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Antileishmanial and cytotoxic activity of dillapiole *n*-butyl ether

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ABSTRACT

Among the neglected diseases, American cutaneous leishmaniasis (ACL) still remains highly endemic in some tropical regions. The currently available drugs for treatment are highly toxic, prompting the search for new therapeutic options. The aim of this study was to evaluate the toxic potential of dillapiole *n*-butyl ether (DBE) against *Leishmania amazonensis* and *L. guyanensis*, as well as its toxicity on human peripheral blood mononuclear cells (PBMCs) *in vitro*. For cell cytotoxicity, concentrations of DBE that ranged from 7.8 to 500 µM were used for 48 and 72 h. For the evaluation of the antileishmanial activity, DBE was tested at concentrations of 0.28 to 18 µM for 24, 48, and 72 h. A value of 36 µM was used for the amastigote assay. The selectivity index (SI) was determined by dividing the CC_{50}/IC_{50} (macrophages/promastigotes). DBE exhibited a CC_{50} of 203.9 ± 0.5 µM in 72 h. DBE inhibited promastigote forms with an IC_{50} of 3.0 µM for both *Leishmania* species for 72 h. The standard, Pentacarinat®, showed an IC_{50} of 2.9 µM and 0.3 µM, respectively. The SI of DBE for both species was 67.9 for 72 h. DBE inhibited intracellular forms of *L. amazonensis* by 65.5% after 48 h. In molecular modeling, DLpOl-F showed two hydrogen bonds (SER418 and 421). DBE demonstrated promising *in vitro* antileishmanial potential.

KEYWORDS: *Leishmania*; dillapiole analog; semi-synthesis; molecular modeling

Atividade antileishmania e citotóxica do éter *n*-butil dilapiol

RESUMO

Dentre as doenças negligenciadas, a leishmaniose cutânea americana (LCA) continua sendo altamente endêmica em algumas regiões tropicais. Os medicamentos disponíveis atualmente para o tratamento apresentam elevada toxicidade, o que torna a busca de novas opções terapêuticas necessária. O objetivo do estudo foi avaliar o potencial tóxico do éter *n*-butil dilapiol (EBD) contra *Leishmania amazonensis* e *L. guyanensis* e a sua toxicidade em células mononucleares do sangue periférico humano (CMSP) *in vitro*. Para a citotoxicidade celular, utilizou-se concentrações de 7,8 a 500 µM por 48 e 72 h. A atividade antileishmania do EBD foi testada em concentrações de 0,28 a 18 µM, por 24, 48 e 72 h. Para o ensaio em amastigotas usou-se um valor de 36 µM. O índice de seletividade (IS) foi determinado pela divisão CC_{50}/CI_{50} (macrófagos/promastigotas). O EBD mostrou em células uma CC_{50} de 203,9 ± 0,5 µM em 72 h. O EBD inibiu as formas promastigotas com uma CI_{50} de 3,0 µM para ambas espécies de *Leishmania* por 72 h, já o padrão Pentacarinat® apresentou uma CI_{50} de 2,9 µM e 0,3 µM, respectivamente. O IS do EBD para as duas espécies foi de 67,9 por 72 h. O EBD inibiu as formas intracelulares de *L. amazonensis* com 65,5% por 48 h. Na modelagem molecular, o DLpOl-F aparece com duas ligações de hidrogênio (SER418 e 421). O EBD demostrou um potencial antileishmania promissor *in vitro*.

PALAVRAS-CHAVE: *Leishmania*; análogo do dilapiol; semissíntese; modelagem molecular

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INTRODUCTION

Leishmaniases are anthropozoonoses caused by the protozoa of the genus *Leishmania*, which are transmitted through the blood supply of female sandflies (BRASIL 2017). They are considered a neglected public health problem since the pharmaceutical industry has little interest in investing in new drugs for the treatment of these diseases (WHO 2020). These diseases are divided into cutaneous, mucocutaneous and visceral leishmaniasis. The American cutaneous leishmaniasis (ACL) is distributed in 88 countries on four continents (the Americas, Europe, Africa and Asia), and about 1,5 million cases occur each year (WHO 2019). In 2019, more than 41,000 new cases of cutaneous and mucosal leishmaniasis were reported in the Americas, with Brazil having the highest concentration of cases at around 37% of the total (OPAS/OMS 2020).

The current treatment of this disease is based on N-methylglucamine antimonate (Glucantime®), and other drugs, such as pentamidine isethionate, miltefosine, amphotericin B and liposomal amphotericin B, constitute therapeutic options for cases of low efficiency of antimonate treatments (Carvalho *et al*. 2019). These existing drugs have serious drawbacks in terms of safety, resistance, stability and cost, as well as low tolerability, long duration of treatment and difficulty to administer (DNDi 2018). Therefore, new, more effective and more affordable alternatives are being sought for the treatment of leishmaniasis. One approach is to survey the chemical diversity of molecules found in natural sources and employ new technologies, such as the semi-synthesis technique, to discover new drugs (De Oliveira *et al*. 2012).

Piper aduncum L. (Piperaceae) is popularly known as matico and long pepper (Lorenzi and Matos 2008). It is widespread in South and Central America and grows naturally in the Amazon and the Atlantic Forest of Brazil (Santos *et al.* 2013). The essential oil extracted from its leaves is commonly characterized by a high concentration of phenylpropanoids such as dillapiole, which is its main component (De Almeida *et al*. 2009). This compound shows a number of biological activities, such as insecticidal (De Almeida *et al*. 2009; Volpe *et al*. 2016), larvicidal (Oliveira *et al.* 2013), antiparasitic (Gaínza *et al*. 2016), antifungal (De Almeida *et al*. 2009; Ferreira *et al.* 2016), and antileishmanial (Dal Picolo *et al.* 2014), and can be used as a natural chemical base to obtain various new molecules (Katz and Baltz 2016). Farah *et al.* (2010) demonstrated the antileishmanial activity of dillapiole and its isodillapiole analogue against *Leishmania chagasi* Chagas and Cunha 1937. Parise-Filho *et al.* (2012) demonstrated the antileishmanial activity of dillapiole and its analogs dihydrodillapiole and isodillapiole against promastigote forms of *Leishmania brasiliensis* Vianna 1911 and *Leishmania (L.) amazonensis* Lainson and Shaw 1972. The analogs isodillapiole, dillapiole propyl ether and dillapiole isopropyl ether inhibited the promastigote forms of *L. amazonensis* and presented a non-toxic profile in J774 macrophages (Barros *et al*. 2021).

The design of antiparasitic drugs is based, especially, on the investigation of the biochemical pathways of the parasite and, when appropriate, as a leishmanolysin, or GP63, which is an important protease of the surface of *Leishmania* that is capable of hydrolyzing a wide variety of substrates both in the parasite and in the host (Shao *et al*. 2019). These are products that are involved in the adhesion and internalization of these parasites in host macrophages and have been related to the resistance of the parasite to lysis by the complement system and increased virulence of *Leishmania* (Rhaiem and Houimel 2016). In this context, molecular docking studies are predictive approaches for designing new drugs against parasite-specific targets (Reguera *et al.* 2014).

Among 15 dillapiole derivatives extracted from *P. aduncum* and evaluated for *in vitro* antileishmanial activity, dillapiole *n*-butyl ether (DBE) showed better results at the 50% inhibitory concentration (IC_{50}) (Barros *et al*. 2021). With the aim of further advancing the knowledge on the biological activity of DBE, the aim of this study was to evaluate the *in vitro* antileishmanial and cytotoxic activity of DBE obtained through semi-synthesis of dillapiole extracted from *P. aduncum.*

MATERIAL AND METHODS

Acquisition of plant material and DBE synthesis

Leaves of *P. aduncum* L. were taken from plants cultivated at Embrapa Amazônia Ocidental (Manaus, Amazonas state, Brazil) (2°53'36.2"S, 59°58'22.8"W) from plants identified in the herbarium of Instituto Nacional de Pesquisas da Amazônia (INPA) (voucher exsiccate # 10.480/2013, collector: E.S.Silva). The essential oil was extracted in the Laboratory of Medicinal Plants and Phytochemistry at INPA using the steam drag technique and dillapiole was isolated from the essential oil using a vacuum fractional distillation system according to the protocol of Pohlit *et al*. (2008).

Dillapiole was identified via (a) ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectral data (Varian, Model INOVA 500 spectrometer) with internal standard tetramethylsilane (TMS) and the solvents used were chloroform ($CDCl_3$) deuterated, and (b) by means of mass spectrometry (gas chromatograph coupled to a mass spectrometer from Shimadzu, CGMS-QP2010, model AOC-20) with automatic injector at the Centro de Biotecnologia da Amazônia (CBA). (Supplementary Material, Figures S1-S4). DBE was prepared using semi-synthesis of 5.5 g of dillapiole obtained by an oxymercuration reaction (Pinto 2008), which was added to a 100 mL volumetric flask with tetrahydrofuran (5.0 mL, Moderna), suspension of mercury acetate (1.5 g, Fluka) and *n*-butanol

(5.0 mL, Synth) and then homogenized in an ice bath for 72 h with stirring until the reaction occurred. The reaction product was analyzed using thin layer chromatography (TLC) plates using 20 x 20 cm size aluminum backed normal phase silica gel chromatoplates with MERCK fluorescence indicator F254, mobile phase hexane: ethyl acetate (9: 1) and alcoholic anisaldehyde solution developer. After obtaining the analog, a solution of sodium borohydride (2.0 g, Fluka), potassium hydroxide (7.0 g, Moderna) and *n*-butanol (5.0 mL) was added and the mixture was shaken. The solution was then filtered and washed according to the protocol of Tomar *et al.* (1979a, b). The DBE was purified in a silica gel chromatographic column 60 (70 - 230 mesh, Merck) and identified using spectroscopic methods (^{1}H and ^{13}C NMR, Varian, Model INOVA 500 spectrometer) with internal standard tetramethylsilane (TMS) and the solvents used were chloroform ($CDCl_3$) deuterated (Supplementary Material, Figure S5-S7). The density of the DBE was determined by the average weight of the oil mass divided by its volume in triplicate.

Cell acquisition and cultivation

For the assay with human peripheral blood mononuclear cells (PBMC), 10 mL of peripheral blood were collected from two healthy volunteers. Each sample was processed individually, diluted in a conical tube (50 mL) with PBS 1X (1:1) and homogenized according to the protocol of Junior (2013). The project was approved by the human ethics committee of Universidade Federal do Amazonas (Amazonas, Brazil) (CEP/ UFAM, CAAE # 29406319.2.0000.5020). The J774 lineage macrophages were provided by Dr. Paulo Afonso Nogueira (Fiocruz Manaus). Cells were cultured in RPMI-1640 medium containing 20% fetal bovine serum (FBS), 50 U mL^{-1} penicillin and 50 µg mL^{-1} streptomycin (Invitrogen).

Cell cytotoxicity tests

The macrophages were placed in 30 ml at a concentration of 10^6 cells ml^{-1} which were pre-cultured in 96-well plates containing RPMI 1640 medium supplemented with 10% SFBi and incubated at 37 °C with 5% CO_2. After 24 h, the cells adhered to the base of the wells and were treated with DBE for 48 and 72 h in triplicate (three plates) for each time. DBE diluted in 0.2% v/v dimethylsulfoxide (DMSO, Vetec®) was also used, standard positive controls using Pentacarinat® and Glucantime®, and a negative control containing 0.2% DMSO with cells (all controls in triplicate). The bioassay was incubated following Jaffe *et al*. (1984).

For the cytotoxicity test in J774 cells, macrophages were placed in 24 mL of SFBi at a concentration of 5 x 10^6 cells mL^{-1} adjusted in a Neubauer chamber and subjected to DBE concentrations from 7.8 to 500 µM for 48 hours in triplicate, Pentacarinat® Glucantime® and 0.2% DMSO were used as controls. To obtain the 50% cytotoxic concentration (CC_{50}) in cells, the colorimetric dose-response test (Alamar Blue®) was used according to Nakayama *et al*. (1997) with spectrophotometer reading at 590 nm.

Acquisition and maintenance of promastigotes

Leishmania (*L*.) *amazonensis* (MHOM/BR/2009/IM5584) and *Leishmania* (*V*.) *guyanensis* Floch 1954 (MHO/BR/75/M4147) were cryopreserved at the Leishmaniasis and Chagas Disease Laboratory at INPA. Subsequently, the samples were cultivated in RPMI 1640 medium supplemented with 10% of inactivated FBS and placed in an oven at 25 °C.

In vitro promastigote assay

The antileishmanial activity of DBE was evaluated via the inhibition of growth and the mortality of promastigote forms of *L*. (*L*.) *amazonensis* and *Leishmania* (*V*.) *guyanensis* during 24, 48 and 72 h, when incubated at 25 °C. For the bioassay, a Schneider culture medium supplemented with 10% inactivated FBS was used. Promastigotes were centrifuged and adjusted to 10^6 mL^{-1} of parasites. The concentrations tested ranged from 18 to 0.2812 µM for both species. The negative control was parasites plus medium and DMSO and the positive controls were Pentacarinat® and Glucantime®. Bioassays were made in triplicates and repeated twice. Parasite viability was quantified using the dose-response colorimetric test according to Nakayama *et al.* (1997) with spectrophotometer reading.

Amastigote-macrophage assay

After obtaining the PBMCs, they were placed in 100 mL at a cell concentration adjusted to 10^6 mL^{-1} in Schneider culture medium and supplemented with 10% SFBi, then in 24-well plates with a sterile glass coverslips prepared in triplicate (trhee plates) for each *Leishmania* species. Then, 1 mL of cell solution was added to each well, and the plates remained in an oven at 37 °C with 5% CO_2. After 7 days, the promastigote forms of *L*. (*V*.) *guyanensis* and *L*. (*L*.) *amazonensis* infected the cells in a 5:1 ratio (promastigotes: cells) and were incubated with DBE, Pentacarinat®, Glucantime® and dillapiole in the concentration of 36 µM, and cells plus DMSO (0.2%) for 24, 48 and 72 h (one plate for each time and *Leishmania* species). On each plate, trhee wells were assigned to DBE, Pentacarinat®, Glucantime®, dillapiole and DMSO, respectively. At the end of the test, the coverslips were stained with a panoptic kit, removed and analyzed under an optial microscope (1.000 x). Infectivity was determined by counting the presence/absence and number of amastigotes in 100 macrophages per coverslip/well in triplicate (three coverslips per treatment).

Cell selectivity index

To determine the selectivity of concentrations with antileishmanial activity against macrophages, the selective index (SI) was obtained by the ratio of the cellular CC_{50} divided by the 50% inhibitory concentration (IC_{50}) for promastigotes, during 72 h using the following equation:

$$SI = \frac{CC_{50} \text{ in macrophages (cytotoxicity)}}{IC_{50} \text{ against } \textit{Leishmania}}$$

Molecular coupling of dillapiole and DBE

Theoretical computational studies to determine the molecular coupling were performed in the Laboratory of Theoretical and Computational Chemistry at UFAM (LQTC/UFAM), on a computer with 12 Intel(R) Core(TM) i5-10400F processing nuclei, 16 GB-RAM and a Linux MINT 20 operating system. The parent compound, dillapiole (DLpOI), and the derivative, dillapiole *n*-butyl ether (DLpOI-F), were structured in two dimensions (Figure 1) and then adjusted for approximate geometric conformation. Both procedures were performed using MarvinSketch (2017). The structures were optimized as isolated structures (in gas phase), using Gaussian software (Frisch *et al*. 2013), at DFT level with the B3LYP/3-21G method (optimizations together with vibrational frequency calculations to confirm the optimization quality).

After optimization, the structures were subjected to molecular docking (MDk) calculations via AutoDock-Vina (Trott *et al*. 2010), using an exhaustion value of 32 and seed of 1924167872, considering the sites in Protein Data Bank File (PDBF) structures obtained at the Research Collaboratory for Structural Bioinformatics - PDB (RCSB-PDB) (2021). Gp63 (PDB code: 1LML) (Schlagenhauf *et al.* 1998) was selected as a protein target for MDk. The PDBF underwent removal of oxygen from the water molecules, followed by the addition of polar hydrogens with the internal parameters of the AutoDock-Tools (Morris *et al*. 2009). The various centralizations of the boxes (CB) were defined after preliminary MDk of all PDBF, using DLpOl as a binder. In this step, the boxes with maximum dimensions were used to involve the entire protein region, and all positions in which there were couplings were selected as potential sites for further analysis. In the further analyses, the cubic dimensions of 20×20×20 Å (8000 Å^3) were chosen, since they fully met the sizes of the ligands.

Statistical analysis

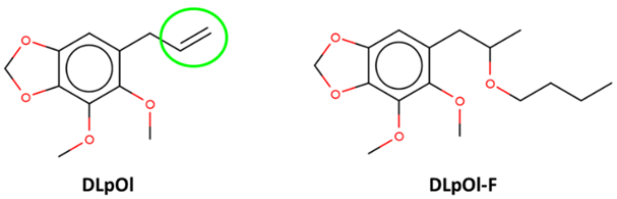

Figure 1. Structure of DlpOl. The green circle marks the carbon where the structural differences occur (the DLpOI-F derivative was explored in S-isomeric form of the labeled carbon). This figure is in color in the electronic version.

Statistical analysis was performed using the GraphPad Prism software (version 6.0) for the *in vitro* data. By means of a dose-response curve, the number of live cells used to calculate the CC_{50} (50% cytotoxic concentration) and the viable parasites to calculate the IC_{50} (inhibitory concentration for 50% of the live parasites) were obtained. The cytotoxicity in PBMC and inhibition of promastigote forms were analyzed with two-way analysis of variance (ANOVA) to compare the antileishmania activity among the experimental treatment (DBE) with standard drugs and dillapiole against different species of *Leishmania*, followed by Tukey's post-hoc test for the pairwise comparison of the means of the different treatment groups, with 95% confidence to be considered significant. All variables that participated in the *in vitro* tests met the requirements for parametric analysis.

RESULTS

Acquisition and characterization of DBE

The dillapiole extracted from the *P. aduncum* presented NMR spectra with the following chemical shifts: NMR ^{1}H ($CDCl_3$; 500 MHz): δ 6.25, s (H-6); 3.31, dd (*J*= 2.0; 7.0 Hz, H-7); 5.91 m (H-8); 5.04 m (H-9a); 5.06 m (H-9b); 5.88 s (H-10); 3.76, s (OMe); 4.01 s (OMe). NMR ^{13}C ($CDCl_3$; 125 MHz): δ 126 (C), 136 (C), 137.6 (C), 144.6 (C), 114.4 (C), 102.6 (CH), 33.8 (CH_2), 137.4 (CH), 115.5 (CH_2), 101.1 (CH_2), 61.2 (OCH_3), 59.9 (OCH_3) (Supplementary Material, Figure S1, S2 and S3). The dillapiole showed a molecular peak at *m/z* 222 (Supplementary Material, Figure S4).

The isolated DBE substance had a yield of 4.1 g, representing 73% of the theoretical yields of the reaction. DBE was a light-yellow oil with the molecular formula $C_{16}H_{24}O_5$, molecular weight of 296.35 g mol^{-1} and density of 1.094 ± 0.06 g cm^{-3}.

The NMR spectra of DBE showed the following chemical shifts: NMR ^{1}H ($CDCl_3$; 500 MHz): δ 6.40 s (H-6); 2.83, dd (*J*= 6.0; 13.5 Hz, H-7a), , 2.53 dd (*J*= 6.5; 13.5 Hz, H-7b), 3.56 sext (*J*= 6.0; 13.0 Hz, H-8), 1.12 d (*J*= 6.0 Hz, H-9), 5.89 s (H-10); 3.48 m (H-11a); 3.38 m (H-11b); 1.51 m (H-12); 1.34 sext (*J*= 7,5; 15,0 Hz, H-13); 0.89 t (*J*= 7,5; 14,5 Hz, H-14); 3.77 s (OMe); 4.02 s (OMe). NMR ^{13}C ($CDCl_3$; 125 MHz): 125.5 (C1), 136.1 (C2), 137.7 (C3), 144.9 (C4), 144.5 (C5), 103.9 (C6), 37.2 (C7), 76.2 (C8), 19.9 (C9), 101.3 (C10), 68.7 (C11), 32.4 (C12), 19.6 (C13), 14.1 (C14), 60.1 (OMe), 61.3 (OMe) (Supplementary Material, Figure S5, S6, and S7).

Cell cytotoxicity tests

After 48 h we obtained the following 50% cytotoxic concentrations (CC_{50}) in J774 macrophages: DBE (477.2 ± 0.8 µM), Pentacarinat® (22.8 ± 1.5 µM). For Glucantime® and dillapiole, it was not possible to obtain the CC_{50} at the concentrations used (Table 1), because dillapiole was not toxic at these concentrations, and because Glucantime® is considered a prodrug. In peripheral blood mononuclear cells,

DBE remained with a less toxic CC_{50} than Pentacarinat® during 48 and 72 h in relation to DMSO 0.2%.

In vitro promastigote assay

In the test of DBE against *L.* (*L.*) *amazonensis* in different concentrations, we observed a reduction in the concentration of viable parasites, with a 50% inhibitory concentration (IC_{50}) of 3.0; 3.2 and 3.0 µM at 24, 48 and 72 h, respectively. IC_{50} for Pentacarinat® was 3.4; 3.0 and 2.9 µM at 24, 48 and 72 h, respectively. For dillapiole and Glucantime® it was not possible to obtain the IC_{50} at the concentrations used, because dillapiole did not inhibit 50% of viable parasites at these concentrations. The same occurred for Glucantime® when compared with DMSO 0.2% (Figure 2).

The tests of DBE against *L.* (*V.*) *guyanensis* in different concentrations also showed parasitic inhibition with an IC_{50} of 12.0; 2.0 and 3.0 µM at 24, 48 and 72 h, respectively. With Pentacarinat®, IC_{50} was 8.0; 0.3 and 0.3 µM at 24, 48 and 72 h, respectively. For dillapiole and Glucantime®, it was not possible to obtain the IC_{50} at the concentrations used since they did not inhibit 50% of the viable parasites when compared with DMSO 0.2%. DBE showed significant inhibition in the first three concentrations ($p < 0.0001$) and Pentacarinat® showed significant inhibition at all concentrations ($p < 0.0001$) (Figure 3).

Cell selectivity index

DBE showed less toxicity to PBMC than to *L. amazonensis* and *L. guyanensis* parasites, with an SI of 67.9 for both species, suggesting that DBE is less toxic than Pentacarinat® (Table 2).

Amastigote-macrophage assay

After 48 h, DBE showed a significantly higher percentage of parasitic death of the amastigote forms of *L. guyanensis* when compared to Glucantime®, dillapiole, DMSO (0.2%)

Table 1. The 50% cytotoxic concentrations (CC_{50}) (mean ± standard deviation of three replicates) of dillapiole *n*-butyl ether (DBE) and Pentacarinat® for human peripheral blood mononuclear cells (PBMC) and J774 macrophages, obtained through serial concentrations of 7.8 to 500 µM after 48 h (J774) and 48 and 72 h (PBMC). The mean CC_{50} are represented in µM.

Test compound	J774	PBMCs	
	48 h	48 h	72 h
DBE	477.2 ± 0.8[a]	350 ± 0.4[a]	203.9 ± 0.5[a]
Pentacarinat®	22.8 ± 1.5[b]	188.2 ± 1.8[b]	17.6 ± 0.8[b]
DMSO 0.2%	> 500	> 500	> 500

Different superscript letters within the table indicate significant statistical differences according to the Tukey test. DMSO = dimethyl sulfoxide (negative control).

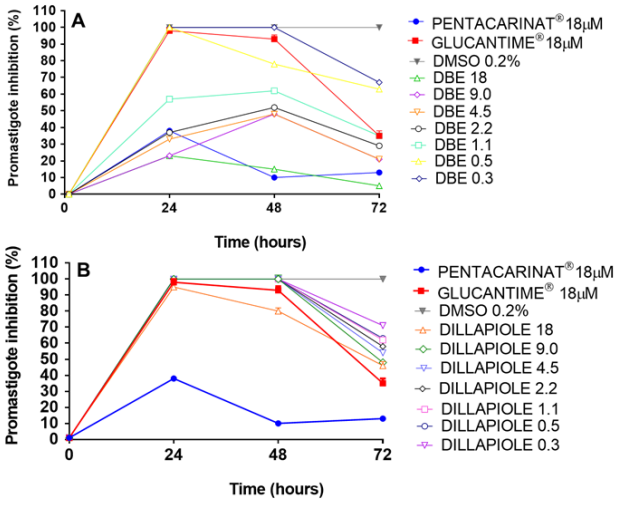

Figure 2. Evaluation of the antileishmanial activity of DBE (A) and dillapiole (B) against the promastigote forms of *Leishmania* (*L.*) *amazonensis* through the quantification of the viable forms of the parasite at 24, 48 and 72 h, Pentacarinat® and Glucantime® (positive controls) in µM and DMSO (0.2%) (negative control). The symbols indicate the mean of three replicates. This figure is in color in the electronic version.

Table 2. IC_{50} values of dillapiole *n*-butyl ether (DBE) and Pentacarinat® from promastigotes of *Leishmania* spp. and CC_{50} in human peripheral blood mononuclear cells (PBMC) for 72 h (mean ± standard deviation of three replicates). The means of IC_{50} and CC_{50} are represented in µM.

Test compound	IC_{50}		CC_{50}
	Leishmania amazonensis	*Leishmania guyanensis*	PBMCs
DBE	3.0 ± 1.0	3.0 ± 0.5	203.9 ± 0.5
Pentacarinat®	2.9 ± 3.0	0.3 ± 1.0	17.6 ± 0.8

CI_{50} = concentration capable of inhibiting the number of promastigotes in culture media by 50%; CC_{50} = cytotoxic concentration for 50% of the cell layer; SD = standard deviation; PBMCs = peripheral blood mononuclear cells.

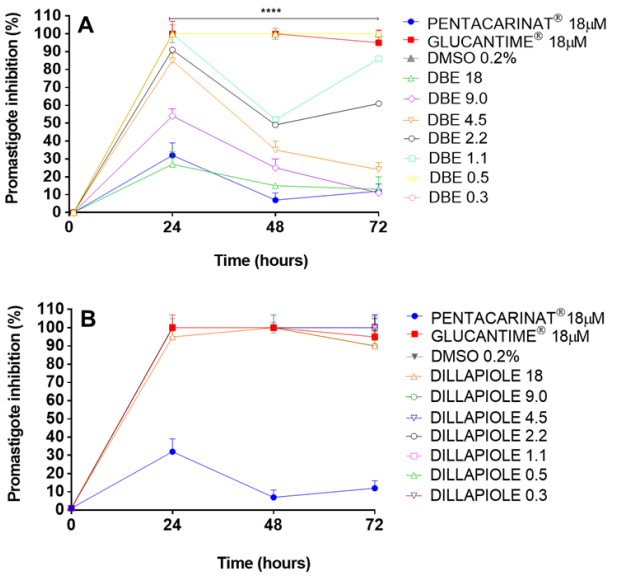

Figure 3. Evaluation of the antileishmanial activity of DBE (A) and dillapiole (B) against the promastigotes forms of *Leishmania* (*V.*) *guyanensis* through the quantification of the viable forms of the parasites at 24, 48 and 72 h, Pentacarinat® and Glucantime® (positive controls) in µM and DMSO (0.2%) (negative control). The symbols indicate the mean of three replicates. This figure is in color in the electronic version.

($p < 0.000.1$), and Pentacarinat® ($p = 0.0045$) (Figure 4a). Against *L. amazonensis*, DBE caused the death of 65.5% of the intracellular forms, significantly higher than Pentacarinat® ($p = 0.0011$) and dillapiole ($p < 0.0001$) and did not differ significantly from Glucantime® (61%) (Figure 4b).

Molecular coupling of dillapiole and DBE

In most cases, the affinity energies in the MDk ($E_{MDk\text{-}Aff}$) for binding the derivative to GP63 show significant differences when compared to DLpOl. Such results suggest that the applied structural modification would affect the activity in biological organisms that are highly dependent on this target protein.

Considering the $E_{MDk\text{-}Aff}$ values (Figure 5), the best centering for the DLpOl coupling would be at C01, and DLpOl-F would couple better in the C01 and C03 regions. In the centralizations C01, C02 and C03, the $E_{MDk\text{-}Aff}$ value is higher for DLpOl-F, which indicates that this derivative has greater affinity in these positions, and only in C04 does DLpOl have greater affinity. At C01 (Figure 6), DLpOl appears with only one hydrogen bond, while DLpOl-F appears with two hydrogen bonds (one of them involving the oxygen of the derivatization), in addition to having more apolar interactions involving the alkyl groups. This difference in the number of interactions justifies the better $E_{MDk\text{-}Aff}$ value of the derivative. This result also suggests the importance of increasing the chain, since new apolar interactions involving alkyls will be more highly favored. Thus, it is normal that this derivative would have better coupling with respect to DLpOl.

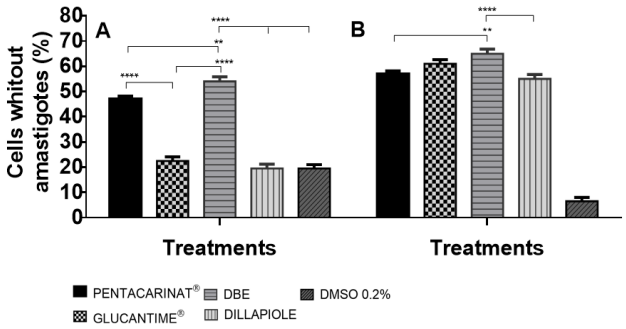

Figure 4. Evaluation of DBE, dillapiole, Pentacarinat®, Glucantime® (36 µM) against amastigotes of *Leishmania* (*V.*) *guyanensis* (A) and *L.* (*L.*) *amazonensis* (B) after 48 h. Columns are mean and bars the standard deviation of three replicates. The asterisks above the columns indicate significant differences according to a post-hoc Tukey test with 95% confidence. Dados considerados significativos **P < 0.01; ****P < 0.0001.

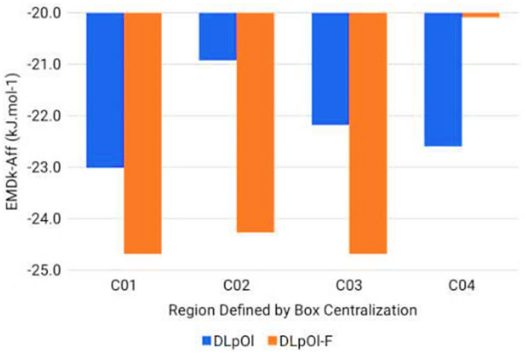

Figure 5. Best couplings of DLpOl-F (blue bars) were to almost all the PDBF-1LML coupling sites (except for C04), demonstrating that the derivation tends to enhance molecule-protein interactions (only M1 modes were considered). This figure is in color in the electronic version.

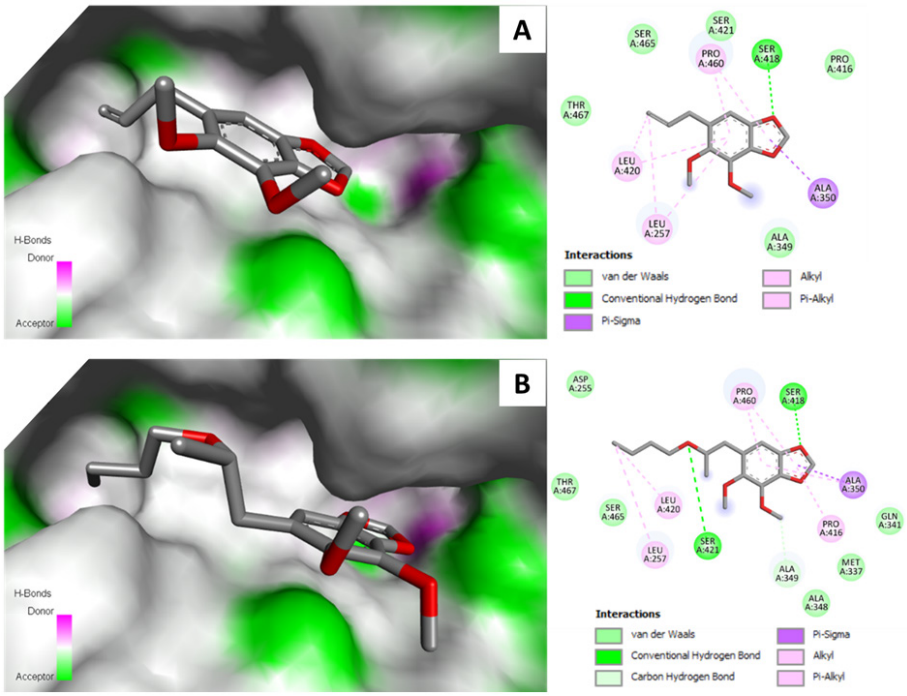

Figure 6. Depictions of surfaces with tendencies to hydrogen bond and the specific interactions for M1 couplings of DLpOl (A) and DLpOl-F (B) in the C01 region of PDBF-1LML. This figure is in color in the electronic version.

DISCUSSION

The NMR spectra of the DBE showed the chemical shifts as described in the literature and in the study by Tomar *et al*. (1979a,b). This demonstrated a viable reproductive process.

In this study, we evaluated the cytotoxic effect of DBE in contact with J774 macrophages for 48 h and obtained a CC_{50} that was less toxic than Pentacarinat®. For Glucantime® and dillapiole, it was not possible to obtain the CC_{50} at the concentrations used. Farah *et al*. (2010) demonstrated a viability in macrophages when exposed to dillapiole and its analogue isodillapiole, at a concentration of 50 μg mL^{-1} after 72 h. This corroborates our study for 48 h. In PBMC cells, DBE showed a less toxic CC_{50} than the standard drug (Pentacarinat®) for 48 h. After 72 h, CC_{50} was 203.9 ± 0.5 μM for DBE and 17.6 ± 0.8 μM for Pentacarinat®, thus showing that DBE remained less toxic when compared to the negative control (DMSO 0.2%). Parise-Filho *et al*. (2012) observed a CC_{50} of 22 μM for dillapiole using human 3T3 fibroblasts for 24 h. However, *P. aduncum* oil showed a high margin of safety of 240 mg kg^{-1} and 120 mg kg^{-1}, with minimal toxic effects on biochemical and hematological parameters *in vivo* (Sousa *et al.* 2008). In our study, DBE presented a satisfactory safety margin in PBMCs, thus corroborating Barros (2018), who showed that peritoneal macrophages exposed to DBE exhibited a lower cytotoxic profile *in vitro*.

DBE showed a reduction in the concentration of viable parasites in promastigotes with an IC_{50} of 3.0 μM against 2.9 μM for Pentacarinat® after 72 h. It was not possible to obtain the IC_{50} for dillapiole and Glucantime®. One isodillapiole derivative showed antileishmanial activity at a concentration of 50 μg mL^{-1} in promastigotes of *L. chagasi* for 72 h, which inhibited 96% of viable parasites (Farah *et al*. 2010). The inhibition of promastigotes and amastigotes of *L. amazonensis* was achieved with an IC_{50} of 1.6 μM using a DBE derivative for 72 h (Barros 2018), likewise demonstrating the potential of DBE against *L. amazonensis*, with an IC_{50} of 3.0 μM after 72 h, when using a new batch.

DBE showed parasitic reduction of the promastigote forms of *L.* (*V.*) *guyanensis* with an IC_{50} of 3.0 μM, the same obtained for Pentacarinat® after 72 h. We found no publication using dillapiole analogs from *P. aduncum* with antileishmanial activity for the inhibition of *L. guyanensis*.

DBE was shown to be less toxic to PBMCs than to *L. amazonensis* and *L. guyanensis* parasites, with SI of 67.9 for both species, showing a less toxic profile than that of Pentacarinat®. In another study, DBE showed a SI of 76.5 and was more toxic to *L. amazonensis* than to peritoneal macrophages after 48 h (Barros 2018). This corroborates our findings since, for a prototype to be considered promising to become a drug, it must have an SI equal to or greater than 10 (Chiaradia 2007).

At 48 h, DBE showed a significantly higher performance against amastigote forms of *L. guyanensis* when compared to Glucantime®, dillapiole, and Pentacarinat®, as well as against amastigotes of *L. amazonensis* compared to Pentacarinat® and dillapiole. DBE inhibited amastigote forms of *L. amazonensis* by around 88% at a concentration of 4.5 μM when tested in infected peritoneal macrophages for 48 h (Barros 2018), which was corroborated by our results. We found no publications on dillapiole analogues against *L. guyanensis*, both in the promastigote and amastigote forms, thus our study is the first to describe the antileishmanial activity of DBE in this species, showing that DBE may be a molecule that has broad-spectrum activity for the treatment of cutaneous leishmaniasis.

The most important interactions in the formation of a protein-ligand complex are intermolecular interactions, such as Van der Waals forces, hydrophobic interactions, π-π, ionic or electrostatic interactions, covalent bonds (Guryanov *et al*. 2016) and, especially, the hydrogen bonds that are very important interactions for the biological system because they are responsible for maintaining the structure of proteins (Barreiro and Fraga 2015). Figure 5 compares the $E_{MDk\text{-}Aff}$ values of DLpOl and DLpOl-F when these couple in four different regions of PDBF-1lML. Although these four regions are in the same protein, it is not appropriate to compare the results of one region with another. The important thing in the figure is that, for the same region (or centralization of the box), the compounds couple with different intensities. The best region for DLpOl coupling would be C01, since this is where this compound couples with more negative energy. DLpOl-F would couple with more negative energy in two regions, C01 and C03, with equal values of $E_{MDk\text{-}Aff}$. In the centralizations C01, C02 and C03, the $E_{MDk\text{-}Aff}$ is more negative for DLpOl-F, indicating that this derivative has greater affinity in these regions, and only in C04 does DLpOl have greater affinity.

The crystalline form of leishmanolysin (PDBS-1LML), previously called GP63, was obtained from proteins that were purified from promastigote membranes and may be targeted for drugs for human leishmaniasis (Schlagenhauf *et al*. 1998). Leishmanolysin can act before infection, ensuring that the maximum number of parasites is produced and released from the insect, or shortly after infection, facilitating binding and entry into target cells, in addition to cleaving MHC and macrophage activation factors (McMahon and Alexander 2004). This suggests a possible DBE mechanism of action.

CONCLUSIONS

DBE showed low toxicity in PBMCs and J774 lineage macrophages, and demonstrated an antileishmanial activity similar to the reference drugs against promastigote and amastigote forms of *L. amazonensis* and *L. guyanensis*. It also presented a more viable SI than Pentacarinat®. As for molecular coupling, DLpOl-F showed a better tendency to bind to the

protein target (PDBS-1LML) when compared to DLpOl. This study demonstrated that DBE has promising antileishmanial activity *in vitro*, and can be explored as an leishmanicidal drug, after confirmatory activity tests in *in vivo* models.

ACKNOWLEDGMENTS

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DATA AVAILABILITY

The data that support the findings of this study are not publicly available.

SUPPLEMENTARY MATERIAL (only available in the electronic version)

Barros *et al*. Antileishmanial and cytotoxic activity of dillapiole *n*-butyl ether

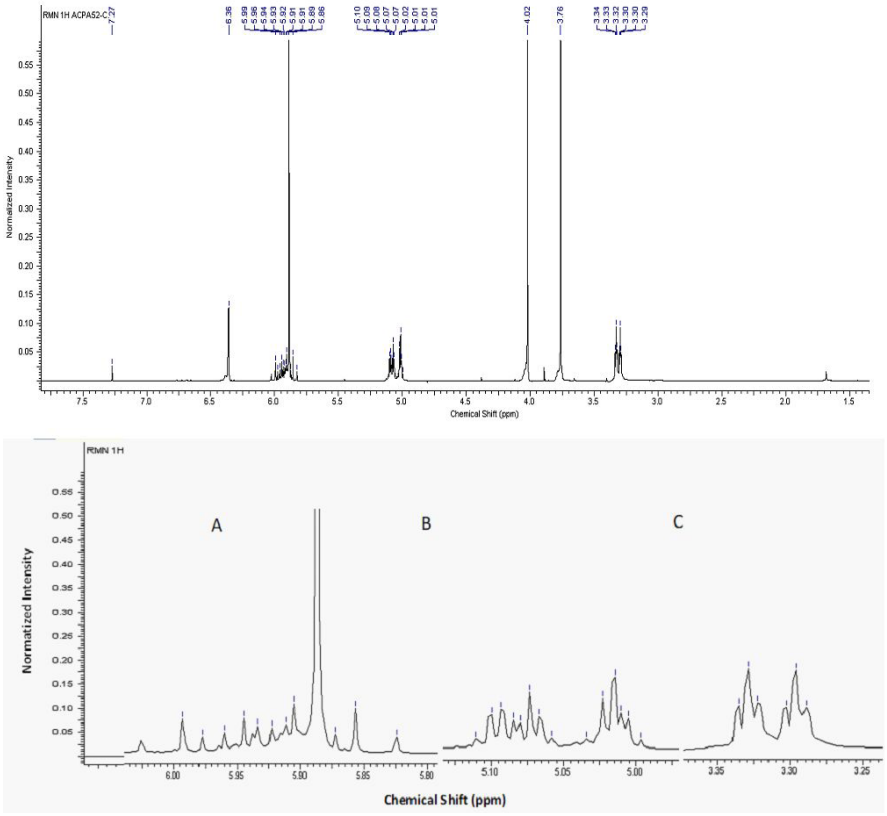

Figure S1. ^{1}H NMR spectrum ($CDCl_3$; 500 MHz) of dillapiole and enlargement of spectrum regions.

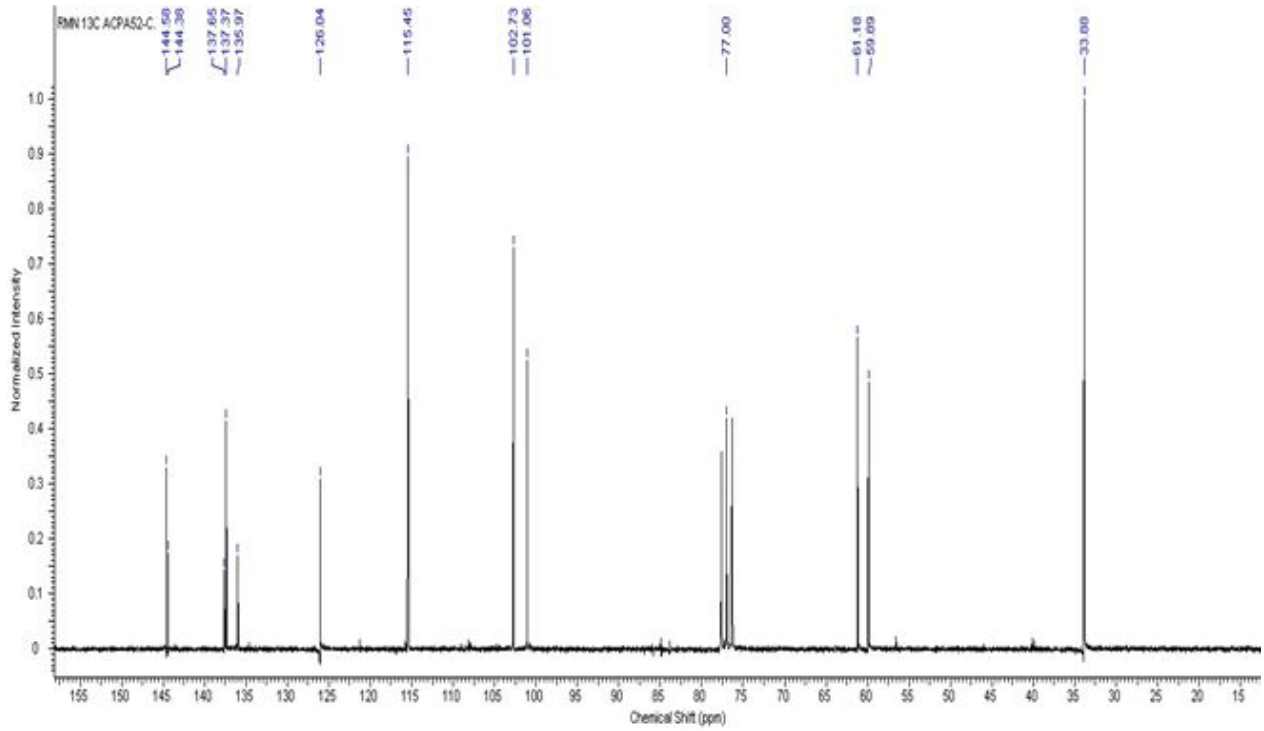

Figure S2. ^{13}C NMR spectrum ($CDCl_3$; 125 MHz) of dillapiole

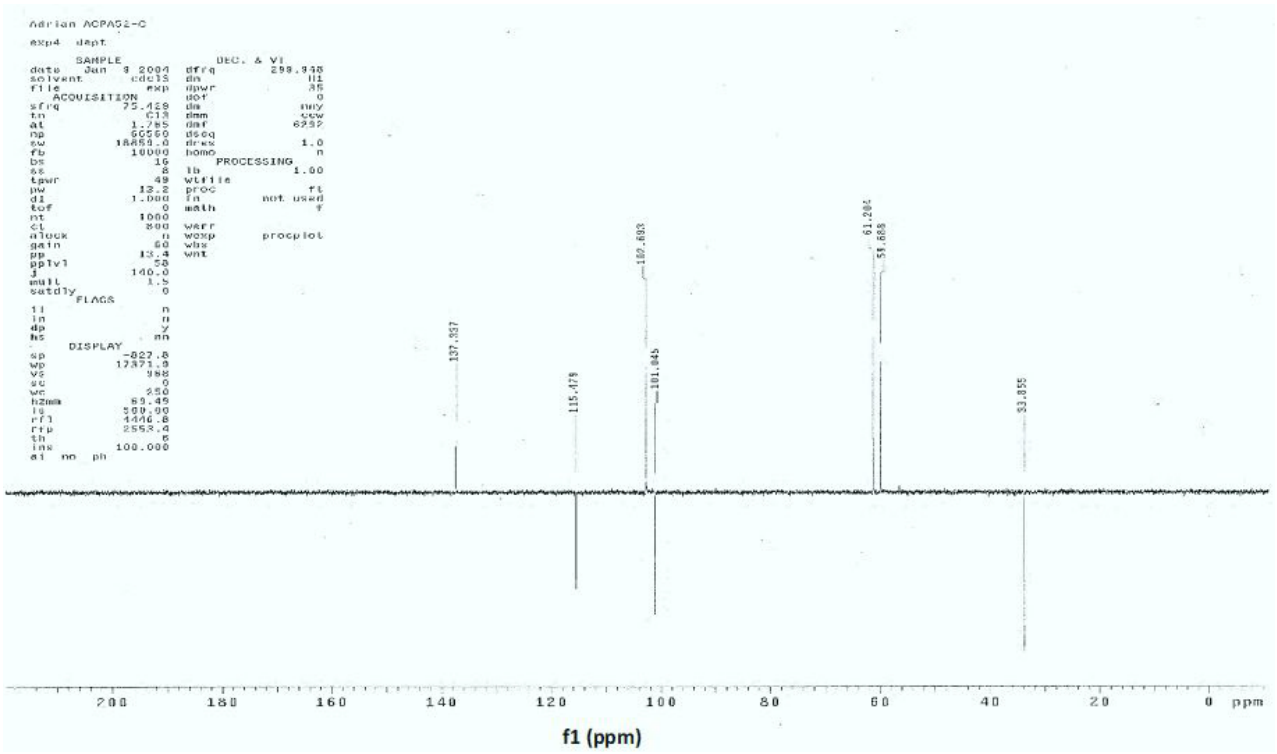

Figure S3. DEPT 135 spectrum ($CDCl_3$; 125 MHz) of dillapiole.

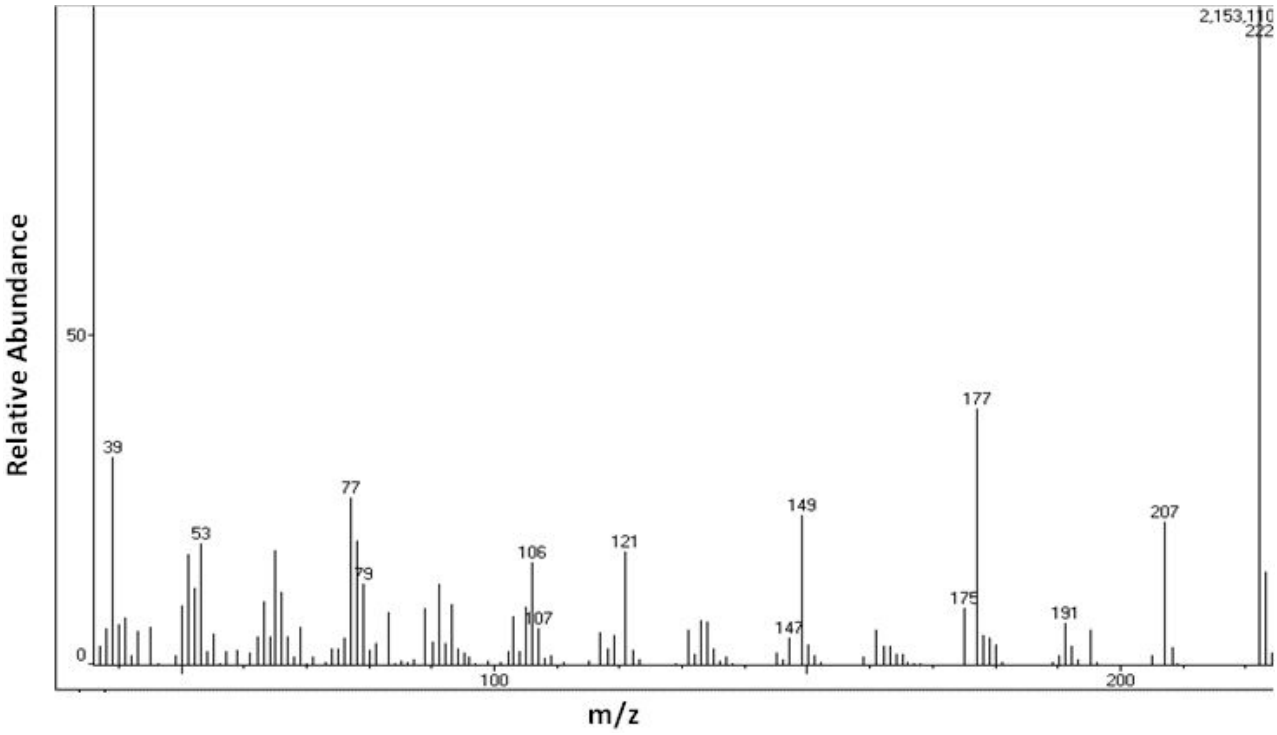

Figure S4. MS/MS spectrum of the ion at *m/z* 222 in the dillapiole.

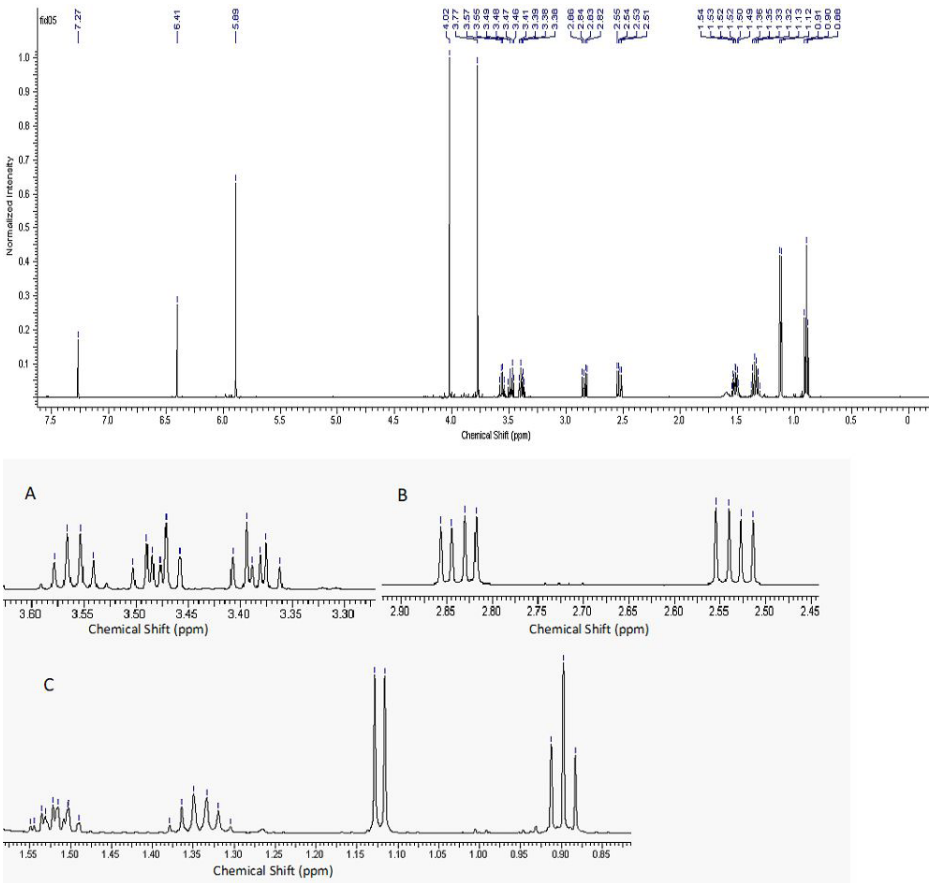

Figure S5. ^{1}H NMR spectrum ($CDCl_3$; 500 MHz) of dillapiole *n*-butyl ether (DBE) and enlargement of spectrum regions.

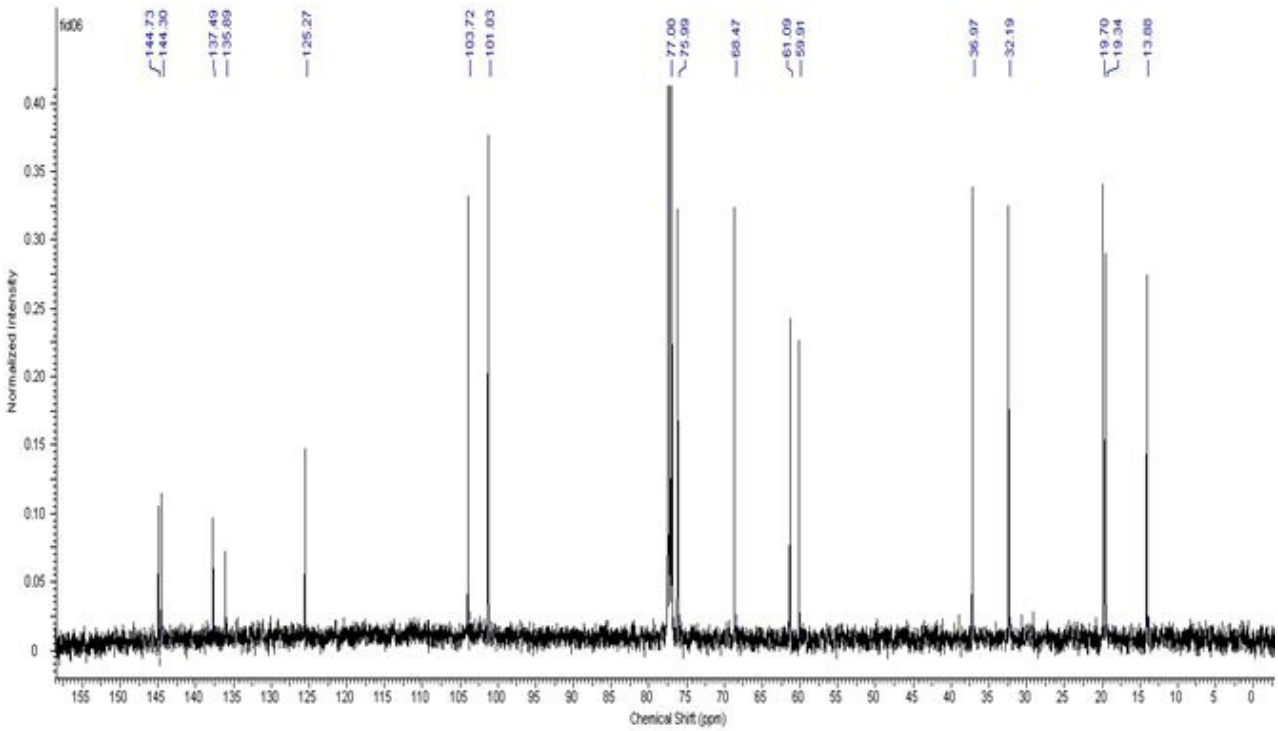

Figure S6. ^{13}C NMR spectrum ($CDCl_3$; 125 MHz) of dillapiole *n*-butyl ether (DBE)

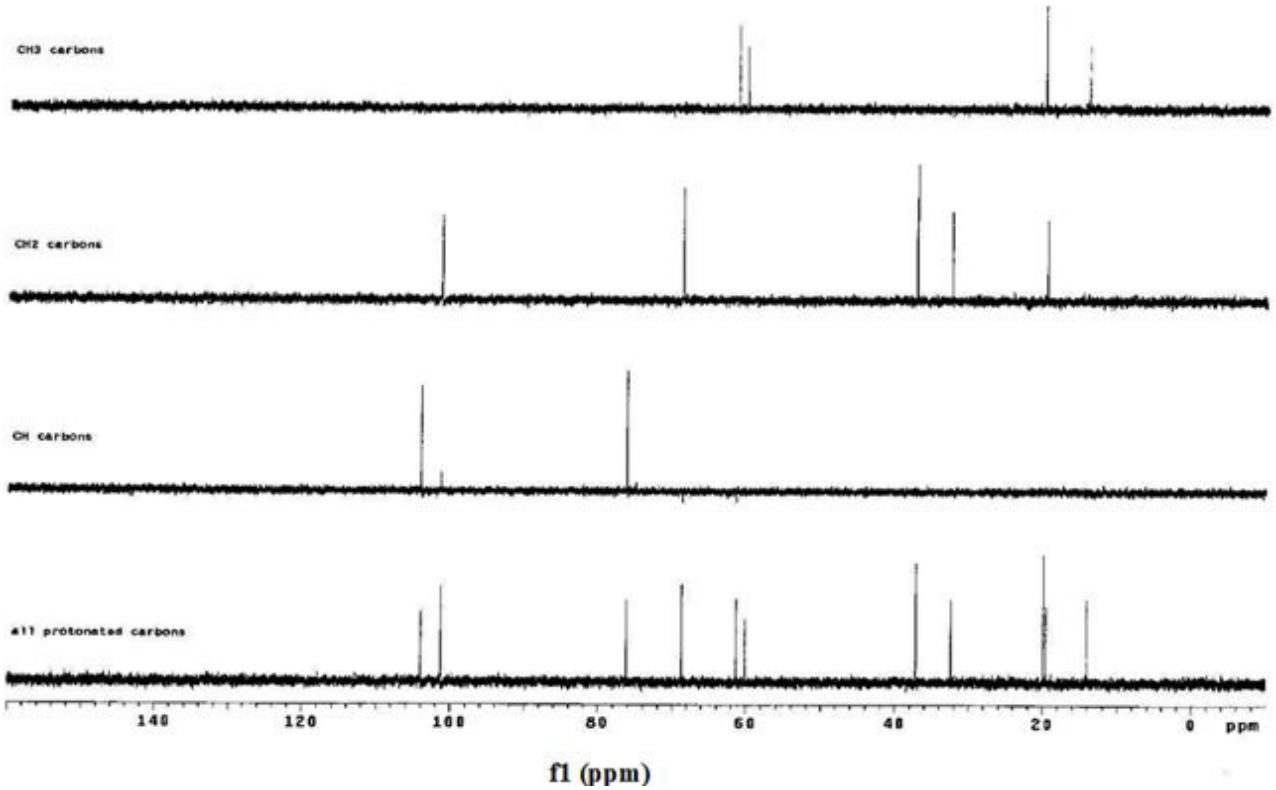

Figure S7. DEPT spectrum ($CDCl_3$; 125 MHz) of dillapiole *n*-butyl ether (DBE).