

Chemical composition, anti-*Trypanosoma cruzi* and cytotoxic activities of the essential oil from green fruits of *Protium ovatum* (BURSERACEAE)

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Abstract - Chagas disease is a major neglected tropical disease caused by *Trypanosoma cruzi*. It has been treated with the antiparasitic drugs nifurtimox and benznidazole, which cause several side effects. The market for natural products has considerably grown lately and the use of plants has become an alternative to the development of novel drugs to cure the disease. Therefore, this study aimed at describing the chemical analysis of the essential oil from green fruits of *Protium ovatum* and evaluating their trypanocidal and cytotoxic potential. The essential oil was obtained by Clevenger hydrodistillation whereas its chemical composition was determined by gas chromatography coupled to mass spectrometry (GC-MS). The major compounds found in the essential oil from green fruits of *P. ovatum* were β -myrcene (62.0 %), α -pinene (11.3 %) and limonene (7.3 %). To the best of our knowledge, this was the first time that the chemical composition of the essential oil from green fruits of *P. ovatum* was described. Results showed that the essential oil had strong trypanocidal activity against trypomastigote forms of the Y strain of *Trypanosoma cruzi* ($IC_{50} = 1.2 \mu\text{g/mL}$). In addition, the essential oil from green fruits of *P. ovatum* did not display cytotoxicity against LLCMK₂ adherent epithelial cell at the concentration range under analysis ($CC_{50} = 550.3 \mu\text{g/mL}$). As a result, it is an excellent option for the development of novel antiparasitic drugs.

Index terms: *Protium ovatum*, β -myrcene, fruits, essential oil, *Trypanosoma cruzi*, cytotoxic analysis.

Composição química, atividades anti-*Trypanosoma cruzi* e citotóxica do óleo essencial dos frutos verdes de *Protium ovatum* (BURSERACEAE)

Resumo - A doença de Chagas é uma das principais doenças tropicais negligenciadas causadas pelo *Trypanosoma cruzi*, e em seu tratamento utilizam-se medicamentos como o nifurtimox e o benznidazol, que causam vários efeitos colaterais. O mercado de produtos naturais tem aumentado consideravelmente nos últimos anos, e o uso das plantas continua sendo uma alternativa para o desenvolvimento de novos medicamentos para cura de doenças. Portanto, este estudo aborda a composição química do óleo essencial dos frutos verdes de *Protium ovatum* e a avaliação de seus potenciais tripanocida e citotóxico. O óleo essencial foi obtido por hidrodestilação, utilizando o aparato do tipo Clevenger. A composição química foi determinada por cromatografia gasosa acoplada à espectrometria de massas (CG-EM). Os principais compostos encontrados no óleo essencial dos frutos verdes de *P. ovatum* foram: β -mirreno (62,0 %), α -pineno (11,3 %) e limoneno (7,3 %). Este é o primeiro relato da composição química do óleo essencial obtido a partir de frutos verdes de *P. ovatum*. Os resultados mostraram que o óleo essencial analisado apresenta forte atividade tripanocida contra as formas tripomastigota da cepa Y do *Trypanosoma cruzi* ($IC_{50} = 1,2 \mu\text{g/mL}$). O óleo essencial exibiu ainda moderada citotoxicidade frente à linhagem LLCMK₂ na concentração avaliada ($CC_{50} = 550,3 \mu\text{g/mL}$). Em suma, o óleo essencial dos frutos verdes de *P. ovatum* pode ser considerado uma fonte alternativa para o desenvolvimento de novos medicamentos antiparasitários.

Termos para indexação: *Protium ovatum*, β -mirreno, frutos, óleo essencial, *Trypanosoma cruzi*, análise citotóxica.

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Introduction

American trypanosomiasis, also known as Chagas disease (CD), is a neglected tropical disease which is endemic in Latin America. The World Health Organization estimates that approximately 6-7 million Latin Americans have been infected with *Trypanosoma cruzi* since CD has been mainly found in endemic areas of Latin American countries (PAULA et al. 2015).

The disease has been treated with drugs which display either high toxicity against the host's body or low efficiency against the pathogen (IZUMI et al. 2012). So far, no vaccine has been developed for CD and the current treatment has been conducted with nifurtimox (Nf) or benznidazole (Bz). However, their well-known toxicity, as well as their limited effect on different parasite isolates and on the chronic phase of the disease, has called for the development of new drugs to treat it (SOEIRO; CASTRO, 2011).

The market for natural products, such as extracts, isolated compounds and essential oils from plants, and the use of plants as an alternative to the development of novel drugs for the treatment of various diseases, including CD, have annually increased in view of the great potential of these compounds (AFFONSO et al. 2012; LEITE et al. 2010). The Cerrado (Brazilian savannah), a natural heritage site owing to its diversity and endemism of biological species, is an important source of novel natural substances with different biological properties (SILVA et al. 2015).

Essential oils from different plant sources carry out several biological activities, such as antibacterial, anticancer, anti-inflammatory, antimutagenic, antifungal, antioxidant and antiprotozoal ones. As a result, the vast arsenal of bioactive compounds found in essential oils has increasingly attracted researchers' intense attention in the last years (CARNEIRO et al. 2017).

The Burseraceae family, for example, comprises 21 genera with 600 species and the genus *Protium* is its main family member with 135 species. In the literature, species of the Burseraceae family have been described as the ones which are commonly used for treating wounds and ulcers. Besides, they act as anti-inflammatory and repellent agents. Triterpenes, mono- and sesquiterpenes from species of the genus *Protium* are well-known for their significant biological properties and their anti-inflammatory and acaricidal activities (MORAES et al. 2013).

Protium ovatum Engl. is a herbaceous plant found in the Brazilian Cerrado. In the literature, several studies have described the anti-inflammatory, antinociceptive, immunostimulant and anticancer properties of resins (SIANI et al. 2011). However, reports have described neither the chemical composition nor the trypanocidal potential and cytotoxicity of the essential oil from specimens of *Protium ovatum*.

This study aims at describing the chemical composition of the essential oil from green fruits of *P. ovatum*, its *in vitro* activities against trypomastigote forms of *Trypanosoma cruzi* and its cytotoxic activity against LLCMK₂ adherent epithelial cells.

Material and Methods

Green fruits of *Protium ovatum* were collected in Rio Verde, GO, Brazil, in September 2015. The plant was identified by the botanist Erika Amaral (Instituto Federal de Educação, Ciência e Tecnologia Goiano, Campus Rio Verde, GO, Brazil). A voucher specimen (no. HJ 7420) was deposited at the Herbarium Jataiense Professor Germano Guarin Neto, which belongs to the Instituto Federal Goiano, Brazil.

The essential oil was extracted from fresh green fruits of *Protium ovatum* (100 g) by a modified cleveger-type apparatus and hydrodistillation for 2 h. The oil was separated and dried over anhydrous sodium sulfate, stored in hermetically sealed glass containers and kept under refrigeration at 5 °C until analysis and trypanocidal and cytotoxicity assays. Total oil yield was expressed as a percentage value (g/100 g of fresh plant material). All experiments were carried out in triplicate.

Gas chromatography – mass spectrometry (GC-MS) analysis was carried out by a Shimadzu QP2010 with an AOC-20i auto-injector and a DB-5MS column (30 m x 0.25 mm, 0.25 mm in thickness). The carrier gas was He with pressure of 57.4 kPa and flow rate of 1.00 mL/min. The split ratio was 1/30, the injector temperature was 250 °C and the injected volume was 1 µL. Temperature programming was the following: 60 – 240 °C, increasing 3 °C/min. MS were recorded on the electron ionization (EI) mode, with ionization energy of 70 eV (scan time: 2 scans/s). Identification of the constituents was based on the retention indices (the calculation used from C₉ to C₂₂ alkanes) and on the comparison of the mass spectra with libraries (Wiley 7 and Nist 62) and references to previously published data (ADAMS, 2007).

¹H- and ¹³C-NMR spectroscopic data were recorded at room temperature in CDCl₃ (Cambridge Isotope Laboratories, Andover, MA, USA) by a Bruker DPX-300 spectrometer (Karlhue, Germany) operating at 300 MHz (¹H)/75 MHz (¹³C). Standard pulse sequences were used for homo- and heteronuclear correlation experiments. Chemical shifts are reported in ppm, using TMS as an internal standard (δ = 0 ppm) whereas coupling constants (*J*) are expressed in Hertz.

To obtain the trypomastigotes of *T. cruzi*, LLCMK₂ cells were cultured in RPMI medium supplemented with 2 x 10⁻⁶ mol/L L-glutamine, 10⁻⁵ mol/L NaHCO₃, 100 U/mL penicillin, 100 µg/mL streptomycin and 10 % inactivated fetal bovine serum. The procedure was accomplished

in culture bottles at 37 °C, under 5 % ambient CO₂ and relative humidity of 95 %. The trypomastigote forms were maintained in RPMI medium and the parasites were transferred to fresh medium every 48 h to furnish free parasite forms. The assay conducted after 24 h was based on the methodology reported by Esperandim et al. (2013). Approximately 1 x 10⁶ trypomastigotes were added to each well in a 96-well microtiter plate. Then, the essential oil was added at concentrations ranging from 12.5 to 200 µg/mL. After 24 h incubation, the biological activity of the samples was evaluated by the colorimetric MTT tetrazolium salt assay (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/mL). Readings were conducted by a microplate reader at 517 nm wavelength. Positive and negative controls were benzonidazole (from 12.5 to 200 µg/mL) and 0.5 % dimethyl sulfoxide (DMSO), respectively. Assays were performed in triplicate.

LLCMK₂ adherent epithelial cells were grown in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 5 % inactivated fetal calf serum. They were kept at 37 °C in 5 % CO₂. A cell suspension was seeded at a concentration of 1 x 10⁶ cells/mL in a 96-well microplate with RPMI 1640 medium. Thereafter, cells were treated with essential oil at different concentrations (6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL). Plates were incubated at 37 °C for 24 h and the biological activity was evaluated by the MTT colorimetric method [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in a microplate reader at 540 nm. RPMI 1640 medium was the positive control whereas DMSO and RPMI 1640 media were the negative ones. All experiments were performed in triplicate. The percentage of cell viability was determined

by the following formula: % cell viability = 1 - [(Y-N)/(N-P)] x 100, where Y = absorbance of wells containing cells and essential oil at different concentrations; N = negative control; and P = positive control (ESPERANDIM et al. 2013).

Results and Discussion

The hydrodistillation of green fruits of *P. ovatum* gave colorless oil with a very strong and fragrant odor. The percentage of oil yielded from the plant was 0.5 % by weight. Altogether, 13 compounds at concentrations above 0.1 % were identified, accounting for 95.0 % of the total oil composition, which was characterized by the predominance of terpenes: β-pinene (5.5 %), sabinene (5.0 %), *p*-cymene (0.3 %), pirillene (0.4 %), pinocaryone (0.2 %), terpinen-4-ol (0.2 %), borneol (0.2 %), α-copaene (0.3 %), *E*-caryophyllene (2.0 %) and α-humulene (0.3 %). Their major components were β-myrcene (62.0 %), α-pinene (11.3 %) and limonene (7.3 %) (Table 1).

β-myrcene, α-pinene and limonene (Figure 1), which are the three major components of the essential oil from green fruits of *P. ovatum*, were found to be among the constituents of the oils from other species of the same genus (ZOGHBI et al. 2005). In previous studies of the efficiency of the essential oil from leaves of *P. ovatum*, the result was 0.10 % and a complex mixture of terpene constituents was determined by ¹H NMR, ¹³C NMR and IR (CASTELO et al. 2010). Other species of *Protium* were analyzed and a complex mixture of monoterpenes and sesquiterpenes were determined in essential oils from resins, foliar rachises, branches and leaves (CARVALHO et al. 2013; PINTO et al. 2010; CARVALHO et al. 2010).

Table 1 - Chemical composition of the essential oil from green fruits of *Protium ovatum*.

Compounds	RI ^a	RI ^b	Composition percentage
β-Pinene	970	969	5.5
Sabinene	971	972	5.0
α-Pinene	928	930	11.3
β-Myrcene	987	991	62.0
<i>p</i> -Cymene	1025	1024	0.3
Limonene	1028	1028	7.3
Pirillene	1103	1102	0.4
Pinocaryone	1164	1163	0.2
Terpinen-4-ol	1173	1178	0.2
Borneol	1165	1165	0.2
α-Copaene	1375	1376	0.3
<i>E</i> -caryophyllene	1420	1420	2.0
α-Humulene	1451	1453	0.3
Total			95.0

RI^a: Linear retention indices found in the literature. RI^b: Retention indices calculated from retention times in relation to those of the series of *n*-alkanes on a 30 m DB-5 capillary column.

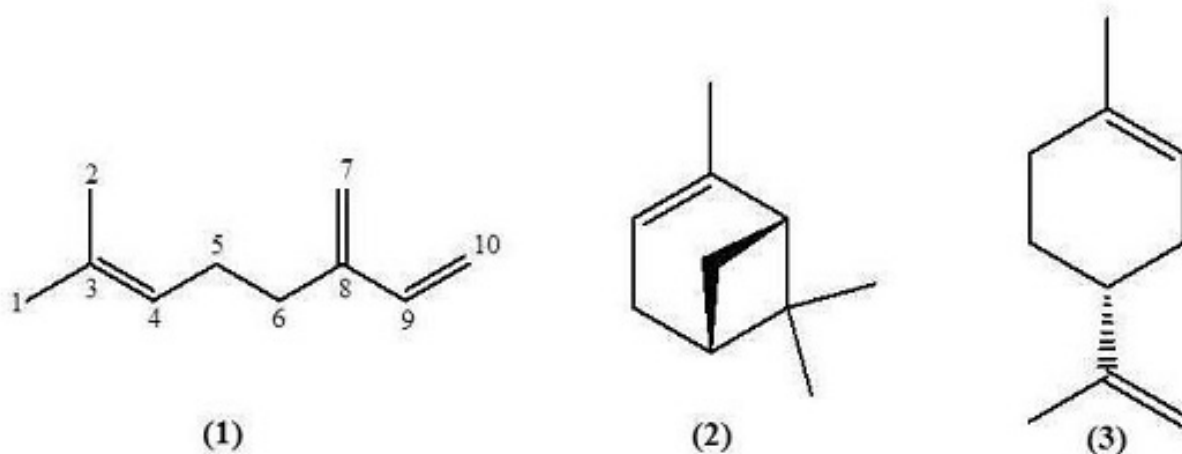


Figure 1 – Structures of main constituents of the essential oil from green fruits of *P. ovatum*: (1) β-myrcene; (2) α-pinene and (3) limonene.

The identification of β-myrcene (62.0 %) in the mixture, the main substance found in the essential oil from fruits of *Protium ovatum*, was carried out by the analysis of ^1H (Figure 2) and ^{13}C (Figure 3) NMR spectra and by comparison with literature data (PESSINI et al. 2005). The spectroscopic data on β-myrcene are as follows:

β-myrcene: NMR ^1H (300 MHz, CDCl_3): δ 6.35 (*dd*, $J = 10.8$ e 17.7 Hz, H-4); 5.43-4.60 (*m*, H-7, H-9 and H-10); 2.24-2.02 (*m*, H-5 and H-6); 1.69 (*s*, H-1); 1.64 (*s*, H-2). NMR ^{13}C (75 MHz, CDCl_3): δ 146.1 (C, C-8); 139.0 (CH, C-9); 131.3 (C, C-3); 124.2 (CH, C-4); 115.5 (CH_2 , C-7); 112.7 (CH_2 , C-10); 31.4 (CH_2 , C-6); 26.9 (CH_2 , C-5); 25.7 (CH_3 , C-1) and 17.7 (CH_3 , C-2).

The essential oil from green fruits of *P. ovatum* has high trypanocidal activity against trypomastigotes of *Trypanosoma cruzi*. Increased infeasibility of trypomastigote cells was observed with increasing concentration of essential oil. High activity was obtained at $\text{IC}_{50} = 1.2$ $\mu\text{g/mL}$ (Table 2) and it was lower than the one of the positive control with benzonidazole, which was $\text{IC}_{50} = 9.8$ $\mu\text{g/mL}$.

The literature has reported that samples with trypanocidal activity of $\text{IC}_{50} < 10$ $\mu\text{g/mL}$, $\text{IC}_{50} > 50 < 100$ $\mu\text{g/mL}$ and $\text{IC}_{50} > 100$ $\mu\text{g/mL}$ are considered highly active, active/moderately active and inactive, respectively (ALVES et al. 2012). The trypanocidal properties of the major components of the essential oil from green fruits *P. ovatum*, β-myrcene (62.0 %), α-pinene (11.3 %) and limonene (7.3 %) were previously reported (SANTOS et al. 2014; SARTORELLI et al. 2012; ZENG et al. 2010). It was proposed that the activity of essential oils against trypanosomatids is mainly due to its terpene composition. Terpenes are responsible for the hydrophobic character of essential oils, thus allowing their diffusion through the parasite cell membrane and affecting intracellular metabolic pathways and organelles (BORGES et al. 2012).

This is the first report of the trypanocidal activity of the essential oil from green fruits of *Protium ovatum*. Despite great advances made by modern medicine in recent decades, plants are still considered very important in regard to health care (CALIXTO, 2000). Several studies of essential oils have shown that some plants have trypanocidal activity against *T. cruzi* (BALDISSERA et al. 2013; ESCOBAR et al. 2010).

Cultures of LLCMK₂ adherent epithelial cells were treated with essential oil at concentrations of 6.25, 12.5, 25.0, 50.0, 100, 200 and 400 $\mu\text{g/mL}$ for 24 h. Results showed that the essential from green fruits did not have toxicity at the concentration evaluated with CC_{50} 550.3 $\mu\text{g/mL}$ (Table 3), by comparison with benzonidazole positive control with CC_{50} 147.3 $\mu\text{g/mL}$.

It is important to point out that the essential oil from green fruits of *P. ovatum* did not display cytotoxicity against adherent epithelial cells at the concentration range under analysis. There is evidence that, owing to their lipid solubility, essential oils have low density and rapid diffusion across cell membranes. As a result, they could damage the parasite cell membrane structure and lead to cellular lysis (ANTHONY et al. 2005). In addition, there could be synergistic and/or additive effects from constituents of the essential oil (CARNEIRO et al. 2017).

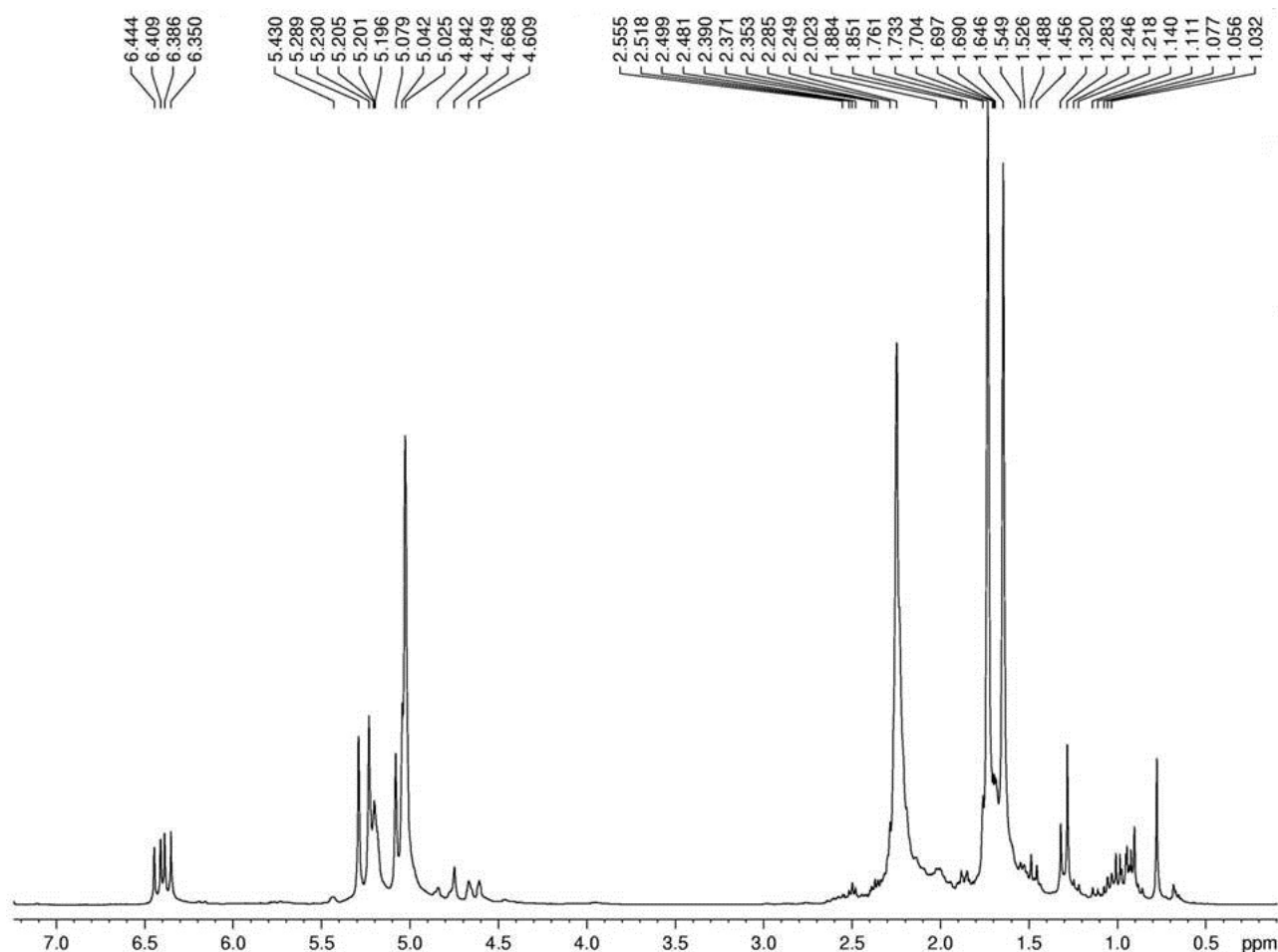


Figure 2 - ^1H NMR (300 MHz, CDCl_3) spectrum of the essential oil from green fruits of *Protium ovatum*.

Table 2 - Trypanocidal activity of the essential oil from green fruits of *Protium ovatum* against trypomastigote forms of *Trypanosoma cruzi*.

	% of lysis \pm S.D./concentration ($\mu\text{g/mL}$)					
	200	100	50	25	12.5	IC_{50} ($\mu\text{g/mL}$)
EO	77.2 \pm 5.8	81.6 \pm 4.9	88.4 \pm 1.0	91.0 \pm 0.4	97.6 \pm 0.4	1.2

EO: essential oil from green fruits of *Protium ovatum*. S.D: standard deviation

Table 3- Cytotoxic activity of the essential oil from green fruits of *Protium ovatum*

	% of lysis \pm S.D./concentration ($\mu\text{g/mL}$)							
	6.25	12.5	25	50	100	200	400	CC_{50} ($\mu\text{g/mL}$)
EO	100 \pm 0	100 \pm 0	100 \pm 0	100 \pm 0	90.2 \pm 5.0	85.7 \pm 1.4	64.3 \pm 2.3	550.3

EO: essential oil from green fruits of *Protium ovatum*. S.D: standard deviation

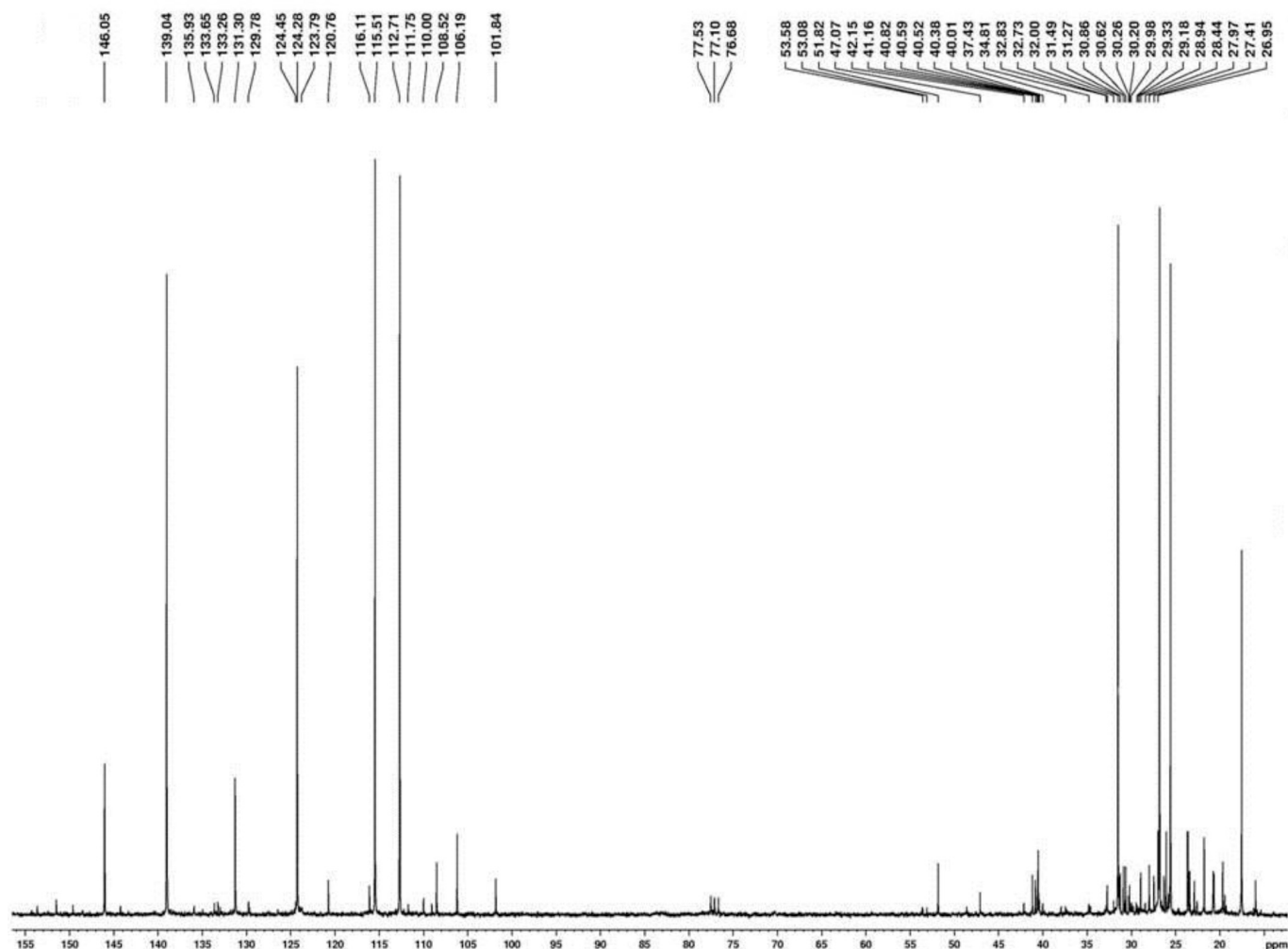


Figure 3 - ^{13}C NMR (75 MHz, CDCl_3) spectrum of the essential oil from green fruits of *Protium ovatum*.

Conclusion

In summary, the results of this study showed that the essential oil from green fruits *Protium ovatum* found in the Brazilian Cerrado, which is located in the central-west region of the country, has promising antiparasitic potential with no cytotoxicity towards LLCMK₂ adherent epithelial cells. The high concentration of β -myrcene (62.0 %) in the essential oil from green fruits investigated by this study is a prospect of a new source of this secondary metabolite as a raw material in the synthesis of new medication. Further studies with *in vivo* and field experiments must be carried out to ascertain its efficiency. However, to the best of our knowledge, this was the first time that the chemical composition of the essential oil from green fruits of *P. ovatum* was described. It is very important to the knowledge of this botanical species.

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