

## Antimutagenic effects of aqueous fraction of *Myristica fragrans* (Houtt.) leaves on *Salmonella typhimurium* and *Mus musculus*

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Natural plant extracts offer a promising hope in the prevention/treatment of cancer arising from genetic mutations. This study evaluated *in vitro* and *in vivo* mutagenic and antimutagenic effects of aqueous fraction of *Myristica fragrans* (AFMF) leaves on TA100 strain of *Salmonella typhimurium* and *Mus musculus* (Male Swiss albino mice), respectively. The antioxidant activity of AFMF against 2,2-diphenyl-1-picrylhydrazyl (DPPH), total phenolic and flavonoid contents were determined, followed by its phytochemical elucidation using the Ultra Performance Liquid Chromatography technique (UPLC). The mutagenicity of AFMF at 4, 20, 50, 100, 200, 500, and 1000 µg/well was <2.0 in *S. typhimurium* and the induced micronucleated polychromatic and normochromatic erythrocytes at 500, 1000, 2000, and 4000 mg/kg were not significantly different from the negative control ( $p \geq 0.05$ ). The mutagenic activity of benzo[a]pyrene and cyclophosphamide was significantly suppressed above 50.0% throughout the tested concentrations. Fifty percent of the free radicals from DPPH were scavenged by AFMF at 0.11 mg/ml. Total phenolic and flavonoid contents of AFMF were 51.0 mg GAE/g and 27 mg QE/g, respectively. Rutin was elucidated by the UPLC technique, and thereby suspected to be the phytochemical responsible for the observed antimutagenic activity. Thus far, AFMF seems to contain a promising chemotherapeutic agent for the prevention of genetic damage that is crucial for cancer development.

**Key words:** benzo[a]pyrene, chromosome, aberrations, cyclophosphamide, micronucleus, *Myristica fragrans*, *Salmonella typhimurium*

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### INTRODUCTION

Cancer is the second leading cause of human death worldwide despite many therapeutic measures being taken to control it. The use of natural products as new chemopreventive agents stands promising in reducing the number of deaths being recorded annually from cancer. Antimutagenic and anticarcinogenic agents offer the most effective procedure for the prevention of cancer and other genetic diseases in humans, as inhibition of the mandatory initiation stage of carcinogenesis is an important antidote to emergence of cancer (Ferguson, 1994). Occurrence of cancer and degenerative diseases has been connected with generation of excess

reactive oxygen species (ROS), inducing cell damage due to imbalance between antioxidants and oxidants (Su *et al.*, 2007; Chatterjee *et al.*, 2007).

Phenolic phytochemical compounds, possessing ability to remove free radicals, chelate metallic catalysts, activate antioxidant enzymes and inhibiting oxidases are given a considerable attention because of their diverse biological and beneficial health effects (Oh *et al.*, 2008). *Myristica fragrans* (Houtt.), a tropical evergreen plant with aromas, gains its popularity among the plants that are being used as spices and in traditional medicines because of its immensely diverse phytochemical constituents (Iyer *et al.*, 2009; Nguyen *et al.*, 2010). Previous studies on evaluation of *M. fragrans* extracts for different activities such as antimicrobial, anxiogenic, antioxidant, cytotoxicity, hypolipidemic and anticarcinogenic effects had employed the fruit, dried seed (nutmeg), aril (mace), and bark of this plant (Sulaiman and Ooi, 2011; Piaru *et al.*, 2011). No known reports had ever considered the leaves of *M. fragrans* for similar investigation before our previously reported remarkable DPPH free radical scavenging activity and suppression of cyclophosphamide-induced chromosomal aberrations by the fruit juice and leaf extract of *M. fragrans* in the *Allium cepa* test (Akinboro *et al.*, 2011a; Akiboro *et al.*, 2011b). The results of our preliminary investigation on mutagenic and antimutagenic activities of methanol leaf extract of *M. fragrans* using *in vitro* and *in vivo* genetic models revealed the presence of phytochemical(s) that could be employed for the development of anticancer therapeutics (Akinboro *et al.*, 2012).

However, the present study sought to evaluate antimutagenic activity of aqueous fraction of methanol leaf extract of *Myristica fragrans* and elucidate its suspected antimutagenic constituent without ignoring the possibility of it being mutagenic in the selected genetic assays for this study.

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**Abbreviations:** AFMF, aqueous fraction of *Myristica fragrans*; AFB1, aflatoxin B1; AAF, acetamido-fluorene; BHA, butylated hydroxyl anisole; BHT, butylated hydroxyl toluene; B[a]P, benzo[a]pyrene; CP, cyclophosphamide; DPPH, 2,2-diphenyl-1-picryl hydrazyl; DNA, deoxyribonucleic acid; FBS, fetal bovin serum; GAE, gallic acid equivalent; HPLC, high performance liquid chromatography; MNPCs, micronucleated polychromatic erythrocytes; MNCE, micronucleated normochromatic erythrocytes; NCE, normochromatic erythrocytes; OECD, organization for economic cooperation and development; PCE, polychromatic erythrocytes; PDA, photodiode array; SR, spontaneous revertant; UPLC, ultra performance liquid chromatography; USM, university sain Malaysia

## MATERIALS AND METHODS

**Chemicals.** Recommended standard chemical reagents for this study were purchased from Sigma Aldrich, Munich, Germany, while the technical grade chemicals were collected at the chemical stores of Schools of Biological and Pharmaceutical Sciences, Universiti Sains Malaysia (USM).

***Salmonella typhimurium* strain (TA100).** The histidine-requiring strain of *Salmonella typhimurium* TA100 (frozen) as part of the strains recommended by the OECD guidelines (OECD, 1997) was kindly supplied by Dr. Sedigheh Mehrabian, Faculty of Sciences, University of Tehran, Tehran, Iran.

**Animals.** The use of mice was approved in the letter with reference number PPSG/07 (A) /044/ issued by the animal ethics committee, USM. This was in line with the internationally accepted guidelines for the treatment of animals in behavioral research and teaching (Sherwin, 2006). Mice were acclimatized to the conditions (temperature,  $29 \pm 1^\circ\text{C}$ ; humidity,  $70 \pm 10\%$ ; 12-hour light-dark cycle) in the transit room in the School of Pharmaceutical Sciences, USM, for one week before the commencement of experiment. They were kept inside plastic cages with wood shavings for bedding, and supplied with standard chow and water *ad libitum*.

Extraction and fractionation of plant material. The plant sample was identified at the herbarium unit of the School of Biological Science where voucher specimen number 11081 was assigned. Fresh leaves were plucked, rinsed with tap water and oven dried at  $40^\circ\text{C}$  for 24 hours. The dried leaves were ground into fine powder and extracted with analytical grade methanol in ratio 1:10 (powder:solvent, w/v) (Akinboro *et al.*, 2012). The methanol extract was concentrated in a rotary evaporator at  $40^\circ\text{C}$ , followed by freeze drying and kept at  $4^\circ\text{C}$  for fractionation.

The freeze dried methanol extract was serially fractionated (1:10, w/v, extract:solvent ratio) to obtain petroleum ether, chloroform, ethyl acetate, *n*-butanol and aqueous fractions as reported (William & Douglas, 2005). The aqueous fraction was filtered through layers of muslin cloth, concentrated in a rotary evaporator at  $40^\circ\text{C}$ , and then freeze dried before it was kept at  $4^\circ\text{C}$  for experimentations.

***In vitro* mutagenicity and antimutagenicity (Ames test).** 80  $\mu\text{L}$  of an overnight bacterial culture of TA100 were added to 1.6 mL top agar, followed by addition of 80  $\mu\text{L}$  of AFMF alone at 4, 20, 50, 100, 200, 500 and 1,000  $\mu\text{g}/\text{well}$  concentrations. Thereafter, 400  $\mu\text{L}$  of S9 mix was added to the mixture in a test tube for the mutagenic test (Flamand *et al.*, 2001). Antimutagenic screening of AFMF was similar to the mutagenic test but with addition of 20  $\mu\text{L}$  of benzo[a]pyrene to the final mixture. The mixture was homogenized, after which 540  $\mu\text{L}$  of the resulting mixture was poured, in triplicates per concentration, into six-well plates containing 5 mL of the glucose minimal agar. This was allowed to solidify, before incubation at  $37^\circ\text{C}$  for 72 hours (Mortelmans and Zeiger, 2000). Distilled water and benzo[a]pyrene (0.1  $\mu\text{g}/\text{well}$ ) served as the negative and positive controls, respectively. Thereafter, the number of histidine-independent revertants obtained at each concentration was counted manually. The results were expressed as the mean of triplicate experiments per concentration. The mutagenicity ratio (R) was calculated as the ratio between the number of revertants at each concentration and the revertants induced by the control solvent. The aqueous fraction of *M. fragrans* was considered mutagenic

if the mutagenicity ratio was  $(R) \geq 2$  at any concentration less than or equal to 100  $\mu\text{g}/\text{well}$  for the tester strain TA 100 (Loh *et al.*, 2009). The antimutagenic efficacy of AFMF was calculated as previously reported (Negi *et al.*, 2003) using the formula:

$$\% \text{ inhibition of B[a]P-induced mutagenicity} = \frac{(R1-SR) - (R2-SR)}{(R1-SR)} \times 100$$

where R1 is the number of revertants recorded with B[a]P alone, R2 is the number of revertants recorded with the mixture of AFMF and B[a]P, and SR is the number of spontaneous revertants in the negative control.

However, the antimutagenic efficacy of AFMF was considered strong if the percentage of inhibition of mutagenicity of B[a]P was 40%, 25–40% for moderate, and less than 25% for weak or absence of antimutagenic effects.

***In vivo* mutagenicity and antimutagenicity (Bone marrow micronucleus test).** In the micronucleus test, mutagenic evaluation was carried out with four male mice (8-week old), per dose level. They were orally administered a solution of the AFMF alone at 500 mg/kg, 1000 mg/kg, 2000 mg/kg and 4000 mg/kg (0.1 ml/10 g v/w, volume:body weight). The antimutagenic test was carried out by orally administering cyclophosphamide (CP) at 50 mg/kg (0.1 ml/10 g v/w, volume/body weight) to the male mice 5 minutes before the administration of AFMF. Distilled water and CP (50 mg/kg, w/v) served as the negative and positive controls, respectively (da Silva *et al.*, 2002). After 24 h, the mice were sacrificed after inhaling diethyl-ether for 1 minute. Both femurs were dissected and bone marrow was flushed into test tubes containing fetal bovine serum (FBS). The mixture was centrifuged at  $300 \times g$  for 10 minutes in an Eppendorf centrifuge (5403; Geratebau Eppendorf GmbH, Engelsdorf, Germany) set at  $0^\circ\text{C}$ . Smear of cell pellets was prepared on glass slides and treated as previously reported (Krishna and Hayashi, 2000). Total of 2000 cells, per mouse, were observed for micronucleated polychromatic and normochromatic erythrocytes (MNPCEs and MNNCEs) using  $100 \times$  oil immersion objective lens in a compound light microscope (Nikon E400). To assess bone marrow cytotoxicity, 200 erythrocytes were evaluated per mouse for the proportion of polychromatic erythrocytes (PCEs) in the total erythrocytes counted. The antimutagenic potency of AFMF against the mutagenicity of CP was calculated as reported (Furtado *et al.*, 2008), but with a slight modification.

$$\text{Clastogenic effects (\%)} = \frac{\text{Number of MNPCEs} + \text{Number of MNNCEs}}{\text{Total number of counted erythrocytes}} \times 100$$

$$\text{Cytotoxic effects (\%)} = \frac{\text{Number of PCEs}}{\text{Total number of counted erythrocytes}} \times 100$$

$$\% \text{ Reduction of CP-induced MN} = \frac{A-B}{A-C} \times 100$$

where 'A' represents frequency of MNPCEs and MNNCEs induced by CP, 'B' represents frequency of MNPCEs and MNNCEs induced by the mixture of AFMF and CP, 'C' represents frequency of MNPCEs and MNNCEs induced by distilled water.

**DPPH free radical scavenging test.** The AFMF was tested for its scavenging activity against DPPH (2,2-diphenyl-1-picrylhydrazyl) following the described methods (Rosidah *et al.*, 2008; Ham *et al.*, 2009). Briefly, 2 mg/ml AFMF was dissolved in HPLC grade methanol, while butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were the reference standards prepared at 1 mg/mL. DPPH solution (0.1 mM) was prepared and dissolved in 100 ml of HPLC grade methanol. In each of the 96-well microplate, the reaction per-

formed in triplicate contained 100  $\mu\text{L}$  of each of AFMF, the reference standard or HPLC methanol, mixed with 200  $\mu\text{L}$  of 0.1 mM DPPH solution. The microwell plate was incubated at room temperature for 30 minutes after which the reaction was measured at 517 nm (wavelength) in a microplate reader (PowerWave  $\times$  TM). The  $\text{IC}_{50}$  values were interpolated on the graph of percentage scavenging activity against concentration (Gulcin, 2009).

$$\text{DPPH scavenging effect (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

**Total phenolic contents.** The total phenolic content in AFMF was determined but with some modifications of the Folin-Ciocalteu's method using the 96-well microplate assay (Sulaiman *et al.*, 2011). The AFMF, at 1 mg/ml, was dissolved in HPLC grade methanol-distilled water (5:5, v/v) in a test tube. Gallic acid, at 1mg/ml, was prepared and further diluted by two folds to obtain 0.004–0.5 mg/ml concentration range to plot the standard calibration curve. Aliquot of 20  $\mu\text{L}$  AFMF and Gallic acid, in triplicate, was added with 40  $\mu\text{L}$  of Folin Ciocalteu reagent in a 96 microwell plate, and left for 10 minutes at room temperature ( $27 \pm 1^\circ\text{C}$ ). Thereafter, 75  $\mu\text{L}$  of 20% (w/v) sodium carbonate solution and 150  $\mu\text{L}$  distilled water were added to the mixture and then incubated at  $40^\circ\text{C}$  for 30 minutes (Jayaprakasha *et al.*, 2007). Reaction of the mixture was measured at 765 nm against the blank using a microplate reader (PowerWave  $\times$  TM). The phenolic content was expressed as milligram of Gallic acid equivalents (GAE) per gram of AFMF (Loh *et al.*, 2009).

**Total flavonoids.** AFMF and quercetin were dissolved separately in HPLC grade methanol at 1 mg/ml. The initial solution of quercetin was serially diluted (two folds) to lower concentrations. In each well of a 96-microwell plate, 35  $\mu\text{L}$  AFMF aliquot and quercetin, in triplicate, was separately mixed with 75  $\mu\text{L}$  of 95% ethanol, followed by addition of 20  $\mu\text{L}$  freshly prepared 10% aluminum chloride, 10  $\mu\text{L}$  of 1.0 M potassium acetate and 140  $\mu\text{L}$  distilled water (Sulaiman *et al.*, 2011; Boubaker *et al.*, 2010).

A blank reaction contained all reagents except Aluminum chloride and Potassium acetate. The reaction mixture was incubated at room temperature ( $27 \pm 1^\circ\text{C}$ ) for 40 minutes, after which reaction of the mixture was measured at 415 nm in a microplate reader (PowerWave  $\times$  TM). The flavonoid content was expressed as mg QE/g dry weight of AFMF.

**Ultra Performance Liquid Chromatography (UPLC).** The ultra performance liquid chromatography (UPLC) technique was adopted to elucidate the possible phytochemical(s) in AFMF. AFMF was dissolved in HPLC methanol at 1 mg/ml, while quercetin and rutin (standards) were prepared separately first, and then the mixture of the two standards was at 0.1 mg/ml. The solutions of AFMF and the standards were separately passed through a syringe filter (0.22  $\mu\text{m}$ ) into 2.0 ml Vials, and covered. Chromatography was carried out with an Acquity-UPLC<sup>TM</sup> system (Waters, MA, USA) in which the separation of AFMF and standards was performed by reversed phase (RP) chromatography, using BEH C18 column (Waters) with 100 mm  $\times$  2.1  $\times$  1.7  $\mu\text{m}$  of particle size. The mobile phases; 'A' consisted of 0.1% formic acid in deionized water while 'B' had 0.1% formic acid in acetonitrile, and were used for the analysis at 60% (A) to 40% (B) proportions. The injected volume was 3  $\mu\text{L}$  at 0.1 mL/minute flow rate. The separation was held for 10 minutes while compounds were detected by a photodiode array (PDA) detector at 280 nm (Dartora *et al.*, 2011). The retention time for the separation of AFMF

was compared with that of rutin and quercetin antimutagenic standards.

The quantity of the suspected antimutagenic compound in AFMF was determined by preparing 0.1 mg/ml of pure rutin compound and diluted by two folds to obtain lower concentrations. The chromatographic separation of rutin and AFMF followed the method (Dartora *et al.*, 2011). The areas under the peaks of chromatograms of rutin were plotted against concentrations. The quantity of rutin in 1 mg of AFMF was obtained from the graph.

## RESULTS

The induction of base pair substitution mutation in *S. typhimurium* TA100 strain was not concentration dependent (Fig. 1). The highest and least mutagenicity ratio of 1.71 and 1.21 were observed at 200  $\mu\text{g}/\text{well}$  and 500  $\mu\text{g}/\text{well}$ , respectively. The mutagenic activity of benzo[a]pyrene was suppressed above 50% at the tested concentrations, with the highest inhibition percentage of 87% recorded at 4 and 50  $\mu\text{g}/\text{well}$  concentrations. However, the suppression of mutagenicity of benzo[a]pyrene at 20, 100, 200, and 1000  $\mu\text{g}/\text{well}$  was less, indicating non concentration-dependent inhibition of the mutagenic action of benzo[a]pyrene by AFMF (Fig. 2).

The clastogenic effects of AFMF on bone marrow cells of the treated mice are presented in Table 1. There was induction of micronucleated polychromatic erythrocytes (MNPCE) in a non-concentration dependent manner. The induced micronucleated normochromatic erythrocyte (MNNCE) at 500 mg/kg was higher than that recorded with the negative control. However, there was

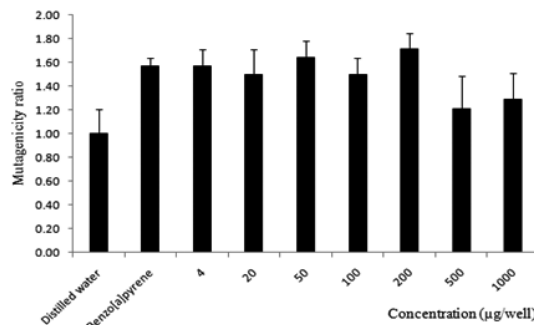


Figure 1. Mutagenic activity of aqueous fraction of *M. fragrans* with metabolic activation in *S. typhimurium* TA100.

Error bars represent standard deviation of average number of re-

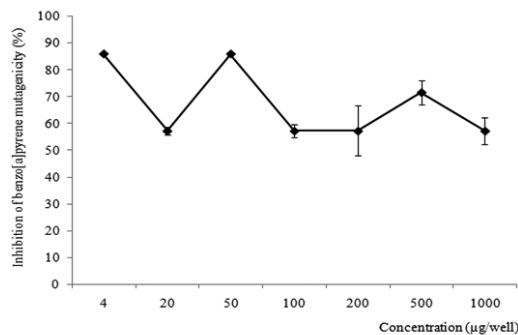


Figure 2. Suppression of benzo[a]pyrene-induced mutagenicity in *S. typhimurium* TA100 by aqueous fraction of *M. fragrans*.

Error bars represent standard deviation of mutagenicity inhibition (n=3)



**Table 1. Clastogenic and proliferative effects of aqueous fraction of *M. fragrans* on bone marrow cells in Swiss albino mice**

Concentration (mg/kg)	MNPCE $\pm$ S.D.	MNNCE $\pm$ S.D.	% PCE	% NCE
Distilled water	0.83 $\pm$ 0.75 <sup>a</sup>	0.17 $\pm$ 0.14 <sup>a</sup>	83.83	16.17
500	1.83 $\pm$ 1.72 <sup>a</sup>	0.33 $\pm$ 0.52 <sup>a</sup>	85.00	15.00
1000	1.17 $\pm$ 1.47 <sup>a</sup>	0.00 $\pm$ 0.00 <sup>a</sup>	85.33	14.67
2000	2.00 $\pm$ 1.90 <sup>a</sup>	0.00 $\pm$ 0.00 <sup>a</sup>	80.83	19.67
4000	2.17 $\pm$ 1.72 <sup>a</sup>	0.00 $\pm$ 0.00 <sup>a</sup>	73.00	27.00

Values with same letter in superscript are not significantly different ( $p \leq 0.05$ ), MNPCE, micronucleated polychromatic erythrocyte; SD, standard deviation; MNNCE, micronucleated normochromatic erythrocyte; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte

**Table 2. Antimutagenic activity of aqueous fraction of *M. fragrans* against CP-induced micronucleated erythrocytes in mouse bone marrow cells**

Concentration (mg/kg)	MNPCE $\pm$ S.D.	MNNCE $\pm$ S.D.	% PCE	% NCE	Reduction of MN (%)
Distilled water	0.50 $\pm$ 0.55	0.17 $\pm$ 0.41	88.00	12.00	–
CP	4.17 $\pm$ 2.48*	1.17 $\pm$ 1.47	81.83	18.17	–
500	2.80 $\pm$ 2.14	0.17 $\pm$ 0.41	82.33	17.67	50.75
1000	2.50 $\pm$ 2.59	0.00 $\pm$ 0.00	82.33	17.17	60.81
2000	4.00 $\pm$ 2.45*	0.83 $\pm$ 0.75	77.67	22.33	10.92
4000	2.83 $\pm$ 1.94	0.17 $\pm$ 0.41	78.83	21.17	50.11

\*Values are significantly different from negative control ( $p \leq 0.05$ ). MNPCE, micronucleated polychromatic erythrocyte; S.D., standard deviation, MN-NCE, micronucleated normochromatic erythrocyte; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte; MN, micronucleus

**Table 3. DPPH scavenging activity, total phenolic and flavonoid contents of aqueous fraction of *M. fragrans***

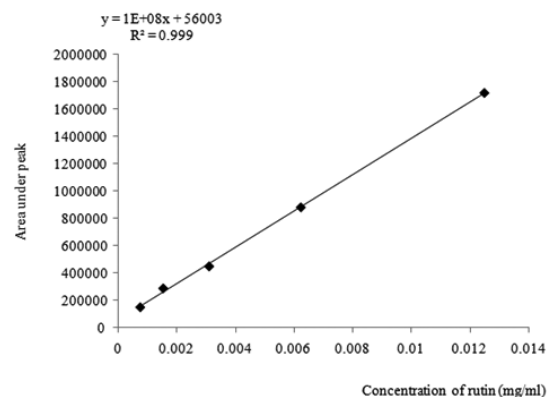
Sample	DPPH scavenging power (IC <sub>50</sub> ) mg/ml	Total phenolic contents (mgGAE/g)	Flavonoid contents (mgQE/g)
Aqueous extract fraction	0.11	51.00	27.00
BHA	0.02	–	–
BHT	0.04	–	–

DPPH, 2,2-diphenyl-1-picrylhydrazyl; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; IC<sub>50</sub>, 50% Inhibitory concentration; mg-GAE: miligram of Gallic acid equivalent; mgQE: miligram of Quercetin equivalent.

no significant difference between the MNPCE and MN-NCE induced at the tested concentrations and negative control ( $p > 0.05$ ). The proliferation of bone marrow cells was not significantly suppressed. However, the mutagenic activity of CP in inducing micronucleated erythrocytes (MNPCE and MNNCE) was suppressed throughout the tested concentrations. The highest inhibition of mutagenic effect of CP was 60.81%, recorded at 1000 mg/kg while the least inhibition of 10.92% was recorded at 2000 mg/kg (Table 2).

The DPPH free radical scavenging power, total phenolic and flavonoid contents of AFMF are presented in Table 3. Butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) were more powerful than AFMF. The IC<sub>50</sub> values obtained for AFMF, BHA and BHT were 0.11 mg/ml, 0.02 and 0.04 mg/ml, respectively. Total phenolic content of 51.0 mg GAE/g was observed in AFMF, while the flavonoid content was 27.0 mg QE/g.

The results of ultra performance liquid chromatographic separation of AFMF and the two standards

**Figure 3. Quantification of rutin in the aqueous fraction of *M. fragrans* using UPLC analysis**

(quercetin and rutin) are presented in Table 4. The retention times of 2.841, and 4.325 minutes were respectively recorded for the separation of the mixture of rutin and quercetin. However, AFMF and the control standard (rutin) had almost similar retention times of 2.09 & 2.837 minutes, and 2.109 & 2.841 minutes, respectively. The quantity of rutin obtainable in 1 g of AFMF was estimated to be 9.0 mg (Fig. 3).

## DISCUSSION

The Ultra Performance Liquid Chromatographic separation of AFMF suggested inclusion of rutin, as evident in similar retention times obtained for the rutin standard and AFMF under the same conditions. The induction of revertants of *S. typhimurium* by AFMF in a non-concentration dependent manner, and obtainment of mutagenicity ratio  $R < 2$  at all the tested concentrations

**Table 4. Chromatographic characteristics of aqueous fraction of *M. fragrans* and two antioxidant standards (quercetin and rutin)**

Sample	Peak	Retention time (mins)	Area	% Area	Height
Aqueous fraction	1	2.09	39618906	72.24	2715508
	2	2.84	15224158	27.76	2383789
Mixture of quercetin and rutin	1	2.15	16245542	22.31	910195
	2	2.84	20966184	28.80	2828805
	3	4.33	35592891	48.89	2770847
Quercetin alone	1	4.321	31308527	100.00	2587250
Rutin alone	1	2.11	6822785	39.19	484356
	2	2.84	10585689	60.81	1831980

suggest non-mutagenic potential of AFMF. A chemical substance is considered mutagenic in *S. typhimurium* tester strain TA100 if it produces a concentration dependent mutagenicity ratio  $R \geq 2$  (Flamand *et al.*, 2001). This was corroborated by the results of the bone marrow micronucleus test in this study. The presence of rutin as the major phytochemical in the AFMF explains its non-mutagenic behavior. Rutin and its metabolites were reported to be non-mutagenic in *S. typhimurium* tester strains (Ben-Rhouma *et al.*, 2012). It neither diminished cell viability nor reduced cell proliferation in the hepatic cells, and was not genotoxic in mice (Hardigree and Epler, 1978; da Silva *et al.*, 2002; Cristina-Marcari *et al.*, 2011). This was attributed to the existence of rutin in a glycosylated form which is probably not absorbed directly in the cell (Manach *et al.*, 1987). The absorption of rutin depends on the action of glycosidases, hydrolyzing rutin to aglycone quercetin which may not be absorbed before being further degraded to non-mutagenic aromatic acids and carbon dioxide (Crebelli *et al.*, 1987).

The results of DPPH free radical scavenging activity of BHA and BHT which were higher than that recorded for AFMF were contrary to our previous data on the DPPH free radical scavenging activity of methanol extract of *M. fragrans* (Akinboro *et al.*, 2012). This may be due to lack of a synergistic interaction in AFMF as might have been the case with methanol extract of *M. fragrans*. The antioxidant activity of rutin using different assays has been reported with obtainment of strong DPPH radical scavenging activity (Yang *et al.*, 2008). Natural flavonoids scavenge free oxygen and nitrogen radicals effectively *in vivo* and *in vitro*, perhaps because of their ability to act as hydrogen or electron donors (Lue *et al.*, 2010).

The antimutagenic effects of AFMF on *Salmonella typhimurium* TA100 showed strong inhibition of B[a]P-induced mutagenicity at all tested concentrations. Our results corroborated the reported antimutagenic activity of the major flavonoids of rooibos (*Aspalathus linearis*) in the *S. typhimurium* mutagenicity assay employing TA98 and TA100 tester strains (Snijman *et al.*, 2007). In their experiment, rutin showed antimutagenic activity against 2-acetamido-fluorene (2AAF) and aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) in a non-concentration dependent manner. In the present study, the suppression of CP-induced mutagenicity in the *in vivo* tests further confirmed the observed antimutagenic activity in the *in vitro* model. The suppression of mutagen-induced mutations in the target cells of *S. typhimurium* and *M. musculus* was non-dose dependent. The lack of dose-dependent suppression of mutagenic action of the two pro-mutagens by AFMF could be the result of activation of different metabolic pathways in the test

organisms. Similar antimutagenic activity of rosmarinic acid and rutin was observed in Swiss albino mice and human lymphocytes in a non-dose dependent manner (Furtado *et al.*, 2008; Undeger *et al.*, 2004).

To the best of our knowledge, this is the first time that the antimutagenic activity of AFMF, suspected to have been brought about by rutin, was reported in *S. typhimurium* TA100 tester strain and in Swiss albino mice. Previous studies showed that mace lignans from *M. fragrans* significantly inhibited radiation-induced DNA damages in skin keratinocytes and splenocytes (Checker *et al.*, 2008; Anggakusuma *et al.*, 2010). Benzo[a]pyrene and cyclophosphamide are pro-mutagens which produce active electrophilic metabolites that are the main inducers of DNA or chromosomal damage (Boyd *et al.*, 1986; Ueng *et al.*, 2001). It has been reported that rutin protects DNA from the action of mutagens/carcinogens by modulating the enzymes necessary for activation of carcinogens or inhibiting the activation of mutagens (Mallet *et al.*, 1989; Webster *et al.*, 1996). Thus, alteration in the metabolic pathways of B[a]P and CP is expected to have prevented these pro-mutagens from being converted to their mutagenic metabolites in the test organisms. The free radical scavenging effects of the AFMF could be due to its phenolics which are capable of donating hydrogen atoms or electrons to the electrophilic mutagenic metabolites of B[a]P and CP in order to make them stable and nonreactive with DNA, hence, suppression of their mutagenicity.

Rutin and its aglycone quercetin have been reported to be effective in inhibiting lipid peroxidation, the action that is possible through the scavenging of free radicals, and consequently prevents damage to DNA. Other possible mechanisms of antimutagenic actions of flavonoids such as carcinogen inactivators, inhibitors of tumor cell growth, modulators of DNA repair, or inducers of apoptosis cannot be completely ruled out. Polyphenols are an important group of pharmacologically active compounds which are most often found with remarkable antioxidant activities (Boubaker *et al.*, 2010). Rutin, a polyphenolic compound, has been shown to possess anticarcinogenic activity against a variety of chemicals, and other healing properties such as antimutagenic, anti-inflammatory, anti-diarrhoea, myocardial protector and immuno-modulator (Webster *et al.*, 1996; Arjumand *et al.*, 2011). It is interesting that in the two assays reported here, the best antimutagenic activity of the AFMF was observed at the lower doses. The lower doses of AFMF in this study might have behaved as DPPH free radical scavengers (antioxidants) capable of suppressing the induction of genetic mutations caused by the two mutagens, hence, the obtainment of higher reduction percentages of B[a]

P and CP-induced mutations. However, the lower reduction percentages of B[a]P and CP-induced mutagenicity obtained at higher doses could have resulted from pro-oxidant activity of AFMF thereby causing oxidative-reodox cycle to induce DNA damage.

The small amount of rutin in AFMF confirmed its strong antimutagenic activity, as suggested by the inhibition of B[a]P and CP-induced mutations above 50% at the lowest concentration of 4 µg/well and 500 mg/kg in the *in vitro* and *in vivo* assays, respectively.

## CONCLUSION

This study revealed that AFMF possessed strong antimutagenic activity in the selected genetic assays, without being significantly mutagenic. The free radical scavenging activity of AFMF, suspected to have been brought about by the rutin phytochemical, could be responsible for the observed antimutagenicity in the *in vitro* and *in vivo* tests. Therefore, the results obtained in this study suggest that AFMF is a promising therapeutic agent in the development of anticancer chemotherapeutics.

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