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Antimutagenic bis (2-ethylhexyl) phthalate isolated from octopus (*Paraoctopus vulgaris*)

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Abstract

In the present study, the antimutagenic activity of methanolic (MP) and hexanic (HP) phase from hexane-soluble extract of *Paraoctopus vulgaris* was evaluated. The HP showed significant concentration-dependent antimutagenic activity ($p < 0.05$) in the Ames assay against aflatoxin B_1 (AFB_1) using *Salmonella typhimurium* TA98 and TA100 tester strains. Seven fractions (F1-F7) were obtained from HP by silica gel-column chromatography and the antimutagenic activity of F6 and F7 caused significant reduction ($p < 0.05$) in the number of AFB_1-induced revertants in both strains. However, high antimutagenic activity was exhibited in TA98 strain (85-97% reversion inhibition) and remained at a fraction-concentration of 3 µg/mL. F6 and F7 composition was analyzed by high-performance liquid chromatography/photodiode array, both fractions shared one peak at 9.42 min suggesting presence of the same compound. ^{1}H-NMR and ^{13}C-NMR analysis results indicate that the bioactive compound is bis (2-ethylhexyl) phthalate. Based on of these results bis (2-ethylhexyl) phthalate exhibit antimutagenic activity, which allows to consider it for further investigation as a potential chemopreventive compound.

Keywords: *Paraoctopus vulgaris*; chemopreventive; antimutagenic; bis (2-ethylhexyl) phthalate.

Practical Application: Bis (2-ethylhexyl) phthalate was isolated from octopus, results suggest a potential for further research in order to be proposed as a chemotherapeutic agent at early stages of carcinogenesis.

1 Introduction

The morbidity and mortality due to cancer incidence has increased in the last years. According to the International Agency for Research on Cancer (IARC), in 2012, there was approximately 8.2 million of cancer deaths in the world: 4.7 million (57%) were males and 3.5 million (43%) females). Projections to year 2030 suggest that the global cancer burden is expected to increase up to 21.4 million of new cancer cases and 13.2 million cancer deaths (Ferlay et al., 2013).

It is estimated that more than one half of all cancer cases and deaths worldwide are potentially preventable and one strategy with enormous potential of use to deal with this issue is chemoprevention (American Cancer Society, 2011). A number of natural agents present in foods such as, flavonoids, carotenoids, isothiocyanates, terpenoids, and omega-3 fatty acids, have been evaluated for their anticancer effect in early stages of carcinogenesis (Vidak et al., 2015; Lawson et al., 2015; Li et al., 2016; López-Saiz et al., 2014; Rovito et al., 2013). However, the oceanic environment has been recognized as a wide source of natural bioactive compounds with pharmacological potential and possible future applications (Montaser & Luesch, 2011).

Marine invertebrates, especially cephalopods, constitute an important source of many biological substances with antioxidant, antitumor, antileukemic, antibacterial, and antiviral activities (Sudhakar & Nazeer, 2015; Jesy et al., 2014; Jayaraj et al., 2008; Rajaganapathi et al., 2000). In particular, *Paraoctopus limaculatus* has been reported as a source of bioactive agents since the isolation of extracts with chemopreventive activity has previously been carried out (Cruz-Ramírez et al., 2015), suggesting the presence of antimutagenic and antiproliferative hexane-soluble compounds. Therefore, the aims of this study were to chemical and structurally characterize the antimutagenic compounds present in the hexane-soluble fraction of octopus tentacles.

2 Materials and methods

2.1 Extraction of bioactive compounds

Octopus (*Paraoctopus vulgaris*) specimens were obtained from Bahía de Kino, Sonora, México (29° 22' 27" N, 112° 34' 08" O). Tentacles were chopped up, separated in homogeneous portions and freeze-dried at -30 °C under vacuum (Unitop 600L, USA).

The compounds isolated from the hexane soluble fraction (HSF), previously reported by Cruz-Ramírez et al. (2015), were partitioned in methanol/hexane (1:1 v:v proportion) for 24 h at 4 °C. The resulting methanolic (MP) and hexanic (HP) phases were physically separated and the solvents were evaporated. Both phases were evaluated by antimutagenic assay.

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2.2 Isolation of bioactive compounds by column chromatography

Since HP was found to be the fraction with the highest antimutagenic activity, it was subjected to open column chromatography with silica gel (3.5 cm × 90 cm, silica gel, 60-120 mesh, Sigma, St. Louis, MO, USA). HP was fractionated using stepwise gradient elution with hexane: ethyl acetate (99:1-85:5), ethyl acetate: methanol (50:50), and methanol (100) to yield seven fractions (F1-F7). These seven fractions were monitored using thin layer chromatography (TLC) testing plates coated with silica gel and they were revealed using an iodide solution and observed under UV light. Fractions with similar *Rf* values were evaluated for antimutagenic activity using the Ames test.

2.3 Antimutagenicity assay (Ames test)

The HP, MP, and seven fractions were evaluated using the mutagenicity assay described by Maron & Ames (1983), using *Salmonella typhimurium* TA98 and TA100 tester strains in presence of metabolic activation (S9 mix). Fractions were reconstituted and serially diluted with dimethyl sulfoxide (DMSO), and spiked with enough pure AFB_1 to achieve a final concentration of 500 ng of AFB_1/100 μL. Seven fractions isolated from HP were evaluated on *S. typhimurium* TA98 and TA100 strain, at 3000, 1000, 300, and 100 µg/mL.

Bis (2-ethylhexyl) phthalate was the antimutagenic compound identified in F6 and F7 and these were evaluated with *S. typhimurium* TA100 using sodium azide as mutagen at 5000, 1000, 500, and 100 ng/mL. The results were determined in terms of the inhibition of sodium azide and AFB_1-induced revertants/ plate for each dilution. All assays were performed by triplicate.

2.4 RP-HPLC analysis

Composition of fractions F6 and F7 was analyzed using an Agilent Technologies 1260 Infinity ultra-high-performance liquid chromatograph equipped with a photodiode detector. A 20-µL aliquot of sample was injected into a Zorbax Eclipse XDB-C18 semi-preparative column (5 µm particle size, 250 × 9.4 mm i.d.). Mobile phase composition and elution gradients employed at times zero and 23 min were 90A:10B and 0A:100B, respectively. Eluent A: ethyl-acetate (HPLC grade); eluent B: hexane (HPLC grade). A 20-µL aliquot was injected at a temperature of 20 °C. Elution of F6 and F7 constituents were monitored measuring their absorption at 350 nm and scanned (190 to 600 nm) to detect maximum absorption values.

2.5 ^{1}H-NMR and ^{13}C-NMR

Antimutagenic fractions F6 and F7 were analyzed using a Bruker NMR spectrometry equipment operated at 400 MHz (Billerica, MA, USA). Each fraction was dissolved in $CDCl_3$ (500 µL; Sigma-Aldrich, Saint Louis, MI, USA) with a small amount of tetramethylsilane (TMS).

2.6 GC-MS

GC-MS analysis was performed using a JMS-GCmatell-GC-MS SYSTEM. Fractions 6 and 7 were injected into the column (HP-5MS 5%-phenyl-methylpolysiloxane, 30 m × 0.25 mm × 0.25 µm, Agilent 19091S-433); elution of components was performed at a flow rate of 1 mL/min. The injector temperature was 310 °C and the initial temperature (40 °C) was maintained for 1 min followed by a ramp of 8 °C/min to 310 °C. The ionization voltage was 70 eV. The identification of compounds was done using the database of National Institute Standard and Technology (NIST, 2016) comparing the mass spectrum obtained with the spectrum of the known components stored in the NIST library.

2.7 Statistical analysis

The statistical analysis was performed using the JMP statistical software (visual statistical discovery software, 10). Data were analyzed using an ANOVA followed by a Tukey-Kramer test ($p < 0.05$).

3 Results and discussion

3.1 Antimutagenic assay

Figure 1 shows the antimutagenic activity of hexanic (HP) and methanolic (MP) phases obtained from the hexanic soluble fraction (HSF) of octopus tentacle, previously reported by

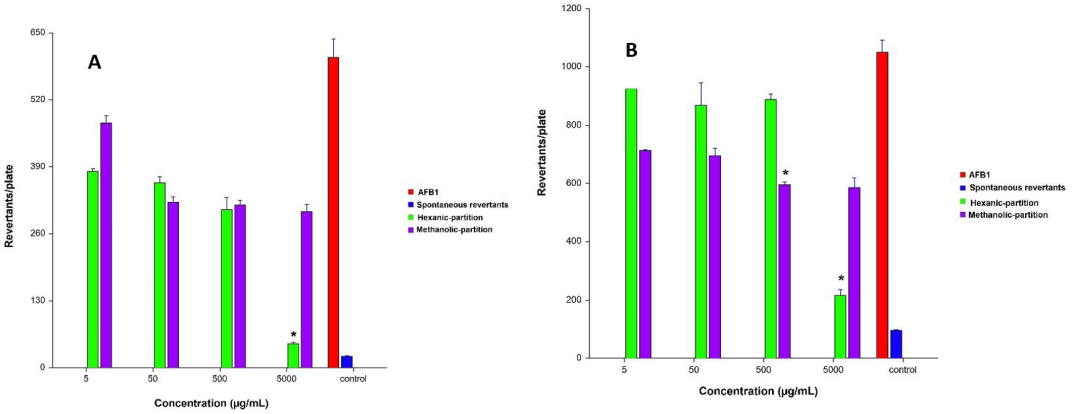

Figure 1. Antimutagenic activity of hexanic and methanolic partition against AFB_1 induced mutation in *Salmonella typhimurium* strain (A) TA98; (B) TA100 (*$p < 0.05$).

Cruz-Ramírez et al. (2015). The HP, at 5000 µg/mL, exhibited the highest antimutagenic effect for TA98 and TA100 strains tested in the presence of S9. The reduction of the mutagenicity induced by 500 ng of AFB_1 in TA100 strain, at the presence of 500 µg/mL of MP, was statistically different ($p < 0.05$) from that observed when same concentration of HP was used. Even though the decrease in the number of revertant/plate, due to the presence of either HP or MP seemed to be concentration-dependent for both strains, no statistical differences ($p > 0.05$) were observed at the two lowest concentrations. These data suggested that the HP contents might protect DNA from damage induced by AFB_1. Recent studies have found that hexane extract from plants (*Astragalus* species) showed antimutagenic activity against sodium azide and 9-aminoacridine using *Salmonella typhimurium* TA1535 and TA1537 tester strains. The mutation inhibition was higher in TA1537 (susceptible to frame-shift type of mutation) than in TA1535 (susceptible to single base-substituted type of mutation) (Gulluce et al., 2010). Similarly, the results in the present study show that HP contains antimutagenic compounds capable of protecting DNA from both types of mutation events, base-pair substitution (TA100) and frame-shifts (TA98). A chemopreventive effect of a hexanic extract from the marine algae *Chondria dasyphylla* was reported by Khanavi et al. (2012), finding evidences of cytotoxic activity against the proliferation of T47D cells. Also, they found that the hexane soluble fraction of *Sargassum angustifolium* showed antiproliferative activity in T47D and HT-29 cell lines.

Results from the antimutagenic assay performed on the fractions obtained from HP by column chromatography are shown in Table 1. Seven fractions were eluted and their antimutagenic activity was found to be concentration-dependent.

Table 1. Antimutagenic activity of fractions of hexanic partition (HP) against AFB_1 induced mutation in *Salmonella typhimurium* strain.

Extract fraction	Concentration (µg/mL)			
	3000	*300*	*30*	*3*
TA98				
F1	390 ± 30^{b}	636 ± 53^{a}	952 ± 36^{a}	1012 ± 85^{ab}
F2	478 ± 20^{a}	660 ± 50^{a}	907 ± 48^{a}	982 ± 26^{bc}
F3	172 ± 21^{cd}	728 ± 59^{a}	718 ± 44^{b}	1030 ± 87^{a}
F4	141 ± 4^{de}	365 ± 44^{b}	571 ± 41^{c}	862 ± 13^{c}
F5	198 ± 13^{c}	295 ± 22^{bc}	359 ± 23^{d}	645 ± 6^{d}
F6	86 ± 9^{e}	145 ± 10^{d}	186 ± 33^{e}	237 ± 38^{e}
F7	110 ± 9^{e}	166 ± 7^{cd}	154 ± 6^{e}	187 ± 19^{e}
TA100				
F1	1595 ± 72^{A}	2192 ± 50^{A}	2207 ± 71^{A}	2066 ± 66^{AB}
F2	1638 ± 69^{A}	2029 ± 81^{B}	2107 ± 65^{A}	2137 ± 56^{AB}
F3	1906 ± 86^{A}	2063 ± 53^{AB}	2201 ± 47^{A}	2208 ± 69^{A}
F4	456 ± 43^{B}	724 ± 34^{C}	1533 ± 67^{C}	2092 ± 42^{AB}
F5	525 ± 88^{B}	781 ± 94^{C}	1071 ± 23^{D}	1610 ± 45^{C}
F6	282 ± 26^{B}	798 ± 14^{C}	1854 ± 29^{B}	1975 ± 38^{B}
F7	296 ± 70^{B}	654 ± 29^{C}	1523 ± 54^{C}	1786 ± 54^{C}

Positive control AFB_1 (500 ng/plate) induced 1302 ± 30 and 2172 ± 25 revertants/plate for TA98 and TA100 tester strains, respectively. Spontaneous revertants were 29 ± 3 and 234 ± 20 for TA98 and TA100 tester strains, respectively. Values are means of three replicates ± standard error mean. Different letters in a column represent significant differences ($p < 0.05$).

Fractions 4, 6, and 7 (yields: 1, 1, and 0.8 % dry base, respectively) showed the lowest reversion rates at 3000 µg/mL concentration ($p < 0.05$) in TA98 tester strain. The inhibition of the AFB_1 mutagenicity in the presence of fractions 6 and 7 remained, even when these fractions were diluted, suggesting a strong antimutagenicity potential at low concentrations of the bioactive compounds present in these fractions. However, the antimutagenic potential tested in the TA100 strain was statistically different ($p < 0.05$) among fractions 4, 5, 6, and 7 at 3000 µg/mL. Recently, Cano-Campos et al. (2011) isolated antimutagenic compounds from the hexane-soluble fraction obtained from *Randia echinocarpa* fruit. They reported high percentage of inhibition of the mutation (> 76 %) induced by 1-nitropyrene in *Salmonella typhimurium* YG1024 tester strain.

Hence, these results showed that fractions 6 and 7 maintain their antimutagenic activity at the lowest concentrations tested in TA98 strains. These fractions were subjected to partial characterization by spectrophotometric techniques.

3.2 RP-HPLC

The chromatogram and UV-Vis absorption spectrum obtained by RP-UHPLC of fractions F6 and F7 are shown in Figure 2. Both fractions showed one major peak at a retention time of 9.42 min, which suggests that the compound responsible of the antimutagenic activity had been parted in both fractions. Both fractions showed absorption in the range of near UV (Figure 2A and 2B) which suggest the presence of compounds containing conjugated double-bond systems.

The presence of conjugated double bond systems in compounds such polyunsaturated fatty acids have been evaluated by Kim et al. (2011). These authors reported hexane-soluble bioactive lipids from *Mytilus coruscus*. Also, they found that these bioactive lipids possess anti-tumour effects by inducing apoptosis in human prostate cancer cells. Based on the above, the presence of compounds containing conjugated double bond systems in their structure is not discarded for fractions 6 and 7.

3.3 Characterization ^{1}H-NMR and ^{13}C-NMR

Figure 3A shows ^{1}H-NMR spectrum for fractions F6 and F7 which evidence similar signals between δ_H 7.5-7.8 corresponding to protons of aromatic ring system, signals between δ_H 4.1-4.3 were associated to protons adjacent to carbons attached to oxygen atoms in ester bonds. Chemical shifts observed between 0.9-2.0 are attributed to methine, methyl, and methylene protons of aliphatic chain of phthalate. The ^{13}C-NMR analysis (Figure 3B) showed signals between δ = 13.16 and 29.32 ppm, which are attributed to aliphatic carbons. The chemical shift observed at δ = 67 ppm is evidence of a carbon involved in a C-O bond, and signals δ= 127-129 ppm are associated to aromatic ring carbons. Finally, the signal of carbonyl function group present in an ester bond was observed at δ=170 ppm. All these results are consistent with the chemical structure of phthalate esters and the presence of this type of compound in marine organisms has been previously reported by Moushumi Priya & Jayachandran (2012) and the isolation of bis (2-ethylhexyl) phthalate from marine *Bacillus pumilus* MB 40 which was found to induce apoptosis and arrest of the cell

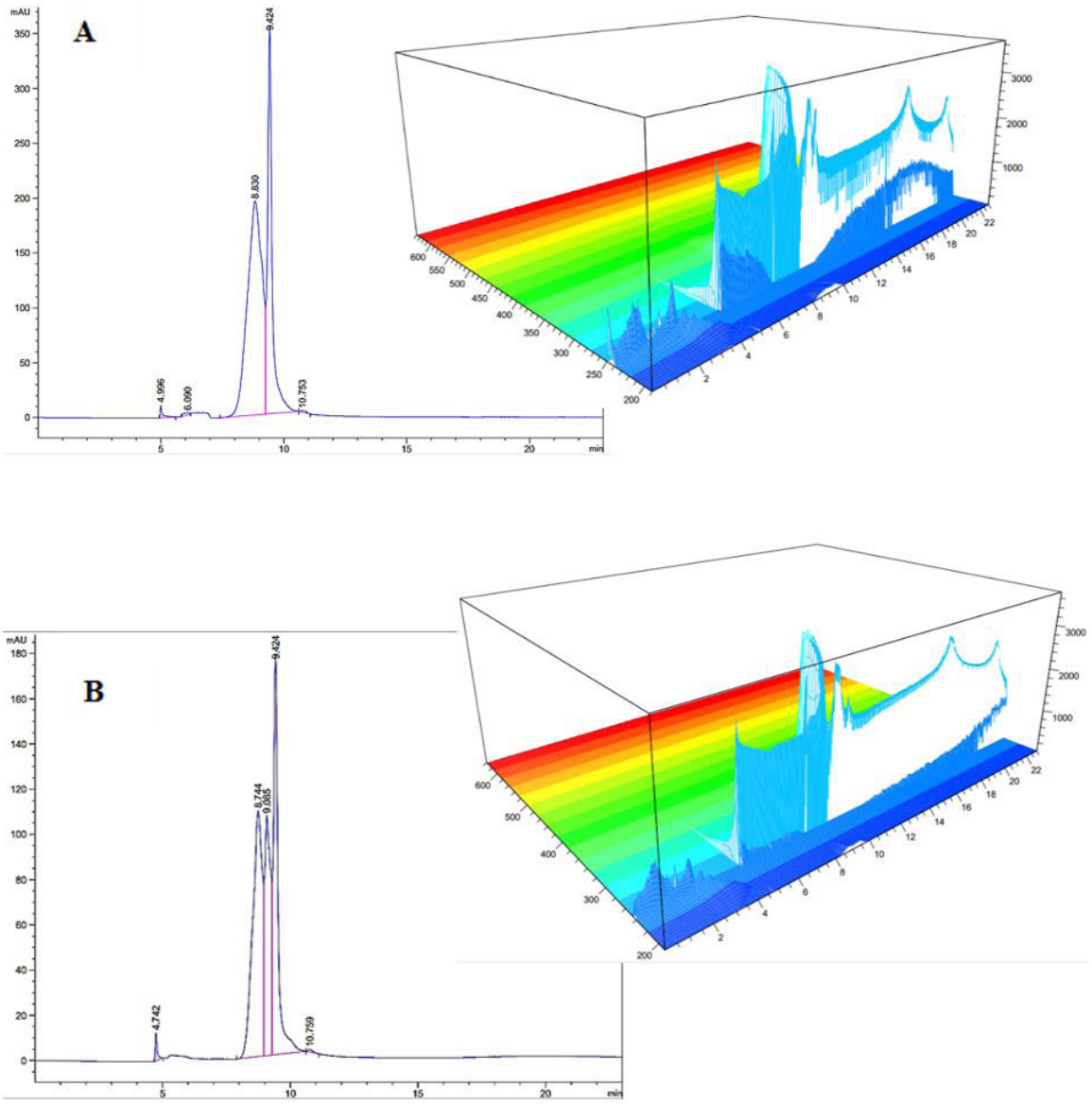

Figure 2. RP-HPLC chromatogram and spectrum of (A) 6 and (B) 7 fraction with antimutagenic activity.

cycle at a sub G0/G1 phase in human erytroleukemic K562 cells. All of this suggested that the bioactive compound(s) responsible for the antimutagenic activity of fractions F6 and F7 might be bis (2-ethylhexyl) phthalate. In order to confirm this approach, GC/MS analyses were carried out.

3.4 GC/MS

The results of GC-MS analysis of fractions F6 and F7 confirmed the presence of four compounds (Figure 4A) with retention times of 19.47, 23.79, 26.03, and 26.13 min. In terms of m/z ratio and % peak area, the results revealed: m/z = 43 (3.7%), m/z = 149 (99.6%), m/z = 59 (3.6%), and m/z = 185 (10.9%). Individual mass fragmentation (Figure 4B) of the main (99.6% abundance) compound produced m/z=113, m/z=279, m/z=167, and m/z=149 ions, is consistent with NIST library (National Institute of Standards and Technology, 2016). The molecular weight shown 390 and molecular formula indicated $C_{24}H_{38}O_4$, these results confirmed the presence of bis (2-ethylhexyl) phthalate.

The structure-activity relationship (SAR) of antimutagenic compounds has been previously studied (Kakinuma et al., 1986; Menon et al., 1999). Based on previous reports on antimutagenic activity, ester-phthalates have been associated to their capability to operate as Michael acceptors within the cell. Thus, the presence of carbonyl group in bis (2-ethylhexyl) phthalate might represent a good nucleophilic site in living systems; therefore, these compounds may act primarily via bioantimutagenic or desmutagenic mechanism. Desmutagen mechanism of bis (2-ethylhexyl) phthalate could be associated to its capability of acting as a nucleophile against an electrophilic promutagen 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-aflatoxin B_1 adduct and prevent the initial DNA damage.

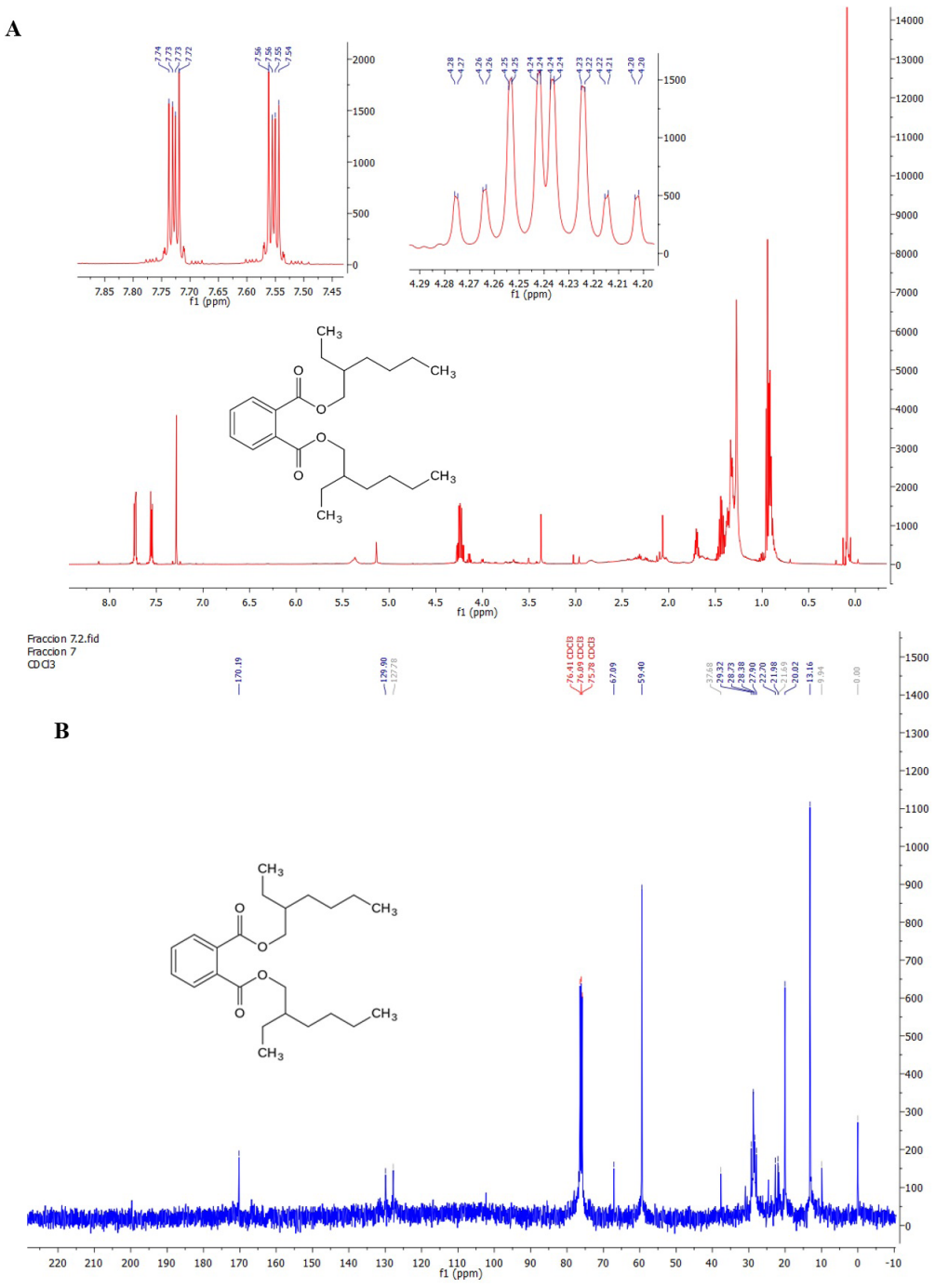

Figure 3. Spectrum (A) ^{1}H-NMR and (B) ^{13}C-NMR of antimutagenic fraction F6 and F7 in $CDCl_3$. Chemical shifts (δ) are in ppm.

3.5 Antimutagenic activity of bis (2-ethylhexyl) phthalate

Figure 5 shows the antimutagenic effect of commercially (sigma-Aldrich, USA) bis (2-ethylhexyl) phthalate on the mutagenicity of 500 ng of AFB_1. A reduction in the number of revertants/plate were observed for 5000 and 1000 ng/mL and represent 60 and 51% inhibition of sodium azide-induced mutations, respectively.

Statistically, mutation inhibition caused by 5000 and 1000 ng/ mL was significantly ($p < 0.05$) different from that observed for 500 and 100 ng/mL. These results are in accordance with those previously reported by Nakamura et al. (1982); lower dosages of bis (2-ethylhexyl) phthalate (2.8 and 28 µg/mL) showed 50% inhibition of the mutation induced by 4-NQO and MNNG in V79 hamster cells. Furthermore, Lee et al. (2000) found that bis (2-ethylhexyl) phthalate decreased the number of revertants/plate in tester strains exposed to AF-2 at concentrations between 100-1 µg/plate, doses-response type of relationships obtained evidenced high percentages of antimutagenic activity (> 46%) as evaluated in *S. typhimurium* TA98 and TA100 strains. Finally, bis (2-ethylhexyl) phthalate did not exhibit toxicity at 5000, 1000, 500, and 100 ng/mL (data not shown) in TA100 strain.

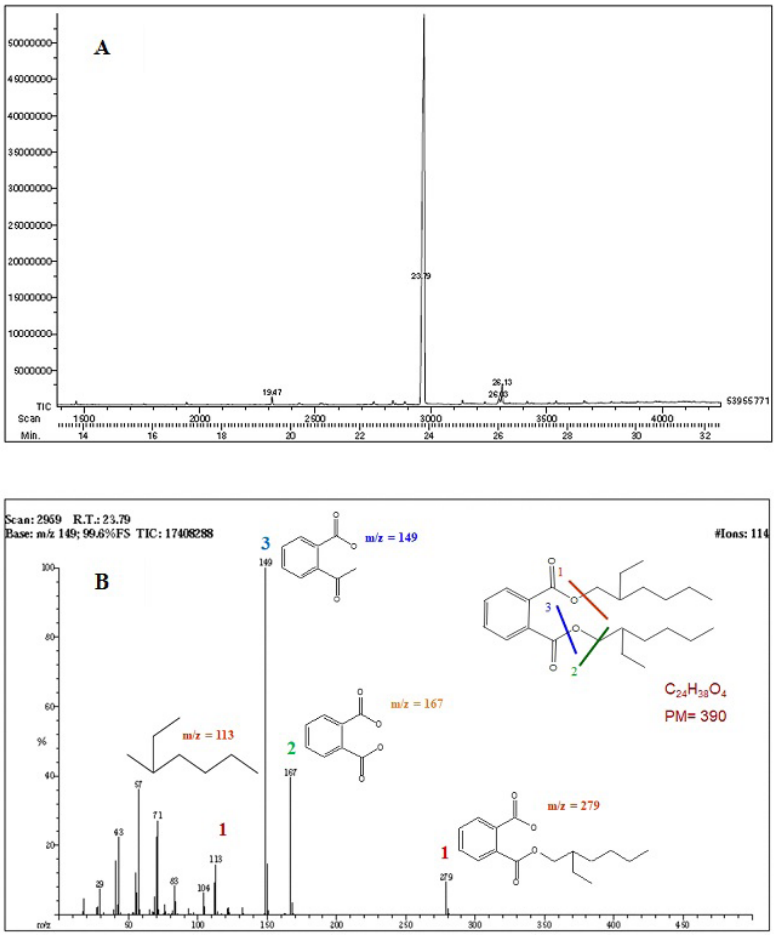

Figure 4. Chromatogram of fractions F6 and F7 (A), mass spectra of peak 23.79 min (B).

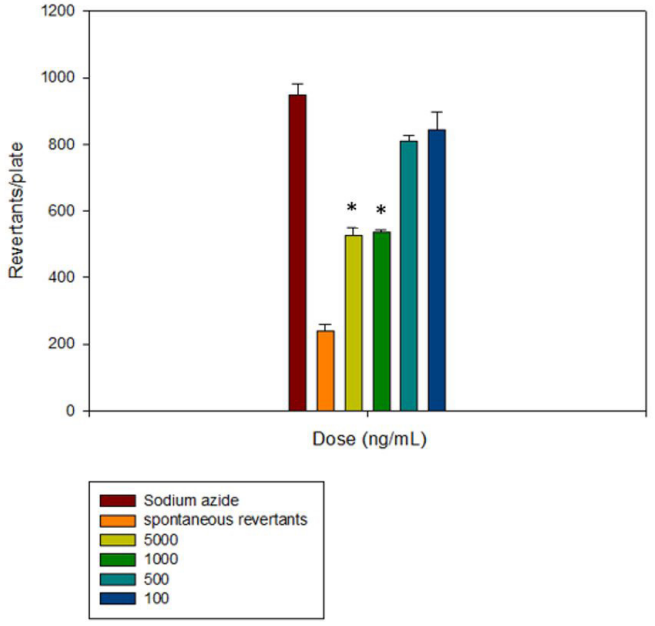

Figure 5. Antimutagenic activity of commercial bis (2-ethylhexyl) phthalate against sodium azide (1.5 µg/mL) induced mutation in *Salmonella typhimurium* strain TA100. Values are means of three replicates ± standard error mean. (*$p < 0.05$).

These results suggest the bis (2-ethylhexyl) phthalate is capable of inhibiting both, frame shift and base pair substitution mutations in *S. typhimurium* tester strains; therefore, this compound might protect DNA from damage caused by both, direct- and indirect-acting mutagens (AFB_1 and sodium azide).

4 Conclusions

In conclusion, fractions F6 and F7, both derived from the hexanic phase of octopus tentacles, are highly antimutagenic *in vitro*, being bis (2-ethylhexyl) phthalate the potential compound responsible for this bioactivity; however, further investigation is necessary to fully assess its chemopreventive properties.

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