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Preclinical efficacy and cellular mechanisms of a polyherbal formulation in doxorubicin nephrotoxicity

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The study aimed to evaluate the efficacy and cellular mechanisms of a standardised polyherbal formulation (PHF) derived from *Asparagus falcatus* L., *Abelmoschus moschatus* Medik. and *Barleria prionitis* L. of Sri Lankan origin in an animal model of doxorubicin nephrotoxicity. *In vitro* studies were carried out for chemical standardisation and determination of shelf life. The efficacy and nephroprotective mechanisms of the standardised PHF were investigated after repeated oral administration of the aqueous PHF at low (200 mg/kg body weight), therapeutic (400 mg/kg body weight), and high (600 mg/kg body weight) doses in the doxorubicin-induced (5 mg/kg body weight) nephrotoxicity model in Wistar rats. Fosinopril (0.09 mg/kg body weight) was used as the standard drug. The PHF derived from the selected medicinal herbs showed satisfactory purity and quality. Treatment of the standardised PHF for 28 days in nephrotoxic rats caused a significant reduction in serum creatinine, blood urea nitrogen, β_2-microglobulin, cystatin C, and urine total protein compared to the doxorubicin model group ($p<0.05$). The biochemical findings on markers of oxidative stress, inflammatory cytokines, and immunohistochemical evaluation of COX-2, BCL-2, and Bax further demonstrated the anti-inflammatory and anti-apoptotic activities of the novel PHF. The findings revealed that PHF was efficacious at the selected doses and its' nephroprotective mechanisms were mediated by mitigating doxorubicin-induced oxidative stress, inflammation, and apoptosis in experimental rats.

Keywords: Chemical standardisation, Doxorubicin-induced nephrotoxicity, Efficacy, Polyherbal formulation, Shelf life

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Polyherbal strategy achieves additional therapeutic efficacy, especially in the occasion of multifaceted diseases, where a combination of herbs may provide desirable therapeutic effects with a simultaneous effect on multiple targets that cause significant relief to the patient. Therefore, a low dose of polyherbal preparations would be sufficient to achieve the desired therapeutic effect due to the presence of multiple active constituents. This may even reduce the risk of harmful side effects of herbal therapies as well. More importantly, treatment with polyherbal mixtures may be convenient for patients, as these eliminate the need to use several single herbal formulations at the same time, leading to increased compliance in patients[1]. This is further supported by the practices of traditional systems of medicine in which diseases have often been managed by using combined medicinal plant extracts in the form of polyherbal remedies rather than using them as single plant extracts. Yet, given consideration to the potential toxic effects and other forms of incompatibilities that occur due to polyherbal formulations, scientific evaluation of their efficacy and safety through clinical validation with chemical standardisation, bioassays, and elucidation of cellular mechanisms in animal models, and clinical trials is crucial[1,2].

The present study is based on a formulation composed of three Sri Lankan medicinal herbs, *Asparagus falcatus* L. (family: Asparagaceae; leaves), *Abelmoschus moschatus* Medik. (Family: Malvaceae; leaves), and *Barleria prionitis* L. (family: Acanthaceae; whole plant). These medicinal herbs were initially selected concerning pharmacopeias and other monographs related to Sri Lankan traditional medicine. The use of selected medicinal herbs in Sri Lankan indigenous and folk medicine for kidney-

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related disorders has been well documented[3]. However, individual aqueous extracts of standardised medicinal herbs showed promising nephroprotective effects in reducing proteinuria, improving kidney function, and reversing structural impairments in kidney tissues during the evaluation of efficacy against nephrotoxicity[4]. Additionally, the administration of hexane, ethyl acetate, butanol, and aqueous extracts of the three plants was able to demonstrate potent antioxidant and anti-inflammatory activities that govern its nephroprotective activity *in vivo*[5-7]. Considering the wide use of the selected medicinal herbs in traditional medicine applications, and the reported efficacy, we hypothesized that a combination of the selected medicinal herbs would be promising in the development of a commercially viable therapeutic agent for patients with kidney disease. The use of a mixture of medicinal herbs would further simulate traditional therapeutic applications and would be beneficial in approaching the population that expects conventional healthcare. Doxorubicin is an anthracycline chemotherapeutic agent with potential nephrotoxicity and the doxorubicin-induced nephrotoxicity model simulates most of the pathophysiological characteristics of human kidney disease[8]. Herein, our objective is to evaluate the efficacy and cellular mechanisms of a standardised polyherbal formulation (PHF) derived from Sri Lankan medicinal herbs, in Wistar rats with doxorubicin-induced nephrotoxicity.

Materials and Methods

Collection, identification, and extraction of medicinal herbs

The botanical raw materials of *A. falcatus* (leaves), *A. moschatus* (leaves), and *B. prionitis* (whole plant) were collected from natural habitats in Galle, Southern province of Sri Lanka. Herbal materials were identified by a certified taxonomist in the National Herbarium and deposited for future reference at the institutional herbarium (archived specimens; PG/2016/55- 01, 02 and 03).

The individual plant materials were dried at 40°C for 72 h, coarsely powdered, and mixed in equal quantities (1:1:1 by weight). The resulting plant mixture (25 g) was extracted with distilled water (500 mL) by refluxing for four hours. The herbal decoction was then evaporated under reduced pressure and freeze-dried to produce the residue, which was later termed polyherbal formulation (PHF) with an extractive value of 29.44% w/w.

In vitro studies

Chemical standardisation, assessment of shelf life, and proximate nutrient composition analysis of the PHF were carried out following standard protocols[9].

In vivo studies

Selection of PHF doses

The human equivalent therapeutic dose in rats (400 mg/kg body weight) was calculated based on the recommended dose of raw herbs in Ayurveda medicine (12 g/day/60 kg) and the extraction yield of the decocted product (29.44% w/w)[10]. Two additional doses of PHF were selected, including a low dose (200 mg/kg body weight) and a high dose (600 mg/kg body weight). The freeze-dried powder was dissolved in distilled water to prepare the selected doses for administration.

Animal studies

Inbred male Wistar albino rats aged 10-12 weeks, at the *vivarium* of the institution were used in the experiments. The procedures were approved by the Institutional Ethics Review Committee (Protocol No.19.09.2018:3.3).

Study protocol

The Wistar albino rats were divided into six groups of six animals each. Except for Group (1) which was treated as the untreated control, the other groups of rats were induced with doxorubicin (5 mg/kg, i.p.; United Biotec, India). The doxorubicin dose was selected based on the findings of pilot studies and considering the reported literature[8]. The development of nephrotoxicity was confirmed based on the urine total protein (UTP) concentration in rats four days after doxorubicin administration, considering the procedures followed in previous studies[11,12]. Based on the findings (greater than x 2 increase in UTP), the rats were included in the intervention either in the doxorubicin model group or treatment of PHF.

Treatment regimens were as follows; Group (1); untreated control group and Group (2); doxorubicin model group, received distilled water in equivalent volumes to the treatment dose. Treatment Groups (3), (4), and (5) received PHF at the low (200 mg/kg body weight), therapeutic (400 mg/kg body weight) and high (600 mg/kg body weight) doses, respectively. Group (6) received fosinopril sodium, the standard drug (0.09 mg/kg; Sigma-Aldrich, USA). The treatment regimens were administered orally for 28

days starting from the second day following doxorubicin administration.

Urine samples were collected for 24 h in diuresis cages on the last day of experiments. On the 29th day, all the rats were sacrificed, and blood was collected by cardiocentesis. One-half of each kidney tissue excised was fixed in 10% formalin to be processed for histopathology and immunohistochemistry. The residual halves of kidney tissues and liver tissues excised were dissected and washed with ice-cold phosphate-buffered saline for the evaluation of biochemical markers of oxidative stress.

The serum was separated in the collected blood samples for the assessment of kidney function parameters including serum creatinine (sCr), blood urea nitrogen (BUN), β_2-microglobulin (B2M), and cystatin C (Cys C). Urine samples were used to estimate the total protein in the urine.

Part of the frozen kidneys was homogenized (1:3) in buffered phosphate saline (pH 7.4) for the evaluation of total antioxidant status (TAS) and selected *in vivo* antioxidant markers; glutathione peroxidase (GPx), glutathione reductase (GR). The remaining part of the frozen kidney tissues and the collected liver tissues were homogenized in 1.15% ice-cold potassium chloride and malondialdehyde (MDA) concentration was estimated[13]. Further, the frozen kidney tissues were homogenized (1:9) in ice-cold 0.1 M phosphate-buffered saline (pH 7.4) for the estimation of inflammatory cytokines; tumor necrosis factor-α (TNF-α; Catalog No: E-EL-H0109), and interleukin-1 beta (IL-1β; Catalog No: E-EL-H0149).

Histopathological evaluation of kidney tissues was performed according to a semi-quantitative score system[6]. Immunohistochemical evaluation of kidney tissues was carried out using three selected primary antibodies including Cyclooxygenase 2 (Anti- COX-2; M3617; Dako, Denmark), B-cell lymphoma gene product 2 (Anti- BCL-2; M0887; Dako, Denmark) and B-cell associated X protein (Anti- Bax; ab216494; Abcam, Cambridge, UK).

Statistical analysis

All animals allocated for the study were accounted for in the data analysis. Significance was assessed through a one-way analysis of variance to make comparisons (IBM SPSS Statistics 22, New York, USA). A p-value less than 0.05 was interpreted as statistically significant. Quantitative estimations were made in triplicates.

Results

In vitro studies

The findings on chemical standardisation and proximate nutrient analysis are shown in Table 1. The LC/MS chromatogram of the proposed PHF is shown in (Fig. 1(a)). No microorganisms were detected at the beginning and end of the study period of 30 days during shelf-life determination. The aerobic plate count was below the detection limit at the beginning and was reported as 1.5×10^1 CFU/g at the end. Furthermore, no substantial change in the phytochemical composition was observed in the TLC fingerprints of the PHF at the beginning and end of the study period as shown in (Fig. 1(b)).

Preclinical efficacy of PHF in DOX-induced rats

PHF on kidney function markers in DOX-induced rats

The effect of aqueous PHF on markers of kidney function is shown in Table 2. BUN (51%) and serum

Table 1 — Ash values, extractive value, heavy metal analysis, and proximate nutrient analysis of the polyherbal formulation

Specification	Results
Ash values	
Total	12.15±0.16% (w/w)
Water-soluble	4.84±0.19% (w/w)
Acid-insoluble	0.06±0.02% (w/w)
Extractive Values	
Cold water	4.52±0.03% (w/w)
Cold ethanol	3.09±0.10% (w/w)
Hot water	7.60±0.04% (w/w)
Hot ethanol	4.47±0.03% (w/w)
Heavy metal analysis	
Arsenic (Not more than 0.01 mg/day)	Not detected
Cadmium (Not more than 0.006 mg/day)	Not detected
Lead (Not more than 0.02 mg/day)	0.0096 mg/day
Mercury (Not more than 0.02 mg/day)	0.0048 mg/day
Proximate analysis of the nutritional composition	
Crude protein	15.03±1.70% (w/w)
Carbohydrates	53.56±0.00% (w/w)
Crude fat	0.05±0.00% (w/w)
Energy	274.81±0.00 Kcal/g
Iron	189.00±0.00 mg/kg
Calcium	1.90±0.00 mg/kg
Vitamin composition	
B_2	1.3±0.00 mg/100 g
B_6	1.8±0.00 mg/100 g
C	Not detected (LOD 0.1 mg/100 g)
E	0.64±0.00 mg/100 g
β carotine	Not detected (LOD 0.1 mg/100 g)

Values are expressed as mean±SEM.

sCr (29%) showed a significant increase in values in the doxorubicin model group of rats compared to the untreated control rats, confirming a significant induction of kidney injury ($p<0.05$). In rats treated with the PHF, BUN (41%, 60%, and 61%) and sCr (24%, 30%, and 30%) concentrations showed a dose-dependent reduction in values. Rats treated with the therapeutic and high doses of the PHF showed a better reduction in both parameters compared to the standard drug ($p>0.05$). Interestingly, the BUN values reported for the therapeutic and high doses were significantly different from the selected lowest dose as well ($p<0.05$). Furthermore, a 70% increase in UTP was observed in the doxorubicin model group compared to the untreated control group ($p<0.05$). However, significant improvement in kidney function could be observed only with the therapeutic and high doses of PHF, with reference to urine protein (48%, and 44%).

Administration of the plant mixture at three doses resulted in a significant reduction in B2M values dose-dependently (6%, 7%, and 12%) ($p<0.05$). On the contrary, Cys C levels showed a dose-dependent increase (44%, 43%, and 39%) in values, although the results showed a significant reduction compared to the doxorubicin model group ($p<0.05$). However, the fosinopril-treated rats showed better nephroprotection as evidenced by both parameters than the nephrotoxic rats treated with the PHF at low and therapeutic doses.

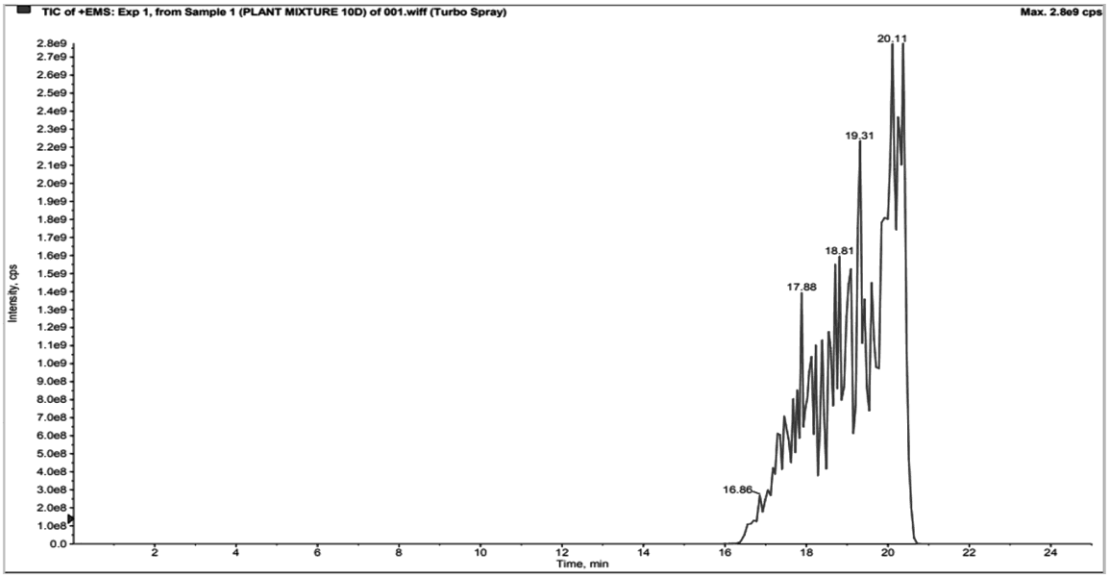

Fig. 1(a) — Liquid chromatography mass spectrometry chromatogram of the polyherbal formulation

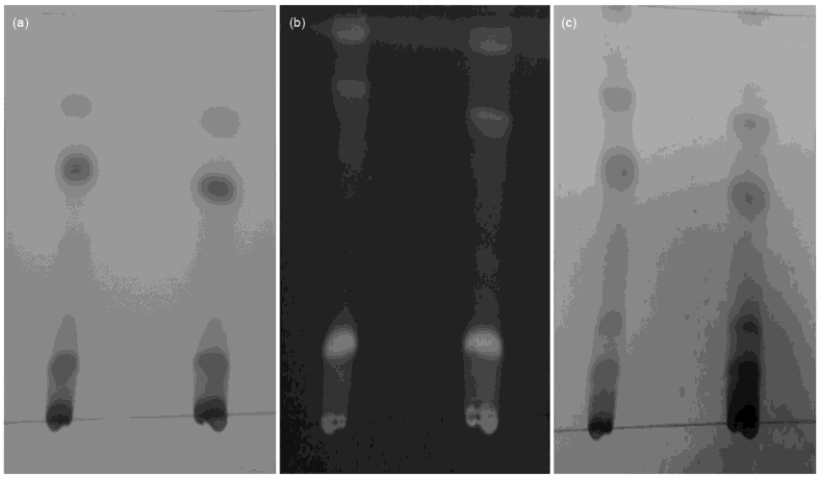

Fig. 1(b) — Phytochemical comparison of thin layer chromatography profiles of the dichloromethane fraction of the polyherbal formulation at the beginning and end of the study period of 30 days, visualized under UV 254 nm (a), UV 366 nm (b) and with vanillin/sulfuric acid under white light (c)

Table 2 — Effect of polyherbal formulation on kidney function and antioxidant, lipid peroxidation, anti-inflammatory markers

	Untreated control	Doxorubicin model	PHF- Low dose	PHF- Therapeutic dose	PHF- High dose	Fosinopril
Kidney function tests						
Blood urea nitrogen (mmol/L)	0.49±0.06*	1.00±0.13†	0.59±0.04*	0.40±0.03*	0.39±0.04*	0.53±0.05*
Serum creatinine (μmol/L)	70.84±2.07*	99.90±5.36	75.56±4.22*	70.19±6.10*	69.70±6.40*	76.22±5.40*
Urine total protein (g/dL)	36.08±8.45*†	122.11±15.27†	84.48±23.34	63.90±9.46*	67.94±14.40*	77.89±3.54*
Serum β_2-microglobulin (μg/mL)	3.34±0.01*	3.86±0.15†	3.62±0.09*	3.60±0.06*	3.39±0.03*	3.42±0.05*
Serum cystatin C (ng/mL)	19.05±2.19*	41.87±9.13†	23.35±0.37*	24.01±0.66*	25.67±0.66*	19.79±1.42*
Antioxidant markers						
Total antioxidant status (mmol/L)	8.26±0.11*	7.00±0.19†	7.73±0.33	8.06±0.43*	8.49±0.31*	8.40±0.31*
Glutathione reductase (U/L)	22.77±2.62	15.88±1.46†	16.69±3.17	25.50±4.27*	32.06±3.86*	25.04±3.16*
Glutathione peroxidase (U/L)	93.51±11.62*	49.14±8.74†	49.82±3.06†	57.76±10.64†	71.43±11.31†	121.79±20.57*†
Lipid peroxidation						
Kidney (nmol malondialdehyde /g protein)	12.83±0.35*†	20.60±1.12†	16.09±0.60*	15.40±0.10*	15.29±0.21*	15.33±0.74*
Liver (nmol malondialdehyde /g protein)	17.12±0.91*	23.61±2.37†	19.49±1.25*	19.06±0.43*	17.96±0.33*	19.79±1.05*
Anti-inflammatory markers						
Tumor necrosis factor -α (pg/ mL)	1386.73±130.92*	1991.28±112.71†	1476.61±125.47*	1193.21±96.19*	1299.04±72.40*	1456.43±39.57*
Interleukin -1β (pg/ mL)	7608.43±676.03	11529.41±3556.61	8347.43±1909.40	7363.00±622.04	6644.08±1856.51	8755.72±929.47

Results show * $p<0.05$ vs. doxorubicin model group and † $p<0.05$ vs. Fosinopril. PHF: polyherbal formulation

PHF on the antioxidant potential in DOX-induced rats

Doxorubicin-induced kidney injury significantly reduced the TAS (18%) and GPx activity (90%) in doxorubicin model rats ($p<0.05$) (Table 2). Although a 43% reduction in GR activity was observed with the doxorubicin model group, the results were statistically insignificant ($p>0.05$). However, oral administration of the PHF to rats with kidney injury restored *in vivo* antioxidant potential, as shown by the increased values of TAS, GR, and GPx. A dose-dependent increase in the three selected markers of oxidative status was observed upon treatment with increased doses of PHF. However, significant changes could be observed with therapeutic and high doses of PHF concerning TAS (15% and 21%) and GR activity (61% and 102%) ($p<0.05$). Interestingly, the GR activity shown by the high dose of PHF was significantly different from the low dose of PHF ($p<0.05$). The elevation of GPx activity in the PHF-treated rat groups was not statistically significant. The results are shown in Table 2.

Oral administration of the PHF in rats with kidney injury further resulted in a significant dose-dependent attenuation of MDA formation in the kidney (22%, 25%, 26%) and the liver (17%, 19%, and 24%) tissues (Table 2). Although the three selected doses of PHF were more effective in suppressing lipid peroxidation in liver homogenates than fosinopril, only the therapeutic and high doses showed similar or better suppression of lipid peroxidation in the kidney homogenates.

PHF on anti-inflammatory potential in kidney homogenates of DOX-induced rats

Treatment with the PHF at selected low, therapeutic, and high doses reduced inflammatory cytokines TNF-α (26%, 40%, and 35%) and IL-1β (28%, 36%, and 42%) compared to the doxorubicin model group. Interestingly, treatment with the therapeutic dose significantly improved the concentration of TNF-α compared to the selected lowest dose of PHF. The findings are shown in Table 2.

PHF on histopathology in kidney tissues of DOX-induced rats

Kidney tissues of rats intoxicated with doxorubicin demonstrated early features of acute tubular necrosis with intertubular hemorrhage, marked loss of tubular brush border, cast formation, pyknosis, and glomerular congestion, as shown in (Fig. 2(a)). The microscopic observations were further supported by the significant differences reported in the average histological scores according to the semi quantitative scoring system followed (Fig. 2(b)). However, no features of inflammatory infiltration were observed under high power (x400) in either group of experimental rats.

The findings revealed a significant reduction in the average histological scores in rats administered with the low, therapeutic, and high doses of the PHF (21%, 27%, and 30%). The loss of brush border, cytoplasmic vacuolization, and glomerular congestion were reduced in a dose-dependent manner with an increase

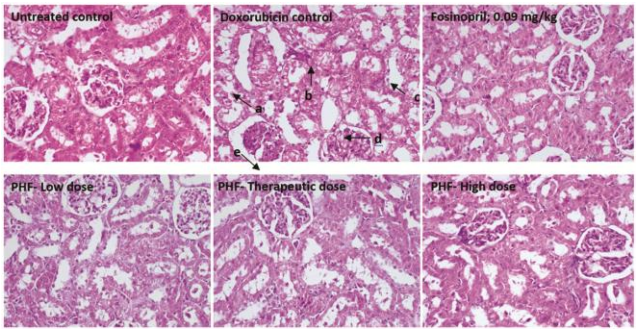

Fig. 2(a) — Histopathological analysis of kidney tissues following treatment regimens of polyherbal formulation at low (200 mg/kg body weight), therapeutic (400 mg/kg body weight), and high (600 mg/kg body weight) doses (x400). (a), cytoplasmic vacuolization, (b), intertubular hemorrhage, (c), pyknosis of tubular epithelial cells, (d), deficit brush border in the tubular epithelium, (e) and glomerular congestion. PHF: polyherbal formulation.

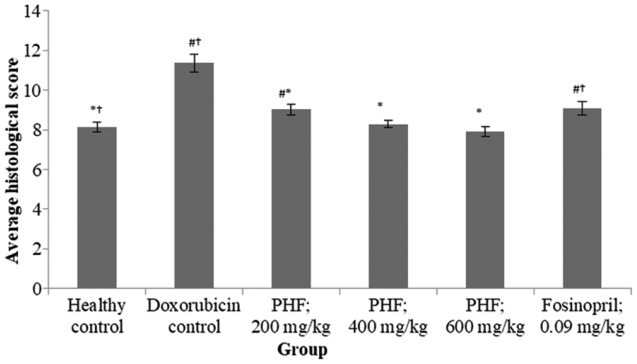

Fig. 2(b) — Average histological score following treatment regimens of polyherbal formulation at low (200 mg/kg body weight), therapeutic (400 mg/kg body weight), and high (600 mg/kg body weight) doses. Results show # $p<0.05$ vs. healthy control, * $p<0.05$ vs. doxorubicin model group, and † $p<0.05$ vs. Fosinopril. PHF: polyherbal formulation

in the dose of PHF. The therapeutic and high doses restored the tissue architecture to the level of the healthy control group as depicted by the improved average histological scores. Interestingly, all three doses of PHF showed better nephroprotection than fosinopril (20%).

Anti-inflammatory and anti-apoptotic effects of PHF by immunohistochemical studies

Treatment with the three selected doses of PHF resulted in a reduction in cyclooxygenase-2 immunohistochemical expression compared to the doxorubicin model group. A significant reduction in immunostaining could be observed with the high dose of PHF (Fig. 3(a)).

A reduction in Bax (proapoptotic protein) immunoreactivity was observed with the increased dose of PHF (Fig. 3(b)). Both the cytoplasm and luminal surfaces of tubular epithelial cells stained brown in the kidney sections of rats treated with low and therapeutic doses of PHF. However, the high dose of PHF showed a positive reaction mainly on the luminal surface of the proximal tubular epithelial cells.

BCL-2 expression was comparatively high in tubular epithelial cells of the experimental rats treated with PHF compared to the doxorubicin model group (Fig. 3(c)). However, in the present study, no dose-related improvement in BCL-2 expression was observed.

Discussion

Herbal medicines can cause harmful health effects if not properly assessed. This is further fortified by recent case reports of serious adverse events of herbal products due to potential contamination with toxic metals, adulteration with Western pharmaceuticals, and deliberate substitution of other herbs[14]. The widespread variation and deterioration in compositions are a few more risks associated with herbal medicine that could lead to adverse health consequences[15]. Hence, the assurance of safety, consistency, and quality control aspects of commercially viable herbal formulations should be a priority in herbal research. Standardisation is the first step in ensuring the safety of medicinal herbs in terms of setting up a consistent chemical profile before incorporating them in pharmaceuticals and evaluating potential bioactivities[15].

The physicochemical parameters provide useful information and control required in producing materials of reasonable consistency. The low value reported for acid-insoluble ash in PHF excludes the potential contamination of plant materials with silica and signifies satisfactory purity. The hot water extract showed the highest extractive value, substantiating its use in evaluating the therapeutic efficacy of the PHF.

Consumption of medicinal herbs contaminated with heavy metals and microbial from soil and water sources in the natural environment has been identified as a leading cause of herb poisoning[16]. Scientific evaluation of the heavy metal content and microbial limits in the PHF disclosed the presence of said heavy metals within the standard safe range and the absence of microbial contamination, safeguarding the usage.

Assessment of the preclinical efficacy of the novel PHF revealed significant nephroprotective effects on

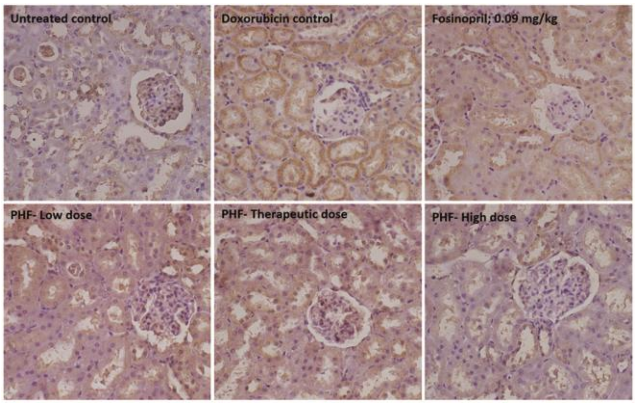

Fig. 3(a) — Cyclooxygenase-2 immunohistochemical expression upon the treatment of polyherbal formulation on doxorubicin-induced experimental kidney injury in Wistar albino rats (x400 magnification). PHF: polyherbal formulation

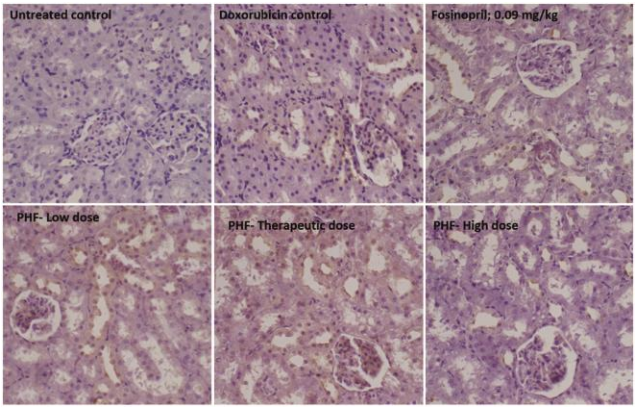

Fig. 3(b) — B-cell-associated X protein immunohistochemical expression upon treatment of polyherbal formulation on doxorubicin-induced experimental kidney injury in Wistar albino rats (x400 magnification). PHF: polyherbal formulation

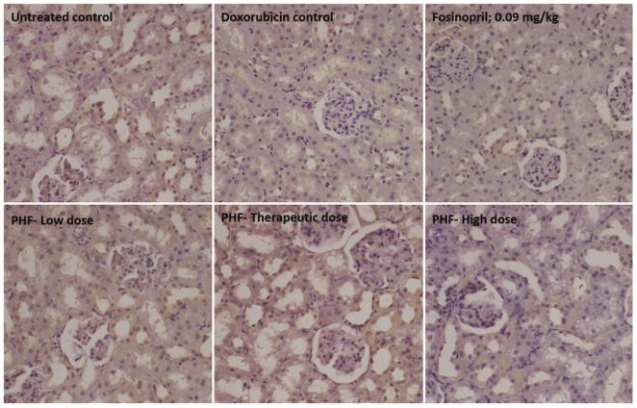

Fig. 3(c) — B-cell lymphoma gene product-2 immunohistochemical expression upon treatment of polyherbal formulation on doxorubicin-induced experimental kidney injury in Wistar albino rats (x400 magnification). PHF: polyherbal formulation

the doxorubicin-induced nephrotoxicity model. Treatment with the PHF in the established doxorubicin-induced nephrotoxicity model caused significant nephroprotection, as evidenced by significant changes observed in kidney function parameters compared to the model group. Interestingly, the therapeutic and high doses of PHF were able to restore the BUN and sCr values to the level of untreated control rats. Similarly, these two doses shared better nephroprotection than fosinopril, concerning sCr, BUN, and total protein concentration in urine. BUN concentrations observed in these two doses were significantly different from the low dose of PHF as well ($p<0.05$). These findings suggest significant nephroprotective effects of PHF at the therapeutic dose and above.

Similarly, the ameliorative effect of PHF on the histopathological changes induced by doxorubicin was parallel to the biochemical alterations reported. The mean histological scores reported further demonstrate the dose-related nephroprotective effects of the studied PHF. However, the absence of significant inflammatory infiltration in the doxorubicin model group excluded the hypothesis that the disease progressed into a chronic stage. The short duration used in the present study considering the mortality of experimental animals might be the reason for the absence of the particular characteristic feature in nephrotoxic animals. This is further confirmed by the findings of Egger *et al.*[17]

Oxidative damage and inflammation are the main principles that explain doxorubicin-induced nephrotoxicity[18]. Treatment with selected doses of PHF reversed the doxorubicin-induced oxidative damage, as manifested by significant elevations in TAS, GPx, and GR activities compared to the doxorubicin model group. MDA formation was significantly reduced in both kidney and liver tissues with all three doses of PHF, further supporting the potential antioxidant effects.

Treatment with PHF suppressed the elevation of TNF-α and IL-1β, induced by doxorubicin, suggesting potential anti-inflammatory effects. Interestingly, the observed concentrations of TNF-α and IL-1β in the experimental rats treated with therapeutic and high doses of PHF were lower than those of the standard drug, suggesting a better anti-inflammatory potential of PHF compared to the standard drug. The immunohistochemical findings on COX-2, further substantiate the anti-inflammatory potential of PHF and the findings further corroborate the results of TNF-α and IL-1β.

BCL-2 and Bax play important roles in the endogenous apoptotic pathways as potential anti-apoptotic and proapoptotic proteins respectively[19]. PHF was found to down-regulate the expression of Bax and up-regulate the expression of BCL-2. These findings demonstrate the anti-apoptotic potential of PHF that could lead to protective effects against doxorubicin-induced nephrotoxicity.

The use of a blend of medicinal herbs with varying efficacy for the preparation of PHF simulates the traditional therapeutic applications in Ayurveda. The reported nutritional value would be an additional advantage in approaching the population who expects conventional healthcare as a potential nutraceutical. The nutritional significance of the selected individual medicinal herbs is also well-reported. *A. falcatus* is one of the most popular food-cum-medicines in Sri Lanka, which is often used in the traditional herbal gruel called "Kola Kanda". In addition, the "mallung", made of the leaves of *A. falcatus* is a popular vegetable dish among rural Sri Lankans and is often recommended for improving eyesight by traditional healers. Even though *A. moschatus* is not commonly consumed as a diet food, *Abelmoschus esculentus* (common name: Okra) of the same genus is one of the foremost widely consumed vegetable crops in tropical and sub-tropical parts of the world. Moreover, the potential nutraceutical significance of *A. esculentus* has already been published[20]. Despite the numerous medicinal properties reported for *B. prionitis*, its dietary benefits have not been reported to date.

Furthermore, the shelf life, in terms of the absence of microbial growth and unchanged phytochemical composition, would be a plus point in the development of a commercially elegant herbal mixture that would target the management of kidney disease. The addition of nutritional value to nephroprotective agents also helps to increase potential therapeutic effects which would be advantageous[21]. The findings on the standardisation parameters would be further beneficial in maintaining the appropriate quality of the raw plant materials during the synthesis of the herbal formulation for commercialization.

Treatment with PHF for 28 consecutive days does not simulate the actual treatment regimens used in patients with kidney diseases, which could be a limitation of the study. Furthermore, a detailed investigation of nephroprotective effects for an extended period (more than 3 months) was not possible with the mortality of experimental animals experienced with the selected dose of doxorubicin (5 mg/kg). Nevertheless, the proven nephroprotective effects and the nutritional composition of the PHF suggest safe and promising protective effects of the proposed composition in the management of patients with kidney diseases.

Conclusions

The PHF was found to be efficacious against doxorubicin-induced nephrotoxicity in Wistar albino rats. Further, the aqueous PHF counteracts oxidative stress and inflammation induced by doxorubicin via antioxidant, anti-apoptotic, and anti-inflammatory pathways. The therapeutic and high doses of PHF showed significant nephroprotection compared to the low dose of PHF and the standard drug. However, the general findings of the present study conclude that PHF made of *A. falcatus*, *A. moschatus*, and *B. prionitis* would be beneficial as a therapeutic/ nutraceutical agent for the treatment of drug-induced nephrotoxicity and other kidney-related diseases.

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contribution

Conceptualization; SSA, APA, LKBM, Formal analysis; SSA, LKBM, Funding acquisition; APA, Resources; APA, LDAMA Software; SSA, Supervision; APA, LDAMA, LKBM, KAPWJ, Roles/Writing - original draft; SSA, Writing - review & editing- APA, LDAMA, LKBM, KAPWJ.

Ethics Approval

Ethics approval was obtained from the Ethical Review Committee of the Faculty of Medicine, University of Ruhuna, Sri Lanka (Protocol No.19.09.2018:3.3). The experiments were performed in accordance with the 'Guide for the Care and Use of Laboratory Animals (NIH Publication No.85-23, 1985).

Data Availability

The authors confirm that the data supporting the findings of this study are presented within the article.

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