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Antimutagenic activity of methanolic extracts of four ayurvedic medicinal plants

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Methanolic extracts of *Acorus calamus* (Rhizome), *Hemidesmus indicus* (Stem), *Holarrhena antidysenterica* (Bark) and *Plumbago zeylanica* (Root), were tested for their antimutagenic potential. These extracts, at tested concentrations, showed no sign of mutagenicity to *Salmonella typhimurium* tester strains. The extracts of the plants exhibited varying level of antimutagenicity. At a dose of 100 µg/plate, the extracts exhibited the inhibition of His+ revertants from 18.51% to 82.66 % against direct acting mutagens, methyl methanesulphonate (MMS) and sodium azide (NaN_3) induced mutagenicity in *Salmonella* tester strains TA 97a, TA 100, TA 102 and TA 104. However, at lower concentrations (25 and 50 µg/ plate) of the plant extracts, a decrease in antimutagenic activity was recorded. Dose dependent antimutagenic activity of the extracts is also evident from linear regression analysis of the data. The over all antimutagenic potential of above four extracts was found to be in order of *A. calamus* > *H. indicus* > *H. antidysenterica* > *P. zeylanica*. Further, total phenolic content of these extracts did not correlate with its antimutagenic activity in *A. calamus* and *P. zeylanica*.

Keywords: *Acorus calamus*, Antimutagenic activity, *Hemidesmus indicus*, *Holarrhena antidysenterica*, Medicinal plants, Phenolics, Plant extracts, *Plumbago zeylanica.*

Mutations are the cause of innate metabolic defects in cellular system, triggering the morbidity and mortality in living organisms. A plethora of synthetic and natural substances, apart from various genotoxic physical and biological agents, are known to act as mutagenic, co-carcinogenic and/or carcinogenic agents[1]. Since, the mutagens are involved in the initiation and promotion of several human diseases including cancer, the significance of novel bioactive phytocompounds in counteracting the promutagenic and carcinogenic effects are gaining credence. Such chemicals that reduce the mutagenicity of physical and chemical mutagens are called as antimutagens. The antimutagens have been first reported almost four decades ago, and since then numerous studies have been carried out in order to identify compounds, which might protect humans against DNA-damage and its consequences. Antimutagenic and anticarcinogenic properties of a wide variety of dietary constituents and plant secondary metabolites have been reported[2-7].

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The antimutagenic or protective effect has been attributed to many classes of phytocompounds mainly flavonoids and phenolic compounds present in foods. However, such compounds have also been reported to exhibit a wide range of other biological activities such as antimicrobial, anti-inflammatory, antiallergic, antioxidant and free radical scavenging[7-9]. Natural antimutagens from edible and medicinal plants are of particular importance because they may be useful for human cancer prevention and have no undesirable xenobiotic effects on living organisms[5,10].

The rich diversity of Indian medicinal plants have not yet systematically screened for antimutagenic activity[11-14]. More than 800 plants are used in the treatment of various ailments in the traditional systems of Indian medicine (Ayurveda, Siddha and Unani). Ayurvedic preparations are mainly polyherbal preparations sometime with other minerals. Based on the chemical diversity of known active phytoantimutagens[6], many traditionally used Indian medicinal plants may exhibit such desired properties due to similarity in the major class of phytocompounds. Therefore, four common traditionally used medicinal plants namely *Acorus calamus* (Sweet flag, Bach), *Hemidesmus indicus* (Indian sarasaparilla, Anantamul), *Holarrhena antidysenterica* (Conessi, Kurchi), *Plumbago zeylanica* (Ceylong-Leadwort, Chita) were selected in this study. The ethnobotanical and

phytochemical data of these plants are well documented in literature[19-22]. The parts of these plants selected were used in a variety of Ayurvedic formulations. We have previously evaluated several medicinal plants including above four plants for their antibacterial, antifungal and antioxidant activities[15-18]. The antimutagenic property of three of the selected plants has not previously reported. In this study, antimutagenic activity of methanolic extracts of above four plants using Ames *Salmonella* test assay against two direct acting mutagens has been reported.

Materials and Methods

Bacterial strains and chemicals—Salmonella typhimurium strains TA 97a, TA 100, TA 102 and TA 104 were kindly provided by Prof B N Ames, University of California, Berkeley, USA. Sodium azide was purchased from Hi-Media lab. Ltd, Mumbai, India. Methyl methane sulphonate was purchased from Sisco Research laboratories Pvt. Ltd Mumbai. All other chemicals and media used were of analytical grade.

Plant materials and preparation of extracts—Four authenticated plant samples *Acorus calamus* L. (rhizome), *Hemidesmus indicus* R.Br. (stem), *Holarrhena antidysenterica* Wall, (bark) and *Plumbago zeylanica* L. (root) were kindly provided by the Himalaya Drug Company, 20 Najafgarh Road, New Delhi, India, now shifted to Dehradun, India. All the plant materials were further identified in the Department of Botany, Aligarh Muslim University, Aligarh, India. The Voucher specimens *Acorus calamus* L. (Acoraceae)/ HDCO-166/297, *Hemidesmus indicus* R.Br. (Asclepiadaceae)/ HDCO-204/76, *Holarrhena antidysenterica* Wall, (Apocynaceae)/ HDCO-120/235 and *Plumbago zeylanica* L. (Plumbaginaceae)/ HDCO- 43/64 have been deposited in the Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, India.

The plant extracts were prepared as described earlier[17] with little modification. Dry plant powder (100g) was soaked in 250 ml of 97% methanol for 8-10 days and stirred after every 18 hr using a sterilized glass rod. At the end of extraction, it was passed through Whatman filter paper No.1 (Whatman Ltd., England). The methanolic extract was concentrated to dryness under vacuum on rotary evaporator at 40°C and then reconstituted in minimum amount of DMSO and stored at 4°C for further use. The stock fraction was further diluted in known amount of DMSO to get methanol extract of known concentration.

Antimutagenicity assay—The *Salmonella* histidine point mutation assay of Maron and Ames [23] was used to test the antimutagenic activity of the extracts, with some modifications as described by Kaur *et al*[13]. The medium was prepared according to the procedure of Maron and Ames[23]. In the pre-incubation experiment, a mixture of test plant extract and mutagen, each having a volume of 0.1 ml of varying concentration, was preincubated at 37°C for 30 min before addition to the bacterial culture. For the experiments, the order of addition was 0.1 ml of bacterial culture of 1×10^7 CFU/ml density, 0.1 ml of mutagen and 0.1 ml of test extract followed by the addition of 2.5 ml of top agar at 45°C (containing 0.5% NaCl and 0.6% agar) supplemented with 0.5 mM histidine-biotin. The combined solutions were vortex-mixed and poured onto minimal glucose plates (having 40% glucose solution and Vogel Bonner medium). The plates were incubated at 37°C for 48 hr, after which the numbers of histidine-independent revertant colonies were scored. Bacterial survival was routinely monitored for each experiment. To check the toxicity of the test sample, parallel controls were run with extracts alone at all concentrations tested with mutagens. The concentrations of the test sample for investigating the antimutagenicity were 25, 50 and 100 μg/ 0.1 ml/ plate. These were tested against sodium azide (1.5 μg/ 0.1 ml/plate) in TA 97a, TA 100 and TA 104 tester strains and MMS (1.0 μg/ 0.1 ml/plate) in TA 102 tester strain. All the test samples and mutagens were dissolved in DMSO. In each case, there was no over toxicity observed and the number of spontaneous revertants were identical to the DMSO vehicle control. Non-toxic concentrations were categorized as those where there was a well-developed lawn, almost similar size of colonies and no statistical difference in the number of spontaneous revertants in test and control plates. Triplicate plates were set up with each concentration and the entire experiment was repeated twice. Inhibitory activity was expressed as percentage decrease of reverse mutation:

$$\text{Percent inhibition} = [(a-b)/(a-c)] \times 100,$$

where

a = number of histidine revertants induced by mutagen,

b = number of histidine revertants induced by mutagen in the presence of plant extract and c = number of revertant induced in negative control.

Determination of total phenolic content of plant extracts—The total phenolic content in the plant extracts was determined with the Folin-Ciocalteau reagent using the method of Spanos and Wrolstad[24], as modified by Lister and Wilson[25]. To 0.50 ml of each sample (containing 25, 50 and 100µg/ml of plant extract), 2.5 ml 1/10 dilution of Folin-Ciocalteau's reagent and 2 ml of Na_2CO_3 (7.5%, w/v) were added and incubated at 45°C for 15 min. Each experiment was performed in three replicates. The absorbance of all samples was measured at 765 nm using a Spectronic 20D+ spectrophotometer (Spectronic 20 $D^{+,}$ Milton Roy & Co./Thermo Electronics ,USA) Results were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g dw).

Statistical analysis—The results are presented as the average and standard error of three experiments with triplicate plates/ dose/ experiment. The data were further analyzed for statistical significance using analysis of variance (One way ANOVA) using MINITAB 11. The regression analysis carried out using Microsoft Excel 2000.

Results and Discussion

Methanolic extracts of *Acorus calamus* (rhizome), *Hemidesmus indicus* (stem), *Holarrhena antidysenterica* (bark) and *Plumbago zeylanica* (root) were non mutagenic to *S. typhimurium* tester strains TA 97a, TA 100, TA 102 and TA104 when tested in pre-incubation method. The extract of all four plants exhibited non toxicity in *Salmonella* tester strains at all tested concentrations (25, 50 and 100 µg/plate).The plant extracts were tested for their antimutagenic activity against sodium azide (NaN_3) induced mutagenicity in the tester strains TA 97a, TA 100 and TA104 while methyl methane sulphonate (MMS) induced mutagenicity was tested in TA 102 strain.

Varying effects of plant extracts on the direct acting mutagens (NaN_3 and MMS) induced mutagenicity in one or more tester strains are presented in the Table 1.

Acorus calamus extract showed decrease in the number of revertants colonies against NaN_3 induced mutagenicity in all three tester strains. Similar antimutagenic activity was also demonstrated against MMS induced mutagenicity in TA102 strain. At 100µg/plate extract concentration, percent inhibition of mutagenicity against NaN_3 and MMS ranged from 60.92% to 83.60%. Antimutagenic activity of the extract was found to be concentration dependent. The linear regression analysis between extract dose and antimutagenic response showed strong correlation with respect to dose dependent response in TA97a (R^2=0.9696), TA100 (R^2=0.9956), TA104 (R^2=0.9687) against NaN3 and in TA102 (R^2=0.9103) against MMS.

The antimutagenic effect of *Hemidesmus indicus* could be demonstrated against NaN_3 and MMS with percent inhibition ranges from 55.30% to 82.66% at 100µg/plate concentration. Concentration and strain dependent response are also evident. Linear relationship between extract dose and antimutagenic response is strong in the strain TA102 (R^2=0.993) followed by TA100 (R^2=0.992), TA104 (R^2=0.9872) and TA97a (R^2=0.9743) against respective test mutagen.

Holarrhena antidysenterica extract showed similar concentration dependent antimutagenic potential against both direct acting mutagens in their respective tester strains used. Antimutagenic response in terms of % inhibition varied (48.3% to 75.8%) in different strains at 100 µg/plate extract concentration. Linear relationship between extract dose and antimutagenic response is strong in the strains TA102 (R^2=0.9912) followed by TA97a (R^2=0.9833), TA104 (R^2=0.9761) and TA100 (R^2=0.8321) against tested mutagen.

The data obtained on antimutagenic potential of *Plumbago zeylanica* extract showed its poor activity against both direct mutagens induced mutagenicity. Even at 100µg/plate, the percent inhibition of mutagenicity was ranged from 18.51% to 28. 28%. The

Table 1—Effect of methanolic plant extracts on the mutagen induced mutagenicity in *Salmonella typhimurium* tester strains

Treatment (Pre-incubation)	D ose (µg/plate)	% inhibition of His^+ revertants [values are mean of 3 experiments]			
		TA 97a	TA 100	TA 102	TA 104
Acorus	25	24.5	29.6	26.9	39.2
calamus +	50	37.4	43.5	68.2	68.3
Mutagen	100	61.6	60.9	80.3	83.6
Hemidesmus	25	43.5	20.2	36.2	33.3
indicus+	50	57.5	36.3	53.7	42.2
Mutagen	100	82.7	58.4	77.1	55.3
Holarrhena	25	30.4	19.3	29.4	35.5
antidysenterica	50	44.7	45.0	56.4	51.7
+ Mutagen	100	67.3	48.3	75.8	60.9
Plumbago	25	5.79	10.03	22.46	13.53
zeylanica +	50	8.78	10.89	25.91	19.65
Mutagen	100	18.51	21.35	28.28	22.91

Negative control (Plant extracts) showed no sign of mutagenicity at the above tested concentrations.

Mutagens used are methyl methane sulphonate (1.0 µg/plate) for TA 102 and sodium azide (1.5 µg/plate) for TA 97a, TA 100 and TA 104

overall antimutagenic activity of above four extracts were in the order of *A. calamus* >*H. indicus* > *H. antidysenterica* > *P. zeylanica.* All four extracts exhibited concentration dependent antimutagenic activity.

The variations in the antimutagenic activity in these plant extracts might be due to the differences in the active constituents and their combinations in the extract[6]. Our findings are in agreements of other workers who have reported concentration dependent antimutagenic activity in other plants[12,13]. On the other hand, Edenharder and workers[26] reported that antimutagenesis of flavonoids and structurally related compounds may be dependent or independent of concentration.

The significant antimutagenic activity in above three plant extracts except *P. zeylanica* against direct acting mutagens suggests that these extracts may directly protect DNA damage from mutagen. However, the inhibition of mutagenesis is often complex, acting through multiple mechanisms[27-29].

The present results are the first attempt to analyse antimutagenic activity of at least three plants viz *A. calamus*, *H. indicus and H. antidysenterica*. The mutagenicity and antimutagenicity reports on *Plumbago zeylanica* and its active constituents are contradictory in the literature. The crude extracts of *Plumbago zeylanica* are known for their protective effect against cyclophosphamide-induced genotoxicity and oxidative stress in Swiss Albino mice[30]. Some workers reported the antimutagenic and anticancerous activity of pure active compound Plumbagin[31,32].While others workers have reported mutagenicity and toxicity of the plumbagin[33,34].Therefore, we presumed that the weak antimutagenic activity of crude extracts observed in our study is probably due to the presence of active constituents plumbagin.

Presence of more than one major phytocompounds like phenols, alkaloids, glycosides, flavonoids, tannins and saponins in these plants has been already reported[17]. Total phenolics concentration equivalent to gallic acid was observed maximum in the extract of *H. indicus* (109.00 ± 13.53 mg/g) followed by *P. zeylanica* (107.33 ± 18.17 mg/g), *A. calamus* (71.93 ± 6.90 mg/g) and *H. antidysenterica* (59.50 ± 5.89 mg/g). Flavonoids and phenolics are the most likely candidate among the methanol extract for providing the antimutagenic effect and preventing the oxidative damage[28]. However, no direct correlation was established between total phenolic concentration and antimutagenic activity of all tested plant extracts. The significant antimutagenic activity showed by *Acorus calamus, Hemidesmus indicus, and Holarrhena antidysenterica* provide a scientific validation for the traditional use of these plants. Further work on isolation and identification of active compounds or standardized extract and its efficacy needs to be conducted against both direct and S9 dependent mutagens *in vitro* and *in vivo* systems.

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