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Preliminary study on the toxicological impacts of *Pinus roxburghii* and *Nauplius graveolens* extracts on albino mice

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Abstract

The objective of this study was to evaluate the potential toxicity of methanolic extracts of *Pinus roxburghii* branch (*P. roxburghii)* and *Nauplius graveolens* (*N. graveolens).* Single oral dosage was administered to male mice at increasing concentrations of 100, 250,500,1000 and 2000 mg/kg body weight to assess the median LD_{50}, which causes the death of 50% (one half) of the mice. Blood samples were collected at the end of the 14 days toxicological trial for the liver and kidney function tests. The results showed that the LD_{50} of *P. roxburghii* was 1708.3 mg/ kg body weight and of *N. graveolens* 1562.5 mg/kg body weight. The acute toxicity had detrimental effects on the liver and kidney function. Based on this finding, a second trial was designed, and the mice were administered day after day intragastrically one tenth (1/10) the LD_{50} of each plant extract for a whole month. At the termination of the 30 days trial, blood samples and organs were collected for liver and kidney function tests. The long-term administration of 1/10 the LD_{50} dosages had no significant adverse effects neither on the liver and kidney functions nor on the histopathological examination. In conclusion, further studies are warranted to determine the potential application of these plant as natural therapeutic products.

Key words: *P. roxburghii* branch, *N. graveolens*, Acute toxicity, LD_{50}, Liver and kidney function

Introduction

Recently, there has been growing intention for the usage of natural products derived from plant origin as health supplements in disease prevention and treatment. Medicinal plants have been widely used in conventional medicinal practices as a reliable cure to fight illness and maintain health. According to The World Health Organization, Medicinal plants has been recognized as a reliable source of therapeutic agents [1]. Medicinal plants contain many diverse bioactive compounds which are less harmful compared to the synthetic drugs [2]. Thus, the medicinal plants offer great scope to discover novel ways for disease control and treatment. However, various medicinal plants used in conventical therapy can cause adverse health effects because studies have been shown to contain toxic components [3,4, 5]. Therefore, there is urgent need to assess the toxicity of medicinal plants to ensure safety usages. The various process including, extraction, handling and type of solvents applied resulted in chemical changes in natural products derived that causing the alteration of the biological profile [6, 7]. Different studies focus on maximizing the efficiency of the extraction methods as a consequence extracting the highest amount of biologically active components. Evaluating the toxicity profile of extracted products is of vital importance [8], as it is important to have proper biochemical, toxicological, and safety data for

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the usage of plants with conventional claims on health benefits. Unfortunately, the chemical and toxicological profile of plant derived products are not yet investigated. Toxicity assessment studies are of vital importance which could clarifies the general safety profile of a substance. Acute toxicity assessment can be defined as a single high dose administration of a material to animals and observing their behavioral, motor-neuronal changes and mortality [9,10]. Therefore, it is necessary to determine the median lethal dose (LD_{50}). *Pinus roxburghii* belongs to Pinaceae family, is known as chir pine. *P. roxburghii* is a large tree up to 30-50m with a trunk diameter of up to 2 m, exceptionally 3 m. The bark is red-brown, thick and deeply fissured at the bottom of the trunk, thinner and flaky within the upper crown. The leaves are needle-like, in fascicles of three, very slender, 20-35 cm long, and distinctly yellowish green. *P. roxburghii* is cultivated in El-Orman Botanical Garden, Egypt [11]. *P. roxburghii* has long been considered for its medicinal and pharmaceutical use such as hepatoprotective, analgesic and anti-inflammatory, antimicrobial, anticancer, anticonvulsant, antiasthmatic, antioxidant, antidyslipidemic and antidiabetic activities [12,13,14,15,16,17 and 18]. *Nauplius graveolens* (synonyms. *Asteriscus graveolens, Bubonium graveolens, Odontospermum graveolens*) belongs to the Asteraceae family known as Tafss [19]. *Nauplius graveolens* is a bushy herp up to 50 cm height with bright yellow florets in heads [20]. *N. graveolens* was collected in South-Sinai (Egypt). Recent studies reported that *N. graveolens* has medicinal and pharmaceutical applications like antifungal, antidiabetic, hepatoprotective, analgesic, anti-inflammatory, antioxidant, antimicrobial and antitumor activities [21,22,23,24,25,26 and 27]. Even though, the medicinal importance of *P. roxburghii* and *N. graveolens* extracts, the scarcity of studies confirming their toxicity profile have been poorly investigated over past years. Therefore, the objective of the present study was to evaluate the *in vivo* toxicity of *P. roxburghii and N. graveolens* extracts.

2.MATERIAL AND METHODS

2.1. Preparation of plant extracts

P. roxburghii was grown and obtained from the ministry of Agriculture, Orman botanical garden, Giza. The branch after the removal of the foliar leaves was cut with the scissor into small pieces, which were allowed to dry at 40 °C. Aliquots of 75 g of the finely ground flour were extracted with 450 ml with absolute methanol. The methanolic extract was filtered and the filtrate concentrated on Buchi rotary evaporator and the residue was freeze dried and saved in airtight bottle until use at -20 °C. *N. graveolens* was obtained from South-Sinai, Egypt. The whole *N. graveolens* plant was treated in similar way as described above with *P. roxburghii* [28].

2.2. *In vivo* toxicological studies

2.3. Composition of Basal diet

The Basel diet is consisting of corn starch 46.5%, casein 14%, Soybean oil 4%, fiber 5%, mineral mixture 3.5% and vitamin mixture 1% as described in AIN-93M [29].

2.4. Animals

72Male albino mice weighing (25-30g) were used for the study. Animals were purchased from Schistosome Biological Supply Centre (SBSC) at Theodore Bilharz Research Institute (TBRI), Imbaba. All the animals were acclimatized for a week under standard husbandry conditions. The animals had free access to standard pellet diet and water ad libitum was available to the animals for the 14 days of the trial. Animal handling and experimental procedures were approved by the Research Ethical Committee at National Research Centre, NRC.

2.5. Toxicological study

Two separate toxicological trials were carried out according to standard method Wilbrandt [30]. In the first toxicological trial, the median lethal dose (LD_{50}) of the methanolic extract of *P. roxburghii* was assessed, while the lethal dose of the methanolic extract of *N. graveolens* was assessed in the second trial. Briefly, the methanolic extracts of *P. roxburghii* and *N. graveolens* was reconstituted in sterile dimethyl sulfoxide (DMSO). Groups 1, 2, 3, 4 and 5 were orally administered once dosage of 100, 250, 500, 1000 up to 2000 mg in DMSO /kg body weight, respectively. The control group was given orally the respective volume of DMSO. The animals had ad libitum access to standard pellet diet and water throughout the 14 days. The animals were monitored continuously for the first 24 hours for any signs of behavioral changes and mortality rate. At the end of the 14 days feeding trial, the diet was withdrawn, and all animals were fasted overnight (12 hours). The mice were anesthetized by intramuscular injection by ketamine chloride (24 mg/kg body weight). Blood was sampled from the orbital sinus of the eye. Blood was collected in clean dry test tubes and the serum was separated by centrifugation (1500 x g, 10 min, 4°C). Serum obtained was used for various biochemical estimations.

2.6. Chronic toxicity by one tenth the LD_{50}

This mouse trial was based on the lethal dose (LD_{50}) findings in the above-mentioned toxicological trials. The LD_{50} is the amount of methanolic extract, which caused the death of 50% (one half) of the test animals.

2.7. Design of study

The mice consumed basal diet throughout the 30 days of the trial, and they were randomly divided into 3 equal groups (6mice per group).

Group (1) The control mice was administered intragastrically with respective amount of DMSO day after day.

Group (2) was administered intragastrically a dose equivalent to 1/10 of LD_{50} of *P. roxburghii* day after day.

Group (3) was administered intragastrically a dose equivalent to 1/10 of LD_{50} of *N. graveolens* day after day.

At the end of the one month feeding trial, the diet was withdrawn, and all animals were fasted overnight (12 hours). The mice were anesthetized by intramuscular injection by ketamine chloride (24 mg/kg body weight). Blood was sampled from the orbital sinus of the eye and collected in clean dry test tubes and the serum was separated by centrifugation (1500 x g, 10 min, 4°C). Serum obtained was used for various biochemical estimations.

The mice were then sacrificed by cervical decapitation, the abdomen opened, and the liver and kidney organs were excised and washed briefly in cold saline solution.

2.8. Changes in body weight (BW) and feed efficiency ratio (FER)

Body weight and food consumption were recorded according to Hsu *et al* [31]. Using following equation:

Changes in body weight = Final body weight – initial body weight

Feed efficiency ratio (FER) = Body weight gain, g / Food intake, g

2.9. Laboratory investigations

The activities of liver function enzymes; alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) using a kit (Biodiagnostic, Egypt). Serum concentrations of urea and creatinine the two indicators of kidney function were determined using kits (Biodiagnostic, Egypt). The assays followed the manufacturer's protocol based on published techniques [32,33,34 and 35].

2.10. Histopathological examination

The liver and kidney organs were separated, fixed in 10% formalin in saline for twenty-four hours. The organs were then washed with tap water, followed by sequential soaking in diluted methanol, diluted ethanol and finally absolute ethyl alcohol for dehydration. The specimens were cleared in xylene and embedded in paraffin and left in hot air oven at 56°C for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness at room temperature by slidge microtome (SLEE medical, Germany). The sections were embedded on glass slides, deparaffinized, stained by hematoxylin &eosin stain for examination through the light electric microscope [36].

Statistical analysis

Data obtained from the experiments were analyzed statistically on SPSS 17.0 computer software package. Data for body weight, food intake and biochemical parameters were presented as mean ± standard error of mean (S.E.M). Differences between the treatment and control groups were compared using one-way analysis of variance (ANOVA). Differences between the various groups were considered statistically significant at $P < 0.05$. Graphs were plotted using GraphPad Prism software, version 5, for Windows (San Diego, CA, USA).

3.Results and Discussion

3.1. Acute toxicity trail

Table 1. Lethal dose (LD_{50}) of *P. roxburghii* extract in mice.

Dose (mg / kg body weight)	Number of mice	Number of dead mice	Z	d	(Z) x (d)
100	6	0	0	100	0
250	6	0	0	150	0
500	6	0	0	250	0
1000	6	1	0.5	500	250
2000	6	2	1.5	1000	1500

Z is the mean of dead animals in two successive groups.

D is the constant factor between two successive groups

Σ (Zxd) = 1750 LD_{50}= Dm-Σ (Zxd)/n

LD_{50}= 2000-(1750/6) = 1708.3mg /kg BW.

Table (1) and (2) presented the median lethal dose (LD_{50}) of the methanolic extracts of *P. roxburghii* accounted to 1708.3 mg /kg body weight, while that of *N. graveolens* was 1562.5 mg /kg body weight.

The 1/10 LD_{50} of *P. roxburghii* and *N. graveolens* extracts, which accounted to 170.83 and 156.25 mg /kg body weight, respectively were used in the second feeding trial to assess the effect of long term sublethal toxicity on the function of the liver and kidney.

Table 2. Lethal dose (LD_{50}) of *N. graveolens* extract in mice.

Dose (mg / kg body weight)	Number of mice	Number of dead mice	Z	d	(Z) x (d)
100	6	0	0	100	0
250	6	0	0	150	0
500	6	1	0.5	250	125
1000	6	1	1	500	500
2000	6	3	2	1000	2000

Z is the mean of dead animals in two successive groups.

D is the constant factor between two successive groups

Σ (Zxd) = 2625 LD_{50}= Dm- Σ (Zxd)/n

LD_{50} = 2000 - (2625/6) = 1562.5 mg /kg BW.

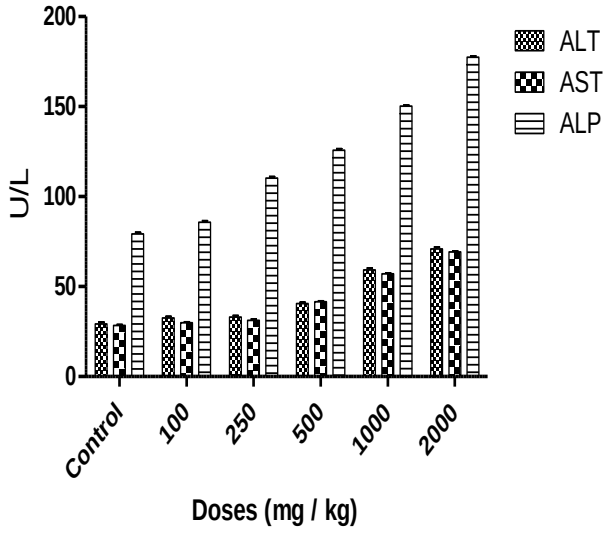

Fig 1. Serum ALT, AST, and ALP activities in different groups for the evaluation of acute toxicity of methanolic extract of *P. roxburghii*.

Values are expressed as mean ± standard error of the mean (SEM).

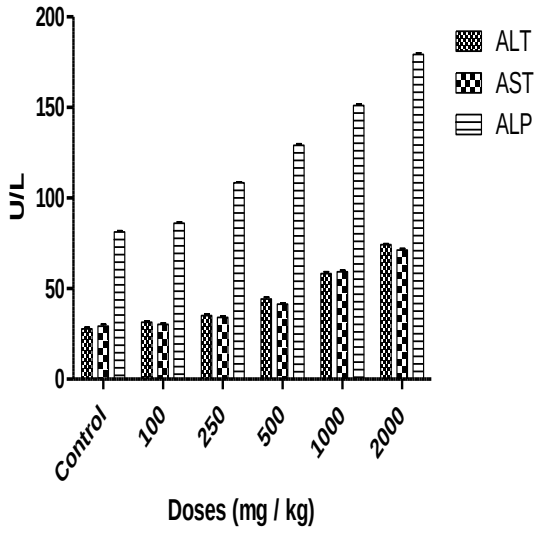

Fig 2. Serum ALT, AST, and ALP activities in different groups for the evaluation of acute toxicity of methanolic extract of *N. graveolens.*

Values are expressed as mean ± standard error of the mean (SEM).

Results of the Liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and Alkaline phosphatase (ALP) of the different experimental groups are shown in Figure (1, 2). There was a significant increase of liver enzymes ALT, AST and ALP levels in the 500,1000 and 2000 mg/kg in *P. roxburghii* extract as compared to control (Fig 1). Moreover, the higher activity of ALT, AST and ALP levels in the 500,1000 and 2000 mg/kg in *N. graveolens* extract as compared to control was observed (Fig 2).

The effect of methanolic extract of *P. roxburghii* on the levels of urea and creatinine in mice are shown in Figure (3, 4). There was significant increase in serum urea level in the dosages 500,1000 and 2000 mg/kg of *P. roxburghii* extract compared to control group (Figure 3). Similarly, serum creatinine level was significantly increased in the same dosages of *P. roxburghii* extract compared to normal control (Figure 4).

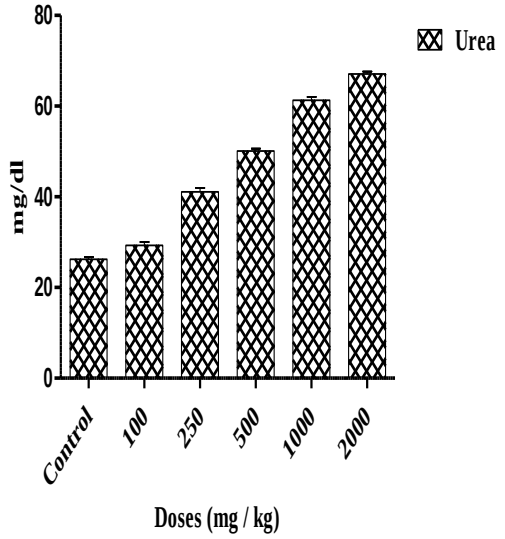

Fig 3. Effect of methanolic extract of *P. roxburghii* on serum urea level in acute toxicity.

Values are expressed as mean ± standard error of the mean (SEM).

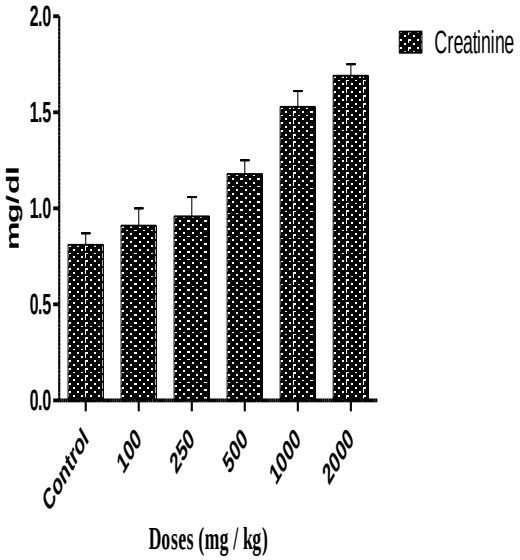

Fig 4. Effect of methanolic extract of *P. roxburghii* on serum creatinine level in acute toxicity.

Values are expressed as mean ± standard error of the mean (SEM).

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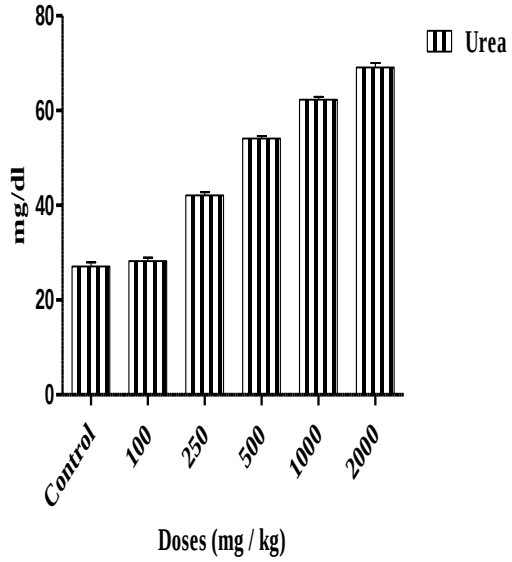

Fig 5. Effect of methanolic extract of *N. graveolens* on serum urea level in acute toxicity.

Values are expressed as mean ± standard error of the mean (SEM).

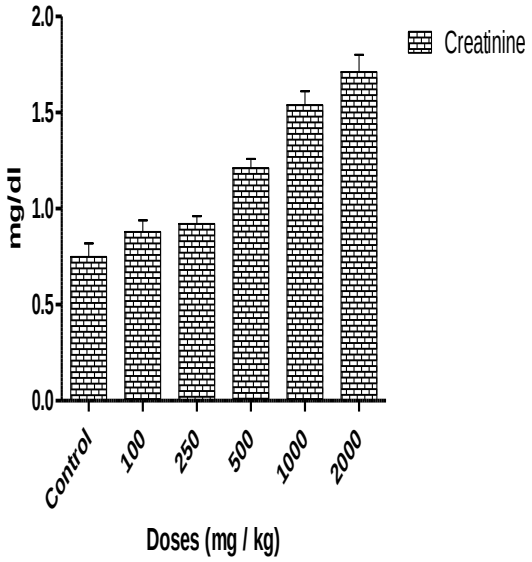

Fig 6. Effect of methanolic extract of *N. graveolens* on serum creatinine level in acute toxicity.

Values are expressed as mean ± standard error of the mean (SEM).

The effect of methanolic extract of *N. graveolens* on the levels of urea and creatinine in mice are shown in Figure (5, 6). There was significant increase in serum urea level in the dosages 500,1000 and 2000 mg/kg of *N. graveolens* extract compared to control group (Figure 5). Similarly, serum creatinine level was significantly increased in the same dosages of *N. graveolens* extract compared to normal control (Figure 6).

3.2. Chronic toxicity trail

Table 3. Nutritional parameters of different experimental groups.

Parameters / Groups	Body weight gain (BWG) (g)	Food intake (FI) (g)	Feed efficiency ratio (FER)
Negative control	26.90±0.37[a]	179.21±0.48[a]	0.15
P. roxburghii	27.17±0.58[a]	180.31±0.70[a]	0.15
N. graveolens	26.69±0.45[a]	178.27±0.87[a]	0.14

All values are represented as mean ± S.E.
Means with different letters are significantly different ($p<0.05$).

Data in Table (3) clearly indicate that administration of 1/10 LD_{50} of the methanolic extracts of *P. roxburghii* or *N. graveolens* had no adverse effects on the mean gain in body weight, the mean food intake and the mean feed efficiency ratio, compared with the respective mean values obtained with the control group.

Body weight is one of the indicators most used in toxicological assessments to indicate the appearance of toxic effects of a substance in the animal body, as well as the reduction in feed intake consumption, behavioral change. The absence of these signals between the groups of animals demonstrate that the extract does not produce toxicity under these conditions [37].

The monitoring of body weight and feed consumption of the experimental mice important while studying the toxicity and safety of a natural product since it reflects the physiological and metabolic status of the mice. In the current study, none of the experimental groups suffered loss in weight or gained overweight. According to Teo et al [38], alteration in the body weight can be considered as markers of adverse effects upon oral administration of drugs and chemicals. Body weight loss >10% from the initial body weight is considered as significant [39]. It was also noted that the pattern of feed consumption was not altered significantly by the administration of 1/10 LD_{50} of *P. roxburghii* and *N. graveolens* extracts which suggested that the mentioned extracts did not induce significant changes in the appetite and did not exert any harmful effects on the general health status and metabolic growth of mice.

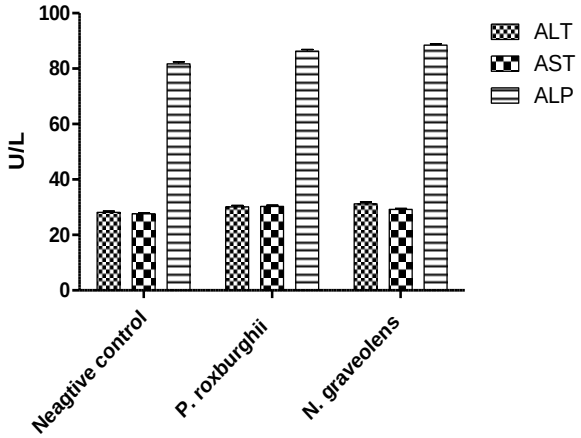

Fig 7. Effect of the administration of 1/10 LD_{50} methanolic extracts of *P. roxburghii* and *N. graveolens* on the mean activities of the serum enzymes ALT, AST and ALP.

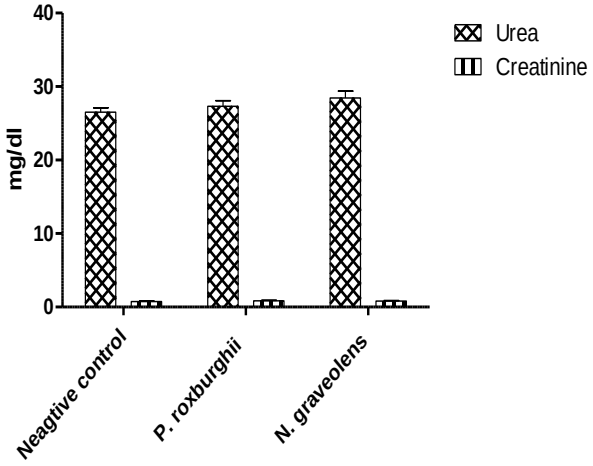

Fig 8. Effect of the administration of 1/10 LD_{50} methanolic extracts of *P. roxburghii* and *N. graveolens* on the mean serum concentrations of creatinine and urea.

Data presented in Fig 7 showed the effect of *P. roxburghii* and *N. graveolens* extracts on liver function tests. No significant differences in liver enzymes were observed between mice treated with 1/10 LD_{50} of *P. roxburghii* and *N. graveolens* extracts and control. It is possible to affirm that *P. roxburghii* and *N. graveolens* extracts didn't promote any hepatic alteration.

Data in Fig 8 illustrated the effect of *P. roxburghii* and *N. graveolens* extracts on renal function. Data showed no significant differences in serum urea and creatinine were observed between mice treated with 1/10 LD_{50} of *P. roxburghii* and *N. graveolens* extracts and control. Therefore, it was suggested that *P. roxburghii* and *N. graveolens* extracts did not cause any renal damage.

Assessment of liver and kidney function is a very vital indicator in evaluating the toxicity of the plant extracts [40]. The primary organs prone to the toxic effects of medicines are the liver and kidney.

The liver is a major target organ of toxicity and its injury affects the integrity of hepatocytes causing the release of membrane bound enzymes (e.g., ALT and AST), destroy hepato-biliary system thereby leading to the release of essential enzymes (e.g., ALP) [41,42]. In the current study, no significant differences were observed in serum ALT, AST and ALP levels between treated and untreated groups after oral administration of 1/10 LD_{50} of *P. roxburghii* or *N. graveolens* extracts.

The kidney plays a major role in excreting waste products like creatinine and urea. Due to the major role of the kidneys in excreting waste products, due to their ability to filter and reabsorb the body needed threshold substance like electrolytes, the levels of these biochemical markers in the serum could be used as renal function tests [43, 44]. Renal function assessment gives insight to the site of cellular tissue damage due to the repeated exposure by the potential toxic agent. Oral administration of 1/10 LD_{50} of methanolic extracts of *P. roxburghii* or *N. graveolens* didn't have any deleterious effects on the parameters of the kidney function compared to the control group.

3.3. Histopathological Observations of Organs.

Figures 9-14

3.4. Histopathological examination

The histopathological examination of the liver tissues of mice orally administered with one tenth the LD_{50} of methanolic extract of *P. roxburghii* illustrated preserved architecture, hepatocytes arranged in thin plates (black arrow) with dilated congested sinusoids (red arrow), central vein, binucleated hepatocytes (Figure 10). Hepatic tissue of mice orally administered with one tenth the LD_{50} of methanolic extract of *N. graveolens* are illustrated in Figure (11) and showed identical preserved architecture, hepatocytes arranged in thin plates (black arrow) with dilated congested sinusoids (red arrow), central vein congested, binucleated hepatocytes.

The renal corpuscle of the Kidney section from mice orally administered with 1/10 the LD_{50} dosage of methanolic extract of *P. roxburghii* had almost normal glomerulus, mildly dilated proximal convoluted and mildly dilated distal convoluted tubules (Figure 13). The Kidney section from mice orally administered with 1/10 the LD_{50} dosage of methanolic extract of *N. graveolens* was also normal and identical to those described above. The corpuscle had almost normal glomerulus, mildly dilated proximal convoluted and mildly dilated distal convoluted tubules (Figure 14).

Histopathological examination revealed no deleterious changes in most animals. The liver and kidney presented no histological changes in any of the animals in the 1/10 of LD_{50} trial.

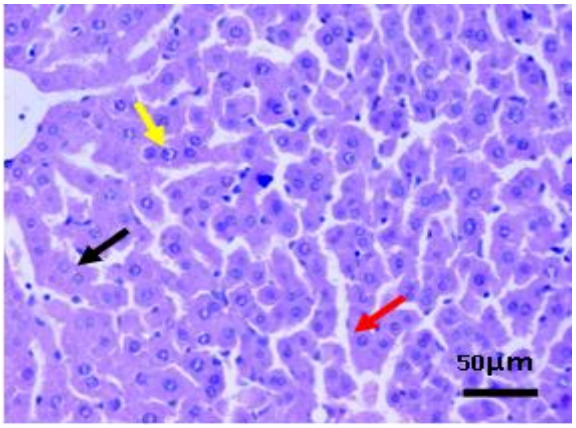

Figure 9. liver section from control group showed hepatic tissue with preserved architecture, hepatocytes arranged in thin plates (black arrow) with dilated congested sinusoids (red arrow), central vein (yell ow arrow), binucleated hepatocytes (yellow arrow) (H&E, x400).

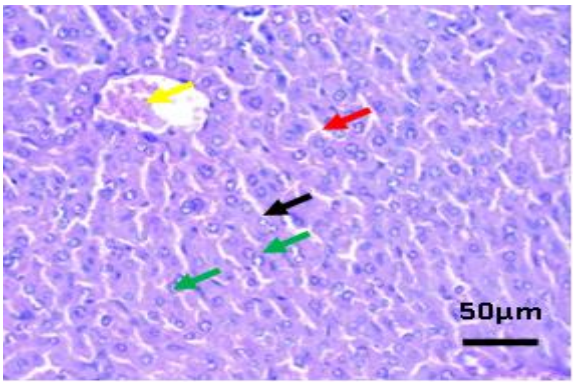

Figure 10. liver section from *P. roxburghii* group showed hepatic tissue with preserved architecture,

hepatocytes arranged in thin plates (black arrow) with dilated congested sinusoids (red arrow), central vein (yellow arrow), binucleated hepatocytes (green arrow) (H&E, x400)

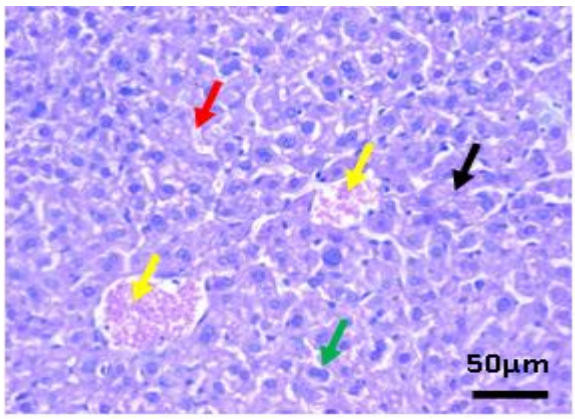

Figure 11. liver section from *N. graveolens* group showed hepatic tissue with preserved architecture, hepatocytes arranged in thin plates (black arrow) with dilated congested sinusoids (red arrow), central vein congested (yellow arrow), binucleated hepatocytes (green arrow) (H&E, x400).

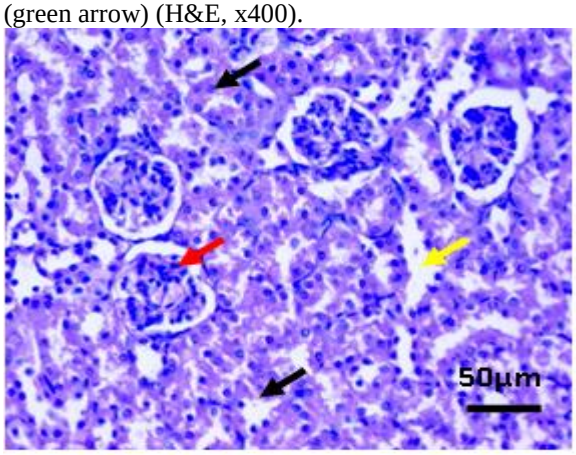

Figure 12. Kidney section from control group showed renal cortex showing renal corpuscle with almost normal glomerulus (red arrow), normal pattern of proximal convoluted (black arrow) and distal convoluted (yellow arrow) tubules (H&E, x400)

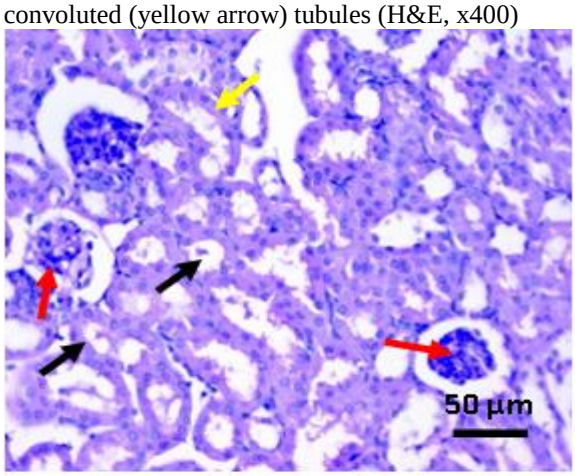

Figure 13. Kidney section from *P. roxburghii* group showed renal cortex showing renal corpuscle with almost normal glomerulus (red arrow), mildly dilated proximal convoluted (black arrow) and mildly dilated distal convoluted (yellow arrow) tubules (H&E,x400)

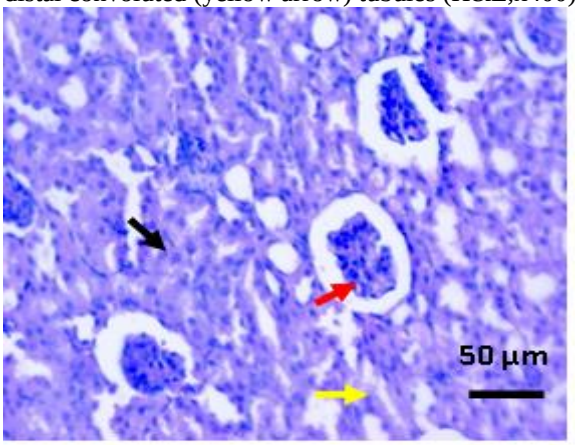

Figure 14. Kidney section from *N. graveolens* group showed renal cortex showing renal corpuscle with almost normal glomerulus (red arrow), mildly obliterated proximal convoluted (black arrow) and distal convoluted (yellow arrow) tubules (H&E, x400)

Conclusion

The acute toxicity study in the mice model revealed that LD_{50} for *P. roxburghii* accounted to 1708.3 mg /kg body weight, while that of *N. graveolens* was 1562.5 mg /kg body weight. The oral administration of 170.83 mg *P. roxburghii* and 156.25 mg *N. graveolens* per kilogram body weight, equivalent to the one tenth the LD_{50} of each plant did not have serious adverse effect on liver and kidney function, the body weights and feed intake. Histopathological examination revealed no histological changes in treated mice groups. Further studies are required to highlight the area of potential application *of P. roxburghii* and *N. graveolens* extracts in cancer treatment and prevention.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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