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# Vernonia cinerea regenerates tubular epithelial cells in cisplatin induced nephrotoxicity in cancer bearing mice without affecting antitumor activity

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## ABSTRACT

**Background:** Traditional Siddha Medicine advises using metal-based formulations to treat cancers. In the case of any toxicities during the therapy, Siddha physicians use *Vernonia cinerea* (VC) whole plant *kashayam* (crude aqueous extract-CAE) to reverse the toxic effects.

**Aim:** To evaluate the nephroprotective activity of CAE and its fractions in cisplatin-induced nephrotoxicity and to assess whether they compromise the anticancer efficacy of cisplatin.

**Materials and methods:** Cisplatin-induced renal damage was induced in Ehrlich Ascites Carcinoma (EAC) bearing mice during mild phase of tumor growth. CAE and its butanol (BF) and aqueous (AF) fractions were administered orally from the 5th day for five days. Nephroprotective potential (serum urea, creatinine, renal histology) and effect of VC on cisplatin anticancer efficacy (tumor volume, viable tumor cells, percentage increase in life span (% ILS)) were calculated.

**Result:** CAE and its fractions significantly reversed the cisplatin-induced renal damage. CAE and BF treated animals showed regeneration of 50%–75% of proximal tubular cells. Compared to EAC control mice, the % ILS of the cisplatin-treated group was 244% and it was further extended to 379% after CAE administration. The % ILS in the CAE treated group was 1.6 times higher than the cisplatin alone treated group. GC-MS study showed the presence of astaxanthin and betulin.

**Conclusion:** CAE of VC reverses cisplatin-induced kidney damage as well as regenerates proximal tubular epithelial cells, without compromising the anticancer effect of cisplatin. When CAE was further fractionated, the nephroprotective activity was retained, but the beneficial anticancer effect of cisplatin was compromised.

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**Abbreviations:** AF, aqueous fraction; BF, n-butanol fraction; CAE, Crude aqueous extract; EAC, Ehrlich Ascites Carcinoma; GC-MS, Gas Chromatography-Mass Spectroscopy; % ILS, percentage increase in life span; MST, Mean Survival Time; US FDA, Food and Drug Administration of the United States; VC, *Vernonia cinerea*; WBC, White blood cells.

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## 1. Introduction

The global cancer burden is high, and still, it is growing bigger. Worldwide, more than 11 million people are newly diagnosed with cancer and the number is expected to rise to 16 million by 2020. Besides, cancer alone is the cause of death in more than 8 million people per year worldwide.<sup>1</sup> It is estimated that there will be a cancer patient or cancer survivors for every 19 people by the year 2020.<sup>2</sup> The most common types of solid tumors are testicular, prostate, ovarian, bladder, cervix, breast, and head and neck cancer.<sup>3</sup> Cisplatin is an important anticancer drug used for the treatment of these solid tumors, that causes many side effects, including acute and chronic renal insufficiency and electrolytes disturbances (hypokalemia, hypomagnesemia, hypocalcemia, and hypophosphatemia), which are due to proximal renal tubular necrosis caused by it.<sup>4</sup> Since cisplatin gets accumulated about five times more than the serum concentration in renal tubular epithelial cells and excreted through the kidneys, the chances for nephrotoxicity is higher during cisplatin administration.<sup>5</sup> Despite all the available preventive strategies, approximately one-third of patients on cisplatin develop nephrotoxicity. Cisplatin-induced nephrotoxicity is accounting for up to 20% of all episodes of acute kidney injury in hospitalized patients with cancer.<sup>6</sup> There are reports on a lack of renal recovery and permanent renal damage with successive cycles of chemotherapy treatment.<sup>7–9</sup>

Amifostine is the only US FDA approved drug to protect kidneys from cisplatin in advanced ovarian cancer patients. Unfortunately, clinicians do not use amifostine as there is a discontinuation rate of 27–41% due to its serious adverse effects such as hypotension and intensive care hospitalization.<sup>10</sup>

To date, there is no drug available to regenerate the renal tubular cells after cisplatin damage. An ideal nephroprotective agent should either reduce renal cell death without impairing the anti-tumor effect of cisplatin or regenerate the renal tubular epithelial cells. Traditional Siddha Medicine, an Indian traditional medical system, advises using metal (mercury, arsenic, copper) based formulations for cancer. In the case of any metal toxicities during the therapy, Siddha physicians use *Vernonia cinerea* whole plant *kashayam* (crude aqueous extract-CAE) to reverse the toxic effects.<sup>11</sup> Since cisplatin is a metal-based anticancer agent, we evaluated the nephroprotective ability of the CAE in cisplatin-induced nephrotoxicity. *Vernonia Cinerea* (*Cyanthillium cinereum*) or the “little ironweed” is a member of the *Astraceae* family of herbs. It is an erect herb which is grown in India and known by the name *sahadevi*. The pubescent stem of this herb is often used in traditional medicine. In our earlier study, we demonstrated that the CAE and its fractions at a dose of 400 mg/kg regenerated around 75% of the damaged proximal tubular epithelial cell in cisplatin-treated non-cancerous mice.<sup>12</sup> However, the effect of CAE and its fraction on the anticancer efficacy of cisplatin has not been explored. Therefore, we evaluated the renal epithelial cell regenerative potential CAE and its fractions in cancer mice treated with cisplatin and also identified the phytochemicals of CAE.

## 2. Materials and methods

### 2.1. *Vernonia cinerea* extract and fractions<sup>11</sup>

*Vernonia cinerea* (VC) whole plant was collected from Manipal Academy of Higher Education, Manipal campus, authenticated by a Taxonomist and the voucher specimen was preserved (no. 604.15042404). Pharmacognostical standardization of VC with microscopic examination, loss on drying, total ash value, water soluble ash, acid insoluble ash, were performed. The data was similar to other reports, in which plant material was collected from

different geographical area of India.<sup>12,13</sup> The CAE was prepared by soaking VC powder (500 g) in distilled water (3.5 L) for seven days and the extract was filtered using Whatman filter paper. This procedure was repeated three times using the same plant materials. The extract was then dried on a water bath, followed by in desiccator until there was no further weight loss. The CAE was further subjected to fractionation in separating funnel from non-polar to polar fractions using different solvents such as petroleum ether, chloroform, ethyl acetate, n-butanol, and water. Only n-butanol (BF) and aqueous fractions (AF) were obtained in this process, and other solvents did not yield fractions. Hence CAE, BF and AF were used for the in-vivo study. In our earlier study, 400 mg/kg of CAE and BF demonstrated regeneration of tubular epithelial cells in non-cancerous mice with cisplatin induced nephrotoxicity. Hence this dose of extract or fractions was chosen for the study.<sup>10</sup>

### 2.2. Chemicals and consumables

Cisplatin was purchased from Fresenius Kabi India Pvt. Ltd., India. The assay kits for serum urea and creatinine evaluation were purchased from Agape Chemicals, India. Chemicals required for histology were purchased from Sigma Aldrich. Other consumables were purchased from Tarsons Product Pvt. Ltd., India and Falcon, Oxnard, CA, USA.

### 2.3. Animal experiment

#### 2.3.1. Animals

Male albino mice aged 9–12 weeks, bred in the Central Animal Research Facility of Kasturba Medical College, Manipal Academy of Higher Education, Manipal, India were used after obtaining the Institutional Animal Ethics Committee approval (No.: IAEC/KMC/08/2014). Guidelines given by the Government of India for the use of laboratory animals were followed for the maintenance and breeding of animals.<sup>14</sup> Animals were maintained in propylene cages throughout the study and the paddy husk was used as bedding material, which was changed every 24 h. The temperature was kept at  $25 \pm 1$  °C along with the humidity of  $45 \pm 1$ %. To avoid overcrowding and stress, a maximum of three mice were kept in the cage and adequate ventilation was provided.

#### 2.3.2. Standardization of cisplatin-induced nephrotoxicity in Ehrlich ascites carcinoma bearing mice

The aim of this experiment was to derive the optimal cisplatin dose required for inflicting renal damage in cancer mice. We had five groups ( $n = 6$ ) of mice in this experiment. Ehrlich Ascites Carcinoma (EAC) was induced in groups 2, 3, 4 and 5. Group 1 served as normal control and group 2 served as EAC control. Group 3, 4, and 5 served as cisplatin-induced nephrotoxic groups. EAC was induced in mice by injecting viable EAC cells ( $1.8 \times 10^6$  cells/mouse) intraperitoneally under aseptic conditions. The day of tumor inoculation was considered as day 0 and day five as a ‘mild phase of tumor growth’.<sup>15</sup> On day six, the nephrotoxicity was induced in groups 3, 4, and 5 by administering a single dose of cisplatin (8, 10, 15 mg/kg body weight, i. p, respectively) and five days were allowed to develop maximum renal damage.<sup>5,16</sup> Bodyweight, food intake, diarrhea, and abnormal behaviors were observed daily. The body weight was found to be directly proportional to tumor development.

After the five days of cisplatin injection, under anesthesia, the right kidneys were collected from three mice in each group for histopathological examination. The hematoxylin and eosin-stained kidney slides were blinded before sending for histological examination by two pathologists. The difference in opinion between two observers was finalized by consensus. During the histological

examination, the scoring of renal tubular epithelial necrosis was done as follows<sup>17</sup>; 0: normal, 1:  $\leq$ 10% renal tubular epithelial (mild) necrosis, 2 : 11–25% renal tubular epithelial (moderate) necrosis, 3: 26–50% renal tubular epithelial (moderate to severe) necrosis, 4 : 51%–75% renal tubular epithelial (severe) necrosis, 5:  $>$ 76% renal tubular epithelial (very severe/extensive) necrosis. The remaining three animals in each group were observed for changes in body weight, food intake, diarrhea, abnormal behaviors, and spontaneous death due to cancer. Based on the results of this experiment cisplatin dose of 8 mg/kg, i. p was chosen for further experiments.

### 2.3.3. Reversal of cisplatin-induced nephrotoxicity by VC in EAC mice

We had six groups with 12 mice in each. Group 1 and 2 served as normal control and EAC control. EAC was induced on day 0, as described in section 2.2.3. After the mild phase of tumor growth, the renal damage was induced in groups 3–6 by injecting a single intraperitoneal injection of cisplatin (8 mg/kg, i. p) on day six. Group 3 served as cisplatin control and Group 4, 5, and 6 received CAE (400 mg/kg), butanol fraction (400 mg/kg) and aqueous fraction (400 mg/kg) respectively. This effective dose was chosen based on our earlier study.<sup>10</sup> The extract and fractions were administered orally for five days from day 11 to day 15. Bodyweight was noted daily.

## 2.4. Parameters assessed

### 2.4.1. Parameters for reversal of renal damage and regeneration

On day 16 (after 5 days of VC treatment), under ketamine anesthesia (50 mg/kg i. p), blood was withdrawn from six animals from each group to evaluate the blood urea and creatinine. The histology of kidney and renal necrosis grading were done as described under section 2.3.2.

### 2.4.2. Parameters to evaluate whether VC compromises anticancer action of cisplatin

**2.4.2.1. Determination of tumor volume.** On day 16, under anesthesia, the abdominal cavity was opened and peritoneal ascites fluid was collected to measure tumor volume.

**2.4.2.2. Estimation of viable tumor cell count<sup>18</sup>.** About 0.1 mL of ascites fluid was thoroughly mixed with 0.1 mL of trypan blue (0.4% in normal saline) dye. The diluted ascites fluid suspension was then charged into white blood cells (WBC) hemocytometer. Under the microscope, the viable (unstained) cells were counted in four WBC chambers. The mean number of viable tumor cells in all four chambers was calculated using the formula, Mean number of viable cells  $\times$  dilution factor  $\times 10^4$ .

**2.4.2.3. Determination of mean survival time and % increase in life span.** Six mice in each group were monitored twice daily for 120 days and mortality was recorded. Mean survival time (MST) for a particular group was calculated as follows; [survival days of animal 1 + animal 2 ... +6]/6. The percentage increase in life span (% ILS) was calculated using the formula, [(Mean survival time of test group/Mean survival time of EAC control group) – 1]  $\times$  100.

## 2.5. Statistical analysis

All the data were expressed in mean  $\pm$  SD or median (interquartile range 25%, 75%). The data were analyzed using SPSS 11.0 version by One way ANOVA, followed by Dunnet's Post hoc test and a p-value of  $<$ 0.05 was set as statistical significant. For data that did not follow normality, the Mann-Whitney *U* test was performed.

## 2.6. Qualitative gas chromatography-mass spectroscopy (GC-MS)

The GC-MS analysis of CAE was carried out using Shimadzu Make QP-2010 with a non-polar 60 M RTX 5MS Column. The carrier gas used was helium. The temperature programming was as follows; the initial oven temperature was 40 °C for 3 min and the final temperature was 480 °C with the rate at 10 °C [min.sup.-1]. A 2  $\mu$ L CAE sample was injected with splitless mode. The mass spectra were recorded over 35–650 atomic mass unit (amu) range with electron impact ionization energy 70 eV and the total running time was 27.12 min. Interpretation of the mass spectrum of GC-MS was made using the database from National Institute Standard and Technology (NIST) that was having more than 62,000 patterns. The name, molecular weight, chemical structure, and retention time of the phytochemicals were ascertained.

## 3. Results

### 3.1. Standardization of cisplatin-induced nephrotoxicity in EAC mice

EAC bearing mice that received 10 and 15 mg/kg of cisplatin died within ten days of cisplatin injection. This could be due to the burden of cancer as well as cisplatin toxicity. Hence, further study was performed with 8 mg/kg of cisplatin.

### 3.2. Reversal of nephrotoxicity from cisplatin

#### 3.2.1. Bodyweight

Mice were matched for body weight while grouping before any intervention. EAC mice gained weight of 7.5 g within 16 days, which was due to the tumor burden in the ascites. A single dose of cisplatin (8 mg/kg) injection significantly ( $p = 0.001$ ) reduced the bodyweight of EAC bearing mice. Both BF and AF further reduced the bodyweight to a certain extent, whereas CAE improved the bodyweight compared to the cisplatin group. Mice treated with AF developed diarrhea on the fourth day, which might have contributed to severe weight loss in this group.

#### 3.2.2. Blood parameters

Eleven days after a single dose (8 mg/kg, i. p) of cisplatin injection, the blood urea and creatinine were not significantly elevated. This could be due to the spontaneous recovery of altered renal function induced by cisplatin (Table 1).

#### 3.2.3. Histopathology

EAC bearing mice had normal nephrons with inflammation on the renal capsule (serositis). Whereas, cisplatin produced maximum renal damage in the cortico-medullary junction area with 60% proximal tubular damage. The damage was not observed in the outer cortex or inner medullary region. Cisplatin-induced renal damage was significantly reversed by CAE ( $p = 0.002$ ), BF ( $p = 0.002$ ) and AF ( $p < 0.001$ ) to 30%. Besides, CAE and BF treated animals showed regeneration of 50%–75% of proximal tubular cells. AF treated animals did not show regeneration. So, this study revealed both CAE and BF significantly reversed the cisplatin-induced proximal renal tubular necrosis and regenerated tubular epithelial cells (Figs. 1 and 2A).

### 3.3. Parameters to evaluate whether VC compromises short term anticancer benefit of cisplatin

#### 3.3.1. Hematology

EAC bearing mice showed a significant increase in white blood cells, especially lymphocytes, due to severe inflammation in the

**Table 1**  
Effect of *Vernonia cinerea* on blood urea, creatinine, tumor volume and % viable tumor cells in cisplatin injected EAC mice.

Groups	Urea (mg/dL) mean $\pm$ SD	Creatinine (mg/dl) mean $\pm$ SD	Tumor volume (ml) median (25%, 75%),	% Viable cell median (25%, 75%),
Normal mice	35.19 $\pm$ 6.02	0.54 $\pm$ 0.10	–	–
EAC control	46.21 $\pm$ 9.76	0.46 $\pm$ 0.06	12 (10, 15)	98 (97, 100)
EAC + cisplatin	38.00 $\pm$ 12.49	0.53 $\pm$ 0.08	0 (0, 0) *	17 (0, 50) *
EAC + cisp + CAE 400 mg/kg	37.57 $\pm$ 8.55	0.39 $\pm$ 0.12	0 (0, 1.25) *	0 (0, 93) *
EAC + cisp + BF 400 mg/kg	39.86 $\pm$ 13.28	0.43 $\pm$ 0.10	0 (0, 1.10) *	0 (0, 98) <sup>#</sup>
EAC + cisp + AF 400 mg/kg	43.21 $\pm$ 13.17	0.44 $\pm$ 0.11	1.55 (1.50, 2.00) *	96 (94, 100)

EAC - Ehrlich ascites carcinoma, Cisp – cisplatin (8 mg/kg, i.p), CAE - Crude aqueous extract, BF – Butanol fraction, AF – Aqueous fraction, \*p = 0.002, #p = 0.015 vs. EAC control (statistics - Mann-Whitney U test).

peritoneum and it was significantly lower in cisplatin-treated mice. Concurrent administration of CAE, BF, and AF with cisplatin did not alter the efficacy of cisplatin on WBC and lymphocyte levels. The reduced hematocrit % (HCT) in EAC control mice was found to be increased in cisplatin, CAE, BF, and AF treated animals (Fig. 2).

### 3.3.2. Tumor volume

The peritoneal ascites tumor fluid volume in EAC bearing mice was 12 mL. Cisplatin treatment in the EAC mice significantly reduced ( $p = 0.002$ ) the tumor volume. CAE, BF, and AF administration did not significantly alter the efficacy of cisplatin on tumor volume (Table 2).

### 3.3.3. Viable tumor cells

EAC bearing mice had 98% viable tumor cells in the peritoneal ascites fluid, whereas the cisplatin treatment significantly ( $p = 0.002$ ) reduced viable tumor cells by 83% compared to the EAC mice. CAE and BF administration did not increase the viable tumor cells after cisplatin treatment. Whereas, the AF treatment increased the viable tumor cells which was similar to EAC control (Table 1).

## 3.4. Parameters to evaluate whether VC compromises long term anticancer benefit of cisplatin

### 3.4.1. Survival analysis

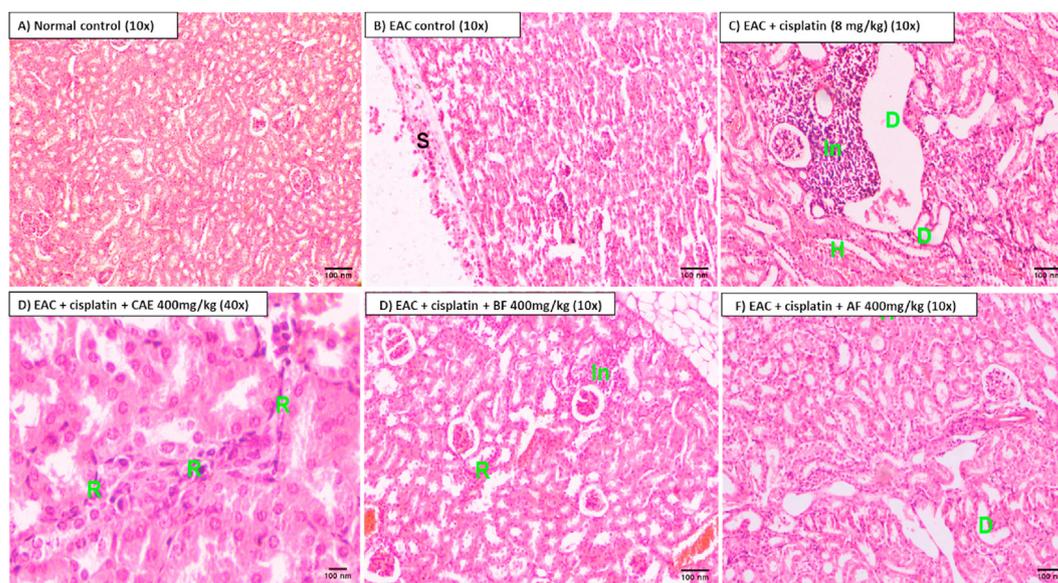
All animals in the EAC control group died by 20 days. Four EAC mice treated with cisplatin died during 120 days of the observational period. Three animals survived in a group that received additional treatment of CAE after cisplatin therapy. Only one animal survived in BF and AF treatment groups. From this study, it could be inferred that the CAE improved the life span of EAC bearing mice after cisplatin therapy (Fig. 3).

### 3.4.2. Mean survival time

Cisplatin treatment increased the mean survival time (days) of EAC mice. It was further increased to 83 days in EAC mice which received CAE. However, BF and AF administration reduced the mean survival time compared to the cisplatin-treated group (Fig. 3).

### 3.4.3. Percentage increase in life span (% ILS)

The % ILS for a group was calculated by summarizing the mean survival time of each animal; hence, this is a more reliable parameter than the mean or median survival time. Compared to



**Fig. 1.** Effect of *Vernonia cinerea* on cisplatin-induced nephrotoxicity in EAC mice.

A) Normal kidney with normal glomerulus, proximal tubules, and vasculature.

B) EAC control kidney showed normal nephrons with serositis (S) in kidney capsules.

C) EAC + cisplatin (8 mg/kg) control kidney showed 60% damage in proximal tubules of the cortico-medullary junction, which was characterized by necrosis, proximal tubular dilatation (D), inflammation (In), and hemorrhage (H).

D) EAC + cisplatin (8 mg/kg) + crude aqueous extract (400 mg/kg) showed 30% proximal tubular damage, regeneration (R) in proximal tubular cells.

E) EAC + cisplatin (8 mg/kg) + butanol fraction (400 mg/kg) showed 30% proximal tubular damage and regeneration (R) in proximal tubular cells, with mild inflammation (In).

F) EAC + cisplatin (8 mg/kg) + aqueous fraction (400 mg/kg) showed 30% proximal tubular damage including proximal tubular dilatation (D).



**Fig. 2.** Effect of Vernonia cinerea on renal necrosis grade and hematology parameters in cisplatin induced nephrotoxicity EAC bearing mice. **A-** Renal necrosis grade, **B-** blood cells, **C-** Differential counts, **D** – hematocrit values EAC - Ehrlich ascites carcinoma, Cisp – cisplatin (8 mg/kg, i. p), CAE - Crude aqueous extract, BF – Butanol fraction, AF – Aqueous fraction, HCT – hematocrit, MCV – Mean corpuscular volume, MCH - Mean Corpuscular Hemoglobin, MCHC - Mean Corpuscular Hemoglobin Concentration, RDW - Red blood cell distribution width.

EAC control mice, % ILS of the cisplatin-treated group was 244% and it was further extended to 379% after CAE administration. The % ILS in CAE treated group was 1.6 times higher than the cisplatin alone treated group. The % ILS seen with BF and AF administrations was lower compared to the cisplatin-treated group, which indicated that these fractions might have a protective action on cancer cells too, hence reducing the life span of cisplatin-treated mice. These results inferred that the CAE, in addition to its nephroprotective action extends the life span of cancer animals treated with cisplatin.

#### 3.4.4. Qualitative gas chromatography and mass spectroscopy of CAE

Since CAE significantly reversed cisplatin-induced renal damage without compromising anticancer efficacy of cisplatin, CAE was further subjected to phytochemical standardization. The qualitative GC-MS chromatogram of the CAE of VC showed the presence of 38 compounds. All the compounds were already listed in the Chemical Abstract Services (CAS) registry (Fig. 4, Table 2).

## 4. Discussion

In a clinical setting, an ideal nephroprotector is expected to protect the kidney or reverse the renal damage during chemotherapy without compromising the beneficial anticancer efficacy of cisplatin. Our earlier study demonstrated that the VC at a dose of 400 mg/kg/day reversed the renal damage to the maximum extent when administered therapeutically.<sup>10</sup> The current study evaluated whether VC reverses the renal damage without compromising the anticancer efficacy of cisplatin in EAC mice. In our study, cisplatin-induced 60% proximal tubular damage in corticomedullary junction, which was significantly reversed by CAE and its two fractions BF and AF. Besides, CAE and BF showed regeneration of 50%–75% of proximal tubular cells, whereas AF treatment did not show any

regeneration.

The CAE, BF, and AF did not compromise the beneficial effect of cisplatin in the EAC mice in terms of normalization of elevated WBC, lymphocytes, monocytes, and granulocytes. Both CAE and BF treatments did not compromise the cisplatin mediated reduction in viable cancer cells, whereas AF treatment increased the percentage of viable cancer cells and it was similar to EAC control, which means that AF nullified the anticancer efficacy of cisplatin. This could be due to the extension of protective activity to cancer cells as well.

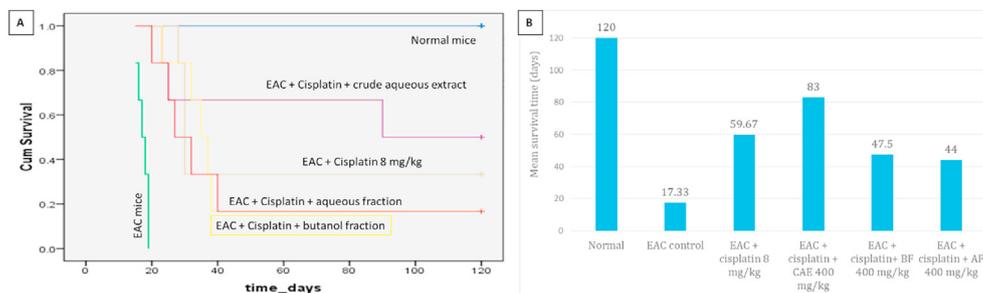
Cisplatin therapy increased the life span of EAC bearing mice. The CAE administration further extended the % ILS, which was 1.6 times higher than the cisplatin alone group. Whereas BF and AF reduced the % ILS seen with cisplatin, indicating the antagonizing anticancer activity cisplatin, possibly by extending their protective action on cancer cells too. This indicated that CAE reversed renal damage without compromising the anticancer effect of cisplatin and also increased the life span of EAC mice treated with cisplatin, which is an added benefit. This supports the traditional Siddha medical literature claim of using the VC whole plant decoction (CAE) to reverse the metal-based drug toxicities during cancer therapy.<sup>9</sup>

This is the first report on the phytochemical profile of CAE of VC. The qualitative GC-MS chromatogram showed the presence of 38 compounds and these compounds have not been reported in other extracts of VC. In a study bioassay-guided fractionation of ethanol extract led to the isolation of vernolide-A, an active principle with anticancer activity. Vernolide-A inhibited lung metastasis of melanoma cells in mice and increased its life span.<sup>19</sup> The results of our study clearly showed that the CAE has a different phytochemical profile than ethanol extract and also suggests that different extracts of the same plant might have ironical actions such as anticancer as well as protective actions.

Biological activities of some of the compounds of CAE, including,

**Table 2**  
List of phytochemicals along with their chemical formula from Qualitative Gas Chromatography–Mass Spectroscopy of crude aqueous extract of *Vernonia cinerea*.

S.N	RT	Probability	Compound name	Formula	Mol. weight	CAS number
1	5.09	36.83	Acetanilide	C8H9NO	135	103-84-4
2	5.19	15.65	D:A-Friedoleanan-3a-ol	C30H52O	428	5085-72-3
3	5.32	69.10	Syringol OR Pyrogallol 1,3-dimethyl ether	C8H10O3	154	91-10-1
4	5.41	81.48	1,2,4,5-Benzene tetracarboxylic 1,2,4,5-diimide, N,N'-bis(o-chlorophenyl)-	C22H10Cl2N2O4	436	6626-72-8
5	5.49	70.75	1,2,4,5-Benzene tetracarboxylic 1,2,4,5-diimide, N,N'-bis(o-chlorophenyl)-	C22H10Cl2N2)4	436	6626-72-8
6			5a'-Cholestan-3-one, oxime	C27H47NO	401	2735-21-9
7	5.54	5.29	N-p-Tosylpirrolidin	C11H15NO2S	225	6435-78-5
8	5.58	36.81	Roridin A	C29H40O9	532	14729-29-4
9	5.76	15.12	1-Naphthalene isocyanide	C11H7N	153	1984-04-9
10	5.85	14.36	1,10-Di-(decahydro-1- naphthyl)decane	C30H54	414	55268-64-9
11	5.96	9.06	1,2,3,6-Tetrahydrophthalimide	C8H9NO2	151	85-40-5
12	6.12	20.47	Isovanilic acid	C8H8O4	168	645-08-9
13	6.34	5.41	Benzanilide, N-methyl-4-nitro-	C14H12N2O3	256	961-61-5
14	6.46	97.37	Cycloheptasiloxane, tetradecamethyl-	C14H42O7Si7	518	107-50-6
15	6.63	22.77	Xanthine, 3-methyl-OR 3-methylxanthine	C6H6N4O2	166	1076-22-8
16	6.78	15.63	Amiphenazole OR Daptazile	C9H9N3S	191	490-55-1
17	6.87	55.92	Octadecamethyl-cyclononasiloxane	C18H54O9Si9	666	556-71-8
18	7.05	26.52	Toluene, 3,4,5-trimethoxy-	C10H14O3	182	6443-69-2
19	7.17	55.08	Guaiacylacetone	C10H12O3	180	2503-46-0
20	7.30	8.42	Equilin	C18H20O2	268	474-86-0
21	7.39	18.03	<b>Astaxanthin</b>	<b>C40H52O4</b>	<b>596</b>	<b>472-61-7</b>
22	7.57	14.24	Benzoyl-L-tyrosine ethyl ester	C18H19NO4	313	3483-82-7
23	9.23	91.97	Hexadecamethyl-cyclooctasiloxane	C16H48O8Si8	592	556-68-3
24	11.20	30.98	Cyclobutane, phenyl	C10H12	132	4392-30-7
25	11.38	15.12	2-chloro-4-cyclohexylphenol	C12H15ClO	210	3964-61-2
26	11.53	33.97	Leuco malachite green	C23H26N2	330	129-73-7
27	11.69	33.69	2-(4-tetra-Butyl-2-methyl phenoxy) ethanol	C13H20O2	208	54934-87-1
28	12.27	75.34	Octadecamethyl-Cyclononasiloxane	C18H54O9Si9	666	556-71-8
29	12.77	18.37	N,N-Dimethyl-4 nitro-3-trifluoromethylaniiline	C9H9F3N2O2	234	41512-62-3
30	13.36	53.26	4' 5 6 7-tetramethoxyflavone	C19H18O6	342	1168-42-9
31	13.53	6.85	<b>Betulin</b>	<b>C30H50O2</b>	<b>442</b>	<b>473-98-3</b>
32	13.95	34.13	Dibutyl azelate	C17H32O4	300	2917-73-9
33	14.48	22.11	Palmitin, 1,2-di-	C35H68O5	568	761-35-3
34	14.61	32.47	1,2-Benzenedicarboxylic acid, dibutyl ester	C16H22O4	278	84-74-2
35	15.88	19.92	Dodecamethylcyclohexasiloxane	C12H36O6Si6	444	540-97-6
36	16.66	18.75	Linoleocyl chloride	C18H31ClO	298	7459-33-8
37	17.21	16.15	5-n-Butyl-6-n-hexylindan	C19H30	258	55030-45-0



**Fig. 3.** Effect of *Vernonia cinerea* on survival period (120 days of observational period) of EAC mice treated with cisplatin. A-Survival graph for 120 days, B- mean survival time EAC - Ehrlich ascites carcinoma, Cisplatin - cisplatin (8 mg/kg), cisp - cisplatin, CAE-crude aqueous extract, BF- butanol fraction, AF - aqueous fraction. Died, alive status: Normal control (0,6), EAC control (6,0), EAC + Cisplatin control (4,2), EAC + cisp + CAE (3,3), EAC + cisp + BF (5,1), EAC + cisp + AF (5,1).

acetanilide, amiphenazole, equilin, octadecamethyl-cyclononasiloxane, betulin, and astaxanthin have been already reported. Among them, betulin, and astaxanthin have pharmacological actions on the kidney. Betulin significantly reduced the size of renal calculi and enhanced the excretion of calcium, phosphate, and oxalate in ethylene glycol induced urolithiasis rats.<sup>20</sup> In another study, betulin reduced the kidney injury by inhibiting inflammation and TLR4/NF- $\kappa$ B signaling in sepsis-induced renal damage in rats developed by caecal ligation and puncture.<sup>21</sup> Cisplatin induces renal epithelial injury by releasing damage-associated molecular pattern molecules (DAMPs), which activate Toll-like Receptor 4 (TLR4), results in the production of a number of cytokines and chemokines, including TNF- $\alpha$ . These cytokines and chemokines

upregulate the adhesion molecules and also attract inflammatory cells like neutrophils into the injured region.<sup>22</sup> By inhibiting the inflammation and TLR4/NF- $\kappa$ B signaling, betulin could have protected the cisplatin-induced renal damage.

Astaxanthin is an orange-red carotenoid present in many plants and seafood, approved in 1999 by the US FDA as a dietary supplement. It prevented the mesangial matrix expansion in rats with diabetic nephropathy<sup>21</sup> and improved glomerular and interstitial fibrosis in adriamycin-induced glomerulosclerosis in mice.<sup>22</sup> In mice with unilateral ureteral obstruction-induced renal fibrosis, it prevented pathological changes in the kidney tissues, decreased the peritubular capillary density through downregulating levels of thrombospondin 1 via inactivation of TGF  $\beta$ 1/Smad signaling

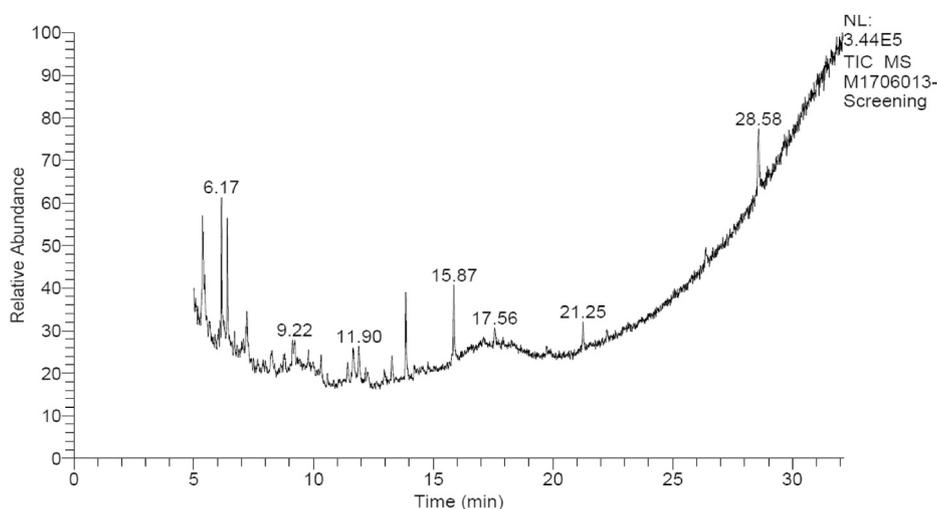


Fig. 4. Qualitative gas chromatography-mass spectroscopy of crude aqueous extract of *Vernonia cinerea*.

pathway and upregulating vascular endothelial growth factor.<sup>23</sup> In another study, mice were pretreated for 14 days with astaxanthin before the induction of ischemia/reperfusion-induced renal injury. Astaxanthin prevented tubular apoptosis/necrosis, inflammation, and epithelial-to-mesenchymal transition via scavenging free radicals.<sup>24</sup> Astaxanthin has been proven for its protective effect on the lung by suppressing elevated inflammatory factors, MAPK phosphorylation, and NF- $\kappa$ B activation, and also significantly improved survival rate in mice with lipopolysaccharide-induced sepsis and acute lung injury.<sup>25</sup> Cisplatin nephrotoxicity is associated with ROS dependent increased expression of TNF- $\alpha$  in the kidney, activation MAPK and NF $\kappa$ B.<sup>26,27</sup> Hence, astaxanthin might offer protective action in cisplatin-induced nephrotoxicity too.

Astaxanthin gets accumulated substantially in mitochondria and reduces mitochondria-associated lipid peroxidation and ROS production.<sup>22,25</sup> Since proximal renal tubules have the highest densities of mitochondria and cisplatin accumulates 4–6 fold higher in mitochondria than plasma, mitochondrial DNA is an important target for cisplatin. Besides, DNA repair mechanisms are less efficient in mitochondria. Hence, proximal tubules are more susceptible to cisplatin-induced damage.<sup>27–30</sup> The astaxanthin present in CAE could have prevented mitochondrial DNA damage caused by cisplatin.

*Vernonia cinerea* is popularly known as an anti-smoking Thai medicinal plant. Supplementation of VC decoction along with exercise among smokers reduced the smoking rate through modulating oxidative stress and releasing beta-endorphin.<sup>31</sup> A meta-analysis of five clinical trials in 347 smokers has concluded that VC is efficacious for smoking cessation with the same rate of adverse events such as dizziness, tongue numbness, or dislike of smell or taste of a cigarette, as placebo without any serious adverse event.<sup>32</sup> With this background, further clinical studies could be carried out using a CAE of *Vernonia cinerea* in cancer patients undergoing cisplatin treatment. The mechanism of nephroprotective activity of betulin and astaxanthin in cisplatin-induced renal damage could be studied in detail. In addition, phytochemical profiling of fractions including BF and AF and its comparison with that of CAE might provide better insight on the possible mechanism of nephroprotective action of CAE.

## 5. Conclusion

CAE of *Vernonia cinerea* reversed the cisplatin-induced renal

damage and also regenerated the necrosed proximal tubular epithelial cells without compromising the anticancer action of cisplatin. When it was further fractionated, the reversal activity of renal damage was retained, but the beneficial anticancer effect of cisplatin was compromised. Hence, it could be concluded that the collective effect of all phytochemicals present in CAE was responsible for its beneficial effect. Astaxanthin and betulin, two well-known nephroprotective compounds present in the CAE of VC, could have protected the renal tubules by modulating cisplatin-induced oxidative stress, preventing mitochondrial DNA damage, inhibiting the inflammation and TLR4/NF- $\kappa$ B signaling and reducing MAPK phosphorylation in renal tubules. Further comprehensive studies are needed to elucidate the nephroprotective mechanism of CAE of VC.

## Conflicts of interest

We declare there is no conflicts of interest in this research.

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## Declaration of competing interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtcm.2020.08.004>.

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