INTRODUCTION

Pain is a bad feeling and sensation that can be linked to possible or present tissue damage. Analgesic substances selectively reduce pain by affecting the central nervous system or the peripheral pain mechanism, all without significantly changing awareness. Steroids and non-steroidal anti-inflammatory drugs (NSAIDs) are often used to lessen pain and inflammation. NSAIDs reduce pain and inflammation by blocking the actions of the cyclooxygenase (COX) enzymes, particularly COX-II. Long-term NSAID use still has been connected to a number of unfavorable liver and gastrointestinal (GI) issues. They also affect the cardiovascular system and the kidneys. Researchers have stressed how important antioxidants are as natural ways to treat the harmful effects of NSAIDs since NSAIDs are harmful to pathways and cause oxidative damage. For this reason, the use of herbal medications to relieve pain and inflammation has increased.

Pteridophytes, also known as palm leaf and their associates were a group of non-flowering vascular plants that have been around for millions of years. From the previous literature, it is supposed that of the primitive vascular plants. Pteridophytic floras were one of the globally distributed species estimated at about 13600 species. The biodiversity-rich India shares an outstanding number of more than 1000 species comprising 67 families and 191 genera.

ABSTRACT

Pteris quadriaurita, a highly significant underutilized plant species that was widely recognized for its ethnobotanical and ethnomedicinal properties. The work aimed to estimate the flavonoid-rich fraction of P. quadriaurita (PQFRF), which was typically used to alleviate pain, for its antioxidant profile and associated analgesic effectiveness. In-silico molecular docking and PRIME MM-GB/SA studies were performed to analyze the binding pattern of nine bioactive substances identified by gas chromatography-mass spectrometry (GC-MS). The plant’s antioxidant activities were tested in-vitro using DPPH, ferric-reducing antioxidant power, Superoxide anion radical, and hydroxy radical scavenging tests. For in-vivo analgesic research, the hot plate model, acetic acid-induced writhing, and tail flicking models were utilised. PQFRF was found to be equipotent to the reference medication in the studied peripheral and central analgesic models at a maximal dose of 50 mg/kg.

Keywords: Pteris quadriaurita, Phytochemicals, Docking, Analgesic activity, Antioxidant activity.


Source of support: Nil.

Conflict of interest: None

INVESTIGATING THE ANALGESIC EFFECT OF FLAVONOID RICH FRACTION FROM PTERIS QUADRIAURITA: EVIDENCE FROM IN-SILICO, IN-VITRO AND IN-VIVO STUDIES

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was *Pteris quadriaurita*. Among various species of *Pteris*, this plant was chosen for the study as it was commonly spread in the geographical region and little of the research work has been done in the field of ethnobotany of this species. The plant *P. quadriaurita*, which is in the family Pteridaceae, was a common land herb that grew in partly shaded, dry places in the plains and Ghats at lower elevations. The selected plant was used as an antihelmenth.15 Decoctions of fresh fronds and rhizomes of this particular species were employed in the management of chronic illnesses caused due to obstructions of viscera, including spleen16. Recent years have seen a resurgence of interest in flavonoid’s unique, multifaceted properties. At first, flavonoids were shown to have protective properties against inflammation, hepatotoxicity, and cataract development.17 Recent studies have demonstrated the considerable analgesic action of certain flavonoids, including gossypin and hydroxyethyl rutoside (HR).18,19 Upon examination of these studies, it becomes apparent that the flavonoids under investigation might not employ a shared mechanism to produce an analgesic effect. Furthermore, the activity of very few flavonoids has been investigated in the current study.

Authors examined the analgesic and antioxidant prospective of *P. quadriaurita*’s flavonoid-rich fraction using gas chromatography–mass spectrometry (GC–MS) analysis, *in-vitro*, *in-vivo*, and *in-silico* studies. Previous literature on flavonoids’ properties motivated this research due to limited prior studies on this plant’s bioactivity, emphasizing its potential for pain relief and antioxidant effects.

**MATERIALS AND METHODS**

**Materials**

The compounds used in this work were α-diphenyl-β-picrylhydrazyl (DPPH), tripyridyl triazine (TPTZ), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide hydrogen (NADH), phenazine methosulfate (PMS), thio-barbituric acid (TBA) of analytical grade and were purchased from Sigma Aldrich Pvt. Ltd., India. Other reagents like phloroglucinol, chloral hydrate, saffranine, methyl orange, etc, and were purchased from Himedia Pvt. Ltd. India.

**Collection and Authentication of Plant Material**

Andhra Pradesh’s hills and moist Sathyavedu forest region yielded the chosen plant. Dr. K. Madhava Chetty, Assistant Professor, Sri Venkateswara University, Tirupati, validated the plant. The Department of Pharmacognosy, Sree Vidyanikethan College of Pharmacy, Tirupati, received the plant voucher specimen (*P. quadriaurita* -SVC/2020/22). The plant was cleaned with water and desiccated under indirect sunlight for 15 to 20 days and then crushed to a coarse powder (Sieve No. 44) using a grinder. The powdered dried herb was kept in a bottle sealed with air.

**Extraction of the Plant Material**

Plant powder weighing 100 g was defatted by soaking for 72 hours in pet ether (60–80°C). Utilizing a soxhlet extraction device, the defatted plant powder was extracted with ethanol (99% v/v) for 3 to 4 cycles of 6 hours each until clear solvent was obtained in the extraction flask. To obtain two pure ethanol extract, it was filtered and dehydrated in a rota vapor at 40°C. The extracted substance has been kept in a glass vial at a freezer temperature of 4°C till used again.

**Determination of Arsenic Content**

As the *Pteris* species was one of the hyperaccumulators of arsenic, the ethanol extract of the plant was exposed to inductively coupled plasma optical emission spectrometry (ICP-OES) analysis for the estimation of arsenic content in the plant extract at a wavelength of 188.979 nm using Perkin Elmer Optima instrument.

**Phytochemical Screening of the Plant Extracts**

The plant ethanol extract was tested qualitatively for chemical components using conventional procedures.

**Separation of flavonoid rich fraction**

The crude plant extract is fractionated using n-hexane, chloroform, ethyl ether, and ethyl acetate based on solvent polarity.21 The dried extracts of each portion were stored at 4°C.

**Determination of total phenolic content**

Using varying doses (0.15–0.02 mg/mL) of gallic acid, total phenolic content (TPC) was investigated using the Folin-Ciocalteu (FC) technique for all the fractions and ethanol extract.22 TPC was determined using the gallic acid calibration curve.

**Determination of total flavonoid content**

The aluminum chloride colorimetric method was used to determine the total flavonoid content (TFC) of each fraction.23 TFC was expressed using the quercetin calibration curve in the form of mg of quercetin equivalent (QE).

**Gas Chromatography-Mass Spectrometry Analysis**

In accordance with a previously published protocol, the phytochemicals in the extract were identified by gas chromatography-mass spectrometry (GC-MS) and compared to the NIST database. By comparing each component’s relative quantity to the entire peak area, the percentage of the peak area was provided.

**Molecular Docking**

In this study, 3D X-ray crystal structures were obtained for peripheral pain targets (Aspirin-acetylated human COX-2, 5F19.pdb, 2.04Å), central pain targets (delta opioid 7TM receptor bound with benzofuro [2,3-a] pyrido [4,3-b] carbazole derivative, 4N6H.pdb, 1.8Å), and derivative of naphtha [2,1-g] indol-10-ol bound mu- The following approach was used for molecular docking and PRIME binding free energy experiments.24,25

**In-vitro Antioxidant Activity**

Flavonoid-rich fraction has been dispersed in purified water to a level equivalent to 1-mg/mL, followed by diluting to prepare concentrations of 100 mg/mL using models such as DPPH radical scavenging, superoxide radical scavenging, hydroxyl radical scavenging, and reducing power activity.
DPPH radical scavenging activity
This test as described in previous studies. The test’s ability to liberate the DPPH radical was expressed as a percentage inhibition and computed using the equation:

\[
\{(A_0 - A_1)|A_0\} \times 100
\]

\( A_0 \) = absorbance of the control and \( A_1 \) = absorbance of the sample.

Ferric reducing antioxidant power (FRAP) activity
The capacities of ferric ions to be reduced have been investigated using method. Increased absorbance at 593 nm indicates antioxidant activity, given in micromolar \( \text{Fe}^{2+} \) equivalents or relative to an antioxidant reference.

Superoxide radical scavenging (SRS) activity
It was estimated using the technique. The percent inhibition was estimated by using the following equation:

\[
\text{SRS} = \{(A_0 - A_1)|A_0\} \times 100
\]

\( A_0 \) = absorbance of the control and \( A_1 \) = absorbance in the presence of the extract and reference. All the \( \text{in-vitro} \) antioxidant tests were done in triplicates and the findings were taken as the mean.

Hydroxyl Radical Scavenging (HRS) Activity
It was determined using the revised procedure given. The herb’s capacity to neutralize hydroxyl radicals was assessed as a percent decrease in deoxyribose degradation utilizing the following equation:

\[
\text{HRS} = \{(A_0 - A_1)|A_0\} \times 100
\]

\( A_0 \) = absorbance of the control and \( A_1 \) = absorbance, considering the extract and reference both exist. All \( \text{in-vitro} \) scavenger experiments were carried out in a trio, with the findings taken as the mean.

Statistical Analysis
The results are presented as mean ± standard deviation (SD). One-way ANOVA and Dunnet’s test showed statistical significance. The significance level was set at \( p < 0.05 \).

In-vivo Studies

Experimental animals
Male Swiss Albino mice, 8 weeks old and 25 to 30 gm, were used for the investigation. They were kept in a room with a temperature of 22 ± 3°C and relative humidity of 50 ± 20%. Artificial lighting and unlimited water were provided for the mice. The Institutional Animal Ethics Committee (IAEC) approved the application SVCP/IAEC/I-002/2018-19.

Acute toxicity
The selected animals were fasted overnight and the dose was fixed according to OECD guidelines No. 423. The extract proved safe in mice at 2000 mg/kg oral dosages. So 25 and 50 mg/kg extracts were employed for the investigation.

Acetic-induced writhing test
It followed the stated procedure. In Figure 3, intraperitoneal injection of 0.6% acetic acid solution at 10 mL/kg 30 minutes after pre-treatment elicited writhing. Each mouse was put in a transparent cage and counted for 10 minutes for writhing.

Eddy’s hot plate method
This test was done following procedure. The commencement of reaction or response time was when animals paw-licked or jumped in response to pain. The response cut-off was 15 seconds to prevent tissue injury. Antinociceptive responses were marked by a considerable decrease in reaction time in test animals compared to controls.

Tail flick method
Previously, a technique was used for this test. Rapid tail removal from hot water was the reaction time with a 15-second cut-off.

RESULTS AND DISCUSSION

Extraction and Fractionation
The yield of the ethanolic extract (EE) obtained per 100 g of dry plant material was found to be 44.0 ± 0.86 g. The ethanolic extract was partitioned into various fractions and yields of the fractions with different solvents were given in Table 1.

Estimation of Arsenic
Despite being a hyperaccumulator of arsenic, the plant’s therapeutic benefits ensure its safe use. Analysis revealed arsenic levels below detection limits, reinforcing its safety for consumption.

Total Phenolic and Flavonoid content
Quantitative estimation of phenolic compounds and flavonoids have indicated the occurrence of sufficient quantity of these bioactive composites in ethyl acetate extract and thus considered as flavonoid-rich fraction (FRF), when compared to other fractions and ethanol extract of the plant as shown in Table 2.

GC-MS Analysis of FRF (Figure 1)
FRF chromatogram showed nine phytoconstituent peaks. Table 3 shows retention times (RT), compound names, molecular formulas (MF), molecular weights (MW), %peak area, compound nature, and activity for the detected compounds.

Total nine chemical compounds were determined through GC-MS analysis, including hydrocarbons, phenolic, flavonoids, amines, etc. It has been recommended that phenolic moiety has been found to be important for antioxidant activity. The hydroxyl groups found in phenolic substances improve their capacity to scavenge free radicals and stimulate their antioxidant properties. Because of their numerous biological benefits as antibacterial, antioxidant, anti-inflammatory, and anticancer agents, phenolic compounds are highly prevalent.
Since *Pteris* species have traditionally been recommended to treat a diversity of illnesses, and moreover, the identified lead compounds in the present study have the potential for analgesic activity, we looked at the binding ability of one bioactive chemical F3 (7-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) from PQFRF with central and peripheral pain targets. The flavonoid F3 exhibited a good glide g score and binding energy values against three targets when compared with standard compounds.

**In-silico Studies**

We initiated our research work by exploring the binding modes of GC-MS-identified compounds of ethyl acetate fraction. Molecular docking followed by binding free energy calculation studies were performed for 8 GC-MS identified compounds against a set of peripheral pain targets and central pain targets using GLIDE and PRIME MM/GB-SA of Schrodinger, respectively (Tables 4-7). The XP docking results were compared with standard aceclofenac for peripheral pain targets and oxycodone for central pain targets. As our focus was a flavonoid-rich fraction of *P. quadriaurita*, we highlighted the binding pattern of one flavonoid F3.

The GC-MS subjected ethyl acetate fraction or flavonoid-rich fraction of *P. quadriaurita* resulted in the identification of different classes of organic compounds (Table 3). Molecular Docking and MM/GBSA Studies of GC-MS Identified Compounds Against Peripheral and Central Pain targets

The identified compounds showed glide g score (-4.77--10.26 kcal/mol) and binding energy (-2.07--70.95 kcal/mol)
with catalytic residues of aspirin-acetylated human cyclooxygenase-2 (5F19.pdb) (Tables 4 and 5). The identified compounds exhibited hydrogen bonding and hydrophobic interactions with Thr212, Tyr385, Glu206 and His207 (Table 6). In Flavonoid F3 (Glide G score: -7.33 kcal/mol and ΔG bind: -61.58 kcal/mol), the -OH group of 4-hydroxyl phenyl group formed a hydrogen bond with Tyr385, whereas the hydrophobic part of the benzopyran ring formed π-π contact with His207. The flavonoid F3 was completely buried in the hydrophobic pocket, showing one hydrogen bond (Figure 2A).

Glide score and binding energy values for the identified compounds in the active site of the human P2X3 ion channel (5SVM.pdb) range between -1.79 to -2.88 kcal/mol and -27.59 to -54.37 kcal/mol, respectively. The identified compounds formed hydrogen bonding and hydrophobic contacts with catalytic residues Glu156, Arg281, Glu109, Glu111, Glu102, Tyr114, Trs406, Asp158, and Lys299 (Table 6). A network of hydrogen bonds was observed for F3 (Glide G score: -5.63 kcal/mol and ΔG bind: -39.36 kcal/mol), where the phenolic oxygen of 4-hydroxy phenyl ring, the carbonyl oxygen of benzopyran ring and 7th substituted OH formed three hydrogen bonds respectively with Glu102, Arg281 and Tyr114 (Figure 2B).

The Glide G scores and ΔG values for identified compounds against δ-opioid receptor (4N6H.pdb) were observed in the range of -3.89 to -7.02 kcal/mol and -88.42 to -43.4 kcal/mol, respectively. The identified compounds formed hydrogen bonding and hydrophobic interactions with Cys210, Tyr139, Tyr312, His291, Ile316, Asp138, Tyr313 and Tyr219 (Table 7). As shown in Figure 2C, the hydroxyl group at 7th position of F3 formed two hydrogen bonds by accepting and donating electron pairs with His278 and the 4-hydroxy phenyl ring formed π stacking interaction with Tyr308.

The Glide G scores and ΔG values for identified compounds against μ-opioid receptor (5C1M.pdb) were observed in the range of -3.89 to -7.02 kcal/mol and -88.42 to -43.4 kcal/mol, respectively. The identified compounds formed hydrogen bonding and hydrophobic interactions with Ser53, Lys303, Tyr148, His54, Ser33, Ser55, Asp147 and His297 (Table 7). In F3 (Figure 2D) the –OH group of 4-hydroxy phenyl ring shared one hydrogen bond with Ser55.

Table 4: Glide G score and PRIME MM/GBSA values (kcal/mol) of GC-MS identified phytoconstituents against COX-2 (5F19. pdb) and P2X3 (5SVM.pdb)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Compounds</th>
<th>COX-2 (5F19)</th>
<th>P2X3 (5SVM)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Glide G score</td>
<td>ΔG bind</td>
</tr>
<tr>
<td>1</td>
<td>2-(9-octadecenoyloxy)-(Z)-Ethanol</td>
<td>-10.26</td>
<td>-44.4</td>
</tr>
<tr>
<td>2</td>
<td>3-Eicosyne</td>
<td>-5.7</td>
<td>-59.34</td>
</tr>
<tr>
<td>3</td>
<td>Phytol</td>
<td>-7.97</td>
<td>-70.95</td>
</tr>
<tr>
<td>4</td>
<td>2-[2,4-dimethoxyphenyl]-3,5,7-trimethoxy-4H-1-benzopyran-4-one (F1)</td>
<td>-5.52</td>
<td>-50.78</td>
</tr>
<tr>
<td>5</td>
<td>2',3',5',7-tetramethoxy flavone (F2)</td>
<td>-8.47</td>
<td>-58.01</td>
</tr>
<tr>
<td>6</td>
<td>7-hydroxy-2-(4-hydroxyphenyl)-4H-1-Benzopyran-4-one (F3)</td>
<td>-7.33</td>
<td>-61.58</td>
</tr>
<tr>
<td>7</td>
<td>2,5-di-tert-butylaniline</td>
<td>-4.75</td>
<td>2.07</td>
</tr>
<tr>
<td>8</td>
<td>2-methoxy-9a-(morpholin-4-yl)-5a,6,7,8,9,9a-hexahydro dibenzofuran-3-ol</td>
<td>-5.02</td>
<td>-43.17</td>
</tr>
<tr>
<td>9</td>
<td>Aceclofenac</td>
<td>-6.23</td>
<td>-38.84</td>
</tr>
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Table 5: Glide G score and PRIME MM/GBSA values (kcal/mol) of GC-MS identified phytoconstituents against Delta opioid (4N6H.pdb) and μ opioid (5C1M.pdb)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Compounds</th>
<th>Delta opioid (4N6H)</th>
<th>µ opioid (5C1M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glide G score</td>
<td>ΔG bind</td>
</tr>
<tr>
<td>1</td>
<td>2-(9-octadecenoyloxy)-(Z)-Ethanol</td>
<td>-6.31</td>
<td>-53.09</td>
</tr>
<tr>
<td>2</td>
<td>3-Eicosyne</td>
<td>-2.92</td>
<td>-65.08</td>
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<tr>
<td>3</td>
<td>Phytol</td>
<td>-4.16</td>
<td>-71.63</td>
</tr>
<tr>
<td>4</td>
<td>2-[2,4-dimethoxyphenyl]-3,5,7-trimethoxy-4H-1-benzopyran-4-one (F1)</td>
<td>-5.05</td>
<td>-65.46</td>
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<tr>
<td>5</td>
<td>2',3',5',7-tetramethoxy flavone (F2)</td>
<td>-8.92</td>
<td>-79.95</td>
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<tr>
<td>6</td>
<td>7-hydroxy-2-(4-hydroxyphenyl)-4H-1-Benzopyran-4-one (F3)</td>
<td>-8.8</td>
<td>-82.27</td>
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<td>7</td>
<td>2,5-di-tert-butylaniline</td>
<td>-5.65</td>
<td>-32.56</td>
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<tr>
<td>8</td>
<td>2-methoxy-9a-(morpholin-4-yl)-5a,6,7,8,9,9a-hexahydro dibenzofuran-3-ol</td>
<td>-7.108</td>
<td>-59.701</td>
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<tr>
<td>9</td>
<td>Oxycodone</td>
<td>-7.853</td>
<td>-87.82</td>
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Analgesic Effect of Flavonoid Rich Fraction from *Pteris quadriaurita*

**In-vitro Antioxidant Studies**

The percentage inhibition of reactive oxygen species for studied fractions at 100 mg/mL concentration was given in Table 8. In DPPH, FRAP, SRS, and HAS scavenging assays, PQEE and PQFRF showed comparable radical scavenging abilities to Ascorbic acid. PQFRF exhibited significantly stronger antioxidant activity than PQEE across all four models compared to ascorbic acid.

Oxygen-based free radicals implicated in diseases and aging, pose threats to health. The extract demonstrated strong scavenging abilities across four antioxidant models, assessed via colorimetric techniques measuring total phenolic and flavonoid content. This efficacy is attributed to the extract’s abundant phenolic components, offering potential health benefits.

**Analgesic Activity of FRF of *P. quadriaurita***

**Acetic-induced writhing test**

Figure 3 shows the FRF’s influence on mouse writhing. FRF at 25 and 50 mg/Kg inhibited acetic acid-induced writhing. The nociceptive response was maximally inhibited at 50 mg/kg. Similar inhibition was seen with diclofenac sodium.

Acetic acid increases capillary permeability and releases molecules that stimulate pain nerve terminals, causing inflammation. NSAIDs block peripheral nociceptors or inhibit cyclooxygenase, lipoxygenase, and other inflammatory mediators to relieve peripheral analgesia. *P. quadriaurita* FRF may have comparable analgesic properties, but further study is required to understand the specific mechanism.

**Hot plate test**

Table 9 shows that two doses of FRF boosted Swiss-albino mice’s heat stimulation response time. A higher dose of 50 mg/kg FRF showed the highest pain inhibition against thermal stimuli, with a response time of 15.44 ± 0.36 seconds,
comparable to tramadol (16.28 ± 0.26 seconds) and statistically significant (p < 0.001 and p < 0.01) compared to the standard drug.

Tail-flick test

Table 10 shows the analgesic efficacy of FRF methanol extract. Normal saline-treated mice had no significant tail-flick response time difference over 60 minutes. Compared to baseline values among the same treatment groups, response time increases at various time periods varied substantially (p-value < 0.05) for standard alone. Standard and FRF-treated mice had longer response times than saline-treated animals, except for the FRF (25 mg/Kg) group at 60 minutes.

Also in hot plate and tail flick tests, the extract demonstrated strong antinociceptive effects versus the standard medication. Hot plate reaction is more complicated, and supraspinally structured. In both thermal models, mu (μ), delta (δ), and kappa (κ) opioid receptors are crucial for analgesic effects. A receptor known as the μ receptor is used for pain alleviation. It’s a powerful thermal pain receptor. Opioids activate these receptors, which block pain-signaling neurotransmitters, including substance P and glutamate. This inhibition slows pain-sensing neuron activity and nociceptive signal transmission to higher brain areas. Moreover, μ2 opioid receptor activation causes spinal analgesia. The results of the in-silico docking studies were correlated with experimental data obtained from in-vivo tests. Compounds predicted from the PQFRF to interact strongly with central and peripheral targets implicated in pain modulation were the likely candidates responsible for the observed analgesic activity. Corroborating experimental and computational findings strengthens the empathetic of the mechanism of action of the studied plant extract.
CONCLUSION
Combining in-vivo research with in-silico profiling offers strong proof for the analgesic properties of the flavonoid-rich fraction that was separated from P. quadriaurita. PQRF shows promise as a natural source for the creation of highly sophisticated painkillers. This study’s thorough investigation offers insightful information about P. quadriaurita’s possible therapeutic uses in pain management. These studies suggest that more investigation and utilization of this natural resource can further enhance the benefits of pain management in clinical settings.

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REFERENCES


