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Original Article

SAMBUCUS NIGRA LINN: *IN VIVO* STUDIES ON RABBITS

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ABSTRACT

Objective: The research work was carried out to explore the safety and efficacy of a low dose of *Sambucus nigra* Linn [*S. nigra*] extract on hematological, biochemical and histopathological parameters of rabbits organs (kidney, liver, stomach and heart).

Methods: *Sambucus nigra* extract was given orally for 90 d to test group rabbits [Test female (SF); Test male (SM)]. Then blood samples of control [C (female); C (male)] and test rabbits were collected by cardiac puncture and changes in hematological, biochemical and histopathological parameters were observed and interpreted.

Results: Gender-based differences were observed in hematological, kidney function, liver function, cardiac enzymes and lipid profile investigations. Urine analysis showed similar results as that of standard and control drug. No noteworthy pathology was observed in heart, stomach, liver and kidney tissues of rabbits, treated with *Sambucus nigra* in a dose of 25 mg/kg/day.

Conclusion: Our results justify the well-documented safe and effective use of *Sambucus nigra* in medicine for curing various pathologies.

Keywords: Black Elder, Anthocyanins, Anti-oxidant, Anti-viral, Europe

INTRODUCTION

Sambucus nigra Linn. (Caprifoliaceae) has been used in the traditional system as a diaphoretic, diuretic, astringent, laxative, anti-inflammatory, anti-oxidant, anti-spasmodic, anti-diabetic, immune stimulant, emetic as well as for the treatment of gastrointestinal, kidney, liver and skin diseases. It is reported that it contains flavonoids, quercetin, rutin, anthocyanin, cyanogenic glycosides, vitamin A and C [1].

German Commission E approved its flowers utilization as medicine for colds whereas bark, leaves, and berries utilization is not approved by World Health Organization (WHO), German Commission E and European Scientific Cooperative on Phytotherapy (ESCOP) for any medicinal use [2].

The purpose of this research work was to carry out chronic toxicity studies of *S. nigra* extract on both genders of rabbits due to lack of toxicity data on it. Chronic toxicity studies included evaluation of hematological, biochemical and urine parameters, as well as, estimation of histopathological changes occurring in liver, heart, kidney and stomach tissues of treated groups in comparison with their control groups.

MATERIALS AND METHODS

Chemicals

Ethanol, acetic acid, formalin, diagnostic kits, xylene, paraffin wax, eosin, hematoxylin and canada balsam were purchased from Merck, Germany. All the chemicals were of analytical grade.

Crude drug extract

S. nigra mother tincture (Lot # 0012188808) was purchased from Bioron suppliers (France). *S. nigra* extract was obtained by concentrating under reduced pressure by rota-evaporator (Buchi-Rotary Evaporator, Switzerland, model # B490) at 40 °C. The extract obtained was stored in cool, dry place for further studies.

Experimental animals

Twenty-four male and female rabbits weighing between 1000 and 1,200 g were purchased from animal house of Dow University of Health Sciences, (DUHS), Karachi. They were kept in the animal house for a period of 15 d to acclamized in separate cages. They were fed commercial feed and water *ad libitum*. Their weights were checked on a random basis. Blood (6 ml) was collected from rabbits for analyses of hematological and biochemical parameters by cardiac puncture at the end of three months. Blood samples collected into clean non-heparinised bottles were allowed to clot, and serum was separated from the clot and centrifuged according to groups into clean bottles for the biochemical analyses. After the collection of blood samples, urine analysis and histopathology was carried out. Animal studies were carried out according to the NIH guide for the care and use of laboratory animals [3].

Drug dosing

Four groups were made (male control–6 rabbits), (female control–6 rabbits), (male test (SM)–6 rabbits) and (female test (SF)–6 rabbits). Male and female control groups were given distilled water, while test groups SM and SF was given 25 mg/kg *S. nigra* extract. All the administrations were given orally. The treatment continued for 90 d. Blood (6 ml) was collected by cardiac puncture with 10 ml sterile syringe using 1 mg/1 ml EDTA as anti-coagulant for the determination of blood and biochemical parameters.

Hematological evaluation

Hematological examination of the collected blood samples was performed according to standard procedures listed as follow. Total erythrocyte counts were counted using a Neubar chamber under a light microscope at 40 x 10 magnifications. Blood samples were diluted to 200 times by Hayem's reagent before counting. Blood hemoglobin concentration was determined using a Sahli's hemometer. Micro Wintrobe hematocrit tubes and hematocrit centrifuge were used to determine the (PCV). Total leucocyte counts were detected using a Neubar chamber under a light microscope at 10 x 10 magnification after diluting blood samples to 10 times with Turk's solution. Mean erythrocyte volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) for particular blood samples were also calculated [4-8].

Biochemical evaluation

Serum samples were obtained by centrifugation of blood at 1300 x g for 15 min. The Menarini Classic Chemistry Analyzer was used to

determine the calcium (Ca), phosphorus (P), blood urea, creatinine, total bilirubin, total protein, albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine phosphokinase (CPK), cholesterol, glucose, amylase, and gamma-glutamyl transferase (GGT). The globulin concentration was determined by subtracting the albumin concentration from the total protein concentration [9-10].

Urine analysis

Voided sample of urine was collected by placing a clean, empty box in the site where the animals usually urinates [11].

Histopathological analysis

After blood collection, the liver, kidney, heart and stomach of the male control and test group were carefully dissected from the abdominal region and were immediately fixed in 10% neutral buffered formalin. Fixed samples were trimmed and processed for paraffin embedding. Sections (5–7 µm) were cut, and the tissues were dehydrated with alcohol of graded concentrations and allowed to dry. The sample slides were subsequently stained in haematoxylin-eosin and examined under a light microscope; photomicrographs of the samples were recorded [12-14].

Statistical analysis

All the results were presented as a mean plus or minus standard error of mean (M±SEM). Differences between control and treatment groups were analyzed by student t-test [15].

RESULTS

Effects of *S. nigra* extract on hematological parameters of male and female rabbits were observed (table 1). In female group (SF) treated with *S. nigra* slightly raised erythrocyte count (6.03±0.0073) was observed while MCV (70.75±0.0836) and MCH (19.85±0.0836) levels were found somewhat reduced. In male treated group (SM), hemoglobin (10.808±0.063), hematocrit (37.75±0.0836), MCV (70.25±0.0836), MCH (20.25±0.0836) and total leucocyte count (6.723±0.02) levels were found elevated, and only slight decrease was observed in erythrocyte count (5.35±0.051). Platelet count was significantly lowered (322.5±0.836) in SF group whereas in SM was found to be significantly raised (590.05±0.32). Changes in the male and female test groups' evaluated parameters may be due to changes in their physiology.

In female test group (SF) urea level was observed to be significantly lowered (19.5±0.836). Creatinine (0.545±0.0083), total protein (7.48±0.0063), albumin (4.985±0.022) and A/G ratio (2.043±0.018) were found towards lower side in SF group as compared to control group. Phosphorus (5.425±0.0083), uric acid (0.0267±0.0048) and globulin (2.485±0.0083) levels were found raised in SF group in comparison with control group. In male test group (SM) creatinine (0.571±0.01), phosphorus (4.625±0.0083), uric acid (0.015±0.002) and globulin (2.65±0.0063) levels were reduced while urea (28.5±0.836), calcium serum (15.285±0.008), total proteins (7.6±0.384), albumin (4.983±0.02) and A/G ratio (1.88±0.0083) were found elevated as compared to control group (table 2).

Significant ($p<0.01$) decrease in cardiac enzyme parameters (LDH-149.33±0.96; CPK-567.5±0.836 and CK-MB-487.33±0.83) were observed in the female test group (SF) as compared to the control female group. CPK and CK-MB enzymes levels were found significantly ($p<0.01$) lowered whereas LDH level was observed to be significantly raised in male test group (SM) as compared to the control male group (table 3).

Triglycerides and VLDL levels were found significantly ($p<0.01$) elevated in female (SF) and male (SM) test groups as compared to their control groups. Triglycerides level was found to be significantly lowered in both the species as compared to their respective control groups. Cholesterol level was observed to be significantly ($p<0.01$) lowered in the male test group (SM) as compared with its respective control group; whereas in female test group (SF) $p<0.05$ elevation was observed in cholesterol level as compared with the control group. HDL level (3.25±0.418) was found to be significantly lowered in the female test group (SF) while slightly raised (3.25±0.418) in male test group on comparison with their respective control groups (table 4).

Table 1: Chronic toxicity test: Effect of *S. nigra* extract on complete blood count of rabbits

Blood parameter	Control C (female)	Test female (SF)	Control C (male)	Test male (SM)	Reference range
Haemoglobin	12.15±0.0836	12.183±0.1036	10.05±0.0836	10.808±0.063*	10.75±0.689
RBC (Erythrocyte Count)	5.895±0.00836	6.03±0.0073*	5.485±0.0083	5.35±0.051*	3.916±0.277
Hematrocrit (HCT/PVC)	42.835±0.0739	42.808±0.063	34.2±0.0632	37.75±0.0836*	38.67±1.932
MCV	72.416±0.0658	70.75±0.0836*	62.5±0.836	70.25±0.0836*	89±3.183
MCH	20.835±0.0739	19.85±0.0836*	18.15±0.0836	20.25±0.0836*	30.167±1.180
MCHC	28.783±0.0658	28.008±0.063	29.05±0.0836	28.835±0.0739	32.5±0.836
Total Leucocyte Count	6.05±0.0836	6.25±0.0836	5.5±0.0632	6.723±0.02*	11±1.673
Platelet Count	353.5±0.836	322.5±0.836 **	140.5±0.836	590.05±0.32**	275±41.83

SF = female rabbit treated with drug; SM = Male rabbit treated with drug, All values are mean±SEM; n = 6; * = Significant ($p<0.05$), ** = Highly significant ($p<0.01$).

Table 2: Chronic toxicity test: Effect of *S. nigra* extract on kidney function parameters of rabbits

Biochemical parameters	Control C (female)	Test animal (SF)	Control C (male)	Test animal (SM)	Reference Range
Urea	72.5±0.83	19.5±0.836**	23.5±0.83	28.5±0.836*	29.167±6.39
Creatinine	0.85±0.008	0.545±0.0083*	0.85±0.0083	0.571±0.01*	0.8167±0.127
Calcium (serum)	14.59±0.063	14.475±0.0083	14.17±0.0083	15.285±0.008*	10.03±0.318
Phosphorus	3.825±0.068	5.425±0.0083*	6.195±0.0083	4.625±0.0083*	3.53±0.318
Uric acid	0.0175±0.004	0.0267±0.0048*	0.165±0.0083	0.015±0.002*	3.916±0.639
Total proteins	8±0.02	7.48±0.0063*	7.495±0.0083	7.6±0.384*	7.467±0.347
Albumin	5.83±0.013	4.985±0.022*	4.305±0.0083	4.983±0.02*	4.5±0.28
Globulin	2.153±0.0096	2.485±0.0083*	3.18±0.0083	2.65±0.0063*	2.35±0.146
A/G ratio	2.715±0.0083	2.043±0.018*	1.35±0.016	1.88±0.0083*	0.75±0.052

SF = Female rabbits treated with drug; SM = Male rabbits treated with drug, All values are mean±SEM; n = 6; * = Significant ($p<0.05$), ** = Highly significant ($p<0.01$).

Table 3: Chronic toxicity test: Effect of *S. nigra* extract on cardiac enzymes of rabbits

Biochemical parameters	Control C (female)	Test animal (SF)	Control C (male)	Test animal (SM)	Reference Range
LDH	163.5±0.836	149.33±0.96**	270.5±0.83	737.5±0.836**	331.67±40.34
CPK	729.5±0.83	567.5±0.836**	421.5±0.83	278.67±0.78**	90.33±25.03
CK-MB	852.5±0.83	487.33±0.83**	194.5±0.83	13.5±1.224**	16.67±2.46

SF = Female rabbits treated with drug; SM = Male rabbits treated with drug, All values are mean±SEM; n = 6; * = Significant ($p<0.05$), ** = Highly significant ($p<0.01$).

Table 4: Chronic toxicity test: Effect of *S. nigra* extract on lipid function parameters of rabbits

Biochemical parameters	Control C (female)	Test animal (SF)	Control C (male)	Test animal (SM)	Reference Range
Cholesterol	30.5±0.83	38.5±0.836*	58.5±0.83	27.5±0.836**	109.16±22.24
Triglycerides	0.5±0.83	289.83±1.036**	131.5±0.83	159.5±0.836**	111.67±13.68
HDL	12.5±0.83	3.25±0.418**	6.5±0.83	8.5±0.836*	19.67±3.18
LDL	16.5±0.83	2.25±0.484**	38.5±0.83	3.75±0.418**	103.33±15.14
VLDL	7.5±0.83	57.5±0.836**	26.5±0.83	50.5±0.836**	30±5.83

SF = Female rabbits treated with drug; SM = Male rabbits treated with drug, All values are mean±SEM; n = 6; * = Significant ($p<0.05$), ** = Highly significant ($p<0.01$).

Table 5: Chronic toxicity test: Effect of *S. nigra* extract on liver enzymes parameters of rabbits

Biochemical parameters	Control C (female)	Test animal (SF)	Control C (male)	Test animal (SM)	Reference Range
SGOT	26.5±0.83	26.5±0.836	42.5±0.83	14±0.632**	21.83±3.11
Total Bilirubin	0.27±0.0083	0.24±0.0063	0.265±0.0083	0.175±0.0083*	1.75±0.083
Direct Bilirubin	0.021±0.005	0.14±0.019*	0.041±0.0065	0.118±0.015*	0.029±0.0008
SGPT	41.5±0.83	67.5±0.836**	68.5±0.83	31.5±0.836**	27.5±4.18
Alkaline Phosphatase	37.5±0.83	213.83±1.036**	228.5±0.83	52.5±0.836**	91.67±17.30
Gamma GT	6.5±0.83	8.5±0.836*	9.5±0.83	13.5±0.836*	29.16±6.39

SF = Female rabbits treated with drug; SM = Male rabbits treated with drug, All values are mean±SEM; n = 6; * = Significant ($p<0.05$), ** = Highly significant ($p<0.01$).

Table 6: Chronic toxicity test: Effect of *S. nigra* extract on urine parameters of rabbits

Urine parameters	Control animal C (female)	Test animal (SF)	Control animal C (Male)	Test animal (SM)	Reference range
Urine physical					
Volume	30.08±0.11	10.18±0.29	25.01±0.136	10.33±0.48	179.17±61.81
Colour	Yellow	Yellow	Yellow	Yellow	Pale yellow-red brown
Appearance	Turbid	Turbid	Turbid	Turbid	Clear
Sp. Gravity	1.0045±0.00037	1.004±0.00065	1.0045±0.00037	1.004±0.00065	1.019±0.007
pH	9±0.063	9.03±0.096	9±0.063	9.1±0.1	8.53±0.195
Urine chemical					
Protein	Nil	Nil	+1 (30 mg/dL)	Nil	Negative
Glucose	Nil	Nil	Nil	Nil	Negative
Ketone Bodies	Negative	Negative	Negative	Negative	Negative
Urobilinogen	Normal	Normal	Normal	Normal	Negative-weak positive
Blood	Negative	Negative	Negative	Negative	Negative
Bilirubin	Nil	Nil	Nil	Nil	Negative
Urine microscopy					
RBC	Nil/HPF	Nil/HPF	Nil/HPF	Nil/HPF	Nil/HPF
WBC	Nil/HPF	Nil/HPF	Nil/HPF	Nil/HPF	Nil/HPF
Epithelial Cell	Nil/HPF	Nil/HPF	Nil/HPF	Nil/HPF	Nil/HPF

Values are mean±SEM; n = 6; * = Significant ($p<0.05$), ** = Highly significant ($p<0.01$)

SGPT (67.5±0.836) and alkaline phosphatase (213.83±1.036) levels were found to be significantly elevated in the female test group (SF) as compared to the female control group. SGPT (31.5±0.836) and alkaline phosphatase (52.5±0.836) levels were found to be lowered in the male test group (SM) with respect to its control group. SGOT levels (14±0.632) were observed to be considerably lowered in male test group (SM) as compared to its control group (table 5). No significant changes were observed in urine parameters of the female test group (SF) and male test group (SM) in comparison to their respective control groups (table 6).

Effects of *S. nigra* on histopathological parameters of male and female rabbit's liver, kidney, heart and stomach tissues were studied and are shown in fig. 1 and 2.

DISCUSSION

German Commission E has documented several medicinal uses of *S. nigra* [16]. *S. nigr*a may inhibit influenza virus types A and B according to the pre-clinical studies conducted by Serkedjieva *et al.* (2010) and Zakay-Rones *et al.* (1995) [17-18]. *S. nigra* medicinal usage is attributed to its following pharmacological activities: anti-viral, anti-inflammatory,

anti-oxidant, diuretic, anti-allergic, antitussive, bronchodilators, anti-cancer and laxative effect. These pharmacological effects of *S. nigra* may be due to the presence of active phenolic constituents comprising of phenolic acids, flavonoids, catechins, and proanthocyanidins [19-21]. The active constituents of *S. nigra* are claimed to provide beneficial effects in reducing the incidence of cardiovascular diseases, cancer, hyperlipidemia and other chronic diseases, as well as ocular deficiencies [22-26].

According to German Commission E, Committee on Herbal Medicinal Products (HMPC) assessment report on *Sambucus nigra* and the American Botanical Council clinical guidelines documents; no adverse effects and toxicity of *S. nigra* were reported [27-30]. The only reported toxicity is of ingestion of unripe or insufficiently cooked elderberries [28-29]. In one repeated dose study carried out on rabbits by Chibanguza *et al.* (1984) on combination product (Sinupret) containing *S. nigra*; no significant toxicity was observed [31]. No acute or chronic toxicity data is available on *S. nigra*.

S. nigra berries are reported in Readers Digest (1986), to be used in the preparation of delicious wine and jam. On the other hand different parts of *S. nigra* have been reported to contain alkaloids that might produce toxicity [32]. As different parts of *S. nigra* have been found to be effective and safe for treatment of different pathological conditions but no toxicity studies data available on it.

Our research work was an endeavor to explore the chronic toxicity by low dose administration of *S. nigra* extract in rabbits for three months and then evaluating their hematological, biochemical and urine parameters as well as carrying out histopathological studies to observe the extract's effects on vital organs.

Significant effects of *S. nigra* extract, low dose administration for three months were observed in the following biochemical parameter: platelet count of male rabbits was elevated from 140.5±0.836 to 590.05±0.32.

Urea level of female rabbits were lowered from 72.5±0.83 to 19.5±0.836. LDH enzyme in male rabbits were elevated from 270.5±0.83 to 737.5±0.836. CPK of female rabbits were lowered from 729.5±0.83 to 567.5±0.836 and in male rabbits CPK level were reduced from 421.5±0.83 to 278.67±0.78. CK-MB enzyme level was found lowered in both female (852.5±0.83 to 487.33±0.83) and male rabbits (194.5±0.83 to 13.5±1.224) respectively. Cholesterol level was found lowered in male rabbits only from 58.5±0.83 to 27.5±0.836.

No significant histopathological changes, that is, no granuloma or malignancy were observed in examined heart, stomach, liver and kidney tissues of male and female rabbits on low dose treatment with *S. nigra* extract for three months, may be due to the presence of polyphenolic constituents in it.

No chronic toxicity effects were observed in hematological, biochemical and histopathological parameters of rabbits in current research work, hence confirming the safety profile of *S. nigra* extract for its use in medicine for alleviating the sufferings of mankind.

More large scale pre-clinical and clinical trials need to be carried out to authenticate the safe and effective use of *S. nigra* preparations for the already sought out clinical indications in order to get the approval of World Health Organization.

Microscopic examination of heart:
Sections show wall of heart composed predominantly of thick myocardium consists of bundles of cardiac muscle fibers separated by a fibrous band, forming a syncytium. Nuclei of myocytes are centrally located. The endocardium is lined by single layer of mesothelial cells resting on a basement membrane. No significant pathology is seen in any of the sections examined.

Microscopic examination of stomach:
Sections show a wall of gastric mucosa with intact architecture. The gastric mucosa is thrown into gastric pits and folds revealing well organized glandular structures. The underlying submucosa is scanty and in unremarkable. Well organized muscular layer is seen beneath, lined externally by serosa. No significant pathology is seen in any of the sections examined.

Microscopic examination of liver:
Sections show liver tissue with overall preserved lobular architecture. Portal tracts are within normal limits, containing portal triad and scanty fibrous tissue. Sinusoidal dilatation and congestion is seen. No significant portal or lobular inflammation was seen. Diffuse lipofuscinosis is noted. No cholestasis. No evidence of granuloma or malignancy is seen

Microscopic examination of kidney:
Sections show renal tissue composed of cortex and medulla. Glomeruli are within normal limits. Severe degree of ATN is seen. Vascular structures are distributed evenly. No evidence of granuloma or malignancy is seen in any of the sections examined.

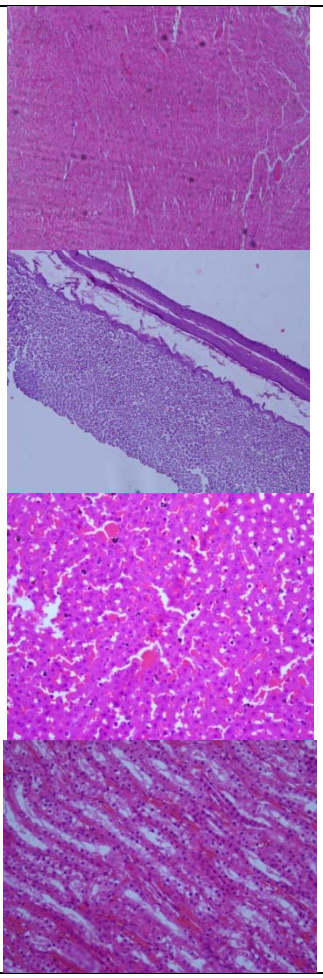

Fig. 1: Microscopic examination of male rabbit's heart, stomach, liver and kidney tissues treated with *S. nigra* extract

Microscopic examination of heart:
Sections show wall of heart composed predominantly of thick myocardium consists of bundles of cardiac muscle fibers separated by fibrous band, forming syncytium. Nuclei of myocytes are centrally located. Endocardium is lined by single layer of mesothelial cells resting on a basement membrane. No significant pathology is seen in any of the sections examined.

Microscopic examination of stomach:
Sections show wall of gastric mucosa with intact architecture. The gastric mucosa is thrown into gastric pits and folds revealing well organized glandular structures. Underlying submucosa is scanty and in unremarkable. Well organized muscular layer is seen beneath, lined externally by serosa. No significant pathology is seen in any of the sections examined.

Microscopic examination of liver:
Sections show liver tissue with overall preserved lobular architecture. Portal tracts are mildly expanded with lymphocytic infiltrate and minimal fibrosis. No significant lobular inflammation seen. Foci of macrovesicular steatosis are seen. No siderosis. No cholestasis. No evidence of granuloma or malignancy is seen.

Microscopic examination of kidney:
Sections show renal tissue composed of cortex and medulla. Glomeruli are within normal limits. Patchy lymphocytic inflammatory infiltrate is seen in tubulointerstitial compartment. Mild tubular injury is also seen. Vascular structures are distributed evenly. No evidence of granuloma or malignancy is seen in any of the sections examined.

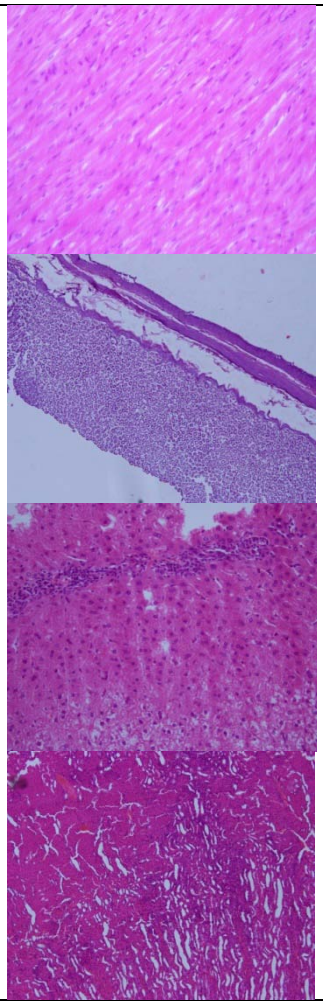

Fig. 2: Microscopic examination of female rabbit's heart, stomach, liver and kidney tissues treated with *S. nigra* extract

CONCLUSION

The present study justifies the safety profile of low-dose administration of *S. nigra* extract for the period of three months.

CONFLICT OF INTERESTS

Authors have no conflict of interest

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