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STUDY ON ANTIGENOTOXIC EFFECTS OF MOROCCAN MEDICINAL PLANTS AND SPICES USING THE *WHITE/WHITE+* SOMATIC ASSAY IN *DROSOPHILA*

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Abstract

The antigenotoxic action of a selection of medicinal plants and spices from Morocco including laurel (*Laurus nobilis*), rosemary (*Rosmarinus officinalis*), verbena (*Verbena triphylla*), fenugreek (*Trigonella foenum-graecum)* and nutmeg (*Myristica fragrens*) was assessed using the eye *white/white*$^{+}$ *(w/w*$^{+}$*)* Somatic Mutation and Recombination Test (SMART) assay of *Drosophila melanogaster*. Methyl methanesulfonate (MMS) was used as a positive mutagenic compound inducing high frequencies of spots in *Drosophila* larvae. The frequencies of spots per eyes of a treated series are compared to those of its concurrent positive control series using χ^2- test for evaluation of the antigenotoxic effect. The nutmeg at 1% (w/v) demonstrated a marked decrease in MMS-induced spots with an inhibition rate of 50%. Rosemary, fenugreek seeds and laurel showed different inhibition rates with different level of significance.

Key words: Antigenotoxicity; Medicinal plants; Spices; *w/w*$^{+}$ SMART assay; *Drosophila*; MMS.

Introduction

In the past few years, there has been considerable interest in natural products endowed with antimutagenic and anticarcinogenic properties. Dietary and medicinal natural inhibitors of mutagenesis and carcinogenesis are of particular importance, because they may be potential agents for human cancer prevention (Ferguson, 1994; Surh, 1999). Cancer chemoprevention is regarded as a promising avenue for cancer control (De Flora et al., 2001; Surh and Ferguson, 2003). This strategy is based on the reduction of cancer incidence level by increasing the public consumption of antimutagens and

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anticarcinogens common used. This approach has been supported by several molecular biological studies, in which some DNA functions were elucidated in terms of oncogenes and tumor suppressor genes (Sugimura and Ushijima, 2000). In this context, special attention has been paid to discovery and exploration of natural chemopreventive agents that can repress the mutagenic potency of chemical mutagens.

Antimutagenic agents are natural or synthetic compounds capable of lowering the frequency of mutation by diverse mechanisms of action (De Flora and Ramel, 1988). Natural antimutagenic and anticarcinogenic agents have been identified in fresh fruit, vegetables, coffee and tea (Mitscher et al., 1996; De Flora, 1998; Alekperov, 2002). Traditional medicinal plants as well as their secondary metabolites have also been proposed for their interesting antimutagenic activities (Mitscher et al., 1996; De Flora, 1998; Alekperov, 2002; Idaomar et al., 2002; El Hamss et al., 2003).

Various genotoxicity tests developed over the past years used a variety of models (prokaryotic, eukaryotic, in vitro and in vivo) and endpoints (gene mutation, chromosome aberration, DNA damage). These tests were originally developed to detect genotoxic substances and/or carcinogens, and have influence on the assessment of antigenotoxic and/or anticarcinogenic effects (De Flora et al., 1992; Graf et al., 1998). In the present study we have chosen the *white/white*$^{+}$ somatic assay in *Drosophila* for the detection of antimutagenic activity of some commonly used spices and medicinal plants in Morocco. The somatic mutation and recombination test (SMART) in *D. melanogaster*, using either eye or wing imaginal disc cells are now well established and were validated with all known classes of genotoxic chemicals: over 400 chemical compounds for the *mwh/flr* wing spot test and about 220 chemicals for the *white/white*$^{+}$ eye spot test have been analysed (Vogel et al., 1999). The tests in *D. melanogaster* combined the predictive value of an eukaryotic in vivo test with a rapidity is comparable with that of prokaryotic or unicellular in vitro systems (Vogel et al., 1999). The *white/white+* eye assay is capable of detecting a broad spectrum of DNA modifications, with interchromosomal mitotic recombination representing the major event observed (Vogel and Nivard, 1993). This genotoxic mechanism is known to be involved as an early step in the origin of certain types of human tumours.

The *white/white*$^{+}$ assay in *D. melanogaster* was utilized in this study to investigate the antigenotoxic potential of laurel (*Laurus nobilis*), rosemary (*Rosmarinus officinalis*), verbena (*Verbena triphylla),* fenugreek (*Trigonella foenum-graecum)* and nutmeg (*Myristica fragrens*). The plants selected are largely.

Material and methods

Compounds

Methyl methanesulfonate (MMS) [66-27-3] was purchased from Sigma Company. The plants and spices were purchased locally. The powder of the fenugreek, rosemary, laurel, verbena leaves and nutmeg were each dissolved in distilled water at room temperature by adding the equivalent weight of plant (g) to 100 ml of distilled water and allowing over-night shaking. After filtering, solutions were used immediately in treatments.

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Treatments

Chemical treatments were performed by chronic co-treatment feeding exposure from the egg stage on. The *Oregon k* (*Ok*) was used. Twenty *Ok-yellow* virgin females were crossed to 20 *Ok-white* males for three days and then transferred to 200 ml bottles containing 3g of instant Drosophila medium (Carolina Biological Supply, Burlington, USA) hydrated with equal volumes of the test plant extract and MMS. MMS was assayed alone concurrently with each treatment as a positive control and at two concentrations: 0.5mM and 1mM. For the negative control, the parents were crossed in the prepared medium with distilled water. The flies were permitted to lay eggs for 3 days. Newly hatched flies were counted and the females were transferred to fresh medium; 2-6 days later their eyes were inspected for the presence of white spots. All experiments were carried out at 24±1°C.

Scoring technique

The eyes of adult females heterozygous for white were inspected for mosaic light spots under a dissecting microscope with optical fibber illumination at a magnification of 80x. The scoring of the etherized flies was carried out in a liquid consisting of 90 parts ethanol, 1 part Tween-80 and 9 parts water (ethanol: Tween: Water=90:1:9, v/v). The number of spots in 100 eyes was used as an estimate of spot frequency. Spots separated from each other by at least four non-mutated ommatidia were counted as independent events.

Data analysis and statistics

A distinction was made between small spots (size classes 1-2 and 3-4), large spots (all clones > 4 ommatidia) and total spots. A program employing the chi-square test was used for statistical analysis. The results obtained were compared with the corresponding positive controls. The percentage of genotoxicity inhibition was calculated based on the spot frequencies according to the formula: [(genotoxin alone-genotoxin + plant or spice)/genotoxin alone] x 100 (Abraham, 1994).

Results

The plants and spices tested showed a suppressing effect on the genotoxicity induced by MMS. Each plant was assayed in at least two independent experiments under identical conditions. No differences between repetitions were observed. Therefore, the data were pooled (Table 1). No delay in larval development was observed and no toxicity of the doses used in the treatments was detected. No statistical differences were found between the results of individual repeated experiments. When used alone, the plants tested did not induce mutagenicity at the selected doses. On the basis of this, two or three doses for each plant or spice were chosen for the evaluation of the antimutagenic effect. Expect for nutmeg was tested at only one dose when the MMS is used at 1 mM since 1% of the plant

Compound concentration	Number of eyes analyzed	Distribution of spots size (ommatidia)							Spots per 100 eyes Size classes(-)			% Inhibition
		1-2	**3-4**	**5-8**	**9-16**	**17-32**	**>32**	**Total**	**S**	**L**	**T**	
Pooled water controls	1586	109	49	19	1	4	0	182	9.96	1.51	11.47	***
Fenugreek seeds (*Trigonella foenum-graecum)*												
MMS 0.5 mM	710	167	80	25	14	9	2	297	34.79	7.04	41.83	***
MMS plus 2%	540	108	52	14	11	7	0	192	29.63[a]	5.93[a]	35.56[a]	14.99
MMS plus 3%	658	90	49	20	17	13	1	190	21.13[d]	7.75[a]	28.88[d]	30.96
MMS 1 mM	510	176	79	35	20	13	6	329	50.00	14.51	64.51	***
MMS plus 1%	240	61	32	24	13	7	9	146	38.75[a]	22.08[b]	60.83[a]	5.70
MMS plus 2%	234	62	29	24	8	3	3	129	38.89[a]	16.24[a]	55.13[a]	14.54
MMS plus 3%	314	60	48	20	11	11	3	153	34.39[c]	14.33[a]	48.72[c]	24.48
Nutmeg (*Myristica fragrens*)												
MMS 0.5mM	294	83	51	10	9	4	6	163	45.58	9.86	55.44	***
MMS and 0.5%	318	66	24	6	4	4	2	106	28.30[c]	5.03[b]	33.33[d]	39.88

MMS and 1%	408	69	22	6	3	8	5	113	22.30[d]	5.39[b]	27.69[d]	50.05
MMS 1 mM	280	96	36	14	12	14	0	172	47.14	14.29	61.43	***
MMS and 0.5%	342	61	18	24	8	18	0	129	23.1[d]	14.62[a]	37.72[d]	38.6
Rosemary (*Rosmarinus officinalis)*												
MMS 0.5 mM	254	31	28	13	15	8	18	113	23.22	21.26	44.48	***
MMS plus 0.5%	246	38	15	10	6	2	16	87	21.55[a]	13.82[a]	35.37[a]	20.48
MMS plus 1%	222	22	20	10	9	5	10	76	18.92[a]	15.31[a]	34.23[a]	22.73
MMS 1 mM	320	131	43	8	7	0	0	189	54.37	4.69	59.06	***
MMS plus 0.5%	298	87	34	14	3	1	2	141	40.60[b]	6.71[a]	47.31[a]	19.89
MMS plus 1%	268	83	31	7	3	3	1	128	42.54[b]	5.22[a]	47.76[a]	19.13
MMS plus 2%	294	64	27	14	5	4	0	114	30.95[d]	7.82[a]	38.77[d]	34.35
Laurel (*Laurus nobilis*)												
MMS 0.5 mM	570	103	35	28	17	11	3	197	24.21	10.35	34.56	***
MMS plus 1%	222	30	16	13	5	3	5	72	20.72[a]	11.71[a]	32.43[a]	6.16
MMS plus 2%	402	57	20	11	7	7	7	109	19.15[a]	7.96[a]	27.11[b]	21.56

MMS plus 3%	326	44	13	11	5	4	1	78	17.48[b]	6.44[a]	23.93[c]	30.76
MMS 1 mM	850	212	112	95	58	24	16	517	38.12	22.70	60.82	***
MMS plus 1%	678	169	105	74	34	23	16	421	40.41[a]	21.68[a]	62.09[a]	***
MMS plus 2%	1002	220	113	84	50	33	16	516	33.23[a]	18.26[b]	51.49[c]	15.34
Verbena (*Verbena triphylla*)												
MMS 0.5 mM	274	72	36	17	4	4	1	134	39.42	9.49	48.91	***
MMS plus 2%	230	52	24	16	7	6	1	106	33.04[a]	13.04[a]	46.08[a]	5.79
MMS plus 3%	156	35	16	9	4	2	0	66	32.69[a]	9.62[a]	42.31[a]	13.49
MMS 1 mM	234	84	46	15	11	7	1	164	55.56	14.53	70.09	***
MMS plus 1%	258	81	43	28	19	11	3	185	48.06[a]	23.64[b]	71.70[a]	***
MMS plus 2%	264	70	38	25	15	7	0	155	40.91[b]	17.80[a]	58.71[a]	16.24
MMS plus 3%	204	55	27	15	9	7	5	118	40.20[b]	17.64[a]	57.84[a]	17.48

Table 1: Summary of results of inhibitory effects of fenugreek seeds, rosemary, nutmeg, laurel and verbena on mutagenicity on MMS in the *Drosophila* eyes spot test.
[(-)] Size classes: S, small 1-4 ommatidia affected; L, large>4 ommatidia; T, total spots.
MMS: Methyl methanesulfonate.
[a] significantly not different from MMS $p>0.05$, [b,c,d] significantly different from MMS control at $p<0.05$; $p<0.01$ and $p<0.001$ respectively.

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was toxic in combination with 1 mM but not with 0.5 mM of the genotoxin used. The classification of mosaic light spots was performed according to their size. Although all treatments were chronic from the egg stage on, the majority of clones were small spots.

MMS significantly increased the numbers of small, large and total spots per 100 eyes tested either at both 0.5 and 1 mM ($p<0.01$). The suppressing effect on induced spots varies among the different plants when they were administrated simultaneously with the alkylating agent. The nutmeg was the most active plant decreasing the MMS-induced spots, particularly at the concentration of 1% (w/v) by 50% (Table 1). A significantly higher inhibitory activity was detected for all types of spots induced by 1 mM MMS for both 0.5 and 1% of this spice. In contrast, no differences were observed in the total spot frequencies produced by MMS alone and in combination with verbena even if the inhibition rate reached 17% with 3% of the plant co-administered with 0.5 mM of the MMS. However, 2 and 3% of verbena co-administered with 1mM MMS significantly reduced $(p<0.05)$ the number of small spots generated from 55.56 to 40 spots per 100 eyes, respectively. This inhibition effect was not interpreted as a real protective effect of verbena against MMS since it was limited only to the category of small spots and not reflected in the frequency of total spots. Rosemary significantly ($p<0.001$) reduced the number of MMS-induced spots when incorporated at 2% ($p<0.001$). However, at 0.5 and 1% rosemary inhibited by 19% to 23% respectively, without reaching statistical significance $(p>0.05)$. In contrast, the frequencies of mutations observed with fenugreek seeds and laurel showed a dose-dependent antigenotoxic effects. Laurel at 3% (w/v) showed an inhibitory effect of 30% $(p<0.01)$ and fenugreek seeds at the same dose afforded a 24-30% inhibition $(p<0.01)$.

Discussion

The antimutagenicity potential of some medicinal plants and spices against Methyl methanesulfonate was assessed using *Drosophila*, an organism that is suited for in vivo testing of simple or complex compounds (Graf et al., 1998). MMS, a monofuctional alkylating agent, is know for its ability to interact directly with DNA in vitro and in vivo producing genotoxic damage in different models (Adler, 1980; Rodriquez-Arnaiz et al., 1996). The mutagenicity and carcinogenicity of monofunctional alkylating agents, including MMS, have been associated with the formation of *O*-alkylated and *N*-alkylated DNA bases (Vogel and Nivard, 1994). The mutational spectra induced in *Drosophila* by MMS suggest the involvement of apurinic sites as mutagenic lesions (Vogel et al., 1990). In addition, 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 (Rodriquez-Arnaiz et al., 1996). Against this direct acting mutagen, all the spices and medicinal plants used in this study with the exception of verbena showed a significant antimutagenic activity. Considering the genotoxin co-administered with these plants, the possibility that the antimutagens in these natural products exert their protective effect by interacting with MMS in desmutagenic manner without affecting the genetic material directly (Kuroda et al., 1992). MMS does not require metabolic activation; therefore, the natural compounds present in the plants and spices 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.

The inhibitory effect detected in this study can be attributed to wide range constituents of plant studied such as chlorophyll, fibres and many phytochemicals including simple phenols, phenolic acids, flavonoids, carotenoids, tocopherols and ascorbic acid (Belakhdar, 1997; Duke, 1992). Chlorophyll, ubiquitously distributed in the green leafy plants including laurel, also the fibres that can be found in rosemary; fenugreek and nutmeg seeds (Belakhdar, 1997; Duke, 1992), will act by scavenging of reactive molecules through binding or absorption. The fibres are able to absorb irreversibly the mutagens (Kada et al., 1984). For their parts, chlorophylls and their soluble derivative chlorophyllin inhibit genotoxicity by forming a reversible complex with the mutagenic agent (Bronzetti et al., 1990; Negishi et al., 1990). The phenolic compounds and many flavonoids were reported also to have the capacity to scavenge mutagens or free radicals (Rice-Evans et al., 1996; Yao et al., 2004). The antimutagenic activity of some flavonoids was caused by radical scavenging effects (Edenharder and Grunhage, 2003). Thereafter, some of polyphenols and flavonoids identified from rosemary and fenugreek (Belakhdar, 1997; Duke, 1992) may be responsible for the inhibitory effect detected for this plants.

On the other hand, many antioxidants have been found in spices and herbs studied including carotenoids, ascorbic acid, tocopherols, and a wide range of other various polyphenolics, some of which are well-known strong scavengers of active oxygen radicals (Rice-Evans et al., 1996; Aruoma, 2003; Kim and Lee, 2004).The antimugenic effect detected for the plants studied may be attributed to those constituents since the antioxidant compounds are known to have inhibitory effects on the genotoxic actions of several known mutagens (Ferguson, 1994).

Other desmutagens were reported to be enzymatic modulators that can act through enzyme systems by inducing either phase I and/or phase II enzymes of detoxification or by altering the balance of different enzyme activities. These agents include ascorbic acid (Kohlmeier et al., 1995) found in rosemary and fenugreek (Belakhdar, 1997; Duke, 1992) that may contribute trough this way on the antimutagenic activity detected of these plants. Ascorbic acid was also reported to be antimutagenic in both in vitro and in vivo tests including *Drosophila* assays (Kaya, 2003).

The *Drosophila* eyes SMART assay is a suitable in vivo system for detecting the antimutagenic effects of complex mixture such as spices or medicinal plants. The plants tested showed some antimutagenic activity against monoalkylating agent such as MMS and this could be attributed to the active principles. Thus further research to characterize their molecular mechanism of protection against mutagen and carcinogen attack to DNA is warranted.

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