SUPPLEMENTARY MATERIAL

Chemical composition of essential oils from different parts of Protium heptaphyllum (Aubl.) Marchand and their in vitro antibacterial activity

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In Brazilian folk medicine, Protium heptaphyllum is used to treat inflammatory conditions and to hasten wound repair. This paper aims to investigate the chemical composition and the in vitro antibacterial effects of the essential oils (EOs) obtained from P. heptaphyllum leaves and ripe and unripe fruits against a representative panel of oral pathogens. The GC-FID and GC-MS analysis revealed that the major components determined in P. heptaphyllum essential oils were myrcene (59.0%), β-elemene (17.2%), limonene (12.9%), spathulenol (12.6%), α-cubebene (11.6%), germacrene D (10.6%), \textit{trans}-nerolidol (9.8%), and α-cadinol (8.8%). The essential oils of the ripe and unripe fruits showed the strongest antibacterial activity against the anaerobic bacteria \textit{Prevotella nigrescens} (MIC = 50 µg/mL). The leaf essential oil displayed very promising activity against \textit{Streptococcus mutans} (MIC = 50 µg/mL) and \textit{Streptococcus mitis} (MIC = 62.5 µg/mL). The antibacterial activity of EOs against oral pathogens is also described for the first time.

\textbf{Keywords:} Protium heptaphyllum; essential oils; cariogenic bacteria; periodontopathic bacteria; oral pathogens; antibacterial activity.
**Experimental**

The plant material was collected at “Instituto Federal Goiano – Campus Iporá” in the city of Iporá (16°24’14.9”S and 51°06’40.0”W), State of Goiás, Brazil, in July 2017, at 9 a.m. The plant was identified by the botanist Luzia Francisca de Souza and a voucher specimen of *Protium heptaphyllum* (HJ1036) was deposited at the Herbarium Jataiense Professor Germano Guarim Neto. The essential oils of *P. heptaphyllum* were extracted from leaves and unripe and ripe fruits by hydrodistillation in a Clevenger-type apparatus for 2 h. Hydrodistillation was performed in triplicate. The plant material was divided into three samples of 500 g each, and 500 mL of distilled water was added to each sample. After manual collection of the essential oils (EOs), traces of water remaining in the oil were removed with anhydrous sodium sulfate, which was followed by filtration. The EOs were stored in an amber bottle and kept in a refrigerator at 4°C until analysis. The EO yield was calculated from the weight of the fresh leaves, unripe and ripe fruits and expressed as the average of the triplicate analyses.

EOs were dissolved in ethyl ether and analyzed by Gas chromatography-flame ionization detector (GC-FID) and gas chromatography–mass spectrometry (GC–MS) using the Shimadzu QP5000 Plus and GCMS2010 Plus (Shimadzu Corporation, Kyoto, Japan) systems. The temperature of the column in the GC-FID was programmed to rise from 60 to 240°C at 3°C/min and was held at 240°C for 5 min; the carrier gas was H₂ at a flow rate of 1.0 mL/min. The equipment was set to operate in the injection mode; the injection volume was 0.1 µL (split ratio of 1:10), and the injector and detector temperatures were 240 and 280°C, respectively. The relative concentrations of the components were obtained by normalizing the peak areas (%). The relative areas consisted of the average of triplicate GC-FID analyses. The GC-MS conditions and the identification of the essential oils have been previously reported (Melo et al., 2015). Identification of the volatile components of essential oils of *P. heptaphyllum* (Table S1) was based on their retention indices on an Rtx-5MS (30 m X 0.25 mm; 0.250 µm) capillary column under the same operating conditions used for GC relative to a homologous series of n-alkanes (C₈–C₂₀). The structures were computer-matched with Wiley 7, NIST 08, and FFNSC 1.2, and their fragmentation patterns were compared with literature data (Adams, 2007).

The minimum inhibitory concentration (MIC) values of the EOs were calculated using the broth microdilution method in 96-well microplates. The following ATCC standard strains were used: *Streptococcus sobrinus* (ATCC 33478), *Streptococcus salivarius* (ATCC 25975), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguinis* (ATCC 10556), *Bacteroides fragilis* (ATCC 25285), *Actinomyces naeslundii* (ATCC 19039), and *Prevotella nigrescens* (ATCC 33563). Individual 24-hour colonies formed on blood agar (Difco Labs, Detroit, Mich, USA) were suspended in 10.0 mL
tryptic soy broth (Difco). Standardization of each microorganism suspension was carried out as previously described (Ferreira et al., 2010). The EOs samples were dissolved in DMSO (Merck, Darmstadt, Germany) at 1 mg/mL and diluted in tryptic soy broth (Difco) so that concentrations in the range from 400 to 3.9 µg/mL would be achieved. The final DMSO concentration was 5% (v/v) and this solution was used as negative control. One inoculated well was included to control the adequacy of the broth for organism growth. One non-inoculated well free of antimicrobial agent was also included to ensure medium sterility. Chlorhexidine dihydrochloride (CHD) (C8527 Sigma) was dissolved in tryptic soy broth (Difco) and used as positive control at concentrations ranging from 59.0 to 0.115 µg/mL. The microplates (96 well) were sealed with plastic film and incubated at 37°C for 24h as described above. After incubation, 30 µL of 0.02% resazurin (199303 Sigma, Stl Louis, MO, USA) aqueous solution were poured into each microplate well to indicate microorganism viability. The MIC values were determined as the lowest concentration of the EOs capable of inhibiting microorganism growth. Three replicate assays were carried out for each microorganism.

References
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Table S1. Chemical constituents of the essential oils from *Protium heptaphyllum* (Aubl.) Marchand.

| Compounds              | \( RT_{exp} \) | \( RT_{lit} \) | % RA | Leaf | Unripe fruit | Ripe fruit |
|------------------------|----------------|---------------|------|------|--------------|------------|
| \( \alpha \)-Pinene    | 933            | 939           | 0.3  |      | 59.0         | 31.2       |
| Myrcene                | 992            | 991           | 3.3  |      |              |            |
| 3-Carene               | 1011           | 1011          | 0.4  |      |              |            |
| Limonene               | 1029           | 1031          | 6.8  |      | 3.8          | 12.9       |
| \( cis \)-\( \beta \)-Ocimene | 1041       | 1040          |      | 2.1  | 2.5          |            |
| Perillene              | 1102           | 1099          | 0.8  |      | 1.7          |            |
| \( \alpha \)-Cubebene  | 1355           | 1351          | 9.0  |      |              |            |
| Isoledene              | 1372           | 1373          | 1.3  |      |              |            |
| \( \beta \)-Elemene    | 1378           | 1375          | 17.2 | 8.2  | 1.2          |            |
| \( \alpha \)-Gurjunene | 1403           | 1409          | 0.8  |      |              |            |
| \( trans \)-Caryophyllene | 1414       | 1418          | 6.2  | 2.2  | 1.9          |            |
| \( \beta \)-Humulene   | 1444           | 1440          | 2.2  |      |              |            |
| \( \alpha \)-Humulene  | 1452           | 1454          | 0.9  |      |              |            |
| Germacrene D           | 1474           | 1480          | 2.1  | 10.6 |              |            |
| \( \beta \)-Selinene   | 1479           | 1485          | 3.7  |      |              |            |
| \( \alpha \)-Selinene  | 1488           | 1494          | 2.4  |      |              |            |
| Bicyclogermacrene      | 1490           | 1494          |      | 2.0  |              |            |
| Germacrene A           | 1499           | 1505          | 1.3  |      |              |            |
| \( \gamma \)-Cadinene  | 1507           | 1513          | 0.9  |      |              |            |
| \( \delta \)-Cadinene  | 1516           | 1524          | 3.2  | 1.9  |              |            |
| Caryophyllene oxide    | 1548           | 1549          | 2.1  |      |              |            |
| \( trans \)-Nerolidol  | 1558           | 1564          |      | 9.8  |              |            |
| Palustrol              | 1565           | 1557          | 2.7  |      |              |            |
| Spathulenol            | 1575           | 1576          | 12.6 | 7.8  |              |            |
| Epiglobulol            | 1588           | 1588          | 2.5  |      |              |            |
| Viridiflorol           | 1589           | 1590          | 4.1  | 1.2  |              |            |
| Globulol               | 1600           | 1590          | 0.8  |      |              |            |
| Rosifoliol             | 1604           | 1599          | 7.6  |      |              |            |
| 1,2-Humulene epoxide   | 1605           | 1606          | 3.7  | 0.8  |              |            |
| \( \delta \)-Cadinol   | 1639           | 1636          | 3.9  | 0.7  |              |            |
| \( \beta \)-Eudesmol   | 1650           | 1654          |      | 0.6  |              |            |
| \( \alpha \)-Cadinol   | 1654           | 1653          | 5.0  | 8.8  | 6.5          |            |

| Monoterpene hydrocarbons |            | 3.1  | 64.9 | 46.7 |
| Sesquiterpene hydrocarbons |          | 58.7 | 23.1 | 5.0  |
| Oxygenated sesquiterpenes |          | 34.0 | 8.8  | 37.5 |
| Others                    |            | 0.8  | 1.7  |      |

| Total identified          | 96.6        | 96.8 | 90.9 |

\( RT \): Retention time; \( RI_{exp} \): Retention index determined relative to \( n \)-alkanes (\( C_8-C_{20} \)) on the Rtx-5MS (30 m X 0.25 mm; 0.250 \( \mu \)m) column; \( RI_{lit} \): Retention index from the literature (Adams, 2007); RA\%: relative area (peak area relative to the total peak area in the GC-FID chromatogram), average of three replicates.