Nephroprotective mechanisms of Ambrette (*Abelmoschus moschatus* Medik.) leaf extracts in adriamycin mediated acute kidney injury model of Wistar rats

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ABSTRACT

Ethnopharmacological relevance: Ambrette (*Abelmoschus moschatus* Medik., Family: Malvaceae) is a common Ayurvedic herbal medicine used in the treatment of kidney-related diseases, in the forms of tea, medicated oil, medicated wine, etc., however, its nephroprotective mechanisms remain unexploited.

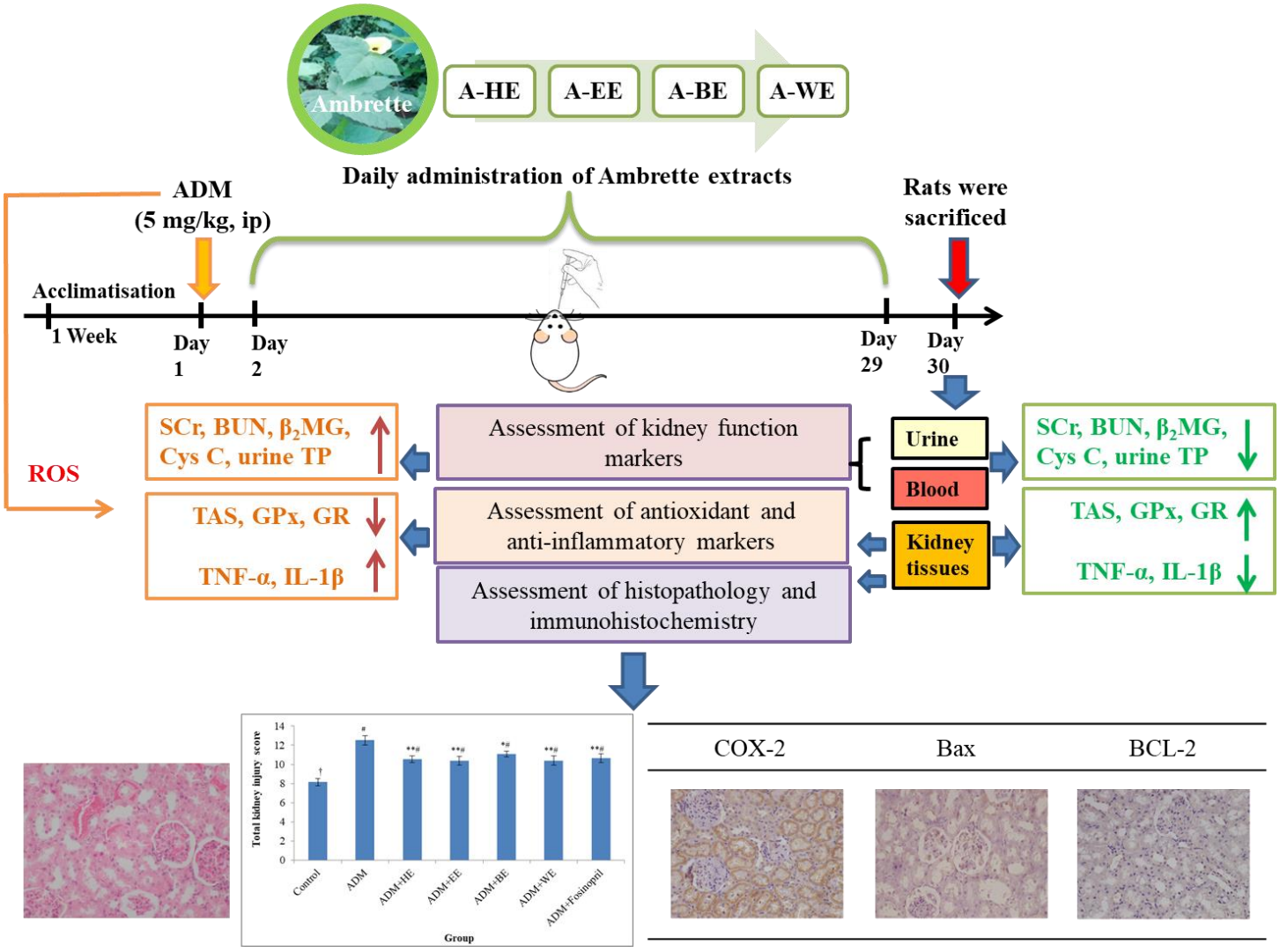
Aim of the study: To investigate the mechanisms by which the hexane (A-HE), ethyl acetate (A-EE), butanol (A-BE), and aqueous (A-WE) leaf extracts of Ambrette protect against the adriamycin-mediated acute kidney injury in Wistar rats.

Materials and Methods: A-HE, A-EE, A-BE, A-WE, and fosinopril sodium were administered at therapeutically effective doses (55, 75, 60, 140, 0.09 mg/kg) to adriamycin-induced (5 mg/kg, ip) Wistar rats for 28 consecutive days.

Results: Oral administration of the selected extracts of *A. moschatus* resulted in amelioration of kidney injury as observed by the significant changes of biomarkers of kidney function in serum and in urine, biochemical parameters of oxidative stress, and inflammation in kidney homogenates ($p<0.05$). Furthermore, the administration of plant extracts caused a significant reduction in total kidney injury scores in H and E stained kidney sections ($p<0.05$). The immunohistochemical expression of the inflammatory marker, COX-2, and the pro-apoptotic marker, Bax, were attenuated and the expression of the anti-apoptotic marker, BCL-2, was increased. A-HE exerted superior nephroprotective effects over the other three extracts and the drug reference standard.

Conclusions: The findings revealed that Ambrette exerts promising protective effects against adriamycin-mediated acute kidney injury through antioxidant, anti-inflammatory, and anti-apoptosis pathways. A-HE might serve as a potential candidate for the development of therapeutic drug leads that will be beneficial in the treatment of acute kidney injury.

Graphical Abstract



Key words: *Acute kidney injury; Ambrette, anti-apoptosis effects; anti-inflammatory effects; antioxidant potential*

Highlights

1. A-HE, A-EE, A-BE, and A-WE ameliorate ADM-induced acute kidney injury.
2. Antioxidant, anti-inflammatory, anti-apoptotic effects accounted for nephroprotection.
3. A-HE showed superior nephroprotective effects over the other three extracts.
4. A-HE might be a potential candidate for future nephroprotective drug leads.

1. Introduction

The use of herbal medicines in the management of human diseases has flourished to date. Traditional medicine systems based mainly on plant-based formulations still play an important role in achieving the primary health care needs of more than two-thirds of the world's population (Joana Gil-Chavez et al., 2013; Jaiswal and Williams, 2017; Kala, 2017). A huge renaissance in the interest of herbal-based medicines as potential objects of scientific analysis has been witnessed over the past few decades (Briskin, 2000). Traditional knowledge gained from doctrines of conventional systems of medicine, folklore claims, and databases serves as powerful search engines in the discovery of novel drug leads (Jaiswal and Williams, 2017). Consequently, a substantial number of modern pharmaceuticals have been derived from natural bioactive compounds of ethnobotanical origin during the last 25 years (Bagnis et al., 2004; Pandey et al., 2011). In fact, the reported success rates of herbal-based therapeutics are comparatively higher than that of their synthetic counterparts (Schulz et al., 2001; Central Council for Research in Ayurvedic Sciences, 2018).

The use of alternative therapeutic approaches in terms of a second option or as an adjunct to avert disease progression and associated health complications is quite common among patients with kidney disease (Huang et al., 2018). Conventional treatment options for kidney injury in the allopathic system of medicine represent primary therapeutic approaches that include volume and haemodynamic management, use of diuretics, and avoidance of nephrotoxins. Norepinephrine, vasopressin, or dopamine is recommended for vasodilation during the events of persisting hypotension after intravascular volume is replenished. Angiotensin II and adenosine antagonists are used to manipulate the renal microvasculature. Renal replacement therapy is implemented through dialysis and kidney transplantation when medical management of the disease becomes ineffective or at the stage of life-threatening kidney injury (Hurtarte-Sandoval and Carlos-Zamora, 2014; Chen and Busse, 2017; Moore et

al., 2018; Mata-Miranda et al., 2019; Kellum et al., 2021). Although these therapeutic approaches are meant to be supportive, most of them are neither specific for kidney damage nor can be used exclusively to produce nephroprotective effects. Furthermore, its therapeutic efficacy has been hampered by the harmful effects of over resuscitation and the associated side effects of angiotensin II and adenosine antagonists (Chen and Busse, 2017). Although modern treatment options such as the supplementation of therapeutic antioxidants including selenium, α-lipoic acid, propofol, curcumin, and inflammatory modifiers such as S1P analogs have shown promising effects in animal models, extensive clinical studies in humans are yet to be performed (Chen and Busse, 2017).

Given the importance of the wide biological activities of bioactive compounds in plant tissues, particularly in terms of antioxidants and anti-inflammatory mediators, plant-based medicines often become a treatment option of choice to cope with free radical pathologies associated with kidney diseases (Joana Gil-Chavez et al., 2013). In the past few decades, researchers have made it a point to concentrate on the development of nephroprotective agents from medicinal plant sources on the basis of practices of traditional medicines (Ahmida, 2012; Wu et al., 2014). Their attention focused mainly on the investigation of the nephroprotective mechanisms of medicinal plant extracts in the protection, prevention, and acceleration of podocyte and tubular cell regeneration against kidney injury (Tavafi, 2012; Nasri and Rafieian-Kopaei, 2013).

Ambrette (*Abelmoschus moschatus* Medik., Family: Malvaceae) is a medicinal plant with a particular interest in the treatment of kidney disease in traditional Ayurvedic practice in Sri Lanka (Jayaweera, 1982). Almost all parts of the plant, including leaves, stems, seeds, flowers, and roots are used in therapeutic applications in traditional Sri Lankan folk medicine. Seeds are diuretic and are often used in the form of tea for the treatment of dysuria. They are the main ingredients of the medicated wine, “Dasamularistaya” and of the

medicated oil, “Maha koranda”, used in the treatment of difficulty and burning sensation in urination (Ayurveda Pharmacopoeia, 1985). In addition, the seeds, roots, and leaves of the plant are used in the treatment of various kidney-related disorders such as renal calculi, cystitis, urethritis, and gonorrheal cystitis due to their anti-inflammatory and antimicrobial effects (Jayawwera, 1982; Anon., 1984; Dwivedi and Argal, 2015; Pawar and Vyawahare, 2017a; Akbar, 2020). The plant is used in the treatment of a variety of ailments in the form of powder (Ashwagandhadi churna, Maha saraswathi churna), tablet (Kunkumadi watiya), concentrated decoction (Wacheekasawaya, Wasakasawaya), medicated wine (Kadirarishtaya, Jeeraka dasarishtaya, Babbularishtaya) and medicated oil form (Brunga raja) in Ayurveda (Ayurveda Pharmacopoeia, 1985).

Ambrette has been studied for its bioactivities and therapeutic potentials by various researchers (Supplementary file 1, Table 1). Ambrette seed, leaf, and whole plant extracts have been reported for diuretic and antiurolithiatic activities *in vivo* (Christina and Muthumani, 2013; Pawar and Vyawahare, 2017a). In addition, various solvent and aqueous extracts of the plant exerted hepatoprotective, antimicrobial, antifungal, antiproliferative, memory strengthening, antidepressant, anti-aging, antidiabetic, anticonvulsant, anxiolytic, anti-inflammatory, antioxidant, and free radical scavenging properties (Pawar and Vyawahare, 2017b; Nandhini et al., 2014; Rahman et al., 2017). Various phytoconstituents have been isolated from the plant such as myricetin (Supplementary file 1, Figure 1), farnesol, palmitic acid, linoleic acid, cannabiscitrin, 2-methylbutyl-2-methylbutanoate, ambrettolide, furfural, cyanidin-3-sambubioside, β sitosterol, cyanidin-3-glucoside, however, potential bioactivities have been reported only for myricetin (Pawar and Vyawahare, 2017b). Our research group reported the efficacy of the aqueous leaf extract of the plant in ameliorating adriamycin (ADM) mediated nephrotoxicity for the first time (Amarasiri et al., 2020 a); however, the possible nephroprotective mechanisms of the plant extract/s after

long-term administration were not addressed in the particular study. Apparently, a 28-day repeated dose oral administration of Ambrette hexane (A-HE), ethyl acetate (A-EE), butanol (A-BE), and aqueous leaf extracts of Ambrette did not produce toxic or adverse effects in healthy Wistar rats (Amarasiri et al., 2020 b).

ADM is an anthracycline antibiotic widely used in cancer chemotherapy. ADM-mediated acute kidney injury in Wistar rats is considered as an appropriate model of experimental nephrotoxicity in the study of the early pathophysiological characteristics of kidney damage (Lee and Harris, 2011). ADM can cause an increase in glomerular capillary permeability and tubular atrophy, resulting in functional and morphological changes related to human kidney disease (Khajavi Rad et al., 2017; Okuda et al., 1986). Histopathological changes in kidney toxicity appear as early as one to two weeks after ADM administration (Lee and Harris, 2011; Wang et al., 2000). Hence, herein we aimed to investigate the potential nephroprotective mechanisms of A-HE, A-EE, A-BE, and A-WE of Ambrette in Wistar rats with ADM mediated nephrotoxicity as a surrogate model of kidney injury.

2. Materials and methods

2.1. Chemicals and reagents

ADM (Generic name: Doxorubicin hydrochloride (CID: 443939) was purchased from United Biotech, India, and used for the induction of kidney injury in experimental animals. Fosinopril sodium (CID: 23681451) a (Sigma-Aldrich, USA) was used as the drug reference standard. The biochemical parameters of kidney function including serum creatinine (SCr; Biorex diagnostics, UK), blood urea nitrogen (BUN; Biorex diagnostics, UK), serum total protein (STP; Stanbio Laboratory, Texas), serum albumin (SAlb; Stanbio Laboratory, Texas) and urine total protein (UTP; Stanbio Laboratory, Texas) were estimated using commercial spectrophotometric assay kits. Serum concentrations of β_2-microglobulin (β_2-MG; DRG

Instruments GmbH, Germany) and cystatin C (Cys C; Elabscience Biotechnology lnc, USA) were measured according to the enzyme-linked immunosorbent assay (ELISA) procedures. Spectrophotometric assay kits for the assessment of antioxidant markers including total antioxidant status (TAS), glutathione peroxidase (GPx), and glutathione reductase (GR) were acquired from Biorex diagnostics (UK). The chemicals for the estimation of malondialdehyde (MDA) concentration in order to assess lipid peroxidation were purchased from Sigma-Aldrich, USA. ELISA kits for the evaluation of anti-inflammatory markers including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were obtained from Elabscience Biotechnology lnc. (USA). The primary markers, anti-Bax (ab216494; Abcam, Cambridge, UK), anti-BCL-2 (M0887; Dako, Denmark), anti-COX-2 (M3617; Dako, Denmark), and the HRP- conjugated secondary antibody (K4061; Dako, Denmark) were used in immunohistochemistry studies.

2.2. Plant material, authentication, and extraction

Ambrette leaves were collected from the Western region of Sri Lanka. The plant materials were authenticated and certified by Mr. N.P.T. Gunawardena, Officer in Charge, National Herbarium, Department of National Botanical Gardens, Peradeniya, Sri Lanka, prior to use in the experiments. A herbarium specimen was deposited under the specimen number PG/2016/55/01 at the mini-herbarium in the Department of Biochemistry, Faculty of Medicine (Supplementary file 1, Figure 2). The standardisation parameters and the liquid chromatography-mass spectrometry (LC-MS) fingerprint of the crude plant extract of Ambrette were previously reported by our research group (Amarasiri et al., 2020 a). The procedure is mentioned in Supplementary file 2.

The preparation of hexane (A-HE), ethyl acetate (A-EE), butanol (A-BE), and aqueous (A-WE) leaf extracts of Ambrette was carried out by the soxhlet extraction method. The oven-

dried (40 ℃), powdered, and weighed (25g) plant materials were placed in an extraction thimble and subjected to sequential soxhlet extraction with hexane, ethyl acetate, butanol, and distilled water. The ratio of material to solvent was 1:20. Once the leachate became colorless (A-HE; 6 h at 70℃, A-EE; 6 h at 80℃, A-BE; 6 h at 120℃, A-WE; 8 h at 100℃) the extraction was discontinued for the particular solvent, and the process was resumed with the subsequent solvent following complete evaporation of the previous. The extracts were collected separately. The solvents in A-HE, A-EE, and A-BE were evaporated using the rotary evaporator (45℃ - 55℃, under vacuum). The concentrated A-WE were freeze-dried (-20 ℃).

2.3. Selection and preparation of the doses of plant extracts

In the present study, the human equivalent therapeutic dose in rats was calculated and adjusted for each extract separately (A-HE; 55 mg/kg, A-EE; 75 mg/kg, A-BE; 60 mg/kg, A-WE; 140 mg/kg) based on the dose recommended in Ayurveda (12 g/day) for an adult human (60 kg body weight). This is equivalent to the selected daily oral doses of A-HE, A-EE, A-BE, and A-WE, calculated based on the percentage yield of different extractions and the dose conversion factor from human to rat (6.2) (Nair and Jacob, 2016). The percentage yields of A-HE, A-EE, A-BE, and A-WE were 4.29, 5.76, 4.53, and 11.40 g/100 g of dry plant material respectively. The A-HE and A-EE were dissolved in corn oil and A-BE was dissolved in 3% polyvinylpyrrolidone (PVP) for administration. The A-WE was dissolved in distilled water.

2.4. Animals and experimental design

The experimental protocols were approved by the Ethical Review Committee of the Faculty of Medicine (Reference Number: 14.12.2015:3.1). Experiments were conducted in accordance with the 'Guide for the Care and Use of Laboratory Animals (NIH Publication No.85–23, 1985). Male Wistar rats (150-175 g) obtained from the in-bred colonies of the

animal house at the Department of Biochemistry, Faculty of Medicine were used in the investigations. The animals were kept under standardised environmental conditions (25±1 ℃, light from 08:00 to 20:00 h) with free access to water and a pelleted meal diet (energy content of 2.7 kcal/g with fat 6%, protein 14.5%, fibre 6%, PO_4^{3-} 0.7%, and Ca 0.9%).

A single intraperitoneal dose of ADM at 5 mg/kg (in normal saline) was used to establish the surrogate model of kidney injury in male Wistar rats. The ADM dose was based on literature data and the findings of our pilot experiments (Lee and Harris, 2011). In pilot experiments, the ADM dose of 2 mg/kg did not induce significant kidney injury, whereas the dose of 10 mg/kg caused premature mortality in some animals.

Healthy male Wistar rats (170 ± 20 g) of 10-12 weeks were used for experiments. Animals were acclimatised to the facility for one week prior to being used in experiments. A total of 42 Wistar rats were randomly divided into seven groups as follows; Group 1- Normal rats (administered with distilled water), Group 2- ADM mediated kidney injury rats (administered with distilled water), Group 3- ADM mediated kidney injury rats administered with A-HE (55 mg/kg), Group 4- ADM mediated kidney injury rats administered with A-EE (75 mg/kg), Group 5- ADM mediated kidney injury rats administered with A-BE (60 mg/kg), Group 6- ADM mediated kidney injury rats administered with A-WE (140 mg/kg), and Group 7- ADM mediated kidney injury rats administered with the drug reference standard, fosinopril (0.09 mg/kg). Six animals were allocated for each group. The development of kidney injury was confirmed by estimating urine total protein concentration in ADM and normal control rats, one week after induction of nephrotoxicity (Supplementary file 3). Rats were administered orally with distilled water, selected Ambrette extracts, and fosinopril once daily for 28 days starting on day 2 (24 h after induction of kidney injury by ADM). A stainless-steel oral gavage feeding needle was used for oral administration of distilled water/plant extracts/drug reference standard. On the last day of the intervention, the rats were individually housed in

diuresis cages and urine samples were collected for 24 h. On day 30 (24 h following the last dose of treatment regimens), the rats were euthanised using carbon dioxide inhalation and blood samples were collected by cardiac puncture. Blood samples were centrifuged for 10 min at 3500 rpm to obtain serum. Urine and serum samples were kept frozen at -80 ° C until processed. The kidneys of sacrificed rats were rapidly dissected, washed with phosphate-buffered saline, and dried with filter paper. One-half of each kidney was fixed in 10% formalin solution for subsequent investigations on histopathology and immunohistochemistry. The remaining kidney tissues were frozen at -80 ℃ for the biochemical assessment of antioxidant and anti-inflammatory markers (Figure 1).

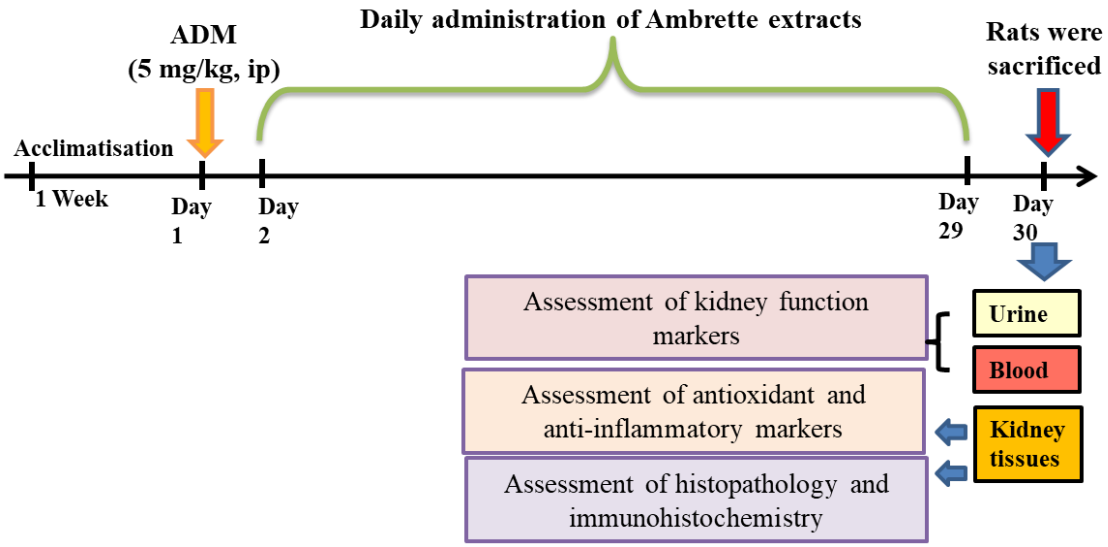


Figure 1. Schematic diagram of the experimental design

2.5. Assessment of kidney function

Kidney function was assessed in terms of SCr, BUN, STP, SAlb and UTP concentrations in blood and urine samples from experimental rats, using commercial spectrophotometric assay kits. Serum β_2-MG and serum Cys C were measured following ELISA procedures The detailed experimental procedures are mentioned in supplementary file 2.

2.6. Assessment of oxidative markers

Kidney tissues were homogenised with phosphate-buffered saline (0.1 M, pH=7.4) and centrifuged (4000 g, 20 min, 4 °C). The resultant supernatant was separated. The concentrations of TAS, GPx (EC 1.11.1.9), and GR (EC 1.6.4.2) were estimated using commercial assay kits following spectrophotometric methods. The MDA concentration was estimated according to the thiobarbituric acid method of Muriel et al. (2001) (Supplementary file 2).

2.7. Assessment of inflammatory cytokines

The concentration of IL-1β and TNF-α were quantified in the kidney homogenates using corresponding commercial assay kits following ELISA principle (Supplementary file 2).

2.8. Quality control in biochemical analysis

The commercially available assay kits, validated for accuracy, analytical sensitivity, analytical specificity, and linearity were used in the investigations. The validated thiobarbituric acid method of Muriel et al. (2001) was used for the estimation of MDA concentration in kidney homogenates. In addition, the analytical precision of some selected parameters was checked using commercially available quality control material. The alternative quality control methods such as duplicate testing, repeated testing with retained samples, etc. were employed in other parameters, in order to assure the precision of test results.

2.9. Assessment of histopathology

Morphometric examination of kidney injury in H&E stained kidney sections was performed blindly by two independent investigators. Each kidney section was examined and recorded across 10 random fields and accordingly, a total of 20 random fields were examined in one

experimental rat for the selected features of kidney injury. Tubular cell vacuolisation, nuclear pyknosis, loss of tubular brush border, and glomerular congestion were recorded on a 4-point scale of 0 = normal, 1 = involvement of < 25% injury, 2 = involvement of 25–50% injury, 3 = involvement of 50–75% injury and 4 = involvement of >75% injury. The presence of tubular casts was assessed on a two-point scale of 0 =no casts, 1 = 1 cast, and 2= >2 casts (magnification: x400). Inflammatory cell infiltration and intertubular haemorrhage were assessed as 0= absence and 1= present (magnification: x400). Individual scores for each morphological feature were added to sum the total kidney injury score for each experimental rat. Subsequently, the average scores for each experimental group were calculated.

2.10. Immunohistochemistry analysis

The formalin-fixed, paraffin-embedded 4 µm thick kidney sections on adhesive slides were deparaffinised, rehydrated, and blocked for endogenous peroxidase activity. The sections were incubated with proteinase K for antigen retrieval for anti-Bax. Heat-induced antigen retrieval was carried out by microwave (100 ℃ for 30 min) in citrate (pH 6) and Tris-EDTA (pH 9) buffers respectively for anti-BCL-2 and anti-COX-2. After cooling and blocking, sections were incubated with primary antibodies of Bax (1:100), BCL-2 (1: 25), and COX-2 (1:100), overnight at 4℃ in a humidified chamber. Subsequent incubation with horseradish peroxidase-conjugated secondary antibody (27 ° C, 2 h) and counterstaining with haematoxylin were carried out prior to examination under a light microscope.

2.11. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analyses were carried out using statistical package for social sciences (SPSS) version 22. Statistical significance was determined by one-way analysis of variance followed by the Least

Significant Difference test after comparing each treatment group and ADM. Statistical significance was defined as # $p<0.05$ (vs. healthy control), * $p < 0.05$, ** $p < 0.01$, and † $p < 0.001$ (vs. ADM).

3. RESULTS

3.1. Ambrette mitigates biochemical parameters of ADM mediated kidney injury in rats

The BUN (46%), SCr (39%), STP (56%), SAlb (36%), β_2-MG (16%), Cys C (56%) and UTP (64%) levels were significantly higher in ADM mediated control group rats, compared to the healthy control group, reflecting significant kidne y injury (Table 1) ($p<0.05$). Treatment with the selected extracts of Ambrette at the equivalent human therapeutic doses (A-HE; 55 mg/kg, A-EE; 75 mg/kg, A-BE; 60 mg/kg, A-WE; 140 mg/kg) exerted significant nephroprotective effects as demonstrated by changes in biochemical parameters of kidney function ($p<0.05$). Interestingly, the experimental rats administered A-EE showed better nephroprotection compared to the other three extracts ($p>0.05$) as indicated by normalisation of BUN (47%), β_2-MG (16%), and Cys C (63%) compared to the ADM group. Moreover, both A-HE and A-EE showed better nephroprotection over the drug reference standard, fosinopril considering the concentration of BUN, β_2-MG, and Cys C, however, the differences were not statistically significant ($p>0.05$). In fact, the experimental rats administered fosinopril showed a better reduction in proteinuria compared to the experimental rats administered Ambrette.

3.2. Ambrette mitigates ADM mediated oxidative stress in rats

A significant reduction in antioxidant potential was observed in ADR-mediated control rats, as reflected in the findings of TAS (86%), GR activity (47%), GPx (81%), and lipid peroxidation (38%) in kidney homogenates ($p<0.05$). The treatment with the selected extracts

of Ambrette was found to attenuate ADM-induced oxidative stress by restoration of antioxidant status as shown by increased values of TAS and the activities of GR and GPx in treated groups (Table 1). The selected extracts were more effective in increasing the activity of antioxidant enzymes compared to the drug reference standard, fosinopril. The experimental rats administered with A-HE showed a statistically significant increase in the GR activity (36%) compared to the rats administered with fosinopril ($p<0.05$).

Interestingly, a significant correlation was observed between MDA formation and all other selected parameters of oxidative stress (TAS ($r= -0.612$, $p=0.00$), GR ($r= -0.622$, $p=0.001$), and GPx ($r= -0.437$, $p=0.033$)) was noted in kidney homogenates. The levels of MDA formation in liver homogenates further corroborate the antioxidant potential of the selected medicinal plant extracts showing significant attenuation in lipid peroxidation in groups of experimental rats administered with the selected extracts of Ambrette ($p<0.05$).

3.3. Effect of Ambrette on inflammatory cytokines in kidney homogenates

The effect of Ambrette extracts on inflammatory cytokines in kidney homogenates is shown in Table 1. Administration of selected Ambrette extracts for 28 consecutive days resulted in suppression of elevations induced by ADM in TNF-α ($p<0.05$) and IL-1β ($p>0.05$). However, neither the experimental group of rats administered the plant extracts nor the drug reference standard showed statistically significant suppression of inflammatory cytokines compared to the control group. Interestingly, the experimental rats administered with A-WE showed better suppression in both TNF-α (41%) and IL-1β (13%) ($p>0.05$) compared to the other three extracts and the drug reference standard.

Table 1: Effect of Ambrette on kidney function tests, antioxidant and anti-inflammatory markers

Parameters	Treatment						
	Control	ADM	A-HE	A-EE	A-BE	A-WE	Fosinopril
BUN (mmol/L)	0.51±0.03†	0.93±0.07$^{\#}$	0.55±0.04†	0.50±0.04†	0.64±0.09†	0.54±0.02†	0.62±0.01†
SCr (µmol/L)	52.53±2.54†	86.20±6.41$^{\#}$	64.83±4.75**	61.17±6.76**	65.63±3.19**	67.51±2.30$^{\#*}$	67.51±2.30**
STP (g/L)	67.35±3.10†	43.07±2.64$^{\#}$	65.64±2.36†	56.03±1.44$^{\#**}$	66.42±1.18†	66.67±4.16†	59.33±2.73$^{\#\dagger}$
SAlb (g/L)	32.61±0.57†	23.96±1.08$^{\#}$	32.52±0.93†	29.70±2.14**	35.44±0.54†	30.46±0.69**	30.23±1.99**
UTP (g/dL)	69.28±11.78**	194.93±32.67$^{\#}$	171.91±45.27$^{\#}$	165.81±47.07$^{\#}$	83.09±15.21**	94.46±31.54*	77.78±1.75**
β_2-MG (µg/mL)	3.55±0.08**	4.25±0.24$^{\#}$	3.61±0.05**	3.58±0.03*	3.71±0.17*	3.86±0.36	3.63±0.07*
Cys C (ng/mL)	18.24±1.43†	41.87±9.13$^{\#}$	15.30±0.43†	15.35±0.30†	16.67±0.99†	16.55±0.66†	19.13±1.29†
TAS (mmol/L)	12.40±1.76†	6.65±0.37$^{\#}$	11.51±0.49†	11.87±0.42†	13.33±0.13†	12.27±0.63†	11.60±0.54†
GR (U/L)	23.30±3.63	15.88±1.46	31.17±2.14**	29.18±2.81**	26.57±5.68*	24.29±1.92	19.89±0.79
GPx (U/L)	163.12±16.38*	90.36±13.40$^{\#}$	210.11±46.08	133.10±20.04**	113.94±22.03	124.22±1.88	113.80±10.02
Lipid peroxidation	12.89±0.36†	20.80±1.03$^{\#}$	14.51±0.62†	14.68±1.17†	14.61±0.74†	16.22±0.59$^{\#\dagger}$	16.62±0.36$^{\#\dagger}$

in kidney (nmol MDA/g protein)							
Lipid peroxidation in liver (nmol MDA/g protein)	16.45±0.73†	23.89±1.45$^{\#}$	18.40±1.12**	20.19±1.54*	18.94±0.41**	20.31±0.78*	19.61±1.01**
TNF-α (pg/ mL)	863.18±76.60†	1410.96±80.80$^{\#}$	931.36±113.41**	905.38±92.60†	894.34±85.93†	836.37±59.56†	904.64±67.41†
IL-1β (pg/ mL)	7456.23± 663.38	12431.82± 4213.39	11718.94± 4435.95	11700.90± 4157.11	12391.45± 3622.38	10762.36± 4914.40	11301.65± 3033.07

Results of the biochemical parameters after 28 days of treatment of the plant extracts are expressed as means ± standard error of the mean (SEM). Statistical significance was defined as # $p<0.05$ (vs. Control), * $p < 0.05$, ** $p < 0.01$, and † $p < 0.001$ (vs. ADM). Control: Healthy Wistar rats, ADM: Adriamycin administered (5 mg/kg, ip) control rats, A-HE: ADM mediated kidney injury rats administrated with hexane extract (55 mg/kg), A-EE: ADM mediated kidney injury rats administrated with ethyl acetate extract (75 mg/kg), A-BE: ADM mediated kidney injury rats administrated with butanol extract (60 mg/kg), A-WE: ADM mediated kidney injury rats administrated with aqueous extract (140 mg/kg), Fosinopril: ADM mediated kidney injury rats administrated with fosinopril (0.09 mg/kg), BUN: blood urea nitrogen, SCr: serum creatinine, STP: serum total protein, SAlb: serum albumin, UTP: urine total protein, β_2-MG: β_2-microglobulin, Cys C: cystatin C, TAS: total antioxidant status, GR: glutathione reductase, GPx: glutathione peroxidase, MDA: malondialdehyde, TNF-α: tumor necrosis factor alpha, IL-1β: interleukin-1 beta.

3.4. Effect of Ambrette on kidney histopathology

Morphometric examination of H&E stained kidney sections showed sub-lethal changes in kidney injury including tubular cell vacuolisation, nuclear pyknosis, loss of the tubular brush border, and sloughing off necrotic cells from renal tubules. Glomerular congestion, haemorrhage, and the presence of tubular casts were the additional striking features observed. Neither tubulointerstitial damage, glomerulosclerosis, inflammatory infiltrations, fibrosis, which signifies chronic kidney injury, nor a full-blown picture of acute tubular necrosis was observed in experimental rats in the ADM group. The morphological findings were further supported by the total kidney injury score (35%) of the particular group.

The administration of the selected extracts of Ambrette for a period of 28 days significantly attenuated ADM-induced tubular and glomerular changes in kidney tissues as observed in a reduction of total kidney injury score ($p<0.05$). Experimental rats administered A-HE, A-EE and A-WE showed a better reduction in total kidney injury score compared to the drug reference standard (15%); however, the differences were not statistically significant ($p>0.05$). The effect of Ambrette on kidney morphology and total kidney injury score is shown in Figure 2(A) and Figure 2 (B), respectively.

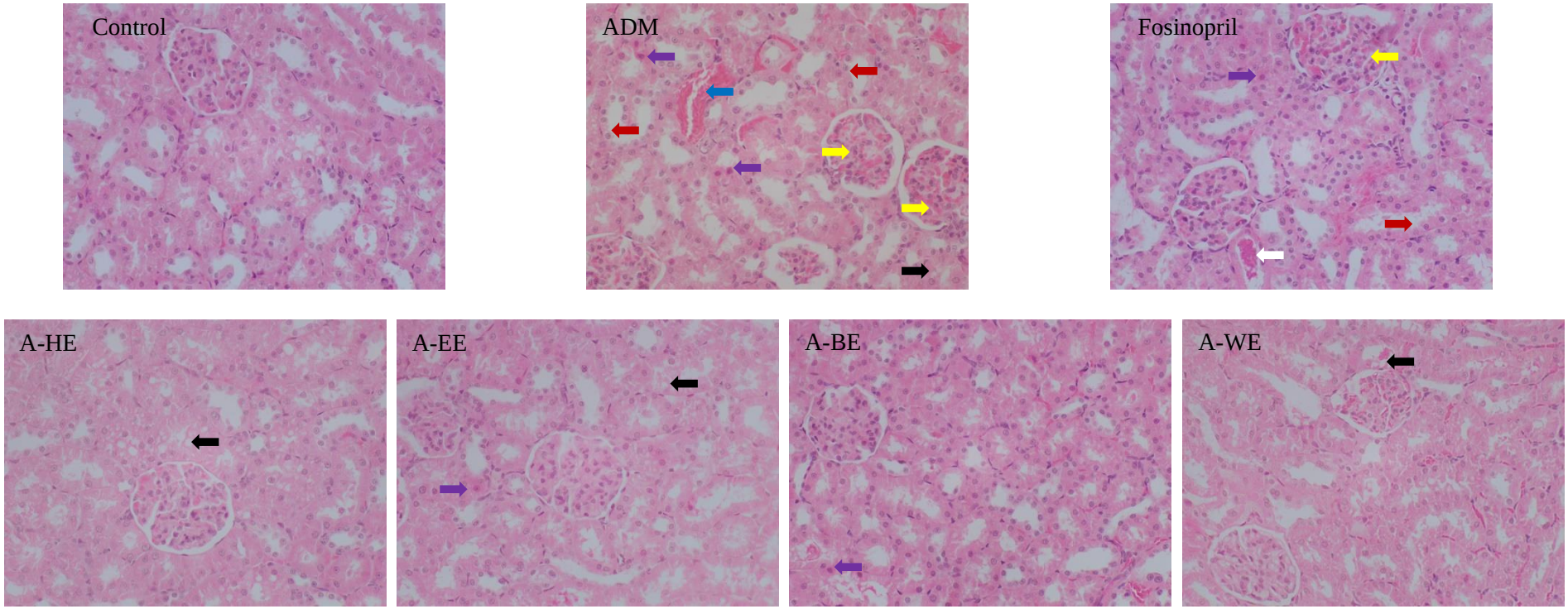


Figure 2 (A): Effects of selected leaf extracts of Ambrette on kidney morphology in H&E stained kidney sections of ADM mediated kidney injury rats (x400 magnification).

Nuclear pyknosis (purple arrow), loss of brush border (red arrow), cytoplasmic vacuolization (black arrow), presence of tubular casts (white arrow), intertubular haemorrhage (blue arrow) and glomerular congestion (yellow arrow). Control: Healthy Wistar rats, ADM: Adriamycin administered (5 mg/kg, ip) control rats, A-HE: ADM mediated kidney injury rats administrated with hexane extract (55 mg/kg), A-EE: ADM mediated kidney injury rats administrated with ethyl acetate extract (75 mg/kg), A-BE: ADM mediated kidney injury rats administrated with butanol extract (60 mg/kg), A-WE: ADM mediated kidney injury rats administrated with aqueous extract (140 mg/kg), and Fosinopril: ADM mediated kidney injury rats administrated with fosinopril (0.09 mg/kg).

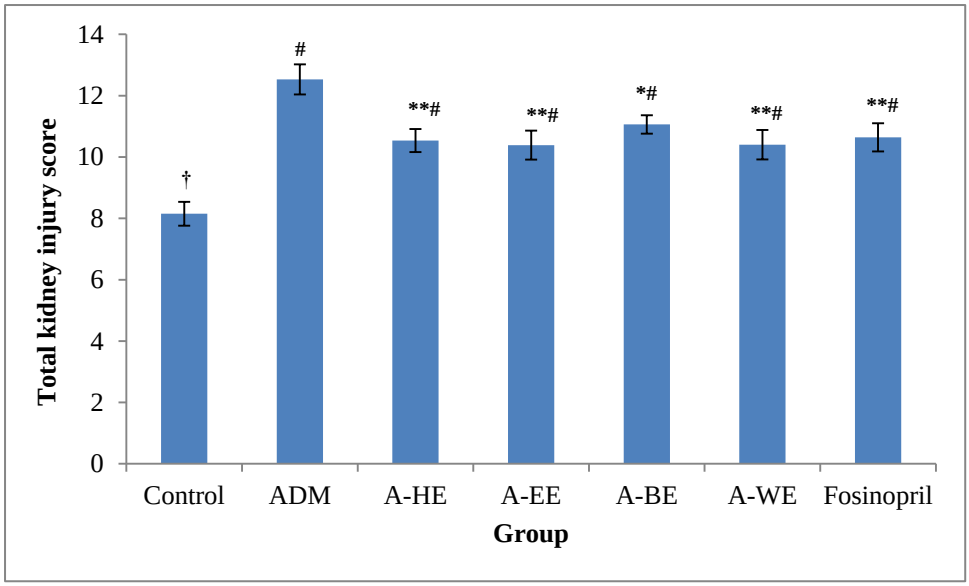


Figure 2 (B): Effects of selected leaf extracts of Ambrette on the total kidney injury score in H&E stained kidney sections of ADM induced kidney injury (x400 magnification).

Results of total kidney injury score are expressed as means ± standard error of the mean (SEM). Statistical significance was defined as # $p<0.05$ (vs. Control), * $p < 0.05$, ** $p < 0.01$, and † $p < 0.001$ (vs. ADM). Control: Healthy Wistar rats, ADM: Adriamycin administered (5 mg/kg, ip) control rats, A-HE: ADM mediated kidney injury rats administrated with hexane extract (55 mg/kg), A-EE: ADM mediated kidney injury rats administrated with ethyl acetate extract (75 mg/kg), A-BE: ADM mediated kidney injury rats administrated with butanol extract (60 mg/kg), A-WE: ADM mediated kidney injury rats administrated with aqueous extract (140 mg/kg), and Fosinopril: ADM mediated kidney injury rats administrated with fosinopril (0.09 mg/kg).

3.5. Effect of Ambrette on the immunohistochemical expression of COX-2, Bax and BCL-2

The anti-inflammatory effect of the plant extracts was further evaluated by assessing the immunohistochemical expression of the inflammatory marker COX-2 (Figure 3A). The highest expression of COX-2 with intense brown staining was observed in experimental rats from the ADM group, whereas the healthy control group showed minimal immunostaining. An attenuation of COX-2 expression was observed in experimental rats administered Ambrette extracts selected to varying degrees, exhibited by a reduction in the intensity of

brown staining. However, the experimental rats treated with A-BE and A-WE showed better attenuation in COX-2 expression compared to the other two treated rats.

As shown in Figure 3B, the immunohistochemical expression of the pro-apoptotic protein, Bax, was relatively high and mainly cytoplasmic in the experimental rats in the ADM group. Reduced expression of the Bax protein was observed in experimental rats of the control group and staining was visible mainly on the luminal surface of tubular epithelial cells. On the contrary, the immunohistochemical expression of the anti-apoptotic protein, BCL-2 (Figure 3C) was comparatively high and of uniform intensity in the tubular epithelial cells of healthy control rats compared to ADM mediated control rats. Focal positivity of BCL-2 staining was observed in experimental rats of the ADM mediated kidney injury control group. Enhanced BCL-2 staining and reduced Bax staining were observed in the experimental rat groups administered Ambrette extracts compared to the ADM group.

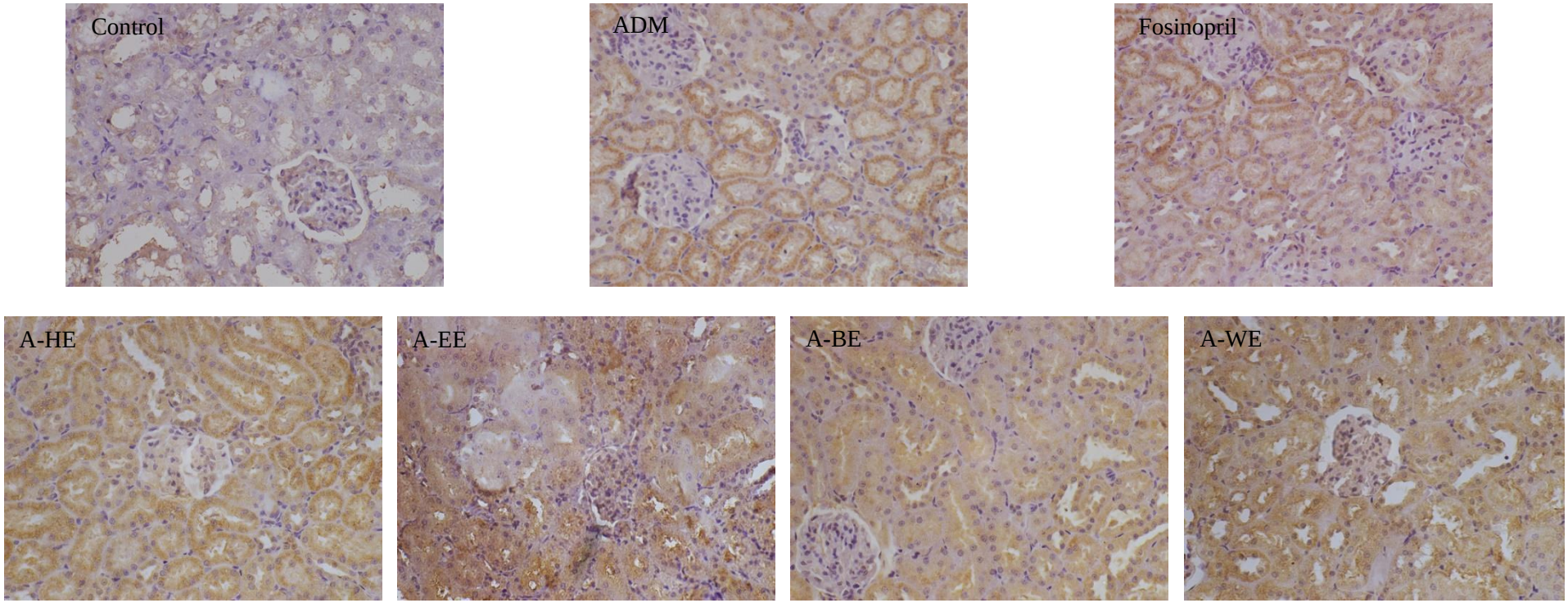


Figure 3(A). Effects of selected leaf extracts of Ambrette on the immunohistochemical expression of cyclooxygenase 2 (COX-2) on ADM mediated acute kidney injury (x400 magnification)

The highest expression of COX-2 with intense brown staining was observed in experimental rats of ADM group whereas, the control group showed minimal immunostaining. An attenuation of the expression of COX-2 was observed in experimental rats administered with the selected extracts of Ambrette signifying potential anti-inflammatory effects. Control: Healthy Wistar rats, ADM: Adriamycin administered (5 mg/kg, ip) control rats, A-HE: ADM mediated kidney injury rats administrated with hexane extract (55 mg/kg), A-EE: ADM mediated kidney injury rats administrated with ethyl acetate extract (75 mg/kg), A-BE: ADM mediated kidney injury rats administrated with butanol extract (60 mg/kg), A-WE: ADM mediated kidney injury rats administrated with aqueous extract (140 mg/kg), and Fosinopril: ADM mediated kidney injury rats administrated with fosinopril (0.09 mg/kg).

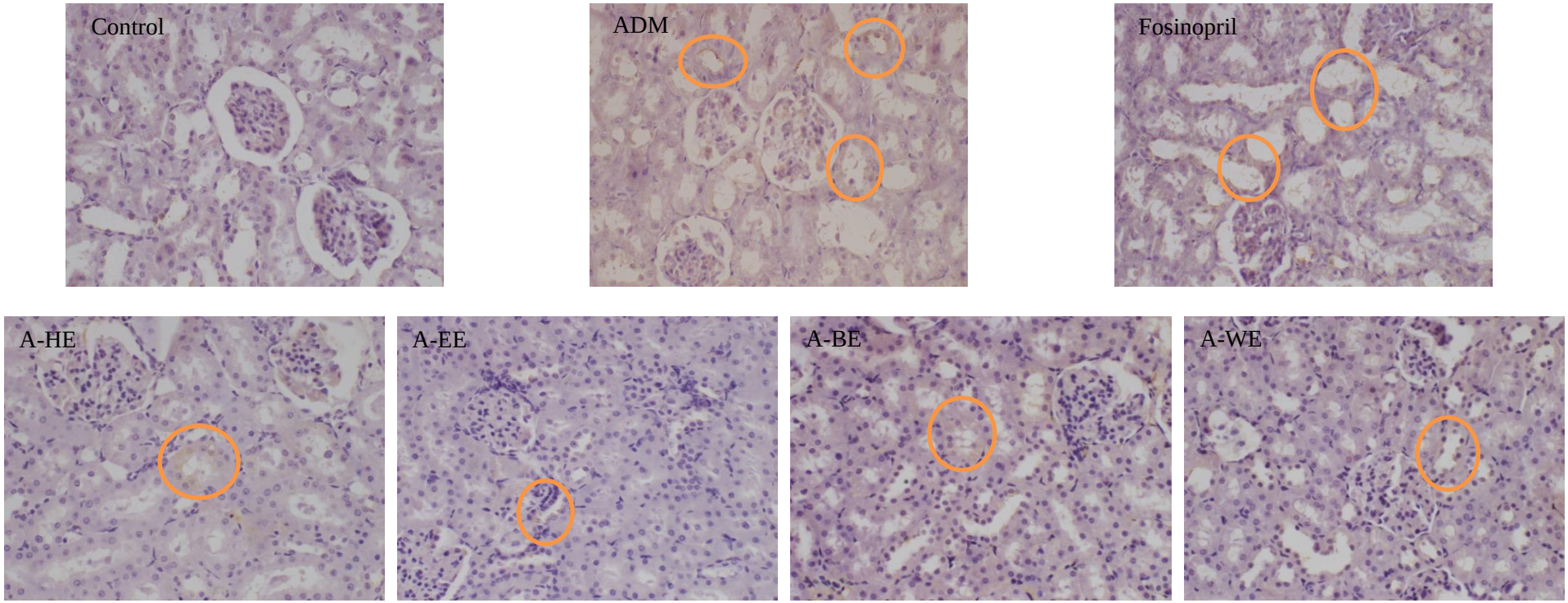


Figure 3(B). Effects of selected leaf extracts of Ambrette on the immunohistochemical expression of B-cell associated X protein (Bax) on ADM mediated acute kidney injury (x400 magnification).

The highest expression of Bax was observed in experimental rats of ADM administered control group whereas, the control group showed minimal immunostaining. An attenuation of the expression of Bax was observed in experimental rats administered with the selected extracts of Ambrette signifying potential anti-apoptotic effects. The tissue areas with positive cytoplasmic staining are circled in the photomicrographs. Control: Healthy Wistar rats, ADM: Adriamycin administered (5 mg/kg, ip) control rats, A-HE: ADM mediated kidney injury rats administrated with hexane extract (55 mg/kg), A-EE: ADM mediated kidney injury rats administrated with ethyl acetate extract (75 mg/kg), A-BE: ADM mediated kidney injury rats administrated with butanol extract (60 mg/kg), A-WE: ADM mediated kidney injury rats administrated with aqueous extract (140 mg/kg), and Fosinopril: ADM mediated kidney injury rats administrated with fosinopril (0.09 mg/kg).

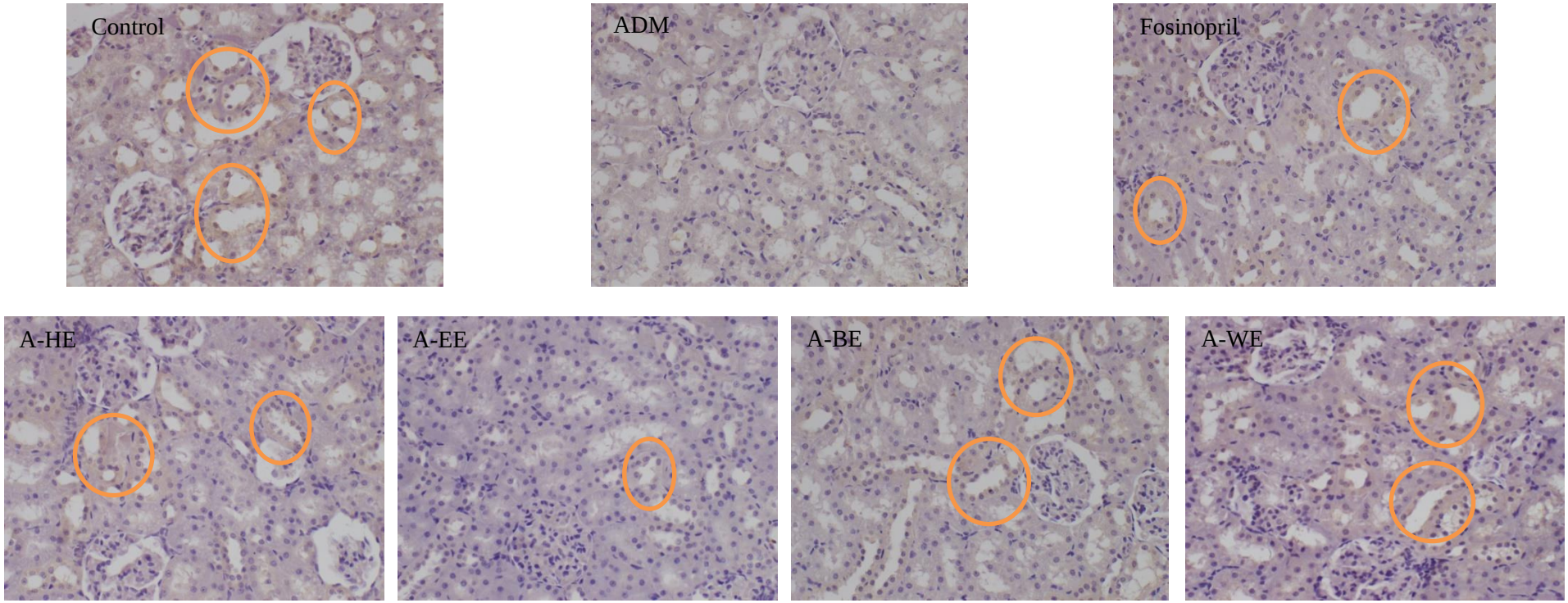


Figure 3(C). Effects of selected leaf extracts of Ambrette on the immunohistochemical expression of B-cell lymphoma gene product 2 (BCL-2) on ADM mediated acute kidney injury (x400 magnification).

The highest expression of BCL-2 with intense brown staining was observed in experimental rats of control group whereas, the ADM administered control group showed minimal immunostaining. An increased expression of BCL-2 was observed in experimental rats administered with the selected extracts of Ambrette compared to the ADM group signifying potential anti apoptotic effects. The tissue areas of positive cytoplasmic staining are circled in photomicrographs. Control: Healthy Wistar rats, ADM: Adriamycin administered (5 mg/kg, ip) control rats, A-HE: ADM mediated kidney injury rats administrated with hexane extract (55 mg/kg), A-EE: ADM mediated kidney injury rats administrated with ethyl acetate extract (75 mg/kg), A-BE: ADM mediated kidney injury rats administrated with butanol extract (60 mg/kg), A-WE: ADM mediated kidney injury rats administrated with aqueous extract (140 mg/kg), and Fosinopril: ADM mediated kidney injury rats administrated with fosinopril (0.09 mg/kg).

4. DISCUSSION

ADM mediated acute kidney injury model is widely used to elucidate nephroprotective principles of herbal medicines that have been used in clinical practice (El-Shitany et al., 2008; Malarkodi et al., 2003; Ouedraogo et al., 2012; Taskin et al., 2014). ADM is a common chemotherapeutic agent used in the treatment of a variety of solid tumors in humans and haematological malignancies for more than 30 years (El-Sheikh et al., 2012; Refaie et al., 2016; Benzer et al., 2018; Heravi et al., 2018). Despite its extensive and comprehensive investigations, the potential mechanisms of the onset of kidney injury by ADM are not fully understood in detail. Interestingly, numerous scientific reports suggest the involvement of oxidative stress, inflammation, and apoptosis in ADM-mediated kidney injury (Supplementary file 4) (Granados-Principal et al., 2010; Lahoti et al., 2012). Therefore, in the present study, the potential nephroprotective mechanisms of Ambrette were evaluated in terms of antioxidant, anti-inflammatory, and anti-apoptotic pathways.

Fosinopril, an angiotensin-converting enzyme inhibitor that is widely used in the treatment of chronic kidney disease (Navis et al., 1996), was used as the drug reference standard in the present study. Fosinopril (0.09 mg/kg) was used in previous studies of evaluating nephroprotective effects in experimental models of nephrotoxicity (Qi et al., 2012). Although angiotensin-converting enzyme inhibitors have not shown a direct advantage in the treatment of acute kidney injury, they have been shown to preserve kidney function in the nephropathy associated with diabetes mellitus, and improve prognosis in chronic kidney disease with significant proteinuria. The effect on glomerular perfusion in terms of reduction of hyperfiltration is the mechanism by which these drugs decelerate the advancement of kidney damage (Jones and Tomson, 2018). Given the importance of the potential nephroprotective effects of the drug as mentioned above and considering the fact that the ADM-mediated

kidney injury is upsurged by the renin-angiotensin system, fosinopril was used as the drug reference standard in the present study.

The solvents that were used in the preparation of extracts significantly affect the biological activities of herbal preparations. The number and quantity of bioactive compounds extracted by different solvents vary based on the differences in the polarity of extraction solvents (Truong et al., 2019). In the present study, the hexane, ethyl acetate, butanol, and aqueous leaf extracts of Ambrette were used at the adjusted equivalent human therapeutic dose in rats, for detailed investigations on nephroprotective mechanisms of ADM mediated kidney injury model in Wistar rats. Repeated dose oral toxicity assessment of the same plant extracts at the same selected doses were published previously by our group (Amarasiri et al., 2020 b), and the results revealed neither mortality nor treatment-related toxic effects in healthy Wistar rats, following repeated dose oral administration of the selected leaf extracts. The findings of biochemical parameters of kidney and liver functions (SCr, BUN, STP, serum alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, and alkaline phosphatase), full blood count parameters, and the relative weight of vital organs were found within the normal physiological range for the species. Further, repeated oral administration of the selected leaf extracts did not cause significant adverse effects on the histomorphology of vital organs, including kidney tissues which revealed normal morphological architecture with no signs of necrosis, inflammatory infiltration, tubular atrophy, or glomerular congestion (Amarasiri et al., 2020 b). Hence, the mechanistic study was preceded with the same extracts at the same selected doses.

Once ADM is cleared from plasma after administration, it is deposited in tissues, particularly in kidneys, and slowly excreted into urine and bile (Lee and Harris, 2011). Therefore, treatment regimens were initiated 24 hours after ADM administration considering the terminal half-life of the drug (20-48 hours), slow rate of drug absorption when administered

intraperitoneally, and based on the experimental protocols followed in previous studies (Oz and Ilhan, 2006; Gokcimen et al., 2007; Turner et al., 2011; Package insert; Doxutec, United Biotech, India). The existence of kidney injury in ADM-induced rats was confirmed by estimating urine total protein concentration one week after induction of nephrotoxicity by using ADM. This was further supported by the findings of published reports which showed a significant increase in urine total protein one week after administration of similar doses of ADM (Ding et al., 2014; Fan et al., 2015). Commencement of the treatments one week after induction of nephrotoxicity was not successful, due to further lengthening of the study duration, and the experienced mortality of experimental rats after one month period. Therefore, treatments were initiated 24 hours after induction of nephrotoxicity, without conducting a biochemical assessment of the kidney injury, which could be considered as a limitation of the present study.

Short-term studies are inadequate to predict the potential protective mechanisms of a therapeutic agent and lack the key parameters required to conduct clinical trials. Further, short-term supplementation of a therapeutic agent may not necessarily reflect the effect of the long-term, low-level consumption of herbal therapeutics in folk medicine. Therefore, the supplementation of plant extracts was continued for 28 consecutive days in the present study and apparently is in line with the OECD guidelines on sub-acute studies (OECD, 2008). The same experimental duration was found in other published reports on the evaluation of the nephroprotective potential of herbal extracts against ADM-induced kidney injury (Rajasekaran, 2019, Mohan et al., 2010).

Present findings on biochemical parameters of kidney function in ADM induced group of rats revealed the potential kidney injury caused by ADM. The significant elevation in SCr in experimental rats of the ADM group reflects substantial damage to the nephrons (Khan et al., 2009; Khan et al., 2010). In fact, the findings on concentrations of BUN, β_2-MG, and Cys C

further substantiate the kidney injury caused by ADM. Increased proteinuria signifies podocyte damage and subsequent reduction in STP and SAlb further corroborates the substantial kidney injury induced by ADM. However, administration of the selected extracts of Ambrette resulted in significant attenuation of biochemical changes induced by ADM indicating potential remission of kidney injury. The superior diminution of proteinuria by the drug reference standard, fosinopril, could be due to its mechanism of action by blocking the renin-angiotensin system (Taskin et al., 2014).

The findings on morphometric evaluation of the H&E stained kidney sections further substantiate the kidney injury caused by ADM. However, the absence of the morphological features of tubulointerstitial damage, glomerulosclerosis, fibrosis, and inflammatory infiltrations excludes the potential induction of chronic kidney injury by ADM. In fact, the presence of sub-lethal changes of kidney injury by ADM implies a state of acute kidney injury in experimental animals.

Oxidative stress plays an important role in ADM-induced kidney injury (Granados-Principal, et al., 2010). Significant attenuation of TAS, reduction in antioxidant enzyme activity, and elevation of MDA formation in kidney homogenates of rats administered ADM demonstrated oxidative stress caused by ADM. Administration of selected extracts of Ambrette resulted in a significant increment in the antioxidant capacity as reflected by increased levels of TAS, GR, GPx, and decreased levels of MDA. The findings on phytochemical analysis of each extract were reported in our previous publication (Amarasiri et al., 2020 b), and the potent antioxidant effects demonstrated by the selected Ambrette extracts can be attributed to the antioxidative phytochemicals identified such as phenolic compounds, flavonoids, tannins, saponins, steroid glycosides, and terpenoids (Amarasiri, et al., 2020 b).

The liver is considered the most frequently targeted organ in drug-induced toxicity. Apparently, it is an organ attacked by reactive oxygen species. Indeed, systemic oxidative stress that arises from the liver is reported to cause damage in kidney tissues, resulting in kidney failure (Li et al., 2015). Hence, in the present study, lipid peroxidation in the liver tissues was assessed via the formation of MDA in liver homogenates. The administration of selected plant extracts was able to restore membrane integrity in liver tissues by attenuating the increased levels of lipid peroxidation. Accordingly, the MDA formation varied in the same order as A-HE>A-BE>A-EE>A-WE in both kidney and liver homogenates of ADM mediated rats treated with the selected medicinal plant.

Administration of A-HE resulted in the highest improvement in kidney function parameters in serum, the activity of antioxidant enzymes in kidney homogenates, and the level of lipid peroxidation in both kidneys and in liver homogenates. The variation in the extraction of phytochemicals by different solvents with different polarities might lead to these discrepancies in the results of antioxidant activity in different extracts (Thavamoney et al., 2018). Hexane extract results in the extraction of less polar secondary metabolites such as less polar flavonoids, terpenoids, lignin, aglycon, sterol, etc. whereas subsequent extraction with ethyl acetate results in the extraction of flavonoids, glycoside compounds, and other medium polar constituents such as alkaloids and sterols (Widyawati et al., 2014; Sharma et al., 2015).

ADM is reported to activate NF-κB pathway, which is associated with the stimulation of the activity of some inflammatory enzymes and cytokines (Heravi et al., 2018). An increased expression of the inflammatory enzyme COX-2 and overproduction of inflammatory cytokines, TNF-α and IL-1β was observed in ADM-induced rats. The suppressed levels of the inflammatory cytokines in kidney homogenates and the decreased expression of COX-2 in immunostained kidney sections of the experimental rats administered with Ambrette revealed

the potent anti-inflammatory effects of the plant. These findings are consistent with the study by Dwivedi et al. (2017) which showed a significant reduction in carrageenan-induced paw oedema after treatment with Ambrette extracts.

The potential effect of Ambrette on ADM-induced cellular apoptosis in renal tubular epithelial cells was evaluated by assessing immunohistochemical expression of the pro-apoptotic marker Bax and the anti-apoptotic marker BCL-2 in the present study. The increased expression of BCL-2 and the decreased expression of Bax compared to the ADM group revealed potential anti-apoptotic effects of the selected Ambrette extracts.

Although there is limited evidence on the potential bioactivities of the phytoconstituents isolated from Ambrette, several studies have shown the therapeutic value of the common phytoconstituents found in the plant. Farnesol is one of those sesquiterpene alcohols found in many plants, including Ambrette, which is reported to alleviate 1,2-dimethylhydrazine-induced colonic mucosal damage by ameliorating oxidative stress, inflammation, and apoptosis (Khan and Sultana, 2011; Ku and Lin, 2015). Furthermore, myricetin, ambrettolide, and β sitosterol have been identified as active ingredients with potential immune and anti-inflammatory effects in a study of the Shufeng Jiedu capsule, in traditional Chinese medicine (Tao et al., 2020). Interestingly, in a study by Rout et al. (2002), both farnesol ((2Z,6E)-farnesyl acetate and (2E,6E)-farnesyl acetate) and ambrettolide ((Z)-7-hexadecen-16-olide) have been isolated from hexane extraction followed by steam distillation of Ambrette seeds, further corroborating the present findings on efficacy of the hexane extract of Ambrette.

5. CONCLUSIONS

The present findings revealed for the first time that Ambrette leaves in A-HE, A-EE, A-BE, and A-WE exert promising protective effects against ADM-induced acute kidney injury. The biochemical, histological, and immunohistochemical findings on oxidative markers,

inflammatory cytokines, and apoptotic markers revealed that the potential nephroprotective mechanisms of the selected leaf extracts of Ambrette could be due to the antioxidant, anti-inflammatory, and anti-apoptosis effects *in vivo*. The A-HE exerted superior nephroprotection over the other three extracts in ADM-induced kidney injury. The findings denote the potency of the development of new therapeutic drug leads using A-HE for the management of acute kidney injury. However, further studies are required to confirm the clinical efficacy of the selected extracts in patients with acute kidney injury.

Conflict of Interests

No conflict of interests was disclosed.

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List of abbreviations

ADM	Adriamycin
A-BE	Ambrette butanol extract
A-EE	Ambrette ethyl acetate extract
A-HE	Ambrette hexane extract
A-WE	Ambrette aqueous extract
BCL-2	B-cell lymphoma gene product 2
Bax	B-cell associated X protein
BUN	blood urea nitrogen
β_2-MG	β_2-Microglobulin
COX-2	Cyclooxygenase 2
Cys C	Cystatin C
GPx	Glutathione peroxidase
GR	Glutathione reductase
IL-1β	Interleukin-1β
MDA	Malondialdehyde
SCr	Serum creatinine
SAlb	Serum albumin
STP	Serum total protein
TAS	Total antioxidant status
TNF-α	Tumor necrosis factor-α
UTP	Urine total protein

Figure captions

Figure 1. Schematic diagram of the experimental design

Figure 2 (A). Effects of selected leaf extracts of Ambrette on kidney morphology in H&E stained kidney sections of ADM mediated kidney injury rats (x400 magnification).

Nuclear pyknosis (purple arrow), loss of brush border (red arrow), cytoplasmic vacuolization (black arrow), presence of tubular casts (white arrow), intertubular haemorrhage (blue arrow) and glomerular congestion (yellow arrow). Control: Heathy Wistar rats, ADM: Adriamycin administered (5 mg/kg, ip) control rats, A-HE: ADM mediated kidney injury rats administrated with hexane extract (55 mg/kg), A-EE: ADM mediated kidney injury rats administrated with ethyl acetate extract (75 mg/kg), A-BE: ADM mediated kidney injury rats administrated with butanol extract (60 mg/kg), A-WE: ADM mediated kidney injury rats administrated with aqueous extract (140 mg/kg), and Fosinopril: ADM mediated kidney injury rats administrated with fosinopril (0.09 mg/kg).

Figure 2 (B). Effects of selected leaf extracts of Ambrette on the total kidney injury score in H&E stained kidney sections of ADM induced kidney injury (x400 magnification).

Results of total kidney injury score are expressed as means ± standard error of the mean (SEM). Statistical significance was defined as # $p<0.05$ (vs. Control), * $p < 0.05$, ** $p < 0.01$, and † $p < 0.001$ (vs. ADM). Control: Heathy Wistar rats, ADM: Adriamycin administered (5 mg/kg, ip) control rats, A-HE: ADM mediated kidney injury rats administrated with hexane

extract (55 mg/kg), A-EE: ADM mediated kidney injury rats administrated with ethyl acetate extract (75 mg/kg), A-BE: ADM mediated kidney injury rats administrated with butanol extract (60 mg/kg), A-WE: ADM mediated kidney injury rats administrated with aqueous extract (140 mg/kg), and Fosinopril: ADM mediated kidney injury rats administrated with fosinopril (0.09 mg/kg).

Figure 3 (A). Effects of selected leaf extracts of Ambrette on the immunohistochemical expression of cyclooxygenase 2 (COX-2) on ADM mediated acute kidney injury (x400 magnification)

The highest expression of COX-2 with intense brown staining was observed in experimental rats of ADM group whereas, the control group showed minimal immunostaining. An attenuation of the expression of COX-2 was observed in experimental rats administered with the selected extracts of Ambrette signifying potential anti-inflammatory effects. Control: Heathy Wistar rats, ADM: Adriamycin administered (5 mg/kg, ip) control rats, A-HE: ADM mediated kidney injury rats administrated with hexane extract (55 mg/kg), A-EE: ADM mediated kidney injury rats administrated with ethyl acetate extract (75 mg/kg), A-BE: ADM mediated kidney injury rats administrated with butanol extract (60 mg/kg), A-WE: ADM mediated kidney injury rats administrated with aqueous extract (140 mg/kg), and Fosinopril: ADM mediated kidney injury rats administrated with fosinopril (0.09 mg/kg).

Figure 3 (B). Effects of selected leaf extracts of Ambrette on the immunohistochemical expression of B-cell associated X protein (Bax) on ADM mediated acute kidney injury (x400 magnification).

The highest expression of Bax was observed in experimental rats of ADM group whereas, the control group showed minimal immunostaining. An attenuation of the expression of Bax was observed in experimental rats administered with the selected extracts of Ambrette signifying potential anti apoptotic effects. The tissue areas with positive cytoplasmic staining are circled in the photomicrographs. Control: Heathy Wistar rats, ADM: Adriamycin administered (5 mg/kg, ip) control rats, A-HE: ADM mediated kidney injury rats administrated with hexane extract (55 mg/kg), A-EE: ADM mediated kidney injury rats administrated with ethyl acetate extract (75 mg/kg), A-BE: ADM mediated kidney injury rats administrated with butanol extract (60 mg/kg), A-WE: ADM mediated kidney injury rats administrated with aqueous extract (140 mg/kg), and Fosinopril: ADM mediated kidney injury rats administrated with fosinopril (0.09 mg/kg).

Figure 3 (C). Effects of selected leaf extracts of Ambrette on the immunohistochemical expression of B-cell lymphoma gene product 2 (BCL-2) on ADM mediated acute kidney injury (x400 magnification).

The highest expression of BCL-2 with intense brown staining was observed in experimental rats of control group whereas, the ADM group showed minimal immunostaining. An increased expression of BCL-2 was observed in experimental rats administered with the selected extracts of Ambrette compared to the ADM group signifying potential anti apoptotic effects. The tissue areas of positive cytoplasmic staining are circled in photomicrographs. Control: Heathy Wistar rats, ADM: Adriamycin administered (5 mg/kg, ip) control rats, A-HE: ADM mediated kidney injury rats administrated with

hexane extract (55 mg/kg), A-EE: ADM mediated kidney injury rats administrated with ethyl acetate extract (75 mg/kg), A-BE: ADM mediated kidney injury rats administrated with butanol extract (60 mg/kg), A-WE: ADM mediated kidney injury rats administrated with aqueous extract (140 mg/kg), and Fosinopril: ADM mediated kidney injury rats administrated with fosinopril (0.09 mg/kg).