4. EXPERIMENTAL

MATERIALS AND METHODS

4.1 Plant material

Dried fruits of *Embelia ribes* and roots of *Arnebia nobilis* Rech. f. were purchased from All India Kirana Stores, Paydhonie, Mumbai. Dried roots of *Plumbago zeylanica* were procured from Jaipur, Rajasthan. Roots of *Plumbago auriculata* were procured from gardens of Institute of Chemical Technology. Seeds of *Nigella sativa* were obtained from YUCCA Enterprises, Mumbai. The plant material were dried in tray dryer at 55°C for 24 h and powdered. The Plant materials were authenticated by Prof. H. M. Pandit, Khalsa College, Mumbai; a voucher specimen was deposited in Medicinal Natural Products Research Laboratory, Institute of Chemical Technology, Matunga, Mumbai.

4.2 General experimental procedures

Melting point was determined using a VEEGO India melting point apparatus. IR spectra (KBr) were recorded on a Perkin Elmer FT-IR spectrometer. $^1$H and $^{13}$C NMR spectra were determined in the indicated solvent on a JOEL spectrometer operating at 400 and 100 MHz, respectively. $^1$H and $^{13}$C chemical shifts (δ, ppm) are relative to the solvent signals used as references [CDCl$_3$: δC (central line of t) 77.04; residual CHCl$_3$ in CDCl$_3$: δ H 7.26. HPLC analysis was performed with a JASCO (Hachioji, Tokyo, Japan) system, using Purospher star RP Endcapped 250 mm × 4.6 mm i.d. (5-μm particle size) column, an intelligent pump (PU-1580, PU-2080), a high-pressure mixer (MX-2080-31), a manual sample injection valve (Rheodyne 7725i), Injection volume loop: 20 μL., monitoring by UV (UV-1575), and chromatographic data were processed with software (Borwin). Silica gel (60-120 mesh) (Merck) was used for column chromatography (CC), and silica gel 60 F-254 (Merck) was used for TLC. Ethanol, ethyl acetate, methanol, chloroform were of laboratory grade and were purchased from M/s. S.D. Five Pvt Ltd. Boisar, India. Standard Thymoquinone was obtained from Sigma–Aldrich (USA). A domestic microwave oven from M/s Kenstar Industries Ltd., Mumbai, India, was used for irradiation of dry seed before extraction. Derivatizing agents (DA) like 5% alcoholic Potassium Hydroxide
reagent were prepared as per procedures given by Wagner. Same procedures were followed for derivatization of TLC plates (*Plant Drug Analysis*, 1996).

### 4.3 Studies On Benzoquinones

#### 4.3.1 Embelin

- Extraction and Isolation
- HPLC and HPTLC studies
- Histo-chemical Localization
- Stress Degradation Studies
- Chemical Modification

#### 4.3.2. Thymoquinone

- Synthesis
- HPLC and HPTLC studies
- Micro-wave Assisted Extraction
4.3.1.1. Extraction and Isolation of Embelin from fruits of *E. ribes*

![Berries of *Embelia ribes* (A&B) and Crystals of Embelin (C)](image)

**Figure 4.1. Berries of *Embelia ribes* (A&B) and Crystals of Embelin (C)**

**Chemical test for Embelin:**

*Bontragers’s test for Embelin:* About 1g of *E. ribes* powder was extracted in 5 ml of chloroform. The chloroform layer was reduced to 1 ml. About 1 ml of 5% potassium hydroxide solution was added to this tube. The aqueous layer assumes violet-pink colour.

**Isolation of Embelin from *E. ribes* fruits**

**Extraction technique:**
1. Soxhlet extraction using light petroleum ether (12 hr.)
2. Treatment of petroleum ether extract, Purification and crystallization:

Dried ripe fruits of *Embelia ribes* (1000 g) were ground to 20# size and extracted in Petroleum ether [60-80° C] using soxhlet assembly. The petroleum ether layer was concentrated to half its volume, on cooling orange coloured embelin precipitates out (25 g). Filtered and given washings with cold petroleum ether until the precipitate appears bright orange in colour.

**Recrystallization:**

2 g of crude Embelin obtained from above procedure dissolved with boiling in 50 ml of methanol. Filter immediately using vacuum filtration and filtrate allow standing for 20-30 minutes gives bright orange flakes of Embelin.
4.3.1.2. Characterization of Embelin

Description : Golden Yellow-Orange shiny crystals

Solubility : Freely soluble in light petroleum ether, chloroform and Methanol

Melting point : 144° C

UV $\lambda_{\text{max}}$ (EtOH) : 286 nm

IR $\nu_{\text{max}}$ (KBr) cm$^{-1}$ : 3310 (Ar-OH), 2921 (Ar-H), 2850 (C-H), 1641, 1615 (C=O)

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 0.8 (t, 3H, R-CH$_3$), 1.25-1.29 (br, s, 20H, -CH$_2$), 6.0 (s, 1H, Ar-H), 7.68 (s, 2H, Ar-OH)

$^{13}$C NMR (CDCl$_3$, 100 MHz): $\delta$ 14 (R-CH$_3$), 22-31 (10X-CH$_2$), 102 (C$_{Ar}$-H), 119 (C$_{Ar}$-R), 157, 161 (2 X C$_{Ar}$-O-H), 181, 182 (2 X C$_{Ar}$=O)

Mass (m/z): 293[M-1]
Spectroscopic data of Embelin

Figure 4.2: IR spectra of Embelin

Figure 4.3: $^1$H NMR spectra of Embelin.
Figure 4.4: $^{13}$C NMR spectra of Embelin.

Figure 4.5: Mass spectra of Embelin
Figure 4.6: MS-MS spectra of Embelin

Figure 4.7: MS-MS fragmentation pattern of Embelin
Morphology of Embelin crystals

Images of Embelin obtained by crystallization using methanol (A) as solvent and that of embelin purified by column chromatography using light petroleum ether (B)

Images obtained under compound microscope

Images obtained under Scanning Electron Microscope

Figure 4.8: UV spectra of Embelin.

Figure 4.9: Morphology of Embelin Crystals.
4.3.1.3. HPTLC Analysis of Embelin:

**Preparation of extract:** 2 g of fruit powder was extracted with methanol (10 ml) by shaking for 5 minutes. Allow standing, methanol layer was concentrated upto 2 ml under vaccum. 10 µl was used for TLC (Fig 4.10).

**Preparation of standard:** Embelin prepared as a 0.01% of methanolic solution, 5 µl used for TLC.

**Chromatographic solvent:** Butanol: Propanol: Ammonia (4N) (7:1:2)

**Detection:** Visible light

Visible (Without chemical treatment)

A. UV -254 nm

1. Isolated compound

2. *E.ribes* fruit extract

![Image of TLC profile of Embelin and E.ribes methanolic extract.](image)

**Figure 4.10:** TLC profile of Embelin and *E.ribes* methanolic extract.
4.3.1.4. HPLC studies of Embelin

Standard solution

Take 10 mg of Embelin in methanol in 10-ml volumetric flask and make up the volume. 1 ml of above solution was taken and diluted up to 10 ml (Stock solution 100 µg/ml). From the stock solution prepare standard solutions (10 µg/ml) by transferring aliquots 1 ml of stock solution to 10-ml volumetric flasks and adjust the volume to 10 ml with methanol.

Chromatographic conditions

Instrument : JASCO PU-1580
Column : Purospher, RP-18e, 250 x 4.6, 5 µ
Mobile phase : Acetonitrile: Water (70:30)
Flow rate : 1 ml/min
Detector : UV Detector (JASCO UV-1575) at 286 nm
Injection volume : 20 µl
Run time : 15 min

Figure 4.11: HPLC chromatogram of Embelin.
4.3.1.5. Histochemical localization of Embelin in *E. ribes* fruit and its quantification

**Macroscopic studies:**
The fruits of *Embelia ribes* are brownish black in colour, about 2 to 5 mm in diameter they have warty surface with a beak like projection at apex. They are often short having a thin pedicel and persistent calyx with usually 3 or 5 sepals present. The pericarp is brittle enclosing a single seed covered by a thin membrane. The entire seed is reddish in colour and covered with orange-yellow spots (*chitra tandula*), odour slightly aromatic and has astringent taste. (*Ayurvedic Pharmacopoeia of India*, 1990). These orange-yellow coloured spots present on the external surface of the seed were studied using histochemical staining techniques.

**Microscopic studies:**
Transverse section of fruit shows epicarp consisting of single row of tabular cells of epidermis, usually obliterated, in surface view the cells appear rounded with wrinkled cuticle, the mesocarp consists of a number of layers of reddish-brown coloured cells and numerous fibrovascular bundles and rarely a few prismatic crystals of calcium oxalate, inner part of mesocarp and endodermis composed of stone cells, endodermis consisting of single layered, thick-walled, large, palisade-like stone cells (Fig. 4.12). The seed coat is composed of 2-3 layered reddish-brown coloured cells along with yellow-orange coloured shiny crystalline substance present in the cavities located on outer side of the seed coat, endosperm cells irregular in shape, thick-walled, containing fixed oil and proteinous masses, embryo small when present otherwise most of the seeds sterile.

The shiny orange substance seen on the external surface of seed was scraped carefully from 10 seeds with needle, dissolved in methanol and subjected to thin layer chromatographic studies along with Embelin, using Butanol: Propanol: Ammonia (4N): 7:1: 2 as mobile phase. The Rf of the yellow-orange crystalline substance was found to match with that of standard Embelin which is 0.33. This was further subjected to histochemical testing. The seed, pericarp as well as the T.S of seed were treated with ammonia solution (25%). Embelin is known to form deep pink coloured complexes with ammonia. When ammonia solution was sprayed on the seed and the pericarp of *E. ribes* fruit, the yellow-orange coloured crystalline powder present in the cavities of the seed coat turn deep pink in colour and so does the orange coloured mass seen in T. S. of seed.
thin membrane present on pericarp which encloses the seed also takes up deep pink colour. This membrane covers the seed and hence some amount of Embelin adheres to this portion of pericarp even after decortication. Embelin is principally present in the cavities present externally on the seed and can be seen clearly without the aid of microscope in area between seed and pericarp.
Fig 4.12: Macroscopic and microscopic studies of *Embelia ribes* fruits

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HPLC Analysis:
HPLC analysis of Embelin with a UV Detector was carried out in an isocratic solvent system at a flow rate of 1.0 ml/min and a run time of 15 min. The mobile phase consisted of a mixture of acetonitrile: ortho-phosphoric acid 1% in water (pH to 3.2) (90:10 v/v). The injection volume was 20 µl; the detection wavelength was 286 nm (appropriate to the UV absorption maxima of the compound determined). Based on this HPLC method the Embelin content in different parts of *E. ribes* fruit i.e. pericarp, seed, entirely crushed fruit and decorticated seed along with pericarp was analyzed. About 500 mg of each of seed, pericarp, whole crushed fruit and decorticated whole fruit were extracted using soxhlet assembly for 8 h using methanol. The methanolic extract thus obtained was diluted appropriately and subjected to HPLC analysis. Embelin was found to be present in the cavities present on the outer surface of seed. Embelin content of seed, pericarp, whole crushed fruit and decorticated whole fruit were determined using HPLC and all samples were found to contain Embelin.

4.3.1.6. Stress Degradation Studies on Embelin
Stress testing is a part of development strategy under ICH requirements and is carried out under more severe conditions than accelerated studies. Further, it is suggested that stress studies should be carried out to establish the stability characteristics of the molecule by establishing the degradation pathways and help in validation of the analytical methods to be used in stability studies. The parent drug stability guidelines by ICH (Q1AR) requires that the stress testing of drug substance should include the effect of elevated temperature, humidity, light, oxidizing agents, as well as the susceptibility across a range of pH values (ICH, Q1A Stability Testing of New Drug Substances and Products, 2000). Considering the future applications of Embelin in modern medicine, the objective of the current work was to carry out stress degradation studies to establish the stability characteristics of the molecule and ascertain the degradation pathways.
Embelin was isolated from dried berries of *E. ribes*. A stock solution of 1.0 mg/ml was prepared in methanol and stored in refrigerator. All the stressed samples were subjected to
HPLC analysis for which Embelin shows a retention time of 7.3 min and percent purity on the basis of area under curve was found to be 98.3%.

A set of standard solutions was prepared by diluting aliquots of the stock solution, with methanol to give concentrations ranging from 10.0 to 100.0 μg/ml. The calibration graphs were constructed by plotting the peak areas versus the concentration of standard solutions and were found to be linear. \( y = 82559x - 86459 \) \( (r^2=0.994) \).

In order to determine its stability, a reference standard of Embelin was stressed under different stress conditions to carry out forced degradation studies.

Accurately weighed 10 mg Embelin was dissolved in 100 ml of methanol. 1 ml of this solution was incubated in dark (in order to exclude the possible effects of light on degradation) with methanolic solution of 0.01 N, 0.1 N and 1 N HCl for acid induced degradation and with 0.01 N, 0.1 N and 1 N NaOH for base induced degradation studies. After 2 h the solutions were neutralized and diluted to 10 ml. The resultant solutions were analyzed by HPLC in triplicate.

Accurately weighed 10 mg of drug was dissolved in 100 ml of methanol. Subsequently, 1 ml of hydrogen peroxide of concentrations 0.3%, 3.0% and 30.0% v/v were added to three different tubes containing 1 ml of the stock solution and incubated in dark for 2 h and then the solution was heated in boiling water bath for 1 h to remove the excess hydrogen peroxide. The volume of the resultant solution was made up to 10 ml with methanol and analyzed by HPLC in triplicate for measuring oxidative degradation.

The photochemical stability of the drug was studied by exposing the drug solution (1 mg ml\(^{-1}\)) to direct sunlight and UV radiation at 254 nm for 24 h. The resultant solutions were diluted to 10μg ml\(^{-1}\) and analyzed by HPLC in triplicate for degradation studies. Embelin (10 mg) was stored at 80°C, 90°C and 100°C for 1 h under dry heat conditions in a hot air oven for analysis of degradation when subjected to dry heat. Then it was dissolved in methanol to get a concentration of 10μg ml\(^{-1}\) and analyzed by HPLC in triplicate to study the degradation products. In all degradation studies, the average peak area of Embelin, after application of three replicates was obtained and the content of residual Embelin was calculated.
The Acid degradation product was isolated and purified using silica gel (60#) column chromatography using light petroleum ether and ethyl acetate as eluant. A 5% fraction of ethyl acetate in petroleum ether yielded orange coloured needles which were subjected to spectroscopic studies (Table 4.1).

**Table 4.1. Spectroscopic Data Of Acid Degradation Product Of Embelin**

<table>
<thead>
<tr>
<th>IR ν max (KBr) cm⁻¹</th>
<th>3353 (Ar-OH), 2920 (Ar-H), 2852 (C-H), 1638,1599 (C=O), 1205 (-OCH₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass Spectra</td>
<td>[M+1]⁺ 309</td>
</tr>
<tr>
<td>MS-MS</td>
<td>[M+1]⁺ 169.1,141.1</td>
</tr>
<tr>
<td>¹H-NMR (CDCl₃, 400MHz)</td>
<td>δ 0.5 (t, 3H, R-CH₃), 1.25 (s, 20H, -CH₂), 3.86 (s, 3H, O-CH₃), 7.23 (d, 1H, Ar-H)</td>
</tr>
<tr>
<td>¹³C-NMR (CDCl₃, 125 MHz)</td>
<td>14(C-17), 22 (C-16), 21 (C-7), 27 (C-8) 31 (C-15), 30 (C-14, C-13, C-12, C-11, C-10, C-9), 56.5 (C-18), 102 (C-6), 119(C-3), 151 (C-2), 161 (C-5), 181(C-4), 182 (C-1)</td>
</tr>
<tr>
<td>Melting point</td>
<td>118º</td>
</tr>
</tbody>
</table>

Solutions of two different concentrations (10 and 100 µg.ml⁻¹) were prepared from sample solution and stored at room temperature for 6.0, 12.0, 24.0, 48.0 and 72.0 h, respectively. The solutions were stored in tightly capped volumetric flasks protected from light. They were then analyzed by HPLC and the chromatograms obtained were analyzed for additional peaks, if any. The R.S.D. (%) for the samples analyzed at different elapsed assay times was found to be < 2%, which proved the stability of the drug in solution state. On treating the solution with 1 N HCl, 0.1 N HCl and 0.01 N HCl for 2 h, the area of the peak corresponding to embelin decreased and an additional peak was observed at Rt 8.9
min (Fig. 2-A), indicating that embelin undergoes degradation under acidic conditions. However the drug was found to be stable in alkaline medium. On treating the solution with 1 N NaOH, 0.1 N NaOH and 0.01 N NaOH for 2 h, the area of the drug peaks shows decrease in concentration when treated with 1N NaOH, however no significant decrease was observed when treated with lower concentrations of alkali.

Based on the outcome of stress degradation studies of Embelin that it condenses with primary alcohol to yield corresponding ether, a homologous series of ether were prepared to increase the lipophilicity of molecule and to increase the chemical space. Different salts of bromoembelin were also prepared as colourants. Attempts were made to prepare alkyl ethers using glacial acetic acid, hydrochloric acid and sulphuric acid. Of these best results were obtained using sulphuric acid which shows more than 26% efficiency of conversion as compare to Hydrochloric and glacial acetic acid which show 16 and 12% conversion to alkyl ether.

**4.3.1.7. Chemical Modification of Embelin**

Embelin is known to possess a number of diverse activities such as chemopreventive effect against DENA/PB -induced hepatocarcinogenesis in Wistar rats, anti-fertility effects, wound healing, antibacterial, free radical scavenging and *in vitro* cytotoxic activity against B16 and XC cell lines. From these reports we attempted to synthesize more derivatives of Embelin in attempt to increase the chemical space. Different derivatives of Embelib were synthesized which include its ether analogues and brom-salts. The brom salts of Embelin were prepared with different bases which were intensely coloured and hence studied for their UV absorbtion properties.

- Preparation of Ether derivatives of Embelin.
- Preparation of 6-bromoembelin.
- Preparation of salts of 6-bromoembelin.

**General experimental procedure for synthesis of ethers of Embelin**
Figure 4.13. Reaction scheme for preparation of ethers of Embelin

<table>
<thead>
<tr>
<th>Sr.no.</th>
<th>R</th>
<th>Sr no.</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-CH₃</td>
<td>6</td>
<td>-C₈H₁₇</td>
</tr>
<tr>
<td>2</td>
<td>-C₃H₅</td>
<td>7</td>
<td>-C₁₀H₂₁</td>
</tr>
<tr>
<td>3</td>
<td>-C₃H₇</td>
<td>8</td>
<td>-C₂H₅OC₂H₅</td>
</tr>
<tr>
<td>4</td>
<td>-C₄H₉</td>
<td>9</td>
<td>-CH₂C₆H₅</td>
</tr>
<tr>
<td>5</td>
<td>-C₆H₁₃</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

General experimental procedure for synthesis of ethers of Embelin

To a solution of embelin (294 mg, 0.01 mM) in chloroform was added 1ml of sulphuric acid and the resulting solution was heated to 60° C and stirred for 15 min. Then alcohols (0.02 mM) were added at 60° C. Embelin was etherified with methanol, ethanol, propanol, butanol, hexanol, octanol, decanol and benzyl alcohol to yield corresponding ether derivative. The reaction mixture was stirred for 8-10 hr and the progress of the reaction was monitored by TLC using 30 % ethyl acetate in n- hexane. After completion of reaction the reaction was quenched using cold distilled water and the reaction mixture was extracted with chloroform. The combined organic layers were washed with distilled water and dried over anhydrous sodium sulphate and concentrated to obtain crude products. The crude product was purified by column chromatography using silica gel (60-120 mesh) using Petroleum ether (60-80°C): Ethylacetate (95:5 to 70:30) to give pure compounds in 25-42% yields.
Spectroscopic Studies of Ethers of Embelin

1) 5-hydroxyl-2-methoxy-3-undecyl-1,4-benzoquinone (Methyl ether of embelin)

![Chemical structure of methylether of Embelin](image)

Figure 4.14. Chemical structure of methylether of Embelin

Yield 35 %; yellow amorphous solid; m.p.65° C; IR (cm⁻¹): 3353 (Ar-OH), 2920 (Ar-H), 2852 (C-H),1638,1599 (C=O), 1205 (-OCH₃);¹H NMR (400 MHz, CDCl₃) δ 0.5 (t, 3H, R-CH₃), 1.25 (br, s, 20H, -CH₂), 3.86 (s, 3H, O-CH₃), 7.23(d, 1H, Ar-H);¹³C NMR( MHz, CDCl₃) 14(R-CH₃), 22-31(10X-CH₂), 56.5(-OCH₃), 102(CAr-H), 119(CAr-R), 151(CAr-O-H),161(CAr-O-R), 181, 182 (2 X CAr=O); MS [M+1] m/z 309.
Figure 4.15. Mass Spectra of methylether of Embelin

Figure 4.16. $^1$H- NMR spectra of methylether of Embelin
Figure 4.17. IR spectra of methylether of Embelin

Figure 4.18. UV spectra of methylether of Embelin
2) 2-ethoxy-5-hydroxyl-3-undecyl-1,4-benzoquinone (Ethyl ether of embelin)

![Chemical structure of ethylether of Embelin](image)

Figure 4.19. Chemical structure of ethylether of Embelin

Yield 32%; orange amorphous solid; m.p. 74°C; IR (cm\(^{-1}\)) : 3348 (Ar-OH), 2916 (Ar-H), 2849 (C-H), 1631, 1606 (C=O), 1208 (\(-\text{OC}_2\text{H}_5\)), \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 0.57 (t, 3H, R-CH\(_3\)), 1.25 (br, s, 20H, -CH\(_2\)), 2.4 (t, 3H -OCH\(_2\)CH\(_3\)) 4.0 (q, 2H, O-CH\(_2\)), 5.8 (s, 1H, -OH), 7.06 (s, 1H, Ar-H); \(^13\)C NMR (MHz, CDCl\(_3\)) 13.7(R-CH\(_3\)), 22.6(-OCH\(_2\)-CH\(_3\)), 22-31(10 X -CH\(_2\)), 65 (-OCH\(_2\)-)102(C\(_\text{Ar}\)-H), 119(C\(_\text{Ar}\)-R), 151(C\(_\text{Ar}\)-O-H), 160(C\(_\text{Ar}\)-O-R), 182, 181 (2 X C\(_\text{Ar}\)=O); MS [M+1] \textit{m/z} 323.
Figure 4.20. Mass spectra of ethylether of Embelin

Figure 4.21. $^1$H-NMR Spectra of Ethylether of Embelin

Figure 4.22 $^{13}$C- NMR Spectra of Ethylether of Embelin
Figure 4.23. IR Spectra of Ethylether of Embelin

3] 5-hydroxyl-2-propanyl-3-undecyl-1, 4-benzoquinone (Propyl ether of embelin)

Yield 41 %; orange amorphous solid; m.p. 77 °C; IR (cm⁻¹): 3345 (Ar-OH), 2922 (Ar-H), 2845 (C-H),1643,1604 (C=O), 1205 (-OC₃H₇) ;¹H NMR (400 MHz, CDCl₃) δ 0.57 (t, 3H, R-CH₃), 0.9 (t, 3H –OCH₂CH₂CH₃),1.2 (br, 20H, -CH₂), 1.63 (m, 2H, –OCH₂CH₂-), 4.0 (q, 2H, O-CH₂), 5.8(s, 1H, -OH), 7.06( s, 1H, Ar-H); ¹³C NMR ( MHz, CDCl₃) 13.7(R-CH₃), 22.9(-OCH₂-CH₂-), 19.2(-OCH₂-CH₂CH₃), 21.6-34.0(10X -CH₂), 65(-OCH₂-),
101.9(C_{Ar-H}), 118.7(C_{Ar-R}), 151(C_{Ar-O-H}), 160(C_{Ar-O-R}), 182.5, 181.3 (2 \times C_{Ar=O}); MS [M+1]^+ m/z 337

Figure 4.25. Mass Spectra of Propylether of Embelin

Figure 4.26 ^1_H-NMR Spectra of Propylether of Embelin
Figure 4.27. $^{13}$C-NMR Spectra of Propylether of Embelin

Figure 4.28. IR Spectra of Propylether of Embelin
4) **5-hydroxy-2-butoxy-3-undecyl-1, 4-benzoquinone** (Butyl ether of embelin)

![Chemical structure of butyl ether of Embelin](image)

**Figure 4.29. Chemical structure of butyl ether of Embelin**

Yield 35%; orange amorphous solid; m.p.83 °C; IR (cm⁻¹): 3442 (Ar-OH), 2922 (Ar-H), 2835 (C-H),1734, 1648,(C=O), 1239 (-OC₂H₅), ¹H NMR (400 MHz, CDCl₃) δ 0.89 (t, 3H, R-CH₃), 0.97(t, 3H,-O-R-CH₃), 1.25 (br, s, 20H, -CH₂), 1.35(m, 2H, -O- CH₂-CH₂), 4.0 (q, 2H, O-CH₂), 5.8(s, 1H,-OH), 7.2( s, 1H, Ar-H); ¹³C NMR( MHz, CDCl₃) 13.7(R-CH₃), 17.8(-O-R-CH₃), 19.1 (-O-C₂H₄-CH₂-), 22-31(10 X -CH₂), 34.3(-OCH₂-CH₂-), 61 (-OCH₂-), 102(C₆H₅),116(C₆H₅-R), 150(C₆H₅-O-H), 159(C₆H₅-O-R), 182.7,181.5 (2 X C₆H₅=O); MS [M+1] m/z 349.
Figure 4.30. Mass Spectra of Butylether of Embelin

Figure 4.31 $^1$H-NMR Spectra of Butylether of Embelin

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Figure 4.32. $^{13}$C-NMR of Butylether of Embelin

Figure 4.33. IR Spectra of Butylether of Embelin
5] 5-hydroxyl-2-hexyloxy-3-undecyl-1, 4-benzoquinone (Hexyl ether of embelin)

![Chemical structure of Hexylether of Embelin]

Figure 4.34. Chemical structure of Hexylether of Embelin

Yield 37 %; red amorphous solid; m.p.114° C; IR(cm\(^{-1}\)): 3319 (Ar-OH), 2921 (Ar-H), 2852 (C-H), 1762, 1720, (C=O), 1200 (-OC\(_6\)H\(_{13}\)) \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 0.87 (t, 3H, R-CH\(_3\)), 0.89 (t, 3H, O-R-CH\(_3\)), 1.25 (br, s, 26H, -CH\(_2\)-), 1.6 (m, 2H, -O-CH\(_2\)-CH\(_2\)-), 3.98 (q, 2H, O-CH\(_2\)), 6.0 (s, 1H, -OH), 7.26 (s, 1H, Ar-H); \(^{13}\)C NMR (MHz, CDCl\(_3\)) 14.2 (R-CH\(_3\)), 14.2 (O-R-CH\(_3\)), 21.9 (-O-C\(_4\)H\(_8\)-CH\(_2\)-), 22-31 (12 X -CH\(_2\)), 32.3 (-OC\(_3\)H\(_7\)-CH\(_2\)-), 63 (-OCH\(_2\)-), 102 (C\(_{Ar-H}\)), 116 (C\(_{Ar-R}\)), 152 (C\(_{Ar-O-H}\)), 161 (C\(_{Ar-O-R}\)), 182.7, 181.5 (2 X C\(_{Ar=O}\)); MS [M+1] \(m/z\) 377.

![Mass Spectra of Hexylether of Embelin]

Figure 4.35 Mass Spectra of Hexylether of Embelin

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Figure 4.36. $^1$H-NMR Spectra of Hexylether of Embelin

Figure 4.37. $^{13}$C-NMR Spectra of Hexylether of Embelin
Figure 4.38. IR Spectra of Hexylether of Embelin

6] 5-hydroxyl-2-octyloxy-3-undecyl-1,4-benzoquinone (Octyl ether of embelin)

![IR Spectra of Hexylether of Embelin](image)

Yield 39 %; pink amorphous solid; m.p.120° C; IR(cm⁻¹): 3391 (Ar-OH), 2922 (Ar-H), 2852 (C-H), 1743, 1690, (C=O), 1215 (-OC₈H₁₇); ¹H NMR (400 MHz, CDCl₃); δ 0.87 (t, 3H, R-CH₃), 0.89(t, 3H, O-R-CH₃), 1.25 (br, s, 26H, -CH₂-), 1.6(m, 2H, -CH₂-CH₂-), 3.98 (q, 2H, O-CH₂), 6.0(s, 1H, -OH), 7.26( s, 1H, Ar-H); ¹³C NMR( MHz, CDCl₃) 14.2(R-CH₃), 14.2(-O-R-CH₃), 21.9 (-O-C₄H₉-CH₂-), 22-31(12 X -CH₂), 32.3(-OC₃H₇-)

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CH₂), 63 (-OCH₂), 103(C₆H₅-H), 116(C₆H₅-R), 154 (C₆H₅-O-H), 161(C₆H₅-O-R), 189.7, 180.5 (2 X C₆H₅=O); MS [M⁺] m/z 408.

Figure 4.40. Mass Spectra of Octylether of Embelin

Figure 4.41. ¹H-NMR of Octylether of Embelin
Figure 4.42. $^{13}\text{C}$-NMR of Octylether of Embelin

Figure 4.43. IR Spectra of Octylether of Embelin
7) 2-decyloxy-5-hydroxyl-3-undecyl-1,4-benzoquinone (Decyl ether of embelin)

![Chemical structure of decylether of Embelin]

Figure 4.44. Chemical structure of decylether of Embelin

Yield 37 %; red amorphous solid; m.p.125° C; IR (cm\(^{-1}\)): 3339.6 (Ar-OH), 2925 (Ar-H), 2854 (C-H), 1743, 1726 (C=O), 1239 (-OC\(_{10}\)H\(_{22}\)); \(^1\)H NMR (400 MHz, CDCl\(_3\)); \(\delta\): 0.88 (t, 6H, R-CH\(_3\)), 1.25 (br, s, 26H, -CH\(_2\)), 1.3 (br, s, 6H, -CH\(_2\)), 1.56 (m, 2H, -O-CH\(_2\)-CH\(_2\)-), 3.6 (q, 2H, O-CH\(_2\)), 5.3 (s, 1H, -OH), 7.2 (s, 1H, Ar-H); \(^{13}\)C NMR (MHz, CDCl\(_3\)) 14.2 (R-CH\(_3\)), 14.2 (-O-R-CH\(_3\)), 22.7 (-R-CH\(_2\)-), 25.8 (-O-C\(_8\)H\(_{16}\)-CH\(_2\)-), 29.3-29.7 (15 X -CH\(_2\)-), 31.9 (-OCH\(_2\)-CH\(_2\)-), 63 (-OCH\(_2\)-), 102 (C\(_A\)-H), 116 (C\(_A\)-R), 152 (C\(_A\)-O-H), 160 (C\(_A\)-O-R), 183.

![Mass Spectra of Decylether of Embelin]

Figure 4.45. Mass Spectra of Decylether of Embelin
Figure 4.46. $^1$H-NMR of Decylether of Embelin

Figure 4.47. $^{13}$C-NMR of Decylether of Embelin
Figure 4.48. IR Spectra of Decylether of Embelin

8] 2-ethoxyethoxy-5-hydroxyl-3-undecyl-1,4-benzoquinone (Ethoxyethyl ether of embelin)

![Chemical structure of ethoxyethyl ether of Embelin](image)

Yield 40%; brown amorphous solid; m.p. 70 °C; IR(cm⁻¹): 3310.2 (Ar-OH), 2924 (Ar-H), 2853 (C-H), 1743, 1613, (C=O), 1333 (-OC₂H₄OC₂H₃); ¹H NMR (400 MHz, CDCl₃) δ 0.87 (t, 3H, R-CH₃), 1.25-1.3 (br, s, 20H, -CH₂-), 3.41(q, 2H, -O-CH₂-), 3.72 Q, 2H, -O-CH₂-CH₂-, 3.98 (q, 2H, O-CH₂), 6.0(s, 1H,-OH), 7.26(s, 1H, Ar-H); ¹³C NMR(MHz, CDCl₃); 14.2(R-CH₃), 14.2(-O-R-CH₃), 20-31(10X-CH₂), 69(-CH₂O-), 70(-OCH₂CH₃), 102(C₆-H), 116(C₆-R), 130(C₆-O-H), 131(C₆-O-R), 172.7, 171.5 (2X C₆=O); MS [M-1] m/z 365.

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Figure 4.50. Mass Spectra of Ethoxyethylether of Embelin

Figure 4.51 $^1$H-NMR of Ethoxyethylether of Embelin
Figure 4.52 $^{13}$C-NMR of Ethoxyethylether of Embelin

Figure 4.53. IR Spectra of Ethoxyethylether of Embelin
Preparation of 6-bromoembelin

![Chemical structure](image)

**Figure 4.54. Reaction scheme for preparation of Bromoembelin**

5 g of embelin was dissolved in 400 ml of chloroform in presence of 3g N-bromosuccinamide and 1.4g of benzoyl peroxide catalyst with aid of stirring. The reaction mixture was kept exposed to a 100 W tungsten lamp under reflux conditions for 3 h. The mixture was then allowed to cool & concentrated to obtain the product 6-bromoembelin.

**Figure 4.55. Mass Spectra of 6-Bromoembelin**
Figure 4.56. $^1$H-NMR of 6-Bromoembelin

Figure 4. 57. $^{13}$C-NMR of 6-Bromoembelin
Figure 4. 58. IR Spectra of 6-Bromoembelin
Salts of 6-bromoembelin

Salts of 6-bromoembelin with different bases were obtained by treating 1 mole of bromoembelin with 2 moles of base under stirring and reflux conditions; filtered, concentrated and washings with water.

Table 4.2. UV-Vis Spectroscopic properties of salts of 6-bromoembelin

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{max}}$ in Methanol</th>
<th>$\lambda_{\text{max}}$ in Chloroform</th>
<th>$\lambda_{\text{max}}$ in Glacial acetic acid</th>
<th>$\lambda_{\text{max}}$ in Pyridine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embelin</td>
<td>290</td>
<td>416</td>
<td>400,416</td>
<td>442</td>
</tr>
<tr>
<td>Bromoembelin</td>
<td>496, 304</td>
<td>426</td>
<td>400,422</td>
<td>500</td>
</tr>
<tr>
<td>2- methyl imidazole salt</td>
<td>510, 304</td>
<td>516 (slightly soluble)</td>
<td></td>
<td>500, 508</td>
</tr>
<tr>
<td>Benzimidazole salt</td>
<td>512, 304</td>
<td>476</td>
<td>400</td>
<td>500, 510</td>
</tr>
<tr>
<td>Benzotriazole salt</td>
<td>510, 304</td>
<td>-(slightly soluble)</td>
<td>400,422</td>
<td>514</td>
</tr>
<tr>
<td>Imidazole salt</td>
<td>518, 306</td>
<td>482-(slightly soluble)</td>
<td>398</td>
<td>508</td>
</tr>
<tr>
<td>Indole salt</td>
<td>512, 306</td>
<td>524</td>
<td>400</td>
<td>510</td>
</tr>
<tr>
<td>Piperidine salt</td>
<td>514, 306</td>
<td>516</td>
<td>406</td>
<td>516</td>
</tr>
<tr>
<td>Triazole salt</td>
<td>512, 304</td>
<td>(slightly soluble)</td>
<td></td>
<td>500, 508</td>
</tr>
</tbody>
</table>
4.3.2. Thymoquinone

4.3.2.1. Preparation of Thymoquinone from Thymol

Preparation of Thymoquinone from Thymol was carried out as per the procedure described by Kremers et al., 1941.
Figure 4.59. Reaction scheme for preparation of Thymoquinone from Thymol

1. Procedure
Step I: Preparation of Nitrosothymol from Thymol:
To a solution of 5 g. (0.03 mole) of thymol in 25 ml. of 95 per cent ethyl alcohol is added 25 ml of concentrated hydrochloric acid. This mixture is cooled to 0° in a 250 ml beaker set in an ice-salt bath, and to it is added 3.6 g. (0.05 mole) of commercial sodium nitrite in portions of about 200 mg. each. The mixture is stirred well after each addition. The solution first becomes brown in color, and a green precipitate soon begins to form. After 0.17 g. of nitrite has been added, the mixture becomes pasty; the intervals between the additions must now be lengthened and the stirring made more vigorous. When all has been added, the bulk of the product is transferred to a 500 ml. flask containing 400 ml. of cold water, and the remainder washed in with water. The product, after agitation with water, is now a light-yellow, fluffy solid; it is filtered off by suction and washed well with water.

**Step II: Preparation of Aminothymol from Nitrosothymol**

The crude, wet nitrosothymol so obtained is worked up with a mixture of 4 ml of 28 per cent aqueous ammonia and 16 ml of water; the brown solution is filtered free of a little resinous matter, and hydrogen sulfide is passed into it. The brown color disappears and a white precipitate of aminothymol forms. The passage of hydrogen sulfide is continued for thirty minutes longer, when the base is filtered and washed well with cold water, contact with air being avoided as far as possible.

**Step III: Preparation of Thymoquinone from Aminothymol**

The wet aminothymol thus prepared is immediately dissolved in 5 ml of concentrated sulfuric acid diluted to 200 ml. and contained in a 1 litre flask. To this solution is added 7.5 g. (0.1 moles) of sodium nitrite in 0.5-1 g. portions, with shaking after each addition. The resulting mixture is heated to 60° on a steam bath, with occasional shaking, for 15 mins, and is then distilled in a current of steam, using Dean –Starks Apparatus. All the thymoquinone passes over with the first 3 l. of distillate; it solidifies on cooling, and is filtered with suction, washed, and dried at room temperature. The yield is 1-1.2 g of bright yellow crystals, melting at 43–45°.

**Spectroscopic studies of Thymoquinone**
Figure 4.60. IR Spectra of Nitrosothymol

Figure 4.61. IR Spectra of Aminothymol
Figure 4.62. IR Spectra of Thymoquinone synthesized

Figure 4.63. IR Spectra of Thymoquinone standard (Sigma-Aldrich)
4.3.2.2. HPLC of Thymoquinone

Standard solution

Take 10 mg of Thymoquinone in methanol in 10-ml volumetric flask and make up the volume. 1 ml of above solution was taken and diluted up to 10 ml (Stock solution 100 µg/ml). From the stock solution prepare standard solutions (10 µg/ml) by transferring aliquots 1 ml of stock solution to 10-ml volumetric flasks and adjust the volume to 10 ml with methanol.

Chromatographic conditions

Instrument : JASCO PU-1580
Column : Purospher, RP-18e, 250 x 4.6, 5 µ
Mobile phase : Methanol: 2-propanol: Water (45: 5: 50)
Flow rate : 1.7 ml/min
Detector : UV Detector (JASCO UV-1575) at 254 nm
Injection volume : 20 µl
4.3.2.3. HPTLC Analysis of Thymoquinone:

**Preparation of extract:** 2 g of seed powder was extracted with methanol (10 ml) by shaking for 5 minutes. Allow standing, methanol layer was concentrated upto 2 ml under vaccum. 10 µl was used for TLC.

**Preparation of standard:** Thymoquinone prepared as a 0.01% of methanolic solution, 5 µl used for TLC

**Chromatographic solvent:** Toluene: Petroleum ether (8:2)

**Detection:** UV-254

Visible (Without chemical treatment)

A. UV -254 nm

1. Isolated compound
2. *N. Sativa* seed extract

Figure 4.66: TLC profile of Thymoquinone and *N.sativa* methanolic extract.
4.3.2.4. Extraction of Thymoquinone from *N. sativa* using Microwave Assisted Extraction and its Comparison with conventional methods

![Image of Nigella sativa seeds and flowering twig](image)

**Figure 4.67. The seeds (A) and flowering twig (B) of *Nigella sativa* L.**

In the present study, a microwave-assisted extraction (MAE) technique was developed for the extraction of Thymoquinone from *Nigella sativa* seeds. Various experimental conditions, such as extraction time, use of different solvents, liquid/solid ratios, pre-leaching/prior irradiation time before MAE was investigated to optimize the efficiency of the extraction. In the present work, emphasis is on rapid extraction of Thymoquinone using microwave exposure of dry seed, microwave exposure of solvent-soaked seeds and also extraction of Thymoquinone from seeds in a microwave reactor (MR) under controlled conditions. The aim of this work is to investigate the feasibility of employing MAE as an efficient extraction technique to extract Thymoquinone from *N. sativa* seeds. To the best of our knowledge, no such MAE application on Thymoquinone extraction from *N. sativa* is available in literature. The suitability of microwave assistance for the extraction of Thymoquinone from *N. sativa* seeds was investigated in the connect work for several reasons: (1) to reduce extraction time; (2) to reduce solvent usage and (3) to improve extraction efficiency (4) to decrease losses due to volatility and degradation due to heating. In the present work, extraction efficiency was defined as follows:

\[
\text{Percentage extraction w/w} = \frac{\text{Mass of Thymoquinone (inextracted solution)}}{\text{Mass of material (Nigella seeds)}} \times 100\%
\]
Dried seeds of *N. sativa* obtained from local market were pulverized and the coarse powder was separated by sieving. Ethanol was used as an extraction solvent for all experiments with exceptions stated otherwise.

**Soxhlet extraction**

The objective of the soxhlet extraction was to determine the maximum recoverable content of the solute in the raw material. Powdered Nigella seeds (2 g) were introduced in soxhlet apparatus with 60mL of ethanol and extracted for 24 h.

**Ultra sound assisted extraction**

2 g of Nigella powder was introduced in a 100 ml flask containing 40 ml ethanol and subjected to sonication in ultra sound bath for a period of 30 minutes. Aliquots were drawn at 5, 10, 20 and 30 mins respectively and quantity of Thymoquinone extracted was analyzed using HPLC.

**Heat reflux extraction**

2 g of Nigella powder was introduced in a 100 ml round bottom flask and subjected to heat reflux in 40 ml of ethanol for period of 2 h.

**Extraction at room temperature (ERT)**

2 g of Nigella powder was added 40 ml of ethanol stirred at ambient temperature at 800 rpm using a magnetic stirrer and extracted for 5, 20, 40, 60, 120, 240 min and 24 h.

**Microwave assisted extraction**

The extraction was conducted by three methods in the first method, to study the effect of microwave and the solvent, the extraction of Thymoquinone was carried out in a Microwave extractor (Model: CATA–R, M/s Catalysts Systems, Pune, India) A 100 ml round bottom flask containing drug material was exposed to the microwave radiation. The flask was connected with an overhead condenser. Temperature measurements were made by an external temperature probe. The power of microwave reactor was controlled in the range of 0–500 W. The extraction of Thymoquinone was conducted by adding 1 g of ground dried seeds in 40mL of solvent (ethanol), in the vessel. The vessel was placed symmetrically in the microwave field under agitation condition (600 rpm). The liquid samples were withdrawn after pre-specified time and analyzed for Thymoquinone content by the HPLC method. Experiments were carried out to determine the effect of irradiation.
time (0–4 min), power input (140, 250, 350 and 450W) and solid loading (1:40, 2:40 g of raw material/mL of solvent) on microwave-assisted extraction efficiency.

In the second method; irradiation of dry seeds of Nigella was done by placing the seeds as a single uniform layer of thickness of about 3 mm in a dish of 10cm diameter in the microwave cavity. After irradiation for a pre-specified time, the seeds were suspended in ethanol in a 100 ml round bottom flask, fitted with a magnetic stirrer. The solid loading of 1:40 (g of material/mL of solvent) was kept constant for all the runs. The stirring speed in the solvent extraction was kept constant at 600 rpm to eliminate the effect of external mass transfer. The samples were collected at specified time intervals and analyzed for Thymoquinone content by the HPLC method.

In the third method, dry seed of Nigella was soaked in water for fixed time and then solvent is removed and wet seeds were exposed to microwave. After irradiation for pre-specified time, the seed material was taken for solvent extraction, keeping all parameters constant as mentioned in the first technique.

The percent extraction was based on Thymoquinone present in the feed material, since the amount of Thymoquinone in the raw material was found to vary with different sources. The total Thymoquinone content in the raw seed material was determined separately by soxhlet extraction with methanol as a solvent for 24 h. The percent Thymoquinone in the seed material was determined to be 0.09% w/w of the seed on dry weight basis.

**High performance liquid chromatographic analysis**

Chromatographic separation of Thymoquinone separation was achieved on a Purospher star-ODS column (250mm x4.6 mm MERCK) column and eluted with CH₃OH–Propanol-H₂O (45:5:50 v/v) at a flow rate of 1.7ml min⁻¹ at detection wavelength of 254 nm (Ghosheh *et al.*, 1999) In the present work, the suspensions following MAE and other conventional extraction method were centrifuged and filtered with a membrane filter (0.5 µm) and then analyzed. Retention time of Thymoquinone was about 14.6 min. The chromatogram obtained showed that Thymoquinone was completely resolved from other compounds, with good linearity ranging from 0.010 to 0.100 mg of Thymoquinone. The method adopted was sensitive and accurate with good reproducibility, the relative standard deviation (RSD) was 1.55%. The analytical operation was completed in 20 min.
Effect of extraction time on percentage extraction of Thymoquinone

The effect of extraction time on percentage extraction by MAE and ERT is indicated in Fig. 4.68. The results indicate that extraction yield increases with an increase in extraction time initially, after which a steady decline in content of Thymoquinone was observed. This property can be attributed to volatility of Thymoquinone at higher temperatures. A maximum Thymoquinone content was observed at an extraction time of 5 min when compared to ERT which shows a gradual rise in the content of the analyte up to 24 h.

Effect of solvent on percentage extraction of Thymoquinone

The percentage extraction of Thymoquinone was greatly influenced by the polarity and dielectric constant of the solvent used. Amongst the different solvents employed viz. methanol, ethanol, acetone, ethylacetate and chloroform, the percentage extraction was found to be highest when ethanol was used as extraction media (Fig. 4.69).

Effect of liquid/solid ratio and pre-leaching time before MAE on extraction

The percentage extraction of Thymoquinone increased with an increase in liquid/solid ratio. Since additional energy and time are needed to treat the leaching solution at high ratios, a liquid/solid ratio (ml/g) 40:1 was found to be optimal. Pre-leaching or pre-soaking at room temperature before MAE or irradiation of dry Nigella powder was found to cause a slight deficit in content of quinone, as prior irradiation of dry seed caused a loss of the volatile active.

Figure 4.68 Effect of microwave irradiation (Power) on extraction of Thymoquinone from dry seeds of Nigella sativa

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Figure 4.69 Comparison of different solvents as extraction media in Microwave assisted extraction of Thymoquinone from seed of *Nigella sativa* at 140W and 800 rpm.

Figure 4.70. Comparison between conventional solvent extraction, Ultrasound assisted extraction and Microwave assisted extraction of Thymoquinone from seed of *Nigella sativa* at 33°C, 800 rpm using ethanol as solvent.
4.4. Studies on Naphthoquinones

4.4.1 Plumbagin

- Extraction and Isolation
- Development of HPLC-Fluorimetric analytical method
- Chemical Modification
  - Preparation of Droserone.
  - Preparation of Alkyl derivatives of Plumbagin

4.4.2. Shikalkin

- Extraction and Isolation
- HPTLC & HPLC studies
4.4.1. Plumbagin

4.4.1.1. Extraction and isolation of Plumbagin from *Plumbago zeylanica* roots

**Chemical test for Plumbagin:**

*Bontrager’s test for Plumbagin:* Take 1g of *P. zeylanica root* powder and extract in 5 ml of chloroform. Reduce the chloroform layer to 1 ml. Add about 1 ml of 5% potassium hydroxide solution to this tube. The aqueous layer assumes pink colour.

**Extraction technique:**

Hydrodistillation (2 hr)

To 50 g of powdered Plumbago roots, 200 ml of distilled water was added and subjected to hydrodistillation for 1.5 h using Clavenger apparatus. About 5 ml of petroleum ether (60-80°C) was added in receiver along with distilled water. After 1.5 h the receiver was drained, the aqueous and organic layer separated. The petroleum ether layer was then partitioned with 2% aqueous potassium hydroxide until the aqueous layer ceased to show the characteristic pink colour. The aqueous layer was acidified with hydrochloric acid, and the precipitate taken up in petroleum ether. The petroleum ether layer on drying yielded about 10 mg orange yellow needles of plumbagin.

**Recrystallization:**
Crude plumbagin (200 mg) obtained from above procedure, dissolved with boiling in 20 ml of light petroleum ether. Filter immediately using vacuum filtration and filtrate allow standing for 20-30 minutes gives bright yellow needles of plumbagin.

**Figure 4.72. P. zeylanica roots (A) and Isolated Plumbagin Crystals (B)**

**Isolation of Plumbagin using column chromatography**

Powdered dried roots of *P. zeylanica* 3 kg were subjected to Soxhletion using chloroform. The chloroform fraction thus obtained was concentrated, loaded on silica and subjected to column chromatography using light petroleum ether: ethyl acetate as eluant. 5% fraction of ethyl acetate in light petroleum ether yielded yellow needles of about 1.08 g Plumbagin.

**4.4.1.2. Characterization of Plumbagin**

![Chemical structure of Plumbagin](image)

**Figure 4.73. Chemical structure of Plumbagin**

Thin layer chromatography was performed on pre-coated silica gel G60 F$_{254}$ (E. Merck) using Petroleum ether (60-80°C): Ethyl acetate:: 9:1 as mobile phase, a single band was
seen at \( R_f \) 0.83 which gave a pink colour with Borntrager’s reagent (5% alcoholic potassium hydroxide). Infra red (IR) spectrum of the isolated compound showed a broad peak at 3034 cm\(^{-1}\) (hydroxyl) and 1604, 1641 cm\(^{-1}\) (carbonyl). Molecular ion peak at 188 \( m/e \) gave the molecular weight of the compound. The UV/Vis maxima in methanol were found to be at 214, 264, 340, 400 and 417 nm. The \( ^1\)H NMR (400 MHz, CDCl\(_3\)) shows \( \delta \) 2.1 (s, 3H, R-CH\(_3\)), 5.3 (s, 1H, Ar-OH), 7.2(m, 3H, Ar-H), 7.5(s, 1H, Ar-H); \( ^{13}\)C NMR (MHz, CDCl\(_3\)) 16.4 (C\(_{11}\)), 129 (C\(_{9}\)), 133 (C\(_{10}\)), 119 (C\(_{8}\)), 124 (C\(_{6}\)), 132 (C\(_{3}\)), 136 (C\(_{7}\)), 150 (C\(_{2}\)), 161 (C\(_{5}\)), 184 (C\(_{1}\)), 190 (C\(_{4}\)). The isolated compound melts at 76°C. Plumbagin showed 98% purity by HPLC.

![Figure 4.74. Mass Spectra of Plumbagin](image-url)
Figure 4.75 $^1$H-NMR of Plumbagin

Figure 4.76 $^{13}$C-NMR of Plumbagin
Figure 4.7. IR Spectra of Plumbagin

Figure 4.78 UV-Vis Spectra of Plumbagin.
4.4.1.3. HPTLC studies of Plumbagin

**Preparation of extract:** 2 g of *P. zeylanica* root powder was extracted with methanol (10 ml) by shaking for 5 minutes. Allow standing, methanol layer was separated and concentrated up to 1 ml under vacuum. 10 µl was used for TLC.

**Preparation of standard:** Plumbagin prepared as a 0.01% of Methanolic solution, 5 µl used for TLC

**Chromatographic solvent:** Toluene: Formic acid (99:1)

**Detection:** Alcoholic 5% Potassium hydroxide solution.

A. Visible (Without chemical treatment)
B. 5% Alcoholic KOH solution
C. UV -254 nm
   1. Isolated compound
   2. *P. zeylanica* root extract

![Figure 4.79. TLC Profile of Plumbagin and *P. zeylanica* roots](image)
4.4.1.4. HPLC studies of Plumbagin

**Standard solution**

Take 10 mg of plumbagin in methanol in 10-ml volumetric flask and make up the volume. 1 ml of above solution was taken and diluted up to 10 ml (Stock solution 100 µg/ ml). From the stock solution prepare standard solutions (10 µg/ ml) by transferring aliquots 1ml of stock solution to 10-ml volumetric flasks and adjust the volume to 10 ml with methanol.

**Chromatographic conditions**

<table>
<thead>
<tr>
<th>Instrument</th>
<th>JASCO PU-1580</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Purospher STAR, RP-18e, 250 x 4.6, 5 µ</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Methanol: Water (0.1% v/v of o- phosphoric acid) (85:15)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Detector</td>
<td>UV Detector (JASCO UV-1575) at 264nm</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 µl</td>
</tr>
<tr>
<td>Run time</td>
<td>25 min.</td>
</tr>
</tbody>
</table>

![Figure 4. 80. HPLC chromatogram of Plumbagin](image_url)
4.4.1.5. *Plumbago auriculata*

Alternative source for Plumbagin is *P. auriculata* Lam.

*Plumbago auriculata* Lam. Syn. *Plumbago capensis* Thunb. (Fig 4.81) is a subscandent shrub with oblong or oblong spatulate leaves and pale blue flowers, indigenous to South Africa and grown in gardens of India as an ornamental plant. It bears throughout the year a profusion of umbel-like clusters of pale azure-blue very pleasing flowers. The roots of this plant are reported to contain Plumbagin. A decoction of plant is taken as remedy for blackwater fever. The root is used as styptic in scrofula; an infusion of it is considered emetic. The powdered root is taken as a snuff to relieve headache. It causes warts to disappear when smeared over them. (Wealth of India, 2005)
4.4.1.6. Development of HPLC method using fluorescence detection for Analysis of Plumbagin

A simple, sensitive HPLC fluorescence method for estimation of Plumbagin in *P. zeylanica*, *P. auriculata* and five different polyherbal formulations containing the same was developed. The HPLC analysis was performed with a JASCO (Hachioji, Tokyo, Japan) system, an intelligent pump (PU-1580, PU-2080), a high-pressure mixer (MX-2080-31), a manual sample injection valve (Rheodyne 7725i), with a flow rate of 1.00 ml/min, Injection volume loop: 20 μl. A 250 mm × 4.6 mm i.d., RP-18 (5-μm particle size) Hibar LiChrocart Purospher star RP-18 endcapped column (Merck, Darmstadt, Germany) was used for analysis. The mobile phase was isocratic using 65:35 of Methanol: 0.1% v/v ortho-phosphoric acid in deionized water (pH 3.2). The mobile phase was degassed using sonication and used at a flow-rate of 1.0 ml/min. The run time was 20 min. The HPLC operating pressure was approximately 220 atm and the column temperature was ambient. Component fluorescence detection was achieved using an excitation wavelength of 264 nm and monitoring of the emission wavelength at 605 nm. The fluorescence detector operated at high sensitivity with a 3 sec response time. Fluorescence detection, a fluorescence detector JASCO FP 2020 was used. To compare the sensitivity of the two HPLC detectors for Plumbagin, the UV-Vis detector was connected in series with the fluorescence detector and both detectors were set to a 1 volt full scale output.
Figure 4.82. UV and Fluorescence emission spectra of Plumbagin 80µg/ml at 214, 264 and 405 nm

Standard and control preparation

Standard preparation

A stock standard of Plumbagin (1.0 mg/mL) was prepared in methanol and stored at 4°C. Working standards of Plumbagin were prepared at 1, 2, 4, 6, 8 and 10 µg/mL in methanol. Following preparation, the QC samples were stored frozen at -20°C and demonstrated stability for at least one week.

Sample extraction

Plant material
10 g of *P. zeylanica* root powder and 10 g of *P. auriculata* root powder were subjected to soxhlet extraction using 60 ml of chloroform. The chloroform extract was concentrated, and resuspended in methanol, filtered, diluted to appropriate concentration and 20 µl of it was injected for HPLC analysis.

**Herbal Formulations**

Herbal formulations in form of tablets were powdered and 5 g of each formulation were subjected to soxhlet extraction using chloroform. For herbal formulations in form of capsules, the capsules shells were emptied of its contents and 5 g of this powder mixture was extracted soxhlet assembly using chloroform as extracting solvent. The chloroform extract was concentrated and resuspended in methanol, filtered, diluted to appropriate volume and 20 µl of it was injected for HPLC analysis.

**Validation**

Calibration plots were constructed after triplicate analysis of each calibration solution, by plotting peak area against concentration (µg mL⁻¹) of the corresponding standard solution. To determine the limits of detection (LOD) and quantification (LOQ) standard solutions were further diluted in methanol. LOD and LOQ were defined as the amounts for which signal-to-noise ratios (S/N) were 3 and 10, respectively. Precision was determined as the intra-day and inter-day variation of results from analysis of six different standard solutions. Intra-day precision was determined by triplicate analysis of each solution on a single day. Inter-day precision was determined by triplicate analysis of the solutions on three successive days. To test sample stability, solutions were stored at room temperature and analyzed after 0, 2, 4, 8, 12, 24, and 48 h. The relative standard deviations (RSD) of retention time (Rt) and peak area (Pa) of analyte for both the detectors were calculated as measures of precision, repeatability, and stability. The accuracy of the method was determined by application of the standard addition method. Accurately known amounts of the standards (80, 100, and 120% of the content of 10 g of *P. auriculata* extract) were added to *P. auriculata* extract and then analyzed in duplicate as described above. The total amount of each compound was calculated from the corresponding calibration plot and the recovery of each compound was calculated by use of the equation:

\[
\text{Recovery} \, \% = \frac{\text{amount found} - \text{amount contained}}{\text{amount added}} \times 100
\]
Method Optimizations

A MERCK Purospher star C$_{18}$ endcapped column (25 cm × 4.6 mm ID) was used for estimation, which provided excellent chromatographic resolution. The mobile phase comprising of methanol and 0.1% 0- phosphoric acid in deionized water (pH 3.2), provided good peak shape for Plumbagin. The mobile phase organic modifiers (e.g. acetonitrile and methanol) were evaluated to determine which organic solvent would provide the best chromatographic separation of Plumbagin from other plant constituents. Methanol was chosen as the organic modifier as it provided good peak shape and selectivity from other endogenous compounds. The earlier reported methods for estimation of Plumbagin comprising of UV detection was found to be less sensitive to detect Plumbagin at low ng/ml concentrations without some form of sample concentration step. For this reason, we decided to evaluate fluorescence detection. The fluorescence spectra were studied using 214, 264 and 417 nm as excitation wavelengths for which the spectra obtained are shown in Fig 4.81.

Fig 4.83 HPLC chromatogram of *P. auriculata* root extract using Fluorescence detector
Table 4.3. Concentration and peak area for UV and Fluorescence detector

<table>
<thead>
<tr>
<th>µg.ml⁻¹ of Plumbagin</th>
<th>Peak Area</th>
<th>µg.ml⁻¹ of Plumbagin</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>129338</td>
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<td>315898</td>
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</table>

Figure 4.84 Linearity curves for HPLC using UV (A) and Fluorescence detector (B).

Validation

The calibration plot for Plumbagin was linear in the ranges 1–10 µg mL⁻¹ ($r^2 = 0.997$). The regression equations were $y = 58317x + 76300$. The LOD and LOQ were 0.2 and 0.5 µg respectively (Fig 4.84). Intra-day and inter-day RSD of retention times and peak areas...
were less than 2.1%, showing precision was good. The reproducibility of the method was also good (RSD 2.58%; Table I) and, as shown in Table II, recovery of Plumbagin was in the range 94.5–98.8, with RSD <2.96%, indicating the analysis was accurate.

### Table 4.4. Combined intra and inter-day accuracy and precision for plumbagin.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration (μg/ml)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plumbagin</td>
<td></td>
<td>Rt  Pa</td>
<td>Rt  Pa</td>
</tr>
<tr>
<td>1</td>
<td>0.3 0.9</td>
<td>0.5 1.2</td>
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<td>4</td>
<td>0.5 1.1</td>
<td>0.8 1.9</td>
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</tr>
<tr>
<td>10</td>
<td>0.2 0.7</td>
<td>0.9 1.3</td>
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</table>

### Table 4.5. Recovery studies of plumbagin

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Contains (µg)</th>
<th>Added (µg)</th>
<th>Found (µg)</th>
<th>Percent recovery (%)</th>
<th>Mean (%)</th>
<th>RSD (%)</th>
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<tr>
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<td>384.0</td>
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<td>97.25</td>
<td>98.43</td>
<td>0.93</td>
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<tr>
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<td>480.0</td>
<td>384.0</td>
<td>843.8</td>
<td>97.66</td>
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<tr>
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<td>480.0</td>
<td>480.0</td>
<td>947.2</td>
<td>98.67</td>
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<td></td>
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<tr>
<td></td>
<td>480.0</td>
<td>480.0</td>
<td>952.6</td>
<td>99.22</td>
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<td></td>
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<tr>
<td></td>
<td>480.0</td>
<td>576.0</td>
<td>1036.5</td>
<td>98.15</td>
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</tr>
<tr>
<td></td>
<td>480.0</td>
<td>576.0</td>
<td>1052.1</td>
<td>99.63</td>
<td></td>
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</table>

These results revealed the method enables rapid, precise, and highly accurate for quantification of Plumbagin. When the method was subsequently used for quantification of Plumbagin in *P. zeylanica* and *P. auriculata*, the concentration found were 0.22 and 0.48 % w/w respectively. The Plumbagin content of five herbal formulations containing the same was found to be: Hepatovin 0.009%, *Supachan vati* 0.0085%, *Medhoter guggul* 0.044%, Digestin 0.0002 % and *Chitrakadi bati* contains 0.38% w/w respectively.

#### 4.4.1.7. Preparation of Semisynthetic derivatives of Plumbagin

**Preparation of Droserone from Plumbagin**

Plumbagin and Droserone are naturally occurring antifeedant compounds occurring in carnivorous plants like *Drosera burmanii*, *Dionaea muscipula*, *Triphophyllum peltatum*, *Nephthensis khasiana* etc. Amongst the two naturally occurring quinones, the content of
plumbagin is found to be significantly high in these plants. Droserone occurs in a relatively lesser concentration making it difficult to isolate from plant sources and more expensive when compared to plumbagin. Because of its inavailability it is much less studied when compared to plumbagin. Hence an attempt was therefore made to isolate plumbagin and synthesize droserone from the same. Droserone has been reported to be synthesized from plumbagin using epoxidation of C-2 C-3 double bond and subsequent hydrolysis in about 31% yields and from naturally occurring juglone and chlorplumbagin. Plumbagin was first brominated at C-3 which was subsequently substituted with hydroxy group by a nucleophilic substitution to obtain Droserone. The synthesized compounds were characterized by IR, MS, $^1$H and $^{13}$C NMR spectral data. RP-HPLC was used to ascertain the purity of the compound obtained.

**Preparation of 3-bromo-plumbagin from Plumbagin:**

![Reaction scheme for synthesis of 3-bromoplumbagin](image)

**Figur 4.85. Reaction scheme for synthesis of 3-bromoplumbagin**

Plumbagin (120 mg) was added to a 10 ml solution of bromine in chloroform and stirred for 30 minutes at room temperature. A mixture of 5 ml of ethanol and 5 ml of glacial acetic acid was added to it and the reaction mixture was refluxed for additional 2 h. The progress of the reaction was monitored using TLC. The reaction mixture was cooled and transferred into a separating funnel and partitioned with water; the organic layer was separated and allowed to evaporate. The residue so obtained was loaded on silica and purified by column chromatography, using light petroleum ether: ethyl acetate (97: 3) as eluent to yield 80 mg of 3-bromoplumbagin.
Conversion of 3-bromo-plumbagin to Droserone:

![Reaction scheme for synthesis of Droserone from 3-bromoplumbagin](image)

3-Bromo-plumbagin (80 mg) dissolved in 20 ml of ethanol to which a 5 ml of 10% sodium hydroxide solution was added. The reaction mixture was refluxed on water bath for 90 min. The reaction mixture was then allowed to cool and poured in 50 ml of dilute hydrochloric acid solution and kept aside for 2 h. Yellowish brown precipitate was formed. The solution containing the fine precipitate was partitioned with light petroleum ether. The organic layer was concentrated and the residue obtained was loaded on silica and purified using column chromatography using light petroleum ether and ethyl acetate (90:10) as eluent to yield 10 mg droserone.

**Spectroscopic data:**

**Bromo-plumbagin (3-bromo-5-Hydroxy-2-methyl-1,4-naphthoquinone):** Infra red (IR) spectrum of the isolated plumbagin showed a broad peak at 3456 cm⁻¹ (hydroxyl) and 1662, 1636 cm⁻¹ (carbonyl), 1636 characteristic of (C=C) in quinones, 748 (bromo). Molecular ion peak at 266,268 m/e gave the molecular weight of the compound. The ¹H NMR (400 MHz, CDCl₃) shows δ 2.1 (s, 3H, R-CH₃), 5.3 (s, 1H, Ar-OH), 7.2(m, 3H, Ar-H) ¹³C NMR( MHz, CDCl₃) 16.4 (C₁₁), 129 (C₀), 133 (C₁₀), 119 (C₈), 124 (C₆),124 (C₃),136 (C₇), 150 (C₂), 161 (C₅), 184 (C₁),184 (C₄). The synthesized compound melts at 82°C. 3-Bromoplumbagin showed 98 % purity by HPLC. Yield 47%.
Figure 4.87. Mass Spectra of 3-bromoplumbagin

Figure 4.88 $^1$H-NMR of 3-bromoplumbagin
Figure 4.89. $^{13}$C-NMR of 3-bromoplumbagin

Figure 4.90. IR Spectra of 3-bromoplumbagin

**Droserone (3,5-dihydroxy-2-methyl-1,4-naphthoquinone):** Infra red (IR) spectrum of the synthesized droserone shows a broad peak at 3454 and 3434 cm$^{-1}$ (hydroxyl) and 1744,
1712 cm$^{-1}$ (carbonyl), 1625 characteristic of (C=C) in quinones. Molecular ion peak at 204 \textit{m/e} gave the molecular weight of the compound. The UV/Vis maxima in methanol were found to be at 250 and 402 nm. The $^1$H NMR (400 MHz, CDCl$_3$) shows δ 2.1 (s, 3H, R-CH$_3$), 5.3 (s, 1H, Ar-OH), 6.2 (s, 1H, Ar-OH); 7.2(m, 3H, Ar-H). $^{13}$C NMR (MHz, CDCl$_3$) 19 (C$_{11}$), 115 (C$_2$), 122(C$_8$), 126 (C$_6$) 130 (C$_{10}$), 133 (C$_7$), 140 (C$_9$), 156 ( C$_5$), 160 (C$_3$), 184 ( C$_1$), 188 (C$_4$). The isolated compound melts at 76°C. Yield 16.3%. Droserone shows 95% purity by HPLC.

![Figure 4.91. Mass Spectra of Droserone](image)

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Figure 4.92. $^1$H-NMR of Droserone

Figure 4.93. $^{13}$C-NMR of Droserone
HPLC studies of Droserone:

*Standard solution*

Take 10 mg of Droserone in methanol in 10-ml volumetric flask and make up the volume. 1 ml of above solution was taken and diluted up to 10 ml (Stock solution 100 µg/ml). From the stock solution prepare standard solutions (10 µg/ml) by transferring aliquots 1 ml of stock solution to 10-ml volumetric flasks and adjust the volume to 10 ml with methanol.

*Chromatographic conditions*

**Instrument**: JASCO PU-1580  
**Column**: Purospher STAR, RP-18e, 250 x 4.6, 5 µ  
**Mobile phase**: Methanol: Water (0.1% v/v of o-phosphoric acid) (65:35)  
**Flow rate**: 1 ml/min  
**Detector**: UV Detector (JASCO UV-1575) at 250 nm  
**Injection volume**: 20 µl  
**Run time**: 15 min.
Figure 4.95. HPLC chromatogram of Droserone

Preparation of Alkyl derivatives of Plumbagin

Vitamin K is an essential cofactor in the synthesis of active blood-clotting factors II, VII, IX, X. Plumbagin is also reported to possess anti-coagulant activities. The structure of plumbagin closely resembles that of vitamin K and hence the approach of chemical modification of Plumbagin to produce compounds resembling Vit K was attempted.
Figure 4.96. Chemical Stucture of Plumbagin and Vitamin K₁

General reaction for Alkylation of Plumbagin

Figure 4.97. Reaction scheme for synthesis of Alkyl derivatives of Plumbagin

Procedure:
In a flask containing 100 mg of Plumbagin and 1.5 millimoles of acid and 30 mg silver nitrate was added a mixture of acetonitrile and water (2:1) about 7 ml and 156 mg of ammonium peroxydisulphate was dissolved in 1 ml if water and added to the reaction mixture in parts over a period of 2 h. The reaction was monitored using TLC. After the reaction was completed the reaction mixture was poured in water and partitioned using...
chloroform. The organic washings were pooled and loaded on silica and subjected to column chromatography using silica gel (60-120 mesh) using light petroleum ether (60-80°C): Ethylacetate (100:0 to 90:10) to give pure compounds in 70-98% yields. The reaction of plumbagin was carried out with glacial acetic acid, chloroacetic acid, palmitic acid, stearic acid, oleic acid, cholic acid and isobutyric acid to yield corresponding alkylated derivatives.

Spectroscopic data of the synthesized compounds

1) methyplumbagin (2,3-dimethyl-5-hydroxy-1,4-naphthoquinone)

![Chemical structure of 3-methylplumbagin]

Figure 4.98. Chemical structure of 3-methylplumbagin

Yield 78%; Yellow amorphous powder m.p. 80°C; IR spectra: 3441.5, 2924.7, 1603.9, 1454.1, 1363.4, 1259, 968, 764 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) shows δ 2.1 (s, 6H, 2 x Ar-CH₃), 5.3 (s, 1H, Ar-OH), 7.2 (m, 2H, Ar-H), 7.5 (m, H, Ar-H); ¹³C NMR (MHz, CDCl₃) 16.4 (C-11, C-12), 119 (C-8), 124 (C-6), 129 (C-10), 133 (C-9), 136 (C-7), 147 (C-3), 149 (C-2), 161 (C-5), 184 (C-1), 190 (C4). MS [M+1] m/z 203.
Figure 4.99. Mass Spectra of 3-methylplumbagin

Figure 4.100. $^1$H-NMR of 3-methylplumbagin

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Figure 4.101. $^{13}$C-NMR of 3-methylplumbagin

Figure 4.102. IR Spectra of 3-methylplumbagin
2) 5-hydroxy-2-methyl-3-pentadecyl -1,4-naphthoquinone (3-pentadecyl plumbagin, palmitic acid derivative)

![Chemical structure of 3-pentadecylplumbagin]

Figure 4.103. Chemical structure of 3-pentadecylplumbagin

Yield 95 %; Yellow amorphous powder; m.p 65° C; C_{26}H_{38}O_{3} IR spectra:3685.14, 2953.09, 2851.53, 1731.74, 1577.4, 1455.12 cm^{-1}.^{1}H NMR (400 MHz, CDCl_{3}) shows δ 0.4 (m,3H,R-CH_{3}), 1.29 (m, 22H, -CH_{2}-), 1.33(m, 4H, R-CH_{2}-CH_{3}) 1.93 (m, 2H, Ar-CH_{2}-R), 2.1 (s, 3H, Ar-CH_{3}), 5.3 (s, 1H, Ar-OH), 7.2 (m, H, Ar-H), 7.5 (m, 2H, Ar-H); ^{13}C NMR( MHz, CDCl_{3}) 12.5 (C-11), 14.2 (C-26) 22.1 (C-25), 22.7 (C-12), 26.6 (C-13), 28.8-30.2 (C-14-C-23), 32.01 (C-24), 119.1 (C-8), 124.5 (C-6), 124 (C-10), 132.2 (C-9), 135.9 (C-7), 144.5 (C-2), 147.4 (C-3), 161.3 (C-5), 184 (C-1), 190 (C-4). MS [M-1] 397.

![Mass Spectra of 3-pentadecylplumbagin]

Figure 4.104. Mass Spectra of 3-pentadecylplumbagin
Figure 4.105. $^1$H-NMR of 3-pentadecylplumbagin

Figure 4.106. $^{13}$C-NMR Spectra of 3-pentadecylplumbagin
3) **5-hydroxy-2-methyl-3-heptadecyl-1,4-naphthoquinone** (3-heptadecyl plumbagin)

stearic acid derivative of Plumbagin

![Figure 4.107. IR Spectra of 3-pentadecylplumbagin](image)

Yield 92 %; C_{28}H_{42}O_3, Yellow amorphous powder; m.p.62°C, IR spectra: 3425.9, 2920.3, 1599.5, 1460, 1354.8, 7695 cm^{-1}. \(^1\)H NMR (400 MHz, CDCl\(_3\)) shows δ 0.4 (m,3H,R-CH\(_3\)), 1.29 (m, 24H, -CH\(_2\)-), 1.33(m, 4H, R-CH\(_2\)-CH\(_3\)), 1.8 (m,2H, Ar-CH\(_2\)-R), 2.1 (s, 3H, Ar-CH\(_3\)), 5.3 (s, 1H, Ar-OH), 7.2 (m, H, Ar-H),7.5(m, 2H, Ar-H); \(^{13}\)C NMR( MHz, CDCl\(_3\)) 12.8 (C-11),14.2 (C-28) 22.7 (C-27), 22.7 (C-12), 26.6 (C-13), 28.8-30.2 (C-14-C-).
25), 32.05 (C-26), 115.0 (C-8), 118.9 (C-6), 123.3 (C-10), 132.2 (C-9), 135.9 (C-7), 144.5 (C-2), 147.4 (C-3), 161.2 (C-5), 184 (C-1), 190 (C-4). MS [M+] m/z 426.

Figure 4.109. Mass Spectra of 3-heptadecylplumbagin

Figure 4.110. $^1$H- NMR of 3-heptadecylplumbagin
Figure 4.111. $^{13}$C-NMR of 3-heptadecylplumbagin

Figure 4.112. IR Spectrum of 3-heptadecylplumbagin
4] 3-(8,9-heptadecenyl)-5-hydroxy-2-methyl-1,4-naphthoquinone (3-[8-heptadecenyl]-plumbagin)

Yield 87 %; C_{28}H_{40}O_3, Yellow amorphous powder; m.p.56°C. IR spectra; 3429.8, 2925.1, 2854.2, 1605.19,1460.7, 1359.7, 1294.6, 773.4 cm \(^{-1}\). \(^1\)H NMR (400 MHz, CDCl\(_3\)) shows \(\delta\) 0.94 (m,3H,R-CH\(_3\)), 1.29 (m, 14H, -CH\(_2\)_2), 1.33(m, 4H, R-CH\(_2\)-CH\(_3\)), 1.33 (m, 4H, =C=C-CH\(_2\)_), 1.33 (m, 2H, Ar-C-CH\(_2\)_2), 1.93 (m,2H, Ar-CH\(_2\)-R), 1.96 (m,4H, =C-CH\(_2\)_2), 2.1 (s, 3H, Ar-CH\(_3\) ), 5.3 (s, 1H, Ar-OH), 5.34 (H=C=C-H), 7.2 (m, H, Ar-H), 7.5(m, 2H, Ar-H); \(^{13}\)C NMR( MHz, CDCl\(_3\)) 12.6 (C-11), 14.0 (C-28), 22.7(C-27), 26.5 (C-12), 27.2 (C-13), 29.2-30.2(C-14,C-15,C-16,C-17, C-22,C-23,C-24,C-25), 31.5(C-18) 31.9 (C-21) 118.8 (C-8), 124.5 (C-6), 128.7 (C-10),130.1 (C-19), 130.1(C-20) 132.2 (C-9), 135.9(C-7), 144.5(C-2),147.3 (C-3), 161.2(C-5), 184 (C-1),190 (C-4). MS [M+1] \(m/z\) 425.

**Figure 4.113. Chemical structure of (3-[8-heptadecenyl]-plumbagin)**

**Figure 4.114. Mass Spectra of 3-[8-heptadecenyl]-plumbagin**
Figure 4.115. $^1$H-NMR Spectra of 3-[8-heptadecenyl]-plumbagin

Figure 4.116. $^{13}$C-NMR Spectra of 3-[8-heptadecenyl]-plumbagin
5] 5-hydroxy-3-(1-methyl-ethyl)-2-methyl-1,4-naphthoquinone (Isopropyl plumbagin)

![Chemical structure of Isopropyl plumbagin.](image)

**Yield 83 %; C_{14}H_{14}O_{3}** Yellow amorphous powder; m.p 68. °C, IR spectra: 3429.8, 2925.1, 2854.2, 1605.19, 1460.7, 1359.7, 1294.6, 773.4 cm\(^{-1}\). \(^1\)H NMR (400 MHz, CDCl\(_3\)) shows δ 1.2 (s, 6H, R-CH\(_3\)), 2.1 (s, 3H, Ar-CH\(_3\)), 2.3 (m, 1H, Ar-C-H), 5.3 (s, 1H, Ar-OH), 7.2 (m, H, Ar-H), 7.5 (m, 2H, Ar-H); \(^1^3\)C NMR (MHz, CDCl\(_3\)) 16.4 (C-11), 23.7 (C-12), 26.7 (C-13, C-14), 119 (C-8), 124 (C-6), 129 (C-10), 130 (C-9), 133 (C-2), 136 (C-7), 161 (C-3), 162 (C-5), 184 (C-1), 190 (C-4). MS [M+1] \(m/z\) 231.
Figure 4.119. Mass Spectroscopic data of Isopropylplumbagin

Figure 4.120. $^1$H-NMR of isopropylplumbagin
Figure 4.121. $^{13}$C- NMR of isopropylplumbagin

Figure 4.122. IR spectra of Isopropylplumbagin
6) Cholic acid derivative of Plumbagin (5-hydroxy-2-methyl-3-[3-(3R,7R,12R,14S)-3,7,12-trihydroxy-5,10,13-trimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)butyl)naphthalene-1,4-dione)

![Chemical structure of Cholic acid derivative of Plumbagin](image)

**Figure 4.123. Chemical structure of Cholic acid derivative of Plumbagin**

Yield 84 %, C_{34}H_{46}O_6, IR spectra: 3442.7, 2925.05, 2831.9, 1597.2, 1362.8, 1259.2, 774.83 cm\(^{-1}\) ¹H NMR Spectra shown in Table 1. \(^{13}\)C NMR( MHz, CDCl\(_3\)) \(\delta\) 190.3(C-1), 136.2 (C-2)149.7(C-3), 184.8 (C-4), 161.2(C-5), 124.2(C-6), 136.2(C-7), 124.0(C-8), 135.5(C-9), 132.1(C-10),16.6(C-11), 22.2(C-12), 32.9(C-13), 29.8(C-14), 18.6(C-15), 33.0(C'-1), 29.6(C'-2), 65.6(C'-3), 36.2(C'-4), 35.2 (C'-5), 36.7(C'-6), 71.9 (C'-7), 41.6 (C'-8), 42.9 (C'-9), 37.6 (C'-10), 28.4 (C'-11), 81.3 (C'-12), 50.4(C'-13),45.6 (C'-14), 25.2(C'-15), 28.1(C'-16), 50.7(C'-17), 11.4(C'-18), 12.6 (C'-19) MS [M+1] \(m/z\) 551.

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Table 4.6. $^1$H NMR and $^{13}$C- NMR Shifts (δ) of Compound Cholic acid derivative of Plumbagin in CDCl$_3$

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<th>Carbon no</th>
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<th>$\delta$ C$^{13}$ Observed</th>
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Figure 4.124. Mass spectra of cholic acid derivative of Plumbagin

Figure 4.125 $^1$H-NMR Spectra of Cholic acid derivative of Plumbagin
Figure 4.12. $^{13}$C- NMR of cholic acid derivative of Plumbagin

Figure 4.127. IR of cholic acid derivative of Plumbagin
4.4.2. Shikalkin

4.4.2.1. Extraction and Isolation of Shikalkin from *Arnebia nobilis* Rech. f. roots:

**Figure 4.128. Roots of *Arnebia nobilis* Rech. f.**

Chemical test for Shikalkin:

Take 1g of *A. nobilis* root powder and extract in 5 ml of chloroform. Reduce the chloroform layer to 1 ml. Add about 1 ml of 5% potassium hydroxide solution to this tube. The aqueous layer assumes deep blue colour.

Isolation of Shikalkin from *Arnebia nobilis* roots.

Extraction with chloroform (2 hr.)

Saponification with sodium hydroxide: 4 hr

Treatment of different solvents, Purification and crystallization:

100 g of *A. nobilis* roots were powdered and extracted with 750 x 3 ml chloroform. The chloroform layer was evaporated and the residue obtained was subjected to base hydrolysis using 200 ml of 5% sodium hydroxide and stirred for 4 h. This was acidified using 10% hydrochloric acid till the colour of the solution changed from deep blue to pink and extracted in light petroleum ether. The organic layer was allowed to dry and the residue was then fractionated in methanol. The methanol layer was given washings with light petroleum ether to remove polymeric pigments and dried to obtain Shikalkin (22mg).

Recrystallization:
About 22 mg of crude Shikalkin obtained from above procedure dissolved in 5 ml of light petroleum ether. Filter immediately using vacuum filtration and filtrate allow standing for 20-30 minutes gives deep pink coloured needles of shikalkin.

4.4.2.2. Characterization of Shikalkin

\[
\begin{align*}
\text{Alkannin} & \\
\text{Shikonin} & 
\end{align*}
\]

Figure 4.129. Chemical structure of Alkannin and Shikonin

UV absorption spectrum exhibits absorption maxima $\lambda_{\text{max}}$ (MeOH) at 558, 542, 520, 488, 276 and 220 nm. The IR spectrum showed the presence of -OH stretching at 3446 cm$^{-1}$, the isohexenyl aliphatic stretching at 2926 cm$^{-1}$ and the quinone carbonyl groups at 1604 cm$^{-1}$ (Fig. 2). The isolated compound showed the following NMR signals: $^1$H NMR (CDCl$_3$) $\delta$: 1.66 and 1.76 [each 3H, s, =C(CH$_3$)$_2$]; 2.34–2.65 [2H, H-2’a, 2’b]; 5.2 [1H, m, H-3’]; 4.9 [1H, m, H-1’], 7.12 [1H, d, H-3]; 7.21 [1H, d, H-7] and 7.26 [1H, d, H-6]; 12.51 and 12.61 [each 1H, s, -OH] (Table 1). The isolated shikalkin showed molecular peak, m/z at 287 (M-1) (Fig. 3). Shikalkin showed a purity of 99.6% by analytical HPLC which was performed with flow rate of 0.5 ml/min and elution program: 40 min, isocratic using Acetonitrile: Water: Methanol (54.4: 16.2: 29.4) as mobile phase. The retention time of isolated compound was found to be 22 min (Fig. 4). The isolated shikalkin was applied on TLC silica gel 60 F$_{254}$ plates using CAMAG LINOMAT IV automatic spotter. Among the different solvent systems employed in the mobile phase, the isolated shikalkin resolved at $R_f$ 0.24 in Toluene: chloroform: acetone (50:50:1) which shows a single spot of shikalkin. The spot of shikalkin shows a pink coloration in visible light and turns blue when treated with 5% alcoholic potassium hydroxide.
Figure 4.130. Mass Spectra of Shikalkin

Figure 4.131. $^1$H-NMR of Shikalkin
Figure 4.132. IR Spectra of Shikalkin

Figure 4.133. UV-Vis Spectra of Shikalkin
4.4.2.3. HPTLC Analysis of Shikalkin:

**Preparation of extract:** 2 g of *A. nobilis* root powder was extracted with chloroform (10 ml) by shaking for 5 minutes. Allow standing, methanol layer was separated and concentrated upto 2 ml under vaccum. 10 µl was used for TLC.

**Preparation of standard:** Shikalkin prepared as a 0.01% of methanolic solution, 5 µl used for TLC

**Chromatographic solvent:** Toluene: chloroform: acetone (50:50:1)

**Detection:** 5% alcoholic KOH

A. Visible (Without chemical treatment)

B. 5% Alcoholic Potassium Hydroxide solution
   1. Isolated compound
   2. *A. nobilis* root extract

![TLC Profile](image)

**Fig. 4.134. TLC Profile of standard Shikalkin and *A. nobilis* methanolic extract.**

4.4.2.4. HPLC studies on Shikalkin

**Standard solution**

Take 10 mg of shikalkin in methanol in 10-ml volumetric flask and make up the volume. 1 ml of above solution was taken and diluted upto 10 ml (Stock solution 100 µg/ml). From the stock solution prepare standard solutions (10 µg/ml) by transferring aliquots 1ml of stock solution to 10-ml volumetric flasks and adjust the volume to 10 ml with methanol.
**Chromatographic conditions**

<table>
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<tr>
<th>Parameter</th>
<th>Details</th>
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<td>Instrument</td>
<td>JASCO PU-1580</td>
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<tr>
<td>Column</td>
<td>Purospher, RP-18e, 250 x 4.6, 5 µ</td>
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<tr>
<td>Mobile phase</td>
<td>Acetonitrile: Water: Methanol (54.4:16.2: 29.4)</td>
</tr>
<tr>
<td>Flow rate</td>
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<tr>
<td>Detector</td>
<td>UV Detector (JASCO UV-1575) at 280 nm</td>
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<tr>
<td>Injection volume</td>
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</table>

![HPLC chromatogram of Shikalkin](image)

**Figure 4.135. HPLC chromatogram of Shikalkin**