5. Isolation of active fraction(s) from Annona squamosa. L leaf by chromatographic techniques, physical data interpretation.

5.1. Introduction.

The ethanol leaf extract of Annona squamosa. L is found to be active against antioxidant (Free radical scavenging) activity.

Based on antioxidant (Free radical scavenging) activity results an effort was made to isolate and identify the active principal present in the leaf of the plant which was taken up as the basis for bioactivity guided isolation from Annona squamosa. L.

5.1.1. Collection, Extraction and Phytochemical Tests of Annona squamosa. L

- Collection and drying.
  
  Discussed in chapter -2

- Extraction of different parts of plant.
  
  The powdered leaf, (100 g) material were extracted by hot extraction using soxhlet extractor successively with solvents in order non polar to polar solvents like pet-ether (40-60°C), chloroform, ethanol and water. Extracts were concentrated to dryness under reduced pressure and controlled temperature (30-60°C). On concentration, it yielded respective solvent extract of Annona squamosa. L extracts.
  
  The extracts were preserved in a refrigerator. The extracts found to be active were further used to isolate the active constituent(s) present in it.

5.1.2. Phytochemical screening:

  Discussed in chapter -4
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The ethanol extracts was only taken up for isolation of active constituent(s). The ethanol extract was column chromatographed on silica gel (60-120) mesh as adsorbent. Column was run with non-polar solvents as their different ratios with hexane and methanol. The column chromatography details are given in the Table No. 5.2.1

Precoated TLC plates (Merck) were used for checking the chemical separation in the column of ethanol extract. Solvents (Qualigens) used for elution were distilled before use. The details of fractions are tabulated (Table No. 5.2.2)

5.2.1. Column chromatography of ethanol extract of leaf of Annona squamosa L.

**Column detail**

<table>
<thead>
<tr>
<th>Column packing material</th>
<th>Solvent used for packing column</th>
<th>Length and</th>
<th>Diameter of the column</th>
<th>Weight of sample for packing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica gel 60-120 mesh</td>
<td>Pet-ether</td>
<td>40cm</td>
<td>2cm</td>
<td>5 gm</td>
</tr>
</tbody>
</table>
5. Isolation of active fraction(s) from Annona squamosa L leaf by chromatographic techniques, physical data interpretation.

Table No 5.2.1. The details of fractions.

<table>
<thead>
<tr>
<th>SL.No.</th>
<th>Mobile phase</th>
<th>Ratio</th>
<th>Fractions</th>
<th>Nature and weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pet ether</td>
<td>100</td>
<td>1</td>
<td>Nil</td>
</tr>
<tr>
<td>2</td>
<td>Pet ether: Methanol 95:5</td>
<td>95:5</td>
<td>2-3</td>
<td>Dark brown mass (400mg)</td>
</tr>
<tr>
<td>3</td>
<td>Pet ether: Methanol 90:10</td>
<td>90:10</td>
<td>4-7</td>
<td>Brown mass (800mg)</td>
</tr>
<tr>
<td>4</td>
<td>Pet ether: Methanol 85:15</td>
<td>85:15</td>
<td>8-9</td>
<td>Reddish brown powder (360mg)</td>
</tr>
<tr>
<td>5</td>
<td>Pet ether: Methanol 80:20</td>
<td>80:20</td>
<td>9-10</td>
<td>Reddish brown powder (475mg)</td>
</tr>
<tr>
<td>6</td>
<td>Pet ether: Methanol 75:25</td>
<td>75:25</td>
<td>11-12</td>
<td>Brick red powder (650mg)</td>
</tr>
<tr>
<td>7</td>
<td>Pet ether: Methanol 70:30</td>
<td>70:30</td>
<td>13-14</td>
<td>Dark brownish red powder (400mg)</td>
</tr>
<tr>
<td>8</td>
<td>Pet ether: Methanol 65:35</td>
<td>65:35</td>
<td>15-16</td>
<td>Dark brown powder (275mg)</td>
</tr>
<tr>
<td>9</td>
<td>Pet ether: Methanol 60:40</td>
<td>60:40</td>
<td>17-18</td>
<td>Dark brown powder (175mg)</td>
</tr>
<tr>
<td>10</td>
<td>Pet ether: Methanol 55:45</td>
<td>55:45</td>
<td>19-20</td>
<td>Dark brown mass (100mg)</td>
</tr>
<tr>
<td>11</td>
<td>Pet ether: Methanol 50:50</td>
<td>50:50</td>
<td>21</td>
<td>Dark brown mass (120mg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3755 mg)</td>
</tr>
</tbody>
</table>
5. Isolation of active fraction(s) from Annona squamosa. L leaf by chromatographic techniques, physical data interpretation.

- A total 21 fractions were collected in 250 ml portion. Individual fractions were tested (Shinoda test) for presence of the active bioflavonoid compounds.

- Fractions 4-7 were active for the active bioflavonoid compounds. The fractions 4-7 were analyses using TLC and HPLC to known number of compound.

- **Isocratic analytical profile of methanol extract (fraction 4-7) of Annona squamosa. L. leaves.**

  The chromatographic separation of active ethanol extract (fraction 4-7) of leaves of the *Annona squamosa*. L was attempted using isocratic run, using reverse phase liquid chromatography with Isocratic solvent system.\(^{4-10}\)

  The samples were prepared at a concentration of 1mg/ml in 30:70 v/v Methanol: Water and was eluted with mobile phase 30:70 v/v Methanol: Water, the wavelength was adjusted at 280nm, flow rate 1ml/min with these condition the chromatogram was resolved a good separation.

- **Analytical profile of fraction from 4-7 of ethanol extract of Annona squamosa. L. leaves**

  Thin layer chromatography.\(^{11-19}\)

  The chromatographic separation of active ethanol extract of leaves of the *Annona squamosa*. L was attempted using normal and thin layer chromatography with different solvent ratios.

  The sample was prepared at a concentration of 1mg/ml in ethanol and was developing the fractions on pre coated TLC plate with butanol: acetic acid: water in ratio of 7:2:1 solvent
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systems. There were five different spots on TLC plate, when illuminated with UV light. The active component (shinoda test) had Rf value of 0.38 from the point of origin of the sample.

- **Active fraction collection.**

The semi purified fractions of ethanol extract of *Annona squamosa*. L leaves which were collected from column chromatography were again subjected for collecting active fraction by column chromatography using butanol: acetic acid: water in ratio of 7:2:1 solvent systems.

The column flow rate 10ml/min with these condition the chromatogram was resolved a good separation. The individual fractions were collected and the solvent allowed evaporating. Natures of fraction are tabulated in table.

**Table – No. 5.2.2. Nature of fractions.**

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>fractions No</th>
<th>Nature of the fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>fractions -1</td>
<td>Yellowish brown gummy</td>
</tr>
<tr>
<td>2.</td>
<td>fractions -2</td>
<td>Creamy white gummy</td>
</tr>
<tr>
<td>3.</td>
<td>fractions -3</td>
<td>Brownish compound</td>
</tr>
<tr>
<td>4.</td>
<td>fractions -4</td>
<td>Cream gummy</td>
</tr>
<tr>
<td>5.</td>
<td>fractions -5</td>
<td>Cream gummy</td>
</tr>
</tbody>
</table>
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Hence the purified 3rd fraction of ethanol extract of *Annona squamosa* L leaves which were collected from conventional column chromatography were again subjected for analysis by HPLC using Isocratic mobile phase condition for the purity showed in table No-5.2.1 and 5.2.2.

- **Characterization of isolated fraction 3rd of ethanol extract (Table 5.2.2 and 4-7 fraction of table 5.2.1) of Annona squamosa. L leaf.**

Further HPLC and TLC analysis of the isolated fraction 3rd of ethanol extract (fraction from 4-7) of *Annona squamosa* L leaves for the purity exhibited single spot at different solvent systems.

- **Melting point:** 220-225°C (rough)

- **The sample designated as ASR**

Sample ASR was brownish yellow crystal.

In HPLC Rf value of the isolated compound (crude fraction) peak eluted at time (3.44 min). This compound subjected for physiochemical examinations, the isolated compound (crude fraction peak) gave positive shinoda test, confirm the compound might be a flavonoid with the presence of -OH group in the 5th position of A ring.
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Fig No-5.2.1. Analytical profiles of active fractions (4-7) from ethanol extract of Annona squamosa. L leaf

DAD1 E, Sig=260, 16
Ref=750,100 (2)
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Fig No-5.2.2: Analytical profiles of isolated active fraction from ethanol extract of Annona squamosa. L leaf
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- **UV- Spectrum**
  - The UV spectrum recorded in methanol.
  - Polyphenolic compounds reveal two characteristic UV absorption bands with maxima in the 240 to 285 and 300 to 550 nm range.
  - The UV spectrum of compound ASR in methanol indicated the presence of chromophoric group with an extended conjugation (In Fig.-5.2.3,). The UV spectrum is in good agreement with that of the flavonoid (Rutin).

- **IR spectrum.**
  - The IR spectrum recorded in (FT-IR (Jasco-5300) (KBR) V max/cm) (In Fig -5.2.4).
  - The IR spectrum of AS-R indicated the broad peak at 3414 cm\(^{-1}\) due to the –OH stretching.
  - The peak at 2928 cm\(^{-1}\) is due to asymmetric stretching of methyl group of the sugar moiety.
  - The peak at 2852 cm\(^{-1}\) might due to symmetric stretching of the methyl group of the sugar moiety.
  - The peak at 1738 cm\(^{-1}\) is due to keto group of the flavonoid.
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- **$^1$H NMR spectrum.**
  The $^1$H NMR recorded in the CD$_3$OD in 500 MHz (In Fig-5.2.5).
  - The H of the –OH group of the phenol group of flavones, when shifted down field as 12.33 δ ppm as single peak.
  - The multiple peak when is find in between 6.22 δ to 7.68 δ ppm is due to aromatic protons.
  - The broad peak as 4.89 δ ppm is due to the –OH groups of the sugar moiety, which resonate at up field.
  - The peak representing 3.30 δ to 3.83 δ ppm is due to the –CH$_2$ group of the sugar moiety.
    The single peak at 1.13 δ is due to –CH$_3$ group of the sugar moiety.

- **$^{13}$C NMR spectrum.**
  The $^{13}$C NMR recorded in the CD$_3$OD in 500 MHz (In Fig-5.2.6).
  - The peak at 178 is due to the carbonyl carbon of the flavone.
  - The peak at 164.56, 161.47, 148.34, and 144.40 is due to carbon atom attached with –OH groups in the flavones of phenols.
  - The peak at 157.94, 157.08, 134.22, 129.21, 122.22, 121.25, 116.37, 114.25, 104.26, 103.28, and 101.01 is the aromatic carbon.
  - The peak at 72.55, 74.33, 75.77, 76.76, 70.88, 70.68. is due to the carbon atoms attached with –OH group in the glycosidic ring.
  - The peak at 98.62, 93.58, 70.00, 68.34, 67.18, and 29.35 is due to the –CH$_2$ carbons of the glycosidic ring.
  - The peak at 16.9 is due to methyl group associated to the glycosidic ring of the Rutin.
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- The Mass Spectrum. LC-MS (SHIMADZU) (In Fig-5.2.7).

- The Mass Spectrum of ASR by LC-MS indicted the molecular ion peak at m/z 611. This corresponds to molecular weight of ASR isolated from the Annona Squamosa . L leaves ethanol extract.

The structure assign to ASR (Rutin).

![Chemical structure of Rutin]

Conclusion

From the ethanol extract, the compound was isolated by TLC and column chromatography methods of separation. The compound designated as ASR, on the basis of its physiochemical measurement it is good agreement as flavones containing glycoside linkage. The name is ‘Rutin’ on the basis of literature reports. It is isolated in good quantity and taken for further biological screening.
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5.3. References.


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11. P. Jandera, L. Petra’nek, M. Kucerova, Characterisation and prediction of retention in isocratic and gradient-elution normal-phase high-performance liquid chromatography on
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