Antigenotoxic capacity of beta-caryophyllene in mouse, and evaluation of its antioxidant and GST induction activities

Isela Álvarez-González, Eduardo Madrigal-Bujaidar and Seydi Castro-García

Laboratorio de Genética, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México

(Received June 7, 2014; Accepted October 1, 2014)

ABSTRACT — The present report was designed to determine the antigenotoxic capacity of beta-caryophyllene (BC) on the damage induced by benzo(a)pyrene (BaP) in mouse. We found no genotoxic potential of BC, and a significant inhibitory effect on the number of sister-chromatid exchanges (SCE) and chromosomal aberrations induced by BaP. The three tested doses of the agent (20, 200, and 2,000 mg/kg) produced a dose-dependent decrease of the two evaluated cytogenetic parameters. In comparison with the effect induced by BaP, the best inhibitory effect (about 80%) was obtained with the high tested dose of BC considering the two evaluated parameters. Other aim of the study was to explore whether in this effect participated the BC antioxidant capacity and/or its effect as inducer of GST activity. We found a dose-dependent decrease induced by BC in regard to both the oxidation of lipids and proteins produced by BaP. In the case of GST, when BC was administered alone we found a mean increase of 64% of the enzyme activity, respect to the control level, and when BC was administered in mice treated with BaP the increase obtained with the high dose of BC reached 27%. Therefore, our data established no in vivo genotoxicity by BC, and a significant antigenotoxic potential of the compound, which may be related with its capacity to block the molecular oxidation and to stimulate the GST activity.

Key words: Beta-caryophyllene, Benzo(a)pyrene, Antigenotoxic, Antioxidant, GST

INTRODUCTION

Epidemiological studies have consistently shown that regular consumption of fruits and vegetables as well as whole grains is strongly associated with a reduced risk of developing chronic diseases, including cancer and cardiovascular disorders, which are the top two causes of death in most industrialized countries (Pomerleau et al., 2005). These studies also highlighted the unique chemical feature of plant-based foods to provide numerous primary and secondary metabolites to the human diet that are absorbable into the body. These phytochemicals can be bioactive non-nutrient compounds that exert diverse biological activities, including some that help to prevent the development of diseases and others that improve human health (Calleja et al., 2013).

Furthermore, the close relationship between the presence of mutations and the development of cancer has promoted the search of antimutagenic phytochemicals which could, eventually, be proposed as chemopreventive agents. An outstanding point in regard to these agents is the knowledge about the types of molecular and cellular actions that they have to carry out toward mutagens and carcinogens to prevent their damage, which may, otherwise, depend on variables such as the involved doses, the route of penetration into the organism, the sequence of intake, chemical interactions, and the biotransformation/detoxification processes in the organism, among others (De Flora and Ferguson, 2005).

In this context, it has been reported that essential oils from different plants have a number of biological activities which may be beneficial for the human health, such as the antimutagenic and antioxidant effects, although it has also been reported oxidative and genotoxic actions. These effects have been observed in plants of the genus Salvia, Piper, and Eugenia, among others (Ramos et al., 2012; Péres et al., 2009; Vieira et al., 2012). A component usually found in the essential oil of these plants is beta-caryophyllene (BC) (Fig. 1), an aromatic, volatile terpenoid, which corresponds to the bicyclic sesquiterpenes and is used in the cosmetic and food industries (Sköld et al., 2006). Regarding this chemical, various biomed-
cal effects has been reported such as anti-inflammatory, antibacterial, hepatoprotector, and neuroprotector (Calleja et al., 2013; Chang et al., 2013); moreover, its action as antioxidant and glutathione S transferase (GST) inducer has also been determined (Calleja et al., 2013; Zheng et al., 1992). In the genotoxicity area, authors have found no damaging effect by using the Salmonella revertant test, as well as by the analysis of micronuclei in cultured human lymphocytes and in mice in vivo (Di Sotto et al., 2008, 2010; Molina-Jasso et al., 2009). In the case of its anti-mutagenic capacity, BC has been studied only in in vitro assays with results showing, a reduction of the DNA damage, as well as no effect (Di Sotto et al., 2010).

According to the above-mentioned information, it seems relevant to further explore the antigenotoxic potential of BC; therefore, for such a purpose we examined whether the sesquiterpene was able to reduce the number of sister chromatid exchanges (SCE) and the number of chromosomal aberrations (CA) induced by benzo(a)pyrene (BaP) in mice. BaP is a polycyclic, aromatic hydrocarbon, known as a strong mutagen in in vitro and in vivo assays, as well as a human carcinogen (IARC, 2010; Halder et al., 2005). Another aim of this study was to explore whether the antigenotoxic capacity of BC show a relation with its antioxidative potential and/or with the induction of the GST activity. The selected mutagen is adequate to achieve these two purposes because in the phase I of biotransformation is principally metabolized by the cytochrome P450 1A1 (CYP1A1), in a process that gives rise to a variety of carcinogetic metabolites that, in a next step, are conjugated with glutation (GSH) by means of the GST in order to be eliminated from the organism (Hodek et al., 2013; Mantey et al., 2014; Gelboin, 1980; Kumar et al., 2012). Furthermore, the metabolic activation of BaP by CYP 1A1 enzyme can produce ROS, which are capable to react with DNA, lipids, and proteins (Miller and Ramos, 2001; Kumar et al., 2012).

**MATERIAL AND METHODS**

**Chemicals and animals**

Corn oil, colchicine, BC [(-)-trans-caryophyllene], BaP, 5-bromodeoxyuridine (BrdU), bis-benzimide, thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2,4-dinitrophenylenihydrazine (DNPH), ethyl acetate, guanidine, and the Glutathione-S-Transferase Assay Kit were acquired from Sigma Chemicals (St Louis, MO, USA). Ether, potassium chloride, methanol, acetic acid, trisodium citrate dihydrated, monobasic sodium phosphate, sodium chloride, monobasic potassium phosphate, dibasic sodium phosphate, and ethanol were obtained from J.T Baker (Mexico City, Mexico). Finally, the Giemsa stain was purchased from Merk (Mexico City, Mexico).

Forty-eight Swiss-Webster male mice of 28 g were obtained from the animal house of the National School of Biological Sciences (Mexico City, Mexico), and maintained in polypropylene cages at 22 ± 2°C, 12 hr dark-light cycles, 50% relative humidity, with free access to water and food (Rodent Laboratory chow 5001, Purina). Seven days after the conditioning period, the experimental protocol was started, once approved by the Committee of Ethics and Biosecurity of the National School of Biological Sciences.

**Genotoxicity/antigenotoxicity: SCE and CA determinations**

Eight groups with six mice each were orally administered with the tested chemicals, with exception of BaP that was intraperitoneally (ip) injected. The selected doses of BC were based on a previous report that used similar experimental conditions (Molina-Jasso et al., 2009). Three groups of mice were treated with 20, 200, and 2,000 mg/kg of BC each; a control group was administrated with corn oil (10% w/v); another group was injected with 200 mg/kg of BaP; and finally, three other groups were treated with 20, 200, and 2,000 mg/kg of BC, respectively, and 30 min later they were ip injected with 200 mg/kg of BaP. Thirty minutes after this step, a BrdU tablet of 45 mg was 60% coated with paraffin, and then, subcutaneously implanted to each mouse under inhaled ether anesthesia. Twenty-one hours later, colchicine (5 mg/kg) was ip injected to each mouse to deter cell division, and 3 hr later the animals were cervicaly dislocated; the bone marrow of both femurs were obtained and placed in a solution of KCl 0.075 M, where the cells were
incubated for 30 min at 37°C, then, they were centrifuged for 10 min at 1,000 g, the supernatant was discarded and the remaining cells were fixed with a solution of methanol-acetic acid (3:1), this fixation process was repeated twice. Subsequently, two or three drops of the suspension were placed on a slide and covered with a cover slip. Then, the chromatic differential staining was made by applying the Hoescht-Giemsa method (Goto et al., 1975; Alvarez-González et al., 2010). With this technique, we were able to differentiate mitosis in the first, second, and third cellular division. In the obtained mitotic cells, the following determinations were performed: a) the number and types of CA in 100 first-division metaphases; b) the SCE frequency in 30 second-division metaphases; c) the determination of cell proliferation kinetics in 100 cells by quantifying the rate of cells in first (M1), second (M2), and third (M3) cellular division. With these data, the average generation time (AGT) was obtained according to the formula \[ \frac{a}{b} \cdot \frac{1}{1} + \frac{a}{2} \cdot \frac{1}{2} + \frac{a}{3} \cdot \frac{1}{3} \times 100 \] and d) the mitotic index in 1,000 cells. The obtained data were statistically analyzed with the ANOVA and the Tukey tests.

**Mechanisms of antigenotoxicity**

The same groups of mice used to evaluate the antigenotoxic potential of BC were also utilized to determine the oxidative/antioxidative capacity of the tested chemicals, as well as its effect on the activity of GST. For these two purposes, the liver of each mouse was dissected immediately after the cervical dislocation and 500 mg of each organ were homogenized in 5 mL of PBS at 4°C, and pH 7. The sample was then centrifuged for 20 min at 13,000 g, and the supernatant was used to determine the total protein content according to the method of Bradford (1976), as well as the GST activity. Also, by using the liver crude homogenate we determined the lipid and protein oxidation. The obtained results were statistically analyzed with an ANOVA test followed by the Student Newman-Keuls test.

**1-Lipid peroxidation assay**

This assay was carried out according to the method of Buege and Aust (1979) with slight modifications. Initially, we added 1 mL of TBA 0.0375% dissolved in 16% formaldehyde 30 min at 37°C. The content of oxidized carbonyls was determined by means of the procedure described by Levine et al. (1990) with slight modifications. We added 300 μL of 20% TCA to 300 μL of liver crude homogenate; the sample was placed on ice for 10 min, centrifuged for 5 min at 11,500 g, the supernatant was discarded, and then, 150 μL of DNPH 10 mM dissolved in HCl 2M was added to the remaining cellular sediment. The mix was incubated for 1 hr at 37°C and centrifuged 10 min at 11,000 g. The sediment was washed three times with ethyl acetate/ethanol 1:1 and the obtained precipitate was dissolved in 1 mL of guanidine (6 M, pH 2.3). Finally, each sample was incubated 40 min at 37°C, centrifuged at 11,000 g (5 min at 4°C), and the absorbance was measured in the supernatant at 360 nm. The results were expressed in nM of reactive carbonyls/mg protein. Molar absorptivity was considered 21,000 M⁻¹ cm⁻¹.

**2-Protein oxidation assay**

The content of oxidized carbonyls was determined by means of the procedure described by Levine et al. (1990) with slight modifications. We added 300 μL of 20% TCA to 300 μL of liver crude homogenate; the sample was placed on ice for 10 min, centrifuged for 5 min at 11,500 g, the supernatant was discarded, and then, 150 μL of DNPH 10 mM dissolved in HCl 2M was added to the remaining cellular sediment. The mix was incubated for 1 hr at 37°C and centrifuged 10 min at 11,000 g. The sediment was washed three times with ethyl acetate/ethanol 1:1 and the obtained precipitate was dissolved in 1 mL of guanidine (6 M, pH 2.3). Finally, each sample was incubated 40 min at 37°C, centrifuged at 11,000 g (5 min at 4°C), and the absorbance was measured in the supernatant at 360 nm. The results were expressed in nM of reactive carbonyls/mg protein. Molar absorptivity was considered 21,000 M⁻¹ cm⁻¹.

**3-GST activity assay**

The activity of this enzyme was determined according to the method described by Habig et al. (1974). For such purpose, we used the GST Assay Kit. Briefly, 1 μL of the supernatant previously obtained from the homogenized and centrifuged liver tissue was added to 99 μL of a previously prepared reaction mixture (980 μL Dulbecco’s phosphate buffered saline, 10 μL of reduced glutathione 200 mM, and 10 μL of 1-chloro-2,4-dinitrobenzene 100 mM). Absorbance of the supernatant was measured once every minute at 340 nm during 5 min. The enzymatic activity is directly proportional to the increase in absorbance. Calculations were made according to the manufacture instructions.

**RESULTS**

**Sister chromatid exchanges, average generation time, and mitotic index**

Fig. 2 shows the results obtained in regard to the number of SCE. Control mice had a mean of 3.8 SCE/metaphase, in contrast with the significant increase in animals treated with 200 mg/kg of BaP (11.6 SCE). With respect to the three tested doses of BC, we determined a mean of 4.1 SCE, a result that clearly establish non-SCE inducing potential of BC, and a result that was contrary to the strong genotoxic capacity shown by BaP. The same figure demonstrated the anti-SCE effect of BC. The reduction of such event in comparison with the level obtained in BaP treated mice was 31.5, 72.4 and 82.3% with 20, 200, and 2,000 mg/kg of BC, respectively; however, only the two high doses were statistically significant. With
In respect to the cellular proliferation kinetics, it is known that this measurement is represented by the proportion of cells in M1, M2 and M3, and that can be expressed as the AGT (Table 1). In this parameter, the results obtained in bone marrow cells showed a homogeneous AGT in the studied groups with a mean of 12.30 hr. Although the two high doses of BC in combination with BaP had a certain cell cycle delay, this effect was no statistically significant.

Finally, data about MI are also shown in Table 1 and indicated a significant inhibition (2.3%) caused by the mutagen respect to the control level. In this parameter, the three doses of BC were found to induce a MI in the same range as observed in the control group, demonstrating no cytotoxic potential by the tested chemical. The two low doses of BC had no protective effect against the cytotoxicity induced by BaP; however, with the high dose of BC (2,000 mg/kg), we determined a MI increase statistically significant with respect to the value obtained with BaP, suggesting certain cytotoxic protection with this dose.

Chromosomal aberrations

Our analysis was based in the recommendations made by the OECD (2013) with slight modifications. Chromosome type aberrations refer to alterations including the two chromatids, while chromatid type aberrations refer to those present in only one chromatid. Fragments were those clearly separated from the chromosome axis and with a size that corresponded to about the double of that of a minute. Breaks were those clearly displaced from the chromatid axis.

Table 2 shows the results observed respect to the number and the types of chromosomal aberrations. We found that the registered aberrations induced with the three doses of BC-treated animals were in the range of the control mice, indicating no genotoxic potential for the compound in the evaluated cytogenetic parameter. In contrast, mice administered BaP had a pronounced elevation in the number of all evaluated alterations, being the presence of fragments highly increased. When BC was administered with the mutagen, we found a protection with the three tested doses of the sesquiterpene; however, statistical difference with respect to results of BaP-treated mice was achieved only with the two high doses. In the case of the numerical anomalies, we only detected the presence of polyploidy. In this case, the results were similar to those described before: a low number of polyploid cells in the control and BC-treated mice, a significant increment in BaP treated animals, and a significant reduction of such damage with the two high doses of BC. Table 3 shows a summary of the obtained data, and demonstrate a similar trend with or without the incorporation of gaps; moreover, in this Table it also shown that various aberrations could be present in a single metaphase as indicated by the lower number of cells with aberrations respect to

Fig. 2. Effect of beta-caryophyllene (BC) and benzo(a)pyrene (BaP) on the number of sister chromatid exchanges (SCE) in mouse bone marrow cells. Each bar represents the mean ± S.D. of 6 mice per group. *Statistically significant difference with respect to the control value, and †with respect to the value of BaP. ANOVA and Student Newman – Keuls tests, P ≤ 0.05.
the number of registered aberrations.

Oxidative/antioxidative determinations

The lipid peroxidation induced by the studied chemicals is shown in Fig. 3. We found that the groups administered with the three doses of BC showed a mean of 12.3 nM MDA/mg, which is practically the same value obtained in the control mice (12.2 nM MDA/mg protein); however, the administration of BaP gave rise to almost a duplication of the control value. Results obtained with the administration of BC in mice treated with the mutagen, showed a dose-dependent decrease of the lipoperoxidative damage. The best MDA inhibitory effect (68%) respect to the value registered for the administration of BaP was determined with the high dose of BC (2,000 mg/kg).

In the case of the results obtained on oxidized proteins (Fig. 4), we found a similar trend as that observed for the lipoperoxidation assay. Almost a duplication of the level induced with BaP respect to the determined for the control animals, an absence of protein oxidation induction by the three tested doses of BC, whose results were found in the range determined for the control mice, and a statistically significant decrease with the two high doses of BC when tested together with the mutagen.

The activity of GST is presented in Fig. 5. In this assay, it was observed that the three tested doses of BC were inducers of the enzyme activity giving rise to increases of 64, 68, and 63 % with 20, 200, and 2,000 mg/kg of BC, respectively, in comparison with the value found in the control group. When we combined BC with the mutagen, our results showed a dose-dependent increase in the GST activity respect to the level observed with BaP: 6, 16, and 27% with 20, 200, and 2,000 mg/kg of BC, respectively; however, only the high dose was statistically significant.

### Table 1. Cellular proliferation kinetics (CPK), average generation time (AGT) and mitotic index (MI) in mice treated with beta-caryophyllene (BC) and benzo(a)pyrene (BaP).

<table>
<thead>
<tr>
<th>Agents</th>
<th>Dose (mg/kg)</th>
<th>M1 (%)</th>
<th>M2 (%)</th>
<th>M3 (%)</th>
<th>AGT (hr)</th>
<th>MI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil 10% p/v</td>
<td>29.5 ± 2.75</td>
<td>62.5 ± 3.9</td>
<td>8.2 ± 1.2</td>
<td>12.1 ± 0.3</td>
<td>4.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>BaP 200</td>
<td>39.2 ± 3.2</td>
<td>50.4 ± 3.5</td>
<td>10.4 ± 1</td>
<td>12.2 ± 0.2</td>
<td>3.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>BC 20</td>
<td>32.4 ± 4.5</td>
<td>56.2 ± 4.2</td>
<td>11.4 ± 0.9</td>
<td>12.1 ± 0.5</td>
<td>5.3 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>BC 200</td>
<td>38.6 ± 2.3</td>
<td>51.2 ± 2.6</td>
<td>10.2 ± 0.8</td>
<td>12.2 ± 0.7</td>
<td>4.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>BC 2000</td>
<td>49.2 ± 5.4</td>
<td>40.6 ± 3.5</td>
<td>10.2 ± 1.3</td>
<td>13.0 ± 0.6</td>
<td>5.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>BC + BaP 20+200</td>
<td>47.5 ± 5.9</td>
<td>34.5 ± 2.5</td>
<td>18 ± 2.1</td>
<td>12.3 ± 0.4</td>
<td>3.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>BC + BaP 200+200</td>
<td>48.0 ± 6.0</td>
<td>40.2 ± 4.6</td>
<td>11.8 ± 2.4</td>
<td>13.2 ± 0.6</td>
<td>3.9 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>BC + BaP 2000+200</td>
<td>53.2 ± 5.7</td>
<td>38 ± 5.2</td>
<td>7.8 ± 1.1</td>
<td>13.3 ± 0.5</td>
<td>4.3 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 100 cells (CPK) and 1,000 cells (MI) per mouse. Six animals per group. CPK correspond to the proportion of metaphases in first (M1), second (M2), and third (M3) division. AGT was obtained according to the formula 21/(1)(M1)+(2)(M2)+(3)(M3) X100. aStatistically significant difference with respect to the control value, and bwith respect to the value of BaP. ANOVA and Student t tests, P ≤ 0.05.

### Table 2. Types and amount of structural and numerical aberrations induced with benzo(a)pyrene (BaP) and beta-caryophyllene (BC) in mouse bone marrow cells.

<table>
<thead>
<tr>
<th>Agents</th>
<th>Dose (mg/kg)</th>
<th>g</th>
<th>ig&quot;</th>
<th>ctb</th>
<th>csb&quot;</th>
<th>f</th>
<th>m</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>-</td>
<td>1.3 ± 0.4</td>
<td>-</td>
<td>0.1 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>BaP 200</td>
<td>7.5 ± 0.3</td>
<td>2.8 ± 0.5</td>
<td>11.6 ± 0.3</td>
<td>6.5 ± 0.7</td>
<td>28.0 ± 7.6</td>
<td>4.6 ± 0.7</td>
<td>2.8 ± 0.6</td>
<td>3.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>BC 20</td>
<td>0.3 ± 0.1</td>
<td>-</td>
<td>0.5 ± 0.1</td>
<td>-</td>
<td>1.2 ± 0.3</td>
<td>-</td>
<td>-</td>
<td>0.2 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>BC 200</td>
<td>0.6 ± 0.1</td>
<td>-</td>
<td>0.5 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>1.3 ± 0.3</td>
<td>-</td>
<td>-</td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>BC 2000</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>1.5 ± 0.4</td>
<td>-</td>
<td>-</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>BC + BaP 20+200</td>
<td>4.3 ± 0.8</td>
<td>3.2 ± 0.7</td>
<td>10.1 ± 1.8</td>
<td>4.6 ± 0.9</td>
<td>14.8 ± 2.5</td>
<td>3.1 ± 0.8</td>
<td>2.0 ± 0.2</td>
<td>2.8 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>BC + BaP 200+200</td>
<td>2.8 ± 0.4</td>
<td>0.3 ± 0.1</td>
<td>1.8 ± 0.7</td>
<td>1.0 ± 0.1</td>
<td>4.8 ± 1.3</td>
<td>0.8 ± 0.7</td>
<td>1.1 ± 0.1</td>
<td>1.5 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>BC + BaP 2000+200</td>
<td>2.1 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>2.1 ± 0.7</td>
<td>0.8 ± 0.4</td>
<td>5.5 ± 1.2</td>
<td>1.1 ± 0.8</td>
<td>1.0 ± 0.3</td>
<td>1.16 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 100 metaphases per mouse. Six animals per group. aStatistically significant difference with respect to the control value, and bwith respect to the value of BaP. ANOVA and Tukey test, P ≤ 0.05.

g = gaps, ig = isogaps, ctb = chromatidic break, csb = chromosome break, f = fragments, m = minutes, r = rings, p = polyploidy.
DISCUSSION

BaP is an omnipresent environmental pollutant known as a genotoxic carcinogen, and classified as a carcinogenic agent to humans by the International Agency for Research on Cancer (IARC, 2010). The chemical has a complex biotransformation process through the initial action of CYP1A1, which generates a number of reactive, toxic metabolites, including epoxides, phenols and quinones that can be further metabolized to other reactive compounds or enzymatically conjugated to be eliminated (Gelboin, 1980). The produced metabolites may damage...
Antigenotoxicity of beta-caryophyllene in mouse

Fig. 4. Oxidized proteins in the liver of mice treated with beta-caryophyllene (BC) and benzo(a)pyrene (BaP). Each bar represents the mean ± S.D. of the reactive carbonyls (CO°) deteminate in the liver of 6 animals per group. °Statistically significant difference with respect to the control value, and °with respect to the value of BaP. ANOVA and Student Newman – Keuls tests, P ≤ 0.05

Fig. 5. Glutation-S-Transferase activity in mice treated with beta-caryophyllene (BC) and benzo(a)pyrene (BaP). Each bar represents the mean ± S.D. of 6 animals per group. °Statistically significant difference with respect to the control value, and °with respect to the value of BaP. ANOVA and Student Newman – Keuls tests, P ≤ 0.05

1 = control agent, corn oil (10 % w/v), 2 = 200 mg/kg of BaP, 3 = 20 mg/kg of BC, 4 = 200 mg/kg of BC,
5 = 2000 mg/kg of BC, 6 = 20 mg/kg of BC + 200 mg/kg of BaP, 7 = 200 mg/kg of BC + 200 mg/kg of BaP,
8 = 2000 mg/kg of BC + 200 mg/kg of BaP
DNA by forming adducts, but also through the induction of ROS that, besides DNA, may also damage proteins and lipids. These molecular alterations can be the subjacent origin of the presently observed increase in the number of SCE and CA, result which is in line with the genotoxic potential of BaP previously demonstrated in in vitro and in vivo short-term tests (Alvarez-González et al., 2011; Bhagavathy and Sumathi, 2012; Sehgal et al., 2012).

In the case of BC, our results showed no capacity of the chemical to induce SCE and CA in mouse, a finding which is concordant with previous studies using bacterial tests, and the analysis of micronuclei in vitro and in vivo (Di Sotto et al., 2008, 2010; Molina-Jasso et al., 2009). So far, such absence of genotoxicity of BC suggests that it may be safe regarding its industrial use as saborizant and cosmetic; and it is also encouraging to extend research about its potential biomedical activities, including its capacity as antimutagen/chemopreventive agent. During its metabolism, BC can originate hydroperoxides as primary oxidation products; which, however, correspond to low reactive and highly stable product (Sköld et al., 2006). This, together with efficient DNA repair and detoxification systems by mouse may be a factor involved in the observed absence of genotoxicity (Molina-Jasso et al., 2009).

To our knowledge, the antigenotoxic potential of BC in mouse was evaluated for the first time. Our results clearly showed a significant dose-dependent inhibitory effect of BC on the number of SCE and CA induced by BaP. The results indicated that the highest protection was exerted with the two high doses (200 and 2,000 mg/kg of BC). Although little is known about the SCE molecular basis, it is known that such lesion corresponds to the interchange of DNA replication products at apparently identical loci of the sister chromatids in response to a damaged DNA template. The exchange process involves DNA breakage and reunion, possibly at the replication fork (Albertini et al., 2000; Painter, 1980; Wilson and Thompson, 2007). On the other hand, structural chromosomal aberrations may result from a direct DNA breakage in the replication of a damaged DNA template or by inhibition of DNA synthesis among other mechanisms. Numerical aberrations refer to changes in chromosome number that occur due to abnormal cell division, and that can be related with damage to the mitotic spindle and/or its associated elements, damage to chromosomal sub-structures, alterations in cellular physiology or to mechanical disruption (Obe et al., 2010)

In previous studies, BC was shown to decrease the number of mutations induced by 2-nitrofluorene, sodium azide, methyl methane sulfonate and 2-aminoanthraquinone in a bacterial reverse mutation assay (Di Sotto et al., 2008). Furthermore, it was also reported that the terpene decreased the genotoxicity produced by ethyl methanesulfonate in cultured lymphocytes, although only when BC was added before or together with the mutagen (Di Sotto et al., 2010). These data, together with our present results, suggest a wide protective spectrum for BC; however, the last mentioned authors found no in vitro protective effect against the aneugenic agent colcemid, a finding which diverge from our BaP polyploid inhibiting results, and could be related with differences in the in vitro/in vivo biotransformation and detoxification processes of xenobiots.

The present knowledge on chemoprevention has revealed numerous activities that can cope with the molecular/cellular effects of damaging agents, and also that one chemopreventive agent may have various abilities, which can be exerted separately against a specific form of damage, or that can act together against a mutagen with different forms of molecular damage; moreover, such action can be carried out in a sequential form. This complex and wide spectrum of protective activities can be exemplified in the well-studied chemical, N-acetylcysteine. This compound (that acts against BaP damage) has been reported to possess four generic types of activities (besides its inhibition of malignancy) (De Flora et al., 1995). These authors describe extracellular effects, trapping and detoxification activities, cytoplasmic effects, and nuclear effects. In regard to specific mechanisms the authors included the scavenging of ROS, the replenishment of GSH stores, and the modulation of procarcino-gen metabolism coordinated with their detoxification. Moreover, in this aspect, concerning to curcumin it has been proposed that its genoprotective mechanism refer to its enhancement of both, the antioxidant and the phase II metabolizing enzymes (Iqbal et al., 2003)

Among the described antigenotoxic mechanism, the blocking or modulating effects on CYP activity has shown efficacy to inhibit the toxicity of metabolites derived from procarcinogens; in the case of BC in particular, a study has found an inhibitory effect on CYP3A4 (Zhang and Lim, 2008); however, no reports have been published about effects on CYP1A, which is the enzyme involved in BaP biotransformation. Therefore, this line of research, as well as others, is an open field for the evaluation of BC chemopreventive potential. Moreover, the present assay, and other investigations have suggested that BC may have various chemopreventive activities. Regarding this point, Di Sotto et al., (2008) considered some possible actions of BC in a bacterial system, and suggested no participation of the agent in DNA repair modula-
tion, however, in the case of the mutagen 2-nitrofluorene, they suggested a protection related with its deactivation, as well as with the reduction of nitroreductases/O-transferases. Besides, Amiel et al., (2012) demonstrated that the sesquiterpene was a potent apoptotic inducer in tumor cell lines, so it could be interesting to explore whether the agent may also have the capacity to trigger BaP heavily damaged cells toward cell death.

The present work contributes to the chemoprevention field by exploring two possible routes of BC: its antioxidant capacity and its effect as inducer of the GST activity. Respect to the first mentioned mechanism, it is pertinent to stress that a single administration of the mutagen resulted in a significant increase of liperoxidation and protein oxidation, suggesting an initial induction of oxidative stress in hepatic tissue that could be disseminated to all cells of the organism. In fact, BaP has been previously reported to increase liperoxidative products either by producing ROS or by decreasing the activities of antioxidant enzymes, as well as to increase the level of oxidized proteins in the liver of mice (Emre et al., 2007; Sehgal et al., 2012). These effects may lead to serious cellular damage including modification of cell structure, enzymatic activity, signaling pathways, and DNA damage (Kumar et al., 2012; Kryston et al., 2011). In our present assay, we demonstrated an antioxidant potential of BC on the damage induced by BaP, as indicated by the significant reduction in the lipid and protein oxidation levels. The inhibition of malondialdehyde content is particularly significant in light that such chemical is known to be involved in DNA damage (Marnett, 1999). In in vitro assays, other authors have also determined similar reduction of lipid peroxidation, as well as scavenging activity against hydroxyl radical and superoxide anion (Vinholes et al., 2014; Calleja et al., 2013). According to their findings, the last authors proposed that BC can act as a highly effective chain-breaking antioxidant agent, and that possesses greater scavenging activity against ROS than against stable organic radicals. These characteristics of BC may apply in our in vivo conditions.

In addition, the chemical structure of BC may provide clues for its antioxidant capacity. As a bicyclic molecule with double rings, it may allow the radical insertion on the π ring of both olefinic systems favouring the generation of tertiary radicals and alilic system of high stability. Other adequate site for radical insertion is in the carbon bridge of the bicyclic system. Also, with the migration of a substituent methyl in C-8 or alpha to the carbon bridge a new tertiary radical with less tension can be formed. Tertiary radicals in the carbon bridge can promote the aperture of the bicyclic system, favouring then the presence of radical structures of secondary, tertiary and alilic carbon which may confer high capacity to react with free radicals, such as peroxyl, hydroxyl, and superoxide radicals.

The second suggested mechanism of DNA damage prevention refers to the participation of BC to stimulate the activity of GST, which is a critical detoxification enzyme family that primarily functions to conjugate toxic metabolites with GSH, as part of a process that ends in their elimination from the organism (Hayes and Pulford, 1995). In the present assay we observed an increase in the GST activity with the administration of BC (a mean of 64% over the control level), as well as a significant 17% increase (in comparison with the value obtained with BaP alone) determined with the high dose of BC in BaP-treated mice. These results, obtained at 24 hr postadministration, seems to agree with the variable elevations reports for GST activity (from about 21 to 130%) obtained after exposures of 28 days (Steinkellner et al., 2001; Zheng et al., 1992; Iqbal et al., 2003). Therefore, the enzymatic stimulation observed in the present assay may suggest an accelerated conjugation of BaP toxic metabolites derived from the CYP1A1 activity with GSH, and, consequently, a more rapid elimination from the system.

In conclusion, in the present work we demonstrated a strong in vivo antigenotoxic potential of BC, as well as the possible involvement in such an effect of both its antioxidant capacity and its activity as GST inducer. Future studies should be carried out to confirm our initial conclusions, as well as to determine the BC effect towards other mutagens, and to examine the participation of other chemopreventive mechanisms.

**Conflict of interest——** The authors declare that there is no conflict of interest.

**REFERENCES**


Amiel, E., Ofir, R., Dudai, N., Soloway, E., Rabinsky, T. and


Steinkellner, H., Rabot, S., Freywald, C., Nobis, E., Scharf, G.,


**Antigenotoxicity of beta-caryophyllene in mouse**