2.1 General Information

Figure 2.1  
(a) Geographical Distribution in Indian Subcontinent
(b) *C. vestita* Wall ex C.B.Clarke Plant with Berries

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Tracheophyta</td>
</tr>
<tr>
<td>Class</td>
<td>Magnoliopsida</td>
</tr>
<tr>
<td>Order</td>
<td>Lamiales</td>
</tr>
<tr>
<td>Family</td>
<td>Lamiaceae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Callicarpa</em> L.</td>
</tr>
<tr>
<td>Species</td>
<td><em>C. vestita</em> Wall. ex C.B.Clarke</td>
</tr>
</tbody>
</table>

**Synonyms Frequently Used For the Plant-**

*C. hookeri* C.B.Clarke

*C. lanata* Gamble

*C. vestita* Wall.
2.2 Present Status of Knowledge

*Callicarpa vestita* Wall. Ex C.B. Clarke (*C. vestita*) belongs to the genus *Callicarpa*. The genus *Callicarpa* comprises of small trees and shrubs with fruits typified as drupaceous with a fleshy exocarp and a hard endocarp (1). A small tree with dark brown stems and brown tomentum leaves ovateacuminate, thick silky white, tomentose beneath, base rounded or shallow cordate. Flowering takes place in the month of April while fruiting takes place in the summer season. Bark and roots of this plant are chewed like betel-nuts (2). This plant is generally known as Chilthiya in vernacular language by local people.

There are different school of thoughts over the status of genus *Callicarpa* to be included in the family Verbenaceae and are of the view that this genus should be considered and included among the Lamiaceae members (3). *C. vestita* is a great source of phytochemicals and traditionally used for the treatment of various ailments in the conventional medicinal system of Kumaun Himalaya region. Plants of this family have been widely explored by various workers around the globe. Yamahara et al., (4) investigated ethanolic extract of the leaves of *C. longissima* for the evaluation of its anticancer activity. *C. longissima* inhibits melanin production in B16F10 mouse melanoma cells by suppressing microphthalmia-associated transcription factor (MITF). Ma et al., isolated (5) new compounds from the plant extract of the *C. nudiflora*. These isolated compounds were further tested for their potential against cancer cells. Some of these isolated compounds such as 6-hydroxyluteolin-7-O-β-glucoside and nudifloside were found to be effective at various concentrations and showed monolithic proliferation inhibitory activities against HELA, A549 and MCF-7 cell lines (6). Liu et al., isolated four new compounds from the *C. longissima*. Out of these four compounds, callilongisins A, when evaluated for its cytotoxicity against a human prostate cancer cell line (PC3), was found to be very effective (7). Mei et al. (8) studied a new iridoid, named nudifloside from the plant extract of the *C. nudiflora*. Isolated compound exhibited remarkable inhibitory effects towards myelogenous leukemia K562 cell line, anticancer efficacy of the isolated natural products was analyzed by the calculation of IC50 values against both the cancer cells.
Keeping in mind potential of *Callicarpa* species, this research work is dedicated to the chemical screening of the plant and to evaluate its pharmaceutical potential against selected microbial stains (bacterial and fungal). The author has reported volatiles from the leaves of the plant. This Chapter also reports isolation of pharmaceutically important flavonoids from the roots of the *C. vestita* plant.

### 2.3 Aims and Objectives

To the best of our knowledge this is the first report on the phytochemical screening of *C. vestita* from Himalayan region and is of great importance. The aims and objectives of the proposed research work can be summarised under the following points.

1. To screen the volatiles of the leaves of *C. vestita* from the Kumaun Himalaya for the first time.
2. Isolation of nonvolatiles from the root of the *C. vestita*.
3. Structural elucidation of the isolate by various techniques GC- FID, GC-MS, H-NMR, C- NMR, and FT-IR spectrometry.
4. To contrive GO based nano-carriers for the enhanced biological activity of the isolates, which has been documented in detail in Chapter four and Chapter five.
5. Evaluation of leaves EO for its biological activity against animal and plant pathogens, which has been documented in detail in Chapter five.

### 2.4 Experimental

#### 2.4.1 Plant Material

Leaves of *C. vestita* were collected from Chandak (District Pithoragarh of Uttarakhand), located at an altitude of 2800 meters above sea level. Leaves were properly cleaned, minced and used for the immediate hydro-distillation for the extraction of EO. Identification of the plant material was done at the *Botanical Survey*
of India, Dehradun. The voucher specimen (Accn No. 113272) is deposited in the High Altitude Plant, Medicinal Chemistry research laboratory, Kumaun University, Nainital.

2.4.2 Extraction of the Essential oil

Finely crushed leaves of the plant C. vestita were hydrodistilled using a Clevenger apparatus with a water-cooled oil receiver for 3 h. The collected oil was transferred into a stoppered tube, dried over anhydrous sodium sulphate and stored in a refrigerator at 4 ºC until analyzed.

2.4.3 Gas Chromatographic Analysis

The oil was analyzed by using a Shimadzu 2010 auto system GC. The column temperature was programmed at 40ºC (hold time for 2 minute) to 210ºC (hold time 5 minute) at 3ºC min⁻¹ and than 210º-300ºC at 20ºC min⁻¹ with a final hold time of 15 minute, using N₂ at 30.0 mL/min column head pressure as carrier gas, the injector temperature was 270ºC and detector (FID, Flame ionization detector) temperature 280ºC.

2.4.4 Gas Chromatographic-Mass Spectrometric Analysis (GC-MS)

The GC-MS used was Autosystem 2010 GC (Rtx- 5, 30m x 0.25mm, i.d. FID 0.25µm) coupled with Shimadzu QP 2010 plus with thermal desorption system TD 20 with (Rtx-5) fused silica capillary column (30 m x 0.25mm with film thickness 0.25µm). The column temperature was 80ºC (hold time for 2 minute) to 210ºC (hold time 5 minute) at 3ºC min⁻¹ and then 210º-300ºC at 20ºC min⁻¹ with a final hold time of 21 minute, using helium as the carrier gas. The injector temperature was 230ºC and 0.2 µL in n-hexane, with split ratio of 1:30 MS were taken at 70 eV with a mass range of 40-650amu. Identification of constituents were done on the basis of Retention Index (RI, determined with reference to homologous series of n-alkanes C8-C28, under identical experimental condition), MS library search (NIST and WILEY), and by comparison with MS literature data (1) The relative amounts of individual components were calculated based on GC peak area (FID response).
2.4.5 Extract of Roots of *C. vestita*

Roots of the plant (2 kg) were dried, powdered and extracted using Soxhlet apparatus with 80% methanol for about 18 h. After extraction, the filtrate was concentrated on a rotary evaporator under vacuum at 20°C till a residual mass was obtained. The alcoholic residue was dissolved in water and partitioned with ethyl acetate, the immiscible mixture was separated with the help of separating funnel; ethyl acetate layer and water layer were concentrated under reduced pressure and on water bath respectively. The ethyl acetate extract was then loaded over a column Silica gel G packed in hexane and eluted with different solvents of increasing polarity, starting from 100% CHCl₃ up to followed by increasing gradient of MeOH up to 100%. The isolate were collected in 25 mL portions and monitored on TLC. Samples (92 to 106) collected by using dichloromethane and MeOH (3:1) when observed via TLC gave single spot. The entire fraction from 94 to 104 was collected. Samples on the border were excluded in order to eliminate any kind of contamination and evaluated for the further analysis. The flowchart for the isolation has been given below.

Fig 2.2 Schematic Representation of the Column Chromatography
2.4.6 Preliminary Screening of the Isolate

1) 1 mL of dilute sodium hydroxide to 1 mL of concentrated plant sample gave a yellow color indicating the presence of flavonoids.

2) With chloroform in presence of Anhy. Aluminium chloride, isolate gave orange red color indicating the presence of aromatic ring.

3) It gave bluish green color when spotted TLC plate was sprayed with alcoholic FeCl₃ indicating the presence of phenolic group.

4) With sodium bicarbonate, no effervescence appeared indicating the absence of –COOH group.

5) With Molisch reagent, Isolate gave no reaction indicating the absence of sugars

2.5 Spectroscopy Investigations

Structural elucidation of the isolated compound was done with the help of H-NMR, C-NMR and FT-IR spectroscopy. Analysis was performed on Bruker 500 MHz instrument, and DMSO was used as the solvent, TMS was used as an internal standard. Chemical shifts (δ) were expressed in ppm shift. NMR and the 13 C-NMR, FT-IR studies were carried out at the Advance Instrument and Research Facility Lab, Jawaharlal Nehru University, Delhi, and CBMR Lucknow.

2.6 Results and Discussion

2.6.1 EO Profile of the C. vestita

A total of 25 compounds, representing 96.59% of the total oil, were identified (Figure 2.3, 2.4 and 2.5). The oil was found to contain markedly different constituents than the previous reports on the leaves oil. Major constituent of the EO was vulgarone “B” (24.26%) and further dominated by sabinene(21.795), β-caryophyllene (15.03%), caryophyllene oxide(5.73%), eucalyptol (3.78%), p-menth-2-en-1-ol (3.43%), germacrene D (2.39%), β-pinene (2.83%) respectively. Details of the identified volatiles have been summarized in table (Table 2.1).
Table 2.1 Chemical Composition of Leaves EO of *C. vestita*

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Compound</th>
<th>KI&lt;sub&gt;exp&lt;/sub&gt;</th>
<th>Area %</th>
<th>Mode of Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>α-thujene</td>
<td>923</td>
<td>0.57</td>
<td>a, b</td>
</tr>
<tr>
<td>2.</td>
<td>α-pinene</td>
<td>933</td>
<td>3.22</td>
<td>a, b</td>
</tr>
<tr>
<td>3.</td>
<td>β-pinene</td>
<td>980</td>
<td>2.83</td>
<td>a, b</td>
</tr>
<tr>
<td>4.</td>
<td>sabinene</td>
<td>974</td>
<td>21.79</td>
<td>a, b</td>
</tr>
<tr>
<td>5.</td>
<td>myrcene</td>
<td>991</td>
<td>0.47</td>
<td>a, b</td>
</tr>
<tr>
<td>6.</td>
<td>p-cymene</td>
<td>1026</td>
<td>2.40</td>
<td>a, b</td>
</tr>
<tr>
<td>7.</td>
<td>eucalyptol</td>
<td>1033</td>
<td>3.78</td>
<td>a, b</td>
</tr>
<tr>
<td>8.</td>
<td>e- β-trans-octimene</td>
<td>1050</td>
<td>0.70</td>
<td>a, b</td>
</tr>
<tr>
<td>9.</td>
<td>p-menth-2-en-1-ol</td>
<td>1285</td>
<td>3.43</td>
<td>a, b</td>
</tr>
<tr>
<td>10.</td>
<td>elemene &lt;delta-&gt;</td>
<td>1337</td>
<td>0.20</td>
<td>a, b</td>
</tr>
<tr>
<td>11.</td>
<td>2,3-pinanediol</td>
<td>1280</td>
<td>0.40</td>
<td>a, b</td>
</tr>
<tr>
<td>12.</td>
<td>4-thujanol</td>
<td>1346</td>
<td>1.92</td>
<td>a, b</td>
</tr>
<tr>
<td>13.</td>
<td>elemene &lt;β-&gt;</td>
<td>1393</td>
<td>1.76</td>
<td>a, b</td>
</tr>
<tr>
<td>14.</td>
<td>β-caryophyllene</td>
<td>1418</td>
<td>15.03</td>
<td>a, b</td>
</tr>
<tr>
<td>15.</td>
<td>α-humulene</td>
<td>1552</td>
<td>1.55</td>
<td>a, b</td>
</tr>
<tr>
<td>16.</td>
<td>germacrene D</td>
<td>1499</td>
<td>2.39</td>
<td>a, b</td>
</tr>
<tr>
<td>17.</td>
<td>germacrene B</td>
<td>1560</td>
<td>0.31</td>
<td>a, b</td>
</tr>
<tr>
<td>18.</td>
<td>caryophyllene oxide</td>
<td>1573</td>
<td>5.73</td>
<td>a, b</td>
</tr>
<tr>
<td>19.</td>
<td>humulene epoxide ii</td>
<td>1608</td>
<td>0.77</td>
<td>a, b</td>
</tr>
<tr>
<td>20.</td>
<td>germacrene D-4-ol</td>
<td>1592</td>
<td>0.50</td>
<td>a, b</td>
</tr>
<tr>
<td>21.</td>
<td>spathulenol</td>
<td>1602</td>
<td>0.35</td>
<td>a, b</td>
</tr>
<tr>
<td>22.</td>
<td>hydroxyl vealerenic acid</td>
<td>1685</td>
<td>0.41</td>
<td>a, b</td>
</tr>
<tr>
<td>23.</td>
<td>vulgarone “B”</td>
<td>1699</td>
<td>24.26</td>
<td>a, b</td>
</tr>
<tr>
<td>24.</td>
<td>aromadendrene oxide</td>
<td>1702</td>
<td>1.35</td>
<td>a, b</td>
</tr>
</tbody>
</table>

Monoterpene hydrocarbons 31.75%
Oxygenated monoterpenes 10.23%
Sesquiterpene hydrocarbons 21.24%
Oxygenated sesquiterpenes 33.37%
Others 3.41%
Total Identified 96.59%

a=Kovats Index (KI)
b=MS (GC-MS) Literature Data
Fig 2.3 GC Profile of Leaves EO of *C. vestita*
Fig 2.4 GC-MS Profile of Leaves EO of *C. vestita*
Fig 2.5 Major Constituents from the Leaves EO of *C. vestita*

### 2.6.2 Non Volatile Constituent from The Extract of *C. vestita* Roots

On the basis of data extracted from various spectroscopic techniques (Fig 2.6, 2.7, 2.8 & Discussion, Table 2.2), the isolate has been confirmed as Quercetin (QSR). Melting point of the isolate was found to be 316 °C. The spectroscopic data was found in complete agreement with the previous studies. Recent pharmaceutical developments have found that the QSR skeleton is active in various biomedical applications including antioxidative, anti-inflammatory and anti-apoptosis activities (9, 10, 1-24).
Fig 2.6 FT-IR- Spectra of the Isolate

Fig 2.7 (A) H-NMR Spectra with Peak of Hydroxyl- H (B) H-NMR Spectra without Peak of Hydroxyl- H
Fig 2.8 A. C-NMR Spectra of the Isolate with Solvent Peak, B. Without Solvent Peak
Table 2.2 Peak Assignment to the Isolated Compound in H-NMR, C-NMR and FT-IR Spectra

<table>
<thead>
<tr>
<th>δ In ppm)</th>
<th>Assigned to</th>
<th>δ values (In ppm)</th>
<th>Assigned to</th>
</tr>
</thead>
<tbody>
<tr>
<td>93.86</td>
<td>C-8</td>
<td>6.191</td>
<td>6-H (Ring A)</td>
</tr>
<tr>
<td>98.65</td>
<td>C-6</td>
<td>6.424</td>
<td>8-H (Ring B)</td>
</tr>
<tr>
<td>103.43</td>
<td>C-10</td>
<td>6.882,6.899 (d)</td>
<td>6'-H (Ring C)</td>
</tr>
<tr>
<td>115.43</td>
<td>C-2'</td>
<td>7.535-7.552(d)</td>
<td>5'-H (Ring C)</td>
</tr>
<tr>
<td>116.03</td>
<td>C-5'</td>
<td>7.655-7.658(d)</td>
<td>2'-H (Ring C)</td>
</tr>
<tr>
<td>120.52</td>
<td>C-6'</td>
<td>8.4-9.6 &amp; 12.5</td>
<td>Broad peaks for OH</td>
</tr>
<tr>
<td>122.39</td>
<td>C-1'</td>
<td></td>
<td>Major FT-IR frequencies (cm⁻¹)</td>
</tr>
<tr>
<td>136.34</td>
<td>C-3 (B-ring)</td>
<td></td>
<td>3000 to 3600 (Broad)- OH group</td>
</tr>
<tr>
<td>145.47</td>
<td>C-2 (B-ring)</td>
<td></td>
<td>1420 cm⁻¹, 1330cm⁻¹ C- OH in plane banding.</td>
</tr>
<tr>
<td>147.24</td>
<td>C-3’</td>
<td></td>
<td>C=O stretching frequency, 1660 cm⁻¹</td>
</tr>
<tr>
<td>148.10</td>
<td>C-4’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>156.58</td>
<td>C-9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>161.10</td>
<td>C-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>164.29</td>
<td>C-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>176.24</td>
<td>C-4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.7 Conclusion

GC and GC-MS analysis of the EO from leaves of C. vestita revealed about volatiles from the species. The major constituents of the EO were found to be α-pinene (3.22%), β-pinene (2.83%), sabinene (21.79%), p-cymene (2.40%), eucalyptol (3.78%), p-menth-2-en-1-ol (3.43) 4-thujanol (1.92%), elemene <β-> (1.76%), β-caryophyllene (15.03%), α –humulene (1.55%) and germacrene D (2.39 %). The major essential constituent was identified as vulgarone “B” (24.26%). Presence of vulgarone “B” as the major constituent of the EO opens a wide window of opportunity to utilize this EO in various medicinal applications. In previous reports,
vulgarone “B” was found to be active against some devastating termite pests such as *Formosan subterranean*, *Coptotermes formosanus Shiraki* and can be used as environment friendly termite controlling agents (25). Vulgarone “B” is also tested and accepted as cost effective alternative for snail control in aquaculture. One such study shows it is active against snails such as *Planorbella trivolvis* which has been recently identified as a significant problem in commercial channel catfish (26).

Various spectroscopic techniques have shown that the isolate from the roots of *C. vestita* is Quercetin (QSR), one of the group of over 4000 naturally available plant phenolics whose isolation and biological identification were first described by Szent-Gyorgyi in 1936. QSR acting as free radical scavengers was shown to exert a protective effect in reperfusion ischemic tissue damage (27-29). QSR prevents free radical induced tissue injury by various ways. One way is the direct scavenging of free radicals. By scavenging free radicals, flavonoid; particularly QSR can inhibit LDL oxidation in vitro (30). This action protects against atherosclerosis. QSR seems to exert antibacterial activity against almost all strains of bacteria known to cause respiratory, gastrointestinal, skin and urinary disorders (31). In various animal and test tube studies, QSR has shown that it inhibits the growth of cancer cells including those from breast, colon, prostate and lung (32). QSR by virtue of its anti-oxidant property prevents reactive oxygen species induced DNA damage, leading to mutational changes. A large clinical study suggested the presence of an inverse association between QSR intake and subsequent incidence of lung cancers (33). In a study done by Caltagirone et al, QSR exhibited the inhibitory effect on the growth of melanoma and also influenced the invasive and metastatic potential in mice (34-35). The bioflavonoid QSR may be a potent alternative to reduce cisplatin induced nephrotoxicity (35). Furthermore QSR seems to inhibit angiogenesis (36). Angiogenesis is a normal and strictly controlled process in the human body(37). Among the angiogenesis inhibitors QSR seems to play an important role (38). However the mechanism behind the anti-angiogenic effect of flavonoids is unclear. A possible mechanism could be the inhibition of protein kinasev (PTKs) (39). As many
of the PTKs are oncogenes, this raised the possibility of QSR being an effective anti-cancer compound. QSR was effective in inhibiting radiation-induced PKC activity. Activation of PKC is one of the means of conferring radioresistance on a tumor cell. Suppression of PKC activity by QSR may be one of the means of preventing the development of radioresistance following radiotherapy (40, 41). In light of the above it can be seen that the phytochemical investigation of the plant species is of utter importance and can be used for more exploration of this plant. In addition to this, presence of such a huge number of biologically active molecules makes it of great pharmaceutical importance.
References:


