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Original Research Paper

Attenuation of Cyclophosphamide-induced Genotoxicity and Oxidative Stress by *Justicia wynaadensis* (Nees) T. Anders - A Study in Swiss Albino Mice

Bioscience

KEYWORDS: Cyclophosphamide; *Justicia wynaadensis*; micronucleus assay; genotoxicity; cytotoxicity;

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ABSTRACT

Justicia wynaadensis (Nees) T. Anders, an endemic plant of Western Ghats having special ethnomedicinal significance has been evaluated for its geno- and cyto-protective effects. Mice were pre-treated with Aqueous extract of the aerial part of J. wynaadensis (Nees) T. Anders (AEAPJW) at different doses (50, 100 and 150mg/kg b.w.) for consecutive 7 days. One hour after the last administration of the extract, animals were injected with CP at 50 mg/kg. Bone marrow micronucleus assay was performed after 30 hours of exposure and results were compared with the CP alone treated group. The extract was subjected to *in vitro* analysis to determine its antioxidant and lipid peroxidation inhibitory potency by employing the standard procedures. The extract imparted protective effects against CP-induced genotoxicity and cytotoxicity as indicated by the significant reduction in the frequency of micronucleated cells (MN-cells) and recovery of P/N ratio. The extract exhibited potential antioxidant and lipid peroxidation inhibitory activities *in vitro* in a dose-dependent manner to certain extent. Thus, AEAPJW possessing potential antioxidant and lipid peroxidation inhibitory properties could minimize the free-radical mediated geno- and cytotoxicities induced by cyclophosphamide.

1.0 Introduction

Cyclophosphamide (CP), an alkylating agent is commonly used for the treatment of a majority of cancers and in chronic regimens of the auto-immune disorders. Despite CP is reliable in various chemotherapy procedures, it has a major limitation as it adversely affect the non-target cells/tissues. The genotoxicity and cytotoxicity of CP in bone marrow are well reported (Arencibia-Arrebola et al. 2013, Pacini and Borziani, 2009). Extensive research is being underway worldwide to explore the natural sources, which can prevent/minimize the toxic effects of non-target cytotoxic drugs like CP. *J wynaadensis*, a seasonal shrub belonging to Acanthaceae family is endemic to Western Ghats, a "Biodiversity hotspots" in the world. It is consumed by the local people of Kodagu (Karnataka State, India) during the *Kataka* or *Adi* month of the Hindu calendar, i.e., July to August with the belief that that the plant acquires 1 medicinal property per day and reaches the maximum on the 17th day (first week of August) with a total of seventeen medicinal properties (Medapa et al., 2011). Ethanomedicinal survey indicated that this plant is used to treat asthma and to boost immune system in Kodagu district (Lingaraju et al, 2013), and rheumatic swelling by the Kurichiar tribes of Tirunelli forest, Wynaad district of Kerala. The plant is found to have certain biomedicinal values; Protein fraction of *J. wynaadensis* has been demonstrated to possess the cysteine protease inhibition and anti-proliferative activity (Nayana and Manjula, 2015), antibacterial property against *Klebsiella pneumoniae* (Ponnamma and Manjunath, 2015), lowering of cellular cholesterol level (Subbiah and Norman, 2002), antioxidant (Medapa et al., 2011) and anti-inflammatory (Vidyabharathi, 2012) activities. The plant has also been demonstrated to exhibit catalase and peroxidase activities (Medapa et al., 2011). Various solvent extracts of *J. wynaadensis* have been analyzed for the compositions through TLC, HPTLC and GC-MS techniques, and are known to contain flavonoids, phenolics, polyphenols, vitamins, alkaloids, proteins, carbohydrates, glycoproteins, etc. (Abhishek et al., 2015; Medapa et al., 2011; Ponnamma and Manjunath, 2012).

Earlier reports indicate that this plant is a rich source of antioxidant molecules (Abhishek et al., 2015), and thus it can be hypothesized that this plant might exert modulatory effects on CP-induced geno and cytotoxicity thereby minimize the drug-induced mitotic depression. It is known that CP apart from its basic mechanism of DNA damage causing cell death/apoptosis; it also induces oxidative stress through free radical generation leading to various adverse effects (Habibi et al., 2015). The present study was taken up to evaluate the genoprotective and cytoprotective effects of the AEAPJW, using the Swiss albino mouse as test system and was also aimed to assess the extract for its antioxidant and lipid peroxidation inhibitory activity through *in vitro* assay.

2.0 Materials and methods

2.1. Chemicals and Reagents

Cyclophosphamide (Cycloxan, CAS No. 50-18-0; Batch No. KB 9124003 Biochem Pharmaceutical Industries LTD. Mumbai, India, was used as the positive control. Phosphate buffered saline (PBS), Giemsa and May-Grunwalds stain powders, reagents of analytical grade for antioxidant/free radical scavenging and lipid peroxidation assays were procured from SRL, India, and from Merck India.

2.2. Collection of the plant

Justicia wynaadensis (Nees) T. Anders (common name: Maddu soppu or Maddu thoppu) was collected from Murnad village, Madikeri taluk of Kodagu district, Karnataka, India (Time zone: IST (UTC+5:30; Altitude: 120 meters above sea level) during the rainy season (1st week of August). The identification of the plant was authenticated taking the help of faculty in the Department of Applied Botany, Mangalore University. A specimen plant is preserved as herbarium and voucher No. is given for future reference. The plant name has been checked with www.theplantlist.org and Indian Biodiversity Portal: http://indiabiodiversity.org.

2.3. Preparation of the plant extract

Maceration method desrcibed by Harborne, 1973 was employed to prepare the aqueous extract of the fresh aerial part (stem and leaves) of the plant taken into consideration of the practical relevance of its traditional utility in healthcare and medicine. The percentage yield of extract was determined (Raaman, 2006).

2.4. Genoprotective and cytoprotective study

2.4.1. Animals

Swiss albino mice (*Mus musculus;* 2n = 40; 8–10weeks old of the body weight 26±2 g) bred and maintained in the animal house of Biosciences department, Mangalore University were used for the experiments. The care, handling and usage of animals for the experiments are in accordance with the iinternationally accepted

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principles for laboratory animal use and care as prescribed by the European Community guidelines (EEC Directive of 1986; 86/609/EEC), and Committee for the Purpose of Control and Supervision on Experiments on Animals; CPCSEA), India. The animals were maintained under absolute hygienic condition in an air-conditioned room at a temperature of 24°C (±10°C) with 12 h light/dark cycle and 50±5% humidity. They were fed with standard mice pellets (Lipton, India) and water *ad libitum*. The present study was carried out after taking the prior approval from the Institutional Animal Ethics Committee (Ref. No.MU/AZ/504 (a)/IAEC/2015-2016, dated 23.09.2015).

2.4.2. Treatment schedule:
Animals were divided into 6 groups, each bearing 5 animals. The treatment was done as follows:

Group 1: Solvent control: 0.2 mL of distilled water (i.p. administration)
Group 2: Positive control: CP – 50 mg/kg (i. p. administration)
Group 3: Extract control: Extract 150 (mg/kg) – oral gavage for 7 consecutive days.
Group 4: CP -50 mg/kg (i.p) + Extract – 50 mg/kg x 7 (oral gavage)
Group 5: CP -50 mg/kg (i.p) + Extract – 100 mg kg x 7 (oral gavage)
Group 6: CP -50 mg/kg (i.p) + Extract – 150 mg kg x7 (oral gavage)

The required concentration of CP prepared in distilled water was administered by i.p. injection in 0.2 ml volume. For the protective effect, the selected doses of AEAPJW was suspended in distilled water administered to animals in 0.3ml volume by oral gavage, for 7 consecutive days followed by a single i.p. treatment of CP 1 hr after the last administration of the extract.

2.4.3. Micronucleus test:
Bone marrow micronucleus assay was performed by employing the standard method Schmid (1973) Formitotic depression effect, PCE/NCE ratio (P/N) was determined following the scoring criteria described by MacGregor et al., 1987. Recovery effect of the extract against CP-induced genotoxicity (micronucleated cells) and cytotoxicity (P/N ratio) was determined by using the formula:
% Recovery = (C – E) ÷ CP x 100; where 139 C is the value obtained for CP, and E is the value obtained for the extract.

2.5. Antioxidant studies/free radical scavenging activity
2.5.1. DPPH free radical scavenging activity
The stable DPPH, i.e., α,α-diphenyl-β-picrylhydrazyl radical scavenging activity of the extract was determined by employing the method of Blois *et.al*., 1958 with minor modification. The ability of the extract and positive control to scavenge DPPH free radical was calculated using the formula: Radical scavenging rate (%) = [1 – (A1 – A2) / A0] × 100, where A0 is the absorbance of the control (without sample) and A1 is the absorbance in the presence of the sample, A2 is the absorbance of sample without DPPH radical.

2.5.2 Lipid peroxidation inhibitory activity (LPIA)
LPIA of the extract was determined by following the standard protocol (Liegeois et al., 2000). Trolox was taken as the reference standard. Rate of the production of conjugated diene hydroperoxide by oxidation of linoleic acid incubated at 37°C for 10 min was monitored by measuring the optical density at 234 nm in UV-visible spectrophotometer. The percentage inhibition of the lipid peroxidation was calculated by using formula: Percentage of Inhibition (%) = (C – T) ÷ C x 100 Where, C is the absorbance of the control reaction and T is the absorbance in the presence of the samples (AEAPJW/trolox).

2.6. Statistical analysis
The statistical analysis of the results obtained for MN test was performed with the help of GraphPadInStat 3 package (GraphPad Software Inc, USA). One-way ANOVA test was applied to compare the differences among the groups. Differences were considered to be statistically significant if P< 0.05. All the antioxidant and LPO assays were performed in triplicates and standard deviation was applied for the mean values.

3.0. Results and Discussion:
Cyclophosphamide, a broad-spectrum anticancer drug despite of its therapeutic potential, is also known to cause severe side effects in non-target cells. An effective alternative to minimize the toxic impact exerted by anticancer drugs is the utilization of natural sources that are enriched with potent bioactive molecules.

In this context, *J. wynaadensis,* which is traditionally known for various medicinal properties was evaluated for its protective effect against geno- and cyto-toxicities induced by CP in bone marrow cells of Swiss albino mice. The treatment of the selected dose of CP (50 mg/kg b.w) induced the formation of micronucleated cells (PCE and NCE) at a significant level when compared with the vehicle control ($p<0.001$) as presented in the table 3.1. The result obtained is in parallel with the previous studies (Kai and Hong-xia, 2005).

Among the 3 selected doses of the plant extract, only the highest dose was taken to check the possible induction of genotoxicity, in order to reduce the number of animals for the experiment in accordance with the "3R's" principle as per the ICMR guidelines (ICMR, 2000). The frequencies of MN-PCE and MN-NCE were almost equal to that of the solvent control group indicating that the extract was not genotoxic at its highest dose (150mg/kg b.wt). There was also no induction of mitotic depression by the extract as indicated by the P/N ratio, which was almost near to 1. Hence, the dose-yield genoprotective and cytoprotective effects were evaluated taking two more doses, viz., 50 and 100 mg/kg/day. The administration of the extract significantly reduced the frequency of MN-cells compared with that of the CP alone treated group. The frequency of MN cells at 150 mg/kg b.wt. was almost similar to that of 100mg/kg b.wt. as indicated by the p value ($p<0.01$; Table 3.1) suggesting that the effect was not purely dose dependent. However, CP significantly induced mitotic depression ($p<0.001$). Upon administration of the extract the P/N ratio almost restored to normal levels. The observed effect was partially dose-dependent (CP+E-50 mg/kg, $p<0.05$; both CP+E-100 and CP+E-150 mg/kg, $p<0.01$; Table 3.1). The antioxidant/free radical scavenging activity of the extract was further confirmed by lipid peroxidation inhibitory activity. The *in vitro* analysis of the AEAPJW conducted in the present study revealed that the extract possesses potential lipid peroxidation inhibitory activity as indicated by the linoleic acid oxidation system. With the increase in concentration, the inhibitory activity was elevated giving the EC50 value, 317.00 ±4.53 µg /mL; which is almost half the potential compared to Trolox taken as the reference standard, whose EC50 value was found to be 157.00±2.84 µg /mL (Table 3.2). The qualitative tests indicate that the extract contains phenolics, polyphenols, alkaloids, carbohydrates, cardiac glycosides, anthraquinones, terpenoids, anthocyanins and proteins (Table 3.3). Among the identified constituents, phenolics (Dai and Mumper, 2010), polyphenols (Pandey et al., 2009), terpenoids (Grassmann, 2005), anthraquinones (Malterud et al., 1993) that have been demonstrated to possess potential antioxidant property. These constituents have also been reported to show modulatory effects on CP-induced genotoxicity *in vitro* and *in vivo* (Delarmelina et al., 2015). The significant reduction in the frequency of micronucleated cells induced by CP by the extract is further justified by the results obtained from *in vitro* free radical scavenging/antioxidant activity studies. The EC50 value derived from the assay employed in the present study, viz., DPPH free radical scavenging activity was found to be 14.56±1.29 µg /mL (table 3.4). Thus, by far the observations documented in the present study suggest that the plant extract has a modulatory influence on CP-induced geno and Cytotoxic effects.

4.0 Conclusion:
AEAPJW possesses potential antioxidant activity/constituents, which are responsible for the mitigation of the CP-induced toxicities at genetic and cellular levels. Therefore, the plant extract may be a considered as a good remedy to minimize the bone marrow

depression, a major side effect of CP-based chemotherapy in cancer patients. Further, it can be said that consumption of dessert / juice prepared from *J. wynaadensis* protects against free radical-mediated various health hazards, and the traditional nutraceutical importance of the plant for health benefits cannot be overruled.

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Author contributions:
All the authors have equally contributed to the present work; the first and corresponding authors are mainly involved in the work.

Table 3.1. Protective effect of AEAPJW on CP-induced micronucleated cells and reduction in P/N ratio:

Treatment Dose (mg/kg)	MN-PCE[A] %±SD	% Inhibition	Total MN-Cells % ±SD	% Inhibition	P/N ratio ±SD	% *Recovery*
Dist. water	0.18±0.05	–	0.15 ± 0.04	–	0.98±0.24	–
E-150x7	0.16±0.04	–	0.14±0.03	–	1.09±0.27	–
CP-50	2.73±0.51[c]	–	1.81±0.42[c]	–	0.54±0.18[c]	–
CP-50+E50x7	1.69±0.39[a]	38.09	1.13±0.32[a]	37.57	0.74±0.22[a]	*27.58*
CP-50+E100x7	0.98±0.32[b]	64.10	0.69±0.27[b]	61.88	0.87±0.25[b]	*43.10*
CP-50+E150x7	1.01±0.29[b]	63.00	0.71±0.26[b]	60.77	0.85±0.23[b]	*46.55*

[A] From 2000 PCE and corresponding NCE/animal; 4 animals/group
AEAPJW: Aqueous extract of aerial part of *Justicia wynaadensis* (Nees) T. Anders
CP: Cyclophosphamide
E: Extract

MN-PCE: Micronucleated polychromatic erythrocytes
MN-NCE: Micronucleated normochromatic erythrocytes

[c]P<0.001 Dist. water *Vs* CP-50
[a]*P*<0.05 , [b]*P*< 0.01 CP-50 *Vs* CP50+Extract

[A] From 2000 PCE and corresponding NCE/animal; 4 animals/group
AEAPJW: Aqueous extract of aerial part of Justicia wynaadensis (Nees) T. Anders
CP: Cyclophosphamide
E: Extract

MN-PCE: Micronucleated polychromatic erythrocytes
MN-NCE: Micronucleated normochromatic erythrocytes

[c]P<0.001 Dist. water Vs CP-50
[a]P<0.05 , bP< 0.01 CP-50 Vs CP50+Extract

Table 3.2. Lipid peroxidation inhibitory activity by AEAJW and trolox

Conc. (µg)	AEAPJW		Trolox	
	% of inhibition (Mean ±SD)	EC50 (µg /mL) (Mean ±SD)	% of inhibition (Mean ±SD)	EC50 (µg /mL) (Mean ±SD)
10	5.33±0.91	317.00±4.53	9.76±0.96	157.00±2.84
100	16.36±1.29		22.23±2.19	
200	29.98±2.06		58.26±3.51	
500	67.53±2.54		81.23±3.34	
1000	84.29±3.19		93.42±4.21	

Table 3.3 Preliminary phytochemical screening of AEAPJW:

Phytochemical classes	Name of the tests	Presence (+) / Absence (-)
Alkaloids	Dragendorff's test	+
	Wagner test	-
	TLC method 1	+
Glycosides	Born Tragers Test	-
Volatile oil	NaOH and HCl Test	-
Carbohydrates	Fehling test	+
Proteins	Biuret test	-
	Millons test	-
Steroids	Liebermann-Burchardt test	-
Flavonoids	Shinoda test	+
	TLC method	+
Phenolics/ Polyphenols	Lead acetate test	+
	Phenol test	+
Tannins	Baraemer's test	-
Anthraquinone	Borntrager's test	+
	Borntrager's (modiefied) test	+
Saponins	Frothing test	-
Cardiac glycosides	Kellar-Kiliani test	+
Anthocyanins	NaoH and HCL tests	+
Terpenoids	Liebermann- Burchardt test	+
	Salkowiski test	+

Table 3.4. Results obtained for DPPH scavenging activity of AEAPJW and of the reference standard:

Conc. (µg)	AEAPJW		L-ascorbic acid	
	% of inhibition (Mean ±SD)	EC50 (µg /mL) (Mean ±SD)	% of inhibition (Mean ±SD)	EC50 (µg /mL) (Mean ±SD)
5	13.2±0.67	14.56±1.29	28.61±1.49	12.26±1.34
10	32.4±1.23		45.21±1.92	
25	75.3±2.93		87.34±2.44	
50	84.9±3.72		93.79±2.43	
75	98.3±2.94		98.76±1.91	

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