CHAPTER V
BIOACTIVE COMPOUNDS FROM MEDICINAL PLANTS;
NEW INHIBITORS FOR MATRIX METALLOPROTEINASES II AND
CARBONIC ANHYDRASE II
This chapter has been divided into three parts:

Part I) Synthesis of pachypodol from onion peel waste,

Part II) *In-silico* modelling studies of arjunolic acid on carbonic anhydrase II system,

Part III) Phytochemical investigation of *Berberis tinctoria*.

**PART I**

**NEW SYNTHESIS OF PACHYPODOL AND ANALOGS FROM ONION PEEL WASTE; DISCOVERY OF NON-ZINC BINDING INHIBITION OF MMP-2**

1. **INTRODUCTION**

Pachypodol (quercetin-3,3′,7-trimethyl ether, 2) is a lesser known quercetin derivative that has been reported to have anti-tumour, anti-picornavirus, anti-fungal, hepato-protective, and other bioactivities\(^1\)\(^2\)\(^3\). It occurs in the plants, *Calycopteris fluoribunda*, *Larrea cuneifolia*, *Croton ciliatoglenduliferus*, *Ballota inaequidens*, *Miliusa balansae* as well as *Agastache rugosa* (Korean mint) and *Melicope ellyrana* (Australian tree)\(^4\)\(^5\)\(^6\). *Calycopteris fluoribunda*, (Sanskrit: Ukshi) is one of the plants used in many Ayurvedic preparations. Though pachypodol (2) occurs in many plants, they are present in small quantities. Multiple bioactivities and scarcity of 2, prompted us to develop a synthesis for 2. To our knowledge synthesis of 2 and its analogs have not been reported so far.

Partial methylation of quercetin (1) was one of the possible choices for the synthesis of 2. However, because of similar reactivities of the hydroxyls, the selective methylation of 3′-OH in the presence of 4′-OH is a difficult process. Therefore, synthesis of 2 from quercetin (1) without protecting its 4′-OH group is not a viable proposition. Spiraeoside (quercetin-4′-glucoside, 3), where the 4′-hydroxyl is protected as glucoside offers a good option for synthesis of 2. Quercetin-4′-glucoside (3) occurs abundantly in waste onion peels\(^7\)\(^8\). Judicious
manipulation of onion peel waste component successfully used for the preparation of 2 as well as 4 and 6, two other quercetin methyl ethers.

Scheme 1. An attempt to the synthesis of pachypodol from quercetin.

1.1. Onion peel waste as a source of flavonoids

Major dietary sources of quercetin (1) include onions (21.4 mg/100 g), apples (3.13 mg/100 g) and tea (1.99 mg/100 mL). Onions (Allium cepa L.) is a primary source of dietary quercetin in the Indian diet. Onion processors remove 40% of the onion peels as coproduct material. It is estimated that 500000 tonnes (globally) of onion waste are generated annually. The onion waste includes the dry outer protective layer and the two outer flesh layers. Non-agreeable smell preclude its use in agriculture, and its disposal present environmental issues. There are limited uses for onion waste (e.g. animal feeds and fertilizer), and the disposal results in high costs for transportation and landfill fees for the industry. In the present study, onion peels waste have been utilized for the preparation of bioactive compounds.

2. PRESENT WORK

The present study (part I) has been sub-divided into four parts;

(i) Isolation of spiraeoside from onion peels,

(ii) Synthesis of pachypodol and analogs,

(iii) MMP inhibitory activities and

(iv) In-silico modelling studies.
2.1. Isolation of spiraeoside from onion waste

Onion peels are reported to contain rich amount of anthocyanins and flavonoids. Extraction using methanol contain a complex mixture of compounds along with anthocyanins. During the optimization of the extraction, it was found that extraction with ethyl methyl ketone at room temperature was an ideal solvent for preferential extraction of flavonoids such as quercetin (1) and spiraeoside (3), since anthocyanins are not soluble in this solvent. Onion peel was collected from the local sources (Vallikkavu, Kollam, Kerala, India).

![Figure 19. Isolation of spiraeoside from onion peel waste.](image)

Onion peels were cut into small pieces and taken in ethyl methyl ketone (MEK). It was shaken in a rotary shaker at room temperature for overnight (12 h). Solvent from the extract was removed by vacuum evaporation. TLC and HPLC-MS analysis of the extract showed, it contain two major compounds (two FeCl₃ positive bands); quercetin (1) and spiraeoside (3). Spiraeoside (3) was conveniently separated by column chromatography (SiO₂) using and 5% MeOH in CHCl₃ as eluent. Spiraeoside (3) was obtained in (600-700 mg), 0.6-0.7% yield (figure 19). The isolated spiraeoside (3) has a UV maximum at [UV (MeOH): λ 253, 364 nm], mass of (M+H)⁺; 465 and fragment of MS/MS; 303, 152; Further, ¹H and ¹³C NMR analyses confirms that the compound was quercetin-4'-glucoside (spiraeoside, 3).
2.2. a) Semi-synthesis of pachypodol (2) from spiraeoside (3)

Selective tri-methylation of 3 was carried out using dimethyl sulphate (3.1 mmol) and potassium carbonate in acetone under refluxed condition (acetone) for 1 h to furnish 4 (scheme 2). 4 gave positive (greenish colour) ferric reaction and showed an UV spectra at [UV (MeOH): \( \lambda \) 255, 294sh, 348 nm]. The compound 4 gave a bathochromic UV shift from 255 to 269 (an increase of 14nm) with AlCl\(_3\) indicating that the compound has free hydroxyl at position-5 (4, scheme 2). Nine methoxyl protons (3 \( \times \) -OCH\(_3\)) were observed in \(^1\)H NMR spectra, revealing tri-methylation has occurred in compound 3. Mass spectral analysis showed gave molecular mass at (M+H\(^+\); 507 and their fragment at MS/MS; 345, 330, 315, 300 & 152, further confirms the assigned structure 4 (Molecular formula; C\(_{24}\)H\(_{26}\)O\(_{12}\)).

![Scheme 2. Synthesis of pachypodol from spiraeoside.](image)

Hydrolysis of 4 with HCl (in methanol) led to the formation of compound 2. The compound 2 absorbed UV spectra at [UV (MeOH): \( \lambda \) 255, 293sh, 355 nm] which was identical with the pachypodol UV spectrum\(^1\). Further, IR (KBr): \( \nu \) 1597 cm\(^{-1}\), \(^1\)H NMR; \( \delta \) 3.82, s, 3H; 3.87, s, 6H (9-methoxy protons) and characteristic mass spectra at (M+H\(^+\); 345 with the molecular formula of C\(_{18}\)H\(_{16}\)O\(_{7}\), confirms that the identity of 2 as pachypodol. Overall yield of from spiraeoside (3) to pachypodol (2) was about 44%.
b) Semi-synthesis of quercetin-3, 7-dimethyl ether (6)

![Scheme 3. Synthesis of quercetin-3,7-dimethyl ether from spiraeoside.]

Reaction of 3 with two equivalents of dimethyl sulphate, (2 mmol, potassium carbonate) refluxed in acetone (1 hr) yielded 5 which was subjected to mild hydrolysis with HCl when 6 was obtained in 50% yield. Compound 6 absorbs UV spectra at [UV (MeOH): λ 255, 371 nm], IR at (KBr): ν 3440, 3243, 1600 cm\(^{-1}\); \(^1\)H NMR; δ 3.80, s, 3H; 3.87, s, 3H (indicated OCH\(_3\) X 2 protons); reveals that the compound 6 was quercetin-3,7-dimethyl ether. The spectral properties of 6 agreed with the reported data\(^{10}\). 6 is a natural product and its occurrence is reported from *Croton schiedeanus* Schlecht (Euphorbiaceae). It possesses vasorelexant properties\(^{10}\).

## 3. MMP INHIBITORY ACTIVITY

Synthesized compounds 1-6 were screened for gelatinases activity (MMP-2 and MMP-9) using HT1080 cells for 24 h. Of the different compounds tested for MMP-2 and MMP-9 activities, 2 showed most significant inhibition of MMP-2 i.e. ~ 40 %, 50% and 90% at concentrations of 5µM, 10µM and 25µM respectively (Figure 21A-D). Though the other compounds, 3, 4 and 6 also showed significant inhibition of MMP-2, they were found to be toxic at the concentrations tested (10µM). Further, HT1080 cells were treated with increasing concentrations (1µM, 5µM, 10µM and 15µM) of 2 for 24 hr, showed a dose-dependent inhibition of MMP-2 activity
Also, the activity of 2 was better when compared to its parent compound 1. Except quercetin 1, no significant activity of MMP-9 inhibition was observed for 2, 4 and 6.

4. IN-SILICO MOLECULAR DOCKING STUDIES

The molecular modelling of pachypodol (2) on MMP-2 was studied (figure 20). Docking of 2 to MMP-2 (catalytic domain) suggested that it is binding to out of active site (S1' site) and not to the zinc in the active site. It is also called specificity site of MMP-2. 2 forms three hydrogen bonds with Ile222, Tyr223 and Thr227 with the docking score of -8.7 gmol\(^{-1}\) (figure 20). 2 Also showed hydrophobic interaction with Leu197, 218, Gly216, Tyr223, Thr229, Asn213 and Arg233. Docking results were comparable with previously reported non-zinc binding inhibitor of MMP-2\(^{11}\). Thus, 2 is the second example of non-zinc binding inhibitor for MMP-2. Unlike, anacardic acid (AA), 2 does not interact with Zn and also remain outside the S1' pocket. The present study suggests that 2 targets the specificity site rather than the active site of MMP-2.

Figure 20. Molecular interaction of pachypodol with MMP-2. Protein indicates in cyanin; Zinc atom in red; pachypodol in yellow; hydrogen bonding are indicated in pink color.
Figure 21. Regulation of MMP-2 activity by Pachypodol: A) zymogram showing MMP-2 activity of conditioned media from HT1080 treated with DMSO (lane 1), Q (lane 2), Q4’G (lane 3), Q3,7 (lane 4), P (lane 5), and P4’G (lane 6), each at a concentration of 10µM. B) a representative plot of percentage inhibition observed in the zymogram. C) zymogram showing MMP-2 activity of conditioned media from HT1080 treated with DMSO (lane 1), Q (lane 2), Q4’G (lane 3), Q3,7 (lane 4), P (lane 5), and P4’G (lane 6), each at a concentration of 25µM. D) a representative plot of percentage inhibition observed in the zymogram. E) zymogram showing MMP-2 activity of conditioned media from HT1080 treated with treated with DMSO (lane 1) and 1, 5, 10, 15µM pachypodol (lanes 2–5, respectively). F) a representative plot of percentage inhibition observed in the zymogram. G) cell viability using a MTT cytotoxicity assay was performed for the cells treated with Q, Q4’G, Q3,7, P, and P4’G, at 10µM and 25µM. H, morphological examination of confluent cells treated with and without pachypodol (15 µM) in serum-free DMEM after 24 h. Each bar represents the mean ± S.E. of triplicate determinations from three independent experiments. ****, P < 0.0001 (one-way analysis of variance with Dunnett’s multiple-comparison post-test).
4.1. MMP-2 catalytic activity

The effect of 2 on MMP-2 catalytic activity was assayed using flurogenic substrate (Mca-Pro-Leu-Ala-Nva-Dap-(Dnp)-Ala-Arg-NH₂). Eight fold increases in fluorescence was observed in the presence of the catalytic domain when compared to the blank. Increasing the dose of 2 failed to inhibit the catalytic activity of MMP-2 (Figure 21B); however AA (zinc binding inhibitor) showed nearly 60% inhibition at a concentration of 15µM (Figure 21C). These results are supporting the docking studies which based on non-zinc binding of 2 with MMP-2.

4.2. Zinc independent activity

To confirm the zinc-independent inhibition of MMP-2 by 2, the cells were treated with the 2 in combination with aqueous ZnCl₂. AA was used as a control. Co-incubation of 2 (15µM) with ZnCl₂ did not reverse the inhibition of gelatinase activity, whereas AA inhibition was reversed by ZnCl₂. The ability of additional Zn²⁺ to overcome the inhibitory effect of AA could be attributed to the chelation of Zn²⁺ by AA, whereas Zn²⁺ has no effect on the inhibition mediated by 2 as it is a zinc-independent process. Thus, it was confirmed that 2 is inhibiting MMP-2 through non-zinc binding interaction.

5. CONCLUSION

In conclusion, pachypodol (2), pachypodol-4′-glucoside (4) and quercetin-3,7-dimethyl ether (6) were synthesized efficiently from spiraeoside (3) obtained from onion peel waste. Amongst compounds tested (1, 2, 3, 4 and 5), 2 was found to be best MMP-2 inhibitor. Pachypodol, 2 was not inhibiting, structurally similar MMP-9. In-silico molecular modelling studies and gelatin zymography assay, employing 2 in combination with zinc chloride suggested that 2 inhibited MMP-2 selectively through non-zinc binding mode. This selective inhibition of pachypodol could be an important feature for the designing of future MMP-2 inhibitors. This method also provides value addition to onion waste.
6. EXPERIMENTAL

6.1. Isolation of spiraeoside (3) from onion peel waste

Onion peels (100gm) were taken in methyl ethyl ketone (MEK, 250ml), and stirred at room temperature for overnight (12 hr). Solvent was removed under reduced pressure. The quantity of MEK extract was 2.5gm. The extract was subjected to column chromatography using silica SiO$_2$ (50gm). Spiraeoside was eluted by a solvent mixture, 0.5 % methanol in ethyl acetate; 700mg of spiraeoside was obtained. The purity and characterization were established by TLC, HPLC-MS, IR, NMR spectral data and compared with those reported in the literature$^8$.

6.2. Synthesis of pachypodol (2)

Spiraeoside (1 mmol) was taken in dry acetone (10 ml). To that K$_2$CO$_3$ (3.1 mmol) and dimethyl sulphate (3.1 mmol) were added. Reaction mixture was refluxed for 1h. After monitored by TLC (toluene 5; ethyl acetate 5, formic acid 2), it was filtered through filter paper. The acetone was removed by distillation. To the reaction mixture, HCl (10%) in methanol (20 ml) was added and refluxed for 1 h. Solvent was removed and product was isolated. The product was purified through SiO$_2$ using a solvent mixture, 5% MeOH; 95% CHCl$_3$. Characterization of compounds are given below.

**Pachypodol: 2** was obtained as pale yellow solid. UV (MeOH): $\lambda$ 255, 293sh, 355 nm; IR (KBr): v 3430, 3241, 1664, 1597, 1496, 1342, 1210, 1126, 1033, 800, 641 cm$^{-1}$. $^1$H NMR 400MHz (DMSO-$d_6$); $\delta$ 3.82, s, 3H; 3.87, d, 3H; 6.3, d ($J = 2.4$ Hz), 1H; 6.7, d ($J = 2.4$ Hz), 1H; 6.9, d ($J = 8.4$ Hz), 1H; 7.62, dd ($J = 2$ Hz), 1H; 7.64, d ($J = 2$ Hz), 1H; 9.9, s, 1H; 12.6, s, 1H. $^{13}$C NMR 100MHz (DMSO-$d_6$); $\delta$ 56, 59.7, 92.6, 98, 112.2, 115.6, 122.2, 147.5, 156.8, 165, 178.3. LC-MS, (M+H)$^+$; 345.

**Pachypodol-4'-glucoside: 4** obtained as light brown solid. UV (MeOH): $\lambda$ 252sh, 268, 294sh, 348 nm; IR (KBr): v 3420, 3285, 2984, 2946, 1665, 1560, 1545, 1506, 1456, 1354, 1287, 1200, 1125, 1096, 1078, 1000, 935, 720 cm$^{-1}$. $^1$H NMR 400MHz (DMSO-$d_6$); $\delta$ 2.7, s, 1H; 2.8, s,
6.3. Synthesis of quercetin-3,7-dimethyl ether (6)

Spiraeoside (1 mmol) was taken in dry acetone (10 ml). To that K₂CO₃ (2.1 mmol) and dimethyl sulphate (2.1 mmol) were added. Reaction mixture was refluxed for 1h. After monitored by TLC (Toluene 5; ethyl acetate 5, formic acid 2), it was filtered through filter paper. The acetone removed by distillation. To the reaction mixture, HCl (10%) in methanol (20 ml) was added and refluxed for 30 min. Solvent was removed and product was isolated. The product was purified using SiO₂ and eluted by a solvent mixture, 2% MeOH; 98% CHCl₃. Characterization of compound is given below.

Quercetin-3,7-dimethyl ether: 6 was obtained as light yellow solid. UV (MeOH): λ 255, 371 nm; IR (KBr): ν 3440, 3243, 1668, 1600, 1499, 1332, 1200, 1125, 1034, 801, 645 cm⁻¹. ¹H NMR 400MHz (DMSO-d6); δ 3.80, s, 3H; 3.87, s, 3H; 6.3, d (J = 2 Hz), 1H; 6.7, d (J = 2.4 Hz), 1H; 6.9, d (J = 8.8 Hz), 1H; 7.5, dd (J = 2.4 Hz), 1H; 7.6, d (J = 2 Hz), 1H; 9.4, s, 1H; 9.8, s, 1H; 12.6, s, 1H. ¹³C NMR 100MHz (DMSO-d6); δ 56, 59.6, 84.2, 92.2, 97.7, 115.5, 120.6, 137.5, 145.2, 148.8, 154.6, 156.3, 160.9, 165, 178. LC-MS, (M+H)⁺; 331.
PART II
TRITERPENOIDS FROM ARJUNA EXTRACT; ARJUNOLIC ACID AS A NOVEL NON-ZINC BINDING CARBONIC ANHYDRASE II INHIBITOR

1. INTRODUCTION

Ayurvedic-biology is one of the important growing areas for metabolic and neurodegenerative diseases\textsuperscript{12}. Many plants are reported to have these activities. Among them, \textit{Terminalia arjuna} is one of the important plant indigenous to India (also known as ‘Arjuna’ tree) reported to have wide range of biological properties. \textit{Terminalia arjuna} belonging to the family-Combretaceae, holds a reputed position in Ayurvedic medicine since ancient times\textsuperscript{13}. \textit{T. arjuna} has been used as a cardio-tonic in heart failure, ischemic, cardiomyopathy, atherosclerosis, myocardium necrosis and also for the treatment of different human diseases like blood diseases, anaemia, venereal and viral diseases\textsuperscript{14}.

\textbf{Figure 22}. Structures of triterpenoids.
Though wide range of bioactivities have been attributed to it, the targets or mechanism of action have not been reported so far. In search of bioactive compounds for the inhibition of carbonic anhydrase II (which is having direct implications in heart disease), we were attracted by the plant *T. arjuna* for its role in cardio-vascular diseases. *T. arjuna* showed two major category of compounds i.e. phenolics and triterpenoids. Literature suggested that most of the bioactivities are attributed to triterpenoids *viz* arjunolic acid and its derivatives.

In the present study, the triterpenoids, arjunolic acid (AJA) and its closely related compounds (figure 22), arjunic acid (AR), asiatic acid (AS), oleanolic acid (OA) and ursolic acid (UA) were studied for their CA II inhibition and their *in-silico* modelling studies were established.

2. PRESENT STUDY

2.1. CA II inhibitory activities by triterpenoids

Arjunolic acid (AJA) and the other related triterpenoids - arjunic acid (AR), asiatic acid (AS), ursolic acid (UA) and oleanolic acid (OA) were assayed, and their respective IC$_{50}$ values were determined (table 1). Acetazolamide was used as a positive control for this study. In spite of the substantial structural similarities, it is remarkable that these triterpenoids exhibited significant differences in their bioactivities with IC$_{50}$ values ranging from 9.11µM to as high as 333µM.

<table>
<thead>
<tr>
<th>Compounds tested</th>
<th>IC$_{50}$ Values (in µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetozolamide</td>
<td>0.48±0.09</td>
</tr>
<tr>
<td>Arjunolic acid</td>
<td>9.11±2.95</td>
</tr>
<tr>
<td>Oleanolic acid</td>
<td>93.74±1.06</td>
</tr>
<tr>
<td>Ursolic acid</td>
<td>125.5±0.86</td>
</tr>
<tr>
<td>Arjunic acid</td>
<td>188.9±0.46</td>
</tr>
<tr>
<td>Asiatic acid</td>
<td>333.7±0.61</td>
</tr>
</tbody>
</table>
In the assay for CA II inhibition, AJA exhibited the maximum potency with an IC$_{50}$ of 9.11μM while AS (IC$_{50}$=333.7μM) was the least potent (Table 1). OA, which lacks hydroxyls at C$_2$, and C$_{24}$, exhibits a 10 fold decrease in activity (IC$_{50}$=93.73μM). UA which is a α-amyrin triterpene series, showed 14 fold decrease in inhibitory activity (IC$_{50}$=125.5μM) as compared to AJA. AR, which is isomeric to AJA, showed a 20 fold decrease in inhibitory activity (IC$_{50}$=188.9μM). AS which also belongs to α-amyrin series of triterpene was the least potent with a 37 fold decrease in inhibitory activity (IC$_{50}$=333.7μM).

### 2.2. Molecular Docking studies of arjunolic acid and closely related compounds on CA II system

The CA II has an active site cleft (15 Å in diameter and 15 Å deep), and contains a Zn$^{2+}$ ion that is coordinated in a tetrahedral geometry with three histidine residues (His94, His96 and His119) and a water molecule/hydroxide ion$^{16}$. The triterpenoids tested in this study were docked into the active site of CA II using Auto Dock tools$^{17}$. The virtual screening results showed estimated free energy of binding between -6.2 and -8.6 kcalmol$^{-1}$. The most potent compound AJA$^{18}$, forms three hydrogen bonds with its hydroxyl group of C$_2$ (A-ring), by interacting with active site residues Asn62, His64 and Asn67.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Binding Energy (K cal mol$^{-1}$)</th>
<th>No of hydrogen bonding</th>
<th>Hydrogen bonding residues</th>
<th>Interaction with Zinc metal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetazolamide</td>
<td>-6.2</td>
<td>1</td>
<td>Glu106</td>
<td>Present</td>
</tr>
<tr>
<td>Arjunolic acid</td>
<td>-7.1</td>
<td>3</td>
<td>Asn-62, His-64, Asn-67</td>
<td>Absent</td>
</tr>
<tr>
<td>Arjunic acid</td>
<td>-8.6</td>
<td>4</td>
<td>His-64, Pro-201, Thr-200 (2).</td>
<td>Absent</td>
</tr>
<tr>
<td>Asiatic acid</td>
<td>-7.4</td>
<td>2</td>
<td>Tyr-7, Val-242.</td>
<td>Absent</td>
</tr>
<tr>
<td>Ursolic acid</td>
<td>-7.8</td>
<td>1</td>
<td>Glu-69</td>
<td>Absent</td>
</tr>
<tr>
<td>Oleanolic acid</td>
<td>-7.6</td>
<td>4</td>
<td>Asp-72 (2), Asp-71, Ser-73.</td>
<td>Absent</td>
</tr>
</tbody>
</table>

The virtual screening results showed estimated free energy of binding between -6.2 and -8.6 kcalmol$^{-1}$. The most potent compound AJA$^{18}$, forms three hydrogen bonds with its hydroxyl group of C$_2$ (A-ring), by interacting with active site residues Asn62, His64 and Asn67.
Interestingly enough, it does not interact with the catalytic Zn$^{2+}$, like the well-known inhibitors of CA II (acetazolamide that forms hydrogen bond with the Zn$^{2+}$). Moreover, AJA is the only triterpenoid that has H bonds with Asn67, a residue that plays a very crucial role in catalysis, as it is involved in proton transfer reaction. In spite of its acidic proton, the carboxyl group (E-ring) of AJA, is not involved in any hydrogen bonding. Additionally, the double bond in the
C-ring forms a π–π interaction with Gln92 and Phe131. AJA also forms hydrophobic interaction with the active site residues Glu69, Phe70, Asp72, His64 and Ile91, stabilizing the overall interaction (figure 23).

2.3. Comparative docking studies of arjunolic acid with the closely related triterpenoids

OA interacts with its hydroxyl at C3 (A-ring) forming hydrogen bonds with Asp72 (2), Asp71 and Ser73. However, it does not interact with Asn67, which is also reflected in the 10 fold increase in its IC₅₀ (figure 24A). In contrast to the A-ring interaction of OA, the E-ring carboxyl group of UA, an isomer of OA, interacts with Glu69 of the CA II.

![Figure 24](image_url). Comparison of arjunolic acid with closely related terpenoids on CA II protein.

The gem-dimethyl groups at C20 of OA and C19, C20 vicinal dimethyl of UA is clearly reflected in the further decrease in activity by 20-25% (figure 24B). AR which is a C19 hydroxyl isomer
of AJA, also interacts with CA II through its E-ring, forming hydrogen bonds with His64, Pro201 and Thr200 (2). Although it interacts with the active site residue His64 and demonstrates a strong binding energy (-8.6 kcal/mole), it still has a significantly reduced (20 fold) inhibitory activity. The isomerization of the OH group from A-ring C₂₄ of AJA to C₁₉ of E-ring of AR (chelation effect on the hydroxyl and carboxyl groups), forces the molecule to interact with the E-ring, instead of the more catalytically favourable A-ring interaction (figure 24C). AS, another isomer of AJA, has vicinal methyl groups at C₁₉ and C₂₀ carbon atoms of the E-ring, reducing its accessibility to interact with the active site. This could probably be due to the 1,3-diaxial steric hindrance of E-ring in the case of AS (C₁₉, C₂₀ methyl groups in the trans position) which is absent in AJA (free rotation of gem-dimethyl group in C₂₀ position). Additionally, since the diameter of the CA II active site is about 15 Å, the rigidity conferred on the E-ring of AS could further prevent its accessibility to the active site (figure 24D). A-ring interaction (H bond) with the Asn67 is crucial for inhibition of CA II activity. The gem-dimethyl group at C₂₀ of the E-ring greatly influences the activity of the compound by conferring flexibility, due to the free rotation and allowing accessibility to the active site of CA II, which is lost in UA and AS. Additionally, isomerization of the OH from C₂₄ to C₁₉ also significantly reduces the activity (AJA vs AR). The hydroxyl of C₂ is also spatially important in hydrogen bonding with the Asn67 (AJA), since OA that lacked this hydroxyl at C₂ exhibited much lower inhibition as compared to AA. The hydrogen bond through the COOH group of the E-ring is not favourable for the inhibitory activity as seen in the case of UA and AR.

3. CONCLUSION

This study reports the effect of triterpenoid, arjunolic acid (AJA), as a potent, non-zinc binding inhibitor of CA II among others. The molecular docking studies, corroborates the inhibition of CA II by AJA, through its interaction with key catalytic residues involved in proton transfer. Future studies are focused towards understanding and improving the specificity of AJA
towards other isoforms of CA. The present study also provides a template for the design of more effective drugs to reverse cardiac hypertrophy. This study gives credence to the use of “Arjuna extract” as cardio-vascular medicines.
PART III
PHYTOCHEMICAL INVESTIGATION OF JAKKALANNU (OOSIKALA) - A PLANT INDIGENOUS TO NILGIRI TRIBES

1. INTRODUCTION

1.1. Ethnomedicines; a valuable source for drug discovery

Ethnomedicine refers to the study of traditional medical practices which are based on traditional knowledge. Research interests and bioactivities in the area of ethnomedicine have increased tremendously in recent times. The components which was used in ethnomedicine have long been ignored by medical practitioner due to various reasons. However, it is interesting to note that using ethnomedicine, some of the important medicines were discovered which includes reserpine from Rauwolfia serpentine for treating hypertension; aspirin from Salix purpurea used for treating inflammation, pain and thrombosis; quinine from Cinchona pubescens used for anti-malarial properties. More recently, artemisinin from Artemisia annua for malaria treatment. Based on these significant aspects into consideration, we would like to explore the indigenous ethnomedicine- Jakkalannu (Oosikala) for its medicinal importance.

1.2. Identification of Jakkalannu (Oosikala)

During the bioprospection of Ooty flora, we discovered that a local plant viz. Jakkalannu (Oosikala) is being used by tribals (i.e. Badugas, Thodas and Kurumbas), for the treatment of stomach-ache, diarrhoea and jaundice. The aqueous root paste along with honey is used as an antimicrobial agent against skin diseases. The plant is located in Kappachi Village (Ooty, The Nilgiris, Tamilnadu, India). The botanical identity of the plant was established as Berberis tinctoria with the help of taxonomist Prof. Ravi, Govt, Arts College, Ooty (Tamilnadu, India). It is also called Nilgiri’s Barberry. Only limited biological studies have been reported on this plant. In the present study, the phytochemical investigation on different parts of B. tinctoria was undertaken.
1.3. Distribution of *Berberis* species

About 77 species of *Berberis* species are reported from India (Anonymous 1988). Most of them are available in Himalayas at altitudes of 1,000-3,000 m and in South India, Nilgiri hills (Ooty) at an altitude of 1,000-2,400 m. The plant *Berberis tinctoria* (Lesch) is a shrub of variable size and form (2 to 3 feet height); in the forests it may attain a height up to 15 feet with thick stem and long scendent branches bearing numerous slender leafy twigs.

1.4. Biological activities of *B. tinctoria*

A very few bioactivities are reported for *B. tinctoria* when compared to other Berberis species (i.e. *B. aristata*). The activities are; berries of *B. tinctoria* mixed with honey, is used for treatment of jaundice$^{19}$. The leaves shown hepatoprotection properties in animals$^{20,21}$. The fruits-barberry is known for anti-haemolytic properties$^{22}$. The root and root bark have antimicrobial properties$^{23}$. Literature suggested that most of the bioactivities are attributed to the presence of alkaloid, berberine (1). So, it is important to know the quantity of berberine (1), present in different parts of *B. tinctoria*.

1.5. Berberine; multitude bioactive compound

Berberine (1) is a quaternary ammonium salt from the protoberberine group of benzylisoquinoline alkaloids. Berberine (1) is an important multitude bioactive compound (figure 25) which are mostly present in the root and stem of *Berberis* species. For example, *Berberis aristata* is an important source for berberine (1) production. It is extensively used in different Ayurvedic preparations. Recent reports on bioactivities of berberine (1) are anti-HIV, anti-tumor, anti-hyperglycemic, anti-inflammatory, anti-malaria and anti-Alzheimer’s disease$^{20}$. More recently, berberine (1) is used for musculoskeletal disorders$^{24}$. The growing interest of berberine (1), made us to investigate *B. tinctoria*, whether an alternate source for this particular compound. A recent study from Srivastava *et al.*$^{25}$ indicated that the berberine (1) content is more (2-5%) in root than the stem (1-2%) of different *Berberis* species. This
study also showed that among *Berberis* herbal samples available in the market, substantial quantity of berberine was found in the roots of *B. chitria* (5.20%) followed by *B. lycium* (3.99%), *B. aristata* (3.55%) and *B. asiatica* (2.25%).

![Berberine, 1](image)

**Figure 25.** Multitude biological properties of berberine.

### 2. PRESENT STUDY

#### 2.1. Collection of plant materials

Plant materials (flowers, leaves, stem and roots) were collected from the Kappachi village, Ooty, Tamilnadu, India. The flowers of *B. tinctoria* was collected in during Jan-Feb 2017 (seasonal). The identity of the plant was established with the help of Prof. Ravi, Department of Botany, Government Arts College, Ooty.

#### 2.2. Extraction of plant materials

Plant materials were dried (at 50°C) and extracted sequentially with petroleum ether (PE), chloroform (CH), methyl ethyl ketone (MEK) and methanol (ME). The solvents were removed under reduced pressure. The dried extracts were taken for further analysis.

#### 2.3. Qualitative analysis of *B. tinctoria*

Qualitative analysis was carried out using different extracts of *B. tinctoria* (table 1). The PE extracts of leaves and flowers gave positive response to Liebermann Burchard (LB’s) test
indicating the presence of steroids/triterpenoids. The MEK and MeOH extracts of leaves and flowers gave yellow precipitate with lead acetate; green colour with neutral ferric chloride indicating the presence of phenolics. LB’s test was negative for ME extracts of stem and root of *B. tinctoria*.

**Table 1.** Colour reactions of *B. tinctoria* extracts.

<table>
<thead>
<tr>
<th>Colour reactions</th>
<th>Leaves</th>
<th>Flowers</th>
<th>Stem</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE</td>
<td>MEK</td>
<td>MeOH</td>
<td>PE</td>
</tr>
<tr>
<td>Liberman’s test</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ferric Chloride test</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lead Acetate test</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Molisch’s test</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Shinoda test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dragon Dorf’s reagent</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The MeOH extract gave positive for Shinoda test (Mg/HCl) which indicates the presence of flavonoids. None of the other extracts (including stem and root) show positive result for Shinoda test. Dragen Dorf’s test\(^{26}\) was positive for both stem and root extracts of *B. tinctoria* which indicates the presence of alkaloid.

**2.4. Phytochemical investigation of *B. tinctoria* flowers**

Flowers were sequentially extracted (figure 26) using non-polar to polar solvents (PE, CH, MEK and ME). PE extract gave positive result for steroids. TLC analysis of PE extract revealed
that this is a mixer of sterols. The column chromatography (SiO₂) was performed to isolate the major components of PE extract. The PE extract contain an orange component, which was identified as β-carotene (240mg; yield-0.36%). The analysis of UV absorption agreed with its characteristics properties [UV (MeOH): λ 427, 451, 478 nm]. The major steroid was separated using solvent mixture, petroleum ether/CHCl₃ (1:1). It was compared with the known standard. It was identified as sitosterol (2, 20mg; yield-0.03%).

![Figure 26. Phytochemical analysis of B. tinctoria flowers.](image)

The MEK extract gave positive reaction with neutral FeCl₃ (indicated as dark green colour). TLC analysis showed that it mainly contain FeCl₃ positive bands (*i.e.* phenolics). The MEK extract was subjected to column chromatography using SiO₂. The major compound was eluted by ethyl acetate. Further, spectral analysis [UV (MeOH): λ 325 nm; IR (KBr): ν 3330, 2955, 1687 cm⁻¹; MASS (M+H)⁺: 355 and MS/MS: 181] suggested that this compound was chlorogenic acid (3, 138mg; yield-0.21%). Chlorogenic acid (3) is reported to have blood pressure lowering effect²⁷. Methanol extract gave positive result for both Shinoda and
Molisch’s test (Acetic anhydride/H₂SO₄) which indicates the presence of flavonoids and glycosides. ME extract was subjected to acid hydrolysis. Two major FeCl₃ positive bands were observed in the TLC. The yellow band corresponds to quercetin. The colourless band was identified as 3,4-dihydroxy cinnamic acid (originated from chlorogenic acid). The ME extract was subjected to column chromatography using SiO₂. The chlorogenic acid was eluted by a solvent mixture, CHCl₃/ethyl acetate with 1:1 proportion. The yellow coloured compound eluted in a solvent mixture, ethyl acetate/MeOH (95:5). Further, spectral data confirms that two major compounds are chlorogenic acid and rutin [4, quercetin-rutinoside; UV (MeOH): λ 256, 355 nm; IR (KBr): ν 3320, 2925, 1656 cm⁻¹; MASS (M+H)⁺: 611, MS/MS: 303, 153]. The alkaloid, berberine was absent in the flower extract of B. tinctoria.

2.5. Chemical investigation of B. tinctoria leaves

The leaves were sequentially extracted (figure 27) with PE, CH, MEK and ME. In PE extract, mainly yellow coloured β-carotene was found. Sterols were identified in CE fraction. MEK extract showed positive results for phenols and negative for flavonoid tests. The major phenolics were cinnamic acid type derivatives (characteristic absorption at UV (MeOH): λ 324-325 nm. Similarly, ME extract also show similar type UV signatures (UV (MeOH): λ 323 nm. The fractions were isolated and subjected UV, MASS, ¹H and ¹³C NMR spectral analyses. The

![Figure 27. Phytochemical analysis of B. tinctoria leaves.](image-url)
identification of the compound is under progress. Alkaloids are absent in both the MEK and ME extracts.

2.6. Chemical investigation of *B. tinctoria* stem and root

![Diagram](image)

**Figure 28.** Phytochemical analysis of *B. tinctoria* stem and root.

ME extracts of stem and root (figure 28) gave positive Dragen Dorf’s (DD) test. TLC analysis revealed that berberine (1) was present in both the extracts (on comparison with standard). For the isolation of berberine (1), column chromatography was tried initially with SiO$_2$. But, berberine (1) was not eluted out from the column easily, this could be because of strong interaction between quaternary ammonium salt and silica (SiO$_2$). Subsequently, the column was tried with neutral aluminium oxide (Al$_2$O$_3$). The berberine (1, 84mg) was eluted by a solvent mixture, MeOH/CHCl$_3$ with 10:90 proportion. The berberine (1) purity was compared with commercial standard (based on HPLC). The berberine (1) content was about 0.8-1% in stem of *B. tinctoria*.

Berberine (1) was also found in the root of *B. tinctoria*. The root extract was subjected to column chromatography using neutral Al$_2$O$_3$. It was found that berberine (1, 190mg) content
was more (3-3.5%) in root when compared to stem (1%). Literature had suggested that so far, roots of *B. chitria* (5.20%) has maximum quantity of berberine (1)\textsuperscript{25}. Our results indicated that in *B. tinctoria*, berberine (1) content is comparable with (3.5-4%) the reported sources. So, this indigenous plant can be utilised for the commercial exploitation of berberine (1).

![Figure 29](image.png)

*Figure 29:* Compounds identified from *B. tinctoria*.

Berberine (1) is generally extracted and separated using mineral acids *e.g.* HCl. Mineral acid removal is one of the major obstacle in berberine (1) separation. So, it is important to have a separation technique for the isolation berberine (1). With this intention and commercialization point of view, we have developed a selective method for the isolation of berberine (1). The details are given below.

### 3. A NEW METHOD FOR THE ISOLATION BERBERINE FROM *B. TINCTORIA*

#### 3.1. Extraction procedure

*B. tinctoria* stem (50 gm) was crushed and grounded in a blender. It was subjected to soxhlet extraction using methanol (150 ml) for 3-4 hrs. Solvent was removed under reduced pressure.
2 gm of extract was obtained. TLC (toluene, 5; ethyl acetate, 5; formic acid 2) was performed to identify berberine (1) content in the extract.

3.2. Isolation of berberine using selective adsorption and desorption method

0.5 gm of stem extract was re-dissolved in methanol and pre-adsorbed with 10gm neutral aluminium oxide (Al₂O₃). The solid matrix was dried until free from the solvent. The dried matrix was taken in a 250 ml conical flask with a magnetic stirrer [After, many experimentations, it was found that a mixture of solvent (methanol-8% and chloroform-92%) selectively elutes berberine] to that solvent mixture (methanol-8% and chloroform-92%, 50 ml) was added and stirred for 10 minutes. The solvent was filtered. The extraction was repeated for 3-4 times with the same proportion of solvents. After evaporation of solvents, the yellow coloured compound was obtained. The purity of the product was established by melting point, TLC, HPLC and compared with its known standard. It was identified as berberine (1). ~110 mg yield of pure berberine was obtained. The column recovery is about 86-90%.

This isolation method was simple, faster and cheaper. So, it can be applied to many other species for berberine (1) isolation and also for the commercial production purposes.

4. CONCLUSION

The phytochemical analysis of B. tinctoria (local name; Jakkalannu) was undertaken with the aim of isolation and quantification of berberine (1). The analysis showed flowers and leaves does not contain berberine (1). The berberine (1) was found in the root and stem of B. tinctoria. The berberine (1) was quantified and results showed that berberine content was more in root (3-3.5%) than stem (0.8-1%). Overall yield of berberine (1) was about 3.5-4%. Additionally, analysis shown that the presence of known constituents viz sitosterol (2), chlorogenic acid (3), rutin (4) and β-carotene (5) (figure 29). A new method was developed for the isolation of berberine from the commercial point of view using selective adsorption and desorption method. This technique can be also applied to different Berberis species.
5. REFERENCES


