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Genotoxicity and anti-genotoxicity of fennel plant (*Foeniculum vulgare* Mill) fruit extracts using the somatic mutation and recombination test (SMART)

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The antigenotoxic action of fennel (*Foeniculum vulgare*) were assessed using the eye (w/w$^+$) somatic mutation and recombination test (SMART) assay of *Drosophila melanogaster*. Fennel is used for various medicinal purposes. Methyl methanesulfonate (MMS) was used as the positive control. Fruit extracts of fennel did not show genotoxicity at the doses used. In the co-treatment, a dose-dependent decrease in mutation frequency was observed for this plant. The fennel at 8% (w/v) demonstrated a marked decrease in MMS with an inhibition rate of 41.16%. The results demonstrate that the fennel exerted a significant and potent anti-mutagenic activity in eyes spot of *D. melanogaster*.

Key words: *Drosophila melanogaster*, somatic mutation and recombination test (SMART), antigenotoxicity, *Foeniculum vulgare*, methyl methanesulfonate (MMS).

INTRODUCTION

Recently, there has been considerable interest in the mutagenicity and antimutagenicity of medicinal plants (Romero-Jimenez et al., 2005; Patenkovic et al., 2009; Ribeiro and Salvadori, 2003). It is sometimes argued that frequently used plants in traditional medicine are safe, due to their long-term use and are considered to have no side effects because they are natural (Van den Berg et al., 2011). This concept is largely circumstantial and it is important to determine toxicology of plant extracts, especially those that are used frequently over long periods.

Foeniculum vulgare Mill. (Apiaceae family) is commonly known as fennel. The plant is native to the Mediterranean region, and temperate regions of Asia. It was introduced and distributed to subtropical regions, Europe and North America and also cultivated worldwide (He and Huang, 2011). The fruits, leaves and roots can be used, but the fruits are most active medicinally and are the part normally used (EFSA, 2009).

Numerous compounds including *trans*-anethole, estragole, fenchone, sesquiterpenoids, coumarins and polyphenolics were isolated from this plant, most of which exhibited significant bioactivities. Fennel is a commonly used household remedy, having been claimed to be useful in the treatment of a variety of complaints, especially those of the digestive system (EFSA, 2009). The plant is said to be analgesic, anti-inflammatory, antispasmodic, aromatic, carminative, diuretic, emmenagogous, expectorant, galactogogous, hallucinogenic, laxative, stimulant and stomachic (El Bardai et al., 2001; Boskabady et al., 2004; Raffo et al., 2011)

The somatic mutation and recombination tests (SMART) system has been used for over 20 years to study anti-genotoxic effects of many mutagens and carcinogens (Zimmering et al., 1990). A wide variety of compounds and complex mixtures have been assayed in

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this test, such as food additives, beverages and insecticides (Osaba et al., 2002). These one generation tests make use of the wing or eye imaginal disc cells in larvae and have proven to be very efficient and sensitive. They are based on the principle that the loss of heterozygosity of suitable recessive markers can lead to the formation of mutant clones of cells that are then expressed as spots on the wings or eyes of the adult flies (Graf et al., 1998).

The purpose of this work was to study the genotoxic activity of fennel and to evaluate its potential anti-genotoxic effect using methyl methanesulfonate (MMS) as a mutagen

MATERIALS AND METHODS

Preparation of the aqueous extract

The fresh fruit of fennel was purchased at the local market in the city of Tetouan, Morocco. Plant material was prepared according to the traditional method used in Morocco**.** Ten grams of dried, ground plant material were extracted in 100 ml of distilled water and allowed for over-night shaking. After filtering, solutions were used immediately in treatments.

Negative and positive controls

Pure distilled water served as a negative control. A 1 mM aqueous solution of MMS [66-27-3] (Sigma) were used as the positive control. MMS was purchased from Sigma and was reagent grade.

Eye spot test

Markets and strains

The following *Drosophila melanogaster* strains were used: *Ok-white*, a strain carrying the X-linked eye color marker *white (w)* and *Ok-yellow.*

Treatment procedure

Plant extract and mutagen were administered by chronic co-treatment feeding exposure from the egg stage. The *Oregon k (Ok)* was used. Twenty *Oregon K- yellow-* virgin female flies were mated with *w* males (20) flies for three days and then transferred to bottles (200 mL) containing 3 g of instant Drosophila medium (Carolina Biological Supply, Burlington, USA) dissolved with equal volumes of the test plant extract and MMS.

Drosophila larvae were exposed to fennel extract at three concentrations: 2, 4 and 8%. This was done by adding the solutions to the flies' medium. The solutions were always freshly prepared, immediately before use. The flies were permitted to lay eggs for three days. Newly hatched flies were counted and the females were transferred to fresh medium; two to six days later, their eyes were inspected for the presence of white spots. All experiments were carried out at 25°C and 65% relative humidity.

Scoring of eyes

The scoring of the etherized flies was carried out in a liquid consistin g of 90 parts ethanol, one part Tween-80 and nine parts water (Smijs et al., 2004). Because of the fast evaporation of this solution, a second solution (90% water + 10% first solution) was added when necessary (Gaivao and Comendador, 1996).

Inspection of the eyes for the presence of white spots was performed using stereomicroscope at a magnification of 80x using a light source that was provided by a glass fiber illumination of 20 W. Large white spots are seen as white parts in the red eyes and small white spots are dark ommatidia in red eye. Spots separated from each other by at least four nonmutated ommatidia were counted as independent events, and the smallest size of white clone expected to be counted accurately is 2 (Vogel and Nivard, 1993). An increase in white clone frequency was only accepted as a positive response if it was significantly higher than that of both the concurrent and pooled experimental controls.

Statistical test

The statistical significance of the differences between spot frequencies in the experimental groups and control was calculated using the Chi-square test for proportions as described by Frei and Wurgler (1988). The percentage of genotoxicity inhibition was calculated based on the spot frequencies according to the formula: [(mutagen alone-mutagen + plant)/mutagen alone] x 100 (Abraham, 1994).

RESULTS

The fennel fruits extract was first analyzed for the evaluation of its mutagenic activity. The numbers of small spots, large spots with the total number of spots are given in Table 1. Fennel extract was not toxic in the chronic feeding. At the concentrations tested, it did not show any effect on the frequencies of somatic mutations when compared with their respective negative controls. The data obtained with this approach showed that the fennel extracts does not induce mutagenicity at the selected doses.

MMS showed to be clearly mutagenic (Figure 1). A high value of genotoxic activity with a high significant response ($p < 0.001$) was detected for all the spot categories: small spot, large spots as well as total spots for all the concentration used when compared with the negative control. MMS at 1 mM showed a highly significant increase in induced somatic mutations (53.03 spot per 100 eyes).

The anti-genotoxic effect was established by means of a chronic co-administration of fruits water extract of fennel with MMS to larvae of *D. melanogaster*. Our result showed a dose-dependent inhibition of genotoxicity in larvae (Table 2). The overall inhibition activity is 31.38, 34.83 and 41.16% for 2, 4 and 8% concentration of fennel, respectively. The chronic co-administration of fennel was effective in significantly reducing the frequencies of large and totals spots induced by MMS.

DISCUSSION

The use of *D. melanogaster* in the evaluation of genotoxicity has been well established as a test-system. Due

Table 1. Mutagenic effect of fennel fruit extract in *Drosophila* eye spot test (w/w$^+$).

Compound concentration (%)	Number of eyes	Distribution of spots size (ommatidia)				Spots per 100 eyes size classes(-)		
		2-4	4-32	>32	T	S	L	T
0	560	40	30	0	70	7.14^b	5.36^a	12.5^a
2	434	47	24	1	72	10.83^a	5.76^a	16.59^a
4	458	37	08	1	46	8.78^a	1.96^b	10,04^a
8	640	43	11	2	56	6.72^a	2.03^b	8.75^b

(-) Size classes: S, small 1-4 ommatidia affected; L, large >4 ommatidia; T, total spots, a: significantly not different from positive control, p>0.05; b, significantly different from control at p<0.05.

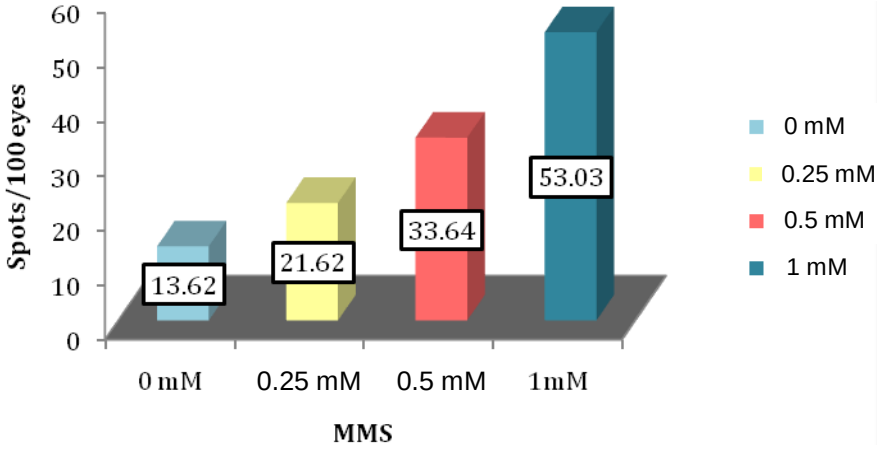

Figure 1. Mutagenic effect of MMS in eye spot test of *D. melanogaster*.

Table 2. Antimutagenic effect of fennel fruit extract on MMS that induced mutagenicity in *Drosophila* eye spot test (w/w$^+$).

	Number of eyes	Distribution of spots size (ommatidia)				Spots per 100 eyes size classes(-)			Inhibition (%)
		2-4	4-32	>32	T	S	L	T	
MMS 1 mM	340	70	48	7	125	20.6	16.17	36.77	-
MMS and 2% Fen	214	16	35	3	54	**7.47^d**	17.75^a	**25.23^b**	31.38
MMS and 4% Fen	242	12	36	10	58	**4.95^d**	19.00^a	**23.96^c**	34.83
MMS and 8% Fen	298	9	46	9	64	**3.02^d**	18.45^a	**21.47^c**	41.16

(-) Size classes: S, small 1-4 ommatidia affected; L, large >4 ommatidia; T, total spots; MMS: methyl methanesulfonate; a: significantly not different from positive control, p>0.05; b, c, d: Significantly different from positive control at p<0.05, 0.01 and 0.001, respectively.

to a genome similarity as compared to mammals and easy maintenance in the laboratory, these flies represent an appropriate organism to run *in vivo* short-term tests (Graf et al., 1996; Vogel et al., 1999). SMART is a simple and fast short-term assay when compared with other *in vivo* tests. Through the use of these test-systems, it is possible to evaluate the genotoxic activity of a single compound as well as complex mixtures (Patenkovic et al., 2009: Schneider et al., 2009; Lehmann et al., 2010).

The somatic assays take advantage of the possibility to expose such large populations of mitotically growing cells in the imaginal discs of larvae. If a genetic alteration occurs in one of these imaginal disc cells, this alteration will be present in all the descendant cells and will form a clone of mutant cells. If the alteration causes a visible change in the phenotype, the mutant cell clone can be detected as a spot of mutant cells on the body surface of the adult flies. Thus, due to its capabilities, SMART was

chosen to evaluate the anti-genotoxic effects of the aqueous fennel extract.

The fennel was first analyzed for the evaluation of its genotoxic/mutagenic activity. Three concentrations of the fennel were tested. The concentrations chosen are not toxic according to progeny mortality relative to the control. Fennel, at the concentrations tested, did not show any effect on the frequencies of somatic mutations when compared with their respective negative controls.

The evaluation of the antimutagenic potential of the aqueous extract from fennel was established by means of a co-treatment protocol in which the extract was administered simultaneously with the mutagens used, MMS a direct-acting genotoxin. MMS is a monofunctional alkylating agent known for its ability to interact directly with DNA, and produces genotoxic damage in different models *in vitro* and *in vivo* (Arnaiz et al., 1996). Under our experimental conditions, MMS showed to be clearly mutagenic. The aqueous extract from fennel displayed significant antimutagenic effect on the somatic mutations induced by MMS.

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 (Arnaiz et al., 1996). Against this direct acting mutagen, the fennel used in this study showed a significant antimutagenic activity. Considering the genotoxin co-administered with the plant, there is possibility that the antimutagens from fennel 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 this plant 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 of constituents of plant including *trans*-anethole, estragole, fenchone, sesquiterpenoids, coumarins and polyphenolics studied. Aqueous extract of fennel fruits contains rich phenolic compounds. Many of them have antioxidant activities, such as 3-caffeoylquinic acid, 4-caffeoylquinic acid, 1.5-Odicaffeoylquinicacid, rosmarinic acid, eriodictyol-7-Orutinoside, quercetin-3-O-galactoside, kaempferol-3-Orutinoside and kaempferol-3-O-glucoside. Besides these compounds, fennel was reported to contain hydroxylcinnamic acid derivatives, flavonoid glycosides and flavonoid aglycones (Parejo et al., 2004). Naturally-occurring antioxidants can be used to protect gainst oxidative stress damage (Scalbert et al., 2005). Fennel was known as excellent source of nature antioxidants and contributed to the daily antioxidant diet (Shahat et al., 2011).

The mutagenicity, antimutagenicity and anticarcinogenicity of fennel and some of its components were subjected to several investigations (Ebeed et al., 2010; Hassan et al., 2011) and indicated that trans-anethole, the main component of fennel oil, does not increase the mutant frequency in the Salmonella/microsome test and it did not induce chromosome aberrations in Chinese hamster ovary cells. In addition, pretreatment with trans-anethole and eugenol led to significant antigenotoxic effects against ethyl methane sulfonate (EMS), cyclophosphamide (CPH), procarbazine (PCB), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and urethane (URE). Both trans-anethole and eugenol exerted dose-related antigenotoxic effects against PCB and URE. There was no significant increase in genotoxicity of trans-anethole and eugenol even when administered at high doses (Abraham, 2001).

Conclusion

This research indicates that fennel may serve as potential dietary sources of natural anti-mutagen for improving human nutrition and health. Purification and identification of the active compounds in these herbs is required for a better understanding of the protective mechanisms involved and for the possible application in the food industry and in medicine.

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