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Antioxidants in aqueous extract of *Myristica fragrans* (Houtt.) suppress mitosis and cyclophosphamide-induced chromosomal aberrations in *Allium cepa* L. cells

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Abstract: In this study, freeze-dried water extract from the leaves of *Myristica fragrans* (Houtt.) was tested for mutagenic and antimutagenic potentials using the *Allium cepa* assay. Freeze-dried water extract alone and its combination with cyclophosphamide (CP) (50 mg/kg) were separately dissolved in tap water at 500, 1000, 2000, and 4000 mg/kg. Onions (*A. cepa*) were suspended in the solutions and controls for 48 h in the dark. Root tips were prepared for microscopic evaluation. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radicals' scavenging power of the extract was tested using butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) as standards. Water extract of *Myristica fragrans* scavenged free radicals better than BHA, but worse than BHT. The extract alone, as well as in combination with CP suppressed cell division, and induced chromosomal aberrations that were insignificantly different from the negative control ($P \leq 0.05$). However, cytotoxic and mutagenic actions of CP were considerably suppressed. The observed effects on cell division and chromosomes of *A. cepa* may be principally connected to the antioxidant properties of the extract. The obtained results suggest mitodepressive and antimutagenic potentials of water extract of the leaves of *M. fragrans* as desirable properties of a promising anticancer agent.

Key words: *Allium cepa*, Antioxidants, Chromosomal aberration, Cyclophosphamide, Mitotic index
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1 Introduction

Cancer is one of the major causes of death, claiming over 6 million lives in a year worldwide (Murias *et al*., 2005). It accounts for more deaths than heart disease in persons younger than 85 years of age (Jemal *et al*., 2009). Deaths from cancer are continuing to escalate, and have been projected to cause 9 million deaths in the year 2015 and 11.4 million deaths in 2030 (Loh *et al*., 2009). Occurrences of cancer and several other degenerative diseases have been partly attributed to the generation of reactive oxygen species (ROS), causing oxidative damage to DNA and other macromolecules in the cell (Mošovská *et al*., 2010). ROS include superoxide radical, hydrogen peroxide, and hydroxyl free radical, all of which have one or more unpaired electrons that potentially cause damage to the respiring cells. All these reactive species are highly toxic and mutagenic (Halliwell, 1994). Many antioxidant compounds, naturally occurring from plant sources, have been identified as free radical or active oxygen scavengers (Kumaran and Karunakaran, 2007). Therefore, antioxidants which can scavenge free radicals have an important role in biological system and may exert

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their effects by different mechanisms, such as suppressing the formation of active species by reducing hydroperoxides (ROO·) and H_2O_2, and also by sequestering metal ions, scavenging free radicals, repairing or removing damage (Tiwari, 2001). Several naturally-occurring antioxidants provide protection to DNA and other macromolecules against damages caused by generation of ROS, leading to lipid peroxidation, protein damage, and DNA strand breakage (Gupta *et al*., 2008). Therefore, the discovery and exploration of compounds possessing antioxidant, antimutagenic, and anticancer properties are of great practical and therapeutic importance. During the search of plants as sources of natural antioxidants having antimutagenic and anticancer potentials, some medicinal plants and fruits have been extensively investigated (Mošovská *et al*., 2010; Zahin *et al*., 2010; Akinboro *et al*., 2011).

Myristica fragrans (Houtt.), commonly called nutmeg, is an aromatic evergreen tree that grows 30–39 ft (1 ft=30.48 cm) high with spreading branches and yellow fleshy fruits, having an appearance like apricot or peach. Many other species of the plant exist, but the most common one is *M. fragrans*. Both the nutmeg and mace have been used as general condiments and to flavour many foods such as soups, gravies, milk products, fruits juices, sweet sauces, gelatins, snacks foods, and breakfast cereals (Nagano, 2008). *M. fragrans* has been used to treat rheumatism and stomach complaints in Indonesia, Malaysia, England, and China, because of its essential oil. Administration of extract of *M. fragrans* into hyperlipidaemia rabbits reduced the level of blood lipoprotein lipids (Chirathaworn *et al*., 2007). Many bioactive compounds including camphene, elemicin, eugenol, isoelemicin, isoeugenol, methoxyeugenol, pinene, sabinene, safrol, myristic acid, myristicin, and lignan were found in *M. fragrans*. Phenolic compounds belonging to the lignans group have been reported capable of scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals as well as chelating with metallic elements to form complexes (Chatterjee *et al*., 2007; Su *et al*., 2007; Gülçin, 2010; Gülçin *et al*., 2010). Argenteane is a dilignan antioxidant that is similar in power to vitamin E isolated from nutmeg's mace (Calliste *et al*., 2010). A great number of spices and aromatic herbs contain chemical compounds having antioxidant properties, most of which are attributed to various phytochemicals including vitamins, carotenoids, terpenoids, alkaloids, flavonoids, lignans, simple phenols, and phenolic acids (Chatterjee *et al*., 2007). Possession of a variety of biological activities such as antitumour, antimitotic, antiviral, and antiatherosclerotic actions by these compounds renders them important plant constituents that confer health benefits on human beings and at the same time may serve as basis for development of modern medicine against certain diseases. Chirathaworn *et al*. (2007), in their in vitro study, reported that the role of *M. fragrans* as an anticancer agent is contained in myristicin which has cytotoxic and apoptotic effects in human neuroblastoma SK-N-SH cells with an accumulation of cytochrome and activation of caspase-3 in the cytosol.

Ames and wing spot tests have been used to examine antimutagenic effectiveness of some natural antioxidants, in the presence of both direct and indirect mutagens such as methyl methane sulphonate (MMS), 2-aminofluorene (2-AF), and benzo(a)pyrene (B(a)P). In these, *Salmonella typhimurium* and *Drosophila melanogaster* were used to detect antimutagenic potential of antioxidants from plant extracts and fruits, which suggest their potentialities in cancer prevention and treatment (Cherdshewasart *et al*., 2008; Parvathy *et al*., 2009; Zahin *et al*., 2010). However, none of these reports have demonstrated potentialities of plant extracts or fruit juices in an in vivo system, using plant as a test organism. This is necessary as any results obtained from an in vitro test have to be further validated using an in vivo test system, so as not to obtain false positive or negative results. *Allium cepa* assay is one of the widely used in vivo plant genetic assays for genotoxicity and antimutagenicity testing of substances (Akinboro and Bakare, 2007; Ragunathan and Panneersel, 2007). It has been utilized for monitoring the potential effects of hydrophilic and lipophilic chemicals (Radić *et al*., 2010). This test is very easy to carry out, in terms of preparation of onion roots on the slides and observation under microscope, because its cell contains only 16 metacentric chromosomes (Türkoğlu, 2008). More importantly, the *A. cepa* test produces similar output as animal bioassays that are being employed in toxicological studies. In addition, it is cheaper to run than any of the previously used tests. Though one of the previous findings has reported in vitro anticancer activity of

M. fragrans (Chirathaworn *et al*., 2007), we believe that there is a need for the establishment of this activity in an in vivo system, and more importantly, to suggest a mechanism by which this possible anticancer effectiveness of *M. fragrans* is accomplished.

Based on these aforementioned justifications, this study therefore sought to evaluate effectiveness of the water extract from leaves of *M. fragrans* in suppressing cyclophosphamide (CP)-induced cytotoxicity and chromosomal damages in cells of *A. cepa*, with a view of establishing the efficacy of its phytochemical constituents in preventing cancer development, and probably forming a baseline for the development of cancer chemotherapeutic agent.

2 Materials and methods

2.1 Source of chemicals

Cyclophosphamide (CP; Chemical Abstracts Service (CAS) No. 6055-19-2), orcein salt (CAS No. 1400-62-0), 2,2-diphenyl-1-picrylhydrazyl (DPPH; CAS No. 1898-66-4), butylated hydroxyanisole (BHA; CAS No. 25013-16-5), butylated hydroxytoluene (BHT; CAS No. 128-37-0), and solvents (of technical grade) were supplied by the Sigma Aldrich, Germany.

2.2 Plant materials and extraction

Fresh leaves, weighing 2000 g, were collected from a *Myristica fragrans* (Houtt.) tree behind the School of Language and Communication, Universiti Sains Malaysia (USM), Penang Island, Malaysia. Herbarium specimens containing leaves, flowers, and fruits were used to authenticate and assign voucher specimen numbers (11080) to the plant at herbarium unit of the School of Biological Sciences, USM. The leaves were washed under tap water and oven-dried at 45 °C until a constant weight was obtained. Dried leaves were ground into fine powder and 100 g was extracted by macerating the powder in 1000 ml of distilled water in a glass jar. The jar was placed inside a water bath set at 40 °C for 24 h. Thereafter, water on the macerated leave powder was decanted and filtered through a white muslin cloth. The filtrate was concentrated at 40 °C using a rotary evaporator (R-215) and stored at −80 °C for 24 h before freeze-drying in a Labconco freeze dryer (Lyph-Lock 6) for 24 h. The freeze-dried extract was kept at 4 °C for evaluations.

2.3 *Allium cepa* test

2.3.1 Mutagenicity

Onions (*Allium cepa* L., 2*n*=16) were purchased from Jusco shopping complex, Penang, Malaysia. They were sun-dried for one week in order to facilitate growth. Forty healthy (without fungal attack) onions were prepared by removing their outer scales without tampering with the primordial root ring. Weight of the onions was taken on a Shimadzu electronic balance. Based on the weight of the onions, the quantity of freeze-dried water extract of *M. fragrans* (FDWEMF) was determined to prepare solution at 500, 1000, 2000, and 4000 mg/kg. Eight onions (per concentration) were suspended in solution of FDWEMF dissolved in tap water for 24 h in the dark. Thereafter, the fresh solution was prepared and the suspension of onions continued for another 24 h. Tap water served as the negative control. Root tips from onions were cut and fixed in ethanol/acetic acid fixative (3:1, v/v) (Akinboro and Bakare, 2007). Further treatment and preparation of fixed roots for microscopic observation followed the method of Saxena *et al*. (2010). Slides (five per concentration) were carefully observed for different stages of mitosis and chromosomal aberrations (CAs) in 5000 counted cells (1000 per slide) under Nikon Eclipse E400 microscope. Average mitotic index (MI) and frequency of CA (f_{CA}) were calculated as stated below:

$$MI=n_d/n_t\times100\%,$$
$$f_{CA}=n_a/n_t\times100\%,$$

where, n_d is the number of dividing cells, n_a is the number of aberrant cells, and n_t is total number of cells counted.

2.3.2 Antimutagenicity

Weight of eight onions (per concentration) and corresponding quantity of FDWEMF at 500, 1000, 2000, and 4000 mg/kg were taken as described for the mutagenic test above. Quantity of CP at 50 mg/kg was calculated and weighed for each onion. FDWEMF and CP were dissolved in tap water to make a solution. Onions were suspended in this solution and further treated as stated for mutagenicity test above. Tap water and CP served as negative and positive controls, respectively. After 48 h of onion growth, roots were

treated and used for slide preparation according to the earlier method (Saxena *et al*., 2010). Stained cells (5000) were observed for stages of somatic cell division and CAs in 1000 counted cells per slide. MI and proportion of CA in the obtained MI at each concentration were determined. Reduction percentage of CP-induced CAs (R) was calculated using the formula of Barcelos *et al*. (2007) with some modifications, as shown below:

$$R=(A-B)/(A-C)\times 100\%,$$

where A is the proportion of CA in MI induced by a known mutagen, B is the proportion of CA in MI induced by a test sample, and C is the proportion of CA in MI induced by a negative control.

2.4 DPPH free radical scavenging test

The potency of the FDWEMF in scavenging free radicals from DPPH was tested following the method of Ham *et al*. (2009). BHA and BHT were prepared at 0.5 mg/ml as the reference standards. The percentages of free radicals scavenged by the FDWEMF and standards were calculated as reported (Öztürk *et al*., 2007; Ak and Gülçin, 2008). Concentrations of the extract and standards that scavenged 50% of DPPH free radicals were interpolated from the graph of percentage of free radicals scavenged against sample concentration:

$$SE_{DPPH}=(A_c-A_s)/A_c\times 100\%,$$

where SE_{DPPH} is DPPH scavenging effect, A_c is the absorbance at 517 nm of the control reaction containing all reagents except the test compound, and A_s is the absorbance at 517 nm of reaction containing test compound (Gülçin, 2009; Talaz *et al*., 2009).

2.5 Statistical analysis

The obtained data were summarized and expressed as MI, frequency of CA, proportion of CA in MI, reduction percentage of CA, and percentage of free radicals scavenged. Duncan's multiple range comparison and Dunnett's tests, under one-way analysis of variance (ANOVA) in SPSS 15.0 version, were used to compare between the parameters and their controls. Significant differences were set at $P\leq 0.05$.

3 Results

Effects of the FDWEMF alone and its combination with CP on cell division in the root tips of *A*. *cepa* were presented in Fig. 1. Lower MI values than that of the negative control were induced by both FDWEMF and its combination with CP. MIs induced by FDWEMF alone were concentration-dependent as opposed those caused by its combination with CP. Higher MI values were obtained at all concentrations of the combined FDWEMF with CP except at 1000 mg/kg, compared to those obtained with FDWEMF alone. At 500 mg/kg, the mixture also induced more dividing cells than those caused by CP alone.

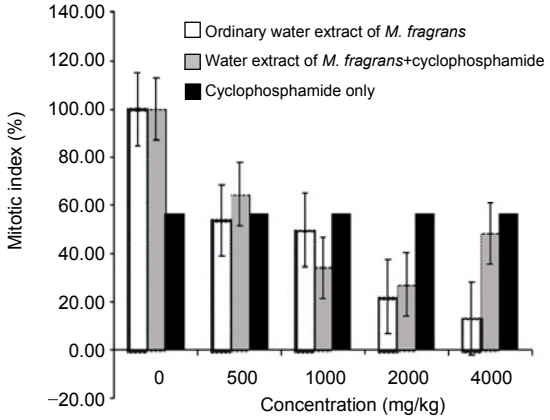

Fig. 1 Mitotic indices induced by water extract of *M. fragrans* alone and *M. fragrans*+cyclophosphamide in root tip cells of *Allium cepa*

Error bars represent standard errors of mean of mitotic index

Table 1 shows the effects of FDWEMF alone and its combination with CP on the chromosomes in *A*. *cepa*. CAs such as spindle disturbance, chromosome bridge, sticky/break chromosomes were observed at 1000 and 4000 mg/kg of the FDWEMF alone. However, frequencies of the observed CAs were not significantly different from the negative control.

Suppression of CP-induced CA by FDWEMF was observed at all concentrations. No CA was observed among dividing cells at 1000 and 2000 mg/kg, representing 107.61% reduction of CA (Table 2). Reduction percentage of CP-induced CA at 500 mg/kg was 7.77% more than 60.55% obtained at 4000 mg/kg. The least reduction percentage of CP-induced CA was obtained at 4000 mg/kg. CP induced highest percentage of CAs, mainly sticky chromosomes.

Table 1 Types and frequencies of chromosomal aberration (CA) induced by the water extract of *Myristica fragrans*

Concentration (mg/kg)	Disturbed spindle	Sticky chromosome	Chromosome breaks	Chromosome bridge	Frequency of CA (%)
Control	0	0	0	0	0.00±0.00[a]
500	0	0	0	0	0.00±0.00[a]
1000	1	1	0	0	0.40±0.55[a]
2000	0	0	0	0	0.00±0.00[a]
4000	1	0	1	1	0.60±0.89[a]

[a] Not significantly different when compared with the negative control ($P \leq 0.05$)

Table 2 Reduction percentages of cyclophosphamide-induced chromosomal aberrations by the water extract of *Myristica fragrans*

Concentration (mg/kg)	Mitotic index (%)	CA	CA/MI	Reduction percentage of CA (%)
Negative control	16.50	0.50	0.03	
Positive control (CP)	9.33	4.00	0.43	
500	10.67	0.16	0.16	68.32
1000	5.67	0.00	0.00	107.61
2000	4.50	0.00	0.00	107.61
4000	8.00	1.50	0.19	60.55

CP: cyclophosphamide; CA: chromosomal aberration; MI: mitotic index; CA/MI: proportion of chromosomal aberrations in MI; Reduction percentage of CA: reduction percentage of chromosomal aberrations caused by the FDWEMF

The efficacies of FDWEMF, BHA and BHT in scavenging free radicals from DPPH are shown in Table 3. The scavenging activity of FDWEMF was observed to be better than that of BHA, as indicated by 0.20 mg/ml against 0.34 mg/ml of half maximal inhibitory concentration (IC_{50}) values, respectively. However, BHT produced the highest DPPH free radical scavenging activity, as it caused 50% inhibition of free radicals at 0.04 mg/ml (Table 3).

Table 3 IC_{50} values showing DPPH scavenging activity of water extract of *Myristica fragrans*, BHA, and BHT

Sample	IC_{50} (mg/ml)
Water extract of *M. fragrans*	0.20
BHA	0.34
BHT	0.04

4 Discussion

Cancer prevention is the most appropriate way for its control, while chemoprevention is a promising alternative of the unavoidable human exposure to environmental and dietary carcinogens (Rauscher *et al*., 1998). Elicitation of cancer by chemicals involves series of events, such as metabolic activation and/or detoxification, interaction of reactive electrophilic metabolites with the DNA, and repair of DNA damages. Since mutations are important early factors in carcinogenesis, short-term genetic tests have been successfully used for detections of mutagen/carcinogens and also of antimutagens/anticarcinogens. In this study, the potential cytotoxic, mutagenic and antimutagenic effects of FDWEMF were evaluated using the *Allium cepa* test. Data on the effects of FDWEMF on cell division suggest inhibition of mitosis, as exemplified in the lower values of MI obtained with the extract compared to negative control. The mitotic inhibitory effect of FDWEMF was cytotoxic because this activity of the extract was induced in a concentration-dependent manner. Our results are in accordance with the previous report on the antimitotic activity of *M. fragrans* (Chatterjee *et al*., 2007). Lignans, which are important class of plant-derived compounds, possess a variety of biological activities such as antitumor, antimitotic, antiviral and antiatherosclerotic activities. The antitumor activity of lignans can also justify the observed cytotoxic effect of FDWEMF, since any antitumor agent is expected to be able to suppress cell division. Obtainment of higher MI value from the mixture of FDWEMF with CP than that from FDWEMF alone implied lowering of activity of mitodepressants in FDWEMF, perhaps due to their interaction with CP. It is possible that these mitodepressants are antioxidants that might react with free radicals generated by CP, thereby reducing cytotoxicity of the mixture unlike in FDWEMF alone. This explanation therefore supports the earlier statement that lignans, antioxidants in the extract of *M. fragrans*, have antimitotic activity. The investigation of Türkoğlu (2008) showed that inhibition of mitosis may occur due to suppression

of DNA/protein (histones) synthesis or an arrest in the G_2 phase of the cell cycle, preventing the cell from entering mitosis.

Induction of CA that was not significantly different from negative control ($P \leq 0.05$), in a non-dose dependent manner by FDWEMF, can be regarded as weak mutagenic effect (Table 1). This effect may not have been induced by one major constituent of the extract. It, therefore, might have resulted from a synergistic reaction between constituents of the FDWEMF. Reports have shown that phenolic compounds, anthraquinones, flavonoids, alkaloids are among the constituents of *M. fragrans* (Olaleye *et al*., 2006). Some of these compounds have been implicated in causing chromosomal damages at certain concentrations. Lopes e Lopes *et al*. (2004) suggested that the mutagenicity of polyphenolic flavonoids may be due to the ROS produced by their oxidation and redox cycling.

Results of the effects of FDWEMF combined with CP on cell division and chromosomes of *A. cepa* showed that cytotoxic effect of CP was suppressed at 500 mg/kg of the extract. Similarly, mutagenic action of CP was suppressed at all concentrations. This was noticed in the lower number of CAs caused at different concentrations of FDWEMF+CP compared to the positive control (CP). This suggests that the water extract of *M. fragrans* has antimutagenic efficacy. CP is an anticancer drug that is metabolized to produce phosphoramide mustard, an antineoplastic component, and acrolein, which is a major mutagenic component of CP. Mutagens and carcinogens such as CP may act through generation of ROS that play a major role as endogenous initiators of degenerative processes, such as DNA damage and mutation, and its promotion (Negi *et al*., 2003). Many plant polyphenols, such as ellagic acid, catechins, chlorogenic, caffeic and ferulic acids, have shown to act as potent antimutagenic and anticarcinogenic agents. Antimutagenic efficacy of FDWEMF in this study might be due to its antioxidant activity. Several reports have been made on the antioxidant activity of *M. fragrans*, attributed to phenolic and polyphenolic compounds, belonging to the group of lignans (Chatterjee *et al*., 2007; Su *et al*., 2007; Calliste *et al*., 2010). Argenteane, a dilignan, isolated from nutmeg is similar in power to vitamin E. This explains the reason for the obtainment of higher free radical scavenging effect of FDWEMF than that of BHA which is a synthetic antioxidant similar to vitamin E. Vitamin A, C, and E have been reported to have antimutagenic activity against several known direct and indirect mutagens (Odin, 1997). Thus, it is logical to say that argenteane, or phenolic compounds, acting as antioxidant may be responsible for the observed antimutagenic effect of this extract against CP-induced CAs. Obtainment of the lowest reduction percentage of CP-induced CA at 4000 mg/kg of FDWEMF+CP might have resulted from a change of activity of constituents of FDWEMF from being antioxidants to pro-oxidants. Antioxidants in this extract might have exerted pro-oxidative effect at 4000 mg/kg as suggested by Vitaglione and Fogliano (2004), leading to generation of free radicals to cause more chromosomal damages than those recorded at lower concentrations of this extract. This is in line with the report that antioxidant vitamins were found to have adverse effects when used in excess (Khan and Sinha, 2008).

5 Conclusions

This study showed that FDWEMF has cytotoxic and antimutagenic effects on cell division and chromosomes of *A. cepa*, and also induced CAs that were insignificantly different from the negative control. These activities may be due to the efficient free radical scavenging properties of FDWEMF. Thus, the obtained data suggest that water extract of *M. fragrans* stands promising in the development of cancer therapeutic agent. Nevertheless, further investigations are needed to confirm these properties of *M. fragrans* in animal test organisms, and towards isolation of the suspected active principle(s).

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