Chapter 4

RESULTS AND DISCUSSION
4. RESULTS AND DISCUSSION

4.1. Morphology studies

The procured plant material was studied with the help of sensory organs to evaluate its morphology and is tabulated in table no. 4.1.

Table 4.1. Organoleptic characteristic of the stem bark

<table>
<thead>
<tr>
<th>Description</th>
<th>Stem bark</th>
<th>Powdered Stem bark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Externally dark brown</td>
<td>Pinkish brown</td>
</tr>
<tr>
<td></td>
<td>and internally light brown</td>
<td></td>
</tr>
<tr>
<td>Odour</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Taste</td>
<td>Astringent</td>
<td>Astringent</td>
</tr>
<tr>
<td>Shape</td>
<td>Curved</td>
<td>Coarse powder</td>
</tr>
<tr>
<td>Surface</td>
<td>Longitudinally wrinkled</td>
<td>....................</td>
</tr>
</tbody>
</table>

4.2. Extractability percentage

The dried powder of stem bark (*Careya arborea*) was subjected to ethanolic, hydroalcoholic and aqueous extraction. The colour, consistency and extractability percentage of the extracts were calculated on the air dried basis.
Table 4.2. Physical characteristics and extractability percentage of *Careya arborea*

<table>
<thead>
<tr>
<th>Content</th>
<th>Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanolic</td>
</tr>
<tr>
<td>Solvent Used</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Quantity (powdered bark)</td>
<td>200 gm</td>
</tr>
<tr>
<td>Color</td>
<td>Dark reddish brown</td>
</tr>
<tr>
<td>Consistency</td>
<td>Solid</td>
</tr>
<tr>
<td>Extractability</td>
<td>89.5%</td>
</tr>
</tbody>
</table>

4.3. **Ash value**

Ash value is the residue left after incineration, which indicates the amount of carbonates, silicates and other organic matter. Also consider to be one of the criteria for evaluating powdered crude drugs.

Table 4.3. Ash value of *Careya arborea* stem bark

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Parameter</th>
<th>Value (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Physiological ash</td>
<td>6.5</td>
</tr>
<tr>
<td>2.</td>
<td>Acid insoluble ash</td>
<td>1</td>
</tr>
<tr>
<td>3.</td>
<td>Water soluble</td>
<td>5</td>
</tr>
<tr>
<td>4.</td>
<td>Sulphated ash</td>
<td>0.8</td>
</tr>
</tbody>
</table>
4.4. **Foaming index**

Froth formation is the specific property of pentacyclic saponins, which is indicative of their detergent property. In the present study the height of the foam is measured with scale and found that the test tube containing 7 ml of decoction shows persistent 1 cm height of foam.

<table>
<thead>
<tr>
<th>Decoction (in ml)</th>
<th>Height of foam (in cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>0.6</td>
</tr>
<tr>
<td>6</td>
<td>0.6</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>0.9</td>
</tr>
<tr>
<td>9</td>
<td>0.9</td>
</tr>
<tr>
<td>10</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Foaming Index is calculated by using the formula as:

\[ FI = \frac{1000}{a} \]

Where ‘a’ volume of decoction in test tube containing 1cm of height of foam

Foaming index = 142.85

4.5. **Phytochemical screening**

The isolated extracts from the stem bark of *Careya arborea* were treated with various reagents for identification of the secondary metabolites. The results
observed were concluded with presence (+) and absence (−) of phytoconstituents of different classes.

Table 4.5. Phytochemical screening of different extracts of Careya arborea

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>Ethanolic extract</th>
<th>Hydro alcoholic extract</th>
<th>Water extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Cardiac glycoside</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) means present and (−) sign means absent

4.6. Microscopic study

Different plant parts possess varying cellular characteristics which can be identified or visualized by performing microscopic studies. It can be performed either by transverse section or by powder studies.

4.6.1. Transverse section of stem bark

Transverse section of stem bark showed 4-5 layers of cork. Beneath the cork, multilayer’s of cortex was present, the cells of which are polygonal parenchyma. Cortex was further followed by phelloderm. In the ground tissue region, pericycle fibres, prominent vessel and medullary rays were
present. Medullary rays were mostly biseriate and rarely multiseriate and present in association with fibres. Phloem region also showed the presence of starch grains and calcium oxalate crystals. Vessels were also present in abundance.

![Image](image_url)

**Fig. 4.1: TS of stem bark of *Careya arborea***

### 4.6.2. Powder microscopy

Powder microscopy of the *Careya arborea* stem bark was carried out and showed peculiar characteristic of lecythidaceae family. Abundance of calcium oxalate crystals were viewed in different shapes of crystal like prism, needle s, but the most common are in rosette form. Starch grains are very few and spherical in shape, either scattered freely or in conjunction with parenchyma. Yellow colored pigment cells were also seen. Cork cells showed different geometry in different views. Sclereids, vessels and fibers were also present. Conducting element medullary rays were observed in bi-seriate or tri-seriate forms. They exist either alone or in fusion with phloem fibres.
Fig. 4.2: Powder characteristics of stem bark of *Careya arborea*

1) Cork cells in surface view; 1a) Cork cells with embedded brownish yellow pigments; 1b) Cork cells in sectional view; 2) & 2a) Acicular crystals; 3) & 3a) Stone cells; 4) Perforated conducting vessels; 5) Fibre; 6) Starch grains; 7) Three sided parenchyma with starch grains; 8) Polygonal parenchymatous cells
Fig. 4.2a: Powder characteristics of stem bark of *Careya arborea*

9) conducting elements; 10) Sclereids; 11) &11a) Collenchyma with stone cells; 12) Phloem fibre tangential-medullary rays along with crystal fibres and brown color pigment; 13) Prismatic crystals; 14) &14a) Medullary rays embedded in phloem fibres and yellow pigment matter; 15) lignified crystal fibre
4.7. Fluorescence analysis

The coarse powder of the stem bark is treated with different solvents and their fluorescence was checked under normal day light, short UV and Long UV.

Table 4.6. Fluorescence characteristics of the stem bark powder

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Visible light</th>
<th>Under UV light</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Short wavelength (254 nm)</td>
</tr>
<tr>
<td>Powder</td>
<td>Pinkish grey</td>
<td>Brown</td>
</tr>
<tr>
<td>Powder + 1N NaOH (aq.)</td>
<td>Rusty brown</td>
<td>Dark brown with green tint</td>
</tr>
<tr>
<td>Powder + Ammonia</td>
<td>Light brown</td>
<td>Pale brown</td>
</tr>
<tr>
<td>Powder + Picric acid</td>
<td>Yellowish brown</td>
<td>Yellowish brown</td>
</tr>
<tr>
<td>Powder + Pet. ether</td>
<td>Light brown</td>
<td>Greenish brown</td>
</tr>
<tr>
<td>Powder + 50% HCl</td>
<td>Light brown</td>
<td>Greenish brown</td>
</tr>
<tr>
<td>Powder + 50% H_2SO_4</td>
<td>Dark yellowish brown</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Powder + HCl</td>
<td>Reddish brown</td>
<td>Brown</td>
</tr>
<tr>
<td>Powder + H_2SO_4</td>
<td>Black</td>
<td>Black</td>
</tr>
<tr>
<td>Powder + 5% KOH</td>
<td>Reddish brown</td>
<td>Brown</td>
</tr>
</tbody>
</table>
4.8. Biological activity

4.8.1 Anti-oxidant activity

Prepared extracts were studied for anti-oxidant activity by DPPH assay at different concentrations ranging from 25-100 µl/ml. Absorbance was recorded spectrophotometrically and readings were taken in triplicate. It was observed that hydroalcoholic and aqueous extracts of stem bark showed dose dependent response of percentage inhibition whereas the alcoholic extract does not showed any trends in comparison to standard rutin.

![Effect of ethanolic extract of Careya arborea in comparison to Rutin](image1)

![Effect of hydroalcoholic extract of Careya arborea in comparison to Rutin](image2)
Fig. 4.5: Effect of aqueous extract of *Careya arborea* in comparison to Rutin

### 4.8.2. Preliminary in vitro anti-cancer activity

The previously prepared extracts after studying antioxidant potential were screened for in vitro anti-cancer activity against PC-3 cell lines at different concentration viz. 25 µg, 50 µg and 100 µg. It was found that the ethanol extract of the drug lowers down the viability rate of cell but that does not form any pattern in accordance with concentration, whereas aqueous extract does not give any reduction pattern in the count of viable cells, inspite the inhibition remains uniform at all concentrations. Interestingly the hydro alcoholic extract shows marked activity and inhibits the % age viability of cells in concentration dependent manner. As the concentration increases the number of viable cells keeps on reducing. Hence, it followed the dose dependant format.
Fig. 4.6: Percentage cell viability of ethanolic, hydroalcoholic and aqueous extract of *Careya arborea* against PC-3 cell lines

4.8.3. Antimicrobial activity

The prepared extracts were screened for *in vitro* antimicrobial activity against one strain for each gram positive and negative as well as against fungus by following microdilution method. Dilutions of different concentrations (1, 2, 4, 8, 16, 32, 64, 128, 256 µl/ml) were prepared and examined for the absence of turbidity which indicates the antimicrobial action.
Table 4.7. MIC for different extracts of *Careya arborea* against microbial strains

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Nature</th>
<th>Ethanolic extract (MIC in μg/ml)</th>
<th>Hydroalcoholic extract (MIC in μg/ml)</th>
<th>Aqueous extract (MIC in μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Gram positive</td>
<td>16</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Gram negative</td>
<td>64</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>Fungus</td>
<td>128</td>
<td>128</td>
<td>-</td>
</tr>
</tbody>
</table>

Hydroalcoholic extract was found to be effective in anti-oxidant, anti-cancer and antimicrobial studies in a particular fashion so the extract is selected and fractionation is done with different solvents of varying polarities like petroleum ether, ethyl acetate, ethanol and aqueous fractions.

4.9. Physical appearance of different fractions

Different fractions obtained by treating hydroalcoholic extract with solvents are then concentrated and their physical appearance was examined.

Table 4.8. Physical appearance of different fractions of hydroalcoholic extract

<table>
<thead>
<tr>
<th>Fractions (EWCA extract)</th>
<th>Color</th>
<th>Consistency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether (PECA)</td>
<td>Pale greenish</td>
<td>Resinous</td>
</tr>
<tr>
<td>Ethyl acetate (EWCA)</td>
<td>Brown</td>
<td>Solid mass</td>
</tr>
<tr>
<td>Ethanol (ECA)</td>
<td>Brown colour</td>
<td>Sticky</td>
</tr>
<tr>
<td>Aqueous fractions (WCA)</td>
<td>Reddish brown</td>
<td>Powdery</td>
</tr>
</tbody>
</table>
4.10. Antioxidant activity of different fractions by DPPH and ABTS assay

Fractions prepared from main hydroalcoholic extract are then subjected to DPPH and ABTS assay separately for accessing its anti-oxidant potential.

Table 4.9. Antioxidant activity of test substances for DPPH radical

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Test Substance</th>
<th>Concentration (μg/ml)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ECA</td>
<td>25</td>
<td>89.13 + 0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>89.28 + 3.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>90.31 + 2.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>91.55 + 0.30</td>
</tr>
<tr>
<td>2.</td>
<td>PECA</td>
<td>25</td>
<td>47.68 + 1.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>66.15 + 1.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>85.31 + 0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>88.65 + 1.54</td>
</tr>
<tr>
<td>3.</td>
<td>EWCA</td>
<td>25</td>
<td>90.03 + 0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>92.72 + 0.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>95.31 + 0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>96.88 + 0.75</td>
</tr>
<tr>
<td>4.</td>
<td>WCA</td>
<td>25</td>
<td>67.09 + 0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>68.47 + 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>56.5 + 0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>60.94 + 0.15</td>
</tr>
</tbody>
</table>
Fig. 4.7: Graphical representation of DPPH inhibitory activity for ethanol fraction

Fig. 4.8: Graphical representation of DPPH inhibitory activity for ethyl acetate fraction
Fig. 4.9: Graphical representation of DPPH inhibitory activity for petroleum ether fraction

Fig. 4.10: Graphical representation of DPPH inhibitory activity for aqueous fraction
Table 4.10. The antioxidant activity of test substances for ABTS radical

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Test Substance</th>
<th>Concentration (µg/ml)</th>
<th>% Inhibition</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ECA</td>
<td>1000</td>
<td>91.44 + 0.46</td>
<td>144.40 + 1.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>85.73 + 0.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>75.22 + 2.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>45.30 + 3.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>29.92 + 1.59</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>PECA</td>
<td>1000</td>
<td>90.82 + 2.69</td>
<td>139.48 + 0.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>77.16 + 1.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>68.94 + 1.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>47.49 + 0.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>47.49 + 0.82</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>EWCA</td>
<td>1000</td>
<td>57.33 + 0.02</td>
<td>634.70 + 0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>47.28 + 0.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>33.80 + 0.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>18.37 + 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>15.29 + 0.18</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>WCA</td>
<td>1000</td>
<td>94.65 + 0.08</td>
<td>123.01 + 0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>93.01 + 0.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>72.67 + 1.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>50.52 + 0.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>34.92 + 0.50</td>
<td></td>
</tr>
</tbody>
</table>
**Fig. 4.11:** Graphical representation of ABTS inhibitory activity for ethanolic fraction

**Fig. 4.12:** Graphical representation of ABTS inhibitory activity for Petroleum ether fraction
Results showed that petroleum ether fraction of hydro alcoholic extract exhibited dose dependent inhibitions in both DPPH and ABTS and it follows trends. The prepared fractions are then screened for cytotoxicity on different cell lines.
4.11. Anti cancer activity of different fractions

Human cancer-derived cell lines are the most widely used models to study the

Fig. 4.15: Cytotoxicity of ECA, PECA, EWCA and WCA on Hep G2 and MCF - 7 cell lines
biology of cancer and to test hypotheses to improve cancer treatment. The test substances ECA, PECA, EWCA and WCA were tested for \textit{in vitro} cytotoxicity studies against Human Breast Cancer (MCF - 7) and Human Liver Cancer (HepG2) cells by MTT assay exposing the cells to different concentrations of test substances. The test substances were taken at concentration ranging from 1000 μg/ml to 62.50 μg/ml to determine the percentage growth inhibition on the cell lines MCF-7 and HepG2.

Table 4.11. Cytotoxic properties of test substances against MCF - 7 cell lines

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Name of Test Substance</th>
<th>Test Conc. (μg/ml)</th>
<th>% Cytotoxicity</th>
<th>CTC50 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ECA</td>
<td>1000</td>
<td>23.30±2.2</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>17.24±0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>11.13±1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>4.81±0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>1.57±1.1</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>PECA</td>
<td>1000</td>
<td>81.91±0.9</td>
<td>543.94±3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>68.66±0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>52.03±1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>48.74±1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>35.65±0.2</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>EWCA</td>
<td>1000</td>
<td>45.77±1.0</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>32.50±1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>28.84±0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>10.71±0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>3.24±1.2</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>WCA</td>
<td>1000</td>
<td>43.47±1.1</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>23.82±1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>19.07±1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>13.69±1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>6.27±3.0</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.12. Cytotoxic properties of test substances against HepG2 cell lines

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Name of Test Substance</th>
<th>Test Conc. (µg/ml)</th>
<th>% Cytotoxicity</th>
<th>CTC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ECA</td>
<td>1000</td>
<td>43.08±1.1</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>39.29±0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>25.54±0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>19.60±1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>7.85±2.1</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>PECA</td>
<td>1000</td>
<td>55.82±0.2</td>
<td>775.55±4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>38.12±0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>25.11±1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>15.40±0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>10.29±1.2</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>EWCA</td>
<td>1000</td>
<td>43.50±1.0</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>33.87±1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>28.59±0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>22.51±0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>4.46±1.2</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>WCA</td>
<td>1000</td>
<td>52.20±0.4</td>
<td>938.96±3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>34.24±1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>29.63±1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>17.34±0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>6.38±1.4</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 4.16: Graphical representation of cytotoxic effect of ECA, PECA, EWCA and WCA on MCF-7 and HepG2 cell lines respectively
4.12. Antimicrobial activity of different fractions

The test substances ECA, PECA, EWCA and WCA were tested for in vitro antimicrobial studies. The microbial stain belongs to gram positive and gram negative strains for bacteria and one strain for fungus.

Table 4.13. Anti microbial effect of different fractions on microbial strains

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Nature</th>
<th>MIC in μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PECA</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Gram positive</td>
<td>16</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Gram negative</td>
<td>32</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>fungus</td>
<td>64</td>
</tr>
</tbody>
</table>

4.13. Isolation of compounds from bioactive fraction

Biological screening revealed that petroleum ether portion exhibited dose dependent pattern in free radical scavenging as well as in inhibiting viable cell count and antimicrobial activity. The same fraction is put forward for the isolation of active phytoconstituents by using column chromatography. This technique is adsorption based separation of components depending upon their affinities to bind with stationary phase and getting eluted with mobile phase. The column of stationary phase (Silica) is eluted first with pure petroleum ether and then by gradually increasing the polarity of mobile phase. Each fraction of 150 ml is collected in drop wise manner and solvent is recovered to concentrate the fraction. TLC of each fraction is performed by using suitable solvent system and ceric ammonium sulphate is used as visualizing agent. Firstly plates were viewed under UV chamber at short UV (254 nm) and at long UV (365 nm). Afterwards precoated silica plates were sprayed and kept in oven which is previously
maintained at 100 °C to get the plates charred and the spots become prominent. TLC plates with similar profile are pooled together. A total of 20 fractions were collected. The plate no. 11-16 showed same TLC pattern consisting of two prominent spots. The solvent system used was petroleum ether: ethyl acetate (9:1).

All these fractions were pooled together and subjected to another column for more precise separation. Column is eluted firstly with pure petroleum ether and then increasing the polarity to 1% ethyl acetate in petroleum ether and so on. Fraction volume is adjusted to 25 ml, solvent is recovered and TLC is run for each fraction. TLC plates with similar profile were pooled together. Solid residue is observed in fraction number 5, 6 from sub-column when eluted with 2% EA in PE. They were pooled together and excess of solvent is added and kept overnight. Solid gets settled at the bottom and separated by decantation and subjected to TLC. The chromatogram shows single spot after spraying visualizing agent. The chromatographic parameters are:

**Solvent system**: Ethyl acetate: Petroleum ether (1:9)

**Spraying agent**: Ceric ammonium sulphate

This compound is marked as CA 2.1

The polarity of the column is further increased and another compound CA 2.2 is isolated from column in 4% EA in PE in fraction 10-12 (pooled).

**Solvent system**: Ethyl acetate: Petroleum ether (3:7)

**Spraying agent**: Ceric ammonium sulphate

Both the isolated compounds were characterized by using spectroscopic techniques like UV spectroscopy, IR, mass spectrometry, C\textsuperscript{13} and H\textsuperscript{1}-NMR.

**4.14. Characterization of the isolated compound by spectral analysis**

The compound CA 2.1 is white needle shaped crystals, having melting point 314-317 °C and insoluble in water and soluble in DMSO. UV absorption spectra show a degree of unsaturation.
Mass spectroscopy (MS) was carried out to determine the molecular weight of isolated phytoconstituents. The MS spectra of CA2.1 is shown in Figure 4.20 and depicts the molecular ion (M+1) peak was obtained at 410.38 m/z which confirms molecular weight of CA 2.1 at 410.38

IR spectrum of isolated compound CA 2.1 gave characteristic signals of groups present in the isolated compound like, IR (KBr, cm \(^{-1}\)) displayed a band at 2926 indicated C-H stretching (due to sp\(^2\) hybridization), 2852 and 2872 (C-H stretching of methylene group due to sp\(^3\) hybridization), C=O stretching vibration of aliphatic acid absorb near the 1732, 1024-1029 (C-O stretching), Sharp peak of tetra substituted benzene ring was observed at 804 cm\(^{-1}\).

Proton NMR spectroscopy was used for the confirmation of structure of isolated compound. NMR gave characteristic signals of protons present in the isolated compound, according to which: \(^1\)H NMR (CDCl\(_3\) at 300 MHz): \(\delta\) 4.16, 4.13, 4.11, and 4.09 (quatret), \(\delta\) 2.049 (singlet, R-CH-CH=O), \(\delta\) 1.706 (singlet, alkylene), \(\delta\) 1.285, 1.261, 1.252, 1.237 (quatret, R-CH\(_2\)-R ; long chain of methylene absorption), \(\delta\) 0.873, 0.866, 0.851, 0.843, 0.834, 0.829, 0.785 (septet, methyl groups) ppm.

\(^{13}\)C NMR (CDCl\(_3\) at 200 MHz) absorption peaks are shown in the region 77, 77.31, 77.107, 77.79, 60.48 (-O-CH\(_2\)), 29.77 (R\(_3\)- C-H), 21.40, 21.13 (R\(_2\)-CH\(_2\)), 14.26 (R-CH\(_3\))

Compound CA 2.2 is white solid, insoluble in water and soluble in DMSO. UV absorption spectra show presence of degree of unsaturation. The mass spectral data of the compound gave m/z 410.38

IR (KBr, cm \(^{-1}\)) spectrum for CA 2.2 displayed a band at 1734 (C=O stretch), 1649 (C=C stretching), 1458-1375 (alkyl C-H stretch), 1248 (C-H bend overtone), 741 cm\(^{-1}\) (-C-H bending, C-O- stretching, primary alcohol).

\(^1\)H NMR (CDCl\(_3\) at 300 MHz): \(\delta\) 4.158, 4.134, 4.110, and 4.086 (quatret), \(\delta\) 2.049 (singlet, R-CH-CH=O), \(\delta\) 1.706 (singlet, alkylene), \(\delta\) 1.285, 1.261, 1.237
(triplet, R-CH$_2$-R; methylene absorption), $\delta$ 0.874, 0.852, 0.844, 0.834 (quatret, methyl groups) ppm.

$^{13}$C NMR (CDCl$_3$ at 200 MHz) absorption peaks are shown in the region 77.431, 77.316, 77.113, 77.922, 76.795 (R-CH$_2$-O), 60.468 (-O-CH$_2$), 31, 30.987 (R$_3$-CH), 21.118 (R$_2$-CH$_2$), 14.259 (R-CH$_3$)

Fig. 4.17: C$^{13}$ NMR of compound CA 2.1
Fig. 4.18: $^1$H NMR of compound CA 2.1

Fig. 4.19: Infra-red spectra of compound CA 2.1
CHAPTER 4

RESULTS AND DISCUSSION

Fig. 4.20: Mass spectra of compound CA 2.1

Fig. 4.21: UV spectra of compound CA 2.1
Fig. 4.22: $^1$H NMR of compound CA 2.2

Fig. 4.23: $^{13}$C NMR of compound CA 2.2
Fig. 4.24: Infra-red spectra of compound CA 2.2

Fig. 4.25: Mass spectra of compound CA 2.2
4.15. Anticancer and anti oxidant activity of isolated compound

*In-vitro* cytotoxicity of the test substances CA 2.1 and CA 2.2 were tested against MCF – 7 (Human Breast Cancer) and HepG2 (Human Liver Cancer) cell lines. The test substances were taken at concentrations ranging from 1000 μg/ml to 62.5 μg/ml to determine the percentage growth inhibition on the cell lines MCF - 7 and HepG2. The test substances CA 2.1 and CA 2.2 exhibited CTC50 value of 955.54±4.4 and >1000 μg/ml respectively on MCF - 7 and whereas on HepG2 cell line CA 2.1 exhibited a CTC50 value of 985.54±4.4 and CA 2.2 exhibited CTC50 value of >1000 μg/ml. Then test substances CA 2.1 and CA 2.2 were evaluated for *in vitro* antioxidant activity by ABTS radical assay using concentrations ranging from 1000 μg/ml to 62.5 μg/ml. CA 2.1 and CA 2.2 exhibited inhibitory activity in dose dependent manner with IC50 values 82.97 ±0.68 and 97.31 ±0.89 μg/ml respectively. For antimicrobial testing none of the isolated compounds showed any inhibition.
Fig. 4.27: Cytotoxicity of CA 2.1 and CA 2.2 on MCF - 7 and HepG2 cell lines respectively
### Table 4.14. Cytotoxic properties of test substances against MCF-7 cell lines

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Name of Test Substance</th>
<th>Test Conc. (μg/ml)</th>
<th>% Cytotoxicity</th>
<th>CTC50 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CA 2.1</td>
<td>1000</td>
<td>51.25±0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>37.36±2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>30.93±0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>28.06±0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>23.62±1.0</td>
<td>955.54±4.4</td>
</tr>
<tr>
<td>2</td>
<td>CA 2.2</td>
<td>1000</td>
<td>40.23±1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>28.16±2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>17.45±1.5</td>
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<td></td>
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<td>125</td>
<td>12.90±1.4</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>3.81±1.2</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

### Table 4.15. Cytotoxic properties of test substances against HepG2 cell lines

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Name of Test Substance</th>
<th>Test Conc. (μg/ml)</th>
<th>% Cytotoxicity</th>
<th>CTC50 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CA 2.1</td>
<td>1000</td>
<td>45.46±1.4</td>
<td></td>
</tr>
<tr>
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<td></td>
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<td>36.10±1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>29.94±0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>21.89±2.8</td>
<td></td>
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<td>62.5</td>
<td>3.53±1.1</td>
<td>985.54±4.4</td>
</tr>
<tr>
<td>2</td>
<td>CA 2.2</td>
<td>1000</td>
<td>47.37±0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>37.34±1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>27.06±0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>18.33±1.2</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>10.11±1.8</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>
Fig. 4.28: Graph of cytotoxic effect of CA 2.1 and CA 2.2 on MCF - 7 cell lines

Fig. 4.29: Graph of cytotoxic effect of CA 2.1 and CA 2.2 on HepG2 cell lines
Table 4.16. Antioxidant activity of isolated compounds for ABTS radical

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Test Substance</th>
<th>Concentration (μg/ml)</th>
<th>% Inhibition</th>
<th>IC50 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CA 2.1</td>
<td>1000</td>
<td>96.42 + 0.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>84.48 + 0.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>71.17 + 0.50</td>
<td>82.97 + 0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>65.10 + 1.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>42.70 + 0.16</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>CA 2.2</td>
<td>1000</td>
<td>90.31 + 0.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>80.25 + 1.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>76.29 + 0.27</td>
<td>97.31 + 0.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>64.31 + 2.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.5</td>
<td>32.06 + 1.90</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 4.30: Graphical representation of ABTS inhibitory activity for CA 2.1

Fig. 4.31: Graphical representation of ABTS inhibitory activity for CA 2.2
4.16. DISCUSSION

To ensure reproducible quality of herbal products, proper standardization of the plant material is utmost essential. Thus in the recent years there has been an emphasis in standardization of medicinal plants of therapeutic potential. Despite the modern techniques, identification and evaluation of plant drugs by pharmacognostical studies is still more reliable, accurate and inexpensive means. According to World Health Organization the macroscopic and microscopic description of a medicinal plant is the initial step in confirming the identity, purity of crude drug and should be carried out before starting any study (Anonymous, 2002). Organoleptic evaluation is a method of qualitative evaluation in which morphological and sensory profiles of the drugs are studied (Kokate, 2007). It gives the easiest and speedy mode to identify and ensure the quality of given drug.

The other evaluation techniques like physical, chemical and biological methods are helpful for proper authentication of crude drugs. The further authentication is carried out by performing anatomical studies. It is performed on appropriate section of selected plant part. The different microscopic features are studied by using TS and also by powder microscopy. Some staining reagents (phloroglucinol, dil. HCl, safranin etc.) also proved to be helpful for determining the chemical nature at cellular level. This study confirms the characters of Careya arborea stem bark with that of lecythidaceae family. The presence of excess of calcium oxalate crystals in different from like raphides, acicular crystals, biseriate medullary rays, lignified fibres confirms the taxonomical relationship.

Physical parameters like moisture content, extractive value, ash value, and foaming index are studied. The loss on drying of plant materials should be determined and the water content is controlled to save the drug from deterioration. This is especially important for materials that absorb moisture easily or deteriorate quickly in the presence of water. The test for loss on drying determines both water and volatile matter (Anonymous, 2002 and Kokate, 2007).
These ash values are important pharmacognostic tool to standardize the crude drugs. The residue remaining after combustion of plant material is the ash content or ash value, which simply represents inorganic salts, naturally occurring in crude drug or adhering to it or deliberately added to it. The ash value was determined by three different methods: total ash, acid-insoluble ash, and water-soluble ash. The total ash method is employed to measure the total amount of material remaining after ignition. This includes both ‘physiological ash’ which is derived from the plant tissue itself, and ‘non-physiological ash’, which is the residue of the extraneous matter adhering to the plant surface. Acid-insoluble ash is a part of total ash and measures the amount of silica present (sand and siliceous matter). Water soluble ash is the water soluble portion of the total ash (Evans, 2002) and estimates the amount of inorganic compound present in the drug. Many herbal drugs have saponins and cause persistent foam when shaken vigorously with water and measured as foaming index. The given drug shows the interminable foam of one cm with seven ml decoction. The foaming index of 142.85 reveals that the high concentration of saponins is present in the drug.

The extracts obtained by exhausting plant materials with specific solvents are indicative of approximate measures of their chemical constituents extracted with respective solvents from a specific amount of air-dried plant material. This parameter is employed for materials for which as yet no suitable chemical or biological assay exists (Anonymous, 1996). The plant material was subjected to preliminary extraction by cold maceration with successive solvent extraction by different solvents to obtain diverse polar and non polar phytoconstituents possessing different solubility pattern. Determination of extractive values give estimate about the amount of active constituent present in given quantity of plant material in respective solvent The ethanol soluble extractive is greater than hydroalcoholic and aqueous extract. The percentage extractability of aqueous, hydroalcoholic and ethanolic extracts found to be 89.5%, 69 and 50%, respectively. These are followed by chemical means of evaluation includes various chemical tests for detection of nature of chemical constituents. The phytochemical screening showed that the ethanol and hydro alcoholic extract
contained alkaloid, glycosides, terpenoids the carbohydrates and the phenolic compounds whereas ethanolic extract was devoid of saponins. The aqueous extract contains saponins, flavonoids and tannins but cardiac glycosides and alkaloids are absent.

Some chemical constituents present in the plant material exhibited fluorescence in day light while others give in ultra violet light (e.g. alkaloids like berberine), which do not visibly fluoresce in daylight. If the substances themselves are not fluorescent, they may often be converted into fluorescent derivatives or decomposition products by applying different reagents. Hence, some crude drugs are often assessed qualitatively by this approach and it is a salient parameter of pharmacognostical evaluation (Gupta, 2006 and Ansari, 2006). The powdered stem bark of Careya arborea gives fluorescence with different reagents as reported in table 4.6.

The therapeutic use of the three extracts is checked by performing antioxidant; anti cancer and antimicrobial activities. The reactive chemicals (free radicals) have the potential to damage the cell components and results into cancer and other health hazards. Antioxidants neutralize free radicals and stop them from causing damage and hence can be proved beneficial for cancer cure. Moreover, DPPH and ABTS methods most acceptable procedures for performing antioxidant studies. Initially the prepared extracts were screened for free radical scavenging activity by DPPH assay and found that hydroalcoholic extract is most effective and shows the trends. The same extracts were further strained for cytotoxic property and the same extract achieved dose dependent inhibition of viable cells. Surprisingly the MIC also seems to be sound for both alcoholic and hydroalcoholic extract but as the hydroalcoholic is common in all activities the same extract was selected to proceed further. The crude extract of plant is subjected to fractionation by suspending in ethanol: water (3:7; v/v) and extracted successively with petroleum ether, ethyl acetate, ethanol and water by using separating funnel. Dried plant extracts of different solvents were stored for future use.
The different fractions ECA, PECA, EWCA, and WCA are tested for antioxidant potential by ABTS assay and exhibited inhibitory activity in dose dependent manner with IC50 values 144.40 ±1.35, 139.48 ±0.89, 634.70 ±0.31, 123.01 ±0.41, µg/ml respectively. These fractions were also studied by DPPH procedure, all the fractions showed inhibition pattern but the results for PECA seemed to be excellent in accordance with dose in both these assays.

Besides that these fractions ECA, PECA, EWCA and WCA are also tested for in vitro cytotoxicity studies against Human Breast Cancer (MCF - 7) and Human Liver Cancer (HepG2) cells by MTT assay exposing the cells to different concentrations of test substances. The test substances ECA and EWCA exhibited CTC50 value of >1000 µg/ml on both MCF - 7 and HepG2 cell line respectively, PECA exhibited CTC50 value of 543.94±3.1µg/ml and 775.55±4.5µg/ml respectively for MCF – 7 and HepG2 whereas WCA exhibited CTC50 value of >1000 µg/ml and 938.96 ±3.9 µg/ml respectively for MCF – 7 and HepG2.

Antimicrobial activity of prepared fractions suggested that WCA the aqueous fraction neither display any antifungal activity against Candida albicans nor against gram positive and gram negative bacteria. While in rest of the fractions EWCA possess mild inhibition whereas PECA and ECA prove to have an inhibitory effect against the bacterial and fungal strains. The MIC is observed at the concentration of 16, 32, 64 µg/ml against Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans respectively for PECA. The concentration of MIC for EWCA is 32, 128, 128 µg/ml and MIC concentration of 32, 32, 128 µg/ml is observed for ECA. Thus, considering the strong therapeutic use of PECA it is significant to investigate the bioactive constituent of petroleum ether fraction of Careya arborea.

The green colored PECA fraction is adsorbed on silica gel and slurry is prepared to introduce into the column. Column is packed with wet packing technique using petroleum ether as mobile phase. Column is run with gradient elution by gradually increasing the polarity by addition of ethyl acetate. Initially pure petroleum ether is used and then 1% ethyl acetate in petroleum ether then with 2% EA in PE and so on. The TLC of fraction 5, 6 showed an intense single
spot with same profile. Both the fractions get combined and kept overnight at room temperature. White coloured solid is obtained and designated as compound CA 2.1. The polarity of the mobile phase is elevated to 4% EA in PE and fractions are collected. The second compound CA2.2 is isolated from fraction 10, 11. On the basis of characterization studies of isolated compounds, it was confirmed that both compounds are closely related and seems to be isomer of each other.

The isolated compounds CA 2.1 and CA 2.2 were also tested for biological activities. In anti oxidant activity two compounds unveil inhibitory activity in dose dependent manner with IC50 values 82.97 ±0.68 and 97.31 ±0.89. For bye CA 2.1 and CA 2.2 exhibited CTC50 value of 955.54 ±4.4 and >1000 μg/ml respectively on MCF - 7 and whereas on HepG2 cell line CA 2.1 and CA 2.2 exhibited a CTC50 value of 985.54±4.4 and >1000 μg/ml respectively. Inspite of this, these compounds also failed to exhibit inhibitory activity on any of the microbial strains.