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Invited Oration

Unraveling the Mystery in Search of Nephroprotective Therapeutics from Sri Lankan Flora

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Introduction

Drug-induced nephrotoxicity has become a serious health problem worldwide due to the wide use of pharmaceutical agents with potential toxicities. Doxorubicin is one such anthracycline drug in which the use in cancer chemotherapy has been largely limited due to its dose-dependent organ toxicities including nephrotoxicity (Heravi et al., 2018). Doxorubicin-induced nephrotoxicity is well explained via the mechanisms of oxidative stress, inflammation, and apoptosis in previous reports (Ibrahim et al., 2020). Despite the fact that doxorubicin chemotherapy has been in practice for almost six decades and associated toxicities are well recognized, no specific antidote has been developed to eliminate its toxic effects. Although the antidote; dexrazoxane has been commonly used to reduce doxorubicin-induced cardiotoxicity, the use of the same drug in alleviating free radical pathologies related to nephrotoxicity is quite challenging due to marked heterogeneity in cell populations (Lahoti et al., 2012). These limitations of current therapeutic approaches led scientists to pay attention to alternative therapeutic strategies, particularly the conventional system of medicine, to prevent disease progression and improving kidney function in doxorubicin-induced nephrotoxicity.

The multifactorial nature of the effects of drug-induced nephrotoxicity often demands therapeutic approaches with multiple protective effects to alleviate associated risks (Sharma et al., 2020). In this context, polyherbal formulations offer an invaluable resource for the development of new pharmaceuticals due to their potential bio-interactions within and between the constituents (Caesar and Cech, 2019). On some occasions, the desired pharmacological effects of the medicinal herbs are generated only when they are potentiated by other plants, rather than using them alone (Sharma et al., 2020). Due to this comparable efficacy and the fewer side effects reported, a revival of interest is being observed worldwide for polyherbal formulations at present.

Hence, the present study is based on a polyherbal combination of Sri Lankan medicinal plants that will be safe and therapeutically beneficial as potential therapeutic agents in the management of kidney-related diseases, particularly for doxorubicin-induced nephrotoxicity. The polyherbal combination was derived based on initial investigations on *Abelmoschus moschatus* Medikus. (leaves), *Asparagus falcatus* L. (leaves), *Barleria prionitis* L. (whole plant), *Gmelina arborea* Roxb. (stem bark), and *Vetiveria zizanioides*

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(L.) Nash (root). These medicinal plants were initially selected with reference to pharmacopeias and other monographs related to traditional medicine (Ayurveda Pharmacopoeia, 1985; Amarasiri, et al., 2020a).

Aims and Objectives

The investigation was planned in three studies. The general objective of this presented work was to investigate the nephroprotective effects of some selected medicinal plant extracts and a new polyherbal formulation derived from selected medicinal plant extracts in Wistar rats with doxorubicin-induced nephrotoxicity

Study 1

To determine the efficacy of five selected plant extracts for nephroprotective activity in rats with doxorubicin-induced nephrotoxicity.

Study 2

To investigate acute and sub-chronic toxic effects and nephroprotective mechanisms of three selected standardized medicinal plant extracts (from the initial five) in rats with doxorubicin-induced nephrotoxicity.

Study 3

To investigate acute and sub-chronic toxic effects and nephroprotective mechanisms of a novel polyherbal formulation in rats with doxorubicin-induced nephrotoxicity.

Methods

Ethical clearance was obtained from Ethics Review Committee, Faculty of Medicine, University of Ruhuna (Ref. no. 14.12.2015:3.1) for the present study. *In vivo* experiments were carried out using Wistar albino rats (10-12 weeks, 175 ± 25 g) under standard laboratory conditions at the Animal House of the Department of Biochemistry, Faculty of Medicine, University of Ruhuna.

Study 1- Determination of the efficacy of selected plant extracts for nephroprotective activity in rats with doxorubicin-induced nephrotoxicity

Materials and methods

The botanical raw materials of five selected medicinal plants were collected from the natural habitats of the Western and Southern regions of Sri Lanka. The selected herbal materials were authenticated at the National Herbarium, Peradeniya. Oven-dried (40°C for 72 hours) and coarsely powdered plant materials (25 g) were extracted with distilled water (500 mL) by refluxing (4 hours) and then freeze-dried.

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***In vivo* study**

The efficacy of the aqueous extracts of the five selected medicinal plants was evaluated for nephroprotective activity at three selected doses *in vivo*. The equivalent human therapeutic dose in rats of each plant was calculated based on the recommended dose of raw herbs (12 g/day) in Ayurveda medicine for an adult human (60 kg body weight). Two additional doses of plant extracts were selected, including a higher and a lower dose than the selected human equivalent therapeutic dose in rats. Consequently, the doses selected for *A. moschatus*, *A. falcatus*, and *B. prionitis* were 200, 400, and 600 mg/kg. The doses selected for *G. arborea* and *V. zizanioides* were 100, 300, 500 mg/kg, and 25, 50, and 100 mg/kg respectively. The lyophilized powder of the plant extracts was dissolved in distilled water to prepare the selected doses for administration.

Potential nephroprotective effects were evaluated after repeated administration of the aqueous extracts of the five selected medicinal plants at the doses mentioned for three consecutive days in doxorubicin-induced (20 mg/kg, ip) Wistar rats (n= 6/group). Fosinopril sodium (0.09 mg/kg) was used as the standard drug. Blood, urine, and kidney tissues were collected for laboratory investigations.

***In vitro* study**

In vitro experiments were carried out for the three selected medicinal plants with better efficacy against doxorubicin-induced nephrotoxicity. Chemical standardization, quantification of total polyphenols and flavonoids, and determination of *in vitro* antioxidant potential were carried out using standard protocols.

Results

***In vivo* study**

Treatment with the selected doses of plant extracts resulted in dose-dependent reduction of serum concentrations of blood urea nitrogen (6-51%), creatinine (11-54%), β_2-microglobulin (17-72%), and cystatin C (6-51%) and urine total protein (8-80%), in groups of rats treated with plant extracts compared to the doxorubicin control group. However, *V. zizanioides* at the selected doses resulted in the least improvement of biochemical parameters in doxorubicin-induced nephrotoxicity showing no significant difference in the concentration of blood urea nitrogen and cystatin C ($p>0.05$). Further, 400, and 600 mg/kg doses of *A. moschatus*, *A. falcatus*, and *B. prionitis* showed similar or better improvement in all the biochemical parameters compared to the standard drug; fosinopril.

Histopathological findings corroborated the results of biochemical parameters. Concomitant administration of the plant extracts improved doxorubicin-induced tubular and glomerular alterations of acute kidney injury in a dose-dependent manner as indicated by the improved mean histological scores of the groups (7-36%). Treatment with equivalent therapeutic dose and the highest selected dose of *A. moschatus*, *A. falcatus*, *B.*

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prionitis and *V. zizanioides* showed more protective effects (≥20%) against doxorubicin-induced nephrotoxicity with reference to histopathological changes (Amarasiri et al., 2020b, Amarasiri, et al., 2020c, Amarasiri, et al., 2021).

The aqueous leaf extracts of *A. moschatus*, *A. falcatus*, and the whole plant extract of *B. prionitis* showed better performance than *G. arborea*, and *V. zizanioides* with respect to the investigated biochemical parameters of kidney function and histopathology. Therefore, *in vitro* studies were carried out on those three plants.

In vitro study

Qualitative analysis of phytochemicals of the standardized three selected medicinal plants revealed the presence of common phytoconstituents as tannins, phenolics, flavonoids, steroid glycosides, terpenoids and saponins. Alkaloids were absent in all three extracts and coumarins were present only in the leaf extract of *A. falcatus* (Amarasiri, et al., 2020b, Amarasiri, et al., 2020c, Amarasiri, et al., 2021).

The total polyphenol and flavonoid contents and the *in vitro* antioxidant activity of the three selected medicinal plants were varied according to the order of *B. prionitis*> *A. falcatus*>*A. moschatus* (Amarasiri, et al., 2020b).

Conclusions

The findings revealed that the aqueous leaf extracts of *A. moschatus*, *A. falcatus,* and the whole plant extract of *B. prionitis* possess significant dose-dependent nephroprotective activity against doxorubicin-induced acute nephrotoxicity, probably mediated through antioxidant properties.

Study 2- Investigation of 28-day repeated dose oral toxicity effects and nephroprotective mechanisms of the three selected medicinal plant extracts in rats with doxorubicin-induced nephrotoxicity.

Materials and methods

The oven-dried (40°C) powdered plant materials of the three selected medicinal plants were sequentially extracted with hexane, ethyl acetate, butanol, and water by the sequential soxhlet extraction method. The human therapeutic dose in rats was calculated for each plant extract based on the percentage yield as follows: *A. moschatus*; hexane (55 mg/kg), ethyl acetate (75 mg/kg), butanol (60 mg/kg), aqueous (140 mg/kg), *A. falcatus*; hexane (55 mg/kg), ethyl acetate (35 mg/kg), butanol (75 mg/kg), aqueous (200 mg/kg) and *B. prionitis* hexane (25 mg/kg), ethyl acetate (80 mg/kg), butanol (70 mg/kg), aqueous (120 mg/kg). The hexane and ethyl acetate extracts were dissolved in corn oil (vehicle 1) and the butanol extracts were dissolved in 3% polyvinylpyrrolidone (PVP) (vehicle 2). The aqueous extracts were dissolved in distilled water.

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Phase 1- Assessment of 28-day repeated dose oral toxicity effects of the selected plant extracts in Wistar albino rats

This phase was performed as per the OECD Guidelines (OECD, 2008). Hexane, ethyl acetate, butanol and aqueous extracts of the three selected medicinal plants were administered orally as a single dose to Wistar rats (n= 5/sex/group) for 28 consecutive days at the equivalent therapeutic dose. Following treatment regimens, blood was collected by cardiac puncture in anesthetized animals (using CO_2 inhalation) for hematological (full blood count) and biochemical analysis of renal and liver profiles. Vital organs (heart, small intestine, liver, spleen, and kidneys) were excised for the evaluation of histopathological changes on H and E stained sections.

Phase 2- Investigation of nephroprotective mechanisms of the selected plant extracts in Wistar rats with doxorubicin-induced nephrotoxicity

A total of 90 healthy male Wistar albino rats were used in the investigation. Nephrotoxicity was induced with a single intraperitoneal dose of doxorubicin (5 mg/kg). The nephrotoxic rats were administered with hexane, ethyl acetate butanol and aqueous extracts of *A. Moschatus, A. falcatus* and *B. prionitis,* and the standard drug; fosinopril (0.09 mg/kg) for 28 consecutive days orally as a daily single dose Urine and blood were collected for biochemical investigations on renal function parameters. The kidney tissues were excised for the semi-quantitative assessment of histopathology, immunohistochemistry studies (Cyclooxygenase 2; COX-2, B-cell lymphoma gene product 2; BCL-2 and B-cell associated X protein; Bax), and assessment of oxidative (total antioxidant status, glutathione reductase (EC 1.6.4.2) activity, glutathione peroxidase (EC 1.11.1.9) activity, lipid peroxidation) and inflammatory markers tumor necrosis factor; TNF-α and interleukin-1 beta; IL-1β) on kidney homogenates.

Results

Phase 1- Assessment of 28-day repeated dose oral toxicity

28-Day repeated dose oral administration of the selected plant extracts in healthy Wistar rats did not cause mortality or treatment-related toxic effects in Wistar rats. The findings of the biochemical parameters of kidney and liver functions, full blood count parameters, and the relative weight of vital organs were found within the normal physiological range of the species. Furthermore, no significant adverse effects on the histomorphology of vital organs were observed (Amarasiri et al., 2020d).

Phase 2- Investigation of nephroprotective mechanisms

Treatment with the selected plant extracts at the therapeutic dose for 28 consecutive days in doxorubicin-induced nephrotoxicity in Wistar rats caused significant nephroprotection, as evidenced by a significant reduction in serum creatinine (18-33%), blood urea nitrogen (22-43%), β_2-microglobulin (10-20%), cystatin C 16-59%) and urine total protein (49-79%) concentrations compared to the doxorubicin control group ($p<0.05$). Biochemical

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results were further corroborated by histopathological findings, which demonstrated significant reduction in mean histological scores compared to the doxorubicin control group. Sub-lethal changes including loss of tubular brush border, sloughing of necrotic cells from renal tubules, tubular vacuolization, and pyknosis were observed in renal tubular epithelial cells. Glomerular congestion, hemorrhage, and the presence of tubular casts derived from protein, tubular debris, and sloughed cells were the additional striking features observed.

The findings on oxidative markers revealed a significant increase in total antioxidant levels and the activity of antioxidant enzymes and a significant suppression of the lipid peroxidation after treatment with plant extracts. The anti-inflammatory potential varied in the order of *A. falcatus>B. prionitis>A. moschatus* considering the concentrations of the pro-inflammatory cytokines; TNF-α and IL-1β. A similar variation of findings could be observed in the immunohistochemical expression of COX-2 in the present study. The immunohistochemical expression of the pro-apoptotic protein, Bax was decreased and the expression of the anti-apoptotic protein, BCL-2, was increased in experimental rats of the plant treatment groups compared to the doxorubicin control group. These findings demonstrate promising anti-apoptotic properties of the selected plant extracts (Amarasiri, et al., 2022a; Amarasiri, et al., 2022b).

Conclusions

The hexane, ethyl acetate, butanol, and aqueous extracts of the three selected medicinal plants at the human equivalent therapeutic dose were found to be safe according to the OECD guidelines for chemical testing and efficacious *in vivo* against the doxorubicin-induced nephrotoxicity model in Wistar rats. A detailed investigation of the nephroprotective mechanisms revealed that the selected plant extracts counteract oxidative stress induced by doxorubicin via antioxidant, anti-inflammatory, and anti-apoptotic pathways. Yet, none of the plant extracts investigated showed significant nephroprotective effects compared to the other extracts of the same plant or different plants, with reference to the biomarkers assessed. Therefore, the aqueous extract of a mixture of similar weights of the three selected medicinal plants was used in the preparation of the polyherbal formulation.

Study 3- To investigate acute and sub-chronic toxic effects and nephroprotective mechanisms of a novel polyherbal formulation in rats with doxorubicin-induced nephrotoxicity.

Materials and methods

The oven-dried (40°C) powdered plant materials of *A. moschatus, A. falcatus* and *B. prionitis* were mixed in equal quantities (1:1:1 by weight) and the resulting polyherbal formulation (25 g) was extracted with distilled water (500 mL) by refluxing (4 hours). The concentrated extract was then subjected to freeze-drying to obtain the lyophilized powder.

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In vitro studies

In vitro studies were carried out for chemical standardization, determination of shelf-life, quantification of total polyphenols, flavonoids, determination of *in vitro* antioxidant potential, and proximate analysis of the nutrient composition of the polyherbal formulation.

In vivo studies

Phase 1- Assessment of 28-day repeated dose oral toxicity effects of the novel polyherbal formulation in Wistar albino rats

The phase was performed as per the OECD Guidelines (OECD, 2008). The aqueous extract of the polyherbal formulation was administered orally as a single daily dose to Wistar rats (n= 5/ sex/group) for 28 consecutive days at three selected doses; 200, 400 (equivalent therapeutic dose), and 600 mg/kg. Following treatment regimens, blood was collected by cardiac puncture in anesthetized animals for hematological and biochemical analysis of renal and liver profile. Vital organs (heart, small intestine, liver, spleen, and kidneys) were excised for the evaluation of histopathological changes on H and E stained sections.

Phase 2- Investigation of the nephroprotective mechanisms of the polyherbal formulation in Wistar rats with doxorubicin-induced nephrotoxicity

A total of 36 healthy male Wistar albino rats were used in the investigation. Nephrotoxicity was induced with a single intraperitoneal dose of doxorubicin (5 mg/kg). The nephrotoxic rats were administered with the aqueous extract of the polyherbal formulation at 200, 400 and 600 mg/kg doses, and the standard drug; fosinopril (0.09 mg/kg) for 28 consecutive days orally as a daily single dose. Urine and blood were collected for biochemical investigations on renal function parameters. The kidney tissues were excised for semi-quantitative assessment of histopathology, immunohistochemistry studies, and for the evaluation of oxidative and inflammatory markers on kidney homogenates.

Results

In vitro studies

No substantial change in the phytochemical composition, moisture content and microbial limits were observed in the standardized polyherbal formulation, at the beginning and end of the study period of 30 days, during determination of the shelf life. Analysis of the nutritional profile revealed relatively high energy content (274.81 kcal/g) as calculated from crude protein (15.03±1.70 %w/w), fat (0.05±0.00 %w/w), and carbohydrate (53.56±0.00 %w/w). Vitamin B_2 (1.3±0.00 mg/100 g), B_6 (1.8±0.00 mg/100 g) and E (0.64±0.00 mg/100g) were present in considerable amounts.

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In vivo studies

Phase 1- Assessment of 28-day repeated dose oral toxicity

The evaluation of the repeated dose oral toxicity of the polyherbal formulation at 28 days caused neither mortality nor treatment-related toxic effects in Wistar rats. The findings of the biochemical parameters of kidney and liver functions, the full blood count parameters, and the relative weight of vital organs were found within the normal physiological range of the species. Additionally, no significant adverse effects on the histomorphology of vital organs were observed.

Phase 2- Investigation of nephroprotective mechanisms

Treatment with the herbal composition for 28 days in nephrotoxic rats caused significant reduction in serum creatinine (24%, 30%, 30%), blood urea nitrogen (41%, 60%, 61%), β2-microglobulin (6%, 7%, 12%) and cystatin C (44%, 43%, 39%) concentrations compared to the doxorubicin control group ($p<0.05$). Interestingly, 400 and 600 mg/kg doses shared better nephroprotection than the standard drug, fosinopril. Histopathological findings demonstrated dose-related nephroprotective effects, with reference to the mean histological scores reported (21%, 27%, 30%). Moreover, the biochemical findings on markers of oxidative stress and inflammatory cytokines in kidney homogenates suggested potential antioxidant and anti-inflammatory effects of the herbal composition *in vivo*. The immunohistochemical findings on the inflammatory enzyme; COX-2, the antiapoptotic protein, BCL-2, and the pro-apoptotic protein; Bax, further demonstrated the anti-inflammatory and anti-apoptotic potential of the novel herbal composition which might lead to the nephroprotective effects.

Conclusions

The polyherbal formulation derived from *A. moschatus, A. falcatus,* and *B. prionitis* was found to be safe according to the OECD guidelines for the testing of drugs, and efficacious against doxorubicin-induced nephrotoxicity in Wistar albino rats. A detailed investigation of the cellular mechanisms revealed that the aqueous extract of the polyherbal formulation counteracts oxidative stress induced by doxorubicin through antioxidant, anti-inflammatory, and anti-apoptotic pathways. However, the general findings of the present study conclude that the polyherbal formulation made from *A. moschatus*, *A. falcatus,* and the whole plant of *B. prionitis* would be beneficial as a therapeutic agent/ potential nutraceutical for the management of drug-induced nephrotoxicity and other kidney-related diseases.

Outcomes

The results have been published in SCI/SCIE journals with high impact factor. A local patent has been gazetted for the proposed polyherbal formulation (Patent no: 21901). Commercilaization has been initiated through UBL, University of Ruhuna.

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