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Modulation of the Micronucleus Formation by Total Extracts from Leaves, Bark and Seeds of *Pongamia pinnata*

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ABSTRACT

Possible anticlastogenic activity of *Pongamia pinnata* leaves, bark and seed extracts, applied at 25, 100, 500 and 1000 mg/kg body weight induced a micronucleus (MN) effect in a dose-dependent manner. A single dose of Positive clastogen, cyclophosphamide (CP) 50 mg/kg body weight – was administered to Swiss Albino mice. Various doses of plant extract showed a marginal increase in the frequency of MN. All three extracts showed an inhibitory effect on CP-induced MN in polychromatic erythrocytes. The petroleum ether extracts of *Pongamia* leaves, bark and seeds induced MN to a lesser extent than those of CP-induced groups. In the treatment of various extracts against CP-induced drug, we observed that the percentage inhibition varied depending on the time interval: in leaves or bark it was higher at 24 h then at 48 and 72 h. The percentage inhibition decreased at all given doses at both 48 and 72 h. This infers that the frequency of inhibition is relatively good at all doses when compared with individual treatment with CP. In the experiment with seeds extract treated with CP, the percentage inhibition was found to be extremely high with respect to doses and time intervals.

Keywords: crude extracts, Karanja, Leguminosae, medicinal plant

INTRODUCTION

In nature numerous environmental and industrial chemicals are capable of inducing genotoxic effects in man and other living organisms. Thus an increased exposure to hazardous chemicals can lead to carcinogenic and mutagenic events. A large number of naturally occurring (Rompelburg *et al*. 1995) compounds of plant origin are known to inhibit chemical mutagenesis and carcinogenesis in *in vitro* and *in vivo* assay systems (Ramel *et al*. 1986; Abraham *et al*. 1993). In recent years, there has been an increased awareness of the protective effects against the toxicity of chemicals and other environmental hazards by many natural compounds derived from plants (Sobti *et al*. 1989; Delmanto *et al*. 2001).

The *in vivo* bone marrow micronucleus (MN) assay allows for an effective assessment of both chromosomal damage and chromosomal loss induced by chemicals (Dass *et al*. 1997) because it is simpler and faster than chromosome analysis (Edwin *et al*. 2002). It also takes metabolism into account, as the genotoxicity of a substance results from the dynamic balance between its enzymic activation and its detoxification. Micronuclei originate from acentric chromosome fragments as well as from whole chromosomes lagging at the anaphase division of the nucleated precursor cells. They persist in the cytoplasm for some time and can be scored at interphase in polychromatic erythrocytes (Ledebur and Schmid 1973; Tucker and Preston 1996). Thus an increase in the frequency of micronucleated polychromatic erythrocytes (MPEs) is an indication of aneuploidy or induction of clastogenecity (Kirsch-Volders 1997).

Pongamia pinnata is a common medicinal plant belonging to the family Leguminosae, grown all over India, especially near the coast and extending from the Central to Eastern Himalayas to Ceylon. The seeds, leaves and oil derived from the seeds are all used in Hindu medicine as remedies for fever, skin diseases, piles, ulcers, bronchitis, whooping cough, etc. Pongam oil showed inhibitory effects on a few bacteria, namely *Bacillus subtillis* (NCIM 2117), *Escherichia coli* (NCIM 2079), *Pseudomonas aeruginosa* (NCIM 2036), and *Staphylococcus aureus* (NCIM 2079) and contains bitter flavonoid constituents, pongapin and karanjin (Duke 1983). The common name of this plant is 'Karanja' and the oil derived from the seeds is known as Karanja oil. The plants have a capacity to produce a large number of organic chemicals of high structural diversity, the secondary metabolites (Castello *et al*. 2002). In *P. pinnata* six compounds (two sterols, three sterol derivatives and disaccharide) together with eight fatty acids were isolated and characterized from the seeds (Shameel *et al*. 1996). Each part of the *P. pinnata* plant i.e. leaves, flowers, seed, roots have been described as useful in Ayurvedic remedy for various diseases, the plant is used in folk remedies for abdominal tumors in India and the seeds for keloid tumors in Sri Lanka, and powder derived from the plant for tumors in Vietnam. In Sanskritic India, seeds were used for skin ailments. Today the oil is used as a liniment for rheumatism. Leaves are active against *Micrococcus*; their juice is used for cold, cough, diarrhoea, dyspepsia, flatulence, gonorrhea and leprosy. A 70% ethanolic extract of *P. pinnata* leaves has potent anti-inflammatory activity against different phases (acute, sub-acute and chronic) of inflammation without side effects on gastric mucosa; the extract also showed significant anti-pyretic action against Brewers yeast-induced pyrexia (Srinivasan *et al*. 2001). Antiplasmodial activity against *Plasmodium falciparum* was reported from leaf extract *P. pinnata* (Simonsen *et al*. 2001). Antioxidant and anti-hyperammomic activity was observed in *P. pinnata* leaf extract on circulatory lipid peroxidation and anti-oxidant status was evaluated in ammonium chloride-induced hyper ammonium rats (Essa and Subrammanim 2006). Anti-diarrhoeal activity and anti-ulcer activity was reported in the methanolic extracts of *P. pinnata* roots (Prabha *et al*. 2003; Brijash *et al*. 2006). Roots are used for cleaning gums, teeth and ulcers. Bark is used internally for bleeding piles. Juices and oil

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Research Note

from the plant are antiseptic (Duke 1983). However, information on antimutagenicity or anticancer effects of the different extracts of this plant is scanty. Therefore the present study deals with the anticlastogenic potency of the leaves, seeds, and bark extracts of this plant in bone marrow cells of mice against the anticancer drug cyclophosphamide (CP) using an *in vivo* MN test.

MATERIALS AND METHODS

Plant materials

Leaves, bark and seeds of *Pongamia pinnata* were collected from the Lakkavalli Reserve Forest Range of the Western Ghats region of Karnataka, India and identified by comparing with the authenticated specimen deposited at the Kuvempu University herbaria (Voucher specimen KUDB/ Ang/822). The leaves, bark and seeds were shade dried, powdered mechanically and sieved (Sieve No. 10/44) and subjected to Soxhlet extraction using the solvent petroleum ether, 60-80 AR (SD Fine Chem Ltd., Mumbai, India).

Phytochemical studies

The extracts from leaves, bark and seeds by Soxhlet extraction method using petroleum ether solvent and concentrated under reduced pressure at 40 ± 5°C using a rotary flash evaporator (Büchi, Flawil, Switzerland). The crude petroleum ether extract was used for the screening studies.

Chemicals used

Cyclophosphamide (CP) at a single dose (50 mg/kg body weight) was used in the experiment. The choice of dose of positive mutagen was based on previous studies on the clastogenic potency of these drugs, where this dose produced a high frequency of MN in mice (Shyama Prasad *et al*. 2002). May-Grunwald and Giemsa stain powder were obtained from E-Merck, India, Bovine Serum Albumin from SRL, India and other Hi media reagents were used in the study.

Experimental animals

Swiss Albino mice of either sex (7-8 weeks old; 25-30 g body weight, *Mus musculus* L., 2n = 40) bred and maintained in the department were used. They were maintained in polypropylene shoe box type cages, bedded with rice husk, maintained 25 ± 2°C and 60 ± 5% RH. Animals were fed with a pelleted diet (Lepton, India) and water was provided *ad libitum*. The Institutional Ethical Committee (Registration No. 144/1999/CPCSEA/SMG) approved the study.

Treatment

The extracts were dissolved in 1% Gum acacia (Singh *et al*. 1996). The anticlastogenic effects of the different extracts were tested at four different concentrations i.e. 25, 100, 500 and 1000 mg/kg body weight. For the anticlastogenic test, different doses of the extract were administered orally for seven days and on the seventh day animals were injected intraperitonally with the selective dose of the positive clastogen (CP: 50 mg/kg body weight). The animals administered with 1% Gum acacia formed the negative control. Three animals per group were used in the experiment.

Bone marrow MN preparation and scoring

Bone marrow preparations were made at 24, 48 and 72 hours time intervals by a modified method of Schmid (1973). Bovine albumin (5%) was prepared in phosphate buffer saline (PBS) was used as the suspending medium. Animals were killed by cervical dislocation at the end of 24, 48 and 72 hours respectively. The air-dried slides were fixed in methanol and stained with May-Grunwald and Giemsa. Two thousand polychromatic erythrocytes (PCE) and corresponding normochromatic erythrocytes (NCE) were scanned from each animal for the presence of micronucleated cells.

Statistical analysis

Despite an insufficient sample size, statistical significance of the experimental results was determined by comparing the MN frequency in CP-only treated groups of animals with that of the drug + extract treated groups by using the student's *t*-test.

RESULTS AND DISCUSSION

The anticancer drug, CP, induced a significant MN whose effect was dose dependent (Vijayalaxmi and Venu 1999; Shyama Prasad *et al*. 2002). As in our experiment a single dose of CP (50 mg/kg body weight) was given to mice. Various doses of the extract showed a marginal increase in the frequency of MN and there was no significant difference between the treatments. Results at 24, 48 and 72 h after administration of the different extracts are described in **Tables 1**, **2** and **3**, respectively. All three extracts showed a significant inhibitory effect on CP-induced MN in PCE's. The petroleum ether extracts of *Pongamia* leaves, bark and seeds induced MN to a lesser extent than CP-induced groups. During the treatment of various extracts against CP-induced drug, we observed that the percentage inhibition varied with the different time intervals.

The percentage inhibition by leaves extract and bark extract with CP at the end of 24 h was high when compared with that at 48 and 72 h. There was a decrease in the percent inhibition at all given doses at 48 and 72 h. This infers that the frequency of inhibition is comparatively good at all doses when compared with that of individual treatment with CP. In the experiment with seed extract treated with CP, the percent inhibition was found to be extremely high with respect to doses and time intervals.

Chromosomal aberrations and MN formation are used as sensitive biological indicators of the clastogenicity of drugs and chemicals (Shyama Prasad *et al*. 2002). The results obtained in the present study revealed that the different extract of *P. pinnata* are capable of imparting protection

Table 1 Inhibitory effect of different doses of the petroleum ether extracts of *P. pinnata* on the induction of micronucleus in polychromatic erythrocytes by cyclophosphamide (50 mg/kg body weight) at 24 hours.

Treatment	Dose (mg/kg)	MN – PCE (%)	P/N Ratio
GA (1%)	-	0.07 (---) ± 0.01	0.99 ± 0.01
CP	50	0.77 (---) ± 0.03	0.86 ± 0.01
LE	25	0.23 (---) ± 0.01	1.43 ± 0.01
	100	0.17 (---) ± 0.01	1.29 ± 0.01
	500	0.23 (---) ± 0.01	1.06 ± 0.01
	1000	0.28 (---) ± 0.02	1.09 ± 0.01
LE + CP	25 + 50	0.17 (77.9) ± 0.02 a	0.91 ± 0.01
	100 + 50	0.22 (71.4) ± 0.01 b	0.92 ± 0.01
	500 + 50	0.23 (70.1) ± 0.01 a	1.06 ± 0.01
	1000 + 50	0.17 (77.9) ± 0.01 b	1.02 ± 0.01
BE	25	0.13 (---) ± 0.03	1.41 ± 0.01
	100	0.28 (---) ± 0.01	0.76 ± 0.01
	500	0.27 (---) ± 0.02	0.94 ± 0.01
	1000	0.27 (---) ± 0.03	0.75 ± 0.01
BE + CP	25 + 50	0.25 (67.5) ± 0.03 b	1.12 ± 0.01
	100 + 50	0.28 (67.5) ± 0.01 b	1.12 ± 0.01
	500 + 50	0.28 (63.6) ± 0.01 b	1.14 ± 0.01
	1000 + 50	0.23 (70.1) ± 0.02 a	0.78 ± 0.01
SE	25	0.18 (---) ± 0.02	0.68 ± 0.01
	100	0.17 (---) ± 0.01	0.74 ± 0.01
	500	0.30 (---) ± 0.01	0.98 ± 0.01
	1000	0.25 (---) ± 0.01	0.95 ± 0.01
SE + CP	25 + 50	0.18 (76.6) ± 0.02 b	0.80 ± 0.01
	100 + 50	0.18 (76.6) ± 0.01 a	0.68 ± 0.01
	500 + 50	0.22 (71.4) ± 0.01 b	1.09 ± 0.01
	1000 + 50	0.22 (71.4) ± 0.01 a	1.11 ± 0.01

Values are mean ± SE of 3 animals in each group. Figures in parentheses are percent inhibition
GA – Gum acacia; CP – Cyclophosphamide; LE – Leaf extract; BE – Bark extract; SE – Seed extract.
P values: a <0.01, b <0.001

Table 2 Inhibitory effect of different doses of the petroleum ether extracts or *P. pinnata* on the induction of micronucleus in polychromatic erythrocytes by cyclophosphamide (50 mg/kg body weight) at 48 hours.

Treatment	Dose (mg/kg)	MN – PCE (%)	P/N Ratio
GA (1%)	-	0.07 (---) ± 0.02	0.95 ± 0.01
CP	50	0.48 (---) ± 0.01	0.81 ± 0.01
LE	**25**	0.08 (---) ± 0.02	1.43 ± 0.01
	100	0.05 (---) ± 0.02	0.95 ± 0.01
	500	0.13 (---) ± 0.02	1.18 ± 0.01
	1000	0.18 (---) ± 0.02	0.87 ± 0.01
LE + CP	25 + 50	0.13 (69.8) ± 0.02 a	0.87 ± 0.01
	100 + 50	0.18 (58.1) ± 0.02 a	0.71 ± 0.01
	500 + 50	0.17 (60.5) ± 0.01 a	0.99 ± 0.01
	1000 + 50	0.20 (53.5) ± 0.01 a	0.95 ± 0.01
BE	25	0.12 (---) ± 0.02	0.89 ± 0.01
	100	0.17 (---) ± 0.01	0.73 ± 0.01
	500	0.17 (---) ± 0.01	0.79 ± 0.01
	1000	0.17 (---) ± 0.03	0.92 ± 0.01
BE + CP	25 + 50	0.18 (58.1) ± 0.03 b	1.21 ± 0.01
	100 + 50	0.17 (60.5) ± 0.01 a	0.95 ± 0.01
	500 + 50	0.15 (65.1) ± 0.02 a	0.91 ± 0.01
	1000 + 50	0.18 (58.1) ± 0.01 b	0.78 ± 0.01
SE	25	0.15 (---) ± 0.01	0.75 ± 0.01
	100	0.08 (---) ± 0.02	1.11 ± 0.01
	500	0.17 (---) ± 0.01	1.09 ± 0.01
	1000	0.17 (---) ± 0.01	0.99 ± 0.01
SE + CP	25 + 50	0.08 (81.4) ± 0.01 b	0.80 ± 0.01
	100 + 50	0.13 (69.8) ± 0.01 b	0.93 ± 0.01
	500 + 50	0.07 (83.7) ± 0.02 b	0.93 ± 0.02
	1000 + 50	0.13 (69.8) ± 0.02 a	1.08 ± 0.01

Values are mean ± SE of 3 animals in each group. Figures in the parentheses are percent inhibition
GA – Gum acacia; CP – Cyclophosphamide; LE – Leaf extract; BE – Bark extract; SE – Seed extract
P values: a <0.01, b <0.001

Table 3 Inhibitory effect of different doses of the petroleum ether extracts or *P. pinnata* on the induction of micronucleus in polychromatic erythrocytes by cyclophosphamide (50 mg/kg body weight) at 72 hours.

Treatment	Dose (mg/kg)	MN – PCE (%)	P/N Ratio
GA (1%)	-	0.03 (---) ± 0.01	0.91 ± 0.01
CP	50	0.32 (---) ± 0.01	0.96 ± 0.01
LE	25	0.07 (---) ± 0.01	1.09 ± 0.01
	100	0.05 (---) ± 0.02	0.95 ± 0.01
	500	0.10 (---) ± 0.01	0.99 ± 0.01
	1000	0.13 (---) ± 0.02	0.96 ± 0.01
LE + CP	25 + 50	0.07 (78.1) ± 0.01 c	0.96 ± 0.01
	100 + 50	0.10 (68.8) ± 0.01 b	0.96 ± 0.01
	500 + 50	0.17 (46.9) ± 0.02 b	1.02 ± 0.01
	1000 + 50	0.20 (37.5) ± 0.01 a	0.91 ± 0.01
BE	25	0.08 (---) ± 0.01	1.04 ± 0.01
	100	0.17 (---) ± 0.01	0.77 ± 0.01
	500	0.13 (---) ± 0.03	0.81 ± 0.01
	1000	0.13 (---) ± 0.01	0.92 ± 0.01
BE + CP	25 + 50	0.18 (43.8) ± 0.02 b	1.28 ± 0.01
	100 + 50	0.12 (62.5) ± 0.01 b	1.07 ± 0.01
	500 + 50	0.15 (53.1) ± 0.02 b	0.93 ± 0.01
	1000 + 50	0.22 (71.4) ± 0.02 b	0.84 ± 0.01
SE	25	0.08 (---) ± 0.01	0.71 ± 0.01
	100	0.08 (---) ± 0.02	0.90 ± 0.01
	500	0.07 (---) ± 0.01	0.95 ± 0.01
	1000	0.03 (---) ± 0.02	0.95 ± 0.01
SE + CP	25 + 50	0.07 (78.1) ± 0.02 b	0.69 ± 0.01
	100 + 50	0.03 (90.6) ± 0.01 c	0.81 ± 0.01
	500 + 50	0.05 (84.4) ± 0.02 b	0.92 ± 0.01
	1000 + 50	0.03 (90.6) ± 0.01 c	1.03 ± 0.01

Values are mean ± SE of 3 animals in each group. Figures in the parentheses are percent inhibition
GA – Gum acacia; CP – Cyclophosphamide; LE – Leaf extract; BE – Bark extract; SE – Seed extract.
P values: a <0.05, b <0.01, c <0.001

against CP-induced clastogenicity as evidenced by the reduction in frequency of MN. Singh *et al*. (1996) demonstrated the anti-inflammatory, analgesic and anit-ulcerogenic activity of petroleum ether, chloroform, acetone and ethanolic extracts of *P. pinnata* seeds in *in vivo* mice. Our study, however, is confined only to the petroleum ether extract of *P. pinnata*.

Mathur *et al*. (1990) described that karanjin (3-methoxy furano-(2'.3'7, 8)-flavone, is the principal furanoflavonoid of *P. pinnata* and was the first crystalline compound isolated from the seed oil. The seed contains 1.25% karanjin and 0.35% pongamol as the major constituents. Flavonoids occur ubiquitously in the plant kingdom and are common components of the human diet (Hertog *et al*. 1993). Flavonoids have been shown to have structurally-dependent, highly specific effects on a variety of enzymes and are able to interfere with numerous cellular processes, including growth and differentiation (Brandy 1992; Jagetia and Reddy 2002). Two flavonoids, known for their antioxidant activity viz. kaempferol and 3,5,6,7,8-pentamethoxy flavone were isolated form the ethanolic extract of flowers of *P. pinnata* and the results suggested that the flowers had a protective effect against cisplatin- and gentamicin-induced renal injury through their antioxidant property (Shirwaikar *et al*. 2003). From our observations we could observe that the percent inhibition of leaf and bark extracts was less than that of the seed extract. A possible reason could be the differences in the chemical composition in different parts of the plants. Thus, based on our observation, it may be concluded that the action of the different extracts is manifested by a combination of different ingredients of a complex mixture or components. Also the degree of protection depends on the interactions of the various components individually or collectively for MAPs (Sarkar *et al*. 1994). Proponents of herbal medicine always claim that mixtures are better than the pure chemicals as dozens of biologically active compounds work together to produce a cumulative effect than any one chemical on its own (Mackenzie 2001).

Madhumita *et al*. (1998) showed that the reduction in the frequency of chromosomal aberrations was significantly less when an *Azadirachta indica* leaf extract was given in combination with CP in mice. The administration of citrus extract reduced the toxicity induced by CP by analysis of liver glutathione in mouse bone marrow cells (Hosseinimehr and Karami 2005). Since the present study was undertaken with petroleum ether extract, which contains a diverse class of compounds, it would be difficult to attribute the protective effect to any one particular compound. The mechanism may be through the diverse effect of ingredients like non-specific redox agents, free radical scavengers, desmutagens, etc. (Abraham *et al*. 1998). Hence the synergistic/additive effects of these compounds may lead to significant anticlastogenic effects as observed in our study.

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