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Original Article

Antimutagenic Effect of *Dioscorea Pentaphylla* on Genotoxic Effect Induced By Methyl Methanesulfonate in the Drosophila Wing Spot Test

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ABSTRACT

Objectives: Plants as dietary sources are known to have several chemoprotective agents. *Dioscorea pentaphylla* is an important medicinal plant, which is often used as edible food. This study was undertaken to evaluate the antigenotoxic potential of *D. pentaphylla* extracts on the genotoxic effect induced by methyl methanesulfonate (MMS) in the Drosophila wing spot test. **Materials and Methods:** The somatic mutation and recombination test (SMART) was carried out in *Drosophila melanogaster*. In transheterogyous larvae, multiple wing hair (mwh 3-0.3) and flare (flr3-38.8) genes were used as markers of the extent of mutagenicity. **Results:** It was observed thatall the three extracts (petroleum ether, choloroform, and ethyl alcohol) in the combined treatment had significantly inhibited the effect of MMS-induced genotoxic effects. When compared to others, the ethanol extract showed a very significant antimutagenic activity. **Conclusion:** The compounds that are present in the extracts may directly interact with the methyl radical groups of MMS and inactivate them by chemical reaction. It is also possible that the compounds in the extract compete to interact with the nucleophilic sites in deoxyribonucleic acid (DNA), thus altering the binding of the mutagen to these sites. Although our results indicate that the compounds present in the extracts may directly interact with the methyl radical groups of MMS and inactivate them by chemical reaction, it may also be quite interesting to investigate through the other different mechanisms by which *D. pentaphylla*could interfere *in vivo* on the effect of genotoxic agents.

Key words: Antigenotoxicity, antioxidant, chemoprotective agents, *diascoria pentaphylla, drosophila melanogaster*, methyl methanesulfonate

INTRODUCTION

Dioscorea yam is a member of the Yam family. The yams are vining plants with 600 known species, 71 of which are native to North America (67 species in Mexico).[1] In many species of yam, the rhizome (tuber) serves as both food and medicine. Many native Americans and South Asians use the syrup of the root to relieve labor pain. The physicians are also known to give wild yam to patients with colic pain, morning sickness, asthma, hiccough, rheumatism, and gastritis-related to alcoholism.[2] Modern herbalists value wild yam to treat intestinal colic, biliary colic, and flatulence as well as menstrual cramps and rheumatoid arthritis.[3-5] Herbalists combine wild yam with black cohosh (and sometimes burdock root and motherwort)[2] for rheumatic complaints. Chinese herbalists use wild yam as a tonic.[2] The Diascoria species includes *Dioscorea pentaphylla,* which

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is known to compose mainly of starch (75–84% of the dry weight) with small amounts of proteins, lipids and mostly vitamins with very rich in mineral content.[6] Reports show that *D. pentaphylla* extracts can reduce blood sugar and blood lipid contents,[7-9] inhibit microbial activity[10] and show antioxidative activity.[9] The active components of Dioscorea species include steroidal sapogenin, glycan and storage protein. Diosgenin, extracted from Dioscorea species, is a natural steroidal sapogenin used as a precursor in the industrial synthesis of steroids. Dioscorin, the storage protein of yam tuber is reported to have scavenging activity towards 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.[11] Dioscorans, a glycan isolated from *D. japonica*, was shown to remarkably inhibit the hypoglycemic affects in normal and alloxan-induced hyperglycemia mice.[7] The antioxidative activities of Dioscorea extracts have been well-documented;[9,11]however, there have been no studies carried out evaluating its genotoxicity, mutagenic, and/or antigenotoxic properties.

With the increase in incidences of cancer associated with human exposure to environmental genotoxins. The search for bioactive products that are both effective and nontoxic in the prevention and/or treatment of cancers and other diseases is of extensive research. Previous studies have underlined the chemo preventive activity of several secondary plant metabolites,[12] and many studies have reported the antimutagenic properties elicited by whole plant extracts[13,14] or with specific compounds, such as polyphenols and triterpenoids.[15] Thus, there has been growing interest in finding and using natural plant products to reduce genotoxic and/or carcinogenic effects.[16,17] Various genotoxicity tests have been developed to detect genotoxic substances and/or carcinogens, which have influence on the assessment of antigenotoxic and/or anticarcinogenic effects.[18,19] The somatic mutation and recombination test (SMART), developed with *Drosophila melanogaster*, has proven to be a good tool for detecting a broad range of genetic alterations quickly and inexpensively.[20] It is an assay that permits the detection of a range of genetic alterations, such as deoxyribonucleic acid (DNA) point mutation, nucleotide deletion in DNA, and mitotic DNA recombination.[20] The markers mwhs (multiple wing hairs) and flr3 (misshapen, flare-like hairs) are recessive wing-hair mutations located on the third chromosome at 0.3 and 38.8, respectively. These are based on the fact that during the early embryonic development of the fruit fly, the cells of the imaginal discsare set apart and they proliferate mitotically during the larval development until they differentiate during metamorphosis into structures of the body of the adult fly (eyes, wings, etc.).[19] The genetic alterations in some of these cells of the imaginal disk result in the formation of descent cells with alterations, forming clones of mutant cells and such alterations are easily detected by phenotypic modifications in the hairs of the wings of the adult fly.[21] Therefore, in this study we have used the wing SMART assay to evaluate the genotoxic and antigenotoxic activity of different extracts (petroleum ether, chloroform, and ethyl alcohol) of *D. pentaphylla* against the genotoxic effects of alkylating agent—methyl methanesulfonate (MMS). So far, to our knowledge the effects of *D. pentaphylla* on alkylating agent genotoxicity have not yet been studied *in vitro* or *in vivo*, and thus this is the first study demonstrating its effects.

MATERIALS AND METHODS

Chemicals

MMS (CAS No. 62-50.0) was purchased from Sigma Co., St Louis, USA, sodium chloride; gum arabic, glycerol, and chloral hydrate from HiMedia Chemicals, Mumbai, India. Distilled water served as a negative control, and 0.1 mM MMS was used as a positive control.

Plant material

Tubers of *D. pentaphylla* were collected from the Lakkavalli reserve forest in and around the area of Bhadra Wildlife Sanctuary of the mid-Western Ghats region of Karnataka, India, and the species was identified by comparing with the authenticated specimen deposited at the Kuvempu University Herbaria (Voucher specimen KUDB/Ang/324). The leaves were washed in running tap water, shade dried, powdered mechanically and sieved (Sieve No. 10/44), and subjected to Soxhlet extraction using different solvents, viz., petroleum ether, chloroform, and ethanol. The extracts were concentrated under reduced pressure at 40 ± 5°C using a rotary flash evaporator (Buchi, Flawil, Switzerland). The extracts were dissolved in 0.2% of dimethyl sulfoxide (DMSO) for its evaluation of antigenotoxic effect.

Phytochemical analysis

Qualitative phytochemical analysis of *D. pentaphylla* tuber extracts was done for the presence of tannins, alkaloids, saponins, flavonoids, terpenoids, and phenols/polyphenols.[22-24]

Strains

Two *Drosophila melanogaster* strains were used: The mwhs strain with genetic constitution mwh/mwh and the flrstrain with genetic constitution flr3/In (3LR) TM3, Bds. The transheterozygous larvae were obtained by crossing ORR: Mwh/mwh males and ORR: Flr3/TM3 females and were obtained from Agarkar Institute, Pune. The more detailed information on the genetic symbols and descriptions can be found in the work of Lindsley and Zimm, (1992).[25] The tests were performed as described in Graf *et al*.[20]

Drosophila SMART test

The SMART was essentially performed as described by Graf *et al*.[20] For this assay, the following cross of *Drosophila*

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*melanogaster*flies was used: ORR (i); ORR (ii); flr3/In (3LR) TM3, BdS virgin females were crossed with mwh males (flies that were kindly provided by Agarkar Institute, Pune). The first strain is characterized by constitutively high cytochrome P-450 activity. The markers mwh and flr3 (misshapen, flr-like hairs) are recessive wing-hair mutations located on the third chromosome at 0.3 and 38.8, respectively. This test is able to detect a wide spectrum of genetic alterations including point mutations, deletions, unbalanced half-translocation and mitotic recombination, chromosomal loss, and non-disjunction as described in Graf *et al*.[20]

Transheterozygous larvae were obtained by parental crosses between *flr3* virgin females and mwh males. Eggs were collected from this cross during 8-h period in culture bottles containing fresh standard Drosophila medium (wheat powder, jaggery, agar agar, propionic acid, and water cooked). After 72 h, third instar larva were floated off with tap water and transferred to plastic vials containing 1.5 g of Drosophila instant medium rehydrated with 9 mL of freshly prepared test solutions (mutagens, mutagens plus extracts, distilled water, and MMS used at positive control at 0.1 and 0.05 mM). For each treatment group in a total of 4,000 larvae, 200 in each vial were employed. The larvae were fed on this medium until pupation of the surviving larvae. All the experiments werecarried out at 24 ± 1°C and at ~60% relative humidity.

Preparation and analysis of wings

The crossing procedure is distinguished phenotypically based on the TM3, Bds marker. Marker-heterozygous flies (mwh/flr3) and balancer-heterozygous (mwh/TM3, Bds) genotypes were mounted on slides with Faure's solutions (30 g gum arabic, 30 mL glycerol, 50 g chloral hydrate, and 50 mL distilled water). Both the dorsal and ventral surfaces of the wings were analyzed under a microscope at × 400 magnification for the presence of clones of cells showing malformed wing hairs, that is, occurrence of small single spots consisting of one or two mwh cells, large single spots consisting of three or more cells, and twin spots consisting of adjacent mwh and flr3 cells (Graf *et al*.).[20] Single spots can be produced by somatic point mutation, chromosome aberration, deletion, or mitotic recombination; and twin spots originate exclusively from mitotic recombination. To determine the recombinagenic activity, the frequencies of mwh clones on the marker-heterozygous wings are compared with the frequencies of mwh clones on the balancer-heterozygous wings. The difference in mwh clone frequency is a direct measure of the proportion of recombination.[26]

Statistical analysis

For the statistical assessment of genotoxicity, the frequencies of each type of spot per fly were compared pair-wise with the corresponding negative control; for the antigenotoxicity of amifostine the frequencies of each type of spot per fly were compared pair wise with the corresponding dose of 8 g/ml fotemustine. A multiple-decision procedure was used to decide whether a result is positive, weakly positive, inconclusive, or negative.[27,28] For the statistical calculations, the conditional binominal test according to Kastenbaum and Bowman[29] was used with $P = 0.05$ significance levels. The frequency of clone formation was calculated based on clone induction frequencies per 105 cells, the recombinogenic activity was calculated as: Mutation frequencies (FM) = frequencies clones mwh/TM3 flies/frequencies clones mwh/flr3 flies; recombination frequencies (FR) =1 − FM. Frequencies of total spots (FT) = total spots in mwh/flr3 flies spots/number of flies; mutation = FT × FM; and recombination = FT × FR.[30,31] Based on the control-corrected spot frequencies per 105 cells, the percentage of amifostine inhibition was calculated as: (Fotemustine alone − amifostine plus fotemustine/fotemustine alone) × 100.[32]

RESULTS AND DISCUSSION

The dietary sources are known to have many chemoprotective agents (Surh, 2003) and *D. pentaphylla* is one of such important medicinal edible food. The antimutagenicity potential of different extracts (petroleum ether, chloroform, and ethyl alcohol) of *D. pentaphylla* against MMS was assessed using *Drosophila*, an organism that is extensively used and suited for *in vivo* testing of simple or complex compounds.[19] When the genotoxic activity of MMS was tested at 0.02, 0.05, and 0.1 mM concentration, it was found that MMS at a concentration of 0.1 mM had significantly increased the number of small, large, twin, and total spots [Table 1]. Therefore, 0.1 mM concentration of MMS was used as positive control; to test the antigenotoxic

Table 1: Comparison of wing spots of different concentration of methyl methanesulfonate in Drosophila larva

Compound conc	Number of wings (*N*)	Small single spots (m=2)		Large single spots (m=5)		Twin spots (m=5)		Total mwh spots (m=2)		Total spots (m=2)		Frequency of clone formation per 10^5 cells
		No.	Fr	No.	Fr	No.	Fr	No.	Fr	No.	Fr	
MMS (0.02 mM)	80	10	0.11	1	0.01	1	0.01	09	0.14	12	0.18	0.16
MMS (0.05 mM)	80	14**	0.16	3*	0.02	2**	0.03	13**	0.23	18**	0.23	0.66
MMS (0.01 mM)	80	32**	0.40	5**	0.06	4**	0.05	37**	0.46	41**	0.51	1.89

MMS = Methyl methanesulfonate, Conc = Concentration, mM = Millimolar, No. = Number of wings, Fr = Frequency, frequency of clone formation per 10^5 cells-clones/wings/24,400, m = Multiplication factor. *Insignificance at 95%, **significance at 95%

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potential of different extracts of *D. pentaphylla* for 48 ± 4 and 72 ± 4 h larvae. When different extracts of *D. pentaphylla* was administered in combined treatment, it was found that all the three extracts inhibited the genotoxic effect of MMS significantly. It is notable that the treatment in both 48 ± 4 and 72 ± 4 h had significantly decreased by three folds the number of twin spots, total spots and frequency of clones per 10^5 cells when compared to control (distilled water) group [Tables 2 and 3]. However, it is to be noted that among the three extracts, the ethanol extract significantly inhibited the genotoxic effects of MMS.

MMS, which is a mono functional alkylating agent, is known for its ability to interact directly with DNA *in vitro* and *in vivo*, producing genotoxic damage in different model syst.[33-35] The mutagenicity and carcinogenicity of monofunctional alkylating agents, including MMS, have been associated with the formation of O-alkylated and N-alkylated DNA bases.[36] The mutational spectra induced in Drosophila by MMS suggest an involvement of apurinic sites as mutagenic lesions.[36] Further, it is to be noted that a clear relationship exists between the extent of the DNA N-alkylation and the efficiency of the MMS to induce mitotic recombination in the Drosophila wing-spot test.[34] In our study, considering the fact that coadministration of plant extracts with genotoxin significantly decreased the genotoxic effect of MMS, therefore it might be possible that the antimutagens in the plant extract exert their protective effect by directly interacting with MMS; however, the components acting in desmutagenic manner without affecting the genetic material cannot be completely ruled out.[37] It is also to be noted that, as MMS does not require metabolic activation; the natural compounds present in the extracts may interact directly with the methyl radical groups of MMS and inactivate them by chemical reaction. It is also possible that these compounds compete to interact with the nucleophilic sites in DNA, thus altering the binding of the mutagen to these sites.

Further, plant components such as polyphenols and flavonoids are known to bring about antimutagenic activity due to its radical scavenging effects.[38] The antioxidant compounds are known to have inhibitory effects on the genotoxic actions of several known mutagens.[39] In the preliminary phytochemical analysis, the different extracts of *D. pentaphylla* were found to possess alkaloids, flavonoids, phenol/polyphenols, saponins, tannins, and terpenoids/steroids [Table 4]. Many antioxidants have been found in Dioscorea species that includes carotenoids, ascorbic acid, tocopherols, and a wide range of other various polyphenolics, some of which are well-known strong scavengers of active oxygen radicals.[40-42] The phenolic compounds and many flavonoids arealso reported to have the potent capacity to scavenge mutagens or free radicals.[40,43] Dioscorin, the storage protein of yam tuber is reported to have

Table 2: Comparison of wing spots of different solvent extracts of *Dioscoreapentaphylla* leaf and methyl methanesulfonate at 48±4 h in Drosophila larva

Compound conc	Number of wings (*N*)	Small single spots (m=2)		Large single spots (m=5)		Twin spots (m=5)		Total mwh spots (m=2)		Total spots (m=2)		Frequency of clone formation per 10^5 cells
		No.	Fr	No.	Fr	No.	Fr	No.	Fr	No.	Fr	
Water	80	16	0.20	1	0.01	1	0.01	18	0.22	19	0.23	0.23
DMSO	80	17	0.21	2	0.02	0	0.00	19	0.23	19	0.23	0.97
MMS (0.1 mM)	80	32**	0.40	5**	0.06	4**	0.05	37**	0.46	41**	0.51	1.89
Petroleum ether extract (1%)	80	25**	0.31	3*	0.03	5*	0.06	28**	0.35	33**	0.41	1.43
Chloroform extract (1%)	80	28**	0.35	2*	0.02	5*	0.06	30	0.37	30**	0.43	1.53
Ethyl alcohol extract (1%)	80	18**	0.22	3*	0.03	2*	0.02	21**	0.26	23**	0.28	0.07

MMS = Methyl methanesulfonate, Conc = Concentration, mM = millimolar, No. = Number of wings, Fr = Frequency, frequency of clone formation per 10^5 cells-clones/wings/24,400, m = Multiplication factor. *Insignificance at 95%, **significance 95%. DMSO = Dimethyl sulfoxide

Table 3: Comparison of wing spots of different solvent extracts of *Dioscoreapentaphylla* leaf and methyl methanesulfonate at 72±4 h in Drosophila larva

Compound conc	Number of wings (*N*)	Small single spots (m=2)		Large single spots (m=5)		Twin spots (m=5)		Total mwh spots (m=2)		Total spots (m=2)		Frequency of clone formation per 10^5 cells
		No.	Fr	No.	Fr	No.	Fr	No.	Fr	No.	Fr	
Water	80	16	0.20	1	0.01	1	0.01	18	0.22	19	0.23	0.92
DMSO	80	17	0.21	2	0.02	0	0.00	19	0.23	19	0.23	0.97
MMS (0.1 mM)	80	29**	0.36	3**	0.37	5**	0.06	32**	0.40	37**	0.46	1.63
Petroleum ether extract (1%)	80	29**	0.36	3**	0.37	5*	0.06	32**	0.40	37**	0.46	1.63
Chloroform extract (1%)	80	28**	0.35	2*	0.02	6*	0.07	30**	0.37	36**	0.45	1.53
Ethyl alcohol extract (1%)	80	18**	0.22	1**	0.03	3**	0.03	19**	0.23	23**	0.28	0.97

MMS = Methyl methanesulfonate, Conc = Concentration, mM = Millimolar, No. = Number of wings, Fr = Frequency, frequency of clone formation per 10^5 cells-clones/wings/24,400, m = Multiplication factor. *Insignificance at 95%, **significance 95%. DMSO = Dimethyl sulfoxide

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Table 4: Phytochemical analysis *Dioscorea pentaphylla* tuber extracts

Phytochemicals	Petroleum ether	Chloroform	Ethanol
Alkaloids	+	–	–
Flavonoids	+	–	+
Phenol/polyphenols	+	+	+
Saponins	+	+	+
Terpenoids/steroids	–	+	+
Tannins	–	+	+

scavenging activity towards DPPH radical.[11] Therefore, it seems that some of polyphenols and flavonoids present in this extract (including Dioscorin) acting as antioxidants may be responsible for the detected genotoxic-inhibitory effect. However, other possibilities or mechanisms cannot be ruled out, as the constituents of extracts may be involved in reducing mutagenicity being caused by mutagensin different ways viz., (i) competition with the nucleophilic sites on DNA for an electrophilic mutagen, (ii) inhibition of promutagen bioactivation by blocking oxidation process, and (iii) reaction with the electrophilic metabolites of a promutagen. Mechanisms one and three might be involved when direct acting mutagens like MMS interacts with DNA. *D. pentaphylla* chemical constituents may possibly play a crucial role in preventing the deleterious interaction between DNA and MMS. *D. pentaphylla* chemical constituents can also block the binding of activated carcinogens to DNA, thus reducing the formation of DNA adducts.[44]

Our study shows that coincubation of different extracts of *D. pentaphylla* with mutagens, leads to significant reductions in the frequency of induced mutations. Although antioxidant potential of the extract and its components seems to be the main mechanism, however, other mechanisms/pathways cannot be completely ruled out. Further in-depth studies on identifying the principal component involved and elucidation its mechanism of antigenotoxic action will help in developing lead components for treatment. This study has demonstrated the suitability of non-mammalian *in vivo* assay for obtaining qualitative and quantitative data on antigenotoxic compounds. Our results are also quite interesting to further investigate other different mechanisms by which *D. pentaphylla*, a medicinal and edible food could interfere *in vivo* on the effect of genotoxic agents.

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