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Editorial

Pharmacognosy Journal is now published by Elsevier: Moving ahead

It is a great pleasure to write an editorial for this issue being published by Elsevier – one of the major publishers in the world. Pharmacognosy Journal is one of the journals published by Phcog.Net and it has grown to become an extremely successful scientific journal in the field of natural products. The journal has a global readership and authorship. It has successfully published 34 issues since 2009 and is one of the most read journals in the fraternity of Medicinal Plants and Pharmacognosy.

Natural products are the most consistent and successful source of drugs and different plant species, and medicinal properties have been assigned to several thousands of them. Many major institutes and research centers are currently involved in exploring this opportunity to investigate newer drugs from medicinal plants. Researchers believe in combining the strengths of active compounds from medicinal plants with modern scientific techniques such as NMR, MS, and chromatography to provide new functional leads with high therapeutic value in a short span of time. But natural products research often faces many hurdles, which hinders drug development. We believe that natural products research information can potentially benefit many researchers involved in this area. We also believe that research on natural products is often delayed by a lack of necessary information about medicinal plants. Phcog.Net – a platform for Natural Product Researchers was created to combat these problems. It makes innovative use of the best tools for information dissemination to overcome the hurdles to Natural Product Research.

Phcog.Net is a non-profit network dedicated to Natural Products Research to develop promising drugs. Our main mission is to make information on herbal drug research readily available in different formats to suit the individual needs of anyone who wants it. Our long term objective is to provide high quality, accurate, and necessary information to enhance herbal drug research. We invite you to join our network and make your research more visible. Visit www.phcog.net for more details.

Currently, we are working on classifying different sections within the journal scope and we will be introducing our section editors in the forthcoming issue. We are also seeking scholars and mentors from around the world who are interested in being a part of various volunteer Editorial and Review Boards of Phcog J. We are constantly working on improved editorial assistance to authors and rapid turnaround for their submitted papers.

We also welcome original research articles, full-length reviews, short reviews, and general articles in the field of natural products. We have also recruited a good number of reviewers, who are committed to timely and high-quality appraisal of articles submitted to the journal. Our main goal is to provide high quality publication and publish the most important research and review articles which fall within the scope of the journal. Finally, on behalf of the Editorial team, we would like to thank all reviewers for their support and dedication toward the growth of *Pharmacognosy Journal*.

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Research article

Cytotoxic effects of *Anagallis arvensis* and *Anagallis foemina* in neuronal and colonic adenocarcinoma cell lines

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ABSTRACT

Anagallis arvensis L. and *Anagallis foemina* Mill. (Primulaceae) have been used in Navarra (Spain) as wound healing remedies within a context of traditional medicine. The species have previously demonstrated antimicrobial and COX-inhibiting properties. Cytotoxic effects of the plants have never been established though they are popularly known to be toxic at high doses and/or long term oral administration. Cytotoxicity was evaluated in PC12 and DHD/K12PROb cells using spectrophotometric methods such as the MTT and LDH assays. Both plants reduced cell survival and induced cell damage (LDH release) in a dose-dependent manner, PC12 cells being more sensitive to the extracts than DHD/K12PROb cells. Methanol extracts were significantly more cytotoxic and doses over 80 μg/ml reduced cell survival above 50%. Results suggest that these plants may be responsible for the toxic effects that have been described in traditional medicine.

1. Introduction

Anagallis arvensis L. and *Anagallis foemina* Mill. (Primulaceae) are known as “the scarlet pimpernel” and “the blue pimpernel” respectively due to its red and blue flowers. However, the scarlet pimpernel (*A. arvensis*) also has a variety with blue flowers (*A. arvensis* L. var. *caerulea* Gouan), which is very similar to the blue pimpernel (*A. foemina*).[1] The taxonomic difference between the species is that the corolla lobe margins in *A. arvensis* is fringed with a large number (35–70) of 3-celled glands where the top cell is enlarged, whereas *A. foemina* has a smaller number of 3 or 4-celled glands (less than 30) with all cells of equal size.[2]

Both species are used in Navarra (Iberian Peninsula, Spain) regarding wound healing properties in human and veterinary ethnomedicine.[3–5] Aerial parts of both species are used to prepare an ointment for the treatment of external infections such as and wounds or infected spots; in some cases, an infusion is prepared with the plants to threat internal or systemic infections though they are popularly known to be toxic at high doses or long term consumption.[6]

In this sense cytotoxicity was evaluated in two cell lines: PC12, which is used as a model of dopaminergic cells in neurosciences, and DHD/K12PROb considered a model of colon adenocarcinoma cells.

2. Material and methods

2.1. Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma–Aldrich. The LDH cytotoxicity detection kit was purchased from Roche (Indianapolis, USA). Culture media, penicillin-streptomycin, fetal bovine serum (FBS), horse serum (HS), sodium pyruvate and Dulbecco’s phosphate buffered saline (PBS) were obtained from Gibco (Barcelona, Spain).

2.2. Plant material

Aerial parts of *A. arvensis* and *A. foemina* were collected in Navarra in spring time 2008 and authenticated by Silvia Akerreta and Rita Yolanda Cavero (Department of Plant Biology, University of Navarra). Voucher specimens have been deposited in the PAMP Herbarium of the University of Navarra: *A. arvensis* (PAMP 18927) and *A. foemina* (PAMP 18718).

2.3. Preparation of extracts

Lyophilized methanol and aqueous extracts prepared for a previous study[7] and kept at −40 °C were used for *in vitro* cytotoxicity assays. Extracts were reconstituted in PBS and filtered before use.

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2.4. Assessment of cell viability

2.4.1. Cell culture

Rat pheochromocytoma (PC12) cells were obtained from the American Type Culture Collection. Cells were grown in DMEM supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, penicillin (10 U/ml), streptomycin (10 μg/ml) and 0.2 mM sodium pyruvate. DHD/K12PROb cells were obtained from a colon adenocarcinoma induced in syngenic BD-XI rats and maintained in a mixture of DMEM and Ham's F-10 supplemented with 10% fetal bovine serum and 0.01% gentamicin. Cultures were incubated in the presence of 5% CO_2 at 37 °C and 100% relative humidified atmosphere.

2.4.2. Cell survival by the MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a yellow tetrazolium salt that is converted into a purple compound (formazan) in viable cells by mitochondrial enzymes. This property is used as an index of cell survival.[8] PC12 cells were seeded in 96-multiwell plates at 2×10^4 cells/well. After 48 h, the medium was replaced by low serum medium (1% heat-inactivated horse serum), the plant extracts were added to the wells at different concentrations and incubated for 24 h. Then, the medium was removed and an MTT-1% DMEM solution (0.3 mg/ml MTT final concentration) was added for 1 h incubation at 37 °C. The MTT was removed and formazan crystals were dissolved in DMSO. Absorbance was measured at 550 nm in a microplate reader (Bio-Tek, USA). DHD/K12PROb cells were also seeded at 2×10^4 cells/well. After 24 h, cells were treated with plant extracts and incubated for 24 h. Cell survival was measured as above but using DMEM-Ham's F-10 supplemented with 10% fetal bovine serum and 0.01% gentamicin as culture medium.

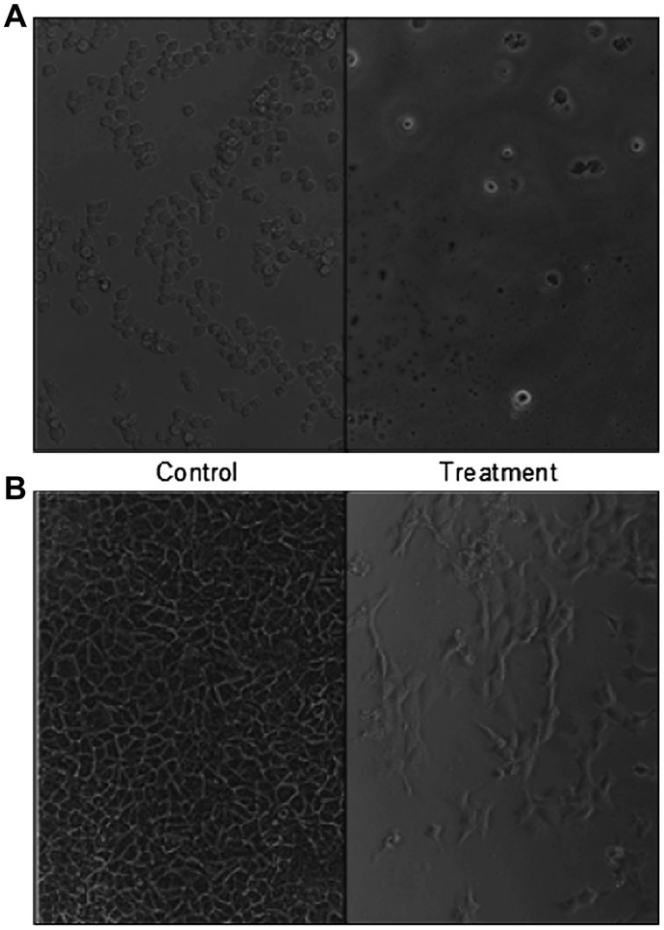

Fig. 1. Changes in morphology and growth of PC12 (A) and DHD/K12PROb (B) cells after treatment with 60 μg/ml of methanolic extract obtained from *Anagallis arvensis*.

2.4.3. Cell death by the LDH assay

Cytotoxicity of extracts was also studied by the determination of lactate dehydrogenase (LDH) into the incubation medium using a commercial kit from Roche. LDH is a cytosolic enzyme released into the medium when the integrity of the cell membrane deteriorates suffering from necrotic cell death.[9] Cells were seeded in 96-multiwell plates at 2×10^3 cells/well. After 48 h incubation, cells were exposed to different concentrations of plant extracts with renewed 1% DMEM for 24 h. DHD/K12PROb were seeded at the same density but treated after 24 h incubation. The assay was carried out following the instructions of the manufacturer. Background interferences were deducted by calculating the LDH activity of the medium. Spontaneous release of LDH was also calculated by measuring LDH activity of untreated cells. Total intracellular LDH was measured in cell lysates obtained by treatment with the manufacturer lysis solution.

2.5. Statistical analysis

Data are expressed as means ± S.D. of three independent experiments. One-way ANOVA followed by Dunnett's multiple comparison tests was used to compare control and treatments.

3. Results

Cellular damage induced by methanol and aqueous extracts after 24 h treatment was observed under a phase contrast

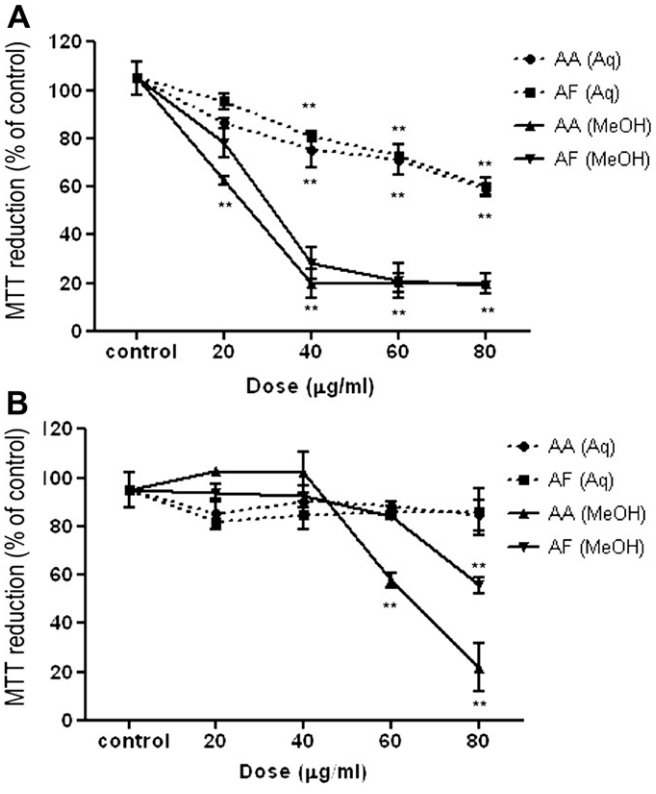

Fig. 2. Cell viability by the MTT assay in PC12 (A) and DHD/K12PROb (B) cells. $^{**}p < 0.01$ versus control. AA: *Anagallis arvensis*; AF: *Anagallis foemina*; MeOH: methanolic extract; Aq: aqueous extract. Triton X-100 (5%) was used as control substance to induce cell death.

microscope (Fig. 1) and quantified by the MTT and LDH assays (Figs. 2 and 3). Both spectrophotometric methods (MTT and LDH) produce complementary data as the MTT assay consist of measuring cell survival whereas the LDH method evaluates cellular damage trough lactate dehydrogenase leakage.

Changes in cell morphology were observed. Most of them lost their polygonal shape becoming smaller and circular and loosing their ability to adhere to the plate surface.

Fig. 2 shows a significant reduction on PC12 and DHD/K12PROb cell survival. The viability of PC12 cells measured by the MTT was greatly reduced in a dose-dependent manner when cells treated with increasing doses (20–80 μg/ml) of methanol and aqueous extracts of *A. arvensis* and *A. foemina*. DHD/K12PROb cells were sensitive to methanol extracts of both species, but not to the aqueous extracts. According to other studies regarding cytotoxicity of medicinal plants, we could state that the cytotoxic events in this case are moderate.

Data of LDH assay (Fig. 3) confirmed what was observed by the MTT method, but in this case in terms of released LDH into the incubation medium. In general, methanol extracts were much more cytotoxic than aqueous and PC12 cells, considered as neuronal-like cells, were more sensitive to the plants than colonic adenocarcinoma cells (DHD/K12PROb). However, significant differences within plant species were not observed.

4. Discussion

A. arvensis and *A. foemina* are traditionally used in the province of Navarra (Iberian Peninsula) for dermatological purposes related to external infections. A previous study by the authors revealed that both species exerted antimicrobial and anti-inflammatory properties as well as presence of saponins and flavonoids in the samples.[7] In certain situations, the plants can be administered orally as infusions to treat internal infections; however, the plants are reported as toxic species during long term oral consumption. Other studies concluded that *A. arvensis* possesses antioxidant,[10] antifungal,[11] molluscicidal,[12] and poisoning[13,14] effects but we demonstrate for the first time the toxic effects of the plants using *in vitro* cell systems.

The extracts were tested in two different cell lines: PC12 cells, commonly used in neurosciences due to its phenotypic characteristics with sympathetic neurons[15] and DHD/K12PROb cells, used as model for antitumor agents against colon adenocarcinoma.[16] The traditional knowledge indicated that those plants can be very toxic and some studies put forward the idea that *A. arvensis* works as a poison in animals.[13] Saponins, that have been previously detected in the extracts[7,17,18] might be involved in the cytotoxic phenomenon as some of them are known to interact with cellular membranes increasing permeability and producing cell damage.[19]

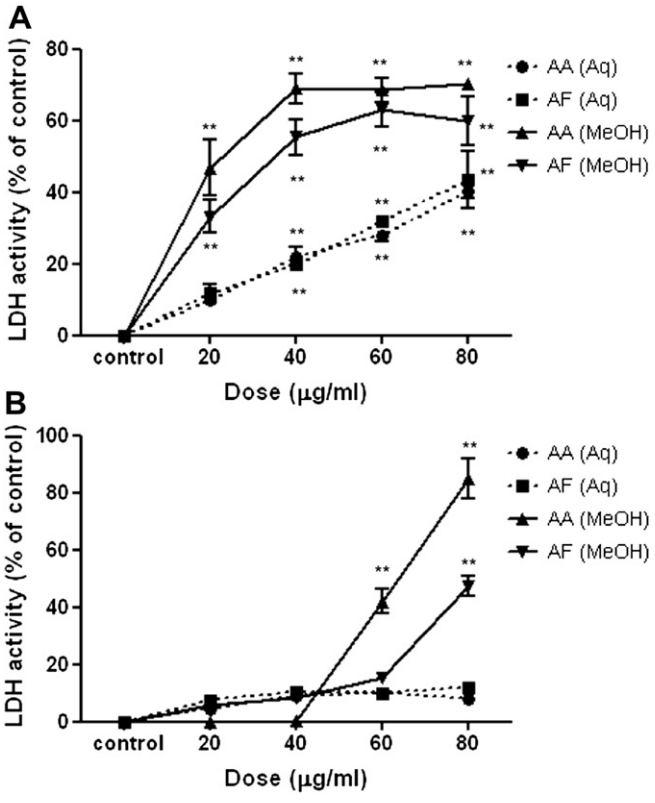

Fig. 3. Cytotoxicity of plant extracts by the LDH assay in PC12 (A) and DHD/K12PROb (B) cells. $^{**}p < 0.01$ versus control. AA: *Anagallis arvensis*; AF: *Anagallis foemina*. MeOH: methanolic extract; Aq: aqueous extract. Triton X-100 (5%) was used as control substance to induce cell death.

5. Conclusions

Results indicate that these plants may be responsible for the toxic effects that have traditionally been described. In general, methanol extracts were much more cytotoxic than aqueous and PC12 cells, considered as neuronal-like cells, were more sensitive to the plants than colonic adenocarcinoma cells. Further studies with the extracts may be of interest to identify new leads and compounds with cytotoxic properties.

Conflicts of interest

All authors have none to declare.

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Research article

Antiedematogenic and antinociceptive effects of leaves extracts from *Protium spruceanum* Benth. (Engler)

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ABSTRACT

Ethnopharmacological relevance: In traditional medicine, gums and oil-resins from *Protium* species have been used for many diseases, however, there are no reports of studies of *Protium spruceanum*' leaves.
Materials and methods: The antiedematogenic and antinociceptive effects of crude ethanol extract (EEB) from leaves of *P. spruceanum* and its fractions were evaluated in biological models. The fractions were obtained with hexane (FHEX) and methanol (FMEOH). Rat paw edema induced by carrageenan, writhing test, formalin test, hot plate test and the toxicity of EEB were performed.
Results: Phytochemical analysis has shown the presence of α and β-amyrins as major constituents of FHEX. Promising results of anti-inflammatory and antinociceptive activity were found for EEB, FHEX and FMEOH. Also were observed the EEB at 6000 mg/kg showed no toxicity.
Conclusions: One might suggest that the activities of FHEX are due to the presence of α and β-amyrins and contributes to the biological activities of EEB.

1. Introduction

The Burseraceae family comprises 21 genera with 700 species divided in three major tribes: Proteae (three genera), Canarieae (eigth genera) and Burseraceae (seven genera).[1] These species are widely spread throughout tropical and subtropical regions, mainly in the Amazon Rain Forest.[2,3] It can be found all over Brazil, where the genus *Protium* makes up 80% of the Burseraceae family.[1]

This family has been a well-known source of exudates and oil-resins rich in aromatic substances that are used in perfumery industries,[4] and appliances by native tribes in the regions where they are found, as anti-inflammatory and insect repellant.[5]

The *Protium* genus (Tribe *Proteae*) has been known as the main family member with 150 species,[1] and it is the most representative. Species of *Protium* are widely found in all countries of South America,[3,6,7] especially in the Rain Forest. The oil-resins and gums produced by many species of this genus are commonly known as "breus".

In traditional medicine, gums and oil-resins from *Protium* species have been used for many diseases, *e.g.* as tonic and stimulant, expectorant, as analgesic and anti-inflammatory agent, healing of ulcers and as insect repellant.[2,6,7]

Phytochemical studies performed on gums and oil resin, obtained from *Protium heptaphyllum* revealed the presence of binary mixtures of triterpenes, especially α- and β-amyrins.[8–10] In the essential oil of *P. heptaphyllum* was detected p-cymene (39.93%) and α-tetradecane (13.38%) as the main constituents.[1] The pharmacological assays using substances isolated from species of *Protium* reveal proprieties as gastroprotective and anti-inflammatory,[11] antiallergic,[12] hepatoprotective effect.[13,14] These biological activities have being attributed to the pentacyclic triterpenes mixture α- and β-amyrin, major component of the resin[10] and also due to the presence of compounds with hydroxyl groups.[11] The mixture of these two triterpenes is frequently found in medicinal plants mainly in species of the genera *Maytenus*.[15] The triterpenes α- and β-amyrin are considered the most important constituents of non-polar fractions and resins obtained from species of the genus *Protium*.[8–10]

P. spruceanum (Fig. 1) grows in abundance in the regions of Amazon, the rain forest, "Mata Atlântica" and in "Cerrado", the most extensive woodland-savanna in South America, characterized by a pronounced dry season. This specie is popularly known as

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Fig. 1. Photograph showing *Protium spruceanum* Benth. aerial parts.

"almécega-de-casca-lisa" or "breu". Reports on its floristic relationships[16,17] and its differences in chemical composition of essential oils obtained from leaves, branches and resin associated to edaphical conditions[18] have been described.

Based on the traditional medicine uses related to this genus, the analgesic activity was evaluated using the hot plate method in mice and acetic acid-induced writhing test. The anti-inflammatory action was studied by rat paw edema induced by carrageenan. This manuscript reports the pharmacological properties of *P. spruceanum* Benth. (Engler) for the first time.

2. Materials and methods

2.1. Plant material and extract EEB preparation

Leaves of *P. spruceanum* were collected around the Lavras City, Minas Gerais, Brazil (between coordinates 21°17′33, 6″S e 44°59′15, 1″W, 21°18′11, 9″S e 44°59′18, 8″W″). A voucher specimen was deposited at the Herbário da Universidade Federal de Lavras (No 16399 HESAL). The leaves were dyed at room temperature and fragmented in a mill. The powder (200.0 g) was subjected to exhaustive percolation with ethanol. The ethanol was recovered using a rotary evaporator, yielding the ethanolic extract [EEB (52.39 g, 26.2%)]. The crude extract EEB (35.05 g) was resuspended in MeOH:H_2O (9:1) and subjected to liquid–liquid partition with hexane to obtain two components: hexanic fraction [FHEX (2.84 g, 08.10%)] and methanolic fraction [FMEOH (27.05 g, 77.16%)].

2.2. Isolation of α- and β-amyrin

Fraction FHEX (2.0 g) was subject to column chromatography on silica gel 60 (70–230 mesh), glass column (2.0 cm internal diameter), using hexane, dichloromethane, ethyl acetate and methanol on a gradient polarity elution technique, producing 166 fractions of 10 mL each. The fractions were analyzed by thin layer chromatography (TLC) using vanillin-perchloric acid solution as the revealing reagent. Fractions with similar TLC patterns based on retention factor (Rf) were combined and selected for subsequent bioassays.

The group of fractions fr97–135 (300.0 mg), eluted with hexane:dichloromethane (70:30 to 50:50), was submitted to silica gel column chromatography using a glass column (diameter 0.5 cm) at the similar conditions mentioned above. Thirty three fractions of 10 mL were collected and the group of fractions fr7–9, after recrystallization in dichloromethane, had presented white solid crystals (melting point: 176.5–177.5 °C). By TLC analysis, only a single constituent was observed both under UV light (254 nm) and after treatment with vanillin-perchloric acid, it produced a pink to purple spot. The solid material of fraction fr7–9 (16.95 mg) was analyzed through ^{1}H and ^{13}C NMR spectrometry.

2.3. Animals

For the animal assays, health male Swiss mice (*Mus musculus*), weighing 25–30 g, were supplied by the Centro de Ciência Animal of Universidade Federal de Ouro Preto. They were randomly divided into groups ($N = 6$ per cage). All experimental animals were kept in controlled conditions of temperature (25 ± 2 °C) and relative humidity (~80%), at 12 h light–dark cycles. All mice were fed with standard pellet diet *ad libitum* and allowed free access to drinking water. The animals were solid food-deprived for 12 h, and housed acclimatized to laboratory conditions 30 min prior the assays. All experiments involving animals were performed with strict adherence to ethical guidelines (protocol N° 2010/07) accepted by institutional Animal Care and Use Committee.

2.4. Preparation of samples

The extract and its fractions were dissolved in saline solution of Tween 80 (3%). Volume solutions of 0.2 mL corresponding to doses of 200.0, 300.0 or 400.0 mg/kg were orally administered in each experimental animal ($N = 6$). The control group was treated only with Tween 80 saline solution.

2.5. Acute toxicity

The LD_{50} (50% lethal dose) was evaluated for the acute toxicity. Doses ranging from 200.0 to 6000.0 mg/kg of EEB were orally administered to the experimental animals ($N = 6$, per group). According to the original protocol suggested by Litchfield et al,[19] all animals were observed during 48 h to detect toxicity signs such as convulsions, diarrhea, alertness, piloerection, sedation, ptosis, urination, spontaneous motor activity and death.

2.6. Paw edema induced by carrageenan

Anti-edema effect was evaluated through induced carrageenan paw edema method according to previously described by Winter et al.[20] Thirty minutes after the administration of EEB, its fractions or the standard indomethacin (10.0 mg/kg, orally, 0.2 mL/animal) each animal was treated by giving an intraplantar injection of 0.1% carrageenan (Sigma, St Louis) in a 20.0 μL volume into the right hind paw using a 26 gauge needle. The opposite paw was treated with 20.0 μL volume of saline solution. The volumes of the carrageenan injected paws edemas of mice (mm) were measured 1 h prior to the injection of carrageenan and at hourly interval for 7 h after the injection using a Starrett digital caliper (300 mm 12″ × 0.005″ 0.01 mm resolution).[21]

The anti-edema effect was estimated based on the difference of right and left paw volume. Relevant difference between treated or non-treated paws volumes, compared with control group (vehicle) was considered as positive anti-edema response.

2.7. Antinociceptive activity

Three models, acetic acid-induced abdominal writhing response,[22] hot plate method[23] and formalin induced hind paw licking[24] were employed to study the antinociceptive effect of the extract EEB and its fractions.

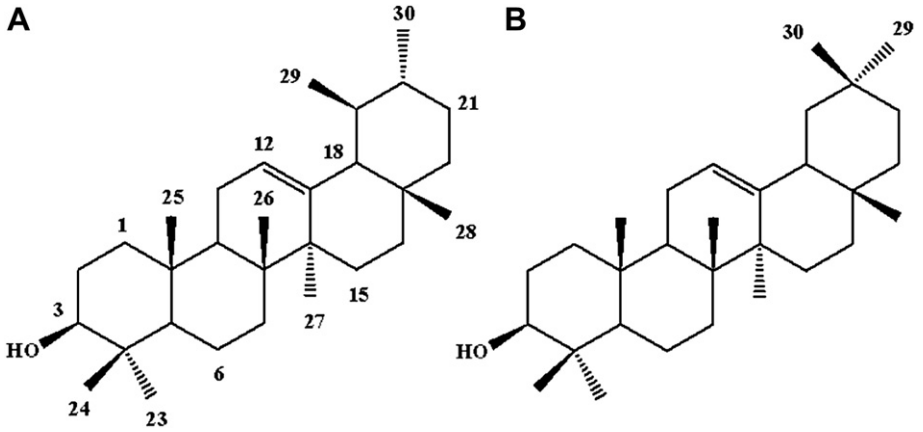

Fig. 2. Chemical structure of α-amirin (A) and β-amirin (B).

2.8. Acetic acid-induced abdominal writhing response

Acetic acid (0.2 mL of 0.6% v/v solution) was administered intraperitoneally to all groups at the dose of 10 ml/kg body weight 30 min after the application of extract EEB, its fractions, and indomethacin (10.0 mg/kg, orally, 0.2 mL/animal) used as standard drug administration.[22] An abdominal constriction is indicated as the full extension of hind limb. The number of abdominal constrictions (writhing) and stretching with a shudder of the hind limb was counted for 25 min after administering acetic acid. Percent protection against writhing movement was taken as index of antinociception. Antinociceptive activity was expressed as the percentage inhibition of abdominal constrictions among control animals and mice pre-treated ($N = 6$) with the EEB, its fractions using the formula (Control mean−treated mean) × 100/Control mean. A statistical reduction in the contortions number compared with control group was considered positive antinociceptive response.

2.9. Analgesic response using hot plate method

Mice were divided into three groups consisting of six animals in each group. After 30 min of oral administration of extract EEB and its fractions, and morphine (7.5 mg/kg, 0.2 mL/animal) used as standard drug. Each animal was placed on the Eddy's hot plate (56 ± 2 °C). Then, the latency time (LT) was recorded as the nociceptive effect in the animals, expressed through its reflex of lick, jump, tap dance or stand on its hind paws. The reaction time in control and treated animals was recorded at 0, 30, 60, 90 and 120 min after the treatment. The maximum animal stay on hot plate was 30 s to prevent tissue damage.[23]

2.10. Formalin induced hind paw licking in mice

The nociception action was induced injecting 30 μL of dilute formalin (1.5% in saline solution) under the skin of the dorsal surface of the hind paw of the mice. Each animal was challenged with formalin 30 min after being pretreated with standard indomethacin (10.0 mg/kg, 0.2 mL/animal); morphine (7.5 mg/kg, 0.2 mL/animal) or dipyrone (200.0 mg/kg, 0.2 mL/animal) and the test extract EEB and its fractions. The animals were placed into a transparent glass container. The licking response was monitored 0–5 min (phase I or neurogenic phase) and 20–25 min (phase II or inflammatory phase) starting immediately after the injection of formalin. The amount of time spent licking the injected paw was considered as indicative of pain. Antinociception was defined as a statistical reduction in the time spent in licking the injected paw in comparison with the control group during the phases I and/or II.

2.11. Statistical analysis

The statistical analysis of the results was carried out using one-way analysis of variance (ANOVA) followed by multiple comparison test of Bonferroni. The results obtained in the study were compared with the control group. P values < 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Chemical analysis of α- and β-amyrins

The signals observed in the ^{13}C NMR spectra of fr7–9 were in accordance to those reported by Mahato & Kundu[25] for α and β-amyrins (Fig. 2). The signal at δ_C 79.31 and δ_C 79.34 were respectively attributed to C-3 of α and β-amyrin. The signals at δ_C 124.71 and 122.01 (C-12), and at δ_C 139.87 and 145.47 (C-13) were associated to double bond present in these two pentacyclic triterpenes. Characteristic signals also were observed at δ_C 40.08 and 31.36 attributed to C-20 of α and β-amyrin, respectively. The signal at δ_C 17.74 and 33.23, associated to C-29, as well as the signal at δ_C 21.66

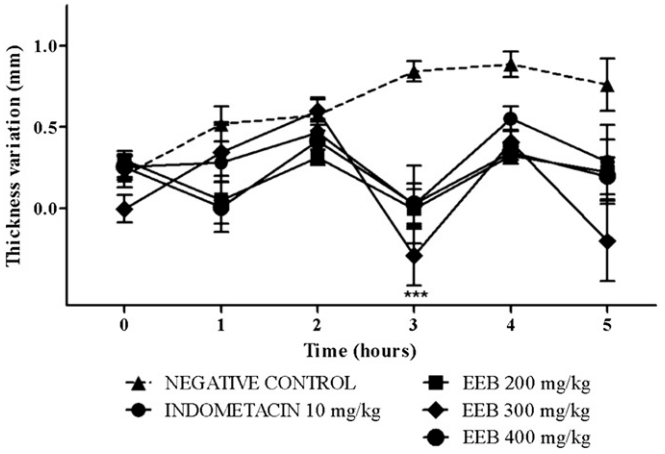

Fig. 3. Effect of EEB (200, 300 and 400 mg/kg, 0.2 mL/animal, v.o.) on mice paw edema induced by carrageenan.

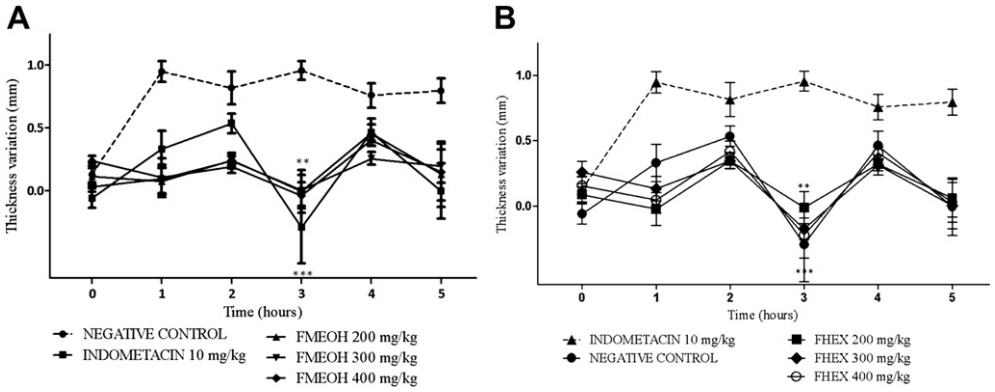

Fig. 4. Effect of FMEOH (A) and FHEX (B) on mice paw edema induced by carrageenan.

and 23.97 attributed to C-30 are other chemical shift assignments used to establish the structures of α- and β-amyrin.

Reports describing phytochemical studies of *Protium* species have evidenced the presence of α- and β-amyrins mainly in non-polar extracts.[2,8,26] The mixture of these triterpenes was considered as being responsible for the anti-inflammatory, analgesic, anti-pruritic, anti-ulcer and hepatoprotective properties found in species of the genera *Protium*.[27] One possible explanation for pharmacological activity of this mixture is related to the presence of the perhydroaromatic ring, which is closely similar to the molecular structure of steroidal drugs.[12]

3.2. Acute toxicity

The extract from leaves of *P. spruceanum* was administered orally to mice ($N = 6$) in order to evaluate the acute toxicity. The extract administration up to a dose of 6000.0 mg/kg had shown no apparent toxicity and did not cause animal death during the assay period. This result suggests a low toxicity for the extract. The data is in accordance to some researchers that attribute the absence of toxicity for *Protium* species.[6,27] Siani and coworkers[6] report the toxicity absence of essential oil from leaves and resin of *Protium* species. By research conduced by Oliveira[27] it was not possible to estimate the LD_{50} of the resin (<5000.0 mg/kg) and a mixture of α- and β-amyrins (<2000.0 mg/kg), obtained from *Protium* sp in rats through oral administration, and also suggested the low toxicity of these substances.[27]

3.3. Paw edema induced by carrageenan

The volume difference of the carrageenan-induced paw edema in animals treated with EEB and indomethacin in relation to negative control group is presented in Fig. 3.

The maximum inflammatory response induced by subplantar administration of carrageenan was observed at the third hour in the control group (0.8 ± 0.06 mm). It was observed that the extract and indomethacin (10.0 mg/kg) administration caused edema inhibition at the third hour. However, only EEB at dosage 300.0 mg/kg (0.3 ± 0.18 mm) was able to inhibit the edema, when compared with the control group (0.8 ± 0.06 mm). Indomethacin was able to inhibit the edema induced by carrageenan (−0.3 ± 0.28 mm) at the third hour, when compared with control group (1.0 ± 0.08 mm) (Fig. 4).

The extract FHEX, rich in α- and β-amyrin, induced an important anti-edematogenic activity at all doses tested, at the third hour (−0.01 ± 0.12 mm, −0.2 ± 0.08 mm and −0.2 ± 0.18 mm for 200.0, 300.0 and 400.0 mg/kg, respectively) in comparison with control group (1.0 ± 0.08 mm) (Fig. 4B). The anti-edematogenic activity of FHEX was attributed to the presence of α- and β-amyrins, isolated from this fraction. This activity has already been reported and

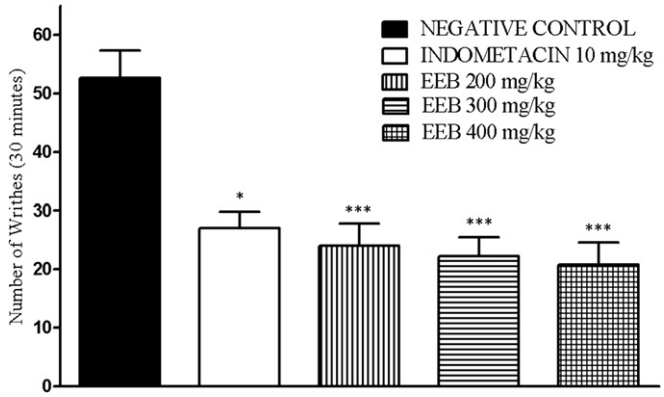

Fig. 5. Effect of EEB (200, 300 and 400 mg/kg, 0.2 mL/animal, v.o.) on mice acetic acid-induced abdominal writhing response.

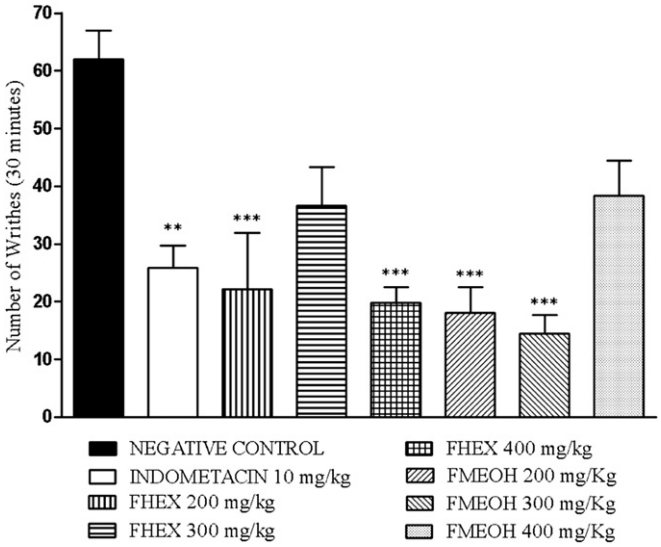

Fig. 6. Effect of FMEOH and FHEX on mice acetic acid-induced abdominal writhing response.

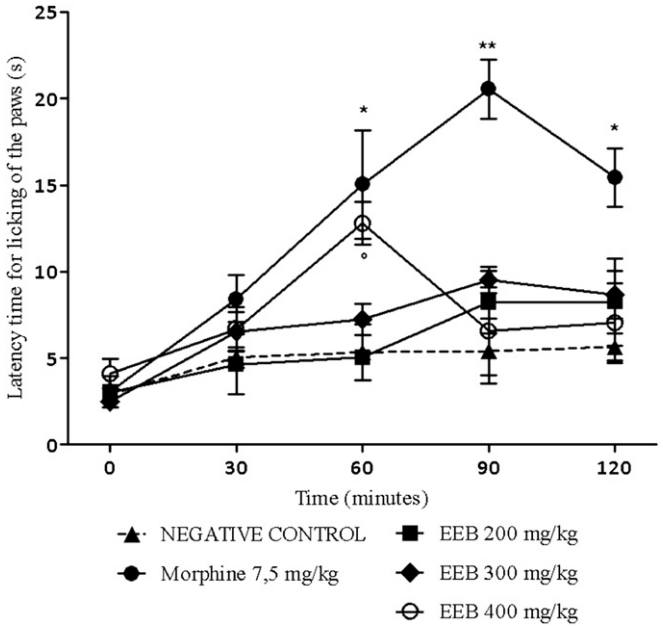

Fig. 7. Effect of EEB control and morphine on analgesic response using hot plate method. The latency time (s) was measured each 30 min, from 0 to 120 min.

attributed to this mixture also found in other species of *Protium* genus.[28,29]

For the first time, the anti-edematogenic activity is reported for FMEOH. The maximum edema inhibition was observed at the third hour (Fig. 4) induced by all doses administered (−0.01 ± 0.17 mm; 0.0 ± 0.12 mm and 0.04 ± 0.14 mm for 200.0, 300.0 and 400.0 mg/kg, respectively) in comparison with control group (1.0 ± 0.08 mm).

3.4. Antinociceptive activity

3.4.1. Acetic acid-induced abdominal writhing response

The abdominal constrictions observed after oral treatment with EEB, FHEX and FMEOH, at doses of 200.0, 300.0 and 400.0 mg/kg and indomethacin (10.0 mg/kg) compared with the control group is respectively showed in Figs. 5 and 6.

The EEB (Fig. 4) decreased the total number of acetic acid-abdominal constrictions in the mice at doses of 200.0, 300.0 and 400.0 mg/kg (24.1 ± 3.78; 22.3 ± 3.26 and 20.8 ± 3.82, respectively). Indomethacin, used as gold standard, also reduced the abdominal constriction number (27.0 ± 2.83). Reduction of the abdominal constriction number was also observed for fractions FHEX and FMEOH (Fig. 5). FMEOH (200.0 and 300.0 mg/kg) decrease the constriction number (18.0 ± 4.55 and 14.4 ± 3.29, respectively) in comparison with control group (62.0 ± 4.93).

Fraction FHEX presented an expressive antinociceptive activity at doses of 200.0 and 400.0 mg/kg (22.1 ± 9.79 and 19.8 ± 2.73, respectively). The antinociceptive activity of this fraction was also attributed to the presence of α- and β-amyrins. Although no antinociceptive activity was considered for FMEOH, the dose of 300.0 mg/kg induced a decrease of animals constrictions (38.3 ± 6.13) when compared with the control group (62.0 ± 4.93).

3.4.2. Hot plate method

The results of hot plate test observed after oral administration of extract and fractions (EEB, FMEOH and FHEX, doses 200.0, 300.0 and 400.0 mg/kg), morphine (7.5 mg/kg) and vehicle are respectively showed in Figs. 7 and 8.

Morphine induces antinociceptive activity at 90 min showing a remarkable enhancement of the latency time (LT) (20.6 ± 1.72 s) when compared to initial LT (3.1 ± 0.89 s). Antinociceptive property was observed for EEB at dose of 400.0 mg/kg (Fig. 7). The initial LT (4.1 ± 0.85 s) was increased in 60 min (12.8 ± 1.24 s). No antinociceptive effect was observed for the other dosages of EEB.

The fraction FHEX (400.0 mg/kg) induced a relevant increase in LT (16.0 ± 3.45 s, Fig. 8B) 90 min after the administration, in comparison with the initial LT (3.5 ± 1.00 s). Morphine (7.5 mg/kg) used as standard drugproduced similar increase: initial LT of 3.1 ± 0.89 s and after 90 min LT of 20.6 ± 1.72 s (Fig. 8B). It was not observed significative reduction in LT induced by the MEOH fraction (Fig. 8A).

3.5. Formalin method

The results of antinociceptive assays determined through the formalin method are showed in accordance with phase I (Fig. 9A) and phase II (Fig. 9B) of inflammatory process. The latency time (LT) observed for EEB and fractions were compared with the results of

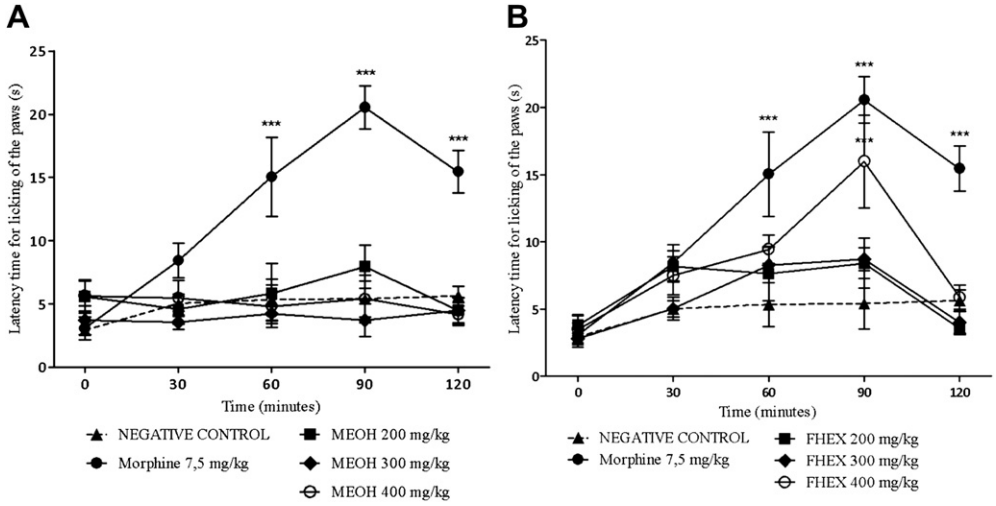

Fig. 8. Effect of FMEOH and FHEX on latency time of analgesic response in mice using hot plate test. Negative control, morphine, and oral doses of FMEOH (A) or FHEX (B) were administered 30 min before the hot plate test. The latency time (s) was measured each 30 min, from 0 to 120 min.

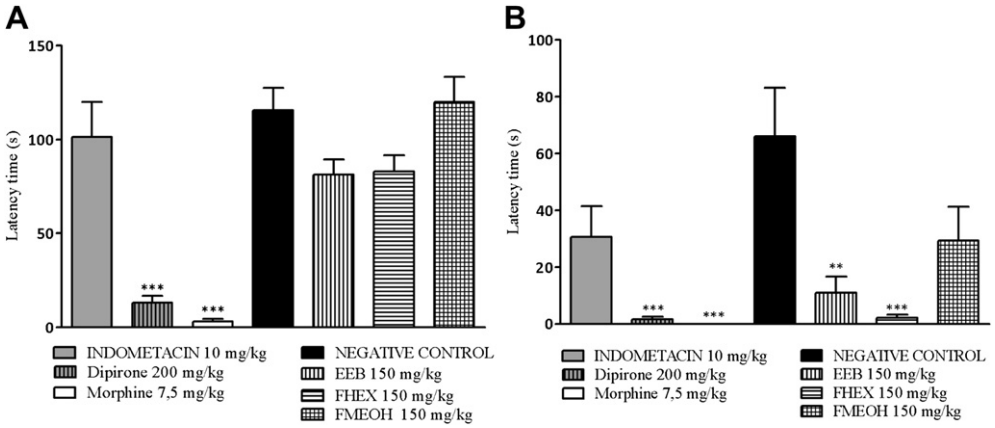

Fig. 9. Effect of oral administration of EEB and fractions (150 mg/kg, v.o.) from leaves of *P. spruceanum* on latency time on formalin induced hind paw nociception. Negative control, indometacin, dipyrone, morphine, EEB, FHEX and FMEOH were administered 30 min before subplantar paw injection of 1.5% formalin solution. The latency time was measured during the first 5 min [phase I (A)] and from 20 to 25 min [phase II (B)] after injection of the nociceptive agent.

control and with indomethacin, dipyrone and morphine, used as positive standard drugs.

Only morphine (LT: 3.0 ± 1.45 s) and dipyrone (LT: 13.0 ± 3.69 s) reduced the initial nociceptive response (phase I). However, at second phase, EEB (LT = 11.0 ± 5.77 s) and FHEX (LT = 2.2 ± 1.13 s) were effective in the reduction of the nociceptive response when compared with control group (LT = 66.1 ± 17.01 s). At phase II, morphine (LT = 10.96 ± 5.77 s) and dipyrone (LT = 1.6 4 ± 0.83 s) presented antinociceptive response in comparison with control group (LT = 66.06 ± 17.01 s). For indomethacin, both in phase I (LT = 101.30 ± 18.75 s) as in phase II (LT = 30.61 ± 10.90 s) was not observed reduction in the animals' reaction time.

The nociceptive behavior induced by formalin is characterized by stimulation of primary afferent sensory fibers leading to irritation in animal paw, inducing the animal to lick or shake the test paw.[30] The initial nociceptive response that occurs at first 5 min after formalin injection is known as phase I, early-phase or neurogenic phase. During this phase occurs the response to tonic pain. Phase II response, also known as late-phase or inflammatory phase, occurs 15–30 min after formalin injection and expresses the response to inflammatory process.[30]

In the first phase, receptors involved on antinociceptive response are constituents of opioids receptor system. At second phase, the inhibition of cyclooxygenase enzyme is responsible to decrease the nociceptive effect.[31]Some compounds that act as analgesics of central action can inhibit both phases. However, compounds with peripheral action, inhibit only the second phase.[32] Holanda Pinto and co-workers[31] using the capsaicin test and formalin test found that pre-treatment with α- and β-amyrins produced pronounced antinociceptive effect in the second phase. This analgesic effect may be associated to pains mediators delivery as neuropeptides and prostaglandins.[31]

The results of formalin test observed in phase II corroborate to the studies of Otuki at al.[29] denoting that FHEX, rich in α- and β-amyrins, has no central activity. However, results for phase II, together with the results of writhing test and hot plate test, implies that FHEX have peripheral and central activity. Studies developed by Holanda Pinto et al.[31] has shown that analgesic action of α and β-amyrin in phase II of formalin test could be reversed by naloxone, suggesting that amyrins might even had direct action on opioid receptors,[31] however, there are no further studies that justify this contradiction. Thus, it is possible to suggest that the analgesic activity of FHEX due to the presence of α- and β-amyrins isolated.

4. Conclusion

The phytochemical study of EEB from leaves of *P. spruceanum* allowed to establish that the mixture of α- and β-amyrins represent the major constituents of FHEX. It was observed that EEB has low acute toxicity. Promising results of anti-inflammatory activity were observed for the EEB and its fractions. The activity observed for FHEX must be attributed to α- and β-amyrins which was further isolated by this fraction. Through the analysis of the results obtained through the methods used to evaluate the analgesic activity, it was possible to suggest that FHEX has relevant central and peripheral antinociceptive activity that can also be attributed to the mixture of α- and β-amyrins. The results of FMEOH by writhing test suggested that this fraction has only peripheral analgesic properties. Then, it is possible to attribute to the mixture of pentacyclic triterpenes α- and β-amyrins is sponsor for anti-inflammatory and analgesic activities of FHEX and contributes to the biological activities of EEB, both extracts isolated from leaves of *P. spruceanum*.

Conflicts of interest

All authors have none to declare.

Acknowledgments

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Research article

Anti-inflammatory and analgesic evaluation of hydroalcoholic extract and fractions from seeds of *Piper cubeba* L. (Piperaceae)

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ABSTRACT

Some of the *Piper* species have been applied for the treatment of several diseases (anti-oxidant, antimicrobial, anti-inflammatory, and analgesic), considering multiple applications used in traditional medicine of different countries. About these, the present study evaluated some biological activities of *Piper cubeba*, as writhing test induced by acetic acid, ear edema induced by croton oil and paw edema induced by carrageenan were used by evaluated the analgesic and anti-inflammatory activities of crude hydroalcoholic extract (PCE) and its fractions of different polarities of *P. cubeba* L. seeds. The lethal dose (LD_{50}) and the effective dose (ED_{50}) were evaluated too. Both the PCE and dichloromethane fraction showed decrease values of edema and abdominal constrictions. The results obtained in this study confirm the low toxicity and analgesic and anti-inflammatory activities of PCE from *P. cubeba* seeds, justifying its use in folk medicine.

1. Introduction

Piper species are widely distributed around the world, especially in tropical and subtropical regions. Some of the *Piper* species have been applied for the treatment of several diseases, considering multiple applications used in traditional medicine of different countries. Species of genus *Piper* are also used as flavor food additive by many communities.[1–3] Some biological activities attributed to this genus involving anti-oxidant, antimicrobial, anti-inflammatory and analgesic effects have been reported.[4,5] Chinese *Piper* species also have been used in traditional medicine for rheumatic diseases, as well as ailments of the respiratory tract. In recent years, *Piper* species have been widely studied by various research groups, leading to the identification of more then 600 different organic compounds.[2,6,7]

Piper cubeba L. is native species from India that has been used in its traditional medicine to treat ailments associated to inflammatory and pain processes. The seeds of *P. cubeba* contain cubebin, a dibenzylbutirolactone lignan, which has been previously described as anti-inflammatory and analgesic[8] with no clastogenicity associated.[9]

The aim of the present work was to study the anti-inflammatory effect of *P. cubeba* L. seed extract (PCE) and its fractions through *in vivo* assays. The effects on rat paw carrageenan-induced edema, the median effective dose (ED_{50}) and on ear edema induced by croton oil were investigated. The median lethal dose (LD_{50}) was also established to guarantee low intoxication risk of the animals used in the experiments.

2. Material and methods

2.1. P. cubeba seeds

Seeds of *P. cubeba* L. were imported from India. The seeds were air dried (60 °C/3 days) and pulverized to a coarse powder (500.0 g). This powdered material was submitted to maceration with water–ethanol (9:1) (6000 ml) by three days. The macerate was filtered and the extraction procedure repeated. The concentrate of combined extracts obtained under reduced pressure furnished 63.65 g (12.73% yield) of crude water–ethanol extract. Part of this *Piper* seed concentrated extract (PCE) (10.0 g) was dissolved in 3% Tween

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80 and 0.9% NaCl solution and the resultant solution was aseptically filtered and administered to the animals.

In parallel, another part of the crude water–ethanol extract (53.0 g) was dissolved in MeOH:H_2O (9:1) and sequentially partitioned with hexane, methylene chloride and ethyl acetate. After solvent removal in a rotatory evaporator, the hexane [7.81 g (14.73%)], methylene chloride [77.24 g (8.18%)] and ethyl acetate [0.62 g (1.16%)] dried fractions were obtained.

2.2. Animals

Male albinos Wistar rats (*Rattus norvegicus*) (150–200 g) and male albinos Swiss mice (*Mus musculus*) (20–25 g), specific pathogen free, were obtained from the Central Biotery of Universidade de São Paulo, S.P., Brazil. Groups of animals were kept at controlled conditions of humidity (53%) and temperature (23 ± 2 °C), and with food and water *ad libitum*. During 12 h before experimental assays, the animals were kept only with water *ad libitum*. The experiments were carried out according to the "Guide for the care and use of laboratory animals" (The National Academic Press, USA, 2011).[10]

2.3. Determination of median lethal dose (LD_{50})

A single dose of PCE (500, 750, 1000, 1250, 1500 and 2000 mg/kg) was administered orally (p.o.) to groups of mice ($n = 8$). The animals of each group were observed during 72 h. The number of death animals was expressed as a percentile, and the LD_{50} was determined by probit test using death percentage *versus* dose's log.[11,12]

2.4. Anti-inflammatory activity

Rat paw edema was induced by Kappa carrageenan type III (Iota-Fluka-Biochemica Co.). The inflammatory agent (100.0 μg/paw) was injected in the right hind paw planter surface of the rat ($n = 8$). Left paw was used as control of edema and sterile saline solution (0.9% NaCl, 0.1 ml) was used as vehicle. The foot volume of all animals was determined by plestimographic method described by Ferreira (1979).[13] The foot volume measurements were taken before and then at hourly intervals during the first 4 h after the injection of the inflammatory stimulus.

Determination of ED_{50} – groups of rats ($n = 8$) were treated orally with *P. cubeba* L. seeds crude extract (PCE) (50, 100, 200 and 300 mg/kg) 30 min before the carrageenan injection. The inhibition of inflammatory process was calculated by measuring the volume difference between the right and left paws of PCE treated animals in comparison to the control group at the third hour of experimentation (edema peak). ED_{50} was determined through the data obtained from the curve of carrageenan-induced edema percent inhibition *versus* dose.[14] This method was used to evaluate the effectiveness of *P. cubeba* hydroalcoholic extract and its fractions in relation to the inhibition of inflammatory process by comparison with the control and indomethacin (MSD Co.), a non steroidal anti-inflammatory drug (NSAID) used as positive control. To establish the ED_{50}, different doses of PCE, indomethacin (5.0 mg/kg, p.o.) and 0.9% NaCl (0.5 ml, p.o.) were administered orally 30 min before the injection of carrageenan and the inflammatory process inhibition was determined.

Similarly, the anti-inflammatory effect of hexane, methylene chloride and ethyl acetate fractions (50.0 mg/kg, p.o.) were compared with the results of indomethacin and 0.9% NaCl.

2.5. Writhing test

Writhing test was carried out as described by Koster et al (1959), modified by Broadbear et al (1994).[15,16] Groups of mice ($n = 8$) were treated orally with different doses of PCE, indomethacin (10 mg/kg) or 0.9% NaCl (0.5 ml) 30 min before the stimulus. The muscular contraction was induced by intraperitoneal injection of 0.6% acetic acid solution (Reagan Co.) (0.25 ml/animal). The number of muscular contractions was counted by a period of 20 min, starting 5 min after the injection, and the results expressed as the average of total writhes observed.

2.6. Ear edema induced by croton oil

The method used was described by Tubaro et al (1985).[17] The cutaneous inflammation was induced by croton oil solution in acetone (10.0 mg/ml) on the right ear surface (0.1 ml, 1.0 mg/ear). The same volume of acetone was applied on the left ear. Thirty minutes after the stimulus, crescent doses of PCE, dexamethasone (MSD Co.) (0.2 mg/kg) and 0.9% NaCl (0.5 ml) were administered orally to different groups of mice ($n = 8$). After 6 h, the animals were sacrificed and a biopsy (8 mm diameter) of each ear was obtained. The weight difference (mg) between the stimulated ear (right) and the control ear (left) represented the inflammatory reference.

2.7. Statistical analysis

The statistical analyses were done using Analysis of Variance (ANOVA) followed by Tukey–Kramer multiple comparison test.[18] Results with $P < 0.05$ were considered to be significant. Data are expressed as mean ± standard deviation (S.D.).

3. Results

3.1. The effectiveness of P. cubeba and lethal median dose (ED_{50} and LD_{50})

PCE has decreased the carrageenan-induced edema in a dose-dependence relationship (correlation coefficient $r = 0.9407$ and linear regression $y = 0.2061x + 4.4297$). ED_{50} for PCE was determined as 150.0 mg/kg (Fig. 1). During 72 h of accurate toxicity assay, no deaths have occurred in function of the doses administrated. The animals showed no stereotypical symptoms associated with toxicity. The calculated value of lethal dose (LD_{50}) was determined to be higher than the highest dose tested (2000.0 mg/kg).

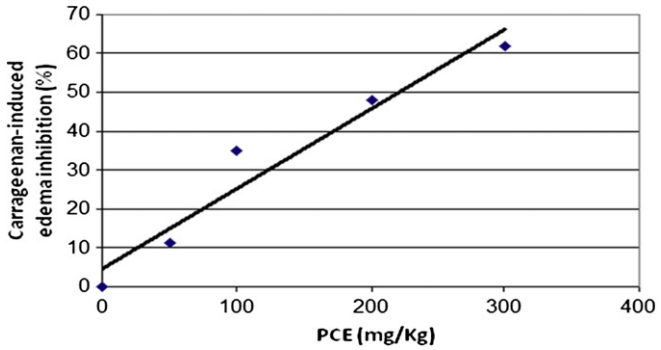

Fig. 1. Effect of PCE (p.o.) on the carrageenan-induced rat paw edema (100.0 μg/paw). The straight line represents the equation $Y = 0.2061x + 4.4297$of the administered doses with $R = 0.9407$.

3.2. Carrageenan-induced rat paw edema

The group treated with PCE in the carrageenan-induced rat paw edema had a significant decrease when compared to the group treated with indomethacin ($P < 0.05$) (Fig. 2). The groups treated with the fractions of PCE (hexane, methylene chloride and ethyl acetate) also have shown statistically significant decrease of the edema ($P < 0.05$).

The treatment with PCE inhibited the formation of the edema by 22.50% in the third hour of experimentation (peak of edema). This result is similar to those observed for the group treated with indomethacin (26.2% of inhibition). Both results are statistically significant when compared to the control ($P < 0.05$), but not between them ($P > 0.05$). In the second and fourth hours of experimentation, the treatment with PCE decreased the edema formation by 28.1% and 30.9%, respectively. The group treated with indomethacin had the edema formation decreased by 51.9% and 48.2%, respectively, after the same period following treatment. Both treatments were significantly different to control group ($P < 0.05$), but not between them ($P > 0.05$).

The organic solvent soluble fractions obtained from PCE (hexane, methylene chloride and ethyl acetate) also produced inhibitory effect at 50.0 mg/kg dose on the carrageenan-induced edema at the third hour. The methylene chloride fraction inhibited the inflammatory process by 28.7% when compared to control ($P < 0.05$). This was comparable to the effect observed for indomethacin, which inhibited the edema by 26.2%. The hexane soluble fraction inhibited the inflammation by 11.2% and the ethyl acetate fraction inhibited it by 20.0% compared to control group (Fig. 3).

3.3. Writhing test

PCE (30.4 ± 5.7 writhes) and indomethacin (29.2 ± 4.3 writhes) were effective in inhibiting the writhing in mice when compared to the control group (41.1 ± 5.4 writhes) ($P < 0.05$). Treated groups did not show significant difference between them. These results are shown in Fig. 4.

3.4. Ear edema induced by croton oil

Compared to the control group, dexamethasone has inhibited the ear edema by 68.4% ($P < 0.01$), and PCE has decreased the edema formation by 20.8% ($P < 0.05$).

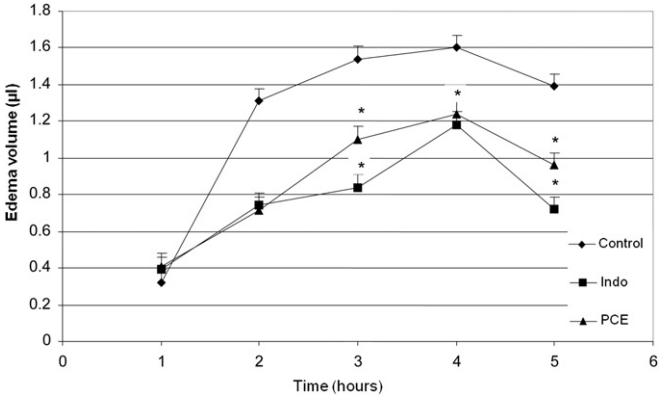

Fig. 2. Effect of PCE (150.0 mg/kg), indomethacin (5.0 mg/kg) or saline solution (0.5 ml) orally administered on the carrageenan-induced rat paw edema. ANOVA followed by Tukey–Kramer *post-hoc* multiple comparison test (*$P < 0.05$).

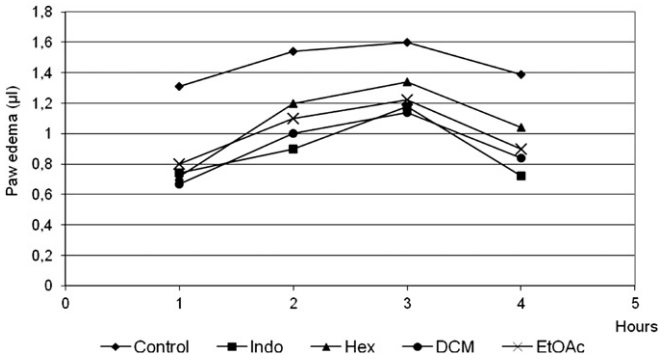

Fig. 3. Effect of PCE fractions (50.0 mg/kg), indomethacin (5.0 mg/kg) or saline solution (0.5 ml) orally administered on the carrageenan-induced rat paw (100.0 µg/paw) over a 4 h period. ANOVA followed by Tukey–Kramer *post-hoc* multiple comparison test (*$P < 0.05$).

4. Discussion

Different *P. cubeba* L. preparations are commonly used in Indian traditional medicine for the treatment of inflammatory processes diseases.[1] In this study, the efficacy of a water-ethanolic extract prepared from seeds of *P. cubeba* L. was evaluated. ED_{50} of PCE was established as 150 mg/kg by carrageenan-induced paw edema for anti-inflammatory evaluation. Using acute toxicity assay, the median lethal dose (LD_{50}) was determined to be higher than 2000.0 mg/kg. In this test, neither deaths nor symptoms associated with toxicity such as convulsion, ataxy, diarrhea or increased diuresis were noticed during the 72 h observation period. These results indicate the efficacy and relative safety of PCE for the treatment of conditions associated with inflammatory processes.

Edema formation in rat paws is the result of a synergism among several inflammatory mediators, promoting the increase of vascular permeability and/or mediators that increase blood flow.[19]

Carrageenan-induced edema is a well-known experimental animal model applied to the studies of acute inflammation. It has been known that at the third hour after carrageenan inoculation the edema reaches its highest volume. The presence of prostaglandins and other mediators are responsible to slow reactions during the inflammatory processes.[20] Carrageenan injection induces the liberation of bradykinin, and also the biosynthesis of PGI_2 and other autacoids, responsible for the inflammatory exudate.[21] The early phase (1–2 h) of the carrageenan effect is mediated by histamine and serotonin and has been associated to the enhancement of prostaglandins synthesis around damaged tissue. The last phase is characterized by prostaglandin release and mediated by

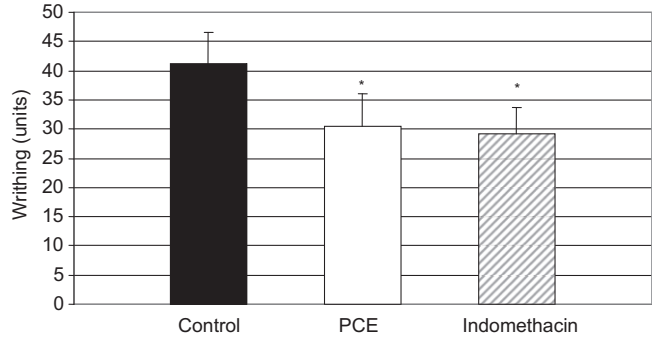

Fig. 4. Effect of PCE (150 mg/kg), indomethacin (5.0 mg/kg) and 0.9% NaCl (0.5 ml) administered orally in writhing induced in mice by intraperitoneal injection of 0.6% acetic acid. ANOVA followed by Tukey–Kramer *post-hoc* multiple comparison test (*$P < 0.05$).

bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages.[22]

The inhibitory activity produced by *P. cubeba* seed extract (150.0 mg/kg) over a period of 4 h in carrageenan-induced rat paw inflammation was very similar to those exhibited by indomethacin treated group, the standard NSAID used as positive control. Results indicate that PCE acts during the third phase, probably involving arachidonic acid metabolites, which produce an edema dependent on neutrophils mobilization.[23] Thus, it is suggested that the mechanism of action of this extract and fractions can be related to prostaglandin synthesis inhibition, as described for indomethacin.[24]

The intraperitoneal administration of irritant agents to serous membranes provokes stereotypical behavior in mice and rats characterized by abdominal contractions, anomalous body movements noted in the hind paws, twisting of dorsoabdominal muscles, and reduction of motor activity and coordination.[25] Although, this model is widely used for analgesic drug screening and involves local receptors (cholinergic and histamine receptor) and acetylcholine and histamine mediators.[26]

Quantification of prostaglandins by radioimmunoassay in the peritoneal exudates of rats obtained after intraperitoneal injection of acetic acid demonstrated that high levels of prostaglandins $PGE_{2\alpha}$ and $PGF_{2\alpha}$ are presented during the first 30 min after stimulus, suggesting that anti-inflammatory substances can be involved in the peripheral analgesic activity.[27] The genesis of carrageenan-induced edema may cause prostaglandins and kinins releases, among other substances.[28] The writhing test has shown results similar to those obtained through edematogenic assay using carrageenan, even probable due to because both assays likely induce the same inflammation mechanisms based in prostaglandin's biosynthesis.

All fractions of PCE obtained with organic solvents showed different degrees of inhibition on the carrageenan-induced edema. Moreover, the methylene chloride fraction showed the best activity, indicating that the active compounds are concentrated in this fraction. These compounds, probably cubebin and other dibenzylbutyrolactones lignans as hinokinin, have been previously described as anti-inflammatory agents.[8] Methylene chloride fraction (50.0 mg/kg) has decreased the inflammatory process by 28.75% in comparison to the negative control group. This effect was similar to those observed for the group treated with indomethacin (5.0 mg/kg), that showed 26.25% of edema inhibition ($P < 0.05$).

Elfahmi et al (2007) have isolated several compounds from the extract of *P. cubeba*. The main constituents are phenolic compounds such as lignans as cubebin, yatein and hinokinin, which have been described as anti-inflammatory.[29,30]

The presence of lignans in the *P. cubeba* extract, such as other compounds as alkaloids and terpenoids (essential oil) are related to the anti-inflammatory and analgesic activities, confirming the ethnopharmacological use of this medicinal plant as food and medicine.

5. Conclusion

The results obtained in this study demonstrated that the seeds extract of *P. cubeba* L. have an anti-inflammatory activity supporting its traditional use for the treatment of inflammatory disorders.

Conflicts of interest

All authors have none to declare.

Acknowledgments

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Research article

Efficacy and structural effects of *Acacia pennata* root bark upon the avian parasitic helminth, *Raillietina echinobothrida*

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ABSTRACT

Introduction: Acacia pennata (L.) Willd. is a well-known shrub in the south and southeast Asian regions, where the natives use it for a wide range of purposes from medicines to culinary cuisines. Among the Mizo tribes of India the plant is used in the treatment of gastrointestinal infections.

Methods: To validate this traditional practice, a defined set of poultry tapeworm, *Raillietina echinobothrida*, was treated with varying concentration *viz*. 1, 2, 5, 10, and 20 mg/mL, of the methanol extract of the root bark. Similar treatment was concurrently performed for a broad-spectrum drug albendazole, in corresponding concentrations.

Results: Assessment of the survival indicated that the plant extract caused concentration-dependent effects comparable to that of albendazole, with significant mortality ($P < 0.5$ by student's *t*-test) of the test worms at all concentrations tested in comparison with those of the control group. Morphological observations using scanning electron microscope revealed that the tapeworms in the 20 mg/mL plant extract treatment group exhibited profound structural damages. The scolex became massively shrunken, with the special attachment organs reduced to crooked appendages. Overall deformation of the tegument was apparent throughout the body surface. All the body segments shrivelled up, deflated and wrinkled into fluffy folds. The fine hairy microtriches completely disappeared, and replaced by an irregular mass of conglomerate tissues.

Conclusion: The study shows that *A. pennata* has an anthelmintic property that requires further investigations as to the nature and mode of action of the active compounds.

1. Introduction

In spite of an enormous development in the discovery and understanding the pharmacology of anthelmintic drugs, helminth infestations continue to be the most debilitating factor of economic losses in animal industry all over the world, and the persistent cause of human morbidity and other health problems. To compound the situation, helminthiasis has not been ameliorated to any significant degree largely due to the rapid evolution of anthelmintic resistance among different parasitic helminths virtually to all types of conventional chemotherapeutic drugs, old and new.[1,2] The consequence will be undesirably alarming if the crisis is not properly checked. To cope up with the inevitable dilemma, a large body of scientific documents has posited that a number of medicinal plants does indeed indicate potent anthelmintic activities, and use of well-established plants are highly advocated.[3–5] However, many of the traditionally acclaimed therapeutic plants are experimentally revealed to have negligible credibility for clinical and veterinary applications, and scientific evidence for the effectiveness of many in use remains obscure.

Acacia pennata (L.) Willd. is a leguminous perennial climbing shrub belonging to the family Mimosaceae, and is native to Bangladesh, Bhutan, India, Myanmar, Sri Lanka and Thailand. It is a common cure for indigestion, especially in infants. The extract of the stem bark is applied as antidote to snake venom and fish poisoning.[6] It is also used as antiseptic for scalding of urine and for curing bleeding gums. The root bark can be used as antiflatulent and to cure stomach pain. It is also used in the treatment of bronchitis, cholera and asthma. A decoction of the leaves is used for general treatment of body aches, headache and fever.[7] The extracts of the dried leaves were experimentally demonstrated to possess analgesic and anti-inflammatory properties in laboratory mice.[8] Burmese and Thai people have long used the shoots in various culinary preparations. In addition, based on the Thai traditional usage, the leaf extract was shown to have mild antimicrobial and antioxidant activities.[9]

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Table 1
Anthelmintic efficacy of the methanol extract of *A. pennata* root bark and albendazole on the survival of *R. echinobothrida*.

Incubation medium	Concentration (mg/mL)	Time (h) taken for death[a]	df	*t* value	Probability level[b]
Control	0	57.70 ± 1.37			
A. pennata extract	1	34.25 ± 2.09	8	20.93281	$P < 0.05$
	2	19.97 ± 2.12	8	33.44	$P < 0.05$
	5	10.28 ± 1.92	8	44.94	$P < 0.05$
	10	06.41 ± 1.35	8	59.49	$P < 0.05$
	20	03.89 ± 1.07	8	69.07	$P < 0.05$
Albendazole	1	19.05 ± 1.42	8	44.78	$P < 0.05$
	2	12.65 ± 1.56	8	48.53	$P < 0.05$
	5	05.94 ± 1.23	8	62.66	$P < 0.05$
	10	03.61 ± 0.86	8	74.69	$P < 0.05$
	20	02.95 ± 1.03	8	77.05	$P < 0.05$

[a] Values are expressed as mean ± SD ($n = 5$).
[b] *P* value considered significant when <0.05 in comparison with control group; level of confidence at 95%.

Among the Mizo tribes of northeast India, the tender root bark, young leaves and shoots are regularly taken as seasonal delicacy and medicine for intestinal infection.[10] A closely related species *Acacia oxyphylla* had been shown to exhibit strong anthelmintic property.[11,12] The present study is thus an attempt to vindicate the use of *A. pennata* as an anthelmintic against a parasitic tapeworm, *Raillietina echinobothrida* Mégnin.

2. Materials and methods

2.1. Preparation of plant extract

The fresh roots of *A. pennata* were collected from the nearby forest of Aizawl, India. The specimens were identified by the botanists at the Department of Botany, Pachhunga University College, India, where voucher specimen (PUC-BOT-A 039) is maintained. The barks were peeled off, vigorously washed with deionized water, chopped off into small pieces, and dried in a hot air oven at 50 °C. The dried parts were pulverized to fine powder and then refluxed with methanol (100 g/L) for 8 h at 60 °C. The resulting solution was filtered through Whatman filter paper (No. 1) and then evaporated to complete dryness at 50 °C. A deep green extract was obtained, which was then refrigerated at 4 °C until further use. An hour before experimental assay, different concentrations *viz.* 1, 2, 5, 10, and 20 mg/mL of the extract were prepared by dissolving in 0.9% neutral phosphate-buffered saline (PBS), supplemented with 1% dimethyl sulfoxide (DMSO). The media were maintained at 37 ± 1 °C in a glass-chambered automated incubator.

2.2. Chemicals and drug

All the chemicals used were of standard analytical grades, obtained either from Merck or S.D. Fine Chemicals Limited, India. The reference drug albendazole (Zentel®) was a product of GlaxoSmithKline Pharmaceutical Limited, India. Similar concentrations to those of the plant extract were prepared for albendazole using the same media.

2.3. Recovery and in vitro treatments of tapeworms

Live fowls (*Gallus domesticus* L.) were sacrificed using an overdose of anaesthesia and upon necropsy, live tapeworms, *R. echinobothrida*, were recovered from the intestines. The tapeworms were collected in PBS and then incubated at 37 ± 1 °C. Actively alive tapeworms with more or less the same body size were selected and introduced into the different plant extract and drug media. One group of tapeworms was maintained in a medium containing only PBS with 1% DMSO to serve as control. Each experimental assay consisted of 5 replicates. Motility and mortality of the worms were observed, and duration of survival was recorded as previously described.[12,13] Death was substantiated by dipping the tapeworms in tepid PBS (~45 °C) that induced movement in sentient worms.

2.4. Scanning electron microscopy

A set of tapeworms was selected from the control and plant extract treated groups, thoroughly washed in PBS, and then immediately fixed in 4% cold-buffered formaldehyde at 4 °C for 12 h. After post fixation in 1% buffered osmium tetraoxide for 1 h, the specimens were dehydrated through ascending concentration of acetone and then air-dried after treatment with tetramethylsilane following the procedure already described elsewhere.[12–14] After coating with gold in a fine-coat ion sputter, JFC-1100 (JEOL) and mounted on metal stubs, electron micrographs were generated using a LEO 435 VP scanning electron microscope at an electron accelerating voltage of 20 kV.

Fig. 1. Scanning electron micrograph of normal *R. echinobothrida* showing the anterior end of the body called the scolex which bears holdfast organs such as four semicircular suckers and a rostellum.

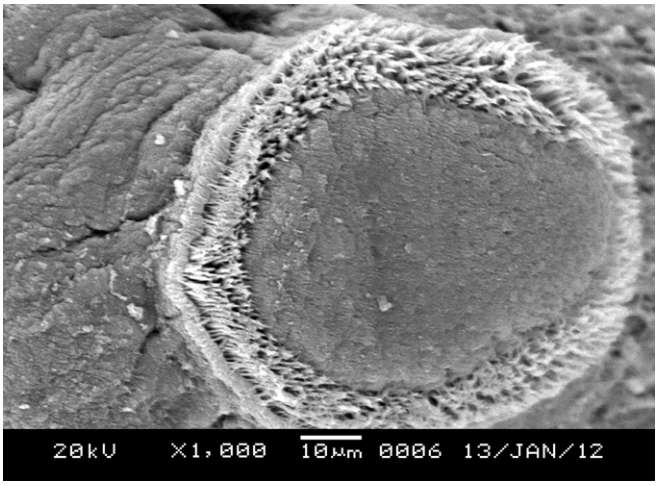

Fig. 2. Scanning electron micrograph of normal *R. echinobothrida* showing a sucker under high magnification; a sucker is lined with rows of sharply pointed spines.

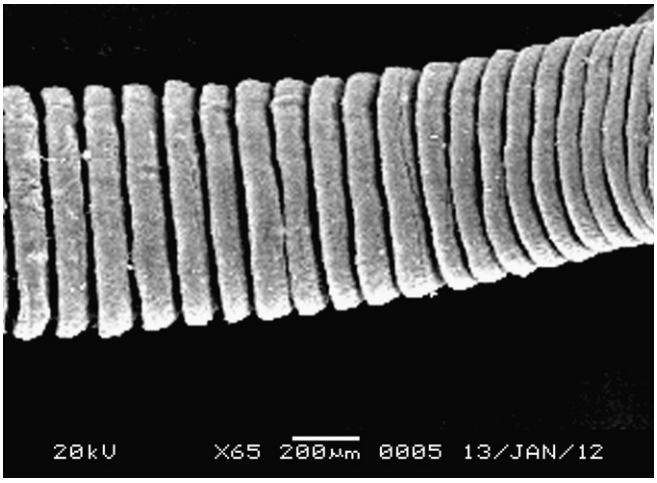

Fig. 3. Scanning electron micrograph of normal *R. echinobothrida* showing the body proper or strobila that is composed of a series of segments called proglottids.

Fig. 5. Scanning electron micrograph of *R. echinobothrida* treated with *A. pennata* extract showing contracted scolex with shrunken suckers.

2.5. Data analysis

All data are presented as means plus or minus the standard deviation (SD) of the mean. Comparison of the mean values between the treated and control groups was made using unpaired Student's *t*-test, and the level of probability value considered significant when $P < 0.05$.

3. Results

The tapeworms maintained in a control medium that consisted of only PBS with DMSO survived well up to 57.70 ± 1.37 h. Observations on the efficacy of the methanol extract of *A. pennata* root bark and albendazole in terms of mortality of *R. echinobothrida* are shown in Table 1. The results indicate that both the plant extract and the reference drug exhibited dose-dependent lethal effects. Treatment of the worms with 1, 2, 5, 10 and 20 mg/mL of both the plant extract and albendazole showed significant efficacy on the survival of the tapeworms. In comparison, albendazole indicated a slightly higher level of potency to the plant extract at all concentrations. Survival of the tapeworms directly related to concentrations of the drug and the plant extract; the higher the concentration the shorter the survival, and vice versa.

For scanning electron microscopic descriptions the tapeworms treated with 20 mg/mL of the plant extract were chosen as the most extensive alterations were shown at this concentration in comparison with the control worms. Normal *R. echinobothrida* is a typical tapeworm with an elongated, flattened, ribbon-like segmented body, having a knob-like anterior end called the scolex. The scolex bears four bulging suckers surrounding a circular opening called rostellum (Fig. 1). These suckers and rostellum constitute the special attachment organs of the parasite to the host's intestinal wall. They are specifically lined with prickly spines (Fig. 2). The body proper called strobila is composed of a chain of conjoined segments called proglottids (Fig. 3). The entire body covering, the tegument, is completely covered with delicate hairs called microtriches, giving the overall surface a velvety appearance (Fig. 4).

The tapeworms treated with the plant extract showed extensive deformity throughout the entire body surface. The scolex is represented by a severe shrinkage with a number of unusual tegumental folds and the prominent suckers were contracted (Fig. 5). Spines demarcating the suckers were sharply crooked and appeared defunct (Fig. 6). The tegument appeared totally disintegrated so much so that the proglottids look like wrinkled textile

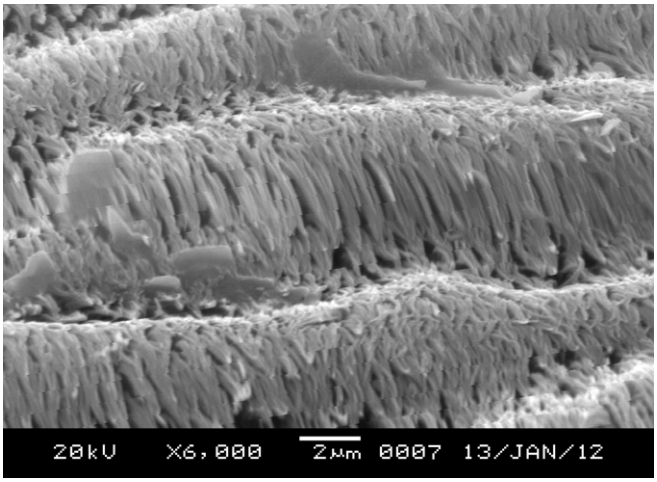

Fig. 4. Scanning electron micrograph of normal *R. echinobothrida* showing the surface of the body called tegument which is made up of dense hair-like microtriches.

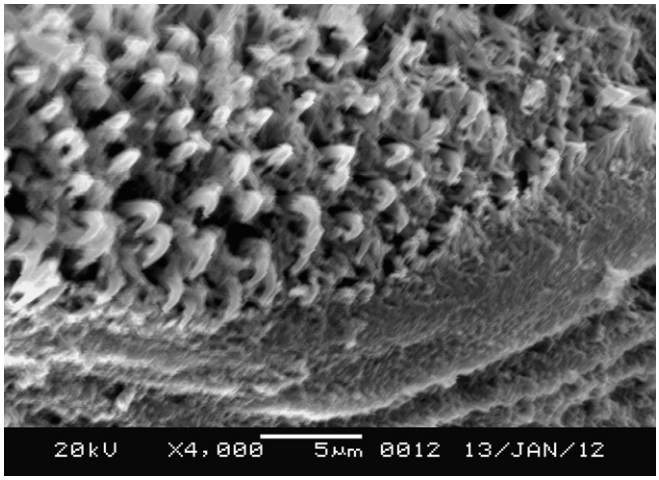

Fig. 6. Scanning electron micrograph of *R. echinobothrida* treated with *A. pennata* extract showing the deformed spines on the sucker.

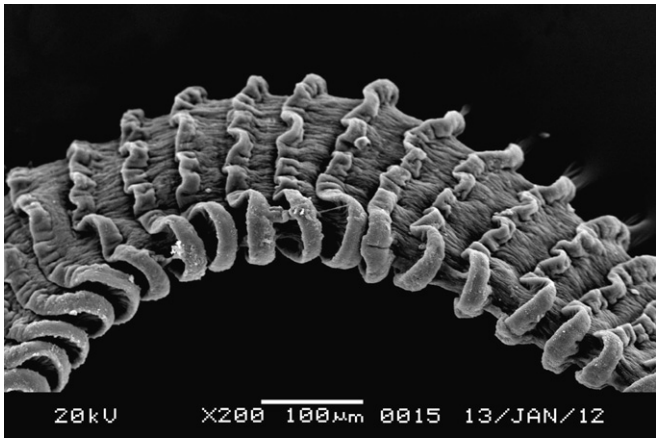

Fig. 7. Scanning electron micrograph of *R. echinobothrida* treated with *A. pennata* extract showing highly deflated proglottids with extensive wrinkles.

(Fig. 7). The fine hair-like microtriches were completely reduced to formless and dishevelled tissues (Fig. 8).

4. Discussion

The data provide the evidence for an anthelmintic potentiality of the extract of *A. pennata* root bark as traditionally used by the Mizo tribes. The concentration-dependent efficacy on *R. echinobothrida* was quite comparable to that of the standard drug albendazole. Interestingly, a closely related species of the present investigation, *A. oxyphylla* stem bark, using different extracts, had been demonstrated to exhibit similar effects on *R. echinobothrida*, including changes in the vital enzymes and trace elements,[15] internal and tegumental damages.[11,12]

Flemingia vestita was also reported to exert severe degenerative effects including clumping of the microtriches, vacuolization of the tegument and shrinkage of the body of *R. echinobothrida*.[16] Root bark extract of *Millettia pachycarpa* was responsible for extensive destruction on the tegument, entire removal of microtriches, complete sloughing off of the scolex surface and metabolic obstruction in *R. echinobothrida*.[17,18] Extract of *Artemisia cina* reportedly caused extensive damage on the scolex and microtriches in the tapeworm *Moniezia extansa*.[19] Formation of tegumental blebs accompanied by destruction of microtriches and shrinkage in the scolex region were described for the effects of *Cassia alata* leaf extract on the tapeworm *Hymenolepis diminuta*.[20]

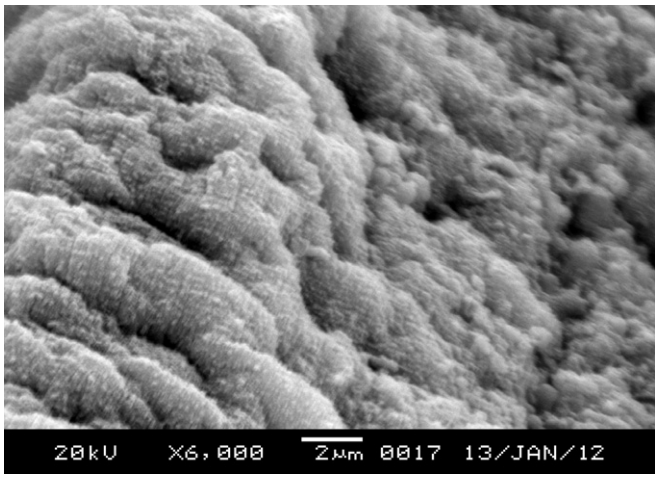

Fig. 8. Scanning electron micrograph of *R. echinobothrida* treated with *A. pennata* extract showing total obliteration of the fine microtriches on the surface of the proglottid.

Albendazole and its related benzimidazoles are established to enter the helminth body by passive diffusion through the tegument in tapeworms, where they directly cause disruption of the tegumental and muscle layers by binding specifically to β-tubulins, thereby, inhibiting assembly and functioning of the cellular motor proteins.[21] The tegument or cuticle is the fundamental interface of the helminth body with its environment, and responsible for selective absorption of nutrients, secretory activities and sensory perception, rendering it specifically susceptible to anthelmintic agents. Consequently, the tegument is the primary target of anthelmintic agents.

Formation of numerous blebs on the tegument, rostellar disorganization and loss of the microtriches were observed for pure albendazole and its sulphoxide combination therapy on the human tapeworm, *Echinococcus granulosus*.[22] Albendazole and praziquantel combination treatment of *E. granulosus* and *Mesocestoides corti* resulted in the loss of sucker concavity, loss of microtriches and destruction of the tegument.[23] Damaging effects described for albendazole, flubendazole and nitazoxanide are highly comparable and typified by reductions in number and length of the microtriches, rostellar degeneration, formation of blebs on the tegument, loss of hooks and destruction of microtriches and vesiculation in *E. granulosus* and *Echinococcus multilocularis*.[24,25] Albendazole also caused complete tegumental disintegration, extensive shrinkage and obliteration of microtriches in *R. echinobothrida*.[26]

It can therefore be concluded that the present study clearly provides the rationale behind the traditional usage of the extract of *A. pennata* root bark as an anthelmintic. Structural changes on the morphology, damages in the suckers and microtriches, and distortion of the fine tegument of tapeworms are the hallmark effects of anthelmintic drugs. Similar structural changes observed in the present investigation, suggests that the plant extract acts transtegumentally to exert anthelmintic effects. However, the active chemical component of the plant extract and the precise mode of action at cellular level are beyond comprehension from the present study, and remain to be further investigated.

Conflicts of interest

The author has none to declare.

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Research article

Characterisation of leaf essential oils of three *Cinnamomum* species from Malaysia by gas chromatography and multivariate data analysis

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ABSTRACT

Identification of chemical composition of essential oils as raw materials is very important for ensuring the quality of finished herbal products. The objectives of the study were to determine the chemical composition of essential oils of the leaves of three *Cinnamomum* species (i.e. *Cinnamomum mollissimum* Hook.f., *Cinnamomum porrectum* (Roxb.) Kosterm. and *Cinnamomum verum* J.S. Presl.) and to characterise the essential oils constituents by using GC and multivariate data analysis. Hydro-distilled essential oils were evaluated using GC–FID and GC–MS techniques. The GC–FID chromatograms were further analysed by multivariate data analysis using principal component analysis (PCA) and hierarchical clustering analysis (HCA) methods. The major compound identified in the oil of *C. mollissimum* was benzyl benzoate (77.69%), whereas that for *C. porrectum* was safrole (93.19%) and *C. verum* was eugenol (93.08%). PCA score and HCA plots revealed that the leaf oils were classified into three separated clusters of *C. verum* (Cluster I), *C. mollissimum* (Cluster II) and *C. porrectum* (Cluster III) based on their characteristic chemical compositions. The combination of GC and multivariate data analysis may be used for identification and characterisation of essential oils from different *Cinnamomum* species that are to be used as raw materials of traditional herbal products.

1. Introduction

Cinnamon belongs to the *Cinnamomum* species of the Lauraceae family. Many of these species yield characteristic volatile oils that are aromatic, depending on nature of the chemical composition. *Cinnamomum verum* J.S. Presl. (synonym *Cinnamomum zeylanicum* Blume) is reported to be one of the most important cinnamon oils in the world trade,[1] and is mainly used as flavouring agents, in perfumery and as medicines. Usually, essential oils from the leaves and bark of the plant have been used in herbal preparation.

The uses of *C. verum* are described in many traditional systems of medicine or folk medicine and indicated in several pharmacopoeias.[2–6] The leaf oil was found to contain eugenol, linalool, benzyl benzoate and (E)-cinnamaldehyde as the major compounds.[7,8] It possessed various activities including antibacterial,[9] antifungal,[8,9] acaricidal,[10] larvicidal,[11] and fumigant against mites.[12] Methanol and ethanol extracts, and essential oil of *C. verum* leaves have high total phenolic content and antioxidant activity.[9,13] The warm and spicy *C. verum* leaf oil is mainly used as insecticides and source of eugenol, as well as in the flavouring and fragrance industries.[1]

Nevertheless, the strong spicy odour of *Cinnamomum mollissimum* Hook.f. leaf oil is potentially used as medicinal and perfumery materials due to the high content of benzyl benzoate.[14] Its leaf oil has been shown to be highly toxic to brine shrimps[15] and possess anti-dermatophytic activity.[8] Additionally, the leaf of *Cinnamomum porrectum* (Roxb.) Kosterm. is traditionally used as carminative, tonic, stomachic and febrifuge.[16] Its oil is rich with safrole that is toxic to brine shrimps,[15] has anti-candidiasis properties and is potentially used as external pharmaceutical preparations.[16,17]

Chromatography is an important and a widely used separation technique of a complex mixture. Essential oils are mainly separated by gas chromatography (GC) combined with usually either flame-ionisation detector (FID) or mass spectrometry (MS). Recently, the application of chromatographic fingerprint analysis has been accepted for quality control of herbal medicines[18] in order to resolve problems with identification and authentication of multi-component materials such as herbal extracts[19] and essential oils.[20] Thus, combination of the chromatographic fingerprint data

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and multivariate analysis provides comprehensive information on the total chemical composition.[21] Principle component analysis (PCA) is a multivariate exploratory data analysis tool that is used to determine similarities and differences among samples, identify groups of samples and study correlations among variables.[22] Whereas hierarchical clustering analysis (HCA) is used to group things according to their similarities based on specified characteristic variables.[22] This method is now gaining popularity as one of approaches for quality control of herbal materials.[23]

In Malaysia, *Cinnamomum* species has been widely used as traditional medicines or herbal-based cosmetics, however, information regarding the volatile composition quality of essential oils from these herbal materials is still limited. Several studies have reported on the quality assessment of essential oils of *C. verum*, *C. mollissimum* and *C. porrectum* using GC–FID and GC–MS techniques. Thus, in this study, we would like to determine the chemical composition of essential oils of the leaves of three Malaysian *Cinnamomum* species and to characterise the essential oils constituents by using GC and multivariate data analysis.

2. Materials and methods

2.1. Plant materials

Fresh leaves of *C. mollissimum* were collected from Terengganu, *C. porrectum* from Kedah and *C. verum* from Selangor. Authenticity of the plant materials was confirmed by Mr. Sani Miran from the Herbarium of Universiti Kebangsaan Malaysia, at which the voucher specimens were deposited (Table 1).

2.2. Extraction of essential oils

Extraction of essential oils was done by continuous hydrodistillation method.[8] The fresh leaves of *Cinnamomum* spp. were hydrodistillated for 8 h using Clevenger's apparatus. The oily layer obtained was manually separated using a pasture pipette and then dried over anhydrous magnesium sulphate to remove traces of water. Every essential oil obtained was kept in a vial, covered with an aluminium foil and stored at 4–5 °C until further analysis. For each sample, the extraction was repeated in triplicate. The percentage yield of essential oil was obtained using the following equation and expressed as percentage of oil based on its dry weight, i.e. free from water content[8]:

$$\text{Percentage yield of essential oil } (\%) = \frac{100a}{bc}$$

Whereby a is the volume of essential oil obtained in mL, b is the weight of fresh sample used in gram, and c is factor of fresh sample free from water content. The latter was determined from the following formula:

Table 1
Information on plant materials used in the study and their collection site, collection date and voucher specimen number.

Species	Collection site	Date of collection	Voucher specimen number
Cinnamomum mollissimum Hook.f.	Bukit Bauk, Terengganu	January 2011	SM 2147
Cinnamomum porrectum (Roxb.) Kosterm	Pulau Langkawi, Kedah	September 2011	SM 2182
Cinnamomum verum J.S. Presl.	Forest Research Institute Malaysia (FRIM), Selangor	January 2011	UKMB 29879

$$\text{Factor of sample} = \frac{100 - d}{100}$$

where d is the water content of the fresh leaves as determined using Dean Stark method and calculated from the following equation[24]:

$$\text{Percentage of water content } (\%) = \frac{100e}{f}$$

whereby e is the volume of water content in mL, obtained from f that is weight in gram of fresh sample used in this analysis. Percentage of oil yield is expressed as mean ± standard deviation (SD) of triplicated extraction ($n = 3$).

2.3. Gas chromatographic (GC) analysis of essential oils

Composition of the essential oils was determined using a Shimadzu gas chromatograph of model GC-2010 (Kyoto, Japan) equipped with a flame-ionisation detector (FID) and a DB-5 capillary column (30 m × 0.25 mm, 1 μm film thickness). The oil was diluted with ethyl acetate in a ratio of 1:5 and 1 μL of which was injected using an auto-injector AOC-20i. Nitrogen was used as a carrier gas at a flow rate of 50 cm/s, whereas temperature of injector and detector was maintained at 250 °C. The column temperature was initially programmed at 75 °C for 10 min, and then increased from 75 °C to 250 °C at the rate of 3 °C/min. The identity of separated components was determined by comparison of their retention indices with literature value and in some cases by co-chromatography with authentic samples,[25] including eugenol, safrole, α-humulene, α-phellandrene, β-caryophyllene, caryophyllene oxide, isoeugenol acetate and linalool (Sigma–Aldrich, Germany), as well as benzyl benzoate and benzaldehyde (Supelco Analytical, USA).

Kovats retention indices of the volatile components were determined using standard homologous series of C_8–C_{22} hydrocarbons (Sigma–Aldrich, USA) as references based on the following equation[8,26]:

$$\text{Retention index} = 100n + 100\left(\frac{t_{R(x)} - t_{R(n)}}{t_{R(n+1)} - t_{R(n)}}\right)$$

Whereby $t_{R(n)}$ is the retention time of n-alkane with n carbon, $t_{R(n+1)}$ is the retention time of n-alkane with n+1 carbon and $t_{R(x)}$ is the retention times of the component of interest. Relative amount of the individual component was calculated based on its peak area and expressed as percentage content. Retention index and relative amount of the component were determined from three injections and expressed as mean ± standard deviation (SD).

2.4. Gas chromatography–mass spectrometric (GC–MS) analysis of essential oils

The essential oils components were identified using a GC–MS system (Agilent 7890A GC with 5975C MSD Chem Station Basic Operation software) and injector series of 7683B with a ZEBRON ZB-FFAP column. The operation parameters were as described above for GC–FID, except for using helium as a carrier gas. The identity of separated components were confirmed by comparison of their mass spectral data with those from the National Institute of Standards and Technology (NIST).[26] Only compounds with composition of 0.05% or more were identified.

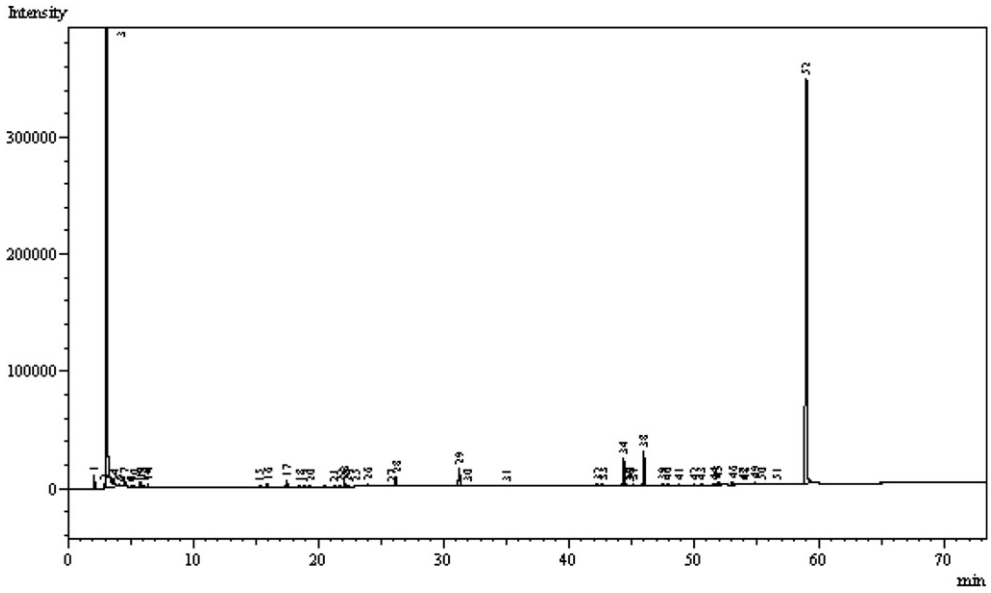

Fig. 1. GC–FID chromatogram of *Cinnamomum mollissimum* leaf oil.

2.5. Multivariate data analysis

The retention index and percentage content of every component for all essential oils were entered into an Excel spreadsheet and the data was analysed using Unscrambler®X version 10.0.1 programme (CAMO Software Inc.). PCA plots of scores, loadings, residuals and influence were analysed and sample outliers were removed prior to generating the HCA using squared Euclidean distance of Ward's method.

3. Results and discussion

3.1. Yield of essential oils

All the investigated *Cinnamomum* species contained essential oils in the range from 1.02 ± 0.05% to 3.33 ± 0.25%. The highest oil yield was obtained from the leaves of *C. verum* (3.33 ± 0.25%), followed by *C. mollissimum* 1.64 ± 0.12% and *C. porrectum* 1.02 ± 0.05%. Our results for *C. verum* leaves are in accordance to the previous reports whereby the essential oil yield was found to be in the range of 1.5–5.5%,[8,9,27] but not quite similar to that reported for *C. mollissimum* (2.65%) and *C. porrectum* (1.95%).[28] Several factors are known to contribute to the variations of essential oil yield such as geographical site of collection, age of plant, time of collection and method of distillation.[29]

3.2. Gas chromatographic analysis of essential oils

Gas chromatograms of essential oils of *C. mollissimum*, *C. porrectum* and *C. verum* leaves are shown in Figs. 1–3, respectively. GC–FID and GC–MS analyses revealed that the oils constituted of a complex mixture of non-terpenes, alcohols, aldehydes,

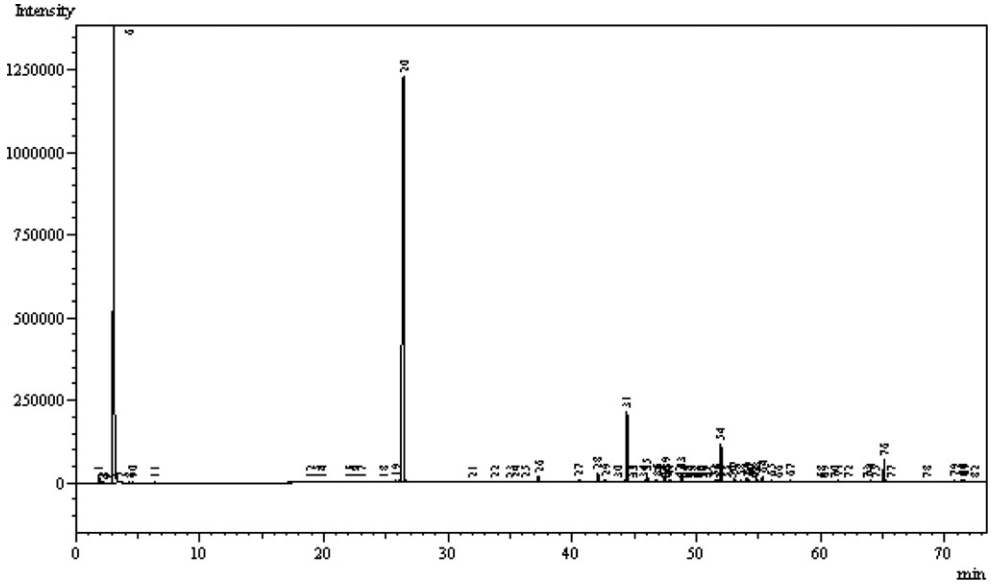

Fig. 2. GC–FID chromatogram of *Cinnamomum porrectum* leaf oil.

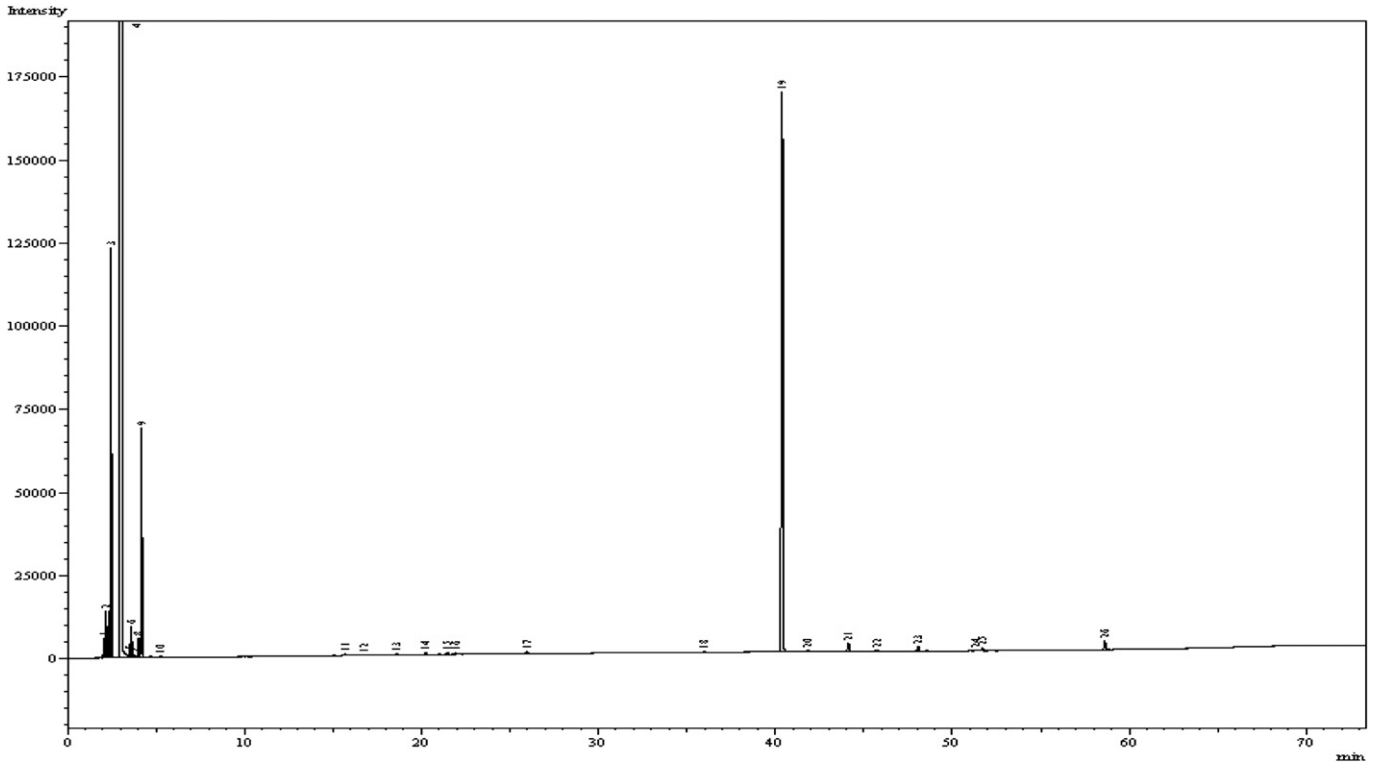

Fig. 3. GC–FID chromatogram of *Cinnamomum verum* leaf oil.

monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpenes and oxygenated sesquiterpenes. The leaf oils of *C. mollissimum*, *C. porrectum* and *C. verum* abundantly contained non-terpenes, oxygenated monoterpene and alcohols, respectively (Fig. 4).

GC analysis revealed the presence of 52 compounds in the *C. mollissimum* leaf oil of which 35 were identified that constituted 99.19% of the total oil content (Fig. 1 and Table 2). Benzyl benzoate (77.69 ± 0.33%), α-humulene (5.38 ± 0.07%), β-caryophyllene (4.59 ± 0.05%), α-terpineol (3.21 ± 0.05%) and linalool (1.43 ± 0.02) were found to be the five main components of the oil. This finding is rather similar to the previous report by Jantan and Goh[14] except 10.71% of benzyl alcohol was detected as compared to that of 0.07% benzyl alcohol from this study. The rich content of *C. mollissimum* leaf oil with naturally-occurring benzyl benzoate could suggest its potential medicinal uses due to the fact that benzyl benzoate is recently reported to inhibit angiotensin II-induced hypertension *in vivo*,[30] and is pharmaceutically utilised for the treatment of scabies, pediculosis and as insect repellent.[31]

The essential oil of *C. porrectum* leaves had 82 compounds but only 17 compounds were identified and accounted for 98.88% of the oil content (Fig. 2 and Table 2). It was found to be highly rich in safrole (93.19 ± 0.05%). Presence of other components in appreciable amounts include β-caryophyllene, tetradecanal, γ-cadinene, δ-cadinene, decanal, α-humulene, farnesyl acetate, dodecal, phytol, eugenol, bisabolol oxide A, germacrone, epi-α-cadinol, isoeugenol

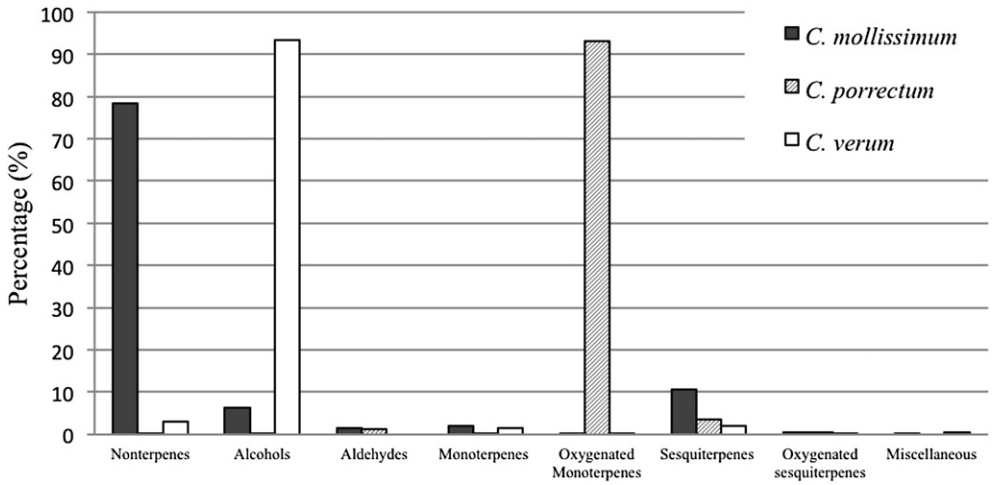

Fig. 4. Chemical composition of the leaf essential oils of three *Cinnamomum* species.

Table 2
Chemical composition of essential oils of three *Cinnamomum* species based on the GC–FID and GC–MS analysis.

Compounds	RI[a]	ID[b]	Percentage of chemical composition (%)[f]		
			Cinnamomum mollissimum	*Cinnamomum porrectum*	*Cinnamomum verum*
α-Thujene	930	[c]	0.30 ± 0.00	–	–
Thiazole	936	[c]	–	–	0.36 ± 0.03
α-Pinene	939	[c]	0.64 ± 0.01	–	0.63 ± 0.07
β-Citronellene	951	[c]	–	–	0.13 ± 0.02
Benzaldehyde	962	[c,d,e]	0.88 ± 0.02	–	–
β-Pinene	979	[c]	0.18 ± 0.00	–	–
Trans-isolimonene	985	[c]	0.07 ± 0.00	0.05 ± 0.00	–
Myrecene	991	[c]	0.10 ± 0.00	–	0.13 ± 0.02
α-Phellandrene	1003	[e]	–	–	0.27 ± 0.00
Pyrazine	1021	[c]	0.11 ± 0.00	–	–
ρ-Cymene	1025	[c]	–	–	0.34 ± 0.06
Limonene	1029	[c]	0.18 ± 0.00	–	–
Benzyl alcohol	1032	[c]	0.07 ± 0.00	–	–
E-β-ocimene	1048	[c]	0.16 ± 0.00	–	–
γ-Terpinene	1060	[c]	0.28 ± 0.01	–	–
Dehydrolinalool	1091	[c]	0.07 ± 0.00	–	–
Linalool	1097	[d,e]	1.43 ± 0.02	–	0.44 ± 0.01
Linalyl oxide	1174	[c]	–	–	0.13 ± 0.02
α-Terpineol	1189	[c]	3.21 ± 0.05	–	–
Safrole	1197	[e]	0.11 ± 0.00	93.19 ± 0.05	–
Decanal	1202	[c]		0.23 ± 0.00	
Benzyl propanoate	1260	[c]	0.10 ± 0.00	–	–
Eugenol	1360	[c,d,e]	–	0.13 ± 0.00	93.08 ± 0.55
Cinnamyl acetate	1389	[c]	0.12 ± 0.00	–	0.34 ± 0.02
α-Damascone	1394	[c]	0.40 ± 0.01	–	–
Dodecal	1406	[c,d]	0.38 ± 0.00	0.15 ± 0.00	–
β-Caryophyllene	1425	[d,e]	4.59 ± 0.05	2.16 ± 0.00	1.57 ± 0.01
β-Farnesene	1443	[c]	–	–	0.29 ± 0.01
α-Humulene	1455	[e]	5.38 ± 0.07	0.22 ± 0.00	–
Dehydroaromadendrene	1463	[c]	0.13 ± 0.00	–	–
β-Bisabolene	1505	[c]	0.27 ± 0.00	–	–
γ-Cadinene	1511	[c]	–	0.84 ± 0.00	–
δ-Cadinene	1520	[c]	0.23 ± 0.01	0.26 ± 0.00	–
Methyl dodecanoate	1526	[c]	–	–	0.79 ± 0.01
α-Cadinene	1541	[c]	0.06 ± 0.00	–	–
Spathulenol	1574	[c,d]	0.05 ± 0.00	–	–
Caryophyllene oxide	1583	[c,e]	–	–	0.13 ± 0.02
Sesquithuriferol	1605	[c]	0.15 ± 0.00	–	–
Geranyl isovalerate	1607	[c]	–	–	0.11 ± 0.01
Humulene epoxide	1608	[c]	–	0.05 ± 0.01	–
Tetradecanal	1613	[c,d]	0.50 ± 0.00	0.98 ± 0.02	–
Isoeugenol acetate	1616	[c,e]	–	0.05 ± 0.00	0.50 ± 0.00
Epi-α-cadinol	1640	[c]	–	0.06 ± 0.00	–
α-cadinol	1654	[c]	0.37 ± 0.00	–	–
Eudesmol	1664	[c]	0.30 ± 0.00	–	–
Bisabolol	1668	[c]	0.18 ± 0.00	–	–
Bisabolol oxide A	1685	[c]	0.35 ± 0.00	0.11 ± 0.01	–
Germacrone	1694	[c]	0.08 ± 0.00	0.10 ± 0.01	–
Farnesyl acetate	1726	[c]	0.07 ± 0.00	0.16 ± 0.01	–
Benzyl benzoate	1760	[d,e]	77.69 ± 0.33	–	1.44 ± 0.04
Phytol	1943	[c]	–	0.14 ± 0.03	–
Total percentage of chemical composition (%)			**99.19**	**98.88**	**100.00**

[a] The retention indices of compounds determined on DB-5 column using GC–FID.
[b] Method of compound identification based on retention indices from.
[c] Literature data.[17]
[d] Mass fragmentation of GC–MS data; or/and
[e] Co-chromatography with reference standards.
[f] Values represent mean ± SD ($n = 3$).

acetate trans-isolimonene and humulene epoxide, in decreasing order of composition. High content of safrole in *C. porrectum* leaf oil is similarly reported.[16,28] Thus, the Malaysian *C. porrectum* leaf oil could possibly be useful alternative to sassafras oils of Brazilian (*Ocotea pretiosa* (Nees) Mez.) and Chinese (*Cinnamomum camphora* Nees) that are utilised as commercial sources of safrole,[32] as well as for the development of topical treatment for candidiasis.[17] However, safrole is found to be a genotoxic hepatocarcinogen *in vivo*.[33] Therefore, its content should be restricted and monitored in traditional preparations containing *C. porrectum* leaf oil intended for internal consumption.

In this study, 17 out of 26 compounds were successfully identified from the essential oil of *C. verum* leaves, giving 100% of the total oil composition (Fig. 3 and Table 2). The predominant constituents were eugenol (93.08 ± 0.55%), β-caryophyllene (1.57 ± 0.01%) and benzyl benzoate (1.44 ± 0.04%). Eugenol is commonly reported as the major component of *C. verum* leaf oils with content ranging from 76.1% and 90.2%.[7,8] However, slight variation in the type and amount of the other components of the oils are observed, for example the leaf oil of *C. verum* collected from Little Andaman, India had eugenol (76.60%), linalool (8.5%) and piperitone (3.31%) as the main components[7] that are rather

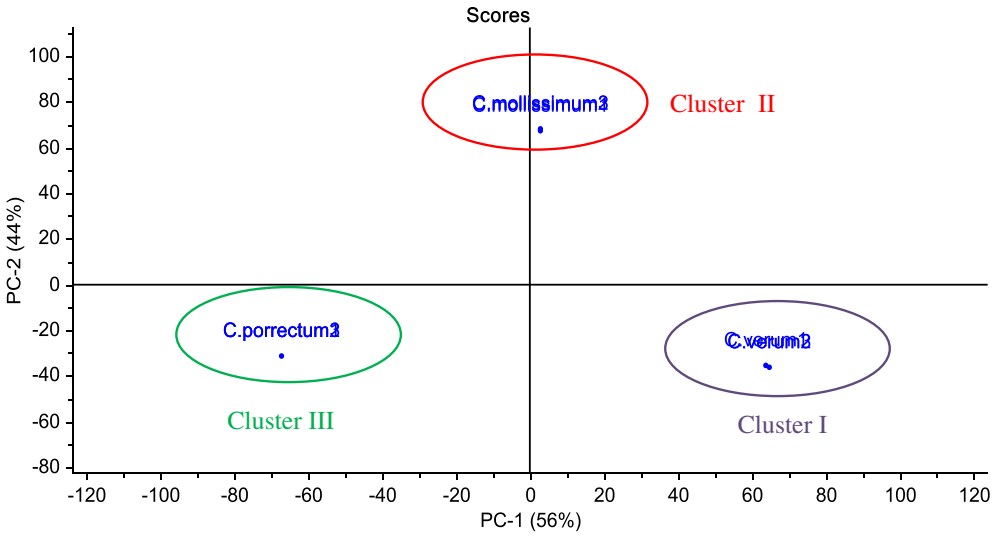

Fig. 5. Principle component analysis (PCA) plot of leaf essential oils of three *Cinnamomum* species.

different from finding of our study. Nevertheless, the previously reported content of eugenol obtained from the leaves of *C. verum* in Malaysia[8] was consistent with our finding (>90%), suggesting the potentiality of the Malaysian *C. verum* to be utilised as a commercial source for eugenol.[34] Eugenol has been used as flavouring agent in cosmetic and food products,[35] and in dental applications.[36]

This study revealed that oil compositions of the three investigated *Cinnamomum* species were greatly varied. Thus, benzyl benzoate, safrole and eugenol could be used as chemical markers for chemotaxonomic characterisation of leaf oils of *C. mollissimum*, *C. porrectum* and *C. verum*, respectively.

3.3. Multivariate data analysis

Qualitatively, the essential oil chromatograms (Figs. 1–3) displayed different profiles that can be used to discriminate *C. mollissimum*, *C. porrectum* and *C. verum*. PCA helps to visually identify pattern of a large number of data and highlight their similarities and differences.

In this study, a total of 459 data set (3 oil samples × 3 replicates × peak area of 51 retention indices in the range of 930–1943) were used in the analysis that generated a PCA score plot having a total sample variance of 100% for the first two principal components (PC) (PC1 = 56% and PC2 = 44%) (Fig. 5). The plot showed distinctive separation of the leaf essential oils into three clusters with *C. verum* grouped in Cluster I (positive score of PC1 and negative score of PC2), *C. mollissimum* in Cluster II (positive score of PC1 and PC2) and *C. porrectum* in cluster III (negative score of PC1 and PC2). Comparison of the PCA score and loading plots allows identification of relationships between the oil samples and the chemical composition variables. The loading plot (Fig. 6) indicated that *C. verum* leaf oil could be differentiated from the other two oils based on the presence of thiazole (RI = 936), β-citronellene (951), α-phellandrene (1003), ρ-cymene (1025), linalyl oxide (1174), eugenol (1360), β-farnesene (1443), methyl dodecanoate (1526), caryophyllene oxide (1583) and geranyl isovalerate (1607). On the other hand, *C. mollissimum* leaf oil could be characterised by α-thujene (930), benzaldehyde (962), β-pinene (979), pyrazine (1021), limonene (1029), benzyl alcohol (1032), E-β-ocimene

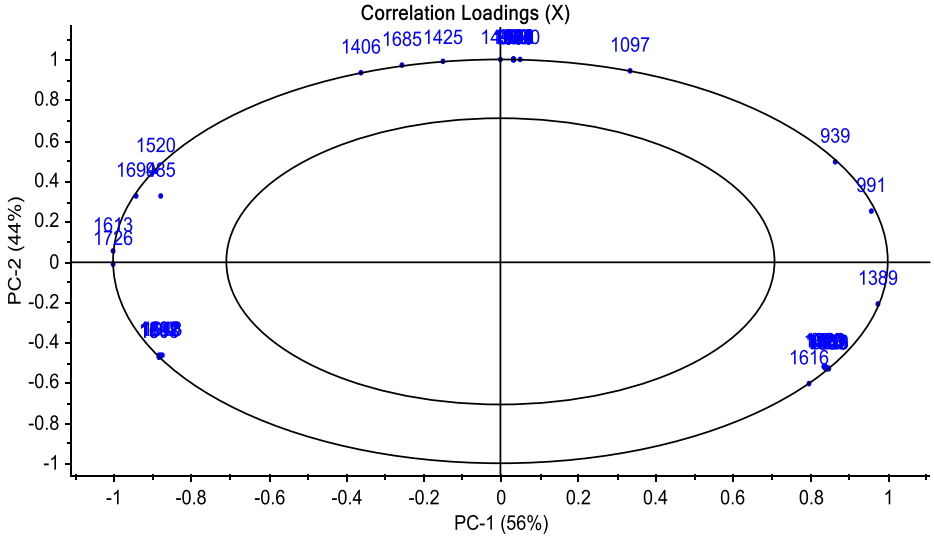

Fig. 6. Loading plot of leaf essential oils of *Cinnamomum mollissimum*, *C. porrectum* and *C. verum*.

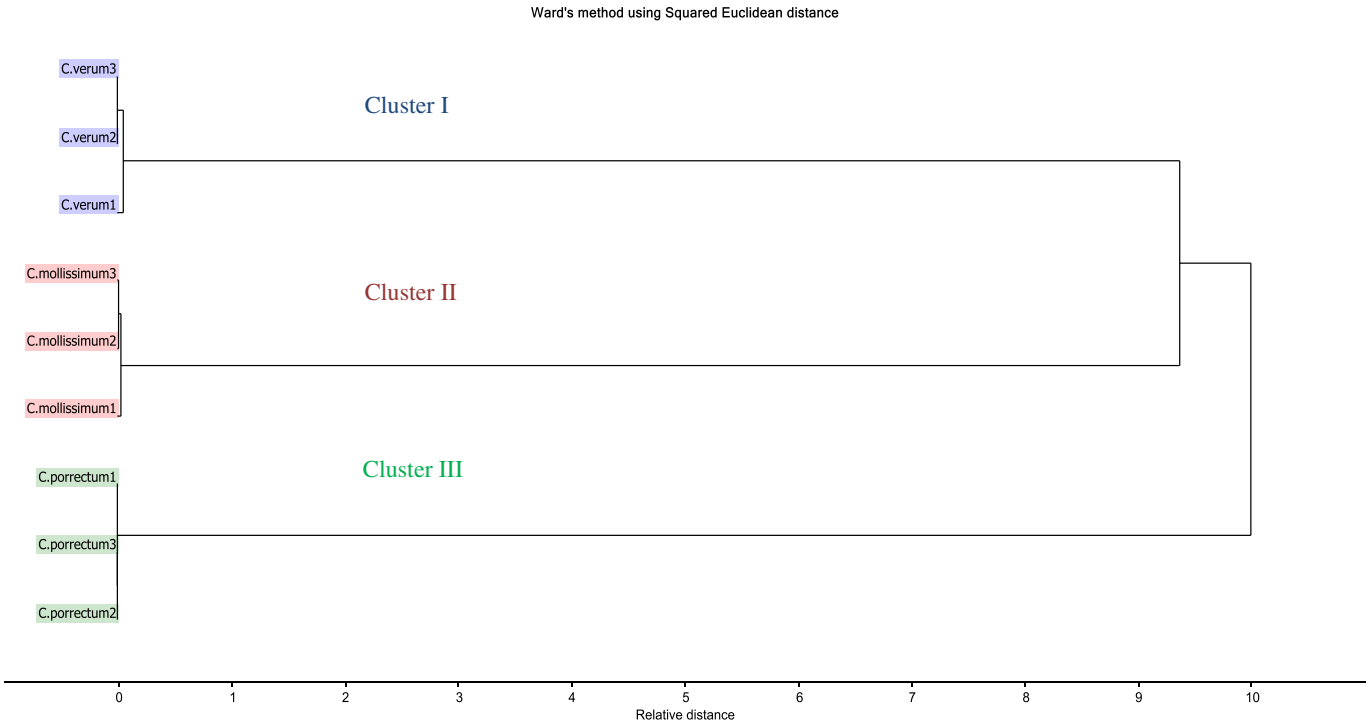

Fig. 7. Hierarchical cluster analysis (HCA) dendrogram of leaf essential oils of three *Cinnamomum* species using ward's method with squared Euclidean distance.

(1048), γ-terpinene (1060), dehydro linalool (1091), α-terpineol (1189), benzyl propanoate (1260), α-damascone (1394), α-humulene (1455), dehydroaromadendrene (1463), β-bisabolene (1505), α-cadinene (1541), spathulenol (1574), sesquithuriferol (1605), α-cadinol (1654), eudesmol (1664), bisabolol (1668), benzyl benzoate (1760). Additionally, safrole (1197), decanal (1202), γ-cadinene (1511), humulene epoxide (1608), epi-α-cadinol (1640) and phytol (1943) could be used to distinguish *C. porrectum* leaf oil. Thus, these chemical constituents could be utilised to characterise and differentiate the *C. mollissimum*, *C. porrectum* and *C. verum* leaf oils.

An HCA dendrogram plot demonstrated that the leaf oils of *C. verum* and *C. mollissimum* were closely related with a relative distance value of 9.3 (Fig. 7). This is also visible from the PCA score plot whereby both oils appear within the positive score of PC1 (Fig. 5) and is further evidenced from the loading plot (Fig. 6), indicating that the similarity was mainly due to the presence of α-pinene (RI = 939), myrecene (991), linalool (1097) and cinnamyl acetate (1389) in both leaf oils (Table 2). On the other hand, β-caryophyllene (RI = 1452) was responsible for the close relationship of the *C. mollissimum* (4.59 ± 0.05%), *C. porrectum* (2.16 ± 0.00%) and *C. verum* (1.57 ± 0.01%) leaf oils (Table 2).

The use of GC fingerprints combined with PCA and HCA has been applied to characterise materials based on their chemical compositions.[20,37,38] The approach is practical and efficient to identify essential oils obtained from different species or varieties; to authenticate oils from adulteration; and to determine quality of the oil based on different cultivation sites, harvesting time, processing method and biological activity.

4. Conclusion

This study provided a preliminary data on the gas chromatographic fingerprinting for characterisation of the leaf oils of *C. mollissimum*, *C. porrectum* and *C. verum*. The fingerprints displayed different profiles that appeared as three distinctively separate clusters with the principal component analysis. This is evidenced by the detection of different major compounds of benzyl benzoate, safrole and eugenol in the leaf oils of *C. mollissimum*, *C. porrectum* and *C. verum*, respectively. However, hierarchical cluster analysis revealed close relationship between *C. mollissimum* and *C. verum* due to the presence of similar components in both oils. Thus, the combination of GC and multivariate data analysis may be used as quality control tools for identification and characterisation of essential oils from different *Cinnamomum* species that are to be utilised as raw materials in traditional herbal products. Further studies need to be carried out to determine fingerprints and chemical compositions of other *Cinnamomum* species and those collected from different origins.

Conflicts of interest

All authors have none to declare.

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