Chapter 6

Isolation of Phytoconstituents
6. Isolation of Phytoconstituents from bioactive extract

6.1 Introduction

The bioactivity guided fractionation and isolation is widely accepted in plant drug research. The crude extracts having biological activity are used as a source for isolation of active molecule(s) using different methods of purification. In bioactivity-guided fractionation, the crude extract of plant material is subjected to several rounds of fractionation with the solvents of different polarity or their mixture. Phytoconstituents are separated based on their solubility. The fractionation and their simultaneously biological evaluation with suitable bioassay are performed to determine the bioactive fraction. The fractions with potent biological activity is proceed for isolation and identification of phytoconstituents with the help of physical and spectral analysis such as Mass spectroscopy, Nuclear magnetic resonance (NMR), and Infrared (IR) spectroscopic analysis. Majority of the biologically active natural products have been isolated using bioactivity-guided fractionation (Pezzuto et al., 1997).

6.2 Experimental

In present investigation, the series of in-vitro, in-vivo antioxidant, hepatoprotective, anti-inflammatory, and immunomodulatory evaluations were undertaken to find out the bioactive extract of selected plant species. The results of study confirmed that the moderately polar extracts i.e. successive ethyl acetate extract of *F. microcarpa* bark and ethanol extract of *L. amara* pericarp was pharmacologically active. These findings suggested the most therapeutically active phytoconstituents accumulated in these two extracts. Therefore, EA-FMB and EO-LAP was subjected for fractionation and isolation of phytoconstituents.

6.2.1 Fractionation and screening of bioactive EA-FMB extracts

The successive EA-FMB extract (8 g) was dissolved in 15 ml of ethyl acetate and was triturated with silica gel (40 g; 100-200 mesh). The adsorbed EA-FMB extract on silica gel was vigorously shaken with successive combination of n-hexane: CHCl₃ followed by CHCl₃: methanol. Eight fractions (Fr.1-8) were collected (Figure 6.3). These fractions were evaluated for antioxidant potential using DPPH assay (Table 6.1).
6.2.2 Fractionation and screening of bioactive EO-LAP extract

The bioactive EO-LAP extract (7 g) was fractioned in similar manner to EA-FMB extract. Eight fractions (Fr.1-8) were collected (Figure 6.4). These fractions were evaluated for antioxidant potential using DPPH assay (Table 6.2).

6.2.3 Isolation of possible bioactive phytoconstituents

6.2.3.1 Isolation of phytoconstituents from EA-FMB

The results of antioxidant studies demonstrated that fraction 1, 3, 6 and 8 have potent antioxidant activity. Thus, these fractions of EA-FMB extract was subjected for column chromatography using silica gel (100-200 mesh). Fr 1 and 3 (0.93 and 0.76 g) was dissolved in little quantity of ethyl acetate and adsorbed on minimum quantity of silica. The adsorbed fraction was subjected to wet packing in a column (25 cm × 1.8 cm) containing silica gel prepared in n-hexane, separately. The Fr-1 column was eluted with n-hexane: chloroform (9:1) to get purified FMC-1 (65 mg). The Fr-3 column eluted with n-hexane: chloroform (7:3) to yield purified FMC-2 (39 mg).

The Fr-6 and 8 (1.03 and 1.1 g) was dissolved in little quantity of ethyl acetate and adsorbed on the minimum quantity of silica. The adsorbed fraction was subjected to wet packing in a column (25 cm × 1.8 cm) containing silica gel prepared in chloroform, separately. The Fr-6 column was eluted with chloroform: methanol (8:2) to get purified FMC-3 (123 mg). The Fr-8 column eluted with n chloroform: acetone (7:3) to get purified FMC-4 (115 mg). The fractionation and isolation scheme is shown in Figure 6.1.

6.2.3.4 Isolation of phytoconstituents from EO-LAP

The results of DPPH assay showed that Fr- 1, 2 and 8 of EO-LAP extract have potent antioxidant activity. Therefore, these fractions were preceded for isolation of phytoconstituents. Fr 1 and 2 (0.72 and 0.93 g) was dissolved in little quantity of methanol and adsorbed on minimum quantity of silica (100-200 mesh). The adsorbed fraction was subjected to wet packing in the column (25 cm × 1.8 cm) containing silica gel prepared in n-hexane, separately. The Fr-1 column was eluted with n-hexane: chloroform (9:1) to get purified LAC-1 (69 mg). The Fr-2 column eluted with chloroform: methanol (9:1) to yield purified LAC-2 (32 mg), on further elution with chloroform: methanol (7:3) to yield purified LAC-3 (48 mg). While, Fr-8 was
dissolved in little quantity of methanol and adsorbed on the minimum quantity of silica gel. The adsorbed fraction was subjected to wet packing in column (25 cm × 1.8 cm) containing silica gel (20 g) prepared in chloroform eluted with chloroform: acetone (6:4) to get purified LAC-4 (29 mg). The fractionation and isolation scheme is shown in Figure 6.2.

The GC-MS spectral study of FMC-2, LAC-2 (7:3) and 4 revealed the presence of mixture in the final isolates. Thus, structural identification of these isolated was not carried out.

6.2.3.4 TLC of isolated phytoconstituents and bioactive fractions

Different combinations of the solvent systems were tried to get better chromatogram for isolated phytoconstituents and bioactive fractions. Bioactive extracts along with isolated compounds were developed in finally optimised mobile phase for *F. microcarpa* (Figure 6.3) and *L. amara* (Figure 6.4)

6.2.3.5 Physical and spectral analysis

The isolated purified compounds initially evaluated for physical properties such as physical state, melting point, UV \( \lambda_{\text{max}} \). Later the spectral analysis was performed for elucidation of isolated structure.
Figure 6.1: Schematic presentation of fractionation scheme for bioactive EA-FMB extract

Fractionation with Silica gel

EA-FMB extract (8 g)

Hexane: CHCl₃ (8:2) Fraction 1 (0.83 g)

Hexane: CHCl₃ (6:4) Fraction 2 (0.62 g)

Hexane: CHCl₃ (4:6) Fraction 3 (0.76 g)

Hexane: CHCl₃ (2:8) Fraction 4 (0.72 g)

CHCl₃: Methanol (8:2) Fraction 5 (0.69 g)

CHCl₃: Methanol (6:4) Fraction 6 (1.03 g)

CHCl₃: Methanol (4:6) Fraction 7 (0.89 g)

Hexane: CHCl₃ (9:1) Fraction 1 (0.62 g)

Hexane: CHCl₃ (6:4) Fraction 2 (0.62 g)

Hexane: CHCl₃ (4:6) Fraction 3 (0.76 g)

CC: Silica gel
CC: Hexane: CHCl₃ (9:1)

CC: Silica gel
Hexane: CHCl₃ (9:1)

CHCl₃: Methanol (8:2)
Hexane: CHCl₃ (9:1)

CHCl₃: Methanol (6:4)
Hexane: CHCl₃ (9:1)

CHCl₃: Methanol (4:6)
Hexane: CHCl₃ (9:1)

Three prominent spots

CHCl₃: Methanol (2:8) Fraction 8 (1.1 g)

CC: Silica gel
CHCl₃: Methanol (2:8)

CC: Silica gel
CHCl₃: Methanol (9:1)

CC: Silica gel
CHCl₃: Methanol (9:1)

CC: Silica gel
CHCl₃: Methanol (9:1)

One prominent spot

Multiple prominent spots

CC: Silica gel
Hexane: CHCl₃ (9:1)

Compound-1
FMC – 1 (9:1)

Compound-2
FMC – 2 (7:3)

Compound-3
FMC – 3 (8:2)

Compound-4
FMC – 4 (7:3)
6.2.4 Physical and spectroscopic characterization of phytoconstituents from EA-FMB extract.

6.2.4.1 Characterization of FMC-1 (oleanolic acid)

Physical data:

State: Colourless crystalline solid
Yield: 65 mg
Molecular Weight: 457
Molecular Formula: C_{30}H_{48}O_{3}
Melting point: 308-311°C

$\text{UV } \lambda_{\text{max}}$ (KBr):

3414 (alcoholic –OH), 2926 (acidic –OH), 1693 (C=C), 1462 (alkane- CH₃).

$^1\text{H NMR (CDCl}_3\text{) (300 MHz) (δ ppm)}$

δ 0.77 (1H, s, H3-28), 0.91 (1H, s, H3-29 and H3-30), 0.98 (1H, s, H3-25), 1.05 (1H, s, H3-23), 1.13 (1H, s, H3-26), 1.18 (1H, s, H3-27), 1.25 (1H, s, H3-24), 1.59 (1H, t, H-9), 1.86 (1H, t, H-18), 3.21 (1H, dd, H-3), 5.27 (1H, m, H-12).

$^{13}\text{C-NMR (CDCl}_3\text{) (75MHz) (δ ppm)}$

δ 15.25 (C-25), 15.47 (C-24), 17.04 (C-26), 18.21 (C-6), 22.82 (C-11), 23.32 (C-30), 23.51 (C-16), 25.88 (C-27), 27.08 (C-2), 27.60 (C-15), 28.07 (C-23), 29.65 (C-20), 30.61 (C-22), 32.35 (C-7), 32.51 (C-29), 33.01 (C-21), 33.71 (C-10), 37.01(C-1), 38.31 (C-4), 38.68 (C-8), 39.18 (C-18), 41.50 (C-14), 45.78 (C-17), 46.45 (C-19), 47.55 (C-9), 55.12 (C-5), 78.95 (C-3), 122.56 (C-12), 143.52 (C-13), 183.54 (C-28).

EI-MS $m/z$ (rel int. %):

457, 439, 411, 393, 248 (100), 217, 203, 177, 163.
6.2.4.2 Characterization of FMC-3 (catechin)

Physical data:

State: Colourless creamish powder
Yield: 123 mg
Molecular Weight: 290
Molecular Formula: C_{15}H_{14}O_{6}
Melting point: 174-178°C

UV $\lambda_{\text{max}}$: 219, 276

$\text{IR } \nu_{\text{max}} \text{ cm}^{-1} \text{ (KBr)}$: 3412-3240 (alcoholic and phenolic -OH), 3059 (aromatic - C-H), 1610 (aromatic C=C), 1514 and 1473 (aromatic ring)

$^1\text{H NMR (CDCl}_3\text{) (300 MHz) (δ ppm)}$:

3.97 (2H, m, H-3), 4.60 (1H, d, H-2), 5.82 (1H, d, H-6), 6.01 (1H, d, H-8), 6.74 (1H, dd, H-6'), 6.83 (1H, d, H-5'), 6.92 (1H, d, H-2'), 7.38 (s, phenolic protons)

$^{13}\text{C-NMR (CDCl}_3\text{) (75MHz) (δ ppm)}$:

δ 27.14 (C-4), 67.74 (C-3), 81.39 (C-2), 94.73 (C-8), 95.81 (C-6), 99.47 (C-10), 114.07 (C-2'), 115.11 (C-5'), 119.16 (C-6'), 130.3 (C-1'), 144.66 (C-3'), 144.79 (C-4'), 155.49 (C-5), 156.15 (C-9), 156.48 (C-7).

$\text{EI-MS } m/z \text{ (rel int. %)}$:

290, 194, 168, 153, 138 (100), 124, 110.
6.2.4.3 Characterization of FMC-4 (p-hydroxycinnamic acid)

Physical data:

State: Creamish coloured powder
Yield: 115 mg
Molecular Weight: 165
Molecular Formula: C_9H_8O_3
Melting point: 188-192°C

UV λ max: 310 nm

IR ν max cm⁻¹ (KBr):
3358 (Phenolic- OH), 2843 (acidic- OH), 1666 (carbonyl- C=O), 1462 (aromatic/alkene C=C).

¹H NMR (CDCl₃) (300 MHz) (δ ppm):
δ 6.31 (1H,d, H-2'; a-position), 6.91 (2H, d, H-3,5), 7.31 (1H, d, H-1'b-position), 7.78 (2H, d, H-2,6).

¹³C-NMR (CDCl₃) (75MHz) (δ ppm):
δ 169.83 (C-3'; -COOH), 156.70 (C-4), 141.01 (C-2'; a-position), 131.06 (C-2, 6), 128.89 (C-1), 118.15 (C-3, 5), 116.36 (C-1'; b-position).

EI-MS m/z (rel int. %):
165 (100), 147, 119, 91, 77, 65.
Figure 6.2 Schematic presentation of fractionation scheme for bioactive EO-LAP extract; CC: column chromatography

- **EO-LAP extract (8 g)**
  - Fractionation with Silica gel
    - **Hexane: CHCl₃ (8:2)**
      - Fraction 1 (0.72 g)
        - One prominent spot
          - CC: Silica gel
            - Hexane: CHCl₃ (9:1)
              - Compound 1 LAC-1 (9:1)
    - **Hexane: CHCl₃ (4:6)**
      - Fraction 3 (0.53 g)
        - Three prominent spot
          - CC: Silica gel
            - Hexane: CHCl₃ (6:4)
              - Fraction 2 (0.93 g)
    - **Hexane: CHCl₃ (2:8)**
      - Fraction 4 (0.76 g)
          - CC: Silica gel
            - CHCl₃: Methanol (8:2)
              - Fraction 5 (0.8 g)
    - **CHCl₃: Methanol (8:2)**
      - Fraction 6 (0.89 g)
          - **CHCl₃: Methanol (4:6)**
            - Fraction 7 (0.73 g)
    - **CHCl₃: Methanol (6:4)**
      - Fraction 8 (0.91 g)
          - CC: Silica gel
            - CHCl₃: aceton (4:6)
              - Two prominent spot
                - CHCl₃: Methanol (2:8)
                  - Fraction 9 (0.91 g)

- **Fractionation with Silica gel**
  - **Hexane: CHCl₃ (6:4)**
    - Fraction 2 (0.93 g)
      - **Hexane: CHCl₃ (4:6)**
        - Fraction 3 (0.53 g)
          - **Hexane: CHCl₃ (8:2)**
            - Fraction 1 (0.72 g)
              - **Hexane: CHCl₃ (4:6)**
                - Fraction 3 (0.53 g)
                  - **Hexane: CHCl₃ (2:8)**
                    - Fraction 4 (0.76 g)
                      - **CHCl₃: Methanol (8:2)**
                        - Fraction 5 (0.8 g)
                          - **CHCl₃: Methanol (4:6)**
                            - Fraction 7 (0.73 g)
                          - **CHCl₃: Methanol (6:4)**
                            - Fraction 8 (0.91 g)
                              - CC: Silica gel
                                - CHCl₃: aceton (4:6)
                                  - Two prominent spot
                                    - CHCl₃: Methanol (2:8)
                                      - Fraction 9 (0.91 g)
n-hexane: ethyl acetate (8:2, \(v/v\)); \(R_f\): 0.78

Toluene: ethyl acetate: formic acid (6:6:1; \(v/v\)); \(R_f\): 0.48

n-Hexane: Ethyl acetate: acetic acid (6:4:1; \(v/v\)); \(R_f\): 0.42

<table>
<thead>
<tr>
<th>FMC-1</th>
<th>EA-FMB; Fr-1</th>
<th>FMC-3</th>
<th>EA-FMB; Fr-6</th>
<th>FMC-4</th>
<th>EA-FMB; Fr-8</th>
</tr>
</thead>
</table>

Figure 6.3: Images of TLC plate for bioactive fractions and isolated compounds from successive ethyl acetate extract of *F. microcarpa* bark

n-hexane: ethyl acetate: acetic acid (6:6:1; \(v/v\)) \(R_f\): 0.55

Toluene: ethyl acetate: formic acid (6:6:1; \(v/v\)) \(R_f\): 0.65

<table>
<thead>
<tr>
<th>LAC-1</th>
<th>EO-LAP; Fr-1</th>
<th>LAC-3</th>
<th>EO-LAC; Fr-6</th>
</tr>
</thead>
</table>

Figure 6.4: Images of TLC plate for bioactive fractions and isolated compounds from successive ethanol extract *L. amara* pericarp
6.2.5 Physical and spectroscopic characterization of phytoconstituents from EO-LAP

6.2.5.1 Characterization of LAC-1 (luteolin)

Physical data:

State: Colourless creamish powder
Yield: 69 mg
Molecular Weight: 286
Molecular Formula: C_{15}H_{10}O_{6}
Melting point: 340-344ºC
UV $\lambda$ max: 258, 346 nm

IR $\nu$ max cm$^{-1}$ (KBr):

3404, 3325, 3290 (alcoholic and phenolic- OH), 3159 (aromatic- CH), 1664 (ketone- C=O), 1610 (aromatic- C=C).

$^1$H NMR (CDCl$_3$) (300 MHz) (δ ppm):

δ 6.18 (1H, d, H-6), 6.37 (1H, d, H-8), 6.62 (1H, s, H-3), 6.82 (1H, d, H-5'), 7.42 (1H, dd, H-6'), 9.41 (1H, s, 7- OH), 11.79 (1H, s, 5- OH).

$^{13}$C-NMR (CDCl$_3$) (75MHz) (δ ppm):

δ 94.07 (C-8), 99.21 (C-6), 103.36 (C-3), 104.17 (C-10), 114.07 (C-2'), 115.11 (C-5'), 119.16 (C-6'), 130.30 (C-1'), 144.66 (C-3'), 148.15 (C-4'), 155.49 (C-9), 156.15 (C-5), 162.37 (C-2), 164.10 (C-7), 181.11 (C-4).

EI-MS m/z (rel int. %):

286 (100), 241, 179, 161, 153, 135, 123, 117.
6.2.5.1 Characterization of LAC-3 (gallic acid)

Physical data:

State: Colourless crystalline powder
Yield: 48 mg
Molecular Weight: 170
Molecular Formula: \( \text{C}_7\text{H}_6\text{O}_5 \)
Melting point: 235-237°C

UV \( \lambda \) max: 272 nm

\( \text{IR} \ \nu \max \ \text{cm}^{-1} (\text{KBr}) \):

3358 (phenolic- \( \text{OH} \)), 1701 (acidic \( \text{C}=\text{O} \)), 1448 (aromatic \( \text{C}=\text{C} \)).

\( ^1\text{H} \text{NMR} \ (\text{CDCl}_3) \ (300 \text{ MHz}) \ (\delta \ \text{ppm}) \):

\( \delta \) 7.26 (2H, s, H-2,6).

\( ^{13}\text{C} \text{NMR} \ (\text{CDCl}_3/\text{DMSO}) \ (75\text{MHz}) \ (\delta \ \text{ppm}) \):

\( \delta \) 170.72 (s, C-7), 163.51 (s, C-3,5), 133.72(s, C-4), 122.59 (s, C-1) and 116.24 (s, C-2,6)

\( \text{EI-MS} \ m/z \ (\text{rel int. \%}) \):

171 (100), 152, 127, 108, 69, 52.
6.2.6 HPTLC standardization of bioactive EA-FMB an EO-LAP with isolated compounds

6.1 Estimation of oleanolic acid, catechin, and \( p \)-hydroxycinnamic acid in ethyl acetate extract of \( F. \) microcarpa bark

<table>
<thead>
<tr>
<th>Chromatographic conditions</th>
<th>FMC-1 (Oleanolic acid)</th>
<th>FMC-3 (Catechin)</th>
<th>FMC-4 (( p )-hydroxycinnamic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development chamber</td>
<td></td>
<td>Camag Twin Trough</td>
<td></td>
</tr>
<tr>
<td>Chamber saturation</td>
<td></td>
<td>20 min</td>
<td></td>
</tr>
<tr>
<td>Stationary phase</td>
<td>Aluminium backed TLC Silica Gel GF(_{254})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Hexane: Ethyl acetate (8:2; v/v)</td>
<td>Toluene: Ethyl acetate: Formic acid (6:6:1; v/v)</td>
<td>Hexane: Ethyl acetate: acetic acid (6:4:1; v/v)</td>
</tr>
<tr>
<td>Standard (conc.)</td>
<td>OA (25 µg/ml)</td>
<td>CAT (25 µg/ml)</td>
<td>PHCA (25 µg/ml)</td>
</tr>
<tr>
<td>Sample (conc.)</td>
<td>EA-FMB extract (100 µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample volume/track</td>
<td>10 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>230 nm</td>
<td>276 nm</td>
<td>310 nm</td>
</tr>
</tbody>
</table>

OA- Oleanolic acid, CAT- Catechin, PHCA- \( p \)-hydroxycinnamic acid

6.2 Estimation of luteolin, gallic acid in ethanol extract of \( L. \) amara pericarp

<table>
<thead>
<tr>
<th>Chromatographic conditions</th>
<th>LAC-1 (Luteolin)</th>
<th>LAC-3 (Gallic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development chamber</td>
<td>Camag Twin Trough</td>
<td></td>
</tr>
<tr>
<td>Chamber saturation</td>
<td>20 min</td>
<td></td>
</tr>
<tr>
<td>Stationary phase</td>
<td>Aluminium backed TLC Silica Gel GF(_{254})</td>
<td></td>
</tr>
<tr>
<td>Standard (conc.)</td>
<td>LUT (25 µg/ml)</td>
<td>GA (25 µg/ml)</td>
</tr>
<tr>
<td>Sample (conc.)</td>
<td>EO-LAP extract (100 µg/ml)</td>
<td></td>
</tr>
<tr>
<td>Sample volume/track</td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>346 nm</td>
<td>272 nm</td>
</tr>
</tbody>
</table>

LUT- luteolin, GA- Gallic acid

After each chromatography, the percentage content of each marker in respective plant extract was calculated as per the following formula.

\[
\text{% content} = \frac{\text{AUC of Sample} \times \text{Conc of Std.} \times \% \text{purity}}{\cdot}
\]
6.3 Result and discussion

6.3.1 Fractionation of bioactive EA-FMB and EO-LAP extract

The *in-vitro* and *in-vivo* results revealed that the EA-FMB and EO-LAP extracts showed antioxidant and hepatoprotective potential. Theses extracts were selected for fractionation and purification. The combination of solvents used i.e. n-hexane-chloroform-methanol to distribute the soluble constituents depending upon their solubility and further to reduce the phytoconstituents load for column chromatography. The eight fractions were obtained from each extracts as shown in fractionation scheme.

6.3.2 Antioxidant screening of bioactive fraction from EA-FMB and EO-LAP extract

Antioxidants are vital substances, which possess the ability to protect the body from damage caused by free radicals (Oyaizu, 1986). However, the imbalance between free radicals generation and antioxidant defence leads to oxidative stress. Oxidative stress is one of the major causes of hepatotoxicity. The drugs with potent antioxidant activity are good hepatoprotective agents. It has been widely that natural antioxidants are safer than synthetic antioxidants. It was often observed through an increased interest of scientific communities for search of natural antioxidant. The effects of natural antioxidants are due to phytoconstituents viz, polyphenols, vitamins and carotenoids, which might help to prevent oxidative damage (Su et al., 2009; Thaiponga et al., 2006). DPPH assay is simple, widely used and scientifically accepted *in vitro* antioxidant test for screening of hydrophilic and lipophilic natural products. Eight fractions isolated from each of EA-FMB and EO-LAP extract, were examined for their scavenging abilities against DPPH free radicals. The results are shown in Table 6.3 and 6.4.

Among the eight fractions isolated from EA-FMB extract, the fraction 1, 3, 6 and 8 showed higher potency in scavenging DPPH radicals than other fractions. In the similar studies, fraction 1, 2 and 8 of EO-LAP extract showed the potent free radical scavenging ability. These bioactive fractions of both plants were considered for the isolation of possible phytoconstituents.
Table 6.3 Free radical scavenging activity of fractions of EA-FMB

<table>
<thead>
<tr>
<th>Fr/ Conc. (µg/ml)</th>
<th>Std (As A)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fr-1</td>
<td>Fr-2</td>
</tr>
<tr>
<td>20</td>
<td>32.15</td>
<td>29.87</td>
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<tr>
<td>40</td>
<td>47.21</td>
<td>54.65</td>
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<td>80</td>
<td>54.27</td>
<td>69.84</td>
</tr>
<tr>
<td>160</td>
<td>66.32</td>
<td>76.12</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>35.17</td>
<td>62.78</td>
</tr>
</tbody>
</table>

Fr- fractions, As A- Ascorbic acid.

The fraction 1, 6 and 8 from EA-FMB yields purified single compounds, identified as oleanolic acid, catechin and <i>p-</i>hydroxycinnamic acid using physical and spectral methods, respectively. The GC-MS analysis of fraction 2 revealed it as mixture of compounds. The mixture composed of three components with molecular weight 501, 319 and 589, however; the final structure could not be established.

Table 6.4 Free radical scavenging activity of fractions of EO-LAP extract.

<table>
<thead>
<tr>
<th>Fr/ Conc. (µg/ml)</th>
<th>Std (As A)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fr-1</td>
<td>Fr-2</td>
</tr>
<tr>
<td>20</td>
<td>32.15</td>
<td>31.54</td>
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<tr>
<td>40</td>
<td>47.21</td>
<td>51.24</td>
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<tr>
<td>80</td>
<td>54.27</td>
<td>61.23</td>
</tr>
<tr>
<td>160</td>
<td>66.32</td>
<td>72.35</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>35.17</td>
<td>72.15</td>
</tr>
</tbody>
</table>

Fr- fractions, As A- Ascorbic acid.

Similarly, fraction 1 and 2 (7:3) of EO-LAP extract yields purified polyphenolic phytoconstituents and identified as luteolin and gallic acid with the help of spectral analysis. The fraction 2 (9:1) and 8 of EO-LAP extract were confirmed as mixture of compounds with the help of GC-MS analysis. The fraction 2 (9:1) showed the presence of mixture of three components with molecular weight of 561, 534 and 550.
while fraction 8 contains two components with molecular weight of 120 and 592, structures were not confirmed.

### 6.3.3 Structure elucidation of isolated compounds from *F. microcarpa*

#### 6.3.3.1 Structure elucidation oleanolic acid (FMC-1)

FMC-1 gave positive colour reaction of triterpenes. The IR spectrum (Figure 6.5) exhibited absorption bands for hydroxyl group at 3414, carboxylic hydroxyl group at 2926, double bond (C=C) at 1693 for methyl group 1462 cm$^{-1}$.

The $^1$H-NMR spectrum showed seven tertiary methyls centered at δ 0.77 (1H, s, H3-28), 0.91 (1H, s, H3-29 and H3-30), 0.98 (1H, s, H3-25), 1.05 (1H, s, H3-23), 1.13 (1H, s, H3-26), 1.18 (1H, s, H3-27), 1.25 (1H, s, H3-24), (all singlets). The carbonylic proton resonated at δ 3.21 (1H, dd, H-3) inferring its α and axial orientation and a multiplet at δ 5.27 (1H, m, H-12) was indicative of the olefinic proton (Figure 6.6).

The $^{13}$C-NMR spectra disclosed the presence of thirty carbon atoms. The experiments indicated eight methyl, ten methylene, five methane and seven quaternary carbons (Figure 6.7).

The EI-MS spectrum showed molecular ion peak at m/z 457 corresponding to molecular formula C$_{30}$H$_{48}$O$_{3}$ (Figure 6.8). The EI-MS spectrum of FMC-1 showed characteristic fragmentation pattern of amyrin skeleton with double bond at C-12 (Budzikiewicz et al., 1963). The above physical and spectral data identified compound FMC-1 as oleanolic acid (Gangwal et al., 2010)
**Figure 6.5** FTIR spectrum of FMC-1 (oleanolic acid)

\[ \text{IR } \nu_{\text{max}} \text{ cm}^{-1} (\text{KBr}): 3414 \text{ (alcoholic –OH), } 2926 \text{ (acidic –OH), } 1693 \text{ (C=C), } 1462 \text{ (Alkane– CH}_3\text{)}. \]

**Figure 6.6** \(^1\text{H NMR spectrum of FMC-1 (oleanolic acid)}\)

\[ \text{\(^1\text{H NMR (CDCl}_3\text{) (300 MHz): } \delta 0.77 \text{ (1H, s, H3-28), 0.91 (1H, s, H3-29 and H3-30), 0.98 (1H, s, H3-25), 1.05 (1H, s, H3-23), 1.13 (1H, s, H3-26), 1.18 (1H, s, H3-27), 1.25 (1H, s, H3-24), 1.59 (1H, t, H-9), 1.86 (1H, t, H-18), 3.21 (1H, dd, H-3), 5.27 (1H, m, H-12).} \]
Figure 6.7 $^{13}$C NMR spectrum of FMC-1 (oleanolic acid)

$^{13}$C-NMR (CDCl$_3$) (75MHz): $\delta$ 15.25 (C-25), 15.47 (C-24), 17.04 (C-26), 18.21 (C-6), 22.82 (C-11), 23.32 (C-30), 23.51 (C-16), 25.88 (C-27), 27.08 (C-2), 27.60 (C-15), 28.07 (C-23), 29.65 (C-20), 30.61 (C-22), 32.35 (C-7), 32.51 (C-29), 33.01 (C-21), 33.71 (C-10), 37.01(C-1), 38.31 (C-4), 38.68 (C-8), 39.18 (C-18), 41.50 (C-14), 45.78 (C-17), 46.45 (C-19), 47.55 (C-9), 55.12 (C-5), 78.95 (C-3), 122.56 (C-12), 143.52 (C-13), 183.54 (C-28).

Figure 6.8: EI-MS spectrum of FMC-1 (oleanolic acid)

EI-MS m/z (rel int. %): 457, 439, 411, 393, 248 (100), 217, 203, 177, 163.
6.3.3.2 Structure elucidation of catechin (FMC-3)

The bioactive fraction Fr-3 of successive ethyl acetate extract of *F. microcarpa* yielded FMC-3 as cream-colored amorphous powder. FMC-3 was confirmed as tannin by chemical test, and its melting point was found to be 174-178°C. Furthermore, it shown the characteristic UV $\lambda_{\text{max}}$ at 219, 276. The IR spectrum of FMC-3 showed a broad band at 3412 cm$^{-1}$ indicating the presence of hydroxyl group. Other features of IR spectrum revealed absorptions at 3059 (C-H, aromatic), 1610 (C=C, aromatic), 1514 and 1473 (aromatic ring) cm$^{-1}$ (Figure 6.9).

The $^1$H NMR spectrum of FMC-3 showed singlet of phenolic proton at $\delta$ 7.38, a doublet of one proton at 6.92 (1H, d, H-2'), a double doublet of proton at $\delta$ 6.74 (1H, dd, H-6'), doublets at one proton at 6.83 (1H, d, H-5'), having one proton integration. The multiplate of alcoholic hydroxyl proton resonates at 3.97 (1H, m, H-3) (Figure 6.10). The $^{13}$C-NMR spectrum of FMC-3 revealed the presence of fifteen carbon atoms in the molecules. The multiplicity assignments were made by spectra; which showed five CH and ten quaternary carbons (Figure 6.11). Consequently, FMC-3 was identified as catechin (Aher et al., 2009). The EI-MS showed the molecular ion peak at m/z 290 (Figure 6.12) corresponding to the molecular formula C$_{15}$H$_{14}$O$_6$, the fragmentation pattern also matches with standard data confirms identity of FMC-3 as catechin (Aher et al., 2009).
Figure 6.9 FTIR spectrum of FMC-3 (catechin)

IR ν max cm⁻¹ (KBr): 3412-3240 (alcoholic and phenolic -OH), 3059 (aromatic- C-H), 1610 (aromatic C=C), 1514 and 1473 (aromatic ring)

Figure 6.10 ¹H NMR spectrum of FMC-3 (catechin)

¹H NMR (CDCl₃) (300 MHz): δ 2.52 (1H, dd, H-4a), 2.93 (1H, dd, H-4e), 3.97 (2H, m, H-3), 4.60 (2H, d, H-2), 5.82 (2H, d, H-6, 8), 6.01 (1H, d, H-8), 6.74 (2H, dd, H-6', 8'), 6.83 (1H, d, H-5'), 6.92 (1H, d, H-2'), 7.38 (s, phenolic protons)
Figure 6.11 $^{13}$C NMR spectrum of FMC-3 (catechin)

$^{13}$C-NMR (CDCl$_3$) (75MHz): $\delta$ 27.14 (C-4), 67.74 (C-3), 81.39 (C-2), 94.73 (C-8), 95.81 (C-6), 99.47 (C-10), 114.07 (C-2'), 115.11 (C-5'), 130.3 (C-1'), 144.66 (C-3'), 144.79 (C-4'), 155.49 (C-5), 156.15 (C-9), 156.48 (C-7).

Figure 6.12 EI-MS spectrum of FMC-3 (catechin)

EI-MS m/z (rel int. %): 290, 194, 168, 153, 138 (100), 124, 110.
6.3.3 Structure elucidation of \( p \)-hydroxycinnamic acid (FMC-4)

The UV \( \lambda_{\text{max}} \) spectrum of FMC-4 showed absorption band at 310 nm, indicating the presence of aromatic nucleus in the molecule. The IR spectrum showed the absorption bands at 3358 (Phenolic- OH), 2843 (acidic- OH), 1666 (Carbonyl- C=O), 1462 (aromatic/alkene C=C) (Figure 6.13). The \(^1\)H-NMR spectrum of FMC-4 displayed two resonances in the aromatic region at \( \delta \) 7.78 (2H, d, H-2, 6), 6.91 (2H, d, H-3, 5), and a doublets of one proton at 6.31 (1H, d, H-2'; \( \alpha \)-position) and 7.31 (1H, d, H-1' \( \beta \)-position) noted (Figure 6.14). The \(^{13}\)C-NMR spectrum of FMC-3 revealed the downfield signals at \( \delta \) 169.83 and 156.70 were assigned to acid carbonyl and aromatic oxygenated quaternary carbon atoms whereas other signals in the aromatic region at \( \delta \) 131.06, 118.15 and 128.89 were assigned to aromatic methanes and aromatic quaternary carbon atoms. The signals for \( \alpha-\beta \) unsaturated carbon were observed at \( \delta \) 141.01 and 116.36 (Figure 6.15). The EI-MS of FCM-3 gave the molecular ion peak at \( m/z \) 165 corresponding to the molecular formula \( \text{C}_9\text{H}_8\text{O}_3 \) (Figure 6.16). Comparison of physical and spectral data with that reported in the literature (Chiang et al., 2003) identified FMC-3 as \( p \)-hydroxycinnamic acid.
Figure 6.13 FTIR spectrum of FMC-4 ($p$-hydroxycinnamic acid)

**IR ν max cm$^{-1}$ (KBr):**
- 3358 (Phenolic- OH)
- 2843 (acidic- OH)
- 1666 (carbonyl- C=O)
- 1462 (aromatic/alkene C=C).

Figure 6.14 $^1$H NMR spectrum of FMC-4 ($p$-hydroxycinnamic acid)

$^1$H NMR (CDCl$_3$) (300 MHz): δ 6.31 (1H, d, H-2'; a-position), 6.91 (2H, d, H-3, 5), 7.31 (1H, d, H-1' b-position), 7.78 (2H, d, H-2, 6).
Figure 6.15: $^{13}$C NMR spectrum of FMC-4 ($p$-hydroxycinnamic acid)

$^{13}$C-NMR (CDCl$_3$) (75MHz): $\delta$ 169.83 (C-3'; -COOH), 156.70 (C-4), 141.01 (C-2'; a-position), 131.06 (C-2, 6), 128.89 (C-1), 118.15 (C-3, 5), 116.36 (C-1'; b-position).

Figure 6.16 EI-MS spectrum of FMC-4 ($p$-hydroxycinnamic acid)

EI-MS m/z (rel int. %): 165 (100), 147, 119, 91, 77, 65.
6.3.4 Structure elucidation of isolated compounds from *L. amara*

### 6.3.4.1 Structure elucidation of LAC-1 (luteolin)

Compound LAC-1 was isolated as a yellow amorphous solid from the ethanolic extract of *L. amara* pericarp. The molecular ion peak in the EI-MS at m/z 286 is in agreement with the molecular formula C\(_{15}\)H\(_{10}\)O\(_6\) (Figure 6.20).

![Luteolin](image)

The \(^1\)H-NMR spectra displayed singlet at \(\delta 6.62\), which was assigned to C-8 proton of the flavone skeleton. Two doublets at \(\delta 6.82\) (1H, d, H-5'), 7.42 (1H, dd, H-6') were assigned to the two aromatic protons of the ring attached to C-2. Two sharp singlet at \(\delta 11.79\) and 9.41 showed the presence of OH-5 and OH-7, respectively (Figure 6.18). The \(^{13}\)C-NMR spectra disclosed the presence of fifteen carbon atoms. The chemical shift of C-5 at 156.15 supported the presence of phenolic group at this position (Figure 6.19). IR spectrum shows the absorption band at 3404, 3325, 3290 (alcoholic and phenolic- OH), 3159 (aromatic- CH), 1664 (ketone- C=O), 1610 cm\(^{-1}\) (aromatic- C=C) (Figure 6.17). A careful comparison of the UV, IR, EI-MS, \(^1\)H-NMR, \(^{13}\)C-NMR data with the reported data indicated that it confirmed as luteolin (Saeidnia et al., 2009; Patora and Klimek, 2002).
IR ν max cm\(^{-1}\) (KBr): 3404, 3325, 3290 (alcoholic and phenolic- OH), 3159 (aromatic- CH), 1664 (ketone- C=O), 1610 (aromatic- C=C).

\(^1\)H NMR (CDCl\(_3\)) (300 MHz): \(\delta\) 6.18 (1H, d, H-6), 6.37 (H, d, H-8), 6.62 (1H, s, H-3), 6.82 (1H, d, H-5\(^\prime\)), 7.42 (1H, dd, H-6\(^\prime\)), 9.41 (1H, s, 7-OH), 11.79 (1H, s, 5-OH).
Figure 6.19 $^{13}$C NMR spectrum of LAC-1 (luteolin)

$^{13}$C-NMR (CDCl$_3$) (75MHz): δ 94.07 (C-8), 99.21 (C-6), 103.36 (C-3), 104.17 (C-10), 114.07 (C-2'), 115.11 (C-5'), 119.16 (C-6'), 130.30 (C-1'), 144.66 (C-3'), 148.15 (C-4'), 155.49 (C-9), 156.15 (C-5), 162.37 (C-2), 164.10 (C-7), 181.11 (C-4).

Figure 6.20 EI-MS spectrum of LAC-1 (luteolin)

EI-MS m/z (rel. int. %): 286 (100); 241, 179, 161, 153, 135, 123, 117.
6.3.4.2 Structure elucidation of LAC-3 (gallic acid)

The IR spectrum showed the absorption bands at 3358 (Phenolic –OH), 1701 (Acidic C=O), 1448 (aromatic C=C) cm\(^{-1}\) (Figure 6.21).

![Gallic acid diagram]

The \(^1\)H-NMR spectrum of LAC-3 displayed only singlet in aromatic region at δ 7.26 (2H, s, H-2, 6) (Figure 6.22). The \(^{13}\)C-NMR spectrum of LAC-3 disclosed the presence of five carbon signals for one methane and four quaternary carbon atoms. The downfield signals at δ 170.72, 163.51 and 133.72 were assigned to acid carbonyl and aromatic oxygenated quaternary carbon atoms whereas other signals in the aromatic region at δ 116.24 and 122.59 were assigned to aromatic methane and aromatic quaternary carbon atoms (Figure 6.23). The EI-MS of LAC-3 gave the molecular ion peak at m/z 171 corresponding to the molecular formula C\(_7\)H\(_6\)O\(_5\) (Figure 6.24). The melting point, UV \(\lambda_{\text{max}}\), along with above data was compared with the literature (Eldahshan, 2011) and showed complete agreement to those of gallic acid.
**IR ν max cm⁻¹ (KBr):** 3358 (phenolic –OH), 1701 (acidic C=O), 1448 (aromatic C=C).

**1H NMR (CDCl₃) (300 MHz):** δ 7.26 (2H, s, H-2, 6).
Figure 6.23 $^{13}$C NMR spectrum of LAC-3 (gallic acid)

$^1$H NMR (CDCl$_3$) (300 MHz): $\delta$ 170.72 (C-7), 163.51 (C-3,5), 133.72(C-4), 122.59 (C-1) and 116.24 (C-2,6).

Figure 6.24 EI-MS spectrum of LAC-3 (gallic acid)

EI-MS m/z (rel int. %): 171 (100), 152, 127, 108, 69, 52.
6.3.5 HPTLC standardization of bioactive EA-FMB an EO-LAP with isolated compounds

Herbal medicines are regaining their position in modern world. The global demand for herbal medicines is increasing exponentially. Despite of long traditional use and time tested therapeutic applications; traditional herbal medicines are not getting accepted in modern scientific world because of lack of standardization. Therefore, it is always advisable to identify and estimate the active constituents with adequate analytical tool which will ensure the quality and purity of crude drug. These analytical tools should withstand with the complexity of natural product mixture (Nicoletti, 2011). Traditional medicinal plants only accepted in modern scientific world through the standardization, identification and documentation of their active phytoconstituents. Because of simplicity, HPTLC has become a very important tool to assess quality, purity, stability, and identity of the complex botanical entities. In the present study, isolated phytoconstituents were quantified in respective extracts of the selected medicinal plants using HPTLC technique.

6.3.5.1 HPTLC estimation of oleanolic acid, catechin and \( p \)-hydroxycinnamic acid in ethyl acetate extract of *F. Microcarpa* bark

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<td>1.72</td>
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<tr>
<td>Catechin</td>
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<td>99.6</td>
</tr>
<tr>
<td>EA-FMB</td>
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<td>280.6</td>
<td>1.94</td>
</tr>
<tr>
<td>( p )-hydroxycinnamic acid</td>
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<td>1562.2</td>
<td>100</td>
</tr>
<tr>
<td>EA-FMB</td>
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<td>229.4</td>
<td>3.66</td>
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</tbody>
</table>

EA-FMB- Ethyl acetate extract of *F. microcarpa*
Figure 6.25 Chromatogram of oleanolic acid, catechin, \( p \)-hydroxycinnamic acid and EA-FMB extract

Department of Pharmacognosy, RCPIPER, Shirpur
6.3.5.2 HPTLC estimation of luteolin gallic acid in ethanol extract of *L. amara* pericarp

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<th>Extract / marker</th>
<th>Rf</th>
<th>AUC</th>
<th>%Content</th>
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<tr>
<td>EO-LAP</td>
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<td>379.6</td>
<td>2.37</td>
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</table>

EO-LAP- Ethanol extract of *L. amara*

Figure 6.26 Chromatogram of luteolin, gallic acid and EO-LAP extract
Identification of the major compounds in the herb or a herbal preparation will be helpful in elucidating pharmacological activity and the underlying mechanisms of action. In present study, the bioactivity guided fractionation, isolated oleanolic acid, catechin, \( p \)-hydroxycinnamic acid from EA-FMB extract. Similarly, luteolin, and gallic acid were isolated from EO-LAP extract.

**Figure 6.27** Schematic presentation of hepatoprotective mechanism of *F. microcarpa* and *L. amara* extracts; MO-micro organism (Huang et al., 2011; Yadav et al., 2008; Jaeschke et al., 2013; Kass, 2006; Ji, 2012)
The compounds from EA-FMB have definite role in hepatoprotection. The earlier studies demonstrated that oleanolic acid involved in the inhibition of toxicant activation and enhancement of the body defence systems. In addition, it has exhibited hepatoprotective activity via modulation of its antioxidant (Elekofehinti et al., 2012) and anti-inflammatory activities (Akkol et al., 2007). The polyphenols like catechin, and p-hydroxycinnamic acid were well documented for free radical scavenging, antioxidant and hepatoprotective potential (Bharrhan et al., 2011).

Similarly, the flavonoids and phenolics from EO-LAP i.e. luteolin and gallic acid have strong antioxidant and hepatoprotective potential. (Qiusheng et al., 2004; Anand et al., 1997)

The few literature were established the molecular mechanism of these phytoconstituents. The plant phenolic like luteolin (Wu et al., 2004), catechin (Bharrhan et al., 2011), p-hydroxycinnamic acid, gallic acid and oleanolic acid (Wu et al., 2004) have cytoprotective effects through nuclear factor-E2-related factor 2 (Nrf2). This has been extensively proven to be a strong activator of the antioxidant-responsive element (ARE)-mediated gene expression pathway (Tanigawa et al., 2007, Chen, et al., 2000). This Nrf2/ARE pathway has been characterized as an important endogenous mechanism for combating oxidative stress by activation of endogenous antioxidants and phase II detoxifying enzymes (Aleksunes et al., 2008).

The results of present study and reports on isolated and identified phytoconstituents suggests, hepatoprotective activity of *F. microcarpa* and *L. amara* might be due to its free radical scavenging ability, up regulation of endogenous antioxidant system (may be through Nrf2/ARE pathway) and inhibition of inflammatory responses. Furthermore, these plants promote the host defence mechanism to combat infective liver diseases through immunomodulatory activity.
6.4 Conclusion

In Indian systems of medicines, the integer of medicinal plants is used for successful treatment of chronic diseases. The very few work has established the correlation between therapeutic activity and chemical constituents present in the plant. In present research, the two plants that are *F. microcarpa* and *L. acutangula var. amara* was selected based on their traditional medicinal claims. Despite of strong recommendation of these medicinal plants in treatment of liver diseases, the plant was neither evaluated for pharmacological activity or phytochemistry. Our study clearly demonstrates that *F. microcarpa* and *L. amara var. amara* possess a significant hepatoprotective effect in chronic and acute hepatotoxicity studies, additionally shown to have potent anti-inflammatory and immunomodulatory activity. The isolation of possible bioactive constituents from *F. microcarpa* bark yields oleanolic acid as triterpenoids and phenolics like catechin and *p*-hydroxycinnamic acid; while *L. amara* pericarp yields flavonoids and phenolics that is luteolin and gallic acid. For the first time, our study reported the presence of luteolin and gallic acid from *L. amara* pericarp, further their correlation with hepatoprotective activity in this plant. With strong literature support, we can claim that isolated phytoconstituents are responsible for pharmacological activities.
6.5 References


