GENOTOXICITY SCREENING OF NUTMEG, OREGANO AND DARK SOY USING AMES FLUCTUATION AND SOS CHROMO TESTS

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ABSTRACT

Nutmeg, oregano and dark soy are among household spices which are consumed in large quantities globally. This study evaluated the mutagenic and genotoxic potential of nutmeg, oregano and dark soy using the SOS Chromotest on Escherichia coli PQ37 and the Ames Salmonella fluctuation test on Salmonella typhimurium strains TA98 and TA100. The concentrations of 0.1, 0.25, 0.5 and 1 g/10 mL distilled water of nutmeg and oregano, and 10, 25, 50 and 100 % dark soy were utilized for the Ames test while six concentrations of two-fold dilutions of 1g of nutmeg and oregano and 100% of dark soy were utilized for SOS Chromotest. The result of the Ames test showed mutagenicity of nutmeg and oregano but not of dark soy, while the SOS Chromotest results showed genotoxicity of nutmeg, oregano and dark soy. E. coli PQ37 system showed better sensitive than the Salmonella assay for detecting genotoxins in the tested samples. The results of this study indicated that nutmeg and oregano are genotoxic while dark soy showed genotoxicity only at low concentration. Long term exposure to these condiments can lead to bioaccumulation which might have varying degrees of genotoxic and mutagenic effects including cancer in exposed individuals.

KEYWORDS: Mutagenicity, genotoxicity, nutmeg, oregano, dark soy.

INTRODUCTION

The discovery of many spices and herbs probably predates the earliest civilizations, when primitive humans were attracted to the aromatic effects produced by what are now called essential oils, which are found nearly in all parts of plants, from the leaves to the roots. Many spices have antimicrobial properties. This may explain why spices are more commonly used in warmer tropical climates, which have more infectious diseases, and why the use of spices is especially prominent in meat preservation. Spices have low
nutritive values and are therefore cannot be grouped as foods; but are known as food adjuncts [1]. Spices stimulate the appetite, increase the secretion and flow of gastric juice, and greatly add pleasure to eating. Sometimes a spice is used to hide other flavors [2]. Among the most commonly used spices in Africa are oregano, dark soy and nutmeg.

Oregano (Origanum vulgare) is a plant native to the Mediterranean region which has been used in traditional medicine and as spice in food for centuries. Oregano is used on meat, fish, poultry and vegetables [3] because of its strong flavor and aromatic leaves.

Dark Soy sauce on the other hand is one of the popular fermented flavouring products, especially in Asia. It is also one of the world’s oldest condiments and has been used in China for over 3,000 years [4]. Soy sauce is a popular flavouring agent which is produced by the fermentation of Aspergillus oryzae with a combination of soybean, wheat flour and salt [5]. The characteristic dark colour of this oriental sauce is as a result of the addition of molasses and caramels.

Nutmeg, the seed of Myristica fragrans, also is used as flavouring agent in food [6,7,8], and in traditional medicine practice for antenatal and postnatal treatments [9]. Nutmeg and its constituents exhibit various biological and pharmacological activities including antimicrobial effects.

Despite the worldwide consumption of these spices, few studies have been carried out evaluating their genotoxic and mutagenic properties (10). Investigation results of spices genotoxicity are rather contradictory. Some reports indicated that spices from various plants were able to induce genetic damages in various test-systems (11). According to other authors, essential oil of such aromatic plants as Origanum vulgare did not show any genotoxic effect [12]. Nevertheless, it must be pointed out that when major components of Origanum such as thymol and carvacrol were tested as individual components, weak but genotoxic effects were obtained in Salmonella (13), rat bone marrow cells (14), and Drosophila (15). The genotoxicity of these individual components does not correspond with their participation in the essential oils or in mixtures of these 2 phenolic monoterpenes. For this reason, it has been assumed that there are no antagonistic phenomena (15). There is therefore need for more genotoxic and mutagenic studies in various test systems to make an informed decision on the potential genotoxic and/or mutagenic effects of these spices. This is because, contrary to popular belief, approval of a natural food ingredient may not be as simple a process as one might think. In fact, unless an ingredient had a history of use in food prior to January 1, 1958, or the substance was deemed “safe by scientific procedures”, the ingredient must be determined Generally Recognized As Safe (GRAS) or be classified as a food additive. Furthermore, approval for one use (such as a flavor) does not constitute approval for any other use (16). Objective of genotoxicity testing of food additives and other food ingredients is the genotoxic hazard identification, with the purpose of minimizing the health risk for consumers through the primary prevention of the exposure to genotoxic substances.

This study was therefore carried out to investigate the genotoxic and mutagenic potentials of oregano, dark soy and nutmeg using Ames and SOS-chromo assays.

**MATERIALS AND METHODS**

**Sample collection**

A 150 mL bottle of Amoy dark soy, 32g oregano (Gel Spice, USA) and 50g nutmeg spice (Gel Spice, USA) were purchased from Shoprite, Ikeja, Lagos, Nigeria. The spices were kept at room temperature in a dry place throughout the period of this study.

**Ames (Salmonella) fluctuation test**

The spices were subjected to Ames test after sterilization by filtration through a 0.22 µm pore-size cellulose nitrate filter (Millipore). Salmonella typhimurium strains TA98 and TA100 obtained from Environmental Bio-Detection Products Inc. (EBPI, Canada) were used in the Ames test conducted according to the method described by Maron and Ames [17]. Tests were conducted under aseptic conditions according to the method described by Rao and Lifshitz [18] and Alabi et al. (19). Four quantities: 0.1, 0.25, 0.5 and 1 g each, of the nutmeg and oregano were dissolved in 10 mL of distilled water each, and 100, 50, 25 and 10 % (v/v, dark soy/distilled water) of dark soy were prepared. 200 µL of each of the concentrations of each was added to 19.8 mL of the reaction mixture (Davis-Mingioli salts composed of D-glucose [ICN Biomedicals, Aurora, OH, USA; CAS 50-99-7] D-biotin [ICN Biomedicals, Aurora, OH; CAS 22879-79-4], L-histidine [Sigma, St. Louis, MO, USA; CAS 7048-02-4], and bromoresorol purple [Fisher Scientific, Nepean, Ont.; CAS 115-40-2]). Reagents were added to sterile culture tubes in the following order: (1) reaction mixture, (2) spices (3) bacteria. The culture tubes were vortexed after each addition and a 200 µL portion was transferred into 96-well flat-bottomed microplates. The microplates were sealed in plastic bags and incubated for five days at 37°C. At the end of this period, the plates were examined for color: all yellow, partially yellow and turbid wells were considered positive, whereas purple wells were deemed negative. The number of positive wells per plate was recorded and compared to the controls. Chi square analysis [20] was used for statistical evaluation of the treated plates versus the control plates. A sample is considered mutagenic when there is a significant increase of the number of positive wells in treated plates over the negative control plates (i.e mutagenic index [MI]). The results were expressed as mutagenicity ratio (number of positive wells in treated plates/number of positive wells in the negative control plates) and are an average of at least three experiments. Sodium azide (NaN₃) and 2-Nitrofluorene (2-NF) were used as positive controls for TA100 and TA98 respectively, while distilled water was used as negative control.

**Sos Chromotest**

The tester strain E. coli PQ37 was obtained from Environmental Bio-Detection Products Inc. (EBPI, Canada). The SOS chromotest was performed without metabolic activation as described by Quillardet and Hofnung [21] with...
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modifications provided by Mersch-Sunderman et al. [22] and Kevekordes et al. [23]. The sample concentrations of 0.0313, 0.0625, 0.125, 0.25, 0.5 and 1 g/mL (sample/dimethyl sulfoxide [DMSO]) of oregano and nutmeg, and 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 % (v/v, dark soy/DMSO) concentrations of dark soy were considered in four replicates without metabolic activation. A 600 µL volume of an appropriate overnight culture dilution were added to a tube containing 20 µL sample volume, and incubated with agitation for 2 h at 37°C and subsequently centrifuged at 700 g for 20 min. The supernatant was discarded and the bacterial pellets were resuspended with 200 µL of SOS Chromogen [p-nitrophenyl phosphate (PNPP, Boehringer Mannheim, Laval, Que.; CAS 4264-83-9) for alkaline phosphatase (AP) and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal, Vector Biosystems, Toronto, Ont.; CAS X100) for Beta-galactosidase (bgal). Plates were re-incubated for 60 min, after which optical density readings were taken at 620 (for b-gal) and 405 nm (for AP) respectively. 4 Nitro-Quinoline Oxide (4 NQO) was used as positive control.

AP reduction factors (RF), b-gal induction factors (IF) and corrected induction factors (CIF = IF/RF) were calculated as described by Legault et al. [24]:

RF = \frac{XOD_{at}}{XOD_{ct}}

IF = \frac{XOD_{at}}{XOD_{ct}}

where X is the mean of four OD readings and t and c refer to test and control dilutions, respectively. As shown above, the RF and IF values account for the background activity of the control. The ratio of IF to RF units yields an estimate of b-gal activity corrected for toxicity. A normalized induction factor of \leq 1.5 was considered to represent significant genotoxic activity [20].

RESULTS

Ames (Salmonella) fluctuation test

The Ames fluctuation test results (TA100 and TA98 strains) on the nutmeg, oregano and dark soy are summarized in Table 1. Two (nutmeg and oregano) of the three samples tested were positive in at least one concentration (1 g/10 mL) when compared with the negative control. No mutagenic activity was detected in dark soy at all the concentrations tested. There was a positive response at 0.5 g in nutmeg with stronger positive responses at 1 g, an indication of concentration-dependent genotoxic response in both TA98 and TA100 strains. In oregano however, positive response was only observed at 1 g in both strains used. The induction of positive response by the condiments showed that nutmeg was more mutagenic than oregano at the tested concentrations. TA98 strain showed better sensitivity to the treatment than TA100 strain making it a better mutagenic indicator. The positive controls induced significant positive responses in both Salmonella strains utilized.

<table>
<thead>
<tr>
<th>Conc (g/10 mL)</th>
<th>Nutmeg</th>
<th>Oregano</th>
<th>MR</th>
<th>Conc (%)</th>
<th>Dark soy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA98</td>
<td>TA100</td>
<td>TA98</td>
<td>TA100</td>
<td>TA98</td>
</tr>
<tr>
<td>0.1</td>
<td>0.48</td>
<td>0.21</td>
<td>0.32</td>
<td>0.27</td>
<td>10</td>
</tr>
<tr>
<td>0.25</td>
<td>0.92</td>
<td>0.88</td>
<td>0.74</td>
<td>0.65</td>
<td>25</td>
</tr>
<tr>
<td>0.5</td>
<td>2.12*</td>
<td>2.03*</td>
<td>0.91</td>
<td>0.82</td>
<td>50</td>
</tr>
<tr>
<td>1.0</td>
<td>5.43*</td>
<td>4.90*</td>
<td>2.04*</td>
<td>1.94*</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2-NF</td>
<td>NaN</td>
<td>2-NF</td>
<td>NaN</td>
<td>2-NF</td>
</tr>
<tr>
<td></td>
<td>4.9</td>
<td>5.3</td>
<td>4.9</td>
<td>5.3</td>
<td>4.9</td>
</tr>
</tbody>
</table>

*Statistically significant at p<0.01

MR = mutagenicity ratio (number of positive wells in treated plates/number of positive wells in the negative control plates)
SOS Chromotest

The results of SOS Chromo genotoxicity tests of nutmeg, oregano and dark soy are presented in Table 2. The SOS Chromotest response was generally more sensitive than the Ames test. The sample is considered genotoxic if β-galactosidase induction coefficient is higher than or equals to 1.5. Induction factor (IF) is defined as the ratio of specific activity of β-galactosidase at a given sample concentration to specific β-galactosidase activity. The SOS Chromotest results showed that both nutmeg and oregano were genotoxic with IF greater than 1.5 at all the concentrations of both nutmeg and oregano utilized. In dark soy however, only 3.13 and 1.56 %, the lowest concentrations tested, were genotoxic, showing an IF greater than 1.5. The IF for 1 g nutmeg and oregano were higher than the value obtained for 4-Nitro-Quinoline Oxide (10 µg/mL), utilized as the positive control. Compared to Ames test, SOS Chromotest appeared to be better indicator of genotoxicity in the assessment of condiments used.

Table 2. Nutmeg, oregano and dark soy genotoxicity as tested by the SOS chromotest at different concentrations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentrations</th>
<th>IF</th>
<th>Genotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (4-Nitro-Quinoline Oxide)</td>
<td>0.31</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.63</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>1.5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>1.7</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>10.00</td>
<td>1.9</td>
<td>+</td>
</tr>
<tr>
<td>Nutmeg (g/mL)</td>
<td>0.0313</td>
<td>1.60</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.0625</td>
<td>1.66</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>1.69</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>2.30</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2.41</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.68</td>
<td>+</td>
</tr>
<tr>
<td>Oregano (g/mL)</td>
<td>0.0313</td>
<td>1.52</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.0625</td>
<td>1.77</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>1.80</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>2.29</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2.70</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.74</td>
<td>+</td>
</tr>
<tr>
<td>Dark soy (%)</td>
<td>1.56</td>
<td>1.68</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3.13</td>
<td>1.54</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>1.20</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>1.42</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.80</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.83</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.45</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = positive for genotoxicity. IF ≤1.5 is considered genotoxic.
- = negative for genotoxicity.
IF = Induction factor.
DISCUSSION

Nutmeg, Oregano and Dark soy have long been valued for their culinary, fragrant, preservative and medicinal properties. As a result of the new attraction for natural products like nutmeg, oregano and dark soy, and their wide use, it is important to develop a better understanding of their mode of biological action and their toxic potential for application in human health. This is especially relevant at a time when there is an increasing interest in finding more natural alternatives to many existing preservatives. This study examined the potential of nutmeg, oregano, and dark soy to be mutagenic and genotoxic. There is considerable interest in determining the risks that these condiments may pose to health. Thus, an assessment of the genotoxic and mutagenic potential of these condiments is necessary to ensure their relatively safe use.

Ames test and SOS Chromotest were used for the analysis of the mutagenic and genotoxic potential of nutmeg, oregano and dark soy. In both microbial assays, nutmeg was shown to be mutagenic and genotoxic. The observed mutagenicity and genotoxicity were believed to be due to nutmeg’s constituents. Myristicin, elemicin and safrole, constituents of nutmeg, have all been reported to be toxic. Myristicin has been shown to have neurotoxic effects on dopaminergic neurons and monoamine oxidase [25,26,27]. Safrole is considered by the European Commission on Health and Consumer Protection to be genotoxic and carcinogenic, while elemicin has been reported to be partially responsible for the psychoactive effects of nutmeg [28], and has been reported to inhibit locomotor activity [29]. The report of Van den Berg et al. [30] showed that myristicin, safrole and elemicin are genotoxic. There are conflicting reports on the mutagenicity and genotoxicity of nutmeg constituents. Mutagenicity and genotoxicity of nutmeg showed in this study are in accordance with the report of Rockwell and Raw [31], where nutmeg extracts were mutagenic. However, Buchanan et al. [32] had reported that myristicin was non-mutagenic when studied alone.

At most of the concentrations tested, oregano did not trigger gene mutations in the mutant strains of Salmonella typhimurium that could have resulted in a reversion in the genome of the strains back to a prototrophic state, indicating that oregano is non-mutagenic, except at a high concentration of 1g/ 10 mL. However, oregano was genotoxic in SOS Chromotest. Oregano was found to induce SOS response as a result of the production of β-galactosidase which gave a blue coloration at the concentrations tested indicating a positive result to genotoxicity. The presence of a blue coloration suggests the presence of certain cytotoxic/genotoxic substances in oregano. The observed genotoxicity of oregano is in accordance with the report of Hamedo and Abdelmigid [11], where oregano induced genotoxic effects in Victa tafa test, with the induction of chromosomal and nuclear abnormalities in mitotic cells. Also, report of Sirnik and Gori’sek [33] showed that oregano has inhibitory activity against E. coli, Proteus vulgaris, Salmonella enteritidis and Pseudomonas fluorescens, which might be due to the destruction of their genetic materials.

The analysis of dark soy using SOS Chromo assay showed both mild and chronic genotoxicity at low concentrations. The Ames test failed to implicate dark soy as a mutagenic substance. This was observed possibly due to the dark coloration of the sample, which was not filtered because when used as a condiment in cooking, it is used in its pure form (not filtered). It could also be due to the lack of reverse mutation from histidine auxotrophy to phototrophy in the bacteria, due to acute toxicity of the sample. Soy sauce contains bioactive components which have various biological functions including antimicrobial activities [34]. Soy sauce has been shown to have a high salt content which limits the growth of bacteria by damaging their DNA [35].

CONCLUSION

In conclusion, our result indicates that nutmeg and oregano are genotoxic while dark soy showed genotoxicity only at low concentration. This is of public health importance considering that these condiments are widely used in varieties of food preparations. Long term exposure can lead to bioaccumulation which might have varying degrees of genotoxic and mutagenic effects including cancer in exposed individuals. Further studies are necessary to arrive at a safe standard for the use of these condiments by the general populace.

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