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Nephroprotective activity of alkaloidal fraction of *Alstonia scholaris*

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Abstract
Aim: This study specifically investigated the possible protective effects of alkaloid fraction of selected plant against oxidative stress and renal impairment induced by suitable agents.
Experimental Work: Around 500 gm dried leaves of *Alstonia scholaris* were coarsely powdered weighed and filled in Soxhlet apparatus for extraction. First the powdered drug was defatted with petroleum ether (60°C-80°C); Defatted drug was then dried and again filled in soxhlet apparatus for successively extraction with methanol and water as solvent. The ethyl acetate soluble fractions were dried and kept in air tight container and it is named as ALKALOID FRACTION (AF) of leaf extract. Cisplatin is the highly effective chemotherapeutic agent widely used in the treatment of a variety of cancers. However, its clinical utility is limited due to its most common adverse effect i.e. nephrotoxicity in many patients. For this method, the samples of the blood was withdrawn, collected in absence of sodium citrate and allowed to clot. The clotted blood was centrifuged for 10 minutes at 2500 rpm. The obtained clean and clear serum was refrigerated at 20^0C for the analysis of blood urea, serum creatinine (CR), and uric acid (UA) level by adopting & utilizing colorimetric assay kits as per given procedure.
Results: There was clear variation and alteration (increase) in the levels uric acid, creatinine and urea levels when compared to normal group animals. 50 and 100mg/kg dose of alkaloidal fraction was able to bring down the elevated values of uric acid, creatinine and urea levels towards normal, when compared to toxic control groups. Alkaloidal fraction showed a most highly significant effect (P>0.001) as compared to other disease control.
Conclusion: The results obtained in this study have shown that alkaloidal fraction displayed significant nephroprotective activity in acute and chronic conditions. Besides from the obvious therapeutic importance, these components would be useful in understanding the mechanism of diseases with higher levels of cellular and molecular level.

Keywords: protective effects, alkaloidal fraction, cisplatin induced nephrotoxicity, bun, uric acid

Introduction
The occurrence of non-dialysis-requiring AKI is around 5000 cases per million individuals per each year and frequency of dialysis requiring AKI is 295 cases per million individuals per every year (Bellomo *et al*, 2012) [2]. AKI has been demonstrated as independent risk factors for mortality and the Intensive Care National Audit Research Center (ICNARC) reported that AKI accounts for 10 % of all intensive care unit beds (Hou *et al*, 2007) [4]. A meta-analysis of 13 cohort study revealed that AKI is one of the risk factor for chronic kidney diseases (CKD) (Joyce *et al*, 2013) [5]. The patients who required dialysis and transplantation due to kidney failure are found to increase from year to year (Andrew *et al*, 2003) [1].
The kidneys are the significant focuses for the poisonous impacts of different chemical substances operators and thus drug-induced AKI is a frequent entity in clinical medicine. Drug induced renal toxicity is recognized as an important contributor to kidney diseases including AKI and CKD. The rate of nephrotoxic AKI is hard to gauge because of inconstancies of patient populaces and criteria of AKI. However, prospective cohort studies of AKI have documented the frequency of drug induced renal toxicity to be approximately 14-26 % in adult population. It is a significant concern in pediatric patients with 16% of hospitalized AKI being attributable primarily to a drug (Schertz *et al*, 2005 and Linda *et al*, 2017) [9, 6].
This study specifically investigated the possible protective effects of alkaloid fraction of selected plant against oxidative stress and renal impairment induced by suitable agents.

Experimental Work
Collection of plants
Dried leaves of *Alstonia scholaris* were procured from the medicinal garden and campus of Pharmacy College in the month of July, Madhya Pradesh, India. Crudes are washed properly through water and dried in shade for the further process.

Drugs and chemicals
Gentamicin- 80mg/2ml was purchased from Ranbaxy, Cisplatin (Cytoplatin)- 50mg/ml was purchased from Cipla Ltd, Bovine serum albumin, DPPH (1,1 Dipheyl,2-Picryl hydrazyl), Sodium citrate, Sulphanilamide, Quercetin, Rutin, Methanol, HPMC-AS, LG from SD Fine Chemicals Ltd; Hyderabad, India.

Kits
Urea Kit, Creatinine Kit from M S Excel Diagnostics Private Limited, Hyderabad.

Equipments
Digital Balance (SHIMA DZU, AX 200), UV/Visible Spectrophotometer (UV/win5 software), Tissue

Homogenizer (REMI MOTOR), Laboratory cooling centrifuge (REMI R- 8C), Serum analyzer (Inkarp,ES-100), Trinocular Microscope (Labomed, IVU-3100), B.O.D incubator (Barath biotech).

Preparation of Alkaloid Fraction

Around 500 gm dried leaves of *Alstonia scholaris* were coarsely powdered weighed and filled in Soxhlet apparatus for extraction. First the powdered drug was defatted with petroleum ether (60°C-80°C); Defatted drug was then dried and again filled in soxhlet apparatus for successively extraction with methanol and water as solvent. The extraction was carried out for a period of 72 hr. The methanolic extract was further dissolved in the water and then partitioned by using ethyl acetate. This process was done consecutively for three times so that all the ethyl soluble alkaloids were extracted out in the ethyl acetate.

The ethyl acetate soluble fractions were dried and kept in air tight container and it is named as ALKALOID FRACTION (AF) of leaf extract. The alkaloid fraction obtained was dried in vacuum to remove excess solvent and were weighed for the determination of % yields (Eddouks *et al*, 2005) [3].

Preliminary *In-Vivo* Nephroprotective Activity

Selection of animals

Wistar albino rats of either sex between 2 and 3 months of age weighing 150-200 gm were used which were procured from the central animal house India. All animals were housed in an animal room under normal condition of 25±1^{0}C, 12 hr light and dark cycle. The animals were allowed free to access commercial rat pallet diet (Lipton India Ltd, Mumbai, India) and water *ad libitum.* The bedding materials of the cages were changed every day. All the experimental procedures were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. The study designs were approved by the Institutional Animal Ethical Committee.

Acute toxicity studies

The acute oral toxicity studies were carried out according to the guidelines set by the Organization for Economic Co-operation and Development (OECD), revised draft guideline 423.

Alkaloidal fraction of selected plant were prepared as a suspension by triturating dried extracts with 1% Tween 80, prepared in distilled water. Wistar rats (150-200 gm) were used for acute toxicity study to determine the acute toxicity.

One tenth and one fifth of the lethal dose was taken as effective dose (therapeutic dose) and cut off value was selected as 50 and 100 mg/kg to evaluate the dose dependent action for the evaluation of nephroprotective activity (OECD guidelines, 2001) [7].

Nephroprotective Activity of Alkaloidal Fraction in Cisplatin Induced Nephrotoxicity

Cisplatin is the highly effective chemotherapeutic agent widely used in the treatment of a variety of cancers. However, its clinical utility is limited due to its most common adverse effect i.e. nephrotoxicity in many patients (Schrier RW, 2002) [10]. Cisplatin (5mg/kg, *i.p*) was administered to the rats. The study was conducted for 15 days and the rats were divided into 4 groups (n=6).

On the 16th day blood was taken out from tail vein method from all groups assessed for renal function tests (Schrier RW, 2002) [10].

The treatment or grouping schedules were as follows.

Group-I: Normal Control

Group-II: Disease Control (Cisplatin induced toxicity, 5mg/kg, *i.p*)

Group-III: Alkaloidal fraction treated rats in a dose of 50 mg/kg+ Cisplatin, 5mg/kg, *i.p*

Group-IV: Alkaloidal fraction treated rats in a dose of 100 mg/kg+ Cisplatin, 5mg/kg, *i.p*

Biochemical Analyses for Estimation of Renal Parameters

For this method, the samples of the blood was withdrawn, collected in absence of sodium citrate and allowed to clot. The clotted blood was centrifuged for 10 minutes at 2500 rpm. The obtained clean and clear serum was refrigerated at 20^{0}C for the analysis of blood urea, serum creatinine (CR), and uric acid (UA) level by adopting & utilizing colorimetric assay kits as per given procedure (Schrier RW, 2002) [10].

Determination of creatinine

Principle

Creatinine produced an orange-yellow colour when reacts with alkaline picrate. The absorbance of the orange-yellow colour which formed by sodium picrate is proportional to the concentration was measured at 500-520 nm photometrically.

Assay procedure

1. Equal volumes of reagent 1 and reagent 2 were mixed. Wait for 15 minutes before use.
2. Serum is preferred but heparinized plasma may be used. We used serum in this case.
3. The solution was mixed well and initial absorbance (A1) was read 20 seconds after mixing and final absorbance (A2) 80 seconds after mixing.
4. The results were calculated as follows:

$$\Delta A = A2 - A1$$

$$\text{Creatinine} = \frac{\Delta A \text{ of Test} \times \text{Concentration of standard (mg/dl)}}{\Delta A \text{ of Standard}}$$

Determination of urea

Principle

The estimation of urea in serum involves the enzyme catalyzed reactions. The decrease rate of absorbance was monitored at 340 nm and was directly proportional to urea concentration in the drug sample.

Assay procedure

1. The reagent and Aqua-4 was allowed to get room temperature (15-300C).
2. The amount of Aqua-4 indicated on the label was added to dissolve the contents of each bottle of substrate and mixed gently. It was not shaking vigorously.

3. The absorbance change (ΔA) for the standard and unknown samples was determined by using the formula:

$\Delta A = A2 - A1$

Determination of uric acid
Methodology
Modified Trinder Method, End Point
The intensity of chromogen was found to be proportional to the concentration of uric acid in the sample when measured at 505 nm.

Assay procedure
The reagents were mixed and kept at incubation for 5 minutes at 37ºc the absorbance of standard and each test sample were read at 505 nm on biochromatic analyzers against reagent blank.

Statistical Analysis
The values are expressed in mean ± SEM. The results were analyzed by using one way analysis of variance (ANOVA) followed by Dunnet's "t" test to determine the statistical significance. $p< 0.05$ was chosen as the level of significance.

Results and Discussion
Extractive value determination

Table 1: Alkaloidal Fraction with their appearance and % yield (in gm)

S. No.	Extracts	Color of dried extracts	Consistency of dried extracts	% Yield (W/W)
1	Alkaloidal Fraction	Dark Green	Sticky	9.4 %

Acute toxicity studies
No toxic effects were observed at a higher dose of 500 mg/kg body weight of Wistar rats. Hence, 1/ 10th dose was selected as effective dose or therapeutic dose. The cut off value of 50 and 1/5 dose double of 100 mg/kg were selected for nephroprotective activity.

Preliminary *In-Vivo* Nephroprotective Activity
Nephroprotective activity of Alkaloidal Fraction in Cisplatin Model
In our protocol single injection (i,p) at a dose of 5mg/kg with Cisplatin resulted in the clear cut Renal toxic symptoms appeared causing acute renal failure. There was clear variation and alteration (increase) in the levels uric acid, creatinine and urea levels when compared to normal group animals. 50 and 100mg/kg dose of alkaloidal fraction was able to bring down the elevated values of uric acid, creatinine and urea levels towards normal, when compared to toxic control groups. Alkaloidal fraction showed a most highly significant effect (P>0.001) as compared to other disease control.

Table 2: Nephroprotective activity of alkaloidal fraction in Cisplatin model

S. No.	Groups & Treatment	Biochemical Parameters		
		Blood Urea (mg/dl)	Serum creatinine (mg/dl)	Uric acid (mg/dl)
1	Normal Control	52.20± 2.22	0.684± 0.06	1.634± 0.11
2	Disease Control, 5 mg/kg	74.16± 2.34	1.971± 0.11	2.78 ±0.33
5	Alkaloidal fraction, 50 mg/kg	54.23 ±4.11**	0.937± 0.04**	1.54 ±0.31**
6	Alkaloidal fraction, 100 mg/kg	51.45±3.55***	0.693±0.12***	1.12±0.15***

Values are representative of Mean ± SEM (n=6). One way ANOVA with Dunnett's test. Where, *P<0.05 vs **P<0.01, Not significant. Toxic control P< 0.05, P<0.01 vs control.

Discussion
The kidneys are the significant focuses for the poisonous impacts of different chemical substances operators and thus drug-induced AKI is a frequent entity in clinical medicine. However, prospective cohort studies of AKI have documented the frequency of drug induced renal toxicity to be approximately 14-26 % in adult population. It is a significant concern in pediatric patients with 16% of hospitalized AKI being attributable primarily to a drug (Schertz *et al*, 2005 and Linda *et al*, 2017) [9, 6].
Plant extracts and alkaloidal fraction are very beneficial in the treatment of various diseases and conditions (Varalakshmi *et al*, 2011). Example of such plant is *Alstonia scholaris* is deciduous plant used medicinally for ulcers, cuts, sores, wounds, general body pains, hemorrhoids, diuretics, malaria and yellow fever (Ouattara *et al*, 2013) [8].
No other sign of toxicity or no mortality was observed and all the animals were survived for 14 days post administration of test drugs. Thus, the dose (500mg/kg, *p.o*) was considered as safe.
Cisplatin is an inorganic complex formed by an atom of platinum surrounded by chlorine and ammonia atoms in *cis* position of a horizontal plane. Since the accidental discovery over four decades ago, cisplatin has been widely used for chemotherapy. Although cisplatin has been a mainstay for cancer therapy, its use is mainly limited by two factors: acquired resistance to cisplatin and severe side effects in normal tissues, which include neurotoxicity, ototoxicity, nausea and vomiting, and nephrotoxicity. Cisplatin is a major anti neoplastic drug commonly used as front-line therapy for cancers such as small cell lung cancer, gut cancer, bladder cancer, stomach cancer, and ovarian cancer and germ cell tumors. The compounds are a class of platinum containing anticancer drugs which induce apoptosis through caspase- 9-dependent pathway. Cisplatin-DNA cross links cause cytotoxic lesions in tumor cells and other regenerative cells, but eventually results in severe adverse effects mainly nephrotoxicity or renal disorder as it is toxic to proximal tubule cells that results in renal damage, through generation of reactive oxygen species (ROS) and this adverse effect is a dose dependent one.
Rats in which nephrotoxicity was induced by intra peritoneal administration of cisplatin (5mg/kg) showed characteristic signs of renal dysfunction and inflammation. The decline in renal function was reflected in the results, showing decrease in bodyweight, decreased urine output, increased levels of serum creatinine, BUN and decreased levels of serum total protein. Similarly decreased levels of urinary creatinine, creatinine clearance were observed.

Both prophylactic and curative treatments with alkaloidal fraction restored body weight, also produced good significance in decreasing serum creatinine, BUN and increase in serum total proteins, urine volume, urine creatinine and clearance of creatinine in urine. Evidences state that in animal models cisplatin damages proximal tubules and cause mitochondrial swelling and nuclear pallor in the distal tubule. Thus the plant extracts have decreased oxidative stress injury.

The findings suggest that the potential use of alkaloidal fraction of *Alstonia scholaris* therapeutically used as a nephroprotective agent. Therefore further studies to explain their mechanisms of action should be conducted to aid the discovery of new therapeutic agents for the treatment of renal diseases.

Conclusions

The results obtained in this study have shown that alkaloidal fraction displayed significant nephroprotective activity in acute and chronic conditions. Besides from the obvious therapeutic importance, these components would be useful in understanding the mechanism of diseases with higher levels of cellular and molecular level.

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