Chemistry and Molecular Characteristics of *Premna flavescens* Wall. ex C. B. Clarke in Vietnam

Le Minh Ha, Ngo Thi Phuong, Nguyen Thi Phuong Trang, Dinh Thi Thu Thuy and Le Ngoc Hung

Abstract

*Premna flavescens* Wall. ex C. B. Clarke, a medicinal plant in the family Lamiaceae, was used for the treatment of arthritis in folk medicine. From the essential oil extracted from the leaves of this plant we have identified 51 components, with β-caryophyllene as the major component (26.3%). The essential oil showed a strong anti-inflammatory effect in vitro with an IC50 of 5.88 µg/mL. DNA barcoding is an useful tool for species identification based on the standardized genomic DNA fragments, but the molecular database of *P. flavescens* is still lacking from Genebank. A molecular species identification tool for *P. flavescens* was developed for the first time using DNA barcoding. The sequences of rbcL, trnH–psbA, internal transcribed spacer, and 18S barcodes of *P. flavescens* were submitted to the Genebank with the accession numbers MW553265, MW553266, MT935698.1, and MW485128.1, respectively. This will be highly useful for the molecular authentication of the marker samples.

Keywords

*Premna flavescens*, conservation, essential oil, anti-inflammatory activity

Introduction

The genus *Premna*, previously classified within the family Verbenaceae, was transferred into the family Lamiaceae. Currently, this genus contains 200 species, which are mainly distributed throughout tropical and subtropical Asia, Africa, Australia, and the Pacific Islands.1,2 *Premna* species can be used in treating various ailments like rheumatism, asthma, dropsy, cough, fever, boils, and scrofulous disease.3 A decoction of the leaves of *Premna flavescens* is taken daily as a tonic.4 In addition, according to the indigenous people in Dong Hy district, Thai Nguyen province, Vietnam, this species was used to treat arthritis effectively. However, up to now, reports on this plant are very limited. There is no research on its pharmacological effect while only a single study on the chemical composition of the essential oil from its leaves was published.5 However, at present, due to overexploitation, the number of this species was significantly reduced. Up till now, study of this plant in Vietnam was mainly focused on ecological characteristics, conservation by propagation, and distribution, but still there is a lack of research on molecular characteristics and molecule-based forensics. DNA barcoding is considered as an efficient and accurate tool for global species identification.6,7 DNA barcodes that are utilized for classification of plants mostly belong either to the internal transcribed spacer (ITS)–rDNA region in the nuclear genome, or the rbcL, MatK, psbA–trnH, and atpF–atpH regions in the chloroplast genome.8,9

In this study, we sequenced rbcL, trnH–psbA, 18S, and ITS–nuclear rDNA regions of *P. flavescens* from Vietnam to obtain additional molecular data for this species. Also, the chemical profile and in vitro anti-inflammatory activity of the essential oil from its leaves were investigated.

Results and Discussion

Total DNA Extraction

Total genomic DNA was successfully extracted and the OD260/280 nm ratio values of these ranged from 1.81 to 1.83.

Efficiency of ITS, rbcL, trnH–psbA—Polymerase Chain Reaction Amplification

Total DNA samples were used as the templates to amplify the ITS–rDNA, rbcL, and trnH–psbA regions. The samples

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gave 100% efficiency for ITS, rbcL, 18S, and trnH–psbA—polymerase chain reaction (PCR) amplification. Electrophoresis of the PCR product from samples showed clear bands with lengths of about 700 bp for ITS, 600 bp for rbcL, 500 bp for 18S, and 400 bp for trnH–psbA, as expected (Figure 1).

Sequencing and Analysis

PCR products were directly sequenced and then the obtained sequences were checked for the accuracy of the obtained PCR products by the Blast tool. The sequences were analyzed using Mega 10. The results showed rbcL, trnH–psbA, ITS, and 18S regions with 589, 412, 681, and 570 bp, respectively. These data will be useful for DNA barcoding based on the authentication of this species.10 The sequences of rbcL, trnH–psbA, ITS, and 18S of P. flavescent were submitted to the Genebank with the accession numbers MW553265, MW553266, MT935698.1, and MW485128.1, respectively.

Chemical Composition

The essential oil of P. flavescent leaves was obtained in a yield of 0.02% based on the fresh material weight. The 51 components of the essential oil were identified using gas chromatography–mass spectrometry (GC–MS), accounting for 98.5% of the composition. The major chemical class present in the essential oil was sesquiterpenoids (83.0%) with β-caryophyllene as the main component (26.3%), followed by α-gurjunene (10.7%), and germacrene D (9.5%), whereas monoterpenoids and other components were 6.5%-9.0%, respectively (Table 1). Interestingly, β-caryophyllene is a common sesquiterpene component, which was proved to possess a variety of biological activities such as antimicrobial, antileishmanial, antioxidant, anticancer, and particularly anti-inflammatory and antiarthritic effects.11-13

Table 1. Chemical Constituents of the Essential Oil.

| No | RIexp | RILit | Chemical name            | %   |
|----|-------|-------|--------------------------|-----|
| 1  | 850   | 854   | Z-Hex-3-en-1-ol          | 0.7 |
| 2  | 976   | 979   | 1-Octen-3-ol             | 3.3 |
| 3  | 977   | 975   | Sabine                  | 0.7 |
| 4  | 983   | 979   | β-Pinene                 | 0.2 |
| 5  | 990   | 991   | Myrcene                  | 0.4 |
| 6  | 1020  | 1026  | α-Cymene                | 0.1 |
| 7  | 1062  | 1060  | γ-Terpine               | 0.2 |
| 8  | 1100  | 1097  | Linalool                | 1.1 |
| 9  | 1184  | 1177  | Terpinen–4-ol         | 0.5 |
| 10 | 1196  | 1189  | α-Terpincol             | 0.1 |
| 11 | 1204  | 1202  | Elsholizia ketone      | 1.7 |
| 12 | 1304  | 1306  | Dihydrocodulain I      | 0.1 |
| 13 | 1307  | 1306  | Dehydrosholizia ketone | 1.2 |
| 14 | 1346  | 1338  | δ-Elemene              | 0.2 |
| 15 | 1359  | 1351  | α-Cubebene             | 0.5 |
| 16 | 1388  | 1377  | α-Copaene              | 4.6 |
| 17 | 1392  | 1385  | β-E-Damascenone        | 0.2 |
| 18 | 1401  | 1391  | β-αr-Elemene            | 3.5 |
| 19 | 1416  | 1415  | Cyperene               | 0.7 |
| 20 | 1424  | 1424  | α-Gurjunene            | 10.7 |
| 21 | 1436  | 1430  | E-Caryophyllene (=β-Caryophyllene) | 26.3 |
| 22 | 1443  | 1434  | β-Gurjunene (≡Calarene) | 0.3 |
| 23 | 1455  | 1451  | Aromadendrene          | 0.3 |
| 24 | 1470  | 1470  | α-Humulene            | 7.7 |
| 25 | 1477  | 1468  | 9-αr-E-Caryophyllene | 2.1 |
| 26 | 1488  | 1480  | γ-Murolone              | 1.1 |
| 27 | 1492  | 1485  | α-Amorphene            | 0.2 |
| 28 | 1496  | 1495  | Germacrene D          | 9.5 |
| 29 | 1502  | 1495  | β-Selinene            | 0.7 |
| 30 | 1510  | 1497  | Viritidiflorene        | 1.3 |
| 31 | 1512  | 1510  | Bicyclogermaocene      | 4.9 |
| 32 | 1527  | 1524  | γ-Cadinene            | 0.3 |
| 33 | 1535  | 1529  | δ-Cadinene            | 2.6 |
| 34 | 1586  | 1581  | Palustrol              | 0.9 |
| 35 | 1594  | 1588  | Spathulanol              | 0.9 |
| 36 | 1601  | 1595  | Caryophyllene oxide    | 1.6 |
| 37 | 1609  | 1601  | Guaiol (≡Champacol)    | 0.1 |
| 38 | 1611  | 1601  | Cubeban–11-ol         | 0.1 |
| 39 | 1619  | 1615  | Rosifoliol            | 0.2 |
| 40 | 1622  | 1617  | Ledol                 | 0.3 |
| 41 | 1628  | 1625  | Humulene Epoxide II       | 0.4 |
| 42 | 1643  | 1640  | 1-αr-Cubenol            | 0.4 |
| 43 | 1655  | 1654  | α-epi-Cadinol (≡Tav-Cadinol) | 0.6 |
| 44 | 1656  | 1652  | α-epi-Murolol (≡T-Murolol) | 0.3 |
| 45 | 1660  | 1658  | α-Murolol (≡β-Cadinol) | 0.4 |
| 46 | 1669  | 1664  | α-Cadinol              | 1.0 |
| 47 | 1673  | 1670  | Neo-intermedeol       | 0.7 |
| 48 | 1703  | 1704  | Eudesma–4(15),7-dien-1-b-ol | 0.1 |
| 49 | 1725  | 1725  | E,E-Farnesiol          | 0.2 |
| 50 | 2114  | 2108  | Phytol                  | 2.8 |

Total identified 98.5
Monoterpenoids 6.5
Sesquiterpenoids 83.0
Others 9.0

Lit: Retention indices from the databases.14-18 The main components has been given in bold.

Figure 1. PCR product of 4 DNA regions checked on 1% agarose gel. Abbreviations: M, DNA ladder 1kb plus; Lane 1, rbcL; Lane 2, 18S; Lane 3, trnH–psbA; Lane 4, ITS; PCR polymerase chain reaction.
Anti-inflammatory Effect

In this study, the essential oil was evaluated for its inhibitory activities on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 cells. L-NMMA was used as a positive control (IC$_{50}$ value of 8.08 µg/mL). Promisingly, the essential oil was found to possess strong NO inhibitory activity with an IC$_{50}$ of 5.88 µg/mL, more than the positive control. This is the first report of the in vitro anti-inflammatory activity of *P. flavescens* essential oil.

Experimental

Plant Materials

*P. flavescens* samples were collected in Dong Hy district, Thai Nguyen province, Vietnam. All fresh leaves were recognized based on the morphology in the field, then transferred to Vietnam Academy of Science and Technology for DNA and chemical analysis. Herbarium samples were prepared and stored in the Department of Ethnobotany.

DNA Extraction

Total genomic DNA was extracted according the method of Doyle and Doyle$^{19}$ under the local laboratory conditions.

PCR Amplification

ITS, rbcL, 18S, and trnH-psbA regions were amplified using universal primers (Table 2).

PCR was performed in 25 µL of reaction system containing 7 µL deionized H$_2$O, 12.5 µL of PCR Master mix kit (2X), 1.25 µL of each primer (10 pmol/µL), and 3 µL of DNA template (10-20 ng). The PCR reaction was performed using PCR Model 9700 (GeneAmp PCR System 9700) for 3 min at 94 °C for denaturation, 35 amplification cycles (45 s at 94 °C for denaturation, 30 s at 55 °C annealing, and 30 s at 72 °C for extension), then 10 min at 72 °C for extension and then held at 4 °C.

Sequence Analysis and Alignment

PCR products were screened by electrophoresis on 1% agarose gel, then sequenced at FirstBase Co. Ltd. Raw sequences obtained were assembled and edited by Chromas-Pro 2.1.6 (Technelysium Pty Ltd). All the sequences were then aligned on BLAST, Genbank (http://www.ncbi.nlm.nih.gov/BLAST). Pairwise distance was determined using Mega 7.0 (Kumar, 2016). The phylogenetic trees were constructed using Maximum likelihood and Bayesian inference with a bootstrap value of 1000.

GC-MS Analysis

GC-MS analysis of the essential oil was carried out on an Agilent Technologies HP7890A GC equipped with a flame ionization detector, Agilent Technologies HP5975C, and a HP5-MS column (60 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies). The injector and detector temperatures were set at 250 °C and 280 °C, respectively. The column temperature progress was initiated at 60 °C, followed by an increase to 240 °C at 4 °C/min. The carrier gas was helium at a flow rate of 1 mL/min. The sample was injected by splitting. The split ratio injected was 1 µL of essential oil. The mass selective detector (MSD) conditions were as follows: ionization voltage 70 eV, emission current 40 mA, and acquisitions scan mass range 35-450 amu under full scan. A homologous n-alkane series was used as the standard to calculate retention time indices (RI) of each component. The relative amounts of individual components were calculated based on the GC peak area (MSD response), without correction. MassFinder 4.0 software connected to the HPCH1607, W09N08 libraries, and the NIST Chemistry WebBook was used to match mass spectra and RI.

Assay for No Inhibitory Effect Using RAW264.7 Cells

RAW264.7 cells were grown in 96-well plates (2 × 10$^5$ cells/well) and incubated in a humidified atmosphere with 5% CO$_2$ at 37 °C for 24 h, then supplemented with Dulbecco’s Modified Eagle Medium (DMEM) (FBS free) for 3 h. The cells were treated with the test sample for 2 h, followed by 1 µg/mL of LPS for 24 h. As a parameter of NO synthesis, the nitrite concentration was measured by the Griess reaction. Briefly, 100 µL of culture medium was incubated with 100 µL of Griess reagent (50 µL of 1% [w/v], sulfanilamide in 5% [v/v] phosphoric acid, and 50 µL of 0.1% [w/v] N–1-naphthylethylenediamine dihydrochloride in water) in a 96-well plate, then incubated at room temperature for 10 min. After incubation, the absorbance was determined using an enzyme-linked immunosorbent assay (ELISA) reader at 540 nm. The DMEM (FBS free) medium was used for blank reading in all experiments, while NG-methyl-L-arginine acetate (L-NMMA) was used as the positive control. The

| Regions | Forward primer (5’-3’) | Reverse primer (5’-3’) | Reference |
|---------|------------------------|------------------------|-----------|
| ITS     | CCTTATCATTTAGGAAAGGAG  | CCGCTTAKGTATAGCTTTAAA  | Cheng et al$^{30}$ |
| rbcL    | ATGTCCCAAAAACAGAAGAAA  | CTTCGGACAAAAATACGAAACGATCTCCTCA  | CBOL Plant Working Group$^8$ |
| 18S     | TCAAAGATTAAGGCCATGCTGCT  | TAGGAGCTTTTTAATCGCAAACAC  | Liu et al$^7$ |
| TrnH-PsbA | GTTATGCAAGCCTAAATGCTC  | CGGGCATGGTGGATTCAATCCC  | Liu et al$^7$ |

Table 2. PCR Amplification.
assay was repeated three times, and the IC_{50} values were identified using TableCurve2Dv4. To evaluate the cytotoxic effect of the sample in RAW 264.7 cells in the assay condition, MTT assay was performed. Briefly, cells were treated with the test sample in a 96-well plate in a humidified atmosphere with 5% CO2 at 37 °C for 72 h; then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added. After 4 h of incubation, the medium was discarded and the formazan blue formed in the cells was dissolved in DMSO. The optical density was measured at 540 nm.

Conclusions

*P. flavescens* is a species with many good therapeutic properties, but currently there is no molecular data available for it, specifically the sequence of DNA regions that are commonly used for barcoding. In this study, we sequenced the four DNA regions that are commonly used for this, which are 18S, ITS, rbcL, and trnH-psbA, with the goal of understanding the molecular characteristics of these DNA regions, as well as submitting them to the Genbank to add to the molecular database of *P. flavescens*, thus helping future research on its classification by barcoding.

The essential oil of *P. flavescens* leaves was obtained in a yield of 0.02% based on the fresh material weight. Sesquiterpenoids (83.0%) were found to be the major components of the essential oil with β-caryophyllene as the major compound (26.3%). Our current study has proven that the essential oil possesses a strong anti-inflammatory activity in vitro, which is considered to be the scientific evidence explaining the use of *P. flavescens* leaves to treat inflammatory diseases. This is the first report of anti-inflammatory activity of *P. flavescens*.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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