

Protective Effect of *Croton macrostachyus* (Euphorbiaceae) Stem Bark on Cyclophosphamide-Induced Nephrotoxicity in Rats

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Muluken Altaye Ayza ¹
Balasubramanian Rajkapoor ^{1,2}
Dawit Zewdu Wondafrash ¹
Abera Hadgu Berhe ¹

¹Department of Pharmacology and Toxicology, School of Pharmacy, College of Health Sciences, Mekelle University, Mekelle, Ethiopia; ²Department of Pharmacology, JKK Nattraja College of Pharmacy, Komarapalayam 638 183, Tamilnadu, India

Background: Cyclophosphamide is an alkylating antineoplastic agent and its major limitation is injury to normal tissue, leading to multiple organ toxicity, including kidney, heart, liver and reproductive toxicity. *Croton macrostachyus* (Euphorbiaceae) has been used in Ethiopian traditional medicine to manage renal diseases.

Objective: The present study aims to assess the protective effect of the stem bark extract and solvent fractions of *Croton macrostachyus* on cyclophosphamide-induced nephrotoxicity in rats.

Methods: Nephrotoxicity was induced using cyclophosphamide 200 mg/kg i.p injection on the first day of the experiment. The negative control groups were administered with cyclophosphamide alone (200 mg/kg, i.p.). The crude extracts were administered at three dose levels (100, 200, and 400 mg/kg), while aqueous and ethyl acetate fractions were given at two dose levels (100 and 200 mg/kg). Excepting the normal control, all groups were subjected to cyclophosphamide toxicity on the first day.

Results: Treatment with crude extract 100 mg/kg and ethyl acetate fraction significantly decreased kidney-to-body weight ratio ($P < 0.001$). In addition, treatment with *Croton macrostachyus* crude extract and solvent fractions significantly decreased serum blood urea nitrogen (BUN) level ($P < 0.001$). Treatment with 100 and 200 mg/kg of ethyl acetate fraction significantly decreased serum creatinine level. Histopathological results confirmed the protective effect of the crude extract and solvent fractions of *Croton macrostachyus*.

Conclusion: *Croton macrostachyus* possesses nephroprotective activities and it could be a possible source of treatment for cyclophosphamide-induced nephrotoxicity.

Keywords: *Croton macrostachyus*, cyclophosphamide, nephrotoxicity, creatinine, blood urea nitrogen

Introduction

Drug-induced kidney damage is not a surprising effect since 25% cardiac output goes to the kidney, which puts kidney at increased exposure to the administered medications.¹ Anticancer drugs, aminoglycoside antibiotics, conventional non-selective non-steroidal anti-inflammatory drugs and amphotericin B are well known to cause renal damage.^{2,3}

Renal damage is a challenging adverse effect that can hinder the clinical use of antineoplastic drugs.⁴ Nephrotoxicity reflects both tubular and glomerular injuries and which will result in acute or chronic functional alterations. According to reports, the frequency of drug-induced nephrotoxicity is around 14–26% in adult populations;

Correspondence: Muluken Altaye Ayza
Tel +251-916707913
Email Mulealto1@gmail.com

about 16% of hospital admitted children are due to kidney damage events, being attributed primarily to drugs.⁵

Cyclophosphamide is an oxazaphosphorine substituted nitrogen mustard, with strong cytotoxic and immunosuppressive activity.⁶ It is the mainstay of most preparative regimens for organ-transplant and broadly active anticancer agent used in combination chemotherapy for Hodgkin's disease, non-Hodgkin's lymphoma, leukaemia, rheumatoid arthritis, Burkitt's lymphoma, lupus erythematosus, multiple sclerosis, neuroblastoma, multiple myeloma, endometrial cancer, breast cancer and lung cancer.⁷

Cyclophosphamide causes damage to the urinary bladder and it might cause renal toxicity.⁸ It has been revealed that generation of oxidative and nitrative stress plays an important role in the pathogenesis of cyclophosphamide-induced nephrotoxicity.³ In addition, cyclophosphamide decreases the activities of lysosomal protein digestive enzymes, which leads to the amassing of abnormal amounts of protein in the kidney; this might be another possible reason for cyclophosphamide-induced nephrotoxicity.⁹

Traditional medicine plays both preventive and therapeutic roles. Plants are the major sources of traditional preparations.¹⁰ Around 80% of humans and 90% of live-stock rely on traditional medicine for primary health-care services in Ethiopia, where modern public health services are limited or not available.¹¹

Croton macrostachyus is a deciduous tree belongs to the family of Euphorbiaceae, a large family with 300 genera and 8000 to 10,000 species.¹² It is commonly known as broad-leaved Croton in English, Bisana in Amharic, Tambush or Tambuk in Tigrigna.¹³ This plant is widely available in secondary forests, on forest edges, along rivers, near to lakes, in moist or dry evergreen upland forests, woodlands, wooded grasslands or clump bushland and around roadsides.¹⁴ *Croton macrostachyus* restores itself naturally in less productive sites, including forest edges, mountain slopes, and waste grounds under a wide range of ecological conditions.¹⁵

Croton macrostachyus has a round crown with slender trunk and immense spreading branches. It has simple and broadly ovate, green, turning to orange before falling and rounded leaves. The bark color of the species ranges from green through light gray to pale-brown. The bark is smooth when young and slightly fissured longitudinally on aging. The height of the plant more commonly ranges from 7 to 15 meters.¹²

The traditional healers use *Croton macrostachyus* to treat various human diseases.¹⁶ In Ethiopia, this plant has different medicinal uses, including kidney problems;¹⁷

wound management,¹⁸ malaria,^{10,19} intestinal parasite and heart failure.²⁰

The ethnomedicinal use of *Croton macrostachyus* in the treatment of various diseases has been validated in several experimental studies. Thus, the plant showed anti-bacterial,²¹ anti-diarrheal,¹³ anti-malarial,²² anti-convulsant,²³ anti-diabetic¹⁶ and cardioprotective activity.²⁴

The phytochemical screening demonstrated that stem bark of *Croton macrostachyus* constituted major secondary metabolites such as, tannins, steroids, alkaloids, phenols, terpenoids, saponins, and flavonoids, which may be the reason for the plants' widespread pharmacological activity.²⁵ In Ethiopian traditional medicine, *Croton macrostachyus* has been widely used to manage renal diseases.¹⁷ Thus, the aim of the present study is to investigate the nephroprotective effect of *Croton macrostachyus* on cyclophosphamide-induced toxicity in rats.

Methods

Plant Material

Fresh stem bark of *Croton macrostachyus* was collected from Abiy-Adi, Tigray, Ethiopia. Identification and authentication of the plant were carried out by Prof. Sileshi Nemomissa, at the Department of Plant Biology and Biodiversity Management, Addis Ababa University, and sample specimen was deposited at the National Herbarium of Ethiopia with Ref. No. ETH/4/2011/2019.

Chemicals and Drugs

Ethyl acetate, n-Hexane (Loba Chemie PVT. LTD), ketamine hydrochloride (Neon Laboratories Limited, India), methanol (Carlo Erba Reagents S.A.S), cyclophosphamide injection IP (Cadila Healthcare Limited, India), formalin 10% (Sheba pharmaceuticals PLC, Ethiopia), normal saline (Addis Pharmaceutical Factory, Ethiopia), distilled water (Jourilabs, Ethiopia) were used. All other chemicals used were also of analytical grade.

Experimental Animals

For the acute toxicity study, female *Swiss albino* mice aged 8–12 weeks were used. Either sex of *Sprague Dawley* (SD) rats (3–4 months age) were used for the main study. Animals were obtained from the Department of Pharmacology and Toxicology, School of Pharmacy, Mekelle University. Animals were housed in a room with 12-hour light/12-hour dark cycles and provided with standard pellet feed and water

ad libitum. The animals were acclimatized to the experimental environment prior to use for the study.

Extraction of Plant Material

The fresh stem bark was washed so as to remove dead materials and allowed to dry for three weeks under a shade. The dried stem bark was then pulverized by using a grinder. The powdered stem bark (1.52 Kg) was macerated in 80% methanol for 72 hrs in maceration jars, extraction was aided by orbital shaker and intermittent stirring. The extract was first filtered using a muslin cloth and then by Whatman filters paper No.1. For exhaustive extraction of the plant material, the residue was re-macerated for another 72 hrs twice and then filtered. The combined filtrates were dried in an oven dryer at a temperature of 40°C and weighed. Then, the dried extract (crude extract) was kept in a tightly closed amber bottle and stored in a refrigerator at 4°C until further use.

Fractionation

Fractionation was carried out using a separatory funnel. Sixty-five grams of the crude extract were dissolved in 325 mL of distilled water. Then, extraction was performed by hexane 325 mL, consecutively for three times, followed by ethyl acetate 325 mL consecutively for three times in order to attain complete fraction. After collecting the hexane and ethyl acetate fractions the remaining residue was considered as aqueous fraction. The fractions were concentrated using oven dryer at 40°C and weighed. Hexane fraction was yellowish oily. Dried powders of the aqueous and ethyl acetate fractions were kept in an airtight container and wrapped with the aluminum foil and stored in a refrigerator at 4°C until further use.

Acute Oral Toxicity Test

Acute oral toxicity test was carried out according to the Organization for Economic Cooperation and Development Guideline No. 425.²⁶ Five healthy, nulliparous, non-pregnant female Swiss albino mice (age of 8–12 weeks) were used.

The mice were fasted for food, but not water four hours prior to dosing and one hour after administration of the extract. A single dose of the extract (2000 mg/kg) was administered using oral gavage to the first mouse and observed for any physical and behavioral changes. After 24 hrs, four mice were fasted and 2000 mg/kg single dose was administered. The mice were observed individually for gross behavioral changes (locomotion, activity, hair texture, pupil size, and feeding) at least once during the

first 30 min, periodically for 24 hours, with special attention given during the first four hours, and daily observation were made thereafter for a total period of 14 days.

Nephroprotective Activity

To evaluate the nephroprotective activity, cyclophosphamide-induced renal damage method was used, as described in previous studies.^{27,28} The rats were divided into eleven groups, each group consist of six animals. Group I served as normal control (NC), administered with normal saline orally for 10 days, group II served as cyclophosphamide (CP) control (administered with a single dose of CP 200 mg/kg, i.p, on the first day of the experimental period), group III–V were administered with CP 200 mg/kg, i. p, on the first day, followed by 100, 200 and 400 mg/kg of crude extract orally for 10 days, respectively. Group VI and VII were administered with 100 and 200 mg/kg of aqueous fraction orally for 10 days, respectively.

For the evaluation of nephroprotective activities of the ethyl acetate fractions of *Croton macrostachyus*, group VIII served as normal control (NC), administered with 2% dimethyl sulfoxide (2% DMSO) orally for 10 days, group IX served as cyclophosphamide (CP) control (single dose of CP 200 mg/kg, i.p, was administered on the first day of the experimental period). Group X and XI rats were treated with the ethyl acetate fractions of *Croton macrostachyus* (100 and 200 mg/kg, respectively). Crude extract and aqueous fractions were dissolved in normal saline, whereas ethyl acetate fraction were dissolved in 2% DMSO (Table 1).

Blood Collection

After 24 hours of the last dose, all rats were anesthetized under ketamine anesthesia (75 mg/kg, i.p,) and blood was collected by the retro-orbital puncture using microcapillary tubes. The blood sample was collected and serum was separated through centrifugation at 5000 rpm and used for the estimation of renal markers.

Determination of Renal Markers

The separated serum was used for the estimation of renal markers, including serum creatinine and blood urea nitrogen (BUN) using BTS 350 semi-automated biochemistry analyzer.

Histopathological Examination

Kidney was immediately removed from animals and washed with water and blotted dry and weighed, then

Table 1 Grouping and Dosing of Rats Used in This Experiment

Treatment Groups (Crude Extract and Aqueous Fraction)	Dose
Normal control (Normal saline)	0.5 mL/100 gm
Cyclophosphamide (CP) control	200 mg/kg
Crude extract (CE100)	100 mg/kg
Crude extract (CE200)	200 mg/kg
Crude extract (CE400)	400 mg/kg
Aqueous fraction (AQ100)	100 mg/kg
Aqueous fraction (AQ200)	200 mg/kg
Treatment Groups (Ethyl Acetate Fraction)	Dose
Normal control (2% DMSO)	0.5 mL/100 gm
Cyclophosphamide (CP) control	200 mg/kg
Ethyl acetate fraction (EA100)	100 mg/kg
Ethyl acetate fraction (EA200)	200 mg/kg

Abbreviations: AQ, aqueous fraction; CE, crude extract; CP, cyclophosphamide; DMSO, dimethyl sulfoxide; EA, ethyl acetate fraction.

fixed in 10% formalin for histopathological analysis. Histological sections of the kidney were stained with hematoxylin and eosin. Then, the tissue was examined for necrosis, multifocal interstitial nephritis, hemorrhage, periglomerular leukocytic infiltration, distorted glomerular tufts and tubular degeneration.

Statistical Analysis

The data were expressed as means $\pm$ Standard Errors of Mean (SEM). The analysis was carried out using the statistical package for social science (SPSS) program (version 20.0). One-way Analysis of variance (ANOVA) followed by Tukey's post hoc test was applied to compare variations among groups. $P < 0.05$ was considered statistically significant.

Ethical Consideration

Animal handling was in accordance with Guidelines for the Care and Use of Animals for Scientific Purposes, which has been developed by the National Advisory Committee for Laboratory Animal Research (NACLAR). Ethical approval was obtained from the Health Research Ethics Review Committee (HRERC) of College of Health Sciences, Mekelle University with protocol number 1536/2018.

Table 2 Percentage Yield of Crude Extract and Solvent Fractions of *Croton macrostachyus*

Extract/Fraction	Actual Mass (g)	Percentage Yield (w/w)
Crude extract	101.5	6.68%
Ethyl acetate	11.4	17.53%
Aqueous	36.7	56.46%

Results

Percentage Yield

The yield of crude extract was 6.68%. Ethyl acetate fraction was 17.53%. Among the fractions, aqueous fraction was the one with the highest yield, with 56.46% (Table 2).

Acute Oral Toxicity Test

Administration of 2000 mg/kg of the *Croton macrostachyus* crude extract and solvent fractions did not produce any sign of toxicity. The crude extract and solvent fractions did not cause changes like breathing, alertness, motor activity, restlessness, diarrhea, coma, convulsions, and appearance in the two weeks of follow-up period. Therefore, the LD50 can be considered as higher than 2000 mg/kg.

Effects of Crude Extract and Solvent Fractions on Kidney Weight and Kidney-to-Body Weight Ratio

In the cyclophosphamide-treated group, both kidney weight ($P < 0.01$) and kidney-to-body weight ratio ($P < 0.001$) were significantly increased compared to normal control, whereas the kidney weight increment was significantly prevented by the treatment of crude extract 400 mg/kg compared with the cyclophosphamide control group ($P < 0.01$). Treatment with the aqueous fraction 100 mg/kg ($P < 0.05$) and 200 mg/kg ($P < 0.01$) significantly prevented kidney weight increment compared with the cyclophosphamide control group. Treatment with 200 mg/kg aqueous fraction significantly decreased kidney-to-body weight ratio compared to the cyclophosphamide control group ($P < 0.05$).

Treatment with ethyl acetate fraction 100 mg/kg and 200 mg/kg significantly decreased kidney-to-body weight ratio compared to the cyclophosphamide control group ($P < 0.001$) (Table 3).

Table 3 Effect of Crude Extract and Solvent Fractions of *Croton macrostachyus* on Kidney Weight and Kidney-to-Body Weight Ratio

Group	Dose	Kidney Weight (gm)	Kidney to Body Wt. Ratio ($\times 10^{-3}$)
NC	NS (0.5 mL/100 gm)	0.88 $\pm$ 0.17	2.71 $\pm$ 0.06
CP control	CP 200 mg/kg	1.02 $\pm$ 0.01**	4.85 $\pm$ 0.04***
CE	CE 100 mg/kg	0.94 $\pm$ 0.02	3.46 $\pm$ 0.04####
	CE 200 mg/kg	0.99 $\pm$ 0.02	3.87 $\pm$ 0.02 [#]
	CE 400 mg/kg	0.90 $\pm$ 0.01 ^{###}	3.84 $\pm$ 0.02 ^{###}
AF	AQ 100 mg/kg	0.90 $\pm$ 0.03 [#]	4.28 $\pm$ 0.15
	AQ 200 mg/kg	0.86 $\pm$ 0.02 ^{###}	3.99 $\pm$ 0.10 [#]
NC	2% DMSO (0.5 mL/100 gm)	0.48 $\pm$ 0.04	1.79 $\pm$ 0.13
CP control	CP 200 mg/kg	0.73 $\pm$ 0.03**	4.83 $\pm$ 0.28***
EA	EA 100 mg/kg	0.67 $\pm$ 0.03	3.57 $\pm$ 0.11####
	EA 200 mg/kg	0.66 $\pm$ 0.03	3.48 $\pm$ 0.07####

Notes: Results were expressed as mean $\pm$ SEM (n = 6). ** p < 0.01, *** p < 0.001 when compared with normal control; and [#] p < 0.05, ^{###} p < 0.01, ^{####} p < 0.001 when compared with CP control.

Abbreviations: AF, aqueous fraction; CE, crude extract; DMSO, dimethyl sulfoxide; EA, ethyl acetate fraction; CP, cyclophosphamide; NC, normal control; NS, normal saline.

Table 4 Effect of Crude Extract and Aqueous Fraction of *Croton macrostachyus* on Renal Biomarkers

Group	Dose	Serum Creatinine (mg/dl)	BUN (mg/dl)
NC	NS (0.5 mL/100 gm)	0.55 $\pm$ 0.05	32.83 $\pm$ 3.72
CP control	CP 200 mg/kg	1.56 $\pm$ 0.08	152.00 $\pm$ 8.50***
CE	CE 100 mg/kg	0.65 $\pm$ 0.05	26.75 $\pm$ 2.75####
	CE 200 mg/kg	0.78 $\pm$ 0.09	41.60 $\pm$ 5.07####
	CE 400 mg/kg	0.60 $\pm$ 0.03	29.60 $\pm$ 3.29####
AF	AQ 100 mg/kg	0.78 $\pm$ 0.03	25.50 $\pm$ 2.48####
	AQ 200 mg/kg	0.76 $\pm$ 0.03	23.00 $\pm$ 1.61####
NC	DMSO (0.5 mL/100 gm)	0.65 $\pm$ 0.08	26.00 $\pm$ 3.35
CP control	CP 200 mg/kg	1.60 $\pm$ 0.14***	51.00 $\pm$ 2.67***
EA	EA 100 mg/kg	1.13 $\pm$ 0.03 [#]	30.17 $\pm$ 1.62####
	EA 200 mg/kg	1.01 $\pm$ 0.05 ^{###}	25.17 $\pm$ 1.08####

Notes: Results were expressed as mean $\pm$ SEM (n = 6). *** p < 0.001 when compared with normal control; and [#] p < 0.05, ^{###} p < 0.01, ^{####} p < 0.001 when compared with CP control.

Abbreviations: AF, aqueous fraction; CE, crude extract; DMSO, dimethyl sulfoxide; EA, ethyl acetate fraction; CP, cyclophosphamide; NC, normal control; NS, normal saline.

Effect of Crude Extract and Solvent Fractions on Renal Biomarkers

Cyclophosphamide administration increased both serum creatinine and BUN levels compared to normal control. Treatment with crude extract and aqueous fraction decreased serum creatinine level compared to cyclophosphamide control group. In addition, crude extract and aqueous fraction administration resulted in a significant

decrease in the serum BUN level compared to cyclophosphamide control group (P < 0.001).

Whereas ethyl acetate fraction of 100 mg/kg (P < 0.05) and 200 mg/kg (P < 0.01) decreased serum creatinine level significantly compared to cyclophosphamide control group. Treatment with ethyl acetate fraction decreased BUN level significantly compared to cyclophosphamide control group (P < 0.001) (Table 4).

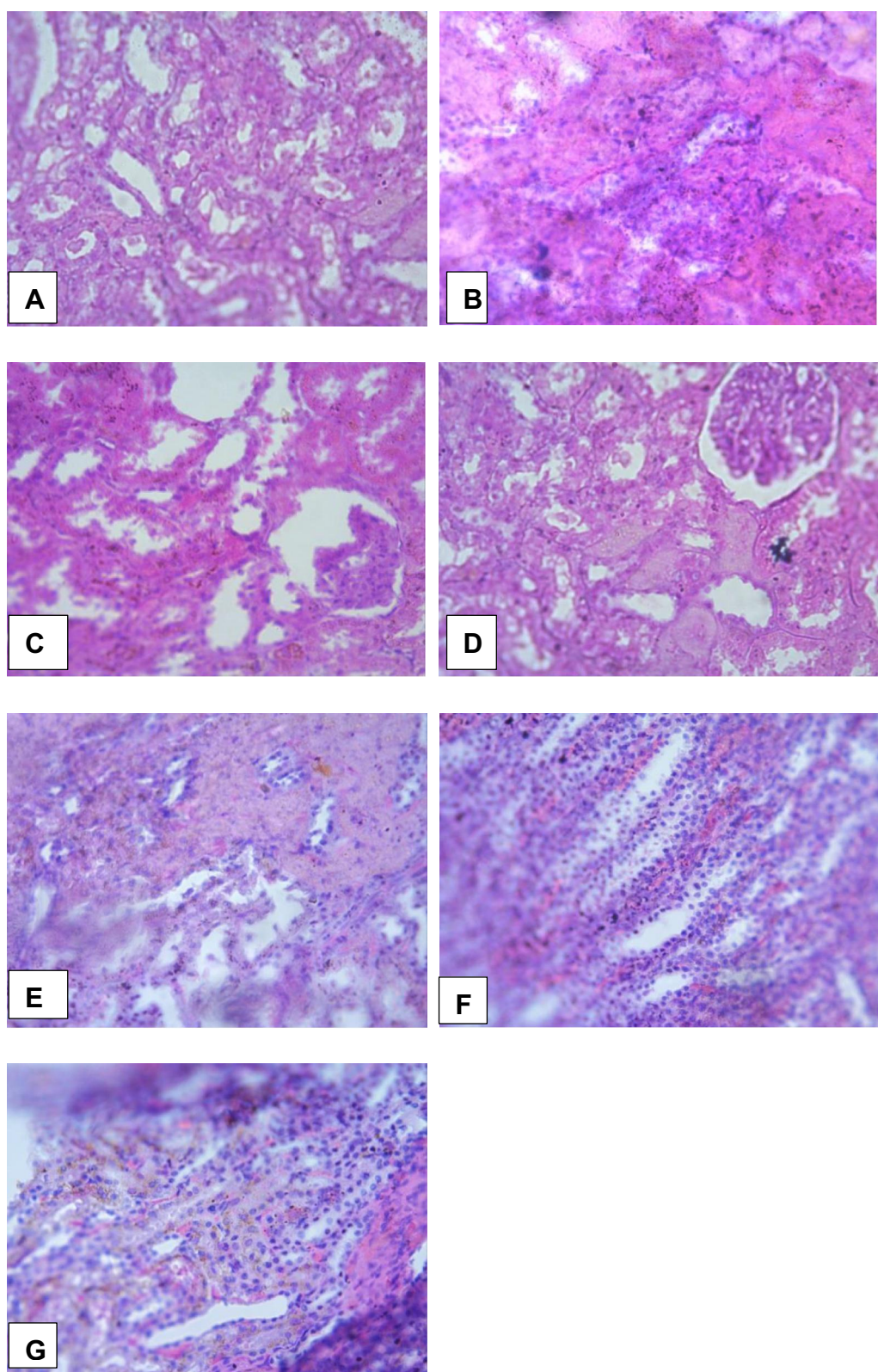


Figure 1 Histopathological changes in the kidney tissue treated with crude extract and aqueous fraction. **(A)** Normal control group (normal structure), **(B)** Cyclophosphamide control group (multifocal necrosis), **(C)** Crude extract 100 mg/kg (damaged glomeruli), **(D)** Crude extract 200 mg/kg (nearly normal glomeruli), **(E)** Crude extract 400 mg/kg (pigment laden macrophage), **(F)** Aqueous fraction 100 mg/kg (increased inflammatory cells), **(G)** Aqueous fraction 200 mg/kg (increased inflammatory cells).

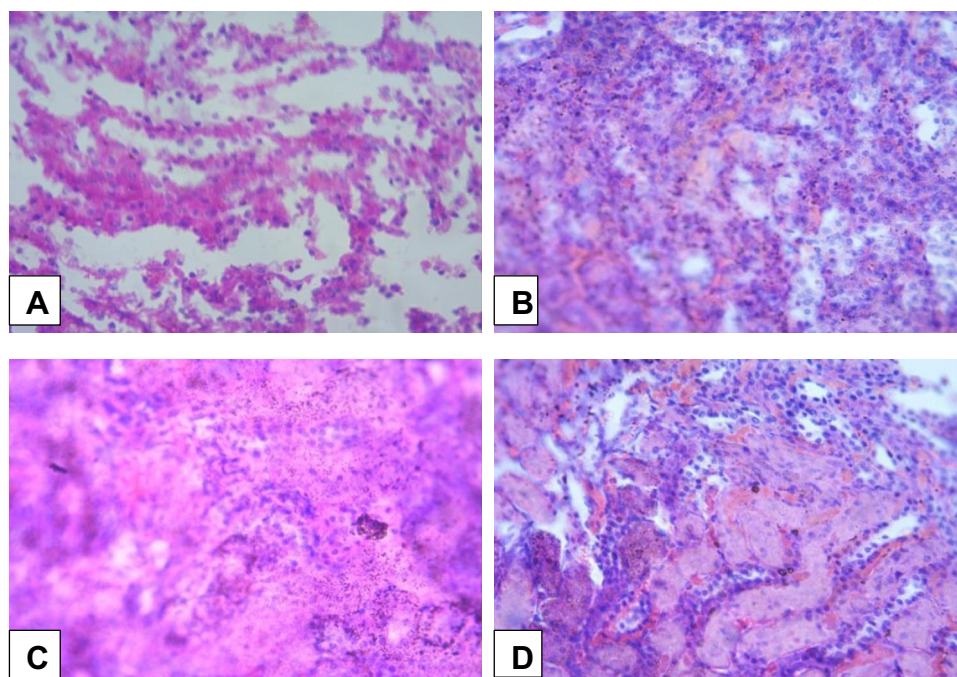


Figure 2 Histopathological changes in the kidney tissue treated with ethyl acetate fraction. (A) Normal control group (normal structures), (B) Cyclophosphamide control group (necrosis), (C) Ethyl acetate fraction 100 mg/kg (necrosis), (D) Ethyl acetate fraction 200 mg/kg (inflammatory cells and congestion).

Histopathologic Findings

Kidney tissues isolated from normal control rats showed intact kidney structures (nephron, basement membranes). While cyclophosphamide-treated group revealed damaged bowman's capsule, inflammatory cells and necrosis, rats treated with *Croton macrostachyus* revealed mild congestion and a decreased number of inflammatory cells (Figures 1 and 2).

Discussion

Cyclophosphamide is an alkylating agent used for cancer management.²⁹ It is broadly used for the treatment of leukemias, lymphomas, multiple myeloma, rheumatic arthritis and prior to bone marrow transplantation.³⁰ Despite its wide array of clinical applications, cyclophosphamide can cause multiple organ toxicity, including nephrotoxicity, cardiotoxicity, hepatotoxicity and reproductive toxicity.^{28,31}

Clinical use of cyclophosphamide is limited due to nephrotoxicity. Increased generation of both reactive oxygen and nitrogen species in kidney tissues, lipid peroxidation and inhibition of superoxide dismutase activity plays a critical role in the pathogenesis of cyclophosphamide-induced kidney damage.^{9,27}

Plants with naturally occurring antioxidant activity can be utilized to control homeostasis between free radicals

and antioxidant stress. Traditionally used medicinal plants might offer important therapeutic effects.³²

In the present study, cyclophosphamide administration resulted in increased kidney weight and kidney-to-body weight ratio. This result agreed with earlier reports by several researchers.^{28,33} This might be due to the formation of cyclophosphamide-induced hydronephrosis and nodules.³⁴ Whereas treatment with crude extract 400 mg/kg and aqueous fractions prevented the increment in kidney weight. All treatment groups prevented increases in kidney-to-body weight ratio compared to cyclophosphamide-treated group. The antioxidant activity²⁴ of *Croton macrostachyus* might be the reason for the prevention of kidney and kidney-to-body weight increment.

Serum creatinine and BUN were used as a measure of kidney function and their increment is associated with kidney damage or failure.³⁵ Nephrotoxicity can be defined as an increase in the serum creatinine concentration by 0.5 mg/dl or more, over individual baseline levels.³⁶ Also, it should be noted that the normal plasma level of creatinine need not necessarily indicates the absence of renal damage after cyclophosphamide treatment and it needs to be confirmed by other parameters like BUN and histological findings.³⁷

In the present study, cyclophosphamide administration increased both serum creatinine and BUN levels compared

to normal group animals. These results were consistent with previously reported studies.^{28,38} Increased levels of serum creatinine and BUN might be due to the cyclophosphamide-induced kidney damage, which resulted in the leakage of these cytosolic enzymes to the circulatory system. Acrolein decreases the activities of antioxidant enzymes by enhancing oxidative stress and results in an increase in lipid peroxidation and the formation of reactive oxygen species including singlet oxygen, superoxide anion radicals, and hydroxyl radicals.³⁹ This is an indication of disturbances in the biosynthesis of these marker enzymes, with alteration in the membrane permeability as a result of kidney damage.⁴⁰ On the basis of the result obtained from this experiment, administration of the *Croton macrostachyus* crude extract and solvent fractions decreased serum creatinine and BUN level back to near normal level. The observed protective effect of *Croton macrostachyus* might be due to its antioxidant activity; mitigating lipid peroxidation and maintaining the renal biomembrane integrity and stability.²⁸

Histopathologic findings from kidney tissues supported the results of the crude extract and solvent fractions of *Croton macrostachyus* on biochemical results. Kidney isolated from the normal control group showed normal structures of the kidney. While cyclophosphamide alone administration resulted in distorted bowman's capsule, inflammation and necrosis, rats treated with *Croton macrostachyus* showed a decreased number of inflammatory cells and mild congestion.

Conclusion

The crude extract, ethyl acetate and aqueous fractions of *Croton macrostachyus* proved to have protective activity against cyclophosphamide-induced nephrotoxicity. We recommend further study on the isolation, identification, and characterization of the active component(s) of the extracts and elucidation of their possible molecular mechanism of action.

Abbreviations

BUN, blood urea nitrogen; OECD, Organization for Economic Cooperation and Development; SCr, serum creatinine; WHO, World Health Organization.

Data Sharing Statement

The outcome of this research was generated from the data collected and analyzed based on the specified methods and materials. The original data supporting these findings will be accessible at the time of reasonable request from the corresponding author.

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Disclosure

The authors report no conflicts of interest in this work.

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