

Ameliorative potential of *Myristica fragrans* extract as hypoglycemic agent on oxidative stress produced by diabetes mellitus in mice

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Abstract: Diabetes mellitus (DM) is a common disease affecting several million individuals worldwide. An increased reactive oxygen species (ROS) and insufficient antioxidant activity is known in DM. Damage has been reported to occur on all components of biological systems (e.g., DNA, RNA, lipids, proteins, carbohydrates, low-molecular-mass species, antioxidants) due to the high reactivity of many oxidants. Antioxidant compounds in the human foods or supplementary diets can be used to counteract several diseases. The treatment of DM with complementary and alternative medicines (CAM) such as dietary supplements and plant-based medicines is increasingly practiced. The myristica fragrans seeds usually used as spice and commonly known as nutmeg have anti-carcinogenic, antioxidant, anti-inflammatory, antidiabetic and hepatoprotective activities. The present study was designed to evaluate the antigenotoxic effects of aqueous extract of *M. fragrans* on DNA damage induced by DM using micronucleus assay (MN) in liver cells and chromosomal aberrations in spermatocyte cells in mice. MN frequency and chromosomal aberrations was significantly increased in diabetic mice compared with the normal mice ($p < 0.05$). Oral administration of aqueous extract of *M. fragrans* (0.1ml/mouse) for 5, 10 and 15 days groups treatment in diabetic mice were significantly decreased MN frequency and chromosomal aberrations in a time dependent manner. According to our knowledge this is the first report on the antigenotoxic capacity of *M. fragrans* against DNA damage induced by DM in vivo.

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1. Introduction

Diabetes mellitus (DM) is a metabolic disorder affecting millions of people worldwide. In 2000, it affected 171 million people around the world and it is expected that in 2030, the number of DM sufferers will reach more than 366 million people corresponding to 4.4% of the world's population. It is projected that the greatest relative increase will occur in the Middle East, sub-Saharan Africa and India (King et al., 1998; Wild et al., 2004).

Recently plant based therapies gains importance as they have been shown to regulate the oxidative complications of DM (Kim et al., 2006). Antioxidants are used as supportive therapy in the treatment of diabetes (Garg and Bansal, 2000). Thus, there is an increasing demand for natural products with antidiabetic and antioxidant activities to attenuate oxidative stress induced complications. In addition, chemoprevention with natural substances aims to reduce insulin resistance and stimulates insulin secretion minimizing the risk of developing DM and its sequels (El Missiry and El Gindy, 2000).

In addition to providing additional taste and flavor to foods, certain spices have been used as remedies in traditional medicine for centuries

(Lampe, 2003; Srinivasan, 2005). Several healthy benefits of the consumption of a number of such spices include a digestion-stimulating action, a hypolipidemic effect, antidiabetic influence, presence of antilithogenic properties, antioxidant potential, anti-inflammatory properties, and antimutagenic and/or anticarcinogenic potential, benefits which appear to have been well documented previously (Lampe, 2003; Rajamani et al., 2005; Srinivasan, 2005).

Myristica fragrans Houtt. (Myristicaceae), usually used as spice and commonly known as nutmeg has been reported to have various biological activities, including antioxidant, antiinflammatory, anticariogenic, antidiabetic and antihepatotoxic properties (Filleur et al., 2001; Sadhu et al., 2003; Jin et al., 2005; Chung et al., 2006; Sohn et al., 2007; Cui et al., 2008; Han et al., 2008).

In the present study, we have evaluated the effect of DM on liver MN frequency and chromosomal aberrations in spermatocytes, as well as, the potential genotoxic/antigenotoxic effect of *M. fragrans* water extract DM-associated DNA damage in mice. In this study, we chose the liver due to its extremely important for the risk assessment of the potential numerical aberration because of the predominantly

high penetration of almost all compounds to this organ (Zhurkov et al.1996). In a relatively short time, hepatic injury has been recognized as a major complication of DM (Harrison, 2006). While, spermatocytes represent the only system in which the transmissible genetic damage from one generation to another takes place (William and Hsu 1980).

2. Materials and Methods

2.1. Animals

Male white Swiss mice aged 9–12 weeks were used in all experiments. The animals were obtained from a closed random-bred colony at the animal's house, National Research Center. The mice used for any one experiment were selected from mice of similar age (± 1 week) and weight (± 2 g). Animals were housed in polycarbonate boxes with steel-wire tops (not more than five animals per cage) and bedded with wood shavings. Ambient temperature was controlled at 22 ± 3 °C with a relative humidity of $50 \pm 15\%$ and a 12-h light/dark photoperiod. Food and water were provided *ad libitum*. Animals were sacrificed after treatment by cervical dislocation.

2.2. Chemicals

Alloxan was obtained from Sigma-Aldrich Inc. (St. Louis, Mo). All other chemicals used were of analytical grade.

2.3. Plant Material and Extract Preparation.

Myristica fragrans used in this study was procured from Agricultural Research Center Giza (Egypt).

Powdered dry seeds (5g) of *M. fragrans* were prepared by adding 100ml of boiling water, let to stand for 30 min. at room temperature, followed by filtration.

2.4. Experimental design

Adult male mice were divided equally into six groups of ten animals in each group:

1) Normal group (control healthy group) received only distilled water.

2) Alloxan induced diabetic mice (a single i.p injection at 120mg/kg b.wt, in citrate buffer 0.1 M, pH 4.5 (Cooperstien and Walkins, 1981). After 72 h, animals with serum glucose levels higher than 250 mg/dl were considered diabetic and were included in the study (Perfumi and Tacconi, 1996).

3) Normal mice received orally by gavage 0.1ml /mouse of water extract of *M. fragrans* for 5, 10 and 15 consecutive days.

4, 5, 6) Diabetic mice treated orally by gavage with 0.1ml /mouse of water extract of *M. fragrans* for 5, 10 and 15 consecutive days.

2.5. Cytogenetic analysis

1. Micronucleus test

Liver-cell suspensions were prepared as

described by (Higgins and Anderson 1931; Tates et al., 1980). One drop of the cell suspension was dropped onto microscope slide using a pipette and then stained with May–Gruenwald and Giemsa at pH 6.8. The smears were dried, and the slides were screened under an oil immersion objective (Olympus, Japan) (Murakami and Horikawa, 1995). Slides were screened for binucleated cells (1000 hepatocytes / mouse, 5 animals/ treatment). The data generated from different doses were statistically compared with solvent controls and between treatments with protection versus treatment alone as described by Kastenbaum and Bowman (1970).

2. Chromosome aberrations

For spermatocytes preparation, animals from each group were injected i.p. with colchicine, 2–3 h before sacrifice. Animals were sacrificed by diethyl ether at end of treatments. Chromosome preparations were made from testes according to the technique of Evans et al. (1964). One hundred well spread diakinase- metaphase I cells were analyzed per animal (5 animals/group) for chromosome aberrations. Metaphases with univalents and chromosome translocations were recorded. The significance of the effect of each treatment dose versus the solvent control and between treatments with protection versus treatment alone was evaluated by t-test.

The percent reduction in the frequency of MN in liver cells and chromosomal aberrations in spermatocytes was calculated according to Waters et al. (1990), using the following formula:

$$\% \text{Reduction} = \frac{\text{frequency of DNA damage in A} - \text{frequency of DNA damage in B}}{\text{frequency of DNA damage in A} - \text{frequency of DNA damage in C}} \times 100$$

Where A corresponds to the group with diabetic mice (positive control), B to the group treated with Diabetes plus water extract of *M. fragrans* and C corresponds to the negative control (vehicle).

3. Results

1. Micronucleus test

Myristica fragrans showed no significant elevation in the frequency of MN in liver cells (Table 1). From the results presented in Table (1) we found that the percentage of MN in binucleated liver cells statistically significant ($p < 0.05$) in DM group compared to negative control. The protective effect of water extract of *M. fragrans* administered as successive dose for 5, 10 and 15 days with diabetic mice reduced MN ($p < 0.05$) in binucleated liver cells in a time dependent matter compared to DM group alone.

The percentage of reduction of MN increased with increasing the time of treatment with water extract of *M. fragrans* (Table 1).

2. Chromosome aberrations

No significant differences between the animals treated with *M. fragrans* up to 15 days and the negative control group was observed in this study (Table 2). The mean percentage of diakinesis metaphase I cells reached to 16.2% with DM and it was statistically significant ($p < 0.05$) compared to the negative control. This percentage was statistically

($p < 0.05$) decreased gradually after oral treatment with the *M. fragrans* (0.1 ml/mouse) for 5, 10 and 15 days. The main types of chromosome aberrations observed were XY univalents and autosomal univalents (Table 2).

The percentage of reduction of chromosomal aberrations was time dependent (Table 2).

Table 1: The effect of total extract of *M. fragrans* on DM-induced elevation of micronuclei in liver cells.

Treatments	Time of Treatment (Day)	NO of MN	MN in binucleated cells Mean± S.E.	Reduction %
I. Control (vehicle).				
II.DM	-	18	0.36±0.50	-
	-	168	3.36±0.53 ^a	-
III. <i>M. fragrans</i>	5	20	0.40± 0.52	-
	10	24	0.48± 0.47	-
	15	26	0.52± 0.60	-
IV. DM+ <i>M. fragrans</i>	5	107	2.14±0.47 ^{ab}	40.67
	10	91	1.82±0.59 ^{ab}	51.33
	15	85	1.60±0.40 ^{ab}	58.67

The total No of scored cells is 5000 (5 animals /group)

a) Significant compared to vehicle control ($p < 0.05$)

b) Significant compared to DM treatment ($p < 0.05$)

Table 2: Number and mean percentage of diakinesis metaphase I cells with chromosomal aberrations in mouse spermatocytes after treatment with DM and *M. fragrans* with DM.

Dose	Time of treatment (Day)	No. of different types of chromosomal aberrations					Total aberrations		II (%)
		XY univalent	Autosomal univalent	XY+ Autosomal univalent	Frag	Chain (IV)	No	Mean±S.E	
I. Control	-	8	6	-	-	-	14	2.80±0.50	-
III. DM	-	40	33	3	1	4	81	16.20±0.43 ^a	-
II. <i>M. fragrans</i>	5	10	5	-	-	-	15	3.00±0.57	-
	10	12	6	-	-	-	18	3.60±0.38	-
	15	12	8	-	-	-	20	4.00±0.64	-
IV. DM+ <i>M. fragrans</i>	5	31	27	1	2	2	63	12.60±0.68 ^{ab}	26.68
	10	32	20	1	1	-	54	10.80±0.72 ^{ab}	40.30
	15	25	18	-	-	-	43	8.60±0.78 ^{ab}	56.72

The total number of scored cells is 500 (5 animals/ group); Frag. : Fragment; II: Inhibitory Index

a) Significant compared to vehicle control ($p < 0.05$)

b) Significant compared to DM treatment ($p < 0.05$) (t-test)

4. Discussion

Oxidative stress occurs when the balance between oxidant and antioxidant systems shifts in favor of the former leading to the generation of free oxygen radicals. Reactive oxygen species are involved in the pathogenesis of many diseases including hypoxia, hypercholesterolemia, atherosclerosis, hypertension, ischemia reperfusion injury and heart failure (Taniyama and Griendling, 2003; Wilcox and Gutterman, 2005). It has been shown that patients with diabetes mellitus have increased oxidative stress and impaired antioxidant defense systems, which appear to contribute to the initiation and progression of diabetes-associated complications (Maritim et al., 2003). There is convincing experimental and clinical evidence that the generation of reactive oxygen species is increased in both types of diabetes. Exposure of the genetic material to ROS could cause DNA strand breaks (Evans et al., 2004) and MN (Shaik et al., 2010). These types of damages could lead potentially serious consequences for the cell (Gomez-Meda et al., 2004; Bonassi et al., 2007).

There are limited reports on the association between diabetes and the occurrence of genotoxic effects. Our results showed that DM induced DNA damage in liver and spermatocyte cells in mice and these observations supported the previous data showed by (Sheth et al. 2006, ;Zuniga-Gonzalez et al., 2007; Celikler et al., 2009; Salah et al., 2011).

DM is a major public health problem worldwide. The management of diabetes without any side effect is still a challenge to the medical system. Natural products have long gained wide acceptance among the public and scientific community because of their effectiveness, fewer side effects and relatively low cost. Wide array of plant-derived active principles were shown to have antidiabetic activity (Prabhakar and Doble, 2008). Even when their contents of the biologically active constituents are unknown (Valiathan, 1998).

The water-insoluble property of a drug limits its therapeutic applications and complicates its administration. Hence, it is necessary to find antigenotoxic agents that are water-soluble, have few side effects and are strong enough to prevent acute cellular rejection (Checker et al., 2008). So, the aim of the present study is to evaluate the genotoxic/antigenotoxic activity of antidiabetic agent *M. fragrans* water extract against DNA damage induced by DM.

Our results showed that *M. fragrans* did not induced DNA damage in liver and spermatocyte cells up to 15 days. In the contrary, it had the ability to reduce the DNA damage induced by DM in a time dependent manner. To the best of our knowledge no

available data about genotoxicity and antigenotoxicity activity of *M. fragrans* on DNA damage induced by DM was observed. Few data published about the antigenotoxicity of other medicinal plants such as *Ulva rigida* (Celikler et al., 2009) and Kafta, Somma, Araar and Doum (Salah et al., 2011) against the genotoxicity induced by DM.

Herbs and spices are among the most important targets in which to search for such antigenotoxicity. *Myristica fragrans* Houtt. (Myristicaceae) is an aromatic evergreen tree cultivated in South Africa, India, and other tropical countries. It has long been used indigenously as a spice in many Western foods (Halliwell and Gutteridge, 2000). *M. fragrans* is also prescribed for medicinal purposes in Asia to treat many diseases, such as rheumatism, muscle spasm, decreased appetite, and diarrhea (Nguyen et al., 2010). As part of an ongoing screening program to search for new AMPK activators from natural plants, the total extract of *M. fragrans* was found to activate the AMPK enzyme in differentiated C2C12 cells. *M. fragrans* is one of the most widely used spices in the preparation of Ayurvedic drugs (Chhabra and Rao, 1994).

Lignans are polyphenolic substances. They are one of the major classes of phytoestrogens and also act as antioxidants. Both nutmeg and mace are known to contain about 2% lignanes (diarylpropanoids), which is water-soluble. Recently Checker et al. (2008) observed that lignans present in aqueous extract of fresh nutmeg mace possess antioxidant, radioprotective and immunomodulatory effects in mammalian cells. Beside it significantly inhibited the radiation-induced DNA damage in splenocytes as indicated by decrease in DNA fragmentation (Checker et al., 2008). Their biological activities like anti-tumor, antimicrobial, antiviral, anti fungal and antiatherosclerotic have been well documented (Mac and Towers, 1984).

Macelignan, a natural compound belonging to a group of lignans isolated from *M. fragrans* has been reported to have various biological activities, including antioxidant, antiinflammatory, anticariogenic, and antihepatotoxic properties (Filleur et al., 2001; Sadhu et al., 2003; Jin et al., 2005; Chung et al., 2006; Sohn et al., 2007; Cui et al., 2008). Macelignan has also been shown to function as an antidiabetic and endoplasmic reticulum stress-relief agent (Han et al., 2008). Macelignan protects the cells from UVB-mediated cell death through its antioxidant activity and, therefore, plays a significant role in ameliorating ROS-related damage (Anggakusuma et al., 2010).

Myristicin (1-allyl-5-methoxy-3, 4-methylenedioxybenzene) is found in *M. fragrans* (NTP, 1983; Bakkali et al., 2008). It is used as

fragrances in the cosmetic and pesticide industry and as flavouring agent (**Hikiba et al., 2005; Slamena et al., 2009**). Myristicin is also used in traditional medicine to treat rheumatism, cholera, psychosis, stomach cramps, nausea, diarrhea, flatulence, and anxiety (**Barceloux, 2008**).

Eugenol one of the main constituent of *M. fragrans* (**Sukumaran and Kuttan, 1995**). Eugenol, a major phenolic component has been widely used in medical practice, due to its potent fungicidal, bactericidal, anesthetic, antioxidant and anti-inflammatory properties (**He and Liu, 2003**). Eugenol ameliorates gamma radiation induced clastogenic effects (**Tiku et al., 2004**) and genotoxin-induced DNA damage (**Abraham, 2001**) in vivo. Eugenol pretreatment prevented DNA strand break and improves the antioxidant status in thioacetamide treated rats (**Yogalakshmi et al. 2010**). It is also reported to induce the detoxifying enzymes namely glutathione-S-transferase in rat liver (**Yokota et al., 1988**).

Murcia et al. (2004) reported that phenylpropanoid compound extracts from nutmeg possessed antioxidant activity. Also, high antioxidant activity has been reported in monoterpenoid rich extracts such as terpinene-4-ol, alpha-terpineol and 4-allyl-2,6-dimethoxyphenol in nutmeg seed (**Maeda et al., 2008**). **Sharma and Kumar (2007)** observed that pretreatment with *M. fragrans* effectively protects the mice against radiation-induced oxidative stress as evident by decrease in lipid peroxidation level and acid phosphatase activity and simultaneous increase in hepatic glutathione and alkaline phosphatase activity.

The ability of *M. fragrans* to ameliorate the chromosome alterations induced by DM may be due to 1- Scavengers of active oxygen species and electrophiles (**Yadav and Bhatnagar 2007**), 2- Antihyperglycemia (**Han et al., 2008**), hyperglycemia- induced protein glycation generates superoxide free radicals (**Raskin et al., 2000**), and 3- Enhancement of antioxidant defense enzymes (**Sharma and Kumar, 2007**).

In conclusion, the present investigation showed that *M. fragrans* have antigenotoxic effect against DNA damage induced in alloxan-diabetic animals. More work is needed to elucidate the mechanisms of action of *M. fragrans* as a preventive agent against diabetes mellitus.

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