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Original article

α-Amylase inhibitory property, antioxidant activity and toxicological study of *Salvia chloroleuca*

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Abstract

Background and objectives: *Salvia chloroleuca* Rech. f. & Allen., is an endemic species growing wildly in north east and center parts of Iran but there is no information about its safety. To provide information about the safety of the species, we evaluated its acute and sub chronic toxicity in rats. **Methods**: In acute toxicity study, the aqueous methanol (80%) extract at a single dose of 2000 mg/kg/day was administered orally to male and female rats and signs of toxicity two weeks after administration were observed. For the subchronic toxicity test, the extract at doses of 250, 500 and 1000 mg/kg/day were orally administered to the rats of both sexes for 45 days. Mortality, clinical signs of toxicity and body weight changes were monitored during the study. Moreover, α-amylase enzyme inhibition, total phenol content, and antioxidant (DPPH and FRAP assays) activity of different fractions of aerial part were evaluated. **Results**: The methanol and aqueous methanol (80%) extracts showed α-amylase enzyme inhibition with IC_{50} values 14.03 mg/mL and 18.05 mg/mL, respectively. The IC_{50} value for ethyl acetate, methanol and aqueous methanol (80%) extracts in radical scavenging assay were calculated as 288.83, 97.93, and 108.02 µg/mL, respectively. Among all extracts, methanol (228.4±12.05) demonstrated the highest FRAP value, followed by methanol (80%) extract (220.4±8.08) and ethyl acetate extract (156.4±10.06). In acute toxicity and subchronic study, neither mortality nor changes in behavior or any other parameter were observed. **Conclusion:** Our findings indicate potent *in vitro* α-amylase and antioxidant activity of *S. chloroleuca* and propose its potential as an anti-diabetic agent for treatment of noninsulin-dependent diabetes mellitus (NIDDM) patients.

Keywords*:* α-amylase, antioxidant, *Salvia chloroleuca*

Introduction

Diabetes mellitus is a serious health and lifestyle concern that is characterized by elevated blood glucose levels. Retarding the absorption of carbohydrates from digestive tract is one of the best therapeutic approaches for reducing postprandial hyperglycemia especially in patients with type II diabetes mellitus [1]. Inhibitors of carbohydrate hydrolyzing enzymes delay

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carbohydrate digestion and cause a reduction in the rate of glucose absorption [2]. α-Amylase is a carbohydrate hydrolyzing enzyme that catalyses the hydrolysis of starch into maltose and finally to glucose, which is the only sugar that can be used by the body. Consequently, these enzymes can be important targets in management of postprandial hyperglycemia in type II diabetic patients [3]. In recent years, there is renewed interest in plants and their constitutions in prevention and treatment of diabetes and many scientists have focused on hypoglycemic agents from medicinal plants [4]. In Iranian Traditional Medicine (ITM) a number of medicinal plants including *Salvia* species have been mentioned for their hypoglycemic effects [5]. *Salvia* is an important genus including about 900 species in Lamiaceae family which is named "Maryam-goli" in Persian language. There are 61 *Salvia* species growing naturally in Iran, 17 of which are endemic [6]. *Salvia* species have been used in folk medicine throughout the world. It has been speculated that Afghanistan is the origin of S*alvia* although the highest number of species (about 250) are found in Mexico [7]. Traditionally, the crude extracts of *Salvia* species have been used for various purposes such as food, drugs and perfumery and have shown different biological activities [8]. *Salvia chloroleuca* Rech. f. & Allen., is an endemic plant in Iran. The chemical constituents of its essential oil has been investigated and the main components have been reported as *α*-pinene, β-pinene, β-caryophyllene, 1,8-cineole and carvacrol [9].

The aim of the present study was to evaluate the *in vitro* α-amylase enzyme inhibition and the antioxidant (DPPH and FRAP assays) activity of different extracts of *S. chloroleuca*. To the best of our knowledge, there is no study about the α-amylase inhibitory effects of *S. chloroleuca*. In addition, the acute and sub chronic oral toxicity of the methanol (80%) extract of *S. chloroleuca* have been determined for the first time.

Experimental

Plant material and extraction

Aerial parts of *S. chloroleuca* were collected from suburbs of Tehran in spring 2012 and were identified by Dr. Y. Ajanii. A voucher specimen was kept at the Herbarium of the Institute of Medicinal Plants (No. ACECR-244). The flowered aerial parts (1 kg) of *S. chloroleuca* were dried in shade, crushed and then extracted with aqueous methanol (80%) by percolation for 48 h at room temperature (thrice). The extract was concentrated using a rotary vacuum evaporator and then freeze dried. The freeze dried extracts (38 g) was submitted to silica gel column chromatography with ethyl acetate and methanol as the mobile phase to give two main fractions.

Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazyl) and butylated hydroxyanisole (BHA), were purchased from Merck, Germany. Folin-Ciocalteu, gallic acid, and TPTZ (2,4,6-tripyridyl-*s*-triazine) were purchased from Sigma, St. Louis, MO, USA. Vitamin E and acarbose were purchesd from Toliddaru, Tehran, Iran. All solvents and chemicals used in the research were of analytical or HPLC grade.

Animals

Four-week-old male and female Wistar rats (weight: 150–260 g; age: 6-8 weeks old) were obtained from Pasteur institute (Tehran, Iran). The rats were clinically healthy and they were at the standard situation of water, food and temperature (22±2 °C) under a 12-hour-dark-light cycle. All experiments were prepared according to the ethical committee for the use of laboratory animals of Tehran University of Medical Science, 12 April 2010.

α-Amylase inhibition study

The α-amylase activity of each extract was measured based on the spectrophotometric assay using acarbose as the reference compound [10]. The extracts were dissolved in DMSO to give concentrations from 10 to 40 mg/mL (10, 20, 30, and 40 mg/mL). The starch solution (0.5% w/v) was prepared by mixing 0.25 g of potato starch in 50 mL of distilled water. The enzyme solution (0.5 unit/mL) was obtained by mixing 0.001 g of

α-amylase (EC 3.2.1.1) in 100 mL of 20 mM sodium phosphate buffer pH 6.9. One mL of enzyme solution and 1 mL of each extract were mixed and incubated at 25 °C for 30 min. After that, 1 mL of this solution was added to the 1 mL of starch solution and was incubated at the same temperature for 3 min. Afterwards, 1 mL of the colored reagent (containing 96 mM 3,5-dinitrosalicylic acid (20 ml), 5.31 M sodium potassium tartrate in 2 M sodium hydroxide (8 mL) and 12 mL of distilled water) was added and placed in 85 °C water bath for 15 min. The solution was cooled and diluted with 9 mL distilled water. The absorbance was measured at 540 nm. The inhibition percentage of α-amylase was calculated by the following equation:

$$\%\text{inhibition} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test}}}{\text{Absorbance}_{\text{control}}} \times 100$$

Free radical scavenging assay (DPPH assay)

Free radical scavenging activity of extracts was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reagent [11]. DPPH solution (4×10^{-2} mg/mL in methanol) was prepared and 5 mL of this solution was mixed with 1 mL of various concentrations of sample solutions in methanol. After 30 min, the absorptions were recorded at 517 nm using a blank containing extract or standards without DPPH radicals. BHA and vitamin E were used as positive standards. The assessment was done for at least 3 times. Inhibition of DPPH radicals was measured using the following equation:

$$\text{DPPH scavenging effect (\%)} = [1-((A_1-A_2)/A_{0)}] \times 100$$

Where, A_1 was the absorbance of the extract and reagents. A_2 was the absorbance without DPPH and A_0 was absorbance of DPPH without extract. IC_{50} values were calculated from plotting inhibition percentage against the extract concentration which expressed the concentration of sample needed to scavenge 50% of DPPH radicals.

FRAP assay

The potential antioxidant ability of different extracts was determined by measuring their abilities to reduce Fe^{3+} (ferric iron) to Fe^{2+} (ferrous iron) by FRAP (Ferric Reducing Antioxidant Power) assay [12]. Briefly, 6 mL of each sample were added to 1.5 mL of the freshly prepared FRAP solution which contained 2.5 mL of 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3.6H_20$ and 25 mL of 0.3 M sodium acetate buffer (pH 3.6) and allowed to remain for 90 min at room temperature then absorbance of the solution was recorded at 595 nm. $FeSO_4.7H_2O$ was used as the standard and the absorbance was recorded. The antioxidant power calculated as the Mmol of $FeSO_4.7H_2O$ equivalent per gram of the sample.

Total phenol assay

Total phenolic compounds of the extracts were calculated using Folin-Ciocalteu procedure [13] with little modification. Lyophilized extract was mixed in methanol then it was added to 2.5 mL of the reagent (diluted 1:10).Then the solution was left for 5 min at room temperature. Finally after 90 min of incubation at 40°C, 2 mL of Na_2CO_3 (7.5% v/v) was added to the mixture. The sample absorbance was read at 760 nm and the standard curve was plotted using 0, 50, 100, 150, 200, 250 mg/L solutions of gallic acid in methanol: water (50:50, v/v). The total phenol content was expressed as mg gallic acid equivalent per 100 g of extracts.

Acute toxicity study

The rats were randomly divided to control and two treatment groups and they were housed in clear plastic cages. Each cage contained five rats of the same sex. The animals were fasted for 4 h prior to dosing. A single dose of 2000 mg/kg body weight of the extract was administered by gavage, to the treatment groups, while the control group received distilled water by gavage in the same volume. The *S. chloroleuca* aqueous methanol (80%) extract suspended in distillated water was administered. The rats were monitored for signs of toxicity, any behavioral changes and mortality continuously for the first 4 h after dosing and then once daily for further 13 days [14]. The number of dead animals was recorded at the end of the study.

Sub chronic toxicity study
Forty wistar rats were randomly divided into four groups of 10 animals each (five males and five females in separated cages), under the same standard conditions as described above. Their initial weights were recorded. Group I received distillated water as the control, Groups II, III, and IV were treated with single doses of 250, 500, 1000 mg/kg/day of *S. chloroleuca* extract by gavage for 45 days. All rats were observed daily for mortality, physiological, and behavioral changes. The animals were weighted weekly during the course of the study. At the end of study the liver and kidney were removed, weighed and fixed in 10% buffered formalin solution for pathological examination.

Hematology and biochemical analysis
After 45 days, animals were fasted 12 h and anesthetized with diethyl ether for blood collection from common carotid artery. Blood samples (1.5 mL) were collected into tubes containing anticoagulant for hematology tests. The following hematological parameters were calculated; red blood cell count (RBC), white blood cell count (WBC), Hemoglobin concentration (Hgb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelets count, platelet distribution width (PDW), and red distribution width (RDW). The non-heparinized blood samples were allowed to coagulate before being centrifuged and the serum was separated. The serum was assayed for glucose, creatinine, uric acid, urea, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), triglycerides, lactate dehydrogenase (LDH), serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), albumin, and total protein.

Statistical analysis
The results were calculated as the mean±SEM for inhibition percentage, body weights, hematological and biochemistry factors. Group comparisons were performed by the analysis of variance using ANOVA followed by Tukey's test. *p* values less than 0.05 were considered significant.

Results and Discussion
α-Amylase inhibition
The *in vitro* α-amylase inhibitory study demonstrated that the methanol and aqueous methanol (80%) extracts of *S. chloroleuca* had a significant α-amylase inhibitory activity (IC_{50} 14.03 and 18.05 mg/ml, respectively; however, the ethyl acetate extract produced weaker enzyme inhibition and it could only demonstrate 44% inhibition of α-amylase in concentration of 40 mg/mL. The IC_{50} value of the reference standard, acarbose, was measured as 0.031 µg/mL.

Antioxidant and total phenolic content assays
The free radical scavenging activity of the extracts was evaluated using DPPH assay. DPPH is a molecule which contains free radicals and has been widely utilized to assess the radical scavenging ability of antioxidants. The IC_{50} values for BHA, vitamin E, ethyl acetate, methanol and aqueous methanol (80%) extracts in radical scavenging were calculated as 8.58, 7.89, 288.83, 97.93, and 108.02 µg/mL, respectively. Total phenol content of the aerial parts of *S. chloroleuca* was measured as 19.8±0.00, 63.66±0.03, 58.87±0.01 (mg gallic acid/100 g) for the ethyl acetate, methanol and aqueous methanol (80%) extracts, respectively. Among all extracts, the methanol extract showed the highest FRAP value (228.4±12.05 Mmol $FeSO4.7H_2O$ /g extract), followed by the aqueous methanol (80%) (220.4±8.08) and the ethyl acetate extracts (156.4±10.06).

Acute toxicity
All rats treated with 2000 mg/kg of extract of *S. chloroleuca* orally were alive during the 14 days of observation. The animals did not show any abnormal gross findings during the observation period. Moreover, no significant changes were observed in body weight between the control and treatment groups.

Sub chronic toxicity

The result of sub chronic toxicity study revealed no significant differences in the mean body weight of groups in comparison with the control group (figure 1). No deaths or significant differences were observed in general behavior or other physiological activities in any of the groups throughout the experimental period. The effects of sub chronic oral administration of *S. chloroleuca* methanol (80%) extract on the hematological parameters have been shown in table 1. The results indicated no significant changes in hematological parameters in the male and female treatment groups compared to the control group. The biochemical analysis (table 2) showed no significant changes in any of the parameters tested in either the control or *S. chloroleuca* treated group of both sexes. Pathological analysis of liver (figure 2) and kidney (figure 3) revealed no signs of toxicity or detectable abnormalities in both sexes.

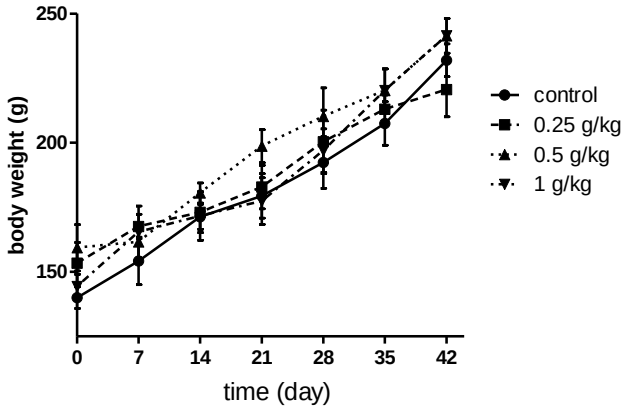

Figure 1. Changes in rats body weight after sub chronic oral treatment with the aqueous methanol (80%) extract of *S. chloroleuca* for 45 days. Each point represents mean±SEM for n=5

The results of the acute toxicity study suggested that the extract from the aerial part of *S. chloroleuca* was not toxic after an acute exposure to the dose of 2000 mg/kg. So, the LD_{50} (lethal dose) of the aqueous methanol (80%) extract in male and female rats could be higher than 2000 mg/kg.

The sub chronic toxicity study of *S. chloroleuca* extract with three doses of 250, 500, 1000 mg/kg/day for 45 days did not show any toxicity effects in treated groups. Changes in body weight were used as a sign of adverse effects of drugs and chemicals [15]. Since no significant differences were detected in the body weight of rats in the treated groups as compared to the control group after 45-day period of daily treatment, it was proposed that administration of these doses had no effect on the normal growth of rats. No significant differences were seen between the control and *S.chloroleuca* treatment groups in hematological and biochemical parameters. Moreover, no signs of toxicity were observed in pathological examinations of liver and kidney. Consequently, pathological studies confirm the safety data of physiological, biochemical, and hematological parameters related to oral administration of *S. chloroleuca* aqueous methanol (80%) extract.

The findings of the present *in vitro* study clearly demonstrated the potent α-amylase inhibitory effects and antioxidant activities of the extracts from the aerial parts of *S. chloroleuca.* Drugs that inhibit carbohydrate hydrolyzing enzymes have shown to decrease post-prandial hyperglycemia and improve glucose metabolism without inducing the insulin secretion of NIDDM patients. Polyphenols and flavonoids as well as their sugar derivatives possess a wide range of biological activities including antioxidant [16] and carbohydrate hydrolyzing enzyme inhibition [17]. Therefore, it is reasonable to assume that the observed α-amylase inhibitory activity of *S. chloroleuca* extract in our study could be in part related to the phenol content. The inhibitory activity of these compounds against digestive enzymes is due to their ability to bind to proteins [18,19]. The results of structure–activity relationship of polyphenols inhibiting α-amylase confirm that hydroxylation of flavonoids improved the inhibitory effect on α-amylase, while glycosylation, methylation, and methoxylation of flavonoids decreased the inhibitory effect [20]. Among the tested extracts, a good correlation between phenolic contents and antioxidant activities with α-amylase inhibitory effects was found.

Table 1. Hematologic values in Wistar rats after 45 days treatment with *S. chloroleuca*

Sex	Group	RBC (10^6/mm^3)	Hgb (g/dL)	HCT (%)	MCV (fi)	MCHC (%)
Male	I	8.52±0.40	16.21±0.23	46.32±0.80	52.22±1.76	35.20±0.30
	II	8.21±0.31	17.20±1.21	48.43±1.23	57.12±3.23	36.42±0.25
	III	8.67±0.44	16.50±0.56	45.76±0.43	54.65±0.87	35.76±0.25
	IV	8.36±0.11	16.76±0.43	42.50±2.44	53.20±0.37	36.20±0.25
Female	I	7.68±0.70	15.00±0.20	37.34±0.56	50.30±1.33	35.22±0.25
	II	7.90±0.02	14.80±0.05	36.22±0.89	50.26±0.45	34.90±0.25
	III	7.41±0.43	16.22±1.10	40.10±2.21	50.87±1.43	36.40±0.25
	IV	7.12±0.31	15.45±0.55	36.52±0.32	51.30±2.22	36.65±0.40
Sex	**Group**	**WBC (10^3/mm^3)**	**Platelets (10^3/mm^3)**	**MPV (fi)**	**RDW (%)**	**PDW (%)**
Male	I	6.26±1.24	1232.00±56.73	6.90±0.20	13.20±0.40	8.23±0.10
	II	7.40±2.43	934.30±115.30	6.81±0.12	13.25±0.09	7.30±0.20
	III	6.25±0.99	988.24±123.43	6.31±0.22	13.33±0.70	7.66±1.00
	IV	7.21±1.23	1158.11±156.11	6.71±0.32	13.10±0.24	7.70±0.10
Female	I	5.76±2.02	997.81±156.13	6.12±0.27	12.27±0.44	7.40±0.10
	II	4.84±2.11	1127.43±161.45	6.00±0.20	12.10±0.50	7.10±0.02
	III	5.32±0.99	947.81±193.76	5.80±0.910	11.81±0.50	7.00±0.10
	IV	6.02±1.56	897.31±221.22	6.03±0.03	11.61±0.70	7.04±0.02

Group I (control), group II (250 mg/kg), group III (500 mg/kg) and group IV (1000 mg/kg) for 45 days. Data presents mean±SEM for 5 rats.

So, it can be concluded that the high amount of total phenols in the methanol extract are responsible for the more considerable antioxidant activity and potent α-amylase inhibitory effects of this portion. The result of clinical and experimental studies proposes that free radicals play an important role in the pathogenesis and progression of diabetes mellitus [21]. Thus, *S. chloroleuca* extracts might be beneficial in control of diabetes and oxidative stresses by inactivation of free radicals.

Table 2. Biochemical values in Wistar rats after 45 days treatment with *S. chloroleuca*

Sex	Group	Glucose (mg/dL)	Creatinine (mg/dL)	Uric Acid (mg/dL)	Urea (g %)	Cholesterol (mg/dL)
Male	I	230.10±21.20	0.45±0.02	4.03±0.23	48.20±4.41	78.30±2.10
	II	198.67±11.60	0.43±0.03	3.81±0.6	50.20±2.75	70.30±5.00
	III	202.50±12.80	0.46±0.05	3.41±0.45	43.00±.3.20	72.10±4.30
	IV	178.40±28	0.46±0.01	3.50±0.71	52.50±3.36	83.70±2.20
Female	I	200.50±22.80	0.41±0.02	4.31±0.30	50.65±2.45	81.20±2.80
	II	193.20±19.76	0.42±0.03	4.01±0.40	44.87±2.50	87.10±3.30
	III	186.45±31.10	0.42±0.08	3.68±0.32	56.34±5.42	92.10±3.30
	IV	208.23±27.60	0.45±0.02	3.50±0.71	52.50±3.36	83.70±2.20

Sex	Group	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	Triglycerides (mg/dL)	LDH (U/L)
Male	I	48.33±4.50	26.03±6.77	9.70±7.20	50.20±12.20	1351.00±180.2
	II	38.00±6.57	37.45±5.30	11.20±3.20	70.14±6.90	1832.60±140.30
	III	44.39±5.60	28.88±4.43	14.40±0.91	55.90±11.00	1553.20±178.40
	IV	36.22±3.91	27.99±4.00	12.40±2.81	66.43±9.66	1946.30±130.30
Female	I	90.20±5.08	33.35±3.98	15.82±1.54	58.55±8.50	1667.30±38.30
	II	77.00 ±7.41	30.00±4.30	12.88±3.90	80.20±17.22	2012.10±165.40
	III	87.33±6.00	29.92±6.30	13.70±4.00	68.43±12.20	1629.30±150.30
	IV	36.22±3.91	27.99±4.00	12.40±2.81	66.43±9.66	1946.30±130.30

Sex	Group	SGOT (U/L)	SGPT (U/L)	Albumin (g/dL)	Total Protein (mg/dL)
Male	I	143.46±16.23	57.20±7.60	3.12±0.36	7.89±0.54
	II	182.61±16.85	48.00±10.20	3.31±0.21	8.33±0.22
	III	152.10±15.70	61.50±8.40	2.93±0.65	9.01±0.22
	IV	193.20±13.10	70.25±5.25	3.31±0.87	8.11±0.10
Female	I	131.20±18.25	65.41±3.50	3.01±0.65	7.73±0.23
	II	153.00±16.30	44.00±9.80	2.64±0.10	7.80±0.12
	III	122.41±15.50	47.20±2.30	2.94±0.43	8.16±0.18
	IV	193.20±13.10	70.25±5.25	3.31±0.87	8.11±0.10

Group I (control), group II (250 mg/kg), group III (500 mg/kg) and group IV (1000 mg/kg) for 45 days. Data presents mean±SEM for 5 rats.

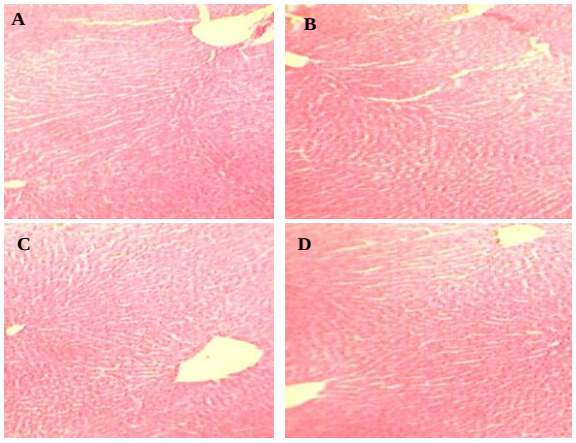

Figure 2. Microscopic panel of liver for control (A) and *S. chloroleuca* treated groups [250 mg/kg (B), 500 mg/kg (C) and 1000 mg/kg (D)] in sub chronic toxicological study. No significant damages were detected after oral administration of the aqueous methanol (80%) extract of *S. chloroleuca* for 45 days in any treatment groups

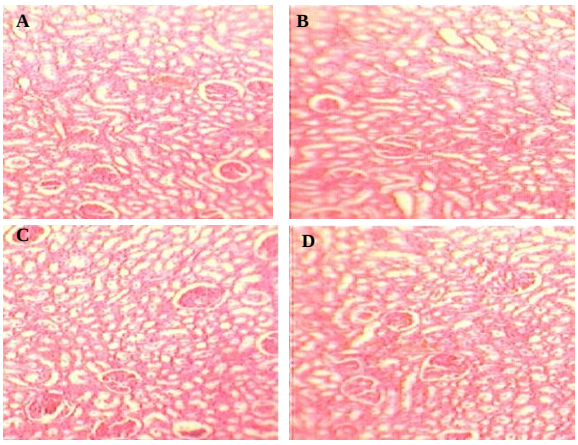

Figure 3. Microscopic panel of kidney from control (A) and *S. chloroleuca* treated groups [250 mg/kg (B), 500 mg/kg (C) and 1000 mg/kg (D)] in sub chronic toxicological study. No significant damages were detected after oral administration of aqueous methanol (80%) extract of *S. chloroleuca* for 45 days in any treatment groups

In conclusion, the aqueous methanol (80%) extract of *S. chloroleuca* was found to be nontoxic during oral acute and sub chronic toxicity studies in male and female rats. Regarding the antioxidant capacity and α-amylase inhibitory activities, it is suggested to evaluate the effect of *S. chloroleuca* extract on type II diabetes although mutagenicity and carcinogenicity studies are required to further confirm the safe use of this plant.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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