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ARTICLE

GC-MS Analysis and Toxicological Evaluation of *Asparagus falcatus* Linn. Extracts in Healthy Wistar Rats Using a Biochemical, Hematological and Histological Approach

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Abstract: The present study evaluates the potential toxic/adverse effects of selected extracts derived from *Asparagus falcatus* Linn. (family; *Asparagaceae*) leaves via acute and sub-acute oral toxicity assessment in healthy Wistar rats. The dried powder of the leaf of *A. falcatus* was sequentially extracted in hexane (AFH), ethyl acetate (AFEA), *n*-butanol (AFNB) and water (AFW), respectively, by the Soxhlet extraction method followed by complete evaporation of the solvents. Investigations were carried out by repeated dose oral administration (daily for 28+ days) of AFH (55 mg/kg), AFEA (35 mg/kg), AFNB (75 mg/kg) and AFW (200 mg/kg) leaf extracts derived from *A. falcatus* in healthy Wistar rats (n = 5/sex/group). GC-MS analysis was also conducted. None of the biochemical and hematological parameters showed clinically significant changes in the sub-acute oral toxicity assessment of the selected leaf extracts at the therapeutic doses. Qualitative analysis of phytochemicals in AFH, AFEA, AFNB and AFW leaf extracts of *A. falcatus* revealed the absence of alkaloids, excluding thus the potential risk of pyrrolizidine alkaloids. The presence of prominent bioactive phytoconstituents identified by GC-MS analysis, including dodecanoic acid, 1,2,3-propanetriyl ester, cyclohexanol,5-methyl-2-(1-methylethyl)-(1.alpha,2.alpha,5.alpha), hexadecanoic acid, butyl ester, octadecanoic acid, ethyl ester, 3,7,11,15-tetramethyl-2-hexadecen-1-ol and hexanedioic acid bis(2-ethylhexyl)ester, further signified the medicinal importance of *A. falcatus*. In addition, dimethyl sulfone, 2,3-butanedioldiacetate, *E*-14-hexadecanal, tetradecane, octadecane, tetracosane,11-decyl, etc., were identified in abundance in the GC-MS analysis. The results might ensure the safe consumption of the plant extracts at the human equivalent therapeutic dose in healthy Wistar rats.

Keywords: *Asparagus falcatus* Linn., GC-MS analysis, Herbal medicine, Repeated dose 28-day oral toxicity assessment.

Introduction

The use of herbal medicines, in terms of self-medications or remedial measures, is considered one of the reasons behind the long life expectancy and low prevalence of certain chronic diseases in the Asian population[1]. *Asparagus falcatus* Linn. (family; *Asparagaceae*), commonly known as 'Hathavariya', is one such popular plant medicine, often consumed by the Asian

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population to manage various ailments[2,3]. The tuberous roots and leaves of the plant are used in the treatment of kidney, liver, respiratory, venereal, and gastrointestinal diseases[3]. The tubers are commonly used for sexual debility and sterility due to their aphrodisiac nature. Moreover, tubers are included in the herbal recipes often given for the treatment of menstrual disorders. The flowers of *A. falcatus* are often included in the treatment regimens for typhoid fever[2]. Similarly, the juice, leaves, tubers, and whole plant of *A. falcatus* have been used in hundreds of herbal therapies in Ayurveda for the treatment of several diseases, including kidney disease[2,3].

Despite the folkloric usage, scientific evidence on the protective effects of *A. falcatus* is limited. Potential bioactivities of the plant have been reported in terms of hepatoprotective effects, anti-angiogenic and anticancer effects to date[4–6]. Even though the phytoconstituents, such as 1-hexacosanol, lupenyl palmitate and furanosesquaterpene diolide, have been isolated from the plant, the potential bioactivities have not been reported in detail to date[7].

Given the consideration of the plant's wide use in managing kidney-related diseases in traditional medicine, our research team assessed the potential nephroprotective effects of the plant in doxorubicin-induced kidney injury in experimental rats. The results revealed that *A. falcatus* substantially attenuates doxorubicin-induced kidney injury via antioxidant, anti-inflammatory, and anti-apoptotic pathways[8]. These findings lead to future research avenues for isolating pharmacologically active compounds for designing potential antidotes/drugs that minimize doxorubicin-induced nephrotoxicity in cancer patients.

Besides the reported clinical efficacy, plant therapeutics still require a well-controlled standard assessment of safety and quality, when investigating them as potential sources for new lead structures[9]. Standardization laid the foundation for developing good quality herbal therapeutics, and accordingly, chemical standardization of the leaves of *A. falcatus* was carried out and the results were published[10]. However, analysis of potential phytochemicals through advanced techniques would add further value to the assurance of the quality of herbal products.

Safety assessment of plant medicines according to international guidelines is crucial in the process of new drug discovery from natural products, to minimize adverse effects of plant metabolites that modulate or modify the effects of active principles, either being toxic or acting as agonists/antagonists to the active principles[11]. The ancient inscriptions provide numerous shreds of evidence of such adverse reactions of medicinal plants used in various treatments[12]. Moreover, quantity is often an important consideration in plant medicines, and in this context, toxicological studies would be informative in assessing the effect of different solvents and extraction methods on the potential level of toxicity, in the process of developing new drug leads[11,13]. Even though previous literature supports the potential bioactivities of the leaves of *A. falcatus*, in terms of anti-angiogenic, nephroprotective and anticancer effects, no scientific data on potential toxic/adverse effects of the leaves of the plant, particularly concerning ethyl acetate, butanol, and aqueous leaf extracts of the plant are available to date[5,6]. Therefore, the present study was designed as a biochemical, hematological, and histopathological approach to evaluate the potentially toxic effects of the hexane, ethyl acetate, *n*-butanol and aqueous leaf extracts of *A. falcatus* in Wistar rats. Further, GC-MS analysis was carried out for the better identification of phytochemicals present in the plant.

Materials and methods

Plant Material and Extraction

A single batch of fresh leaves of *A. falcatus* was collected from the Southern region (6˚04’05”, 80˚13’35” E) of Sri Lanka, in December 2018. Authentication of the plant was done at the National Herbarium, Peradeniya, Sri Lanka. A voucher specimen (PG/2016/55/02) was deposited at the department herbarium.

The leaves of *A. falcatus* were gently washed in tap water and oven-dried at 40 ℃ until the leaves reached a steady weight. The plant material was then pulverized using an electrical grinder to obtain a powder form of suitable consistency. The powdered plant materials were weighed and sequentially extracted in hexane (AFH), ethyl acetate (AFEA), *n*-butanol (AFNB) and water (AFW), respectively, by the Soxhlet extraction method. The material-to-solvent ratio was 1:20. The extraction procedure was continued with each solvent for 6–8 hours (until the leachate became colorless) at the respective boiling temperature (AFH, 70 ℃; AFEA, 80 ℃;

AFNB, 120 ℃; AFW, 100 ℃). The extraction with the subsequent solvent was carried out following the complete evaporation of the previous. The solvent extracts were collected separately. The AFH, AFEA and AFNB were then reduced using a rotary evaporator and a vacuum oven to obtain the dried form of the extracts. The lyophilized powder of the concentrated AFW was obtained by freeze-drying at –20 ℃.

Experimental Animals and Husbandry

Adult healthy male and female Wistar rats with an average weight of 150–175 g (8–10 weeks of age) were used in both single and repeated-dose oral toxicity studies. The animals were acclimatized to standard laboratory conditions (25 ± 3 ℃ temperature, 12/12-hour cycle of dark and light) for two weeks before the experiments. They were fed with a pelleted diet (based on Medical Research Institute rat formulae; gross energy 2700 kcal/kg, protein 14.5%, fat 6%, fiber 6%, Ca 0.9%, $PO4^{3-}$ 0.7%, methionine 0.3% and ash 7%), and tap water *ad libitum*. The experimental protocols were approved by the Ethics Review Committee of the Faculty of Medicine, University of Ruhuna, Sri Lanka (Reference Number: 14.12.2015:3.1). The experimental procedures were conducted as per the International Ethical Guidelines[14].

Experimental Design

The treatments on experimental animals were carried out during the daytime at the vivarium of the Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Sri Lanka. The experimental rats were randomly allotted into five groups, each with male (n = 5) and female (n = 5) rats:

Group 1: Normal control group. The experimental rats were treated orally with an equivalent volume of distilled water.

Groups 2–5: The experimental rats were orally treated with AFH (55 mg/kg, Group 2), AFEA (35 mg/kg, Group 3), AFNB (75 mg/kg, Group 4) and AFW (200 mg/kg, Group 5).

The plant doses were selected based on the theory of Nair and Jacob[15] on calculating the human equivalent therapeutic dose in rats based on the dose recommended in Ayurveda (12 g/day) for an adult human (60 kg body weight) and the respective percentage yield of each extract (AFH, 4.51%; AFEA, 2.80%; AFNB, 6.11%; AFW, 16.88%). The selected doses were therapeutically effective in the attenuation of kidney injury in experimental nephrotoxic rats[8]. The plant extract/distilled water was administered through the oral route using a stainless-steel oral gavage feeding needle to the experimental rats (non-fasting) at a stipulated time during the daytime. The treatment doses were adjusted according to the body weight of the experimental rats, and accordingly, a volume of 0.2 mL was administered to a rat with a body weight of 150 g.

Acute Oral Toxicity Study

Organization for Economic Cooperation and Development (OECD) guideline 420, a fixed-dose procedure was followed with minor modifications for the assessment of acute oral toxicity of the selected plant extracts[16]. A total of ten animals per group (five female and five male) were used in each experimental group. The experimental animals were administered with distilled water (Group 1) and plant extracts (Groups 2–5) once orally according to the experimental design mentioned above. Observations were made at three hours and then daily for 14 days. Changes in the eyes and mucous membranes of the eyes, changes in skin and fur, changes in respiration, convulsions, tremors, lethargy, sleep, coma, salivation and diarrhea were the features examined.

Subacute Toxicity Study

Plant extracts were administered (0.2 mL for an animal with 150 g of average weight) through the oral route as a single daily dose for 28 days according to the experimental design mentioned above[17]. The distilled water (0.2 mL per animal with 150 g of average weight) was administered to the control group, considering the experimental designs followed in similar studies based on the previous literature[18,19].

Bodyweight, Food Consumption and Water Intake

The body weights of the experimental rats were recorded on days 0, 7, 14, 21 and 28. The daily food consumption and water intake were measured in each experimental group and averaged at weekly intervals.

Clinical Pathology

Twenty-four hours following the last treatment, the rats were euthanized using CO_2 inhalation. Blood samples were collected from

sacrificed rats in EDTA and serum collection gel tubes for the assessment of selected hematological and biochemical parameters, respectively. The hematological analysis was carried out by assessing full blood count parameters using Mindray BC 5150 automated hematology analyzer (China). Serum concentrations of glucose, total cholesterol, triacylglyceride, high-density cholesterol (HDL-C), creatinine, blood urea nitrogen, total protein and serum activity of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (γ-GT), were estimated using spectrophotometric methods (UV-1800, SHIMADZU recording double beam spectrophotometer, USA).

Organ Weight

Vital organs (kidney, lung, spleen, small intestine, heart, and liver) were excised from male and female rats from either group, blotted gently using an absorbent paper, and absolute organ weights were measured. The proportion of absolute organ weight and total body weight of each rat was calculated to determine the relative weight of the organs.

Histological Examination

Formalin-fixed (10%) organ samples of the liver, kidney, heart, small intestine and spleen of the experimental rats were processed for the preparation of hematoxylin and eosin-stained microscopic slides for the histological examination[20].

Qualitative Phytochemical Screening

The selected extracts of *A. falcatus* were screened for the presence of various phytochemical metabolites, including alkaloids, steroid glycosides, flavonoids, phenolic compounds, saponins, tannins, and terpenoids, using standard procedures.

GC-MS Analysis

Agilent inert detector operating in EI mode (7890A/5975C-GC/MSD) with HP-5 ms (30 m × 250 μm × 0.25 μm) capillary column was used for the detection and identification of volatile compounds. The sample (1 μL) with a 20:1 split ratio was injected using an autosampler. The injection port temperature was 250 ℃. Temperature programming was performed at a starting column temperature of 70 ℃ which lasted for 4 min and then increased at a rate of 12 ℃/min, to 270 ℃ and held for 20 min. The carrier gas was helium at a flow rate of 1.0 mL/min. The ionic source temperature and quadruple temperature were 230 ℃ and 150 ℃, respectively, and the scanning quality range was set at 50 – 550 amu. The ratio of the average peak area to the total peak areas was used for the calculation of the percentage composition of the extract. Identification of compounds was carried out using the NIST standard spectral library, and the matching percentage is presented as a comparison to the fragment patterns of the library compound to the experimentally observed fragment pattern peak.

Non-isothermal retention indices were calculated and described using the definition of Van den Dool and Kratz as $RI = 100n + 100(t_x - t_n) / (t_{n+1} - t_n)$ where t_n and t_{n+1} are retention times of the reference *n*-alkane hydrocarbons eluting immediately before and after the chemical compound "X". The retention time of the compound "X" is given in t_x[21]. To calculate the retention indices, a standard *n*-alkane series containing C_8–C_{20} hydrocarbons (40 mg/L, Sigma-Aldrich) in hexane was used.

Statistical Analysis

The results were analyzed statistically by one-way analysis of variance (ANOVA) with Tukey's post hoc test, using the Statistical Package for the Social Sciences (SPSS, version 22.0). The level of significance was considered at values of $p < 0.05$.

Results

Toxicity and Adverse Effects

None of the experimental animals died during the study period of 14 days. The experimental animals showed neither noticeable symptoms of toxicity nor adverse signs of distress. The lethal dose (LD_{50}) of the AFH, AFEA, AFNB and AFW extracts was found to be greater than 55, 35, 75, and 200 mg/kg in rats, respectively.

Neither death nor visible signs of adverse/toxicity effects were observed in the experimental rats during or at the end of the study period of 28 days. Further, no significant changes were observed in any of the biochemical, hematological parameters, or histological observations between respective male and female groups ($p > 0.05$).

No significant changes in the body weight measurement, food consumption and water intake were observed in groups of rats administered with AFH, AFEA, AFNB, and AFW com-

pared to the untreated control group ($p > 0.05$). The average change in body weight, food consumption and water intake over the experimental period of 28 days are shown in Figure 1.

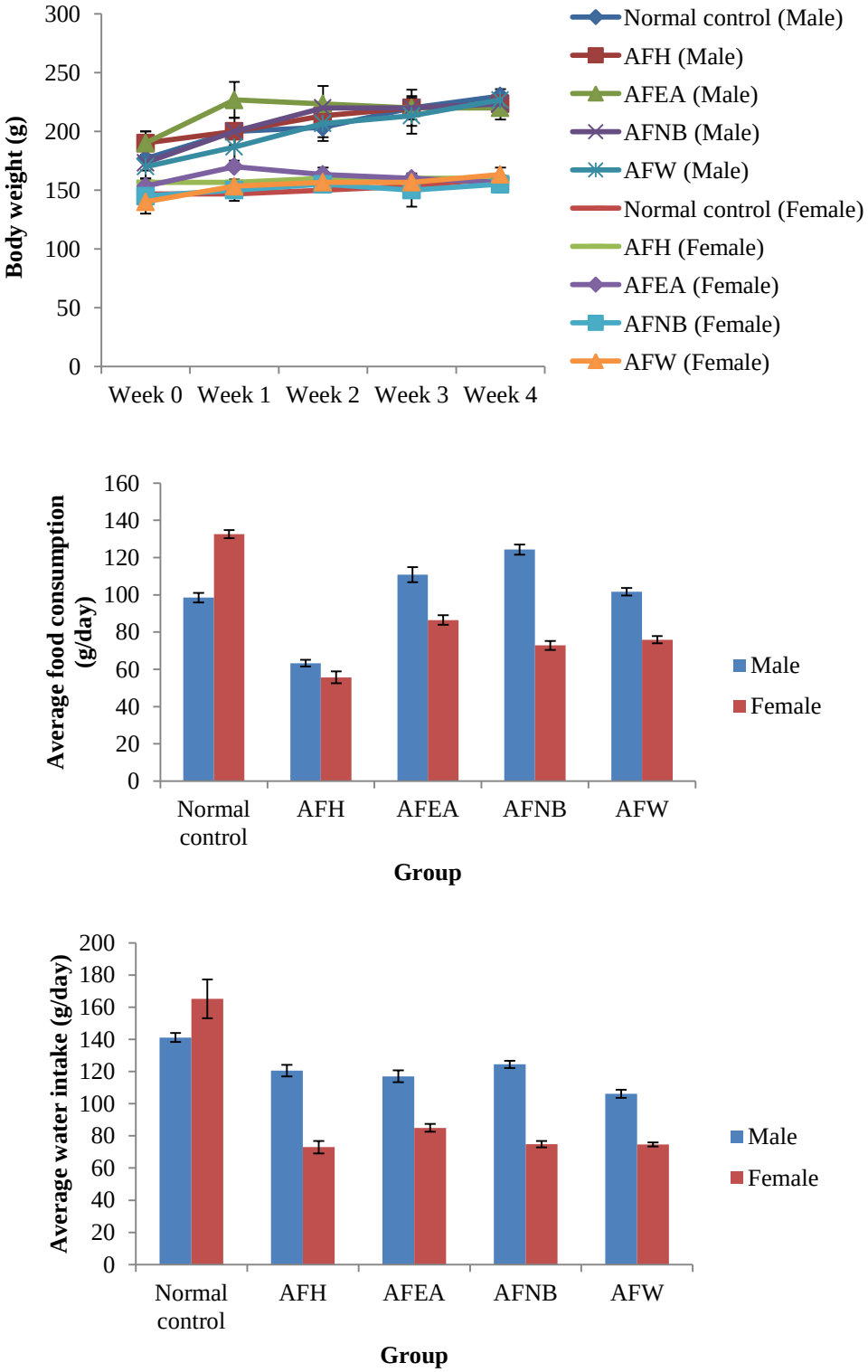

Figure 1. Effect of leaf extracts of *A. falcatus* on average body weight, food consumption and water intake of male and female Wistar rats following subacute administration of AFH (55 mg/kg), AFEA (35 mg/kg), AFNB (75 mg/kg) and AFW (200 mg/kg). Data are expressed as mean ± SEM. Significant differences $p < 0.05$. AFH: hexane extract, AFEA: ethyl acetate extract, AFNB: *n*-butanol extract, AFW: water extract.

Effect of Subacute Extracts Administration on Hematological Parameters

The effect of the investiagted leaf extracts of *A. falcatus* on the hematological parameters of experimental rats is shown in Table 1. Elevated red cell counts, platelet counts, packed cell volumes and hemoglobin concentrations were noted in experimental rats administered with AFH, AFEA, AFNB and AFW compared to the untreated control group. However, a significant increase was noted only in the hemoglobin concentration of male and female rats administered with AFH ($p < 0.05$). Similarly, an increase in white cell counts was observed with the treatment of all four extracts of *A. falcatus* in male rats and with AFH, AFNB and AFW in female rats. However, the changes were not statistically significant ($p > 0.05$). None of the parameters in white cell differential count, red cell indices or platelet count showed significant changes in plant extracts administered rats compared to the normal control group ($p > 0.05$).

Table 1. Effect of investigated leaf extracts of *Asparagus falcatus* on hematological parameters.

Parameters	Normal control	Plant extract treatment			
		AFH	AFEA	AFNB	AFW
Male					
Red cell count ($\times 10^6/\mu L$)	7.15±0.23	8.59±0.39	8.12±0.30	8.21±0.20	8.00±0.15
Hemoglobin (g/dL)	13.80±1.40	15.40±0.76*	14.90±0.67	14.37±0.17	14.40±0.31
Packed cell volume (%)	50.25±3.95	56.60±2.62	55.00±1.50	53.97±0.95	53.67±1.07
Mean corpuscular volume (fL)	67.20±0.30	65.90±0.10	67.77±0.66	65.80±0.72	67.13±0.13
Mean corpuscular Hemoglobin (pg)	18.50±0.60	17.93±0.22	18.37±0.35	17.67±0.39	18.07±0.03
Mean corpuscular hemoglobin concentration (g/dL)	27.40±0.70	27.16±0.28	27.10±0.60	26.87±0.33	26.87±0.13
Platelet count ($\times 10^3/\mu L$)	247.50±45.50	344.00±41.20	225.33±22.57	329.00±20.21	288.33±10.33
White cell count (per mm^3)	1.55±0.85	3.27±1.16	2.80±0.55	4.57±0.09	5.43±0.09
Neutrophils (%)	19.00±4.00	25.00±4.16	22.67±3.84	22.33±1.86	21.67±5.24
Lymphocytes (%)	77.00±4.00	71.33±4.84	72.67±4.18	73.33±1.76	75.67±5.04
Eosinophils (%)	0.50±0.50	0.67±0.33	1.00±0.00	1.00±0.00	0.00±0.00
Basophils (%)	1.00±0.00	2.33±0.88	2.67±0.33	2.00±0.58	2.00±0.00
Monocytes (%)	1.50±0.00	0.67±0.33	1.00±0.58	1.33±0.33	0.67±0.67
Female					
Red cell count ($\times 10^6/\mu L$)	7.57±0.23	8.28±0.14	7.84±0.46	7.70±0.20	7.70±0.25
Hemoglobin (g/dL)	13.53±0.35	15.17±0.32*	13.77±0.26	13.60±0.00	13.83±0.37
Packed cell volume (%)	50.33±1.68	54.77±0.79	52.57±2.77	51.45±0.85	50.73±1.78
Mean corpuscular volume (fL)	66.43±0.22	66.17±0.38	67.33±0.47	67.10±0.80	65.77±0.18
Mean corpuscular Hemoglobin (pg)	17.90±0.12	18.33±0.27	18.30±0.55	17.75±0.45	17.90±0.15
Mean corpuscular hemoglobin concentration (g/dL)	26.97±0.23	27.73±0.29	27.10±0.61	26.40±0.40	27.27±0.29
Platelet count ($\times 10^3/\mu L$)	249.00±45.04	285.67±18.35	293.33±6.06	259.50±17.50	338.67±7.62
White cell count (per mm^3)	1.43±0.58	3.47±0.99	1.30±0.25	1.95±0.45	2.47±0.19
Neutrophils (%)	20.00±6.25	9.00±1.00	12.00±8.00	20.00±12.00	20.00±1.00
Lymphocytes (%)	73.67±6.84	88.33±0.88	82.00±9.00	72.50±9.50	76.33±1.45
Eosinophils (%)	1.00±0.00	1.00±0.58	2.00±0.58	1.00±0.00	2.00±0.00
Basophils (%)	1.67±0.67	1.00±0.58	2.67±1.20	4.00±1.00	1.00±0.58
Monocytes (%)	3.67±1.45	0.67±0.33	1.33±0.67	2.50±1.50	0.67±0.67

Values are presented as mean ± SEM. *: Significant differences ($p < 0.05$) compared to the normal control group. AFH: *A. falcatus* hexane extract, AFEA: ethyl acetate extract, AFNB: *n*-butanol extract, AFW: water extract.

Effect of Subacute Extracts Administration on Biochemical Parameters

The toxic/adverse effects of the investigated leaf extracts of *A. falcatus* were further evaluated using selected biochemical parameters of kidney and liver function and carbohydrate and lipid

metabolism. The results are shown in Table 2. The repeated administration of the plant extracts did not significantly modify the biochemical parameters of kidney function ($p > 0.05$). Both serum creatinine and BUN concentrations showed comparable results to those of the untreated healthy control group. Assessment of liver toxicity by serum total protein, AST, ALT, ALP, and γ-GT revealed slight changes. A reduction in ALT and ALP values was observed in rats administered with plant extracts together with a significant reduction in ALT activity in female rats administered with AFNB. Furthermore, there was a reduction in ALP activity in male rats administered with AFEA, compared to the experimental rats of the normal control group ($p < 0.05$). No significant changes were observed in fasting serum glucose, total cholesterol, triglycerides, and HDL-C in groups of rats administered with the plant extracts.

Table 2. Effect of investigated extracts of *Asparagus falcatus* on biochemical parameters.

Parameters	Normal control	Plant extract treatment			
		AFH	AFEA	AFNB	AFW
Male					
Blood urea nitrogen (mmol/L)	7.96±0.35	6.25±0.45	6.49±0.06	6.96±0.52	7.79±0.16
Creatinine (µmol/L)	68.36±4.90	62.47±13.53	65.71±3.83	73.96±2.06	68.95±4.42
Total protein (g/L)	6.83±0.74	8.25±0.99	7.67±0.21	6.65±0.09	7.24±0.57
Aspartate aminotransferase (U/L)	113.90±39.10	96.20±17.90	98.00±10.17	77.60±10.58	112.70±33.06
Alanine aminotransferase (U/L)	53.93±5.73	40.93±5.88	44.04±3.19	40.55±9.66	41.90±12.96
Alkaline phosphatase (U/L)	351.07±1.91	252.10±10.66	176.45±8.25*	280.00±19.24	344.00±26.58
Gamma-glutamyltransferase (U/L)	5.79±1.33	6.66±4.34	8.11±1.16	5.50±2.61	4.44±1.35
Fasting plasma glucose (mmol/L)	5.27±0.12	4.53±0.53	4.49±1.15	5.51±0.68	6.99±0.44
Total cholesterol (mmol/L)	74.94±6.37	66.70±8.26	48.43±10.04	83.87±2.79	81.50±5.70
Triglycerides (mmol/L)	1.19±0.25	1.21±0.34	0.93±0.03	0.72±0.02	1.00±0.02
HDL cholesterol (mmol/L)	1.72±0.09	1.38±0.03	1.28±0.07	1.57±0.19	1.61±0.24
Female					
Blood urea nitrogen (mmol/L)	6.89±0.27	7.20±0.16	7.43±1.51	5.71±0.00	8.48±1.16
Creatinine (µmol/L)	65.12±6.09	78.58±0.00	81.33±5.30	69.25±2.57	67.18±6.02
Total protein (g/L)	7.28±0.27	6.90±0.42	8.06±0.48	7.75±0.13	7.47±0.17
Aspartate aminotransferase (U/L)	79.33±11.13	79.57±22.22	61.10±1.53	73.35±0.55	89.27±5.78
Alanine aminotransferase (U/L)	45.40±2.10	39.58±5.74	30.65±3.19	25.90±0.87*	33.56±3.05
Alkaline phosphatase (U/L)	351.07±1.91	252.10±10.66	176.45±8.25	280.00±19.24	344.00±26.58
Gamma-glutamyltransferase (U/L)	6.18±1.02	4.05±1.16	6.76±1.35	6.66±0.87	5.60±2.35
Fasting plasma glucose (mmol/L)	4.82±0.53	3.77±0.48	3.55±0.21	4.29±0.19	5.00±0.70
Total cholesterol (mmol/L)	69.00±3.69	60.30±12.78	40.50±11.14	58.75±3.95	64.00±3.73
Triglycerides (mmol/L)	1.04±0.11	0.85±0.11	1.01±0.08	0.93±0.24	0.96±0.03
HDL cholesterol (mmol/L)	1.49±0.04	1.03±0.16	1.27±0.11	1.28±0.09	1.52±0.00

Values are presented as mean ± SEM. *: Significant differences ($p < 0.05$) compared to the normal control group. AFH: *A. falcatus* hexane extract, AFEA: ethyl acetate extract, AFNB: *n*-butanol extract, AFW: water extract.

Effect of Subacute Extracts Administration on Relative Organ Weight

No significant changes were observed in the relative weight of the excised organs in the experimental rats administered with plant extracts compared to the untreated control group ($p > 0.05$). The results are shown in Table 3.

Table 3. Effect of investigated leaf extracts of *A. falcatus* on the relative weight of organs.

Treatment	Relative weight of organs (g/g BW)					
	Heart	Liver	Small intestine	Lung	Spleen	Kidney
Male						
Normal control rats	0.33±0.02	2.78±0.11	1.50±0.06	0.50±0.10	0.27±0.03	0.65±0.02
AFH	0.33±0.01	2.64±0.10	1.43±0.14	0.44±0.03	0.23±0.02	0.52±0.01
AFEA	0.35±0.01	2.78±0.06	1.36±0.07	0.49±0.01	0.24±0.01	0.57±0.01
AFNB	0.35±0.01	2.58±0.05	1.17±0.03	0.49±0.03	0.23±0.01	0.53±0.01
AFW	0.32±0.02	2.74±0.03	1.57±0.25	0.46±0.01	0.22±0.01	0.38±0.14
Female						
Normal control rats	0.34±0.01	2.91±0.08	2.18±0.11	0.59±0.02	0.28±0.02	0.55±0.01
AFH	0.34±0.02	2.53±0.06	1.85±0.11	0.55±0.02	0.26±0.01	0.56±0.02
AFEA	0.37±0.01	2.78±0.08	1.60±0.01	0.53±0.03	0.27±0.01	0.58±0.02
AFNB	0.36±0.03	2.78±0.20	1.73±0.35	0.56±0.03	0.26±0.01	0.57±0.05
AFW	0.33±0.01	2.69±0.08	1.84±0.08	0.54±0.00	0.25±0.01	0.56±0.00

Values are presented as mean ± SEM. *: Significant differences ($p < 0.05$) compared to the normal control group. AFH: *A. falcatus* hexane extract, AFEA: ethyl acetate extract, AFNB: *n*-butanol extract, AFW: water extract.

Effect of Subacute Extracts Administration on the histology of vital organs.

The rats administered with the selected leaf extracts of *A. falcatus* showed no toxicity changes in hematoxylin and eosin-stained sections of the excised vital organs. Photomicrographs are shown in Figure 4(a-e). The heart, liver, spleen, small intestine and kidney tissues of experimental rats did not show visible lesions, necrosis, congestion, or hemorrhages after administering the plant extracts for 28 days. Hepatocytes, sinusoids, and central veins in liver tissues appeared normal. The glomeruli and renal tubules in the kidney tissues showed normal cellular architecture.

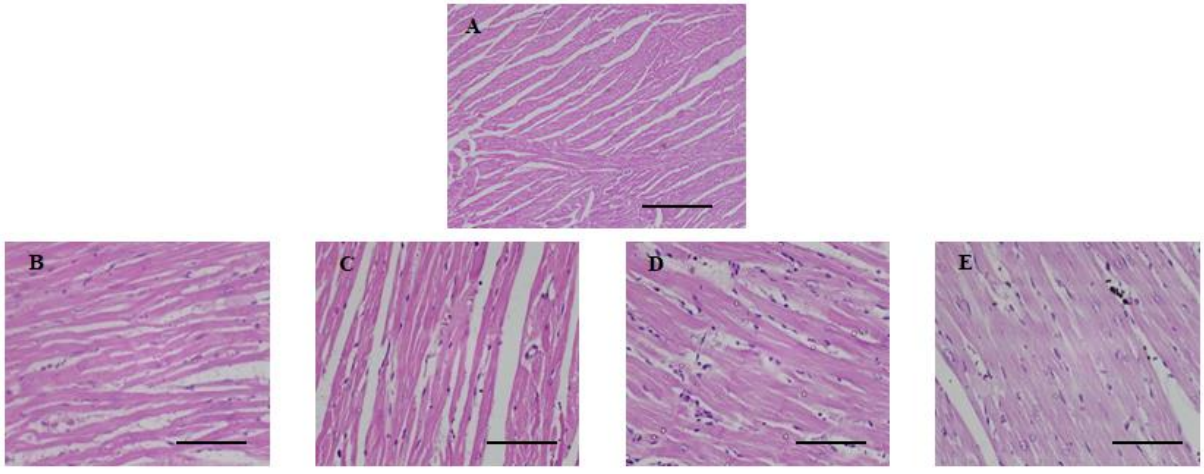

Figure 4a. Histology of hematoxylin and eosin-stained sections of cardiac tissues from representative samples of female rats from normal control (A) and groups of rats administered with AFH (55 mg/kg; B), AFEA (35 mg/kg; C), AFNB (75 mg/kg; D) and AFW (200 mg/kg; E) (×400, scale bar: 50 µm).

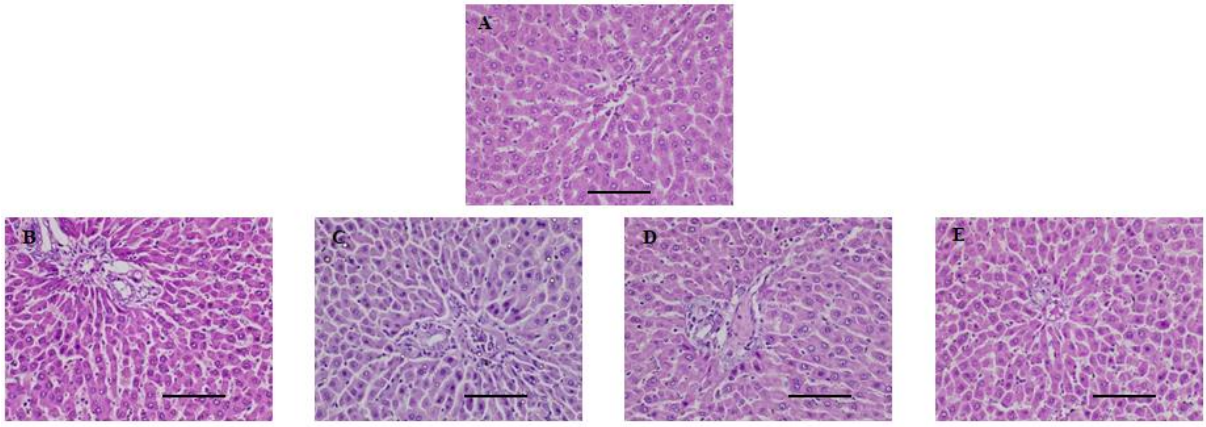

Figure 4b. Histology of hematoxylin and eosin-stained sections of liver tissues from representative samples of female rats from normal control (A) and groups of rats administered with AFH (55 mg/kg; B), AFEA (35 mg/kg; C), AFNB (75 mg/kg; D) and AFW (200 mg/kg; E) (x400, Scale bar: 50 µm).

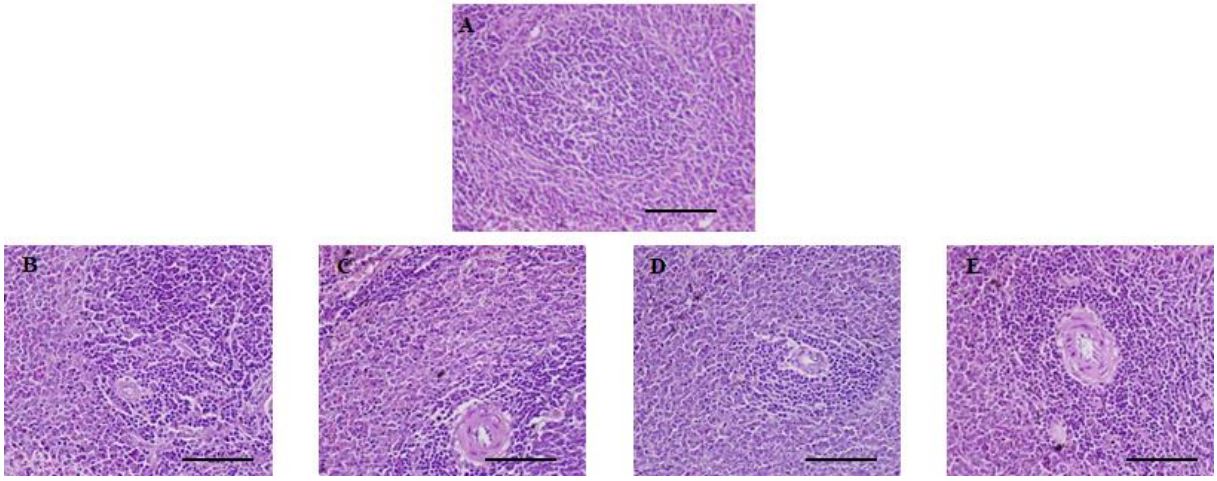

Figure 4c. Histology of hematoxylin and eosin-stained sections of spleen tissues from representative samples of female rats from normal control (A) and groups of rats administered with AFH (55 mg/kg; B), AFEA (35 mg/kg; C), AFNB (75 mg/kg; D) and AFW (200 mg/kg; E) (×400, Scale bar: 50 µm).

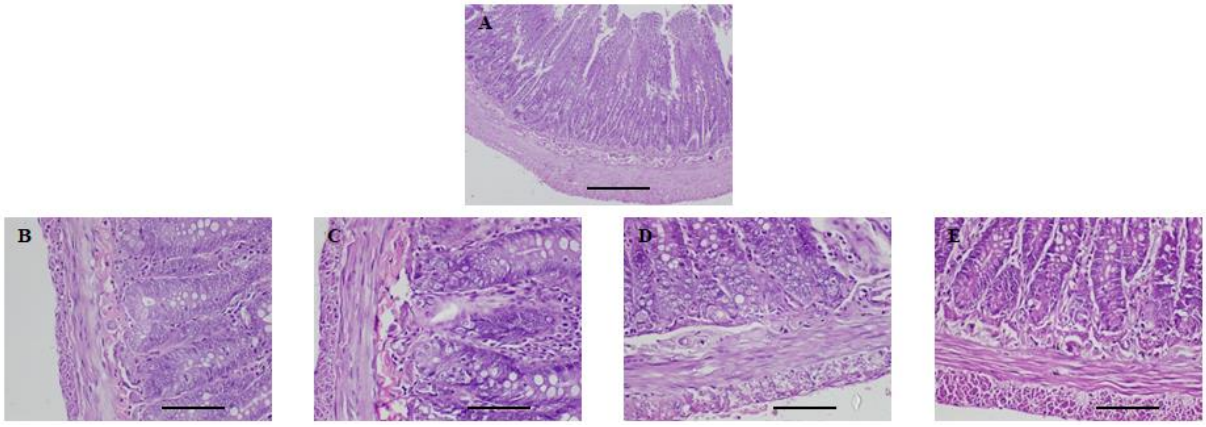

Figure 4d. Histology of hematoxylin and eosin-stained sections of the small intestine from representative samples of female rats from normal control (A) and groups of rats administered with AFH (55 mg/kg; B), AFEA (35 mg/kg; C), AFNB (75 mg/kg; D) and AFW (200 mg/kg; E) (×400, Scale bar: 50 µm).

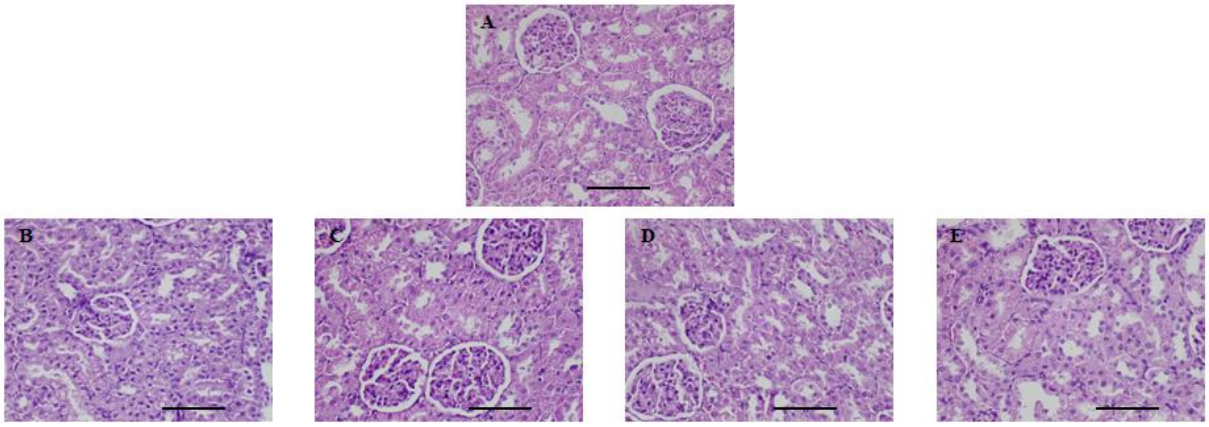

Figure 4e. Histology of hematoxylin and eosin-stained sections of kidney tissues from representative samples of female rats from normal control (A) and groups of rats administered with AFH (55 mg/kg; B), AFEA (35 mg/kg; C), AFNB (75 mg/kg; D) and AFW (200 mg/kg; E) (x400, Scale bar: 50 µm).

Qualitative and Quantitative phytochemical screening

The findings of the preliminary phytochemical screening of AFH, AFEA, AFNB and AFW revealed the presence of phenolic compounds at varying degrees in the selected extracts of *A. falcatus*. Tannins, flavonoids, saponins and coumarins were found only with the AFW of the plant. Steroid glycosides and terpenoids were present in both AFNB and AFW. None of the extracts was positive for alkaloids.

Quantitative analysis by GC-MS was performed on AFEA. A total of 26 major chemical components were detected. The detected compounds are listed in Table 4, where the molecule name, retention time (RT), retention index (RI), matching percentage, molecular formula and the percentage of each compound in the extract are listed. The compounds listed in Table 4 are confirmed by structural analysis and fragment ion analysis of the mass spectrum with library compounds. Thus, the compounds with low matching percentages are not listed in Table 4.

Discussion

Scientific publications on the safety of Sri Lankan medicinal plants provide supportive evidence on various herbs with potential toxic effects. *Cassia auriculata* L. (family: *Leguminosae*), commonly known as 'Ranawara', is one such example. The beverage (Ranawara tea) made of the flowers of 'Ranawara' is a popular diuretic often recommended by traditional healers. The study revealed the presence of pyrrolizidine alkaloids, a category of phytochemicals with toxic, teratogenic, and carcinogenic effects, in the bark, flowers, and seeds of *C. auriculata*[22,23]. A similar study by the same group of researchers identified a few more Sri Lankan herbs with toxic effects on the liver, pulmonary, and renal tissues. The bael fruit (*Aegle marmelos*, family: *Rutaceae*) and Indian sarsaparilla (*Hemidesmus indicus*, family: *Periplocaceae*, local name: *Iramusu*) are a few such examples[24]. More importantly, mild toxicity effects of long-term usage of *Asparagus racemosus*, a plant of the family *Asparagaceae*, have been recently reported by Soren and Yadav[25]. Thus, evaluation of the toxic effects of plant therapeutics according to the standard guidelines is paramount before the development of commercially viable therapeutic drug leads. Hence, acute and subacute oral toxicity studies were carried out in the Wistar rat model for the elucidation of adverse/toxic effects of AFH, AFEA, AFNB and AFW in the present study.

The acute toxicity studies facilitate the selection of appropriate doses for subacute and chronic low-dose toxicity assessments, which could be clinically more relevant[26]. In the present study, the administration of a single dose of the selected extracts of *A. falcatus* at the therapeutic dose showed neither mortality nor clinical signs of toxicity. Therefore, the selected doses were used in further experiments on the detailed evaluation of the toxic effects of *A. falcatus*.

Table 4. Chemical constituents identified in abundance in the GC-MS analysis of ethyl acetate extract of *Asparagus falcatus*

Name	RT	RI	%area	Matching %	Molecular formula
Dimethyl sulfone	4.604	919	0.18%	91	$C_2H_6O_2S$
Decane	6.198	989	0.05%	97	$C_{10}H_{22}$
2,3-Butanedioldiacetate	7.538	1048	0.10%	78	$C_8H_{14}O_4$
1,2,3-Propanetriol,monoacetate	7.892	1064	0.08%	64	$C_5H_{10}O_4$
Ethyl (+-)-3-acetoxybutylrate	8.194	1077	0.07%	86	$C_8H_{14}O_4$
1,3-Butanediol,diacetate	8.421	1087	0.11%	90	$C_8H_{14}O_4$
Undecane	9.494	1138	0.07%	93	$C_{11}H_{24}$
E-14-hexadecanal	11.917	1260	0.04%	72	$C_{16}H_{30}O$
Tetradecane	12.004	1264	0.07%	98	$C_{14}H_{30}$
1-Tetradecene	14.044	1378	0.08%	98	$C_{14}H_{28}$
Hexadecane	14.112	1382	0.07%	95	$C_{16}H_{34}$
5-Amino-1-methyl-phenylpyrazole	14.813	1425	0.04%	46	$C_{10}H_{11}N_3$
Tetradecanoic acid,ethyl ester	15.924	1494	0.09%	83	$C_{16}H_{32}O_2$
Octadecane	15.978	1497	0.05%	91	$C_{18}H_{38}$
Bicyclo(3.1.1.)heptane,2,6,6-trimethyl-(1.alpha-2-beta.5.alpha)	16.34	1521	0.11%	49	$C_{10}H_{18}$
6-Octen-1-ol,3,7-dimethyl	16.553	1535	0.04%	58	$C_{10}H_{20}O$
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-0,9-diene-2,8-dione	17.127	1573	0.04%	90	$C_{17}H_{24}O_3$
Hexadecanoic acid, ethyl ester	17.617	1606	0.14%	89	$C_{18}H_{36}O_2$
Tetracosane,11-decyl	17.659	1609	0.08%	43	$C_{34}H_{70}$
Dodecanoic acid, 1,2,3-propanetriyl ester	18.199	1647	0.25%	50	$C_{39}H_{74}O_6$
Cyclohexanol,5-methyl-2-(1-methylethyl)-(1.alpha,2.alpha,5.alpha)	18.583	1674	0.32%	68	$C_{10}H_{20}O$
Hexadecanoic acid, butyl ester	19.11	1712	0.26%	92	$C_{20}H_{40}O_2$
Octadecanoic acid,ethyl ester	19.16	1716	0.41%	64	$C_{20}H_{40}O_2$
3,7,11,15-tetramethyl-2-hexadecen-1-ol	19.358	1730	0.22%	43	$C_{20}H_{40}O$
Butyl 9,12-octadecadienoate	20.344	1804	0.17%	94	$C_{22}H_{40}O_2$
Hexanedioic acid,bis(2-ethylhexyl)ester	20.636	1826	0.33%	64	$C_{22}H_{42}O_4$

Repeated oral administration of investigated leaf extracts of *A. falcatus* did not reveal clinically significant toxic/adverse effects on the selected animal model concerning the evaluated hematologic, biochemical, and histological parameters. None of the experimental animals died during the study period of 28 days, inferring the fact that the investigated plant extracts at the selected doses did not have any unwanted effects in rats when administered orally for 28 consecutive days. Similarly, none of the experimental animals lost body weight during the study period of 28 days, and the findings ruled out any substantial effect of plant extracts on the metabolism or activity level of experimental rats. The absence of significant changes in food consumption and water intake further indicates the unaffected appetite of experimental rats.

None of the hematological parameters showed significant changes in values compared to the untreated control group, except the increase in hemoglobin concentration in rats administered with AFH. Elevation of hemoglobin concentration in the particular group indisputably excludes the risk of the development of anemia. However, the potential risk of polycythemia could also be omitted, as the values were found within the physiological range for the species[27]. The observed increase in white blood cell counts following treatment with all four extracts directly indicates the strengthen-

ing of the defense systems in experimental rats. These findings further suggest that selected plants may contain biologically active compounds that can boost the immune system by increasing the population of defensive white blood cells[28].

The findings on biochemical parameters of renal function excluded potential nephrotoxicity by the investigated plant extracts at the doses studied. Significant differences were observed only with the levels of ALT and ALP in the groups of rats administered with AFNB and AFEA, respectively, concerning the parameters of the liver functions. The particular groups showed a significant reduction in ALT and ALP values, and the findings also acted in favor of the investigated plant extract, excluding the risk of hepatocellular damage. However, all four extracts of *A. falcatus* resulted in a reduction in both ALT and ALP values compared to the untreated control group, suggesting potential hepatoprotective effects of the plant.

In the present study, the metabolic states of the experimental animals about carbohydrate and lipid metabolism were assessed using fasting serum glucose and lipid profile parameters. None of the experimental groups showed significant changes concerning the serum biochemical parameters assessed. However, a reduction in the concentration of total cholesterol was observed in female rats administered with all four extracts and in male rats administered with AFH and AFEA, further substantiating the health benefits of the selected medicinal plant ($p > 0.05$). Furthermore, none of the groups of experimental rats showed significant changes in the relative weight and histology of the excised vital organs, and these findings rule out the potentially toxic effects of AFH, AFEA, AFNB, and AFW in the Wistar rat model. These findings were further supported by the findings of the toxicity study of Hewawasam and coworkers, which revealed the potential safety of the aqueous extract of *A. falcatus* tubers[29].

Qualitative analysis of phytochemicals revealed the absence of alkaloids in the investigated extracts of *A. falcatus* in the present study. Hence, the findings exclude the potential risk of having pyrrolizidine alkaloids in the leaves of the plant. However, the presence of phenolic compounds and flavonoids in AFW further substantiates the value of the plant as an antioxidant medicine in various disease conditions. This is further corroborated by numerous recent scientific publications which have confirmed the protective effects of phenolic compounds against diseases of oxidative damage and flavonoids in numerous disease conditions such as diabetes, Alzheimer's, gout, hemorrhoids, cardiac, neurodegenerative, eye diseases, etc[30]. The finding on preliminary phytochemical screening was further supported by the results of GC-MS analysis. The present study offers the first evidence of GC-MS analysis of *A. falcatus* and the findings revealed the presence of phytoconstituents, including octadecanoic acid, hexadecanoic acid, tetradecanoic acid, dodecanoic acid, etc. which are known to have antioxidant, anti-inflammatory, hypercholesterolemia, cancer preventive, antiarthritic, and hepatoprotective activities, further substantiating the medicinal value of the plant[31]. Yet a quantitative analysis of potential phytochemicals with therapeutic/toxicity effects was not performed in the plant and is considered a limitation of the present study.

Considering the ethical issues related to animal studies, the investigations on potential acute and subacute toxicity effects of the plant were limited to a single dose in each of the investigated extracts, and the effect of human equivalent therapeutic dose in rats was centered on the investigations. The omission of multiple doses of each extract, as pre-defined by the OECD guidelines is therefore a limitation of the present study. Further, the determination of the maximum tolerated dose of each extract was not carried out in the present study, considering the ethical aspects related to the use of an increased number of experimental animals. Moreover, the experiments were not carried out to assess the potential toxic effects of the plant extracts at different time slots e.g. on day 0 and during the intervention, and this could be considered as another limitation of the study. Yet, the findings of the present study would provide sufficient data on the in vivo safety of the plant, leading future studies on the development of new drug leads with nephroprotective effects.

Conclusions

The findings on biochemical, hematological and histological assessments excluded potential toxic/adverse effects of the investigated aqueous and solvent leaf extracts of *A. falcatus* at therapeutically effective doses in vivo, thus demonstrating their potential safety according to the OECD standards. Qualitative analysis of

phytochemicals in AFH, AFEA, AFNB and AFW revealed the absence of alkaloids, excluding the potential risk of pyrrolizidine alkaloids. The presence of several bioactive phytoconstituents identified by GC-MS analysis further signifies the medicinal importance of *A. falcatus*.

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