Phytochemicals, Antiinflammatory, and Analgesic Effects of the Ethanol Extract From the Leaves of *Premna Flavescens* Wall. ex C. B. Clarke

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Abstract

In the current study, the ethanol extract of *Premna flavescens* leaves (EPF) was evaluated for its in vivo antiinflammatory, analgesic potentials, and acute oral toxicity. The analgesic potential was assessed through the acetic acid-induced writhing test, and the antiinflammatory characteristic was determined through the carrageenan-induced paw edema test in mice. The analgesic experimental results indicated that at a dose of 750 mg/kg, EPF exhibited nearly the same effect as aspirin at a dose of 100 mg/kg. Carrageenan-induced paw edema inflammation in experimental mice treated with EPF at a dose of 750 mg/kg was significantly reduced after 5 h of carrageenan injection. EPF did not show acute oral toxicity at the highest dose of 5 g/kg. Six compounds were isolated from EPF: friedelin (1), friedelinol (2), holoptelin B (3), ethyl β-methoxycinnamate (4), 3β-hydroxy stigmast-5-en-7-one (5), and n-triacontanol (6). Compounds 1, 2, and 6 were isolated from *P. flavescens* for the first time, while compounds 3, 4, and 5 were reported from the genus *Premna* for the first time. These findings give more scientific evidence for the traditional medicinal use of this plant to treat rheumatism and osteoarthritis.

Keywords

*Premna flavescens*, antiinflammatory, analgesic, ethanol extract, phytochemical, friedelin

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Introduction

The genus *Premna* is a big genus belonging to the family Lamiaceae with about 200 species. *Premna* species are distributed mainly in subtropical and tropical Australia, Asia, Africa, and the Pacific Islands.¹ Many *Premna* species have been used to deal with a variety of diseases such as asthma, rheumatism, dropsy, fever, boils, cough, and scrofulous disease.² Phytochemical studies of this genus have resulted in the isolation of more than 100 compounds belonging to classes such as iridoids and their glycosides, diterpenoids, sesquiterpenoids, triterpenoids, flavonoids, isoflavones, lignans, and xanthones. Extracts and pure compounds from *Premna* species displayed various biological activities including antioxidant, antibacterial, antiinflammatory, cytotoxic, immunomodulatory, antidiabetic, antihyperlipidemic, hepatoprotective, and cardioprotective.³

*Premna flavescens* called “Don vo” or “Cach tro vang” in Vietnamese is a precious medicinal plant used by local people. The leaves of the plant have been used as a kind of tea while the whole plant has been used to treat rheumatism and osteoarthritis in folk medicine.¹ Up to now, there have been very few studies on the chemical and biological activities of *P. flavescens*. In our previous study, we reported on the chemical composition of the leaf essential oil of *P. flavescens* and its remarkable in vitro antiinflammatory activity. The essential oil showed a strong antiinflammatory effect with an IC₅₀ value of 5.88 µg/mL. Sesquiterpenoids were found to be the main components of the essential oil, and β-caryophyllene was identified as the key compound (26.3%).⁵ The leaf essential oils of *P. flavescens* and some other *Premna* species displayed mosquito larvicidal activity.⁶ In this study, we continued to look for more scientific evidence to support the use of *P. flavescens*. The acetic acid-induced writhing test has been widely used to screen for antiinflammatory and analgesic agents. Pain is triggered by the

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injection of acetic acid into the peritoneal cavity of mice. The animals reacting to the irritant exhibit a typical stretching action called writhing. The analgesic effect of the test sample is deduced from a reduction in the frequency of writhes.4 Acute inflammation is a short-term process usually lasting only several days involved in the overproduction of free radicals, activating complex enzymes, and stimulating the release of the inflammatory and proinflammatory mediators. The carrageenan-induced paw edema test is a popular animal model to screen and discover novel active antiinflammatory agents. This method is very useful to assess the antiinflammatory effect of components acting on mediators of acute inflammation.5 Therefore, in the present study, we selected the acetic acid-induced writhing test and the carrageenan-induced paw edema model to assess the analgesic and antiinflammatory effects of \emph{P flavescent}, respectively. This study aimed to clarify the chemical composition and evaluate the analgesic and antiinflammatory effects of the medicinal plant.

### Results and Discussion

#### Analgesic Activity

The number of acetic acid-induced writhes in mice is given in Table 1. The findings show that mice treated with 750 and 500 mg/kg of ethanol extract of \emph{Premna flavescent} leaves (EPF) produced a significant reduction in the number of writhes compared to the control group \((P<.05)\). However, treatment with 250 mg/kg of EPF caused an insignificant decrease in the number of writhes in comparison with the control \((P>.05)\). The percentage inhibition of writhing in mice treated with EPF is presented in Figure 1. The best analgesic effect of EPF was achieved at a dose of 750 mg/kg after 25-30 min of acetic acid administration with an inhibition percentage of 52.0%, while EPF at a dose of 500 mg/kg inhibited at most 33.3% of writhing responses after 15-20 min. EPF at a dose of 750 mg/kg caused nearly the same analgesic effect as the standard agent, aspirin, at a dose of 100 mg/kg.

Pain is one of the most commonly reported symptoms of osteoarthritis and arthritis. There is no known cure for these conditions, and hence treatments are used to reduce pain and other symptoms to improve the patients’ quality of life.6 The analgesic effect of EPF was considered as clear evidence to support the use of \emph{P flavescent} as a folk remedy for osteoarthritis and arthritis. Two other \emph{Premna} species, \emph{Premna tomentosa} and \emph{Premna integrifolia}, have also been shown to possess analgesic activity.7,8

#### Antinflammatory Activity

The antinflammatory effect with the percentage inhibition of paw edema of EPF is displayed in Figure 2. The results indicate that EPF at a dose of 750 mg/kg expressed a promising antinflammatory effect with a maximum percentage inhibition of 41.8% after 5 h of carrageenan treatment. However, at a dose of 500 mg/kg, EPF just revealed a moderate antinflammatory effect after 4 and 5 h of injection, while EPF at a dose of 250 mg/kg was almost inactive. The results suggested that \emph{P flavescent} could be used to treat some conditions related to inflammation, especially rheumatism and osteoarthritis. In the genus \emph{Premna}, many species have been reported for their antinflammatory activity, such as \emph{Premna caesmunda}, \emph{Premna herbaeza}, \emph{P integ-rifolia}, \emph{Premna latifolia}, \emph{Premna obtusifolia}, \emph{Premna rerrarifolia}, and \emph{P tomentosa}.3 Hence, antinflammatory activity might be a typical characteristic of \emph{Premna} plants.

#### Chemical Results

Six compounds, friedelin (1), friedelinol (2), holoptelin B (3), ethyl \(p\)-methoxycinnamate (4), 3β-hydroxy stigmast-5-en-7-one (5), and \(\alpha\)-triacontanol (6) were isolated from EPF. Their structures (Figure 3) were identified by analyzing electrospray ionization-mass spectrometry, 1-dimensional, and 2-dimensional nuclear magnetic resonance (NMR) spectra in comparison with reported data.9-11 Compounds 1, 2, and 6 were isolated from \emph{P flavescent} for the first time, while compounds 3, 4, and 5 were first reported for the first time from the genus \emph{Premna}. Remarkably, friedelin was found to be the major compound isolated from EPF with an isolation efficiency of about 0.66% (165.0 mg/25.0 g). Several reported studies indicated that friedelin possessed potential antinflammatory, analgesic, and

### Table 1. Number of Writhes Within Different Periods of Time.

| Treatment      | 0-5 min | 5-10 min | 10-15 min | 15-20 min | 20-25 min | 25-30 min |
|---------------|---------|----------|-----------|-----------|-----------|-----------|
| Control\(^a\) | 31.7 ± 7.2 | 23.7 ± 3.9 | 16.7 ± 3.1 | 13.7 ± 3.3 | 11.2 ± 1.5 | 8.7 ± 1.0 |
| Aspirin\(^b\) (100 mg/kg) | 14.8** ± 3.3 | 14.2** ± 3.5 | 10.3** ± 2.4 | 8.4** ± 1.9 | 6.12** ± 1.4 | 4.1** ± 1.8 |
| EPF (750 mg/kg) | 18.2* ± 5.4 | 14.4* ± 4.4 | 11.0* ± 4.8 | 8.0* ± 3.2 | 6.0* ± 3.5 | 4.2* ± 1.6 |
| EPF (500 mg/kg) | 24.7 ± 7.1 | 18.8 ± 2.28 | 13.2 ± 2.9 | 9.17 ± 2.47 | 8.1* ± 2.0 | 6.0* ± 1.9 |
| EPF (250 mg/kg) | 27.2 ± 4.8 | 20.1 ± 3.90 | 14.0 ± 4.1 | 11.89 ± 3.02 | 9.80 ± 2.8 | 7.3 ± 2.6 |

Abbreviation: EPF, ethanol extract of \emph{Premna flavescent} leaves. Values are shown as the mean ± SD, \(n = 6\), \(^*P < .05\), \(^**P < .01\) as compared to the control.

\(^a\)Mice administered distilled water.
\(^b\)Mice administered aspirin.

\(^\text{No. of writhes}\)^ c Number of writhes after injection of acetic acid solution.

**

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\(^\text{aMice administered distilled water.}

\(^\text{bMice administered aspirin.}

\(^\text{cNumber of writhes after injection of acetic acid solution.}**
antipyretic properties, as well as cytotoxic and antidiabetic effects.\textsuperscript{18-20} This suggested that friedelin might be the main component responsible for the antiinflammatory and analgesic characteristics of EPF.

\textit{Acute Toxicity}

Experimental mice that were treated with EPF at doses of 1, 2, 3, 4, and 5 g/kg lived, ate food, and drank water normally within the monitored periods. There was no significant difference ($P>0.05$) in mice body weight between the control group and the groups taking EPF. The animals did not express any toxicity signs (slow movement, inactivity, vomiting and loss of appetite) at the maximum dose of 5 g/kg of EPF throughout the experiment. Therefore, EPF was considered to be orally nontoxic according to the classification of oral toxins by the World Health Organization (1993), Organization for Economic Cooperation and Development, and Globally.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Percentage inhibition produced by ethanol extract of \textit{P. flavescens} leaves (EPF) at different doses in acetic acid-induced writhing test in mice. *$P<0.05$, **$P<0.01$. Aspirin was used as the standard drug.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Percentage inhibition produced by ethanol extract of \textit{P. flavescens} leaves (EPF) at different doses in carrageenan-induced paw edema model in mice. *$P<0.05$, **$P<0.01$. Aspirin was used as a standard drug.}
\end{figure}
Harmonized System of Classification and Labelling of Chemicals.

Conclusions

In conclusion, the findings of the present study showed promising antiinflammatory and analgesic effects of the EPF and its nontoxicity. Friedelin (1), along with friedelinol (2), holoptelin B (3), ethyl p-methoxycinamate (4), 3β-hydroxy stigmast-5-en-7-one (5), and n-triacontanol (6) were isolated from EPF. These findings provided more novel scientific evidence for using *P. flavescens* in folk medicine and suggested that the EPF may be used as an analgesic and anti-inflammatory agent.

Experimental

Plant Material

The leaves of *P. flavescens* were collected in Thai Nguyen province, Vietnam in February 2021, and authenticated by the botanist Nguyen The Cuong (Institute of Ecology and Biological Resources, VAST). The voucher samples (DV-02.2021) have been deposited in the Institute of Natural Products Chemistry, VAST.

General Procedures

The 1H- and 13C-NMR (125 MHz) spectra were recorded on a Bruker AM500 spectrometer at 500 and 125 MHz, respectively. Column chromatography (CC) was carried out on silica gel (0.040-0.063 mm, Merck). Thin-layer chromatography (TLC) was performed on precoated silica gel 60 F254, Merck. All solvents were distilled before use. Compounds on TLC were observed under ultraviolet light at wavelengths of 254 and 365 nm, and by immersing the plate rapidly in 10% H2SO4 solution, followed by heating. Carrageenan, acetic acid, and aspirin were purchased from Sigma-Aldrich.
Experimental Animals
Albino BALB/c mice of both sexes (20-22 g) were provided by the Institute of Biotechnology, VAST. All the animals were kept in appropriate cages under standard controlled laboratory conditions (25 °C, 12 h light, and 12 h dark cycle). Mice were provided food and water ad libitum. Experiments were performed at the Institute of Biotechnology, VAST following Vietnamese ethical laws and European Communities Council Directives of November 24, 1986 (86/609/EEC) guidelines for the care and use of laboratory animals.

Extraction and Isolation
The powdered leaves of *P. flavescens* (2.0 kg) were extracted 3 times with ethanol at 50 °C to produce the EPF, which was then used to evaluate biological activities. The ethanol extract (25.0 g) was fractionated by CC eluting in turn with *n*-hexane, ethyl acetate, and methanol to obtain *n*-hexane (6.4 g), ethyl acetate (7.2 g), and methanol (9.4 g) fractions after removal of the solvents. The *n*-hexane fraction was subjected to silica gel CC using a gradient of *n*-hexane-ethyl acetate (100:0-0:100, v:v) to give 5 fractions (H1-H5). Fraction H2 (1.2 g) was further subjected to silica gel CC using *n*-hexane-ethyl acetate (95:1) to yield 3 subfractions (H2.1-H2.3). Recrystallizing of the subfraction H2.2 yielded compound 3 (15.0 mg). Fraction H3 (2.5 g) was subjected to a silica gel column using *n*-hexane-acetone (60:1) to give 4 smaller fractions (H3.1-H3.4). Fraction H3.2 (1.1 g) was further chromatographed by silica gel CC using *n*-hexane-ethyl acetate (30:1). Compound 1 (165.0 mg) was obtained after recrystallizing fraction H3.2.2 using a mixture of *n*-hexane-acetone (3:2). Subfraction H3.3 (0.7 g) was further separated on a silica gel column by eluting with a mixture of *n*-hexane-acetone (50:1) to produce 3 smaller subfractions (H3.3.1-H3.3.3). Compound 2 (12.1 mg) was produced from the subfraction H3.3.2 by using a silica gel column and an eluent of *n*-hexane-acetone (20:1). The ethyl acetate fraction was subjected to silica gel CC using a gradient of *n*-hexane-ethyl acetate (100:0-0:100, v:v) to give 4 fractions (E1-E4). E1 (2.5 g) was separated on a silica gel column using *n*-hexane-ethyl acetate (15:1) to obtain 4 smaller fractions (E1.1-E1.4). Subfraction E1.3 (1.3 g) was further separated on a silica gel column using *n*-hexane-ethyl acetate (12:1) to yield compounds 4 (10.5 mg) and 6 (16.5 mg). Fraction E2 (1.2 g) was first subjected to a silica gel column with *n*-hexane-ethyl acetate (10:1) as an eluant to produce 4 subfractions (E2.1-E2.4). Compound 5 (13.0 mg) was produced from fraction E2.3 by silica gel CC with *n*-hexane-acetone (5:1) as the eluant.

Analgesic Assay
Acetic Acid-Induced Writhing Test in Mice. Twenty-four BALB/c mice were separated into 4 groups (each contained 6 mice). After being treated with either EPF (250, 500, and 750 mg/kg) or distilled water or aspirin (100 mg/kg) as a reference standard for 30 min, all experimental groups were administered an intraperitoneal injection of acetic acid solution (10 mL/kg, 0.6%). The number of writhes was counted every 5 min after acetic acid application within 30 min. Percentage inhibition was calculated by comparing the data of the groups taking EPF with the control group taking distilled water.21

Antiinflammatory Assay
Carrageenan-Induced paw Edema Test in Mice. Twenty-four BALB/c mice were separated into 4 groups (each contained 6 mice). After 60 min of dosing with either EPF (250, 500, and 750 mg/kg) or 0.9% saline solution or reference standard, aspirin (100 mg/kg), edema was induced by injection of carrageenan 1% (w/v) in sterile 0.9% saline solution (0.05 mL per mouse) into the right hind paw. The hind paw edema thickness was determined after injection at 0.5, 1, 2, 3, 4, and 5 h.16 The inflammation inhibition percentage was determined at various periods of time according to the formula below:

\[
\text{Percentage inhibition} = \left( \frac{C - T}{C} \right) \times 100
\]

where C is the average paw edema thickness of the control group and T is that of the test groups.21

Acute Toxicity
Thirty-six healthy albino BALB/c mice were separated into 6 groups (each contained 6 mice) and treated with EPF at doses of 1, 2, 3, 4, and 5 g/kg. The control group was administered distilled water. These animals were observed for 7 days after administration of EPF in case of showing signs of abnormality. Their behavior, movements, consuming food and water, and death were especially observed and noted during the first 2 h, then periodically during the first 72 h, and daily thereafter.22

Statistics
All experimental groups consisted of 6 animals. The obtained data were presented as mean ± SD. One-way analysis of variance, followed by the Bonferroni post hoc test was employed for statistical comparison among different experiment groups. Differences with \( P \leq 0.05 \) between groups were considered significant.

Author Contributions
HML, PTN searched the literature and designed the study. Both HTTD and ATN did experiment work. HML, PTN were responsible for data analysis, preparation, editing, and review of the manuscript.

Declaration of Conflicting Interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.
Ethical Approval
Experiments were performed at the Institute of Biotechnology, VAST following Vietnamese ethical laws and European Communities Council Directives of November 24, 1986 (86/609/EEC) guidelines for the care and use of laboratory animals.

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There are no human subjects in this article and informed consent is not applicable.

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Supplemental material
Supplemental material for this article is available online.

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