HESPERIDIN, A CONSTITUENT ISOLATED FROM METHANOL EXTRACT OF ALPINIA CALCARATA RHIZOMES AND ITS ANTI-ARTHRITIC ACTIVITY EVALUATION

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Abstract
The chronic inflammatory and debilitating joint condition of rheumatoid arthritis (RA) causes the joint disease. The nation worldwide. Alpinia calcarata Roscoe (Family: Zingiberaceae), is a perennial rhizomatous herb widely used in typical Sri Lankan medicinal systems. In tropical countries, including Sri Lanka, India, and Malaysia, Alpinia calcarata is cultivated. Alpinia calcarata rhizomes have been found to have anthelmintic, aphrodisiac, antibacterial, antinociceptive, antidiabetic, gastroprotective, anti-inflammatory, antioxidant, and antifungal functions in laboratory forms. To explore the effectiveness and potential mechanism of isolated hesperidin phytoconstituent from methanol extract of A. calcarata rhizomes and well-characterized (HPTLC, UV-Visible, FT-IR, 1H and 13C-NMR) and assessed by percentage inhibition of protein denaturation and HRBC membrane stabilization methods for their antiarthritic function. In addition, molecular docking was carried out to research the binding mode of this compound to the Bruton's Tyrosine Kinase (BTK) mutant active site (PDB ID: 3T9T). With an IC50 value of 254.95 μg/mL, the isolated compound hesperidin demonstrated impressive anti-arthritic behaviour relative to Diclofenac sodium with an IC50 value of 298.34 μg/mL as a percentage inhibition of the process of protein denaturation. Similarly, isolated compound hesperidin demonstrated impressive anti-arthritic behavior with an IC50 value of 310.48 μg/mL relative to Diclofenac sodium with an IC50 value of 351.15 μg/mL in the stabilization process of the HRBC membrane. Compound hesperidin has been shown to have a strong inhibition potential with a binding energy value of -9.4 kcal/mol against Bruton's Tyrosine Kinase (BTK) mutant (PDB ID: 3T9T). Hesperidin is also a likely moiety that carries anti-arthritic action. The findings of the present investigation could make it possible to design potent inhibitors of small molecules against rheumatoid arthritis.

Keywords: Alpinia calcarata Roscoe, Bruton's Tyrosine Kinase, Hesperidin, Molecular docking, Rheumatoid arthritis.

Introduction
One of the furthest most prevalent chronic wellbeing conditions and a significant origin of impairment is arthritis [1]. The name "arthritis" comes from terms "ites" and "arthron" in Greek, implying joint inflammation. It will be able to demarcate as a systemic autoimmune disorder, inflammatory, and persistent marked by synovial rigidity, swelling, and joint pain [2]. More than 100 different conditions are included in arthritis, the most popular types being psoriatic arthritis, gout, fibromyalgia, rheumatoid arthritis (RA), and osteoarthritis (OA) [3]. The utmost communal type of arthritis is osteoarthritis [4]. This deteriorating condition remains marked through injury towards the lower extremity of hip, leg, and other articular cartilage joins. The reported threat on behalf of OA in the knee is around 40% in males, and 47% in women and its pervasiveness is expected to rise due to population ageing and the rising incidence of fatness [5]. Rheumatoid arthritis is a persistent and universal autoimmune inflammatory disease wherein inflammation of the joints results in injury to the cartilage and muscles, weakness, and sometimes systemic problems, as well as elevated morbidity and mortality[6]. A variety of variables play roles in RA pathogenesis, such as hereditary factors, the climate, and autoimmunity.
All of these stimuli cause activation of immune system, which activates autoantigen appearance through activation of antigen-precise B and T cells and abnormal presentation. The formation of inflammatory cytokines contributes to synovitis, cartilage and subchondral tissue. Destroying. There may also be further-articular structures, for instance digestive system and the skin, being active [6]. For all of these main types of arthritis, pharmacological treatment is often symptomatic and tends to reduce inflammation and discomfort. This involves the application of anti-inflammatory oral glucocorticoids and nonsteroidal medications (NSAIDs). The usage of disease-modifying antirheumatic medications (DMARDs) for examplesulphasalazine, hydroxychloroquine, and methotrexate is the foundation of existing therapeutic modalities for RA. These medications, however, may induce severe side effects, with defects in liver function, pneumonitis, and gastrointestinal criticisms. Further, in the treatment of OA and RA, biological medications (soluble receptors or antibodies for TNF-α, IL-6, and IL-1) take increased prominence. These agents are capable of minimizing inflammation and joint damage, but together their advantages and abiding side effects not yet stood completely known, in addition to the advanced expense of such novel proxies, which restrict their application. These medications were not constantly successful and serious side effects, for instance increased cancer risk, and severe infections can occur. Cytotoxic and immunosuppressive medications (e.g., cyclophosphamide, azathioprine, cyclosporine, and leflunomide) can also be cast-off, although a range of toxic side effects are often caused by these medicines [8, 9]. As a result, the usage of dietary supplements and botanicals has become extremely significant, and quantity of studies in the treatment of arthritis on the possible health benefits of plant extracts is growing [10]. In last decade, the antiarthritis ability of extracts and herbalkindswere thoroughly studied and a variety of articles have been published dealing with certain botanicals [11-20].

Alpinia calcarata Rosc is an extensive perpetual herb in tropical and subtropical Asian republics, comprising Malaysia, Sri Lanka, Thailand, Bangladesh, and India [21, 22]. In conventional Sri Lankan and Indian medicine, rhizomes have been widely used to manage chronic inflammatory disorders such as rheumatism and arthritis [23]. In carrageenan-induced mouse models, experiments on rhizome oil extract, Ethanolic and hot water extract, demonstrated significant anti-inflammatory activity [24-26]. Previous results were documented antioxidant, antimicrobial, aphrodisiac, gastroprotective, antidiabetic, antinociceptive, antihelminthic, and antifungal properties of AC extracts [27]. Researchers have previously stated that the biochemical conformation of ACEO mature in Sri Lanka is high in 1,8-cineole oxygenated monoterpenes as a main integral of leaf and rhizome EOS [28]. However this study fails to provide a thorough profile of the volatile AC blossoming ingredients mature in Sri Lanka. Analogous auxiliary gossips were reported in South India with ACEOs from germplasms [29-32]. Furthermore, the key components alpha-terpineol (TPN) and 1,8-cineole (CIN) are recognized to perform in vivo anti-inflammatory agents [33, 34]; their up-to-date anti-inflammatory consequence and mechanism of exploit have never been reported on behalf of skin illnesses for instance atopic dermatitis. Keep this in mind, the present work focuses on the isolation of bio-active compound hesperidin and evaluated for its anti-arthritis and molecular docking studies.

Materials and Methods

Collection of plant material

The Alpinia calcarata rhizome was collected in Kolli Hills, Nammakkal District, Tamil Nadu, India, in the month of July 2016. Dr. S. John Britto, Administrator of the Rapinat Herbarium, St. Joseph College, Tiruchirapalli, and Tamil Nadu, named and authenticated the weed. The specimen voucher No. PP001 was dated 14.07.2016.

Preparation of crude extract

Alpinia calcarata rhizome was eroded consecutively in water, the shade dried at 35-40°C for a week, during which it was machined to a 40 mesh size standardised powder. Using the soxhlet extraction
process, these plant powders were extracted with different solvents according to the growing order of polarity of solvents. 500g of dried plant powder was evenly packed into the soxhlet extractor and constantly collected for 24 hours with 1L of suitable solvent or until the solvent in the syphon tube of the extractor became colourless. To exclude all strong impurities, including cellular materials that are insoluble in the extraction solvent, the extracts were filtered over filter paper Whatmann No. 42. Utilizing a rotary evaporator under decreased heat, the whole extracts were condensed to drying. Final extracted sample was placed in sealed sterile bottles and held at 4 ° C for potential usage in the refrigerator. We calculated the percentage yield.

Isolation of phytochemical compounds by column chromatography

Column chromatography was performed over TLC grade silica gel on the condensed methanol extract of rhizomes (1 kg) of Alpinia calcarata. Elution of the column formed a variety of fractions, former with n-hexane, increasing the volume of ethyl acetate in n-hexane and eventually with methanol. Ethyl acetate-methanol (40:60) from fraction 9 was used to prepare solvent systems for the synthesis of hesperidin (778 mg/486g). Via spraying with Libermann-Burchard reagent, the compounds were found on TLC plates and heated for 10 minutes at 100 °C.

Detection of isolated compounds by high performance thin layer chromatography (HPTLC)

For the study, a CAMAG HPTLC machine fitted with the ATS4 automated TLC analyst, TLC scanner 3 and the Win CATS 3 optimized programme was utilized. Sample was wash away on a Si 60F254 (20 cm x 10 cm) precoated silica gel HPTLC plate with a layer thickness of 200 μm. On the panel, the samples and standards were added as 8 mm large bands by continual request rate of 150Nl s⁻¹, through an automated TLC sampling (ATS4) below N₂ gas flow, 15 mm from the heart, 15 mm starting the edge, and 6 mm in the plate space amongst two locations.

Structural elucidation of isolated compounds

To elucidate the composition of isolated molecules, different spectroscopic approaches have been used, including UV, FTIR, 1H NMR and 13C NMR. With a Shimadzu 160A UV-visible spectrophotometer, the UV-visible spectra of the isolated compound in methanol was reported. Using the KBr pellet technique, the Fourier Transform Infrared (FTIR) spectra were reported with a nominal resolution of 4 cm⁻¹ and a wave number scale of 400 to 4000 cm⁻¹. On Bruker WP 200 SY and AM 200 SY (300 MHz) devices, ¹H and ¹³C NMR spectra were acquired utilizing TMS as the internal normal and CDCl₃ as the solvent.

Anti-arthritic activities

Inhibition of protein denaturation method

Anti-arthritic activity of isolated compound hesperidin through inhibition of protein denaturation method was assessed by a previously described method [35].

Human red blood cell (HRBC) membrane stabilization method

Anti-arthritic activity of compound hesperidin through HRBC membrane stabilization method was assessed by a previously described method [36].

Molecular docking experimental procedure

Based on previous literature, the goal Bruton Tyrosine Kinase (BTK) mutant protein (PDB ID: 3T9T) was selected for molecular docking studies[37]. Molecular docking experiments were used to inspect the binding mode of association between hesperidin compound and 3T9T protein utilising Autodockvina 1.1.2[38]. Bruton’s Tyrosine Kinase (BTK) mutant (PDB ID: 3T9T) crystal structure was retrieved from the Protein Data Bank (http://www.rcsb.org). Through ChemDraw Ultra 12.0 and Chem3D Pro 12.0 software, the 3D structure of the compound hesperidin was drawn and energy minimised. The AutodockVina input files were generated using the AutoDock Software 1.5.6 application bundle. From previous literature[37], the binding pocket was established and in the binding pocket were the amino acid residues Ile369, Gly370, Ser371, Val377, Ala389, Lys391, Leu433,
Thr435, Glu436, Phe437, Gly441, Cys442, Asp445, Leu489, Ser499 and Asp500. The 3T9T protein quest grid was defined as center-x, y, z: -3.258, 2.655, 11.176 with size-x, y, z: 24, 20, 20 by spacing 1.0 Å. The meaning of exhaustiveness has been set at 8. For Vina docking, the other parameters were set to default and not specified. The best-scoring compound is the compound having least binding affinity value, and the findings have been visually evaluated using the application Discovery Studio 2019.

**Results and Discussion**

**Optimization of hesperidin by HPTLC chromatographic technique**

HPTLC fingerprint patterns were assessed for the isolated compound *hesperidin* from methanolic extract *A. Calcarata* rhizomes. The Rf value of standard hesperidin 0.40 was matched with the isolated compound *hesperidin* Rf value of 0.40. From the HPTLC technique the isolated compound was confirmed as *hesperidin*. The HPTLC chromatogram of isolated compound was displayed in Figure 1.

![HPTLC chromatogram of hesperidin](image)

**Figure. 1. HPTLC chromatogram of hesperidin**

(a) standard Hesperidin (b) isolated hesperidin in *Alpinia calcarata* rhizomes

**Structural elucidation of isolated compound hesperidin**

White solid; M.p: 165°C; Mw: 610.56; Mf: C_{28}H_{34}O_{15}: UV \( \lambda_{\text{MeOH max}} \) nm (abs): 345 (6.2); IR (cm\(^{-1}\)): 3429.12 (OH\(_{\text{Str}}\)), 2998.74 (CH\(_3\)), 2993.18 (CH\(_2\)), 1644.08 (C=O), 1513.92 (-C=C-), 1436.82
(CH$_2$)$_3$, 1313.64 (CH$_2$-bend), 1025.48 (C=O), 964.13 (Pyran ring); $^1$H NMR (300MHz, DMSO- d$_6$): δ 12.03 (6H, s, OH), 9.13 (2H, s, OH), 6.13 – 6.96 (5H, m, Phenyl ring), 3.40 – 5.40 (11H, m, -CH), 3.78 (2H, dd, J=9.2, 7.8 Hz, CH$_2$), 4.51 (2H, dd, J=9.2, 7.8 Hz, CH$_2$), 2.51 (6H, s, 2CH$_3$); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 197.53 (1C, C=O), 165.58 (1C, C=C), 165.53 (1C, C=C-O), 163.53 (1C, =C-OH), 148.41 (1C, =C-O), 146.89 (1C, -C=O), 131.33, 118.44, 118.26, 114.52, 96.82, 95.99 (6C, Phenyl ring), 101.06 (1C, -C=O), 96.82 (1C, CH$_2$), 103.77, 70.03, 70.72, 71.14, 72.51, (5C, Pyran ring), 112.51, 73.43, 75.95, 76.71, 78.85, (5C, Pyran ring), 78.91 (1C, CH$_2$), 56.12 (1C, OCH$_3$), 42.50 (1C, CH$_2$), 39.42 (1C, CH$_3$).

**UV spectrum of hesperidin**

UV-VIS profile of isolated compound hesperidin was examined at a wavelength between 200 to 800 nm. The compound hesperidin displayed one signal in UV spectra at $\lambda_{MeOH}^{max}$ value of 345 nm with the absorbance value of 6.2 respectively. The UV spectra of isolated compound hesperidin was shown in Figure 2.

![Figure 2. UV spectra of isolated compound hesperidin](image)

**IR spectrum of hesperidin**

FT-IR spectra of hesperidin was done to recognize the functional groups existent in compound isolated from A. calcarata. The main bands were perceived at V$^{KBr}$ cm$^{-1}$: 3429.12, 2998.74, 2993.18, 1644.08, 1513.92, 1436.82, 1313.64 and 1025.48 (C=O) and 964.13 respectively. The band at 3429.12 cm$^{-1}$ designates the absorption rising from O-H stretching. The band at 2998.74 cm$^{-1}$ designates the stretching vibrations that might be the existence of CH$_3$ group. The band at 2993.18 cm$^{-1}$ specifies the stretching vibrations that might be the occurrence of CH$_2$ group. The band at 1644.08 cm$^{-1}$ is confirms the presence of C=O group. The band at 1513.92 cm$^{-1}$ agrees to the vibration of sp$^2$ C=C bond on the ethylene group. The band around 1313.64 cm$^{-1}$ is corresponds to the existence of CH$_2$ bending frequency. The band at 1025.48 cm$^{-1}$ endorses the bending vibration of C=O bond in the alcohol moiety. The band at 964.13 cm$^{-1}$ indicates the presence of Pyran moiety. Moreover, around weedy absorption bands were also noted in the spectra. The FT-IR spectrum of isolated compound hesperidin was shown in Figure 3.


Figure. 3. FT-IR spectra of isolated compound hesperidin

$^1$HNMR studies of compound hesperidin isolated from *A. Calcarata* at 300 MHz by using CDCl$_3$ as a solvent. The chemical shift values were uttered as ppm and the coupling constant values in Hz. The singlet peak at $\delta$ 12.03 ppm confirms the presence of six hydroxyl protons. Similarly, the peak at 9.13 ppm resembles the structure having two hydroxyl protons in it. The five aromatic protons at 6.13 – 6.96 ppm with multiplet splitting evidents the existance of phenyl ring. Multiplet signal at 3.40 – 5.40 ppm signifies due to the presence of eleven –CH protons. Four protons from two –CH$_2$ groups at chemical shift value of 4.51 and 3.78 shows doublet of doublet splitting with coupling constant values of 9.20 and 7.80 Hz respectively. The peak at 2.51 ppm resembles six protons from two methyl groups. The $^1$H-NMR spectra of isolated compound hesperidin was shown in Figure. 4.

Figure. 4. $^1$H-NMR spectra of isolated compound hesperidin

$^{13}$C NMR spectrum of hesperidin

$^{13}$C NMR assessment of compound hesperidin isolated from *A. Calcarata* at 300 MHz with CDCl$_3$ as a solvent and the chemical shift values were stated as ppm. The key assignment at $\delta$ 197.53 ppm confirms the carbonyl carbon. The peaks at 165.58 and 101.06 ppm represents the presence of ethylenic carbon atoms. Signals at 165.53 and 148.41 ppm confirms two carbon atoms of –C=O were athesperidined with the ethylenic carbon. The peak at 163.53 ppm signifies one of the ethylenic carbon atom was athesperidined to the hydroxyl group. The characteristic peak at 146.89 ppm evident that one of the carbon atom was bonded with hydroxyl group. The six aromatic carbon atoms from phenyl ring were
at 131.33, 118.44, 118.26, 114.52, 96.82 and 95.99 ppm respectively. The peaks at 96.82, 78.91 and 42.50 ppm due to the presence of three –CH₂ carbon atoms. Five carbon atoms from the pyran ring were at chemical shift value of 103.77, 70.03, 70.72, 71.14 and 72.51 respectively. Chemical shift values at 112.51, 73.43, 75.95, 76.71 and 78.85 ppm evidents another pyran ring was present in the structure. The peak at 56.12 ppm confirms the presence of one methoxy carbon atom in the structure and 39.42 ppm signifies the structure having one methyl carbon atom in it. The ¹³C-NMR spectra of isolated compound hesperidin was shown in Figure. 5.

Figure. 5. ¹³C-NMR spectra of isolated compound hesperidin

Anti-arthritic activity
Percentage inhibition of protein denaturation
The phytoconstituent hesperidin isolated from the methanolic extract of medicinal plant *AlpiniaCalcarata* rhizome was assessed for anti-arthritic activity by using percentage inhibition of protein denaturation. The compound Diclofenac sodium was utilized as a standard. The test compounds and standard were prepared at a concentrations of 100, 200, 300, 400 and 500 μg/mL for percentage inhibition of protein denaturation. At 500 μg/mL the standard Diclofenac sodium displayed 107.25 % of protein denaturation. The isolated compound hesperidin shows higher activity with the IC₅₀ values of 254.95 μg/mL than standard Diclofenac sodium with the IC₅₀ value of 298.34 μg/mL respectively. The compound hesperidin isolated from the methanolic extract of *A. Calcarata* rhizomes displayed remarkable activity with the IC₅₀ values of 254.95 μg/mL respectively. The results were summarized in Table 1.

<table>
<thead>
<tr>
<th>Concentrations (μg/mL)</th>
<th>% Inhibition of protein denaturation method</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Hesperidin</td>
</tr>
<tr>
<td>100</td>
<td>40.02±1.59</td>
</tr>
<tr>
<td>200</td>
<td>51.65±1.62</td>
</tr>
<tr>
<td>300</td>
<td>62.57±2.31</td>
</tr>
<tr>
<td>400</td>
<td>73.12±2.57</td>
</tr>
<tr>
<td>500</td>
<td>81.35±2.64</td>
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<tr>
<td>IC₅₀</td>
<td>254.95±3.48</td>
</tr>
</tbody>
</table>

HRBC membrane stabilization method
The phytoconstituent hesperidin isolated from the methanolic extract of medicinal plant *Alpinia Calcarata* rhizome was assessed for anti-arhritic activity by using HRBC membrane stabilization. The compound Diclofenac sodium was used as a standard. The test compounds and standard were prepared at concentrations of 100, 200, 300, 400 and 500 μg/mL for HRBC membrane stabilization. At 500 μg/mL the standard Diclofenac sodium displayed 92.32 % of HRBC membrane stabilization. The isolated compound hesperidin shows higher activity with the IC₅₀ values of 310.48 μg/mL than standard Diclofenac sodium with the IC₅₀ value of 351.15 μg/mL respectively. The compound hesperidin isolated from the methanolic extract of *A. Calcarata* rhizomes displayed remarkable activity with the IC₅₀ values of 310.48 μg/mL respectively. The results were summarized in Table 2.

**Table 2. HRBC membrane stabilization**

<table>
<thead>
<tr>
<th>Concentrations (μg/ml)</th>
<th>HRBC membrane stabilization method</th>
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<tr>
<td></td>
<td>Hesperidin</td>
</tr>
<tr>
<td>100</td>
<td>20.45±0.46</td>
</tr>
<tr>
<td>200</td>
<td>23.52±0.52</td>
</tr>
<tr>
<td>300</td>
<td>41.42±0.13</td>
</tr>
<tr>
<td>400</td>
<td>55.36±0.27</td>
</tr>
<tr>
<td>500</td>
<td>70.89±1.19</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>310.48±2.73</td>
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**Molecular docking**

In demand to spreadinsightkeen on the reasonable mechanism of biological events docking, simulations were accomplished. The compound hesperidin was assessed for docking behavior by protein 3T9T via AutodockVina program. The compound hesperidin shows binding affinity of (-9.4 kcal/mol) and forms five Hydrogen bonds with the receptor 3T9T. Hydrogen bonding shows a key part in the steadiness of protein-ligand bonding, and the favorable bond distance is less than 3.5 Å between H-acceptor and H-donor atoms [39]. The amino acid residues Lys391 (bond length: 2.54), Thr435 (bond length: 2.39), Arg486 (bond length: 2.60), Ser499 (bond length: 2.57), and Asp500 (bond length: 1.83) were involved in hydrogen bonding connections. The amino acid residue Cys442 was involved in hydrophobic interactions. The hydrogen bonding and hydrophobic interactions of residues of amino acids in 3T9T protein with compound hesperidin was shown in Figure 6. The conseqeunces showed that the compound hesperidin devising remarkable inhibition ability in particular target protein. The results were summarized in Table 19.

**Table 19. Molecular docking interaction of compound hesperidin.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bruton’s Tyrosine Kinase (BTK) mutant (PDB ID: 3T9T)</th>
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<tr>
<td></td>
<td>Binding affinity (kcal/mol)</td>
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<tr>
<td>hesperidin</td>
<td>-9.4</td>
</tr>
</tbody>
</table>
Conclusion
It can be inferred from this analysis that the hesperidin compound was extracted from the methanol extract of the Alpinia Calcarata rhizome medicinal plant. The isolated compound was confirmed by HPTLC, UV-Visible, FT-IR, $^1$H and $^{13}$C NMR techniques. In addition, the isolated compound hesperidin was further assessed for anti-arthritis activity through HRBC membrane stabilization and percentage inhibition of protein denaturation methods. The isolated compound hesperidin displayed remarkable anti-arthritis activity with the IC$_{50}$ value of 254.95 μg/mL than standard Diclofenac sodium with the IC$_{50}$ value of 298.34 μg/mL in percentage inhibition of protein denaturation method. Similarly, isolated compound hesperidin displayed remarkable anti-arthritis activity with the IC$_{50}$ value of 310.48 μg/mL than standard Diclofenac sodium with the IC$_{50}$ value of 351.15 μg/mL in HRBC membrane stabilization method. Molecular docking studies revealed compound hesperidin having significant inhibition ability with the binding energy value of -9.4 kcal/mol against Bruton’s Tyrosine Kinase (BTK) mutant (PDB ID: 3T9T). The outcome of the current investigation may include additional clinical opportunities for arthritis care.

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References


