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Genotoxic potential of BM-21, an aqueous-ethanolic extract from *Thalassia testudinum* marine plant

[*Potencial genotóxico del BM-21, un extracto hidroalcohólico obtenido de la planta marina* Thalassia testudinum]

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Abstract

Context: BM-21 is a hydro-ethanolic extract obtained from the leaves of *Thalassia testudinum* marine plant, which is rich in polyphenols, and it has demonstrated antioxidant, anti-inflammatory, cytoprotective and neuroprotective properties.

Aims: To investigate the genotoxicity potential of BM-21.

Methods: *Salmonella typhimurium* Hist. - strains were used in the point-mutation test and *Escherichia coli* cells were used in SOS response test. DNA primary damage was tested in hepatocytes of mice treated with oral dose of the extract (2000 mg/kg). Bone marrow micronucleus assay was used in mice to detect clastogenic damage while serum from the same animals was used to determine MDA levels in order to find out the influence of BM-21 on lipid peroxidation. Positive and negative controls were included in all experimental series.

Results: BM-21 did not increase the frequency of reverse mutations in the Ames test, and it did not induce primary damage in *E. coli*. Comet assay showed that 2 000 mg/kg of BM-21 induced single strand breaks or alkali-labile sites in the hepatocytes from the treated mice. However, no increase in the micronucleus frequency was observed in mice polychromatic erythrocytes and significantly reduced MDA levels were detected.

Conclusions: BM-21 was neither mutagenic nor induces DNA damage to prokaryotic cells. Although, it increased DNA strand breaks *in vivo*, this one was not translated into clastogenic damage to the whole organism. Results suggested that BM-21 was not mutagenic or genotoxic under our experimental conditions.

Keywords: Ames test; Comet assay; genotoxicity; micronucleus assay; SOS Chromotest.

Resumen

Contexto: BM-21 es un extracto hidro-alcohólico de las hojas de la planta marina *Thalassia testudinum*. Este es rico en polifenoles y ha demostrado propiedades antioxidante, anti-inflamatoria, citoprotectora y neuroprotectora.

Objetivos: Investigar el potencial genotóxico del BM-21.

Métodos: Se usaron cepas mutantes de *Salmonella typhimurium* Hist.- en el ensayo de mutaciones puntuales y células de *Escherichia coli* en el ensayo de respuesta a SOS. El daño primario al ADN se probó en hepatocitos extraídos de ratones tratados con el extracto (2000 mg/kg, p.o.). El ensayo de micronúcleos se realizó en médula ósea de ratones para descubrir daños clastogénicos. El suero de los mismos animales se usó para determinar los niveles de MDA y así averiguar la influencia de BM-21 sobre la peroxidación lipídica. Se incluyeron controles positivos y negativos en cada serie experimental.

Resultados: Los datos mostraron que el BM-21 no aumentó la frecuencia de reversión de mutaciones en el ensayo de Ames y tampoco indujo daño primario a células de *E. coli*. El ensayo Cometa demostró que BM-21 (2 000 mg/kg) introdujo rupturas de simple cadena y sitios lábiles a álcali en los hepatocitos extraídos de los ratones tratados. Sin embargo, no se observó aumento en la frecuencia de micronúcleos en eritrocitos policromáticos de ratón y los niveles de MDA se redujeron significativamente.

Conclusiones: El BM-21 no fue mutagénico ni introdujo daño al ADN en células procarióticas. Aunque el extracto aumentó el daño in vivo a la cadena de ADN, este no se tradujo en daños clastogénicos al organismo entero. Los resultados sugirieron que BM-21 no fue mutagénico ni genotóxico bajo nuestras condiciones experimentales.

Palabras Clave: Ensayo de Ames; ensayo Cometa; ensayo de micronúcleos; genotoxicidad; SOS Chromotest.

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INTRODUCTION

Seagrasses are widely distributed in shallow coastal areas of tropics and subtropics of the western Atlantic zone; they are a rich source of secondary metabolites, particularly, phenolic compounds (Garateix et al., 2011; De la Torre et al., 2012).

Thalassia testudinum is a sea grass very abundant in the Cuban coast. The first evidences of the bioactive properties of this plant started from a study of the Cuban marine biodiversity done at the CEBIMAR, Cuba. The extract obtained from the leaves of *T. testudinum* was named BM-21 showing antioxidant, cytoprotective and regenerative properties (Nuñez et al., 2006; De la Torre et al., 2012).

Phytochemical characterization of BM-21 shows the presence of triterpene steroids, tannins, phenols, flavonoids, proanthocyanidins, saponins and reducing sugars. Among these, phenolic compounds were found to be the most abundant (29.5 ± 1.2%) (Regalado et al., 2012).

The pharmacologic properties of BM-21 have been demonstrated in vitro and in vivo models. BM-21 shows potent anti-inflammatory activity in mice using a classic experimental model of acute inflammation as well as inhibitory effects of lipid peroxidation (Fernández et al., 2003). It also has protective effects against t-butyl-hydroperoxide-induced hepatotoxicity, ethanol and LPS in cultured primary rat hepatocytes, decreases malondialdehyde formation and increases the glutathione reduction (Rodeiro et al., 2008). BM-21 has skin protective effects against UVB radiation (Regalado et al., 2009). *In vivo* neuroprotective effects have been recently reported (Menendez et al., 2014), and it also exhibits the antinociceptive activity mediated by the inhibition of acid-sensing ionic channels (Garateix et al., 2011).

One new supplement is under development in Cuba because its proved pharmacologic properties, but genotoxicity tests are required as part of the determination of potential hazards. Since genotoxicity evaluation of *T. testudinum* or other members of the same genus have not been performed yet, a battery of in vitro and in vivo assays was employed in this study to establish the potential genotoxic effect of this extract.

MATERIALS AND METHODS

Chemicals

Nicotinamide adenine dinucleotide phosphate disodium salt, glucose 6-phosphate, L-histidine monohydrate, agarose, 9-aminoacridine (9AA), 2-amino anthracene (2AAC), picrolonic acid (AP), benzo(a)pyrene (BAP), bleomycin (BLM), bovine serum albumin (BSA) fraction V, D-biotin, colchicine, collagenase, daunomycin (DM), dimethylsulfoxide (DMSO), formaldehyde, Hanks balanced salt solution (HBSS), sodium carbonate, silver nitrate and tungstosilicic acid, were purchased from Sigma (St. Louis, M.O., USA). Sodium azide (NaAz), 2-aminofluorene (2AF), sodium phenobarbital and 5,6-ß-naphthoflavone were obtained from BDH (Chemicals Ltd., England). All other reagents used were of analytical grade.

Plant material

Thalassia testudinum Banks and Soland ex. Koenig was collected in April 2009 from "Guanabo" Beach (22° 05' 45"N, 82° 27' 15" W) and identified by Dr. Areces J.A. (Institute of Oceanology, Havana, Cuba). A voucher sample (No. IdO 039) has been deposited in the herbarium of the Cuban National Aquarium. Dried and ground leaves of *T. testudinum* (2.3 kg) were extracted with 30 L of EtOH/H_2O (50:50) at room temperature. The combined aqueous ethanol solutions were filtered, concentrated under reduced pressure, and dried by spray dried to yield 170 g of extract (BM-21).

The chemical characterization of the extract was performed by qualitative and quantitative analysis by combining two standard phytochemical screening tests (Rondina and Coussio, 1969; Schabra et al., 1984). This analysis revealed a higher total phenolic content (29.5 ± 1.2%), flavonoids and proanthocyanidins (4.6 ± 0.2 and 21.0 ± 2.3%, respectively) and proanthocyanidins (condensed tannins) in a range from 10 to 150 mg tannin per g of tissue (Regalado et al., 2012).

The BM-21 sample used in this study was provided by the Chemistry Department of CEBIMAR (Lot 0902001). Its organoleptic

characteristics were: homogeneous and fine powder, green color and characteristic odor (CEBIMAR, Report Results Analytical Service).

For the in vitro and in vivo exposures, BM-21 was dissolved in culture medium or distilled water, respectively (vehicle). In the in vitro experiments, the solutions were filtered by a 0.2 µm millipore membrane.

Standard plate incorporation assay (Ames test)

The *Salmonella typhimurium* strains used in the experiments, TA98, TA100, TA102, TA1535, and TA1537 (Mortelmans and Zeiger, 2000) were kindly supplied by Dr. Javier Espinosa Aguirre, UNAM, Mexico.

Liver cytosolic fractions were prepared from young adult male Sprague Dawley rats. According to INVITTOX Protocol (INVITTOX, 1990), animals were sacrificed after 5 days of receiving daily i.p injections of sodium phenobarbital at 30 mg/kg (day 1) and 60 mg/kg (days 2-5). On the third day, 80 mg/kg of 5,6 β-naphtoflavone were also administrated. The 9000 *g* liver supernatant (S_9) was split into 1 mL aliquots, frozen and stored at –80°C. At the time of the assay an S_9 mix was prepared, which contained 33 mM KCl, 2.8 mM $MgCl_2$, 4 mM NADP, 5 mM glucose 6-phosphate, 4% S_9 fraction and 200 mM sodium phosphate buffer at pH 7.4. This mixture was kept in an ice bath until used. The standard plate-incorporation method in the presence and absence of S_9 was performed according to Maron and Ames (1983). BM-21 extract was prepared at stock concentration of 50 mg/mL and was added to the cultures at 50, 150, 500, 1500 and 5000 µg/plate, the latter being the maximum concentration recommended for this assay (Maron and Ames, 1983; INVITTOX, 1990). Each concentration was done in triplicate and experiments were repeated twice. Negative (vehicle) and positive controls were included. BAP (10 µL/plate), NaAz (1.5 µg/plate), 2AF (10 µg/plate), AP (100 µg/plate), 9AA (2 µg/plate), 2AAC (2 µg/plate) and DM (6 µg/plate) were used as positive controls. For each test, 2 mL of top agar containing 0.6 % agar, 0.5 % NaCl, 0.5 mM biotin and 0.05 mM L-histidine were mixed successively with 0.1 mL of the BM-21 extract solutions (or controls), 0.1 mL overnight culture (about 10^8 cells) and 0.5 mL S9 mix or 0.5 mL of phosphate buffer. Afterward, all the plates were incubated at 37°C for 48 h. After incubation, the number of revertant colonies was determined.

For data analysis, the validity of the test was assessed by comparing the control values with internal historical data. First, an exploratory experiment for studying the toxicity effects of the extract was performed. In this case, only the highest concentration (5000 µg/mL) was used on each strain and after 48 h of exposure no toxicity effects were observed.

SOS Chromotest assay

A toxicity assay was conducted before the SOS Chromotest assay. *Escherichia coli* cells (PQ37 strain, F– thr leu his-4 pyrD thi galE lac ΔU169 srl300::Tn10 rpoB rpsL uvrA rfa trp:: Muc+ sfiA::Mud (Ap. lac)ts) were cultured in Luria-Bertani (LB) medium at 37°C. Colonies were taken at the exponential phase and they were diluted 1:10 in a LB(2X) medium. Aliquots of 500 µL were distributed into sterile tubes. Final concentrations of BM-21 were: 100, 200, 500, 1000 and 2000 µg/mL. Positive control received 150 Gy of gamma irradiation, which were calculated by using a Fricke's dosimeter (Co60 PX-γ-30M Russian irradiator) held at a temperature of 2 ± 0.5°C (Prieto and Cañet, 1990). Negative control was 2% DMSO. To estimate the percentage of cell survival the following formula was used:

$$S = N/N_o \times 100,$$

where S was stood for cell survival; N was the number of colonies in the treatment plates, and N_o was the number of colonies in the negative controls (Iwanami and Oda, 1985).

The alkaline phosphatase assay in treated *E. coli* PQ37 cells (protein synthesis inhibition indices) was also used as a toxicity criterion (Quillardet and Hofnung, 1993). Afterward, the Chromotest assay was conducted (Quillardet et al., 1982) with some modifications to avoid interferences with the data measurement caused by colors present in the plant products. Briefly, fluorescent substrates and a modified substrate

buffer for the alkaline phosphatase assay was used (Salvo et al., 1994). The extract is considered mutagenic if it produces a dose-response effect and a 2-fold increase in the Induction Factor (IF) with respect to control. IF was calculated as:

IF = [β-galactosidase (induced)/ phosphatase (induced)]/[β-galactosidase (control)/ phosphatase (control)] (Quillardet et al., 1989).

In vivo assays

Animals and treatments: NMRI mice (20-25 g) of both sexes were purchased from the CENPALAB (Havana, Cuba) and adapted for five days to laboratory conditions. A standard rodent chow manufactured at CENPALAB was supplied *ad libitum*. All manipulations were performed according to the ethical principles for animal care and management recommended by the Cuban Guidelines and the Standard Operational Procedures (Gámez and Mas, 2007). The assay was performed by using six animals per group. An oral dose of BM-21 (2 000 mg/kg) was evaluated as recommended by ICH (2012). Positive control group was administered with BLM (40 mg/kg, i.p.), which is the recommended dose to induce clastogenic damage in this species (Gámez and Mas, 2007). A control vehicle (distilled water) was also included. Animals were sacrificed by cervical dislocation 24 or 48 h after treatment with BM-21 or vehicle (controls) and 48 h after a single injection for BLM group. Blood samples were collected to assess the malondialdehyde levels as criteria of lipid peroxidation. DNA strand breaks and alkali labile sites were detected in hepatocytes by conducting Comet assay and micronucleus test were carried out in femora bone marrow cells.

Comet assay: The Comet assay was performed as described elsewhere (Singh et al., 1988) with some modifications (Collins et al., 1993; 2003). Briefly, 10 µL of cell suspensions were embedded in 75 µL of 0.8 % low melting point agarose and spread on a slide pre-coated with 150 µL of 1% normal melting point agarose. Two slides were prepared for each sample. A cover slip was added to each slide and allowed to solidify at 4°C. Slides were incubated for 1 h in lysis solution (2.5 M NaCl; 0.1 M EDTA; 10 mM Tris; 1% Triton X-100; 10% DMSO, pH 10) at 4°C. Then, they were placed in an electrophoresis chamber filled with alkaline buffer (0.3 M NaOH, 1 mM EDTA, pH > 13) for 20 min to allow DNA unwinding. Later, it was applied 1.25 V/cm and 300 mA to run electrophoresis during 20 min. After electrophoresis, slides were neutralized with PBS (137 µM NaCl; 2.68 µM KCl; 8 µM HNa_2PO_4; 1.47 µM H_2KPO_4), dried and stained with silver nitrate (Nadin et al., 2001, with some modifications of García et al., 2004). Stained nucleoids were evaluated with an optical microscope NOVEL 40X. Fifty cells from each slide were analyzed and classified in five levels (assigning them a value between 0 and 4) according to the DNA damage (Collins et al., 1995). The DNA damage score was calculated as arbitrary units (AU):

AU= 1 x (Nucleoids in level 1) + 2 x (Nucleoids in level 2) + 3 x (Nucleoids in level 3) + 4 x (Nucleoids in level 4).

Micronucleus test in mouse bone marrow: Both femora were removed from freshly killed animal. The bones were freed from muscle, and the distal epiphyseal portion was torn off by gentle traction with the rest of the tibia and the surrounding muscle. The proximal end of each femur was carefully shortened until a small opening to the marrow canal became visible. Then, 2 mL of fetal calf serum (FCS) were perfused into the femur canal with a syringe. After several aspirations and flushing, FCS was added to achieve 5 mL final volume, and the bone marrow cells suspension was centrifuged at 1000 rpm for 5 min. Two drops of the cell suspension from each animal were placed onto clean, dry slides, they were smeared, fixed in methanol and stained with Giemsa at 5% for 8-12 min. For each animal, 2000 polychromatic erythrocytes (PCE) were analyzed for determining the presence of micronuclei (MN) and to calculate the percentage of cells containing micronucleus over the total number of scored cells. Normochromatic erythrocytes (NCE) from each animal were also scored in 500 erythrocytes in order to determine the PCE/NCE ratio (Hayashi et al., 1994).

Malondialdehyde (MDA): MDA concentration was measured in blood serum. Briefly, 0.65 mL of 10 mM N-methyl-2-phenylindole in acetonitrile was added to 0.2 mL of each sample and vigorously agitated for 3-4 s. Afterward, 0.15 mL of 37% HCl was added, and samples were closed with a tight stopper and they were incubated at 45°C for 60 min. Then, samples were cooled on ice, centrifuged, and the absorbance was measured at 586 nm. A calibration curve of accurately prepared standard MDA solutions (from 2 - 20 nmol/mL) was made. Measurements were performed in triplicate. The standard deviations were less than ± 10% (Esterbauer and Cheeseman, 1990).

Statistical analysis

Mann-Whitney U test (Maron and Ames, 1983) from the SALANAL statistical package software was used to compare revertants/plate data among groups in the Ames test. Data from the SOS Chromotest were analyzed by ANOVA and the Tukey–Kramer multiple comparison tests (Sokal and Rohlf, 1995). Dunnet non-parametric post-hoc test from STATISTICA 6.1 package software was used for the Comet assay, the MN and MDA test data. Statistic significance p-level was *a priori* selected as 0.05. All data were reported as mean ± standard error of mean (SEM).

RESULTS

In vitro assays

The mutagenic potential of BM-21 was evaluated through the Ames test. TA98, TA100, TA1535, TA1537 and TA102 (hist.-) strains of *S. typhimurium* were used in presence or absence of metabolic activation. Table 1 shows no toxicity after exposure to the highest concentration (5 000 µg/mL) of BM-21 in any tested *Salmonella* strain. The frequency of spontaneous reversion did not differ from the historically observed in our laboratory for the same strains, whereas the mutagens used as positive controls significantly increased the frequency of revertants. BM-21 did not induce significant changes in the reversion frequency when compared with controls at concentrations between 50-5000 µg/plate, and no differences were observed with or without metabolic activation. Thus, these results demonstrated that the extract did not induce point mutations in the different strains of *S. typhimurium* under our experimental conditions.

After, SOS Chromotest assay was used to measure DNA primary damage in *E. coli* bacteria. Fig. 1 shows the results for the SOS response IF (SOS IF) in *E. coli* cells following the treatment with BM-21 (100 - 2000 µg/mL). As can be observed, no statistically significant increase in the level of DNA damage (expressed as SOS response induction factor, IF SOS) was induced in cells exposure to BM-21 when compared to negative controls ($p > 0.05$). Thus, it is indicating that the extract did no induce DNA damage in these experimental conditions.

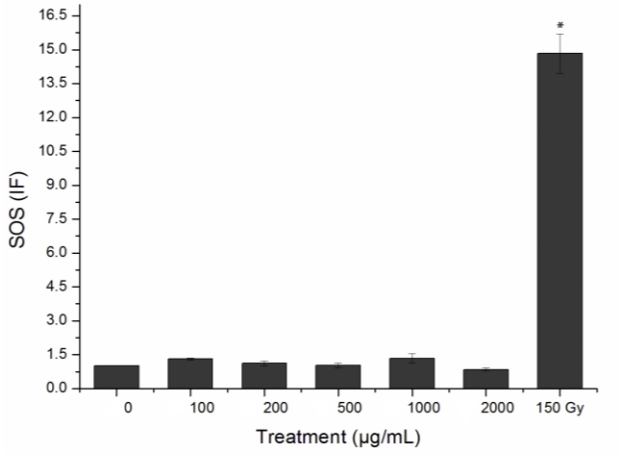

Figure 1. Effects of *T. testudinum* extract (BM-21) on SOS response induction factor (SOS IF) in strains of *E. coli* (mean ± SEM). Effect of primary DNA damage of BM-21 (100, 200, 500, 1000 and 2000 µg/mL) in PQ37 strain of *E. coli* were expressed as response SOS (IF). Positive control: Gamma radiation (150 Gy) and negative control DMSO (0.2%). Each value represents the mean ± SEM of three independent experiments. Statistically significant differences with respect to the negative control (*p <0.05, ANOVA and Tukey–Kramer).

In vivo assays

Mice treated with BM-21 (2000 mg/kg) were tested for primary damage in hepatocytes (Comet assay), MN frequency in bone marrow and for MDA levels in the serum of the blood samples obtained from the animals. Comet assay was used to determine DNA damage measured as strand breaks and alkali-labile sites in hepatocytes of NMRI mice. BM-21 caused a significant increase ($p < 0.05$) of the DNA damage in hepatocytes of NMRI mice of the both sexes treated with BM-21 extract (2000 mg/kg) after 24 and 48 h (Fig. 2).

Micronucleus comprise a portion of chromatin surrounded by a separated nuclear membrane. Said membrane may arise by a) the exclusion of intact centric chromosomes from anaphase segregation or b) by the condensation of acentric chromosomes, which remain separated at anaphase because their inability for attaching to the spindle during mitosis (Mitchell and Combes, 1997). Thus, the existence of increased numbers of micronuclei is evidence of prior induction of structural chromosome damage or of changes in chromosome number. In our study, micronucleus analysis was performed in both human lymphocytes primary culture and on bone marrow in mice. BM-21 did not cause a significant increase ($p < 0.05$) of the micronucleus frequency by comparing treated animal with negative controls (Table 2) and it did not significantly increase the frequency of PCE. Therefore, BM-21 did not show clastogenic or aneugenic activity to bone marrow at the applied dose (2000 mg/kg).

MDA assay was performed in serum from the same animals treated with BM-21 (2000 mg/kg) and tested by the Comet assay and the micronuclei induction. BM-21 caused a significant decrease ($p < 0.001$) in MDA concentration in treated animals in regards to non-treated ones (Fig. 3).

DISCUSSION

Plants are traditionally used in Cuba for the treatment of different ailments. Natural products contain numerous classes of useful chemical constituents including polyphenols, which are responsible for important biological properties (Nuñez et al., 2006). Polyphenols are secondary plant metabolites characterized by the presence of more than one phenol unit or building block per molecule, and generally involved in cell defense mechanisms against UV radiation, tumorigenesis, or aggression by pathogens. Several thousands of polyphenols molecules have been identified, and they can be classified according to their structure as phenolic acids, flavonoids, stilbenes and lignans (Manach et al., 2004; Hooper et al., 2008; Hollman et al., 2011).

In the present work, we have investigated the cytotoxic, mutagenic, genotoxic and antioxidant properties of BM-21, one product with potential benefits demonstrated by in vitro and in vivo pharmacological screening. However, studies about its possible genotoxic activity have not been carry out yet, although this kind of evaluation is essential in the case of products with potential use in humans (MacGregor et al., 2000).

In vitro tests were initially performed as it is commonly recommended by national and international regulatory agencies (OECD 1997; Gámez and Mas, 2007; ICH, 2012). The mutagenic activity was investigated by performing the reverse mutagenic assay, with *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102. Data revealed that the extract was not mutagenic in the different strains of *S. typhimurium* used. SOS Chromotest has been widely used as part of the genotoxicity screenings of medicinal plants (Vidal et al., 2010; Kocak et al., 2010; Cuétara et al., 2012; Rodeiro et al., 2012). The results showed that BM-21 did not induce DNA primary damage in *E. coli*. These results were in agreement with data from the reverse mutagenic assay in *S. typhimurium*. The SOS Chromotest indicated that the BM-21 did not produce DNA lesions, which block DNA synthesis, thus leading to the induction of the SOS system.

Table 1. Results observed of *T. testudinum* extract (BM-21) in Ames test (mean ± SEM)

Strains	S_9	C(-)	BM-21 (µg/plate)					
			50	150	500	1500	5000	C(+)
TA 35	-	5.0 ± 0.5	8.3 ± 1.8	5.6 ± 1.3	8.0 ± 1.1	5.0 ± 1.0	4.6 ± 0.6	267.0 ± 25.7*
	+	8.6 ± 0.9	10.1 ± 1.9	9.6 ± 1.5	9.9 ± 1.6	10.0 ± 1.3	10.6 ± 1.7	324.0 ± 28.8*
TA 37	-	4.3 ± 0.6	4.6 ± 0.6	6.0 ± 0.0	4.6 ± 0.6	4.0 ± 1.0	5.0 ± 0.5	16.0 ± 0.3*
	+	7.3 ± 0.8	11.3 ± 0.6	6.3 ± 0.3	8.0 ± 0.5	10.0 ± 0.5	10.3 ± 0.8	248.6 ± 11.3*
TA 98	-	11.3 ± 1.4	14.6 ± 3.2	13.0 ± 3.0	15.0 ± 1.3	14.6 ± 2.1	15.6 ± 2.1	529.0 ± 14.6*
	+	31.3 ± 2.72	24.6 ± 2.4	21.6 ± 2.1	24.0 ± 1.0	21.3 ± 3.1	19.6 ± 3.1	2000 ± 0.0*
TA 100	-	83.6 ± 2.9	90.3 ± 2.9	75.6 ± 7.4	76.6 ± 5.3	79.6 ± 1.2	83.0 ± 3.7	1264.0 ± 7.3*
	+	90.3 ± 5.5	90.3 ± 1.4	92.6 ± 5.8	99.0 ± 6.6	98.0 ± 3.0	89.6 ± 3.5	167.0 ± 5.8*
TA 102	-	64.3 ± 2.8	58.6 ± 2.8	75.3 ± 1.7	71.0 ± 5.2	69.3 ± 4.0	78.6 ± 0.3	186.0 ± 5.5*
	+	73.3 ± 2.2	69.6 ± 2.1	76.3 ± 2.7	75.4 ± 3.1	70.3 ± 2.3	73.6 ± 0.9	267.0 ± 8.9*

Mutagenic potential the hydro-alcoholic extract BM-21 in strains TA98, TA100, TA35, TA37 and TA102 the *S. typhimurium* in the presence (+ S_9) and absence (-S_9) of hepatic microsomal fraction. Each value represents [a] mean number of revertant colonies by plate in each treatment ± standard error of the mean of three replicates of two independent experiments. C(-): Negative control (water), C(+): Positive controls: TA35 (-S_9) and TA 100 (-S_9): Sodium azide (AzNa) (1.5 µg/plate), TA35 (+S_9): 2-aminofluorene (AF) (5 µg/plate), TA37 (-S_9): 9-aminoacridine (9AA) (2 µg/plate), TA37 (+S_9): 2-aminoanthracene (2AAC) (2 µg/plate), TA98 (-S_9): Picrolonic acid (AP) (100 µg/plate), TA98 (+S_9): AF (10 µg/plate), TA100 (+S_9): Benzo(a)pyrene (10 µL/plate), TA102 (-S_9): Daunomycin (DM) (6 µg/plate), TA102 (+S_9): 2AAC (2 µg/plate). * $p < 0.05$ for comparison between groups by Mann-Whitney U test.

Table 2. Effects of *T. testudinum* extract (BM-21) on micronucleus assay in NMRI mice.

Treatments	Females		Males	
	Micronucleated PCE per 1000 PCE from a total of 2000 PCE	PCE (%) from 250 ET	Micronucleated PCE per 1000 PCE from a total of 2000 PCE	PCE (%) From 250 ET
Vehicle control	0.4 ± 0.19	42.6 ± 2.15	1.6 ± 0.19	42.5 ± 1.94
BM-21 (2000 mg/kg) 24 hours	0.9 ± 0.46	46.3 ± 2.18	1.1 ± 0.33	54.6 ± 2.58
BM-21 (2000 mg/kg) 48 hours	1.3 ± 0.30	51.3 ± 2.17	1.0 ± 0.27	48.4 ± 2.19
BLM (40 mg/mL)	5.4 ± 0.48*	48.1 ± 1.71	6.8 ± 0.8*	42.8 ± 1.65

Each value represents the mean ± SEM. BLM: bleomycin PCE: polychromatic erythrocyte; ET: erythrocytes. *Statistically significant difference compared to negative control $p<0.05$ (Dunnett non-parametric test), n=6.
% calculated as polychromatic erythrocyte/polychromatic erythrocyte + normochomatic erythrocyte x 100.

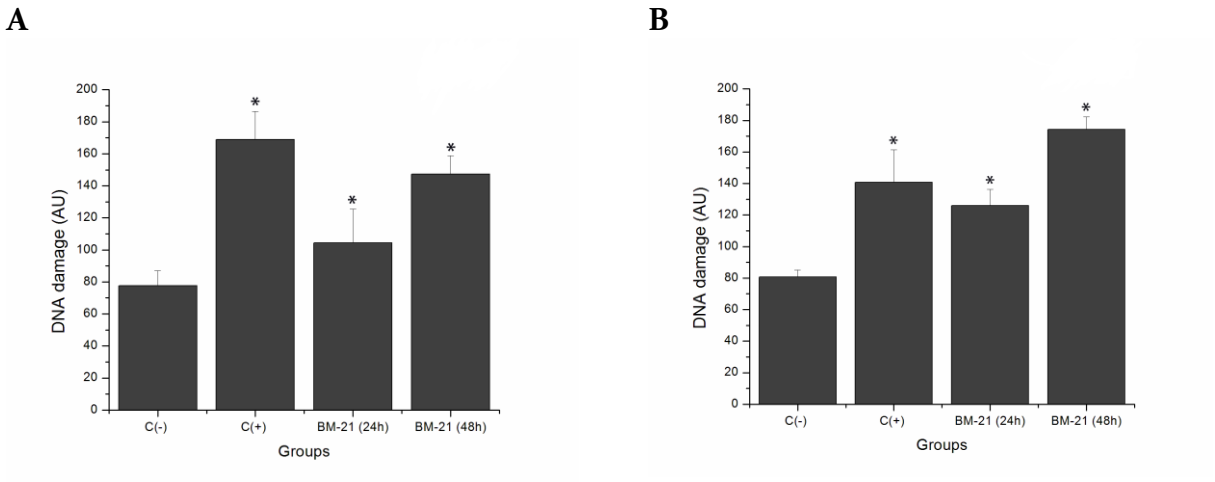

Figure 2. Effects of *T. testudinum* extract (BM-21) on Comet assay in both sexes NMRI mice: females (**A**) and males (**B**) (mean ± SEM). Effect of primary DNA damage of BM-21 (2000 mg/kg i.g.) in mice hepatocytes after the 24 and 48 hours, expressed in arbitrary units (AU). C(+): Positive control, bleomycin (BLM, 40 mg/mL i.p.) and C(-): negative control, water. Each value represents the mean ± SEM, n = 6 animals/group. Statistically significant difference compared to negative control (*p < 0.05 Dunnett test).

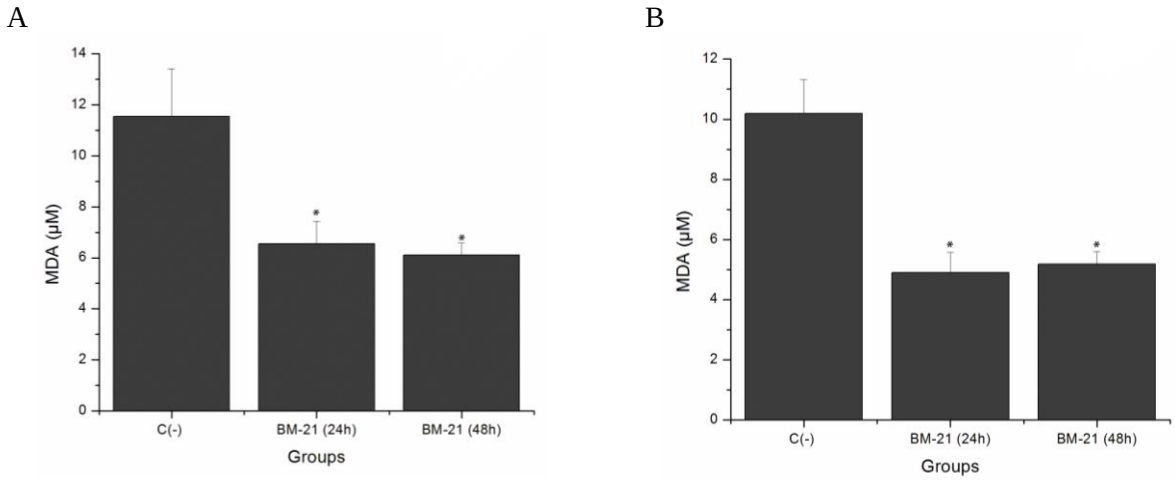

Figure 3. Effects of *T. testudinum* extract (BM-21) on malondialdehyde (MDA) levels in serum of both sexes NMRI mice: **(A)** females and **(B)** males (mean ± SEM). Concentration the MDA of BM-21 (2000 mg/kg i.g.) in serum of mice the both sexes after the 24 and 48 h, expressed in µM. C(-): Negative control, water. Each value represents the mean ± SEM, n = 6 animals/group. Statistically significant difference compared to negative control (*p < 0.05, ANOVA of simple classification and Dunnett test).

Studies on plant extracts with similar chemical composition (rich in polyphenols) than *T. testudinum* have been reported to have no mutagenic effect in bacterial test systems. For example, the aqueous extracts of *Tinospora cordifolia* (Menispermaceae) from India (Chandrasekaran et al., 2009), *Orthosiphon stamineus* (Lamiaceae) from Southeast Asia (Muhammad et al., 2011) and *Mangifera indica* (Anacardiaceae) from Cuba (Rodeiro et al., 2006) have been studied. Others studies have used a combination of SOS Chromotest and Ames test to evaluate the genetic activity. For instance, the study on the alcoholic extract of the Brazilian plant *Annona crassiflora* (Annonaceae), which is rich in flavonoids (Vilar et al., 2011), and the evaluation of the glucosyl xanthone mangiferin, the main constituent of the aqueous extract from *Mangifera indica* bark (Rodeiro et al., 2012). Both

studies demonstrate that neither the extracts, which contains a complex mixture of flavonoids nor the purified flavonoid or xanthones in particular, are mutagenic or inducer of DNA primary damage. Results in prokaryotic models were the starting point for the in vivo studies in NMRI mice.

As we mentioned, at present study mice treated with BM-21 (2000 mg/kg) were tested for primary damage in hepatocytes (Comet assay), MN frequency in bone marrow and for MDA levels in serum. The use of the same animal to explore different endpoints has been already reported (Friedmann et al., 2010). The Comet assay data showed that BM-21 induced DNA damage in hepatocytes. Similar results have been found in extracts of *Sophora flavescens* by Yune-Fang et al. (2009) in comparable experimental conditions. However, the MN test proved that BM-21 was neither cytotoxic nor genotoxic in mouse's bone marrow under our experimental conditions, which was in agreement with some reports regarding other natural products rich in polyphenols (Utescha et al., 2008; Berni et al., 2012; Lina et al., 2012).

The relevance between positive results in the Comet assay and the negative result in the Ames test, SOS Chromotest and MN assay, is important. It may be possible that the genotoxic compound(s) present into the BM-21 extract could be detoxified in the liver and did not reach the bone marrow in its active form, thus resulting in no-genotoxic action. In addition, no cytotoxicity was observed in the bone marrow. ICH guideline S2 (R1) describes that the value of the in vivo results is directly related to the demonstration of an adequate exposure of the target tissue by the tested compound. This is especially true for negative results when in vitro tests have shown convincing evidence of genotoxicity (ICH, 2012).

MDA is one of the best-studied products; it is a marker of lipid peroxidation in cells and one of the best known inductor of ROS-mediated damage. This aldehyde is a toxic molecule which interacts with DNA and proteins. Burton and Ingold (1984) showed that polyphenols may be involved as pro-oxidants in lipid peroxidation. Many studies combine Comet and MN assays with MDA determination to analyze the relationship between genotoxicity and oxidative stress damage (Ortega-Gutiérrez et al., 2009; Patlolla et al., 2009; Link et al., 2010). However, our results demonstrated that doses in which the DNA damage was observed in hepatocytes did not increase MDA in serum. From these results, it could suggest that not enough evidence supports that the induction of oxidative stress is the most likely mechanism of induction of DNA damage. Surprisingly, our results did indicate a reduction of MDA levels in the same animals where the DNA damage in hepatocytes was detected. The latter confirmed the antioxidant activity previously reported for the extract (Rodeiro et al., 2008; Garateix et al., 2011; Regalado et al., 2012; Menendez et al., 2014). Few reports have been performed in regards to the possible toxicological risks of this new natural mixture. In our knowledge, the present study provides the first results about the genotoxicity potential of the BM-21 extract.

CONCLUSIONS

According to our present results, BM-21 is not mutagenic, cytotoxic or clastogenic/aneugenic product. Taking into account that only was observed a positive result on Comet assay and the high con-centration needed to produce the DNA damage in the hepatocytes, the data suggest a very low, if existent, genotoxic risk associated with the exposure to this product. Meanwhile, further studies should be conducted in order to evaluate the full mechanism involved in the damage to DNA observed in the hepatocytes of mice exposure to this product.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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