved in immune activity and is the organ most affected during any immunosuppressive therapy. Loss of stem cells and inability of bone marrow to regenerate new blood cells will result in thrombocytopenia and leucopoenia.

Thus, cyclophosphamide induced myelosuppressed animal model is a reliable, convenient and reproducible experimental system to study the immunostimulatory activity of the plant drugs. Hence, in the present study, cyclophosphamide induced alterations in the haematological parameters and protective effect of aqueous extract of different parts of TC were evaluated. Decrease in the number of neutrophils and lymphocytes in cyclophosphamide treated group were restored to near normal upon administration of selected plant extracts.

Cyclophosphamide induces hypoproteinemia due to its cytotoxic effect (Prasada Rao *et al.*, 1983; Fleming, 1997; Ambali, 2009). It was evident in the present study through decreased levels of total protein and albumin in group II animals which were treated with cyclophosphamide. Animals in herbal extract treated groups had shown drastic improvement in the altered protein level. Liver is an important organ for protein synthesis. Hepatotoxic effect of cyclophosphamide is the reason for reduction in protein level. Presence of hepatoprotective polyphenols in test extracts might have been responsible for bringing back near normal protein level (Venkatalakshmi *et al.*, 2016b).

The liver cytochrome P450 system converts cyclophosphamide to 4-hydroxycyclophosphamide, which is in equilibrium with its acyclic tautomeric form, aldophosphamide. In cells susceptible to cytolysis, nonenzymatic cleavage of aldophosphamide yields phosphoramide mustard and acrolein. These two compounds are highly cytotoxic and may represent active forms of the drug. In spite of its requirement for hepatic metabolism for activity, cyclophosphamide is an uncommon hepatic toxin, and reports of elevated hepatic enzymes are attributed to the drug (Aubrey, 1970; King and Perry, 2001). In our study also, elevated liver marker enzymes in group 2 indicated the hepatotoxicity of cyclophosphamide which was reversed by the herbal extracts.

Cyclophosphamide causes nephrotoxicity by alkylation of renal cell by sulfhydryl group of acrolein which is one of the active metabolites of cyclophosphamide. Renal cell alkylation leads to reduction of glomerular filtration rate as well as tubular dysfunction resulting from acute renal failure. Cyclophosphamide also generates the free radicals that cause renal damage (Singh *et al.*, 2014). Significant elevation of urea and creatinine in the animals treated with cyclophosphamide in our study indicated the nephrotoxicity induced by it. Treatment groups which received aqueous extracts of bark, fruit and wood of TC restored normalcy and exhibited their nephron protective effect which is statistically significant.

5. Conclusion

Taken together, it could be concluded that aqueous extract of bark, fruit and wood of TC is having the potential to restore normalcy in altered hematological and biochemical profiles of cyclophosphamide induced cytotoxicity. Further studies are needed to assess the ameliorating potential of these extracts and also isolated phytochemicals of various parts of this plant in chronic exposure to cyclophosphamide and other such cytotoxicants.

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Conflict of interest

The authors declare that there are no conflicts of interest in the course of conducting the research. Both the authors had final decision regarding the manuscript and decision to submit the findings for publication.

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