Phytochemical Investigation

**Determination of total phenol content** (Mcdonal, 2001).

Total phenolic content was determined by Folin ciocalteu reagent. The dilute extracts (0.5ml of 1mg/ml) were mixed with Folin ciocalteu reagent (5ml, 1:10 diluted with distilled water) and aqueous sodium carbonate (4 ml, 1 M). The mixtures were allowed to stand for 60 min and total phenols were determined using double beam UV-Vis spectrophotometer at 765nm. Total phenolic values were expressed as gallic acid equivalent in g/100g of extract which is a common reference compound. The concentrations of polyphenols in samples were derived from a standard curve of gallic acid ranging from 10 to 50 µg/ml.

**Determination of total flavonoids content** (Chang, 2002).

Aluminium chloride colorimetric method was used for flavonoids determination. Alcoholic and aqueous extracts of the bark (2ml) were mixed with 0.1ml of 10% w/v aluminium chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. It was kept at room temperature for 30 minutes and the absorbance of the reaction mixture was measured at 415nm using double beam UV-Vis spectrophotometer. The calibration curve was plotted using quercetin solutions at concentrations 12.5 to 100 µg/ml in methanol.

**Free radical scavenging activity determinations** (Braca, 2001).

The free radical scavenging capacity of the extracts were determined using DPPH method. Plant extracts and ascorbic acid were weighed and dissolved in methanol to obtain six different concentrations (1, 5, 10, 50, 100 and 500 µg/ml). Aliquots were prepared suitably by diluting with methanol.

DPPH was weighed and dissolved in methanol to make 0.004% w/v solution. 3ml of 0.004% DPPH solution was added to each test tube with the help of calibrated pipette to obtain the desired concentrations. The prepared mixtures were incubated at 37°C for 30 min. The absorbance value of each test tube was determined using UV-Visible spectrophotometer at 517nm. The percentage inhibition values were calculated using equation.

\[
\text{DPPH scavenged(%) = } \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of Control}} \times 100
\]

IC\(_{50}\) was determined from % inhibition vs concentration graph. IC\(_{50}\) expressed the antioxidant activity defined as the concentration in ml that inhibits the formation of DPPH radicals by 50 %. 

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*Evaluation of Pharmacognostical Phytochemical and Antidiabetic Profiles of Some Indigenous Plants* 

...91....
Isolation and Identification of Phytoconstituents.

*Careya arborea* Roxb.

When all the extracts were studied for anti-diabetic study, it was found that alcoholic, petroleum ether and aqueous extracts showed highly potent activity (highly significant) than other extracts. But as per the literature Sterols as well as flavonoids are known to reduce the blood sugar level. Hence petroleum ether extract containing sterols with potent antidiabetic activity, subjected to column chromatography for the separation and isolation of sterols. For flavonoids purpose ethyl acetate fraction of alcoholic extract subjected to column chromatography for separation of active chemical constituents.

i. **Identification of Sterols (Triterpenes) by TLC:**

The petroleum ether extract subjected to thin layer chromatography for the identification and confirmation of sterols.

*Preparation of TLC Plate:*

Thin layer chromatography was performed using silica gel G as adsorbent. Slurry of silica gel was prepared in distilled water. The slurry was applied to get a thin layer of 0.4mm thickness over a clean and dry glass plate of 10 x 20 cms size by an applicator. The plate was activated at 110°C for one hour.

The details of TLC as under:

- **Adsorbent**: Silica gel G (activated)
- **Thickness**: 0.4mm
- **Plate size**: 10 x 20 cms
- **Activation temperature**: 110°C for one hour
- **Solvent system**: Benzene: Ethyl acetate (9.75: 0.25)
- **Detecting reagent**: Vanilline:Sulphuric acid.

ii. **Isolation of Sterols (Triterpenes) from Pet. Ether extract:**

*Column chromatography:*

150g of Alumina for column chromatography was activated in hot air oven at 110°C for one hour. The petroleum ether solvent was used to build the alumina in the glass column. The activated alumina was charged into the column in small portions with gentle tapping after each addition in order to ensure uniform packing. The small quantity of solvent system was allowed to remain on the top of the column (about 2cm). The air
bubbles present in the column were removed by gentle tapping to get a uniform bed of adsorbent.

The 2 gm of Petroleum ether extract was dissolved in petroleum ether. The activated alumina (3 g) was added slowly with continuous stirring to pet. ether solution to adsorbed on the activated alumina. Care was taken for no lumbs in the adsorbed alumina. Then the adsorbed alumina was charged into the column in small portion with gentle tapping after each addition in order to ensure uniform packing. The column was eluted with graded mixture of petroleum ether, Pet.ether: Benzene and chloroform.

Table 17: The details of column chromatography of pet. ether extract of Careya arborea Roxb.

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Alumina for column chromatography activated at 110° for 1 hour.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of the column</td>
<td>41 cms.</td>
</tr>
<tr>
<td>Length of the adsorbent</td>
<td>22 cms.</td>
</tr>
<tr>
<td>Diameter of the column</td>
<td>Outer 3 cms, inner 2.8 cms.</td>
</tr>
<tr>
<td>Rate of elution</td>
<td>10-15 drops /min.</td>
</tr>
<tr>
<td>Volume of the each fraction collected</td>
<td>10-12 ml each</td>
</tr>
<tr>
<td>Total volume of each mixture elute collected</td>
<td>90 - 100 ml.</td>
</tr>
<tr>
<td>Elution</td>
<td>petroleum ether, pet. ether: benzene, chloroform</td>
</tr>
</tbody>
</table>
Fig. 7: Column chromatography of pet. ether extract of *Careya arborea* Roxb.

Table 18: Elution scheme for column chromatography of pet. ether extract of *Careya arborea* Roxb.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Proportion</th>
<th>Solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 09</td>
<td>100</td>
<td>Petroleum ether</td>
</tr>
<tr>
<td>10 to 18</td>
<td>50 : 50</td>
<td>Petroleum ether : Benzene</td>
</tr>
<tr>
<td>19 to 28</td>
<td>100</td>
<td>Chloroform</td>
</tr>
</tbody>
</table>

For each solvent, fractions were collected. Total fractions amounted to 28. TLC of each fraction was carried out to find out homogeneity and detection with various reagents for the separation of sterols. The chromatographic pattern of each fraction was studied thoroughly and the fractions belongs to the same elute solvent which gave identical pattern with respect of Rf value and colour reaction were mixed.
Separation by High Performance Thin Layer Chromatography (HPTLC)

Materials and methods
The alcoholic extract, Pet. Ether extract and isolated compounds were prepared. These samples were used as test samples for the quantification of active compound (terpenoid). Lupeol was used as working standard for quantification of content in extracts. Pure lupeol (sigma-aldrich) was obtained from Anchrom enterprises, Mumbai.

Equipment
A camag HPTLC system equipped with sample applicator, Linomat 5, twin trough plate development chamber, TLC scanner (Camag, Switzerland), TLC plate heater.

Test sample preparation
A. Alcoholic extract preparation
   100 mg of alcoholic extract was weighed and dissolved in 20 ml of alcohol.

B. Pet. Ether extract
   20 mg of Pet ether extract was weighed and dissolved in 10 ml of alcohol.

C. Standard preparation
   1 mg of lupeol was accurately weighed and dissolved in 1ml of methanol.

D. Isolated compound
   1 mg of lupeol was accurately weighed and dissolved in 1ml of methanol.

In HPTLC, the precoated plate of size 10x10 was used. The sample was taken in applicator syringe, the air bubbles were removed and sample was applied on the plate with the help of Linomat 5 sample applicator for uniform distribution of the sample. 10 µl of Pet. ether and alcoholic extract and 3 µl of Std. lupeol (1mg/ml) were applied to the plate. The plate was placed in glass twin trough solvent chamber containing solvent.
system, Toluene: Ethyl acetate (9:1) and was saturated well. Then plate was removed from chamber and sprayed with spraying reagent Anisaldehyde: Sulphuric acid. Photo documentation of TLC plate image and also image scanned at 520 nm was done after derivatisation. The separation was achieved by this HPTLC method.

The isolated compound was also confirmed by

UV-Visible Spectrophotometer.
Isolated compound and standard lupeol were subjected to UV-Visible analysis. The UV spectrum was performed by using UV spectrophotometer at Anchrom test lab.

FTIR
The isolated compound from the column chromatography was taken and dried in dessicator. The dried compound mixed with KBr and pellet was prepared using KBr press and an IR spectrum was obtained. The analysis was carried out by using Jasco FTIR at A. B. College of Pharmacy, Sangli.

¹NMR
The isolated dried compound was subjected to NMR spectroscopic analysis. The compound was soluble in CDCL₃ and the spectrum was obtained at 300 mhz. the NMR was performed at chemistry department of Shivaji University, Kolhapur. The instrument Bruker model AV300 MHZ was used for analysis. ¹NMR study was also confirmed at Central drug research institute, Lucknow.

GC-MS
The isolated dried compound was subjected to GC-MS analysis. GC-MS was performed at IIT, Pawai. The instrument Make- Hewlett Packard, Model- GCD:1800 A, Specifications: EI Source, Quadrupole Analyzer, Mass range: 10-425 amu was used for analysis.
Flavonoids Separation

Thin Layer Chromatography (TLC)

The flavonoids were separated using thin layer chromatographic plates coated with silica gel. The different solvent systems were tried. The ethyl acetate fraction was subjected to thin layer chromatography.

The details of TLC as under:
- **Adsorbent**: Silica gel G (activated)
- **Thickness**: 0.4mm
- **Plate size**: 10 x 20 cms
- **Activation temperature**: 110°C for one hour
- **Solvent system**: Toluene: Ethyl acetate: Formic acid (7:2:4:1)

Identification and separation of flavonoids by High Performance Thin Layer Chromatography (HPTLC).

50 ml alcoholic extract was concentrated to 20 ml then to it 10 ml water and 100 ml ethyl acetate was added. Ethyl acetate was separated and evaporated.

This sample was used as test samples for the quantification of active compound (Flavonoids). Quercetin was used as working standard for quantification of content in extracts. Pure Quercetin (Hi-media) was obtained from Appasaheb Birnale College of Pharmacy, Sangli.

**A. Test sample preparation**

10 mg of ethyl acetate extract was weighed and dissolved in 10 ml of Methanol.

**B. Standard preparation**

1 mg of quercetin was accurately weighed and dissolved in 1 ml of Methanol.

In HPTLC, the precoated plate of size 10x10 was used. The sample was taken in applicator syringe, the air bubbles were removed and sample was applied on the plate with the help of Linomat 5 sample applicator for uniform distribution of the sample. 10 µl of test sample and 2 µl of Std. quercetin were applied to the plate. The plate was placed in glass twin trough solvent chamber containing solvent system, Toluene: Ethyl acetate: Formic acid (7:2:4:1). Then plate was removed from chamber and scanned at 254 nm after derivatisation.
Column chromatography

Table 19: The details of column chromatography of alcoholic extract of *Careya arborea* Roxb.

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Silica gel for column chromatography activated at 110° for 1 hour.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of the column</td>
<td>41 cms.</td>
</tr>
<tr>
<td>Length of the adsorbent</td>
<td>22 cms.</td>
</tr>
<tr>
<td>Diameter of the column</td>
<td>Outer 3 cms, inner 2.8 cms.</td>
</tr>
<tr>
<td>Rate of elution</td>
<td>8-10 drops /min.</td>
</tr>
<tr>
<td>Volume of the each fraction collected</td>
<td>10 each</td>
</tr>
<tr>
<td>Total volume of each mixture elute collected</td>
<td>50 ml.</td>
</tr>
<tr>
<td>Elution</td>
<td>Toluene: Ethyl acetate</td>
</tr>
</tbody>
</table>

The isolated flavonoid is identified using thin layer chromatographic plates coated with silica gel. The different solvent systems were tried. The isolated compound and std. quercetine were subjected to thin layer chromatography.

**Preparation of TLC Plate:**

Thin layer chromatography was performed using silica gel G as adsorbent. Slurry of silica gel was prepared in distilled water. The slurry was applied to get a thin layer of 0.3mm thickness over a clean and dry glass plate of 10 x 20cms size by an applicator. The plate was activated at 110 ± 1°C for one hour.
The details of TLC as:

- **Adsorbent**: Silica gel G
- **Thickness**: 0.3mm
- **Plate size**: 10 x 20 cms
- **Activation temperature**: $110\pm1^\circ C$ for one hour
- **Mobile phase**: Toluene: Methanol: Formic acid (9:1:0.5)
- **Detecting reagent**: Anisaldehyde: Sulphuric acid.

The isolated compound was also confirmed by

**UV-Visible Spectrophotometer**

Isolated compound and standard lupeol were subjected to UV-Visible analysis. The UV spectrum was performed by using UV spectrophotometer at Anchrom test lab.

**FT-IR**

The isolated compound from the column chromatography was taken and dried in dessicator. The dried compound mixed with KBr and pellet was prepared using KBr press and an IR spectrum was obtained. The analysis was carried out by using Jasco FTIR at A.B. College of Pharmacy, Sangli.

**$^1$NMR**

The isolated, dried compound was subjected to NMR spectroscopic analysis. The compound was soluble in DMSO and the spectrum was obtained at 300 mhz. The NMR was performed at chemistry department of Shivaji University, Kolhapur. The instrument Bruker model AV300 MHZ was used for analysis. $^1$NMR study was also confirmed at Central drug research institute, Lucknow.

**GC-MS**

The isolated dried compound was subjected to GC-MS analysis. GC-MS was performed at IIT, Pawai. The instrument Make- Hewlett Packard, Model- GCD:1800 A, Specifications: EI Source, Quadrupole Analyzer, Mass range: 10-425 amu was used for analysis.
**Phytochemical Investigation of Bridelia retusa Spreng.**

**Determination of total phenol content** (Mcdonal, 2001)

Total phenolic content was determined by Folin ciocalteu reagent. The dilute extracts (0.5ml of 1mg/ml) were mixed with Folin ciocalteu reagent (5ml, 1:10 diluted with distilled water) and aqueous sodium carbonate (4 ml, 1 M). The mixtures were allowed to stand for 60 min. and total phenols were determined using double beam UV-Vis spectrophotometer at 765nm. Total phenolic values were expressed as gallic acid equivalent in g/100g of extract which is a common reference compound. The concentrations of polyphenols in samples were derived from a standard curve of gallic acid ranging from 10 to 50 µg/ml.

**Total flavonoids determination** (Chang, 2002)

Aluminium chloride colorimetric method was used for flavonoids determination. Alcoholic and aqueous extracts of the bark (2ml) were mixed with 0.1ml of 10% w/v aluminium chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. It was kept at room temperature for 30 minutes and the absorbance of the reaction mixture was measured at 415nm using double beam UV-Vis spectrophotometer. The calibration curve was plotted using quercetin solutions at concentrations 12.5 to 100 µg/ml in methanol.

**Free radical scavenging activity determinations** (Braca, 2001)

The free radical scavenging capacity of the extracts were determined using DPPH method. Plant extracts and ascorbic acid were weighed and dissolved in methanol to obtain six different concentrations (1, 5, 10, 50, 100 and 500 µg/ml). Aliquots were prepared suitably by diluting with methanol.

DPPH was weighed and dissolved in methanol to make 0.004% w/v solution. 3ml of 0.004% DPPH solution was added to each test tube with the help of calibrated pipette to obtain the desired concentrations. The prepared mixtures were incubated at 37°C for 30 min. The absorbance value of each test tube was determined using UV-Visible spectrophotometer at 517nm. The percentage inhibition values were calculated using equation.

\[
\text{DPPH scavenged(%) = } \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of Control}} \times 100
\]
IC₅₀ was determined from % inhibition vs concentration graph. IC₅₀ expressed the antioxidant activity defined as the concentration in ml that inhibits the formation of DPPH radicals by 50%.

IDENTIFICATION AND SEPERATION OF ACTIVE CONSTITUENTS

When extracts were studied for antidiabetic study, it was found that alcoholic extract of bark of *Bridelia retusa* Spreng. was more potent than other extracts hence; alcoholic extract was used for, separation and isolation of active constituents.

**Preparation of TLC Plate:**

Thin layer chromatography was performed using silica gel G as adsorbent. Slurry of silica gel was prepared in distilled water. The slurry was applied to get a thin layer of 0.3mm thickness over a clean and dry glass plate of 10 x 20cms size by an applicator. The plate was activated at 110 ± 1°C for one hour.

The details of TLC as:

- **Adsorbent**: Silica gel G
- **Thickness**: 0.3mm
- **Plate size**: 10 x 20 cms
- **Activation temperature**: 110±1°C for one hour
- **Mobile phase**: Toluene: Methanol: Formic acid (9: 1:0.5)
- **Detecting reagent**: Anisaldehyde : Sulphuric acid.

**Column chromatography:**

150g of silica gel for column chromatography (laboratory grade) was activated in hot air oven at 110±1°C for one hour and filled column using Toluene. The small quantity of solvent system was allowed to remain on the top of the column (about 2cm). The air bubbles present in the column were removed by gentle tapping to get a uniform bed of adsorbent. The details of column chromatography are as under:
Table 20: The details of column chromatography of alcoholic extract of *Bridelia retusa* Spreng.

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Silica gel for column chromatography activated at 110°C±2 for 1 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of the column</td>
<td>41cms.</td>
</tr>
<tr>
<td>Length of the adsorbent</td>
<td>17.5cms.</td>
</tr>
<tr>
<td>Diameter of the column</td>
<td>Outer 3.4cms, inner 3cms.</td>
</tr>
<tr>
<td>Rate of elution</td>
<td>8 drops /min.</td>
</tr>
<tr>
<td>Volume of the elute collected</td>
<td>50 ml each</td>
</tr>
<tr>
<td>Gradient elution</td>
<td>Mixture of Toluene and Methanol.</td>
</tr>
</tbody>
</table>

This alcoholic extract was added to the activated silica gel with mild trituration to enable proper adsorption of the extract onto the silica gel. Then the sample-adsorbed silica gel was charged on to the column in small portion with gentle tapping after each addition in order to ensure uniform packing. The column was eluted by gradient method with Toluene and methanol. The details of graded mixtures were as under.

Table 21: Elution scheme for column chromatography of alcoholic extract of *Bridelia retusa* Spreng.

<table>
<thead>
<tr>
<th>Proportions</th>
<th>Solvent System</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Toluene</td>
</tr>
<tr>
<td>B</td>
<td>Toluene : Methanol</td>
</tr>
<tr>
<td>C</td>
<td>Toluene : Methanol</td>
</tr>
<tr>
<td>D</td>
<td>Toluene : Methanol</td>
</tr>
<tr>
<td>E</td>
<td>Toluene : Methanol</td>
</tr>
<tr>
<td>F</td>
<td>Toluene : Methanol</td>
</tr>
<tr>
<td>G</td>
<td>Toluene : Methanol</td>
</tr>
<tr>
<td>H</td>
<td>Toluene : Methanol</td>
</tr>
<tr>
<td>I</td>
<td>Toluene : Methanol</td>
</tr>
<tr>
<td>J</td>
<td>Toluene : Methanol</td>
</tr>
<tr>
<td>K</td>
<td>Methanol</td>
</tr>
</tbody>
</table>
Separation by High Performance Thin Layer Chromatography (HPTLC)

Materials and methods

The alcoholic extract, isolated compounds were prepared. These samples were used as test samples for the quantification of active compound (steroids).

β-sitosterol was used as working standard for quantification of content in extracts. Pure β –sitosterol( Sigma-aldrich, Mumbai) was obtained from Appasaheb birnale college of pharmacy, Sangli.

Test sample preparation

A. Alcoholic extract preparation

300 mg of alcoholic extract was weighed and dissolved in 5 ml of alcohol.

B. Isolated

15 mg of Isolated compound was weighed and dissolved in 1 ml of alcohol.

C. Standard preparation

1 mg of β –sitosterol was accurately weighed and dissolved in 1ml of methanol.

In HPTLC, the precoated plate of size 10x10 was used. The sample was taken in applicator syringe, the air bubbles were removed and sample was applied on the plate with the help of Linomat 5 sample applicator for uniform distribution of the sample. 10 µl of Alcoholic extract, isolated compound and 2 µl of Std. β-sitosterol (1mg/ml) were applied to the plate. The plate was placed in glass twin trough solvent chamber containing solvent system, Toluene: Methanol: Formic acid (9:1:0.5) and was saturated well. Then plate was removed from chamber and sprayed with spraying reagent Anisaldehyde: Sulphuric acid. Photo documentation of TLC plate image and also image scanned at 520 nm was done after derivatisation. The separation was achieved by this HPTLC method.

Characterization

The Compound isolated was also confirmed by following spectral analysis.

UV Spectrum

Isolated compound and standard β-sitosterol were subjected to UV-Visible analysis. The UV spectrum was performed by using UV spectrophotometer at Anchorm test lab.
FTIR spectrum
The isolated compound from the column chromatography was taken and dried in dessicator. The dried compound mixed with KBr and pellet was prepared using KBr press and an IR spectrum was obtained. The analysis was carried out by using Jasco FTIR at A. B. College of Pharmacy, Sangli.

$^1$H NMR Spectrum
The isolated, dried compound was subjected to NMR spectroscopic analysis. The compound was soluble in DMSO and the spectrum was obtained at 300 mhz. the NMR was performed at chemistry department of Shivaji University, Kolhapur. The instrument Bruker model AV300 MHZ was used for analysis. The instrument Bruker model AV300 MHZ was used for analysis. $^1$NMR study was also confirmed at Central drug research institute, Lucknow.

GC-MS
The isolated dried compound was subjected to GC-MS analysis. GC-MS was performed at IIT, Pawai. The instrument Make- Hewlett Packard, Model- GCD:1800 A, Specifications: EI Source, Quadrupole Analyzer, Mass range: 10-425 amu was used for analysis.
Identification of flavonoid by High Performance Separation Thin Layer Chromatography (HPTLC).

50 ml alcoholic extract was concentrated to 20 ml then to it 10 ml water and 100 ml ethyl acetate was added, ethyl acetate is separated and evaporated.

This sample was used as test samples for the quantification of active compound (Flavonoid). Quercetin was used as working standard for quantification of content in extracts. Pure Quercetin (Hi-media) was obtained from Appasaheb Birnale College of Pharmacy, Sangli.

A. Test sample preparation

10 mg of ethyl acetate extract was weighed and dissolved in 10 ml of methanol.

B. Standard preparation

1 mg of quercetin was accurately weighed and dissolved in 1 ml of methanol.

In HPTLC, the precoated plate of size 10x10 was used. the sample was taken in applicator syringe, the air bubbles were removed and sample was applied on the plate with the help of Linomat 5 sample applicator for uniform distribution of the sample. 10 µl of test sample and 2 µl of Std. quercetin were applied to the plate. The plate was placed in glass twin trough solvent chamber containing solvent system, Toluene: Ethyl acetate: Formic acid (7.2:2.4:1). Then plate was removed from chamber and scanned at 254 nm after derivatisation.
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*Journal of Natural Products* 2001; 64: 892–895.