Phytochemical analysis: isolation and characterization of Phytoconstituents Aerial parts of Sarcococca saligna extract

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ABSTRACTS
Plant materials and herbal remedies are being used from decades for cure and treatment of various disease and disorders. Finding healing powers in plants is an ancient idea. Herbal medicines have the ability to affect body system. The effects are dependent on the chemical compounds present in the plant used. Human being have been benefited from these compounds for many years in both medical and nutritional context. The plant natural product has been studied by combination of chemical, biochemical, and molecular, biological and genetic approaches. Now days they represent substantial portion of the global drug market. As per the World Bank Report, the international market for herbal drug is estimated to be more than 60 billion US$ which is expected to grow up to 6 trillion US$ by the end of 2050. Medicinal plants still remain the mainstay of primary health care because of better compatibility with human body and lesser side effects. Medicinal plants belong to the oldest known health care products that have been used by the mankind all over the world in the form of folklore medicines. The World Health Organization estimates that 80% of the World’s population still relies on herbal medicines as its major source of medicinal products. For global positioning of herbal drugs, it is necessary to do the standardization and characterization of herbal extracts. A herbal drug may be standardized on the basis of its organoleptic characters, Macroscopy, Microscopy and Chemical constituents. In present investigation the bioactive phytoconstiuents have been made the basis of standardizing selected herbal drugs. In the present study, Isolation of chemical constituents and standardization of plant extracts of Sarcococca saligna was done, Sarcococca saligna is the well-known medicinal plant used from the ancient era to till date for their medicinal values. Standardization was done on the basis of marker compounds isolated from the plant extracts. The plant was collected and analyzed.

Key words: Organoleptic characters, Macroscopy, Microscopy and Chemical constituents etc.

INTRODUCTION
Plant Description
Sarcococca saligna is an evergreen aromatic shrub, widely distributed throughout the northern areas of Pakistan and Kashmir at 5000-9000 ft. altitudes. It is also widely distributed in western Himalayas from Afghanistan to west of Nepal. Sarcococca is a genus of 16-20 species of flowering plants in the family Buxaceae. They are slow growing evergreen shrubs 1-2 m tall.

Leaves: Leaves are borne alternately and are oblong-ovate to lanceolate, acute to acuminate at both ends with entire margin. Leaves are 3-veined at the base, and having petioles 1.5-3mm long.

Flowers: Sarcococca saligna bear fragrant flowers. Flowers are unisexual, sessile, in short auxiliary raceme Petals are long, ovate and acute. Female flowers are inserted basally, bracteoles are several, acute, ovate and densely imbricate.

Fruits: The fruit is a red or black in colour containing 1-3 seeds. The fruit is about 1cm long and ovoid to globose. Exocarp is fleshy or subdry, endocarp is fragile. Fruit are become dark purple when get mature

SEEDS: Membranous testa. Subglobose in shape. Endosperm is fleshy.

Bark: Bark is smooth
Botanical Description
Latin name: Sarcococca saligna Linn.
Family: Buxaceae
Synonyms: Sarcococca trinervia, Buxus saligna, Sarcococca salicifolia, Sarcococca pruniformis var. angustifolia.

Chemical Constituents: Chemical constituents of Sarcococca saligna are pregnane-type steroidal alkaloids. Alkaloids are the organic products of natural or synthetic origin which are basic in nature and contain one or more nitrogen atoms, normally of heterocyclic nature, and possess specific physiological actions on human or animal body, when used in small quantities. Steroidal alkaloids form a class of compounds possessing the basic or modified steroidal skeleton within the nitrogen incorporated as an integral part of the molecule either in ring or inside the chain.

MATERIALS AND METHOD
Procurement of Plant Material
In the present study, the aerial parts of Sarcococca saligna were collected in winter from the forest area of Patnitop, (Jammu) having altitude 7000ft. The plant was identified and authenticated by botanist. After authentication, aerial part was dried at room temperature until it was free from the moisture and subjected to physical evaluation with different parameters such as nature, odour, colour, taste, size, shape, width, length. Finally aerial part was subjected to size reduction to get coarse powder. Then the powder was subjected to extraction and determination of various parameters of Phytochemical screening.

Phytochemical Screening
Chloroform extract was subjected to preliminary phytochemical investigation for detection of Alkaloids, Carbohydrates, Glicosides, Phenolic compounds, Flavonoids, Proteins and Amino acids, Saponins, Phytosterols, Acidic compounds, Resins and Reducing sugars.

Extraction, Fractionation, Isolation of Chemical Constituents and Standardization

Extract preparation: The coarsely powdered aerial parts of Sarcococca saligna were extracted with Ethanol. The method of preparation is described below.

Ethanolic extract: Dry Plant of Sarcococca saligna was ground to coarse powder. The coarsely powdered aerial parts (5.0Kg) were packed in a percolator, soaked in a Ethanol (15litres) and kept for one week. The extract was drained, filtered and concentrated under reduced pressure using rotary film evaporator. The extraction process was repeated three times more under similar conditions. The combined extract was finally dried in vacuum desiccator and weighed.

Weight of plant material taken : 5.0 kg
Weight of extract formed : 495g
Extractive value : 9.9%

Fractionation of Ethanolic extract: The dried Ethanolic extract was dissolved in (750ml) distilled water. The solution thus formed was defatted with n-Hexane (5 litres). The aqueous extract was dissolved in 10% acetic acid to adjust the pH 3.5 and extracted with chloroform three times (3x2liter) to yield alkaloid fraction(A1). The acidic aqueous filtrate thus obtained was basified to pH 9-10 by adding ammonium hydroxide and extracted with chloroform (3x2liter) to afford crude alkaloid gummy fraction(A2).

Chromatography of Chloroform Extract (A2)
Isolation of Markers
Compounds were isolated from the chloroform extract by using column chromatography and fractions were monitored on TLC.

Slurry Formation
Dried chloroform extract was taken and dissolved in the minimum quantity of chloroform and then adsorbed on weighed quantity of neutral alumina, to get free flowing material.

Weight of neutral alumina used : 200 g
Weight of extract: 78 g

**Packing of column**

A neat and dried column was taken. A cotton plug was put at the base of the column. Solvent (chloroform) was poured into the column and packed with slurry of neutral alumina prepared by suspending it into the solvent. The adsorbed extract was then charged into the column.

- Weight of neutral alumina used (column): 1000 g
- Diameter of column used: 6 cm
- Length of column used: 95 cm

**Elution of the column**

The column was first eluted with chloroform. Then column was eluted with the solvent by gradually increasing the percentage of methanol in chloroform.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Fraction no.</th>
<th>Column Solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-25</td>
<td>CHCl₃</td>
</tr>
<tr>
<td>2</td>
<td>26-40</td>
<td>5% Methanol in CHCl₃</td>
</tr>
<tr>
<td>3</td>
<td>41-87</td>
<td>10% Methanol in CHCl₃</td>
</tr>
<tr>
<td>4</td>
<td>88-109</td>
<td>15% Methanol in CHCl₃</td>
</tr>
<tr>
<td>5</td>
<td>110-128</td>
<td>30% Methanol in CHCl₃</td>
</tr>
<tr>
<td>6</td>
<td>129-139</td>
<td>50% Methanol in CHCl₃</td>
</tr>
<tr>
<td>7</td>
<td>140-165</td>
<td>60% Methanol in CHCl₃</td>
</tr>
<tr>
<td>8</td>
<td>166-184</td>
<td>80% Methanol in CHCl₃</td>
</tr>
<tr>
<td>9</td>
<td>185-200</td>
<td>Pure methanol</td>
</tr>
</tbody>
</table>

Each fraction of 250 ml was collected and concentrated on rotavapour. A total of 200 fractions were collected and TLC of all 200 fractions was done using different developing solvents. The fractions were pooled on the basis of the TLC pattern shown by them.

**Developing solvents used for the TLC in various proportions**

1. Chloroform: Methanol
2. Pet Ether: Acetone: Diethyl Amine

**Visualizing agent used in TLC**

The spots were visualized by spraying the chromatogram with Dragendorff’s reagent.

**Preparation of Dragendorff’s reagent:**

(A) Bismuth sub nitrate (0.85g) + water (20ml) + add 2 drops of conc. Nitric acid and heat till mixture become homogenous.

(B) Potassium iodide 8g dissolved in 20ml of water. Mix 5ml of A & 5ml of B + 20ml acetic acid.

Fractions 1-22 eluted in chloroform, which gives mixture of compound in TLC plate. Dry at rotavapour & noted the wt. (26g).and used for further column chromatography.

Fractions 43 eluted in 10% methanol in chloroform, which on concentration and filtration yielded a solid mass, which was recrystallized in methanol to give a crystalline compound. The melting point of the compound was found to be 245-247˚C. The compound was identified as Hookerianamide C, on the basis of TLC pattern, melting point and spectroscopic data in comparison with reported data of Hookerianamide C in the literature.
COLUMN CHROMATOGRAPHY

Slurry Formation
Dried chloroform extract of fr. No.1-22 (26g) was taken and dissolved in the minimum quantity of chloroform and
then adsorbed on weighed quantity of silica gel. The solvent was completely removed to get free flowing material.
Particle size of silica gel used 100-200 mesh
Weight of silica gel used 75g
Weight of extract 26g

Packing of column
A neat and dried column was taken. A cotton plug was put at the base the column. Solvent (petroleum ether) was
poured into the column and packed with slurry of silica gel,(100-200mesh) prepared by suspending it into the
solvent. The adsorbed extract was then charged into the column.
Weight of silica gel used (column) 700 g
Diameter of column used 6cm
Length of column used 95cm

Elution of the column
The column was first eluted with petroleum ether: acetone: diethylamine. Then column was eluted with the solvent
by gradually increasing the percentage of acetone in petroleum ether: acetone: diethylamine

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Fraction no.</th>
<th>Column Solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-80</td>
<td>Petether:Acetone:Diethylamine  (70:25:5)</td>
</tr>
<tr>
<td>2</td>
<td>81-145</td>
<td>Petether:Acetone:Diethylamine  (60:35:5)</td>
</tr>
<tr>
<td>3</td>
<td>146-180</td>
<td>Petether:Acetone:Diethylamine  (50:45:5)</td>
</tr>
<tr>
<td>4</td>
<td>181-190</td>
<td>Petether:Acetone:Diethylamine  (30:65:5)</td>
</tr>
<tr>
<td>5</td>
<td>191-200</td>
<td>Petether:Acetone:Diethylamine  (10:85:5)</td>
</tr>
<tr>
<td>6</td>
<td>201-211</td>
<td>Acetone:Diethylamine  (95:5)</td>
</tr>
<tr>
<td>7</td>
<td>212-220</td>
<td>Pure Acetone</td>
</tr>
</tbody>
</table>

Each fraction of 250 ml was collected and concentrated on rota vapour. A total of 220 fractions were collected and
TLC of all 220 fractions were done using different developing solvents. The fractions were pooled on the basis of
the TLC pattern shown by them.

Developing solvents used for the TLC in various proportion
Petroleum ether: acetone: diethyl amine (14:5:1)

Visualizing agent used in TLC
The spots were visualized by spraying the chromatogram with Dragendorff reagent

Preparation of Dragendorff reagent:
(C) Bismuth sub nitrate (0.85g) + water (20ml) + add 2 drops of conc. Nitric acid and heat till mixture
become homogenous
(D) Potassium iodide 8g dissolved in 20ml of water. Mix 5ml of A & 5ml of B + 20ml acetic acid.
Fractions 35-38 eluted in petroleum ether: acetone: diethyl amine (70:25:5), which on concentration and filtration
yielded a solid mass, which was recrystallized in ethyl acetate to give a crystalline compound. The melting point of
the compound was found to be 233˚C. The compound was identified as Saracocine, on the basis of TLC pattern,
melting point and spectroscopic data in comparison with reported data of Saracocine in the literature.
4.3.2. Chromatography of Chloroform Extract (A1)

**Isolation of Markers**
Compounds were isolated from the chloroform extract by using column chromatography and fractions were monitored on TLC.

**Slurry Formation**
Dried chloroform extract was taken and dissolved in the minimum quantity of chloroform and then adsorbed on weighed quantity of neutral alumina, to get free flowing material.

- Weight of neutral alumina used: 100 g
- Weight of extract: 40g

**Packing of column**
A neat and dried column was taken. A cotton plug was put at the base the column. Solvent (chloroform) was poured into the column and packed with slurry of neutral alumina prepared by suspending it into the solvent. The adsorbed extract was then charged into the column.

- Weight of neutral alumina used (column): 700g
- Diameter of column used: 6 cm
- Length of column used: 95 cm

**Elution of the column**
The column was first eluted with Petroleum ether. Then column was eluted with the solvent by gradually increasing the percentage of ethyl acetate in Petroleum ether.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Fraction no.</th>
<th>Column Solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-7</td>
<td>CHCl$_3$</td>
</tr>
<tr>
<td>2</td>
<td>8-45</td>
<td>2% Ethyl acetate in Petether</td>
</tr>
<tr>
<td>3</td>
<td>46-223</td>
<td>5% Ethyl acetate in Petether</td>
</tr>
<tr>
<td>4</td>
<td>224-305</td>
<td>7% Ethyl acetate in Petether</td>
</tr>
<tr>
<td>5</td>
<td>306-450</td>
<td>10% Ethyl acetate in Petether</td>
</tr>
<tr>
<td>6</td>
<td>451-510</td>
<td>20% Ethyl acetate in Petether</td>
</tr>
<tr>
<td>7</td>
<td>511-538</td>
<td>50% Ethyl acetate in Petether</td>
</tr>
<tr>
<td>8</td>
<td>539-570</td>
<td>60% Ethyl acetate in Petether</td>
</tr>
<tr>
<td>9</td>
<td>571-580</td>
<td>75% Ethyl acetate in Petether</td>
</tr>
<tr>
<td>10</td>
<td>581-603</td>
<td>Pure Ethyl acetate</td>
</tr>
<tr>
<td>11</td>
<td>604-615</td>
<td>5% Methanol in Ethyl acetate</td>
</tr>
<tr>
<td>12</td>
<td>620-633</td>
<td>10% Methanol in Ethyl acetate</td>
</tr>
<tr>
<td>13</td>
<td>634-655</td>
<td>20% Methanol in Ethyl acetate</td>
</tr>
<tr>
<td>14</td>
<td>656-676</td>
<td>30% Methanol in Ethyl acetate</td>
</tr>
<tr>
<td>15</td>
<td>677-704</td>
<td>50% Methanol in Ethyl acetate</td>
</tr>
<tr>
<td>16</td>
<td>705-728</td>
<td>75% Methanol in Ethyl acetate</td>
</tr>
</tbody>
</table>
Each fraction of 250 ml was collected and concentrated on rota vapour. A total of 200 fractions were collected and TLC of all 200 fractions were done using different developing solvents. The fractions were pooled on the basis of the TLC pattern shown by them.

**Developing solvents used for the TLC in various proportion**
Chloroform: Methanol

**Visualizing agent used in TLC**
The spots were visualized by spraying the chromatogram with **Dragendorff** reagent

**Preparation of Dragendorff** reagent:
1. Bismuth sub nitrate (0.85g) + water (20ml) + add 2 drops of conc. Nitric acid and heat till mixture become homogenous
2. Potassium iodide 8g dissolved in 20ml of water. Mix 5ml of A & 5ml of B + 20ml acetic acid.

Fractions 50-54 eluted in 5% ethyl acetate in Petether, which on concentration and filtration yielded a solid mass, which was recrystallized in Ethyl acetate to give a crystalline compound. The melting point of the compound was found to be 159-160°C. The compound was identified as **Epipachysamine E-5-en-4-one**, on the basis of TLC pattern, melting point and spectroscopic data in comparison with reported data of **Epipachysamine E-5-en-4-one** in the literature.

Fractions 550-555 eluted in 60% ethyl acetate in Petether, which on concentration and filtration yielded a solid mass, which was recrystallized in Ethyl acetate to give a crystalline compound. The melting point of the compound was found to be 245-248°C. The compound was identified as **Epipachysamine D**, on the basis of TLC pattern, melting point and spectroscopic data in comparison with reported data of **Epipachysamine D** in the literature.

**RESULT AND DISCUSSION**

**Isolation of phytoconstituents**

**Hookerianamide C (SS-43)**

Elution of the column with chloroform:Methanol (9:1), gave cream coloured crystals of **SS-43**, recrystallised from methanol, 60mg

Rf: 0.6

m.p.: 245-247°C

[α]D: -3.42 (C, 0.585 in CHCl3)

$^1$H NMR (CDCl3):

$^{13}$C NMR (CDCl3):

M.W.: 486.74

![H$^1$NMR spectra of Hookerianamide C](image-url)
$^{13}$C NMR spectra of Hookerianamide C

Mass spectra of Hookerianamide C
Sarcocine \{SS(35-38)\}

Elution of the column with petroleum ether:acetone:diethylamine (70:25:5), white amorphous powder of \textit{SS(35-38)}, recrystallised from Ethyl acetate, 40mg.

\textbf{R}_f: 0.4

\textbf{m.p:} 233\textdegree C

\textbf{[\alpha]_D:} -28 \textdegree (C, 0.12 in CHCl$_3$)

$^1$H NMR (CDCl$_3$):

$^{13}$C NMR (CDCl$_3$):

M.W. : 400

\begin{figure}
\centering
\includegraphics[width=\textwidth]{ss-35-38}
\caption{H$^1$NMR spectra of sarcocine}
\end{figure}
Mass spectra of sarcocine

Epipachysamine E-5-en-4-one (SS(50-54))
Elution of the column with petroleum ether:Ethyl acetate (95:5), gave cream coloured crystals of SS(50-54), recrystallised from ethyl acetate, 60mg
Rf: 0.48  
m.p: 159-160°C  
$\alpha$D: +41.25 (C, 0.4 in CHCl₃)  

$^1$H NMR (CDCl₃):  
$^{13}$C NMR (CDCl₃):  
M.W: 440

H$^1$NMR spectra of Epipachysamine-5-en-4-one  

$^{13}$CNMR spectra of Epipachysamine-5-en-4-one
Mass spectra of Epipachysamine-5-en-4-one

Epipachysamine D \{SS(550-555)\}

Elution of the column with petroleum ether: Ethyl acetate(40:60), white crystals of SS\{550-555\}, recrystallised from Ethyl acetate, 90mg

\(R_f\): 0.3

m.p: 245-248 \(^\circ\)C

\([\alpha]_D\): +16.31 (C, 0.52 in CHCl\(_3\))

\(^1\)H NMR (CDCl\(_3\)):

\(^1^3\)C NMR (CDCl\(_3\)):

M.W.: 450.70

H\(^1\)NMR spectra of Epipachysamine D
$^{13}$C NMR spectra of Epipachysamine D

Mass spectra of Epipachysamine D
STRUCTURES OF ISOLATED COMPOUNDS

Sarcocine

Hookerianamide C
Epipachysamine E-5-en-4-one

Epipachysamine D

REFERENCES


