MATERIALS AND METHODS

The plants selected for the present study are *B. montanum; Drypetes roxburghii and Codiaeum variegatum* were collected and maintained in the medicinal plant garden, Department of Molecular Biology, Bangalore University, Bangalore. Phytochemical qualitative and quantitative test were carried out and phytochemical fingerprint was developed. The flavonoid fractions were extracted from leaf of these medicinal plants and their anti-inflammatory, anti-microbial and antioxidant activity was tested. Knowing the importance of flavonoids in pharmaceuticals and nutraceuticals, the flavonoid synthesis was induced in *in vitro* using different elicitors. GC-MS analysis was carried out, quercetin quantification was done by HPLC method, nanoparticles were synthesized from leaf of medicinal plants and its antimicrobial activity was tested.

3.1 EUPHORBIACEAE FAMILY

Euphorbiaceae (spurge family) is a large syndicate of flowering plants and has a cosmopolitan distribution with five subfamilies, 49 tribes, 317 genera and about 8,000 species. Most spurges are herbs, but some, especially in the tropics, are shrubs or trees. Some are succulent and resemble cacti because of convergent evolution. The leaves are alternate, seldom opposite, with stipules. They are mainly simple, but somewhere compound, they are always palmate, never pinnate. Stipules may be reduced to hairs, glands or spines or in succulent species are sometimes absent. The plants can be monoecious or dioecious. The radially symmetrical flowers are unisexual, with the male and female flowers usually on the same plant. As can be expected from such a large family, there is a wide variety in the structure of the flowers. The number of stamens (the male organs) varied from 1 to 10 (or even more).
The female flowers are hypogynous, with superior ovaries. Milky latex is a characteristic of the subfamilies Euphorbiaceae. This family is considered as one of the top 25 economically important plant family (Beneth). The family contains a large variety of phytotoxins (toxic substances produced by plants), mainly diterpene esters, alkaloids, glycosides, and ricin-type toxins (Betancur-Galvis et al., 2002) Euphorbiaceae consists of species of great economic importance, the source of rubber and medicine (Hevea); staple starch source (Manihot) and fruits (Phyllanthus emblica); seed oils (Ricinus, Vernicia); insecticides, Waxes (Euphorbia antisphilitica), edible seeds (Caryocelondron orinocense) and leafy vegetable (Cnidoscolous aconitifolius).

3.1.1 COLLECTION, DESCRIPTION AND MEDICINAL PROPERTIES OF EUPHORBIACEAE MEMBERS

Three members of Euphorbiaceae family namely Baliospermum montanum (Wild.) Muell-Arg., Codiaeum variegatum (L.) Bl. and Drypetes roxburghii (Wall.) Huresawa were selected for phytochemical and antimicrobial studies.

3.1.1.1 BALIOSPERMUM MONTANUM It is an endangered plant and has been listed in red list of medicinal plant species (Rajshekhar and Ganeshan 2002) and it was collected from Sirsi, Western Ghats of Karnataka.

| Botanical name | Baliospermum montanum (Wild.) Muell-Arg |
| Synonym | Baliospermum solanifolium (Burm.) Suresh |

**Vernacular name**

<table>
<thead>
<tr>
<th>Hindi</th>
<th>Dandi</th>
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<tbody>
<tr>
<td>Kannada</td>
<td>Kaduharalu</td>
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<tr>
<td>Tamil</td>
<td>Pey-amanakku</td>
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<td>Marathi</td>
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<td>Malayalam</td>
<td>Ceriyadanthi</td>
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<td>Telugu</td>
<td>Adi amudamu</td>
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</table>
Description of Plant

Plant is a perennial and woody undershrub grows from 10 cm to 8 m in height with herbaceous branches from the roots. Leaves are simple, toothed with undulations. (Fig. 1), upper ones are small, lower ones are large, sometimes 3-30 cm long, 1.5-15 cm broad, flowers are numerous (Fig. 2), Male and female flowers are separated, seen in the same flowering branch, about 3 mm across, greenish yellow, arranged in axillary and terminal racemes, spikes or fascicles (Fig. 3). Fruit is of capsule shape, 12 mm long, obovoid (Fig. 4), Seeds are egg-shaped.

Classification of *B. montanum*

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
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</thead>
<tbody>
<tr>
<td>Division</td>
<td>Magnoliophyta</td>
</tr>
<tr>
<td>Order</td>
<td>Malpighiales</td>
</tr>
<tr>
<td>Family</td>
<td>Euphorbiaceae</td>
</tr>
<tr>
<td>Subfamily</td>
<td>Crotonoideae</td>
</tr>
<tr>
<td>Tribe</td>
<td>Codiaeae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Baliospermum</em> Blume</td>
</tr>
<tr>
<td>Species</td>
<td><em>Baliospermum montanum</em></td>
</tr>
</tbody>
</table>

Medicinal properties

- Anti-inflammatory (Lalitha and Gayathiri 2013)
- It is used to treat body ache and joints pain (http://herbs.indianmedicinalplants.info/)
- Cathartic and antidropsical (http://herbs.indianmedicinalplants.info/)
- Effectively used in loss of appetite, indigestion, liver disorders, intestinal gas and intestinal worms. (www.efloras.org/florataxon.aspx)
- To treat asthma, bronchitis and purgative (www.efloras.org/florataxon.aspx); (Nadkarni, 1988)
- Roots are used to cure jaundice, leucoderma, skin diseases, wounds, anthelmintic (Ravindra and Raju, 2008); stem decoction for toothache (Bhatt, 1982).
- Treatment of abdominal tumor (Chopra and Chopra, 1994).
- Seeds are used as purgative and in gastric complaints (Goel and Sahoo, 1984).
Materials and methods

Major known chemical constituents of *B. montanum*

The phytochemical studies on the roots of *B. montanum* has revealed the presence of five phorbol esters, viz. montanin (C\textsubscript{32}H\textsubscript{48}O\textsubscript{8}; yield, 0.018%), baliospermin (0.003%), 12 deoxyphorbol-13-palmitate (0.021%), 12-deoxy-16-hydroxyphorbol-13-palmitate (0.001 %) and 12-deoxy-5β-hydroxyphorbol-13-myristate (0.007%) (Ogura *et al.*, 1978) and Axillarenic acid from seeds.

3.1.1.2 *DRYPETES ROXBURGHII* It was collected from Sirsi, Karnataka Western Ghats.

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Drypetes roxburghii (Wall.) Huresawa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synonym</td>
<td>Putranjiva roxburghii Wall</td>
</tr>
</tbody>
</table>

Vernacular name

<table>
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<th>Hindi</th>
<th>Putrajeeva, Putranjiva</th>
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<td>Amani Putrajiva</td>
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<td>Karupala</td>
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<td>Malayalam</td>
<td>Pongalam</td>
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<td>Telugu</td>
<td>Kuduru</td>
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</table>

Description of Plant

A moderate sized evergreen tree grows up to 13 m in height (Fig. 5). Leaves simple, alternate, dark green, shiny, elliptic-oblong, distantly serrulate, main nerves numerous (Fig. 6); Male flowers very short pedicellate in rounded axillary clusters, female flowers 1-3 in an axil (Fig. 7) fruits ellipsoid or rounded drupes, white tomentose; seed normally one, stone pointed, very hard rugose (Fig. 8).

Distribution

*D. roxburghii* is distributed in Indomalaysia; Western Ghats- in dry zones of South, Central and south Maharashtra Sahyadris.
Classification of *D. roxburghii*

<table>
<thead>
<tr>
<th>Kingdom</th>
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<tr>
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<tr>
<td>Group</td>
<td>Dicot</td>
</tr>
<tr>
<td>Family</td>
<td>Euphorbiaceae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Putranjiva</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>D. roxburghii</em></td>
</tr>
</tbody>
</table>

**Medicinal properties** (Archana Singh *et al.* 2013)

- Anti-inflammatory.
- Aphrodisiac, elephantiasis and eye infection.
- Habitual abortion and sterility.

3.1.1.3. *CODIAEUM VARIEGATUM* It has been collected from Lal Bagh Botanical garden, Bangalore.

<table>
<thead>
<tr>
<th>Botanical name</th>
<th><em>Codiaeum variegatum</em> (L.) Bl.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synonym</td>
<td><em>Codiaeum variegatum aureo-maculatum</em></td>
</tr>
<tr>
<td>Vernacular Name</td>
<td>Croton</td>
</tr>
</tbody>
</table>

**Description of Plant**

It is an evergreen shrub grows up to 3-4 ft tall, it is dense and compact when young (Fig.9) and has large, thick, leathery, shiny evergreen leaves, alternately arranged (Fig.10) depending on cultivar, the leaves may be ovate to linear, entire to deeply lobed and variegated with green, white, purple, orange, yellow, red or pink, the colours may follow the veins, the margins or they may be in blotches on the leaf, 5–30 cm long and 0.5–8 cm broad (Fig.11). The stems contain milky sap in plant which bleeds from cut ends. Flowers are inconspicuous, small, yellow to white in color, and star-shaped. Summer is the usual bloom time (Fig. 12).

**Distribution**

*C. variegatum* occurs naturally in southern Asia, Indonesia and other Eastern Pacific islands where it grows in open forests and scrub.
Classification of *Codiaeum variegatum*

<table>
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<tr>
<td>Genus</td>
<td><em>Codiaeum</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>Codiaeum variegatum</em></td>
</tr>
</tbody>
</table>

Medicinal properties
- Anti-inflammatory (Olusola *et al.*, 2007)
- Used to treat irregular menstruation (Bourdya *et al.*, 1992)
- Antifungal, antiamoebic and anticancerous activities (Olusola *et al.*, 2007)

### 3.1.2 IDENTIFICATION OF PLANTS
The collected plants were identified with the help of flora, a botanist and taxonomist.

### 3.1.3 MAINTENANCE OF PLANTS
The collected plants were planted in the natural soil of medicinal plant garden, Department of Molecular Biology, Bangalore University, Bangalore.

### 3.2 PHYTOCHEMICAL QUALITATIVE TEST
#### 3.2.1 PREPARATION OF PLANT EXTRACT
Different plant parts (root, stem, leaf and flowers) were collected from *B. montanum, D. roxburghii* and *C. variegatum*. They were dried for one week (in shade and were grinded). 25 g of air-dried powder of the medicinal plants was mixed separately with 150 mL of organic solvents (water, ethanol, acetone, petroleum ether, chloroform, methanol and hexane) and the mixtures were taken into the soxhlet apparatus which was run up to 48 h. The extracted solution was filtered and concentrated in a rotary evaporator under reduced pressure. The dried extracts (500 mg) were re-dissolved in 100 mL of the respective solvent and stored at 4° C in
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airtight bottles and considered it as test solution. Qualitatively and quantitatively tests were carried on test solution to analyze phytochemical constituents.

3.2.2 COLLECTION OF LATEX

Latex samples of *B. montanum* was collected early in the morning from plant by nipping the leaves near the stem or by incision of the trunk and branches of the plant and allowing the latex to drain in clean glass tube separately, brought to the laboratory and kept in refrigerator (till the experiment start). Latex was homogenized in a homogenizer under chilled condition and filter through four folds of muslin cloth. Filtrate latex samples were used for phytochemical analysis.

3.2.3 ESTIMATION OF PHYTOCHEMICALS QUALITATIVELY

3.2.3.1 QUALITATIVE ESTIMATION OF ALKALOIDS (Gibbs 1974)

The 0.5 mL test solution was dissolved in chloroform and the solution was extracted with 8 mL dil. Hydrochloric acid (HCl) or sulphuric acid (H₂SO₄) and acid layer taken and tested for presence of alkaloids.

I. Dragendorff’s test

To 2 mL of acid layer test solution / 250µL of latex, add 2 mL of Dragendorff’s reagent (potassium bismuth iodide solution) and 2 mL of dil. Hydrochloric acid (HCl). An orange-red precipitate indicates the presence of alkaloids.

II. Mayer’s test

To the 1 mL of acid layer test solution / 250µL of latex, add 1 mL of Mayer’s reagent (potassium mercuric iodide solution). Whitish or cream colored precipitate indicates the presence of alkaloids.

III. Wagner’s test

To the 1 mL of acid layer test solution / 250µL of latex, add 2 mL of Wagner’s reagent (iodine in potassium iodide). Reddish brown colored precipitate indicates the presence of alkaloids.
3.2.3.2 QUALITATIVE ESTIMATION OF ANTHRAQUINONE (Bormtrager’s test)

About 0.5 mL / 250µL of latex of the test solution were taken into a dry test tube and 5 mL of chloroform (CHCl₃) was added and shaken for 5 min. The extract was filtered and the filtrate was shaken with equal volume of 10% ammonia solution (NH₃). A pink violet or red color in the ammoniacal layer (lower layer) indicates the presence of anthroquinone (Aiyelaagbe et al. 2009).

3.2.3.3 QUALITATIVE ESTIMATION OF CARBOHYDRATES

I. Molisch’s test

To 2 mL of the test solution / 250µL of latex, add 1 mL of α-napthol solution (C₁₀H₇OH), add concentrated 1 or 2 mL of sulphuric acid (H₂SO₄) through the sides of the test tube. Purple or reddish violet color at the junction of the two liquids reveals the presence of carbohydrates.

II. Barfoed’s test

To 0.5 mL / 250µL of latex of test solution, add 3 mL of Barfoed's solution, Place the tubes in a boiling water bath A rusty or brownish-red colour will indicate monosaccharides.

III. Benedict's test

To 0.5 mL of test solution / 250µL of latex, add 3 mL of Benedict's reagent. Shake each test tube thoroughly; place the tubes in a boiling water bath for 3 minutes, red, green or yellow precipitate obtained shows presence of reducing sugar.

3.2.3.4 QUALITATIVE ESTIMATION OF CARDIAC GLYCOSIDES (Keller Killiani’s test)

About 1mL of test solution / 500µL of latex were dissolved in 1 mL of glacial acetic acid (C₂H₄O₂) containing one drop of ferric chloride (FeCl₃) solution. This was then under layer with 1 mL of concentrated Sulphuric acid (H₂SO₄). A brown ring obtained at the interface indicated the presence of Cardiac glycosides (Aiyelaagbe, 2009).
Materials and methods

3.2.3.5 QUALITATIVE ESTIMATION OF COUMARINS TEST

1 mL of test solution / 500µL of latex were taken in a test tube and covered with filter paper moistened with dilute sodium hydroxide (NaOH), then heated on water bath for a few minutes. The filter paper was examined under UV light, yellow fluorescence indicated the presence of coumarins (El-Tawil, 1983).

3.2.3.6 QUALITATIVE ESTIMATION OF FATTY ACIDS TEST

5 mL of test solution / 1000µL of latex were mixed with 5 mL of ether. This extract was allowed to evaporate on filter paper and filter paper was dried. The transparency on filter paper indicates the presence of fatty acids (Chandrashekar, 2013).

3.2.3.7 QUALITATIVE ESTIMATION OF FLAVONOIDS

I. Shinoda’s test

1 mL of test solution / 250µL of latex were added to 5 mL of ethanol (C₂H₆O) (98 %). To this a small piece of magnesium foil metal was added, this was followed by drop wise addition of concentrated hydrochloric acid (HCl). Red colour indicates the presence of flavonoids (Brain and Turner, 1975).

II. Lead acetate test

Few drops of lead acetate solution (Pb (C₂H₃O₂)₂) were added to 1mL of the test solution / 250µL of latex in test tubes. Formation of yellow coloured precipitate indicated the presence of flavonoids (Tiwari et al., 2011).

III. Alkaline reagent test

About 1 mL test solution / 250µL of latex was treated with few drops of sodium hydroxide (NaOH) solution and observed for intense yellow coloration which disappeared on the addition of dilute Hydrochloric acid (HCl) (Veena et al., 2013).

3.2.3.8 QUALITATIVE ESTIMATION OF GUM AND MUCILAGE

1mL test solution / 500µL of latex were dissolved in 10 mL of distilled water and to this; 25 mL of absolute alcohol (C₂H₆O) was added with constant stirring. White or cloudy precipitate indicated the presence of gums and mucilages (Sai Koteswar Sarma et al., 2011).
3.2.3.9 QUALITATIVE ESTIMATION OF PROTEIN AND AMINO ACIDS

**Ninhydrin test** Add two drops of freshly prepared 0.2% ninhydrin reagent (C₉H₆O₄) (0.1% solution in n-butanol) to 1 mL of test solution / 500µL of latex and heat. Development of blue color reveals the presence of proteins, peptides or amino acids. (Saxena Mamta *et al.* 2012)

3.2.3.10 QUALITATIVE ESTIMATION OF PHENOLS

**Ferric chloride test** To 10 mL of alcoholic solution of test solution, 2 mL of distilled water followed by few drops of 10% aqueous ferric chloride (FeCl₃) solution was added. Formation of blue colour indicates the presence of phenols (Chandrashekar, 2013).

**Folin Ciocalteu test** 500 µL of latex was mixed with few drops of diluted Folin Ciocalteu reagent and aqueous sodium carbonate solution. The mixture was allowed to stand for 10 min and formation of gray colour indicates the presence of Phenolic groups.

3.2.3.11 QUALITATIVE ESTIMATION OF SAPONINS

About 0.5 mL of the test solution / 500µL of latex were shaken with water in a test tube. Frothing, which persist on warming was taken as a preliminary evidence for the presence of saponin. Few drops of olive oil was added to 0.5 mL of the extract and vigorously shaken. Formation of soluble emulsion in the extract indicates the presence of saponins (Odebiyi and Sofowora, 1978).

3.2.3.12 QUALITATIVE ESTIMATION OF STEROIDS

**Liebermann Burchard Test** To 1 mL of test solution / 500 µL of latex, 1 mL of glacial acetic acid (C₂H₄O₂) and 1 mL of acetic anhydride (C₄H₆O₃) and two drops of concentrated sulphuric acid (H₂SO₄) were added. The solution becomes red, then blue and finally bluish green, indicates the presence of steroids (Seema Firdouse, 2011).

3.2.3.13 QUALITATIVE ESTIMATION OF TANNINS

1mL of test solution / 500µL of latex were treated with 15% ferric chloride (FeCl₃) test solution. The resultant colour was noted. Formation of blue colour indicates the presence of hydrolysable tannin. Or into 10 mL of freshly prepared
Materials and methods

potassium hydroxide (KOH) in a beaker, 0.5 mL of the test solution was added and shaken. A dirty precipitate observed indicates the presence of tannin (Odebiyi and Sofowora, 1978; Sofowora, 1982).

3.2.3.14 QUALITATIVE ESTIMATION OF TERPENOIDS

Salkowski test 0.5 mL of test solution / 500µL of latex were added to 2 mL of chloroform. 3 mL of concentrated sulphuric acid (H₂SO₄) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids (Sofowora, 1982).

3.3 ESTIMATION OF PHYTOCHEMICALS QUANTITATIVELY

The plant samples were subjected to quantitative phytochemical tests for plant secondary metabolites such as alkaloids, flavonoids, saponins, phenolics, tannins and terpenoids.

3.3.1 QUANTITATIVE ESTIMATION OF ALKALOIDS (HARBORNE, 1973)

In 250 mL conical flask, 5 g of the dried fine powdered sample / 2.5 mL of latex are taken and 200 mL of 10% acetic acid (C₂H₄O₂) in ethanol (C₂H₆O) was added and covered; allowed to settle for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide (NH₄OH) was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide (NH₄OH) and then filtered. The residue is the alkaloid, which was dried and weighed.

3.3.2 QUANTITATIVE ESTIMATION OF FLAVONOIDS (BOHAM AND KOCIPAI- ABYAZAN, 1994)

10 g of the dried plant sample / 5 mL of latex were extracted repeatedly with 100 mL of 80% aqueous methanol (CH₃O) at room temperature. The whole solution was filtered through whatman filter paper No 42 (125 mm). The filtrate was evaporated to dryness over a water bath and weighed.
3.3.3 QUANTITATIVE ESTIMATION OF SAPONINS (OBADONI AND OCHUKO, 2001)

In conical flask, 20 g of dried fine particles of plant sample / 5 mL of latex were taken and 100 mL of 20% aqueous ethanol (C₂H₆O) was added. This mixture was heated (55°C) on water bath for 4 h with continuous stirring. Later the mixture was filtered and the residue was re-extracted with another 200 mL of 20% ethanol (C₂H₆O). This extract was further reduced to 40 mL over hot water bath (90°C). The concentrated extract was transferred into a 250 mL separating funnel and 20 mL of diethyl ether ((C₂H₅)₂O) was added and shaken vigorously. Ether layer was discarded and aqueous layer was collected.

This step of purification was repeated. 60 mL of n-butanol (C₄H₉OH) was added. The n-butanol extracts were washed twice with 10 mL of 5% aqueous sodium chloride (NaCl). The remaining solution was heated in a waterbath. After evaporation the samples were dried in the oven and weighed.

3.3.4 QUANTITATIVE ESTIMATION OF TANNINS (VAN BUREN AND ROBINSON, 1981)

In 250 mL conical flask, 5 g of powdered plant sample / 2.5 mL of latex were taken and 50 mL of distilled water was added and shook it vigorously for 1 h. Later this solution was filtered into a volumetric flask and 5 mL of this filtrate is pipetted out into a test tube. The sample were incubated for 1.5 h at 20 – 30°C and the sample was then filled with distilled water up to mark of 50 mL of the volumetric flask.

Tannic acid was used as standard; 0.1g of tannic acid was dissolved in 100 mL of water to form tannic acid solution. Distilled water was used as blank. The absorbance of the samples was measured at 760 nm. The values generated were used to calculate the tannin content.

3.3.5 QUANTITATIVE ESTIMATION OF TOTAL PHENOLS (EDEOGA et al., 2005; JING-CHUNG et al., 2007)

For phenol extraction and determination, the sample was boiled with 50 mL of petroleum ether for 15 min. 5 mL of the extract / 2.5 mL of latex was taken into a 50 mL flask, then 10 mL of distilled water was added. 2 mL of ammonium hydroxide
**Materials and methods**

\[(\text{NH}_4\text{OH})\] solution and 5 mL of concentrated amyl alcohol were also added and left to react for 30 min for colour development. This was measured at 505 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg/L solutions of gallic acid (C\textsubscript{7}H\textsubscript{6}O\textsubscript{5}) in methanol water (5050 v/v).

### 3.3.6 QUANTITATIVE ESTIMATION OF TERPENOIDS (FERGUSON, 1956)

Total terpenoids content in the leaf extracts were assessed by standard method. 1 g of dried sample / 1 mL of latex was taken separately and soaked in alcohol for 24 h. Then filtered, the filtrate was extracted with petroleum ether; the ether extract was treated as total terpenoids.

**Calculation of quantitative percentage**

\[
\% \text{ Dry weight} = \frac{\text{Weight of extracted phytochemical}}{\text{Weight of powdered plant material}} \times 100
\]

### 3.4 DEVELOPMENT OF PHYTOCHEMICAL FINGERPRINT

The presence or absence of phytochemicals such as alkaloids, anthraquinones, carbohydrates, cardiac glycosides, coumarins, fatty acids, flavonoids, gum and mucilage, phenols, proteins and amino acids, saponins, steroids, tannins and terpenoids in root, stem, leaf, flower of *B. montanum, D. roxburghii* and *C. variegatum* using seven different solvents such as water, ethanol, acetone, petroleum ether, chloroform, methanol and hexane; in latex of *B. montanum* was computed on work excel sheet obtaining the phytochemical fingerprint.

### 3.5 ISOLATION OF FLAVONOID FRACTIONS

#### 3.5.1 PREPARATION OF PLANT EXTRACT

Leaf of *B. montanum, D. roxburghii* and *C. variegatum* were collected, dried for one week at room temperature (in shade) and grinded in a blender to fine powder. Crude plant extract was prepared by soxhlet extraction method, 750 g of the powdered leaves of *B. montanum, D. roxburghii* and *C. variegatum* was boiled with 5 L
methanol for 8 h. The extracted solution was filtered and concentrated in a rotary evaporator under reduced pressure (rotary vacuum flash evaporator).

3.5.2 SEPARATION OF FLAVONOID FRACTIONS

The crude methanol extract (28 g) obtained was subjected to chromatography (Silica gel 120 mesh, 500 g) and eluted with ethyl acetate–n-hexane (73) solvent system. A total of 60 fractions were eluted. A Shinoda test was carried out to confirm the presence of flavonoids. Appearance of pink or red colour indicated presence of flavonoids. Fractions 1-25 were negative. Small Fractions 26-30, 35 - 40, 50-55 gave positive test. These fractions were pooled to form fraction I, II and III. The presence of flavonoids in fraction was confirmed through Thin layer chromatography.

3.6 ESTIMATION OF ANTIINFLAMMATORY ACTIVITY OF ISOLATED FLAVONOID FRACTIONS

3.6.1 CYTOTOXIC ACTIVITY

The cytotoxic activity of isolated flavonoid fractions were tested against Mouse leukaemic monocyte macrophage cell line RAW 264.7. The cells of cell line RAW 264.7 were seeded in 96-well plates (4×10⁴ cells/well) with 200 µl Dulbecco’s Modified Eagle Media (DMEM) for 24 h. RAW 264.7 cells were treated with lipopolysaccharide (LPS, 2 µg/mL), following exposure to 50 µg/mL of samples (flavonoid Fractions- I, II, III) for 30 min. After incubation for 4 h, the supernatant was removed, and the purple crystals were dissolved in 200 µL dimethyl sulfoxide (DMSO). After incubation for 24 h, cell viability was estimated by measuring the absorbance at 550 nm using an ELISA plate reader. Fresh culture medium was used as a blank and RAW 264.7 cells with fresh medium as control.

\[
\text{Cell viability} \% = \frac{A_{\text{sample}} - A_b}{A_c - A_b} \times 100
\]

\[
A_b = \text{absorbance of blank}
\]

\[
A_c = \text{absorbance of control}
\]
3.6.2 ANTI-INFLAMMATORY ACTIVITY

The anti-inflammatory activity was detected by Nitric oxide (NO) assay. Mouse leukaemic monocyte macrophage cell line RAW 264.7 was cultured in Dulbecco’s Modified Eagle Media (DMEM) (2 mM L-glutamine, 45 g/L glucose, 1 mM sodium pyruvate) with 10% fetal bovine serum (FBS). The cells were cultured at 37°C with 5% CO₂ and were subcultured twice a week. RAW 246.7 cells were seeded in 96-well plates at a density of 1 x 10⁶ cells /well and incubated for 24 h at 37°C with 5% CO₂ for adherence. The cells were treated with test samples for 1 h and then incubated for 24 h in fresh DMEM with 1g/mL of Escherichia coli lipopolysaccharide (LPS). Fresh culture medium was used as a blank. Two categories of control were used; first one was RAW 264.7 with LPS only and second one was without LPS and test sample.

The level of NO production in the pre-incubation of RAW 264.7 (Mouse leukaemic monocyte macrophage cell line) cell supernatants was determined according to the quantity of the nitrite indicator, using a colorimetric assay based on the Griess reaction; 100 µL of cell culture medium was mixed with 100 µL Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl-ethylenediamine dihydrochloride). Subsequently, the absorbance of the mixture was measured at 550 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader, after incubation at room temperature for 10 min. Percentage inhibition was calculated using the following equation (Srisopa Ruangnoo 2012).

\[
\text{Inhibition} (\%) = \frac{(A - B) \times 100}{(A - C)}
\]

[A LPS (+), sample (−); B LPS (+), sample (+); C LPS (−), sample (−)].

3.7 ESTIMATION OF ANTIOXIDANT ACTIVITY OF ISOLATED FLAVONOID FRACTIONS

3.7.1 ABTS assay [2, 2’-azinobis-ethyl-benzothiozoline-6-sulphonic acid]

Reagents

i. PBS (Phosphate Buffered Saline -125mM NaCl in 10mM Sodium phosphate buffer, pH 7.4); 0.14196 g of Disodium hydrogen orthophosphate (Na₂HPO₄), 0.1560 g of Sodium dihydrogen orthophosphate (NaH₂PO₄) and
0.7305 g of Sodium chloride (NaCl) is dissolved in 25 mL of distilled water and pH is adjusted to 7.4 using dilute sodium hydroxide solution and the volume is made up to 100 mL with de-ionized water.

ii. **ABTS (2,2’-azinobis-ethyl-benzothiazol-6-sulphonic acid) (7mM)** 38.4 mg of ABTS is dissolved in PBS and the volume is made up to 10 mL.

iii. **APS (Ammonium per sulfate) (2.45 mM)** 5.59 mg of APS is dissolved in PBS and the volume made up to 10 mL.

iv. **ABTS (2,2’-azinobis-ethyl-benzothiazoline-6-sulphonic acid) Radical solution**
   a) Mother stock 10 mL of ABTS (7 mM) and 10 mL of APS (2.45 mM) solutions are mixed and allowed to maintain at room temperature in dark for 16 h.
   b) Working solution 1.7 mL of the mother stock is made up to 50 mL with PBS, so as to give an absorbance of 1.000

v. **Quercetin (Reference standard)** 1 mg was made up to 10 mL with PBS.

ABTS assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process which is associated with a change in absorption which can be followed spectroscopically. The relatively stable ABTS radical has a green colour and is quantified spectrophotometrically at 734 nm.

The assay is performed as per Auddy (2003). ABTS radical cations are produced by reacting ABTS and APS (Ammonium per sulfate) on incubating the mixture at room temperature in dark for 16 h. Different concentrations of the test sample and the reference standard (highest volume taken was 50 µL) are added to 950 µL of ABTS working solution to give a final volume of 1 mL, made up by adding PBS. The absorbance is recorded immediately at 734 nm. The percent inhibition is calculated at different concentrations and the IC\textsubscript{50} values are calculated by Graphpad prism analysis.
3.7.2 DPPH assay [1,1-diphenyl-2-picryl hydrazyl]

Reagents

i. **Reference standard** Quercetin, store at room temperature.

ii. **Preparation of working solutions.**
- **DPPH** 1.3 mg/ml in HPLC grade methanol.
- **Quercetin** 5 mg dissolved in 100 mL methanol.

DPPH is a stable free radical with purple colour. Antioxidants reduces DPPH to 1,1-diphenyl-2-picryl hydrazine, colourless compound which is measured at an absorbance of 510 nm.

![DPPH diagram](Purple coloured)

DPPH assay was carried out as per the method of Rajakumar (1984). 75 μL of DPPH solution; various concentration of test solution and 3 mL of HPLC grade methanol were mixed. The reaction mixture was incubated at 25°C for 15 min. The absorbance was measured at 510 nm using semi-autoanalyzer. A control reaction was carried out without the test sample.

3.8. ESTIMATION OF ANTIMICROBIAL ACTIVITY OF ISOLATED FLAVONOID FRACTIONS

3.8.1 BACTERIA USED

The pure cultures of *Escherichia Coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* were procured from the Department of Microbiology,
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Bangalore medical college, Bangalore and were further maintained on nutrient agar slants at 4°C for further use, in the Department of Molecular Biology Bangalore University, Bangalore.

3.8.1.1 PATHOGENESIS OF BACTERIA

1) *Escherichia coli* Castel. and Chalm. *E. coli* was discovered by German paediatrician and bacteriologist Theodor in 1885 (Ryan and Ray, 2004). It is Gram –ve, motile and opportunistic colon commensal. It causes enteric diseases, haemorrhage colitis (blood haemorrhage), urinary tract infections, nosocomial infections, pyogenic infections and septicaemia (Riley *et al*., 1983; Tenover, 2006; Croft *et al*., 2007; Guyot *et al*., 1999 and Feng *et al*., 2002).

2) *Salmonella typhimurium* (Loe) Cast. And Chalm. It is gram –ve, bacillary enterobacteria probably with man is the only known reservoir. It causes enteric fever termed typhoid, gastroenteritis, bacteraemia with or without metastatic infection (Greenwood *et al*., 1998).

3) *Pseudomonas aeruginosa* (Sch) Migula it is Gram –ve, aerobic, non spore forming and capsulated bacillus occurs in soil, water, sewage, the mammalian gut and plants. It causes nosocomial infections including metabolic, haematologic and malignant diseases. Severe epidemic diarrhea of infants, ocular and burn infections, cystic fibrosis, osteomyelitis, otitis externa and varicose ulcers (Lennette *et al*., 1985) it infects the pulmonary tract, urinary tract, burns, wounds and causes blood infections (Fine *et al*., 1996).

3.8.1.2 MEDIA PREPARATION

The ingredients of a medium (Hi-media) (Table 2) were accurately weighed using digital electronic balance and dissolved in the known quantity of distilled water, pH of the medium was adjusted to 7.2 by adding few drops of 0.1N NaOH/HCl buffers using flasks and plugged with non-absorbent cotton. These were then sterilised by autoclaving in an autoclave at 15 lbs, 121°C for 15 min. The flasks were then cooled to 40°C and used in the study.
3.8.1.3 PREPARATION OF INOCULUM

Bacterial broth cultures (18 h old) were used as inoculum after adjusting its population to $10^6$ CFU/mL (Colony forming Unit) using 0.9% (w/v) sterile saline by the method of direct microscopic count (Mandal et al., 2007).

3.8.1.4 AGAR WELL DIFFUSION ASSAY

The Indian Pharmacopoeia (1996) technique depends upon the diffusion of test solution i.e. flavonoids Fractions I, II and III isolated from leaf of medicinal plants viz. *B. montanum, D. roxburghii* and *C. variegatum* and standard drugs from a cavity through the solidified agar layer to an extent such that growth of the added micro organism is inhibited entirely in circular area or zone around the cavity.

20 mL of sterile molten Mueller-Hinton agar medium was poured into a sterile petri dish under aseptic conditions and was allowed to solidify. Likewise a series of agar plates were prepared. Then, each plate was inoculated with 200 µL of 18 h old bacterial axenic cultures of $10^6$ CFU and was evenly spread with a sterile bent glass rod. A five number of agar wells (8 mm, diameter) were made equidistantly using sterile cork in order to load test solutions. Of these, the middle well was loaded with 100 µL of 2 mg/mL (w/v) streptomycin antibiotic solution with the help of a micropipette whereas, the four peripheral wells were loaded with 100 µL of isolated flavonoid Fraction I, II and III and control methanol.

All these plates were incubated after 30 min in BOD incubator at 37°C for a period of 24 h. The observed marked zone of growth of inhibition of bacteria were measured with the help of scale and recorded. This experiment was performed in triplicates in order to confirm the reproducibility.

3.8.2 FUNGI USED

The pure cultures of *Aspergillus fumigates, Aspergillus niger* and *Microsporum gypseum* were procured from the Department of Microbiology, Bangalore medical college, Bangalore and were further maintained on nutrient agar slants at 4°C for further use, in the Department of Molecular Biology Bangalore University, Bangalore.
3.8.2.1 PATHOGENESIS OF FUNGI

1) *Aspergillus fumigatus* Fresinius It is septate mycelial ascomycetous fungus generally found in the compost heap. The inhalation of conidia may result in allergic reactions. Growth of the fungus into tissues leads to ‘fungus ball’ of the sinuses of ear, which is fatal. It also causes bovine abortion in cattle and Pulomonary infection in birds (Cruickshank *et al.*, 1976)

2) *Aspergillus niger* Van Tiegh it is also septate mycelial ascomycetous fungus with characteristic coverings over the pseudoparenchymatous cleistothecia (Curaah, 1974). The nephrotoxins produced by *A. niger* causes kidney and liver damage, convulsions, haemorrhages of lung and brain (Shurtleff *et al.*, 1990; Griffin, 1994). It also causes aspergillosis in ducks, chicken and turkeys.

3) *Microsporum gypseum* (E. Bodin) Gui. And Grig. It is keratinophilous pathogenic deuteromycetous soil fungus whose perfect stage is called Annizia of Ascomycetes. It is the cause of tinea capitis, tinea pedis, tinea barbae, tinea corporis and tinea cruris in man and ringworm in animals (Frey *et al.*, 1979).

3.8.2.2 MEDIA PREPARATION

The composition of media is given in Table 3; media pH was adjusted to 5.6 and was prepared as mentioned earlier in the bacterial media preparation.

3.8.3.3 PREPARATION OF INOCULUM

Fungi were grown in potato broth were used for experimental studies. Cultures of fungi were incubated at 30°C.

3.8.3.3 AGAR WELL DIFFUSION ASSAY

The Rose Bengal Agar (RBA) and Sabouraud Dextrose agar (SDA) plates were prepared as mentioned in the bacterial study. These plates i.e. RBA plates were inoculated with *A. niger* and *A. fumigates* and SDA plates with *M. gypseum*, respectively with a sterilised absorbent cotton swab. The middle agar well is loaded with 100 µL of nystatin (2 mg/mL) while the peripheral four wells were loaded with 100 µL with isolated flavonoid Fractions I, II and III from leaf of *B. montanum*, *D.*
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roxburghii and C. variegatum and control (methanol) respectively. All the plates were incubated at 28°C in BOD incubator for 48 h and were observed for the circular zone of growth of inhibition of fungi around each well. This experiment was carried out twice by keeping triplicates in order to get reproducible data (Indian Pharmacopoeia, 1996).

3.9 ACCELERATION OF FLAVONOID SYNTHESIS

3.9.1 MEDIA PREPARATION

In the present investigation, Murashige and Skoog’s Basal Media (MSBM) was used for inducing callus. The stock solutions of macronutrients, micronutrients and organic constituents were prepared in double distilled water and stored in brown bottles at 4°C in the refrigerator till further use (Table 4). The sequential steps involved in the preparation of media are as follows

- Required quantity of sucrose was weighed and dissolved in distilled water
- Appropriate quantities of the various stock solutions including growth regulators were added
- The final volume of the medium was made with distilled water
- Required quantity of agar was weighed and dissolved in distilled water containing sucrose and stock solutions by uniform heating and constant stirring to avoid lumps
- The pH of the medium was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl.
- About 15 mL of the medium was dispensed into culture tubes and 40 ml into culture bottles
- The culture tubes were plugged with non-absorbent cotton wool wrapped in cheese cloth and culture bottles with polypropylene caps
- The culture tubes and bottles containing medium were sterilized at a pressure of 1.06 kg/cm² at 121°C for 15 minute.

3.9.2 STERILIZATION OF EXPLANTS

Internode of B. montanum, D. roxburghii and C. variegatum were surface sterilized by liquid detergent, Laboline 5% (v/v) for 5-10 minutes and brushed gently with the help of soft painting brush in order to remove all the debris, then washed thoroughly in distilled water and subsequently double distilled water to remove all the
traces of detergent. Further, internodes were disinfected by using mercuric chloride solution (0.5% w/v) under laminar air flow. Finally, internodes were dipped in 70% ethanol twice for 2-3 minutes followed by a rinse for 3-4 times with sterile double distilled water (Monokesh et al., 2014).

3.9.3 CALLUS INDUCTION

Internodal explants of *B. montanum*, *D. roxburghii* and *C. variegatum* were inoculated on Murashige and Skoog’s basal medium (MSBM) consisting of vitamins, 3% sucrose and 0.8% agar supplemented with 2,4-D (2,4-dichlorophenoxyacetic acid) at the concentrations of 2.26 µM, 4.52 µM, 9.05 µM, 13.16 µM and 10.08 µM and BAP (6-benzylaminopurine) at the concentrations of 2.22 µM, 4.44 µM, 8.87 µM, 13.32 µM and 17.76 µM were used for callus initiation and proliferation. MSBM without any plant growth regulators (2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine) was used as control. These cultures were allowed to grow up to 6 weeks. The pH of media was adjusted to 5.8 before autoclaving at 121ºC for 20 min. All cultures were maintained at 25 ± 2ºC in growth chamber with fluorescent light (1500 lux), 16 h light and 8 h dark photoperiod (Monokesh et al., 2014).

3.9.4 SUSPENSION CULTURE

Cell Suspension of *B. montanum*, *D. roxburghii* and *Codiaeum variegatum* were obtained from 6 weeks callus tissue developed from internodal explants. One gram of callus was excised in petri dish containing Whatman No.1 filter paper. Callus was carefully transferred with sterilized forceps to each of 250 mL Erlenmeyer flasks containing 50 mL liquid MSBM, supplemented with 2.26 µM of 2, 4-D alone. They were subcultured after every 14 d, the ratio inoculum to fresh medium was 16. The flasks were kept on a gyratory shaker at 100 rpm to a photoperiod of 16 hours, with fluorescent light (1200 lux) and a temperature of 25º C.

3.9.5 VIABILITY TESTS BY MTT (3-(4,5-DIMETHYTHIAZOL-2-YL)-2,5-DIPHENYL TETRAZOLIUM BROMIDE) ASSAY

Viability was determined by the MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. 1 mL sample of 6 weeks old cell suspensions was placed in micro-centrifuge tubes, centrifuged at 1000 rpm, and 100 µl of MTT
was added to the sample. MTT protocol was based on Tisserat and Manthey method, (1996). 1% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution was used to test the viability, mixture was incubated for 30 min at room temperature or 37°C. Viability was determined by counting the coloured cells in a hemocytometer. Control tests of viability were done with suspension cultures fixed in 70% ethanol.

3.9.6 ELICITOR TREATMENT

1. **The first experiment** Phenylalanine (25-100 µg/mL) was added in the 6 week old cell suspensions. The plant cells were harvested after 24, 48 and 72 h respectively and the liquid media was used to estimate total flavonoid content.

2. **The second experiment** Copper sulphate (CuSO₄) was added in flasks containing 6 weeks suspension cultures to obtain a final copper concentration of 20 µM, 30 µM and 40 µM respectively. The plant cells were harvested after 24, 48 and 72 h respectively (Cristina and Constantin, 2011).

3. **The third experiment** The bacterial cultures (*Pseudomonas aeruginosa*) were maintained on Mueller-Hinton Agar (MH). Maintained cultures were transferred to 100 mL liquid medium in 250 mL flasks and incubated at room temperature. The bacterial liquid cultures were kept on a rotary shaker (90 rpm). The culture was collected after reaching stationary phase (48 h). Flasks which contain bacteria were then autoclaved and the solution obtained was stored at 4°C for future use. The pH was adjusted to 5.8 before autoclaving at 121°C and a pressure of 1.04 kg/cm² for 20 min. The dead cells of *P. aeruginosa* (1, 2, 3 µg/mL) was added in 6 weeks old suspension culture (Buitelaar et al., 1992).

3.9.5 EXTRACTION OF FLAVONOIDs

For the extraction of flavonoids, plant cells from suspension cultures were oven dried at 45°C and pulverized. Powdered samples (1.0 g) was soaked in 100 mL of methanol for 72 h and filtered through a Whatman No. 1 filter paper. The filtrates were concentrated using a rotary evaporator.
3.9.6 TOTAL FLAVONOIDS ESTIMATION

Aluminum chloride colorimetric method was used to estimate total flavonoid content. 1 mL of plant cells methanol extract was mixed with 1mL of methanol, 0.5 mL aluminum chloride (1.2%) and 0.5 mL potassium acetate (120 mM). The mixture was allowed to settle for 30 min at room temperature, and then the absorbance was measured at 415 nm. Quercetin was used as standard. Flavonoid is expressed in terms of quercetin equivalent (mg/g of extracted compound). Each of the tests was carried out in triplicate and the results are expressed in Mean ± STD (Chang et al., 2002).

3.10 GAS CHROMATOGRAPHY-MASS SPECTROMETRY PROFILING

3.10.1 PREPARATION OF PLANT MATERIAL

The fresh leaf of *B. montanum*, *D. roxburghii* and *C. variegatum* were collected and washed individually under running tap water to remove any traces of soil particles and other dirt. The samples were dried at 60°C for 2 d in an oven. They were then macerated to powder form with a mixer grinder.

3.10.2 PREPARATION OF SAMPLE FOR GC-MS STUDY

About 20 gm of the powdered plant sample were soaked in 100 mL methanol separately. It was left for 24 h so that phytoconstituents will get dissolved. The methanol extract was filtered using Whatman No.1 filter paper and the residue was removed. It was again filtered through sodium sulphate (Na₂SO₄) in order to remove the traces of moisture.

3.10.3 GC-MS

The soxhlet extracted methanolic leaf solution was filtered and concentrated in a rotary evaporator under reduced pressure (rotary vacuum flash evaporator). Methanolic extracts (1 µL) were injected for GC-MS analysis.

GC-MS analysis of the methanolic extract of *B. montanum* (sample name-SB3), *D. roxburghii* (sample name- SB2) and *C. variegatum* (sample name-SB1) were performed using a Perkin–Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS).
equipped with a Restek RtxR (5% diphenyl/95% dimethyl poly siloxane) fused a capillary column (30 × 0.25 μm ID × 0.25 μm). For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 1 μl was employed. The injector temperature was maintained at 280°C, the ion-source temperature was 200°C, the oven temperature was programmed for 40°C (isothermal for 5 min), with an increase of 10°C/min to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC/MS running time was 60 min. The mass-detector used in this analysis was Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-5.2.

3.10.4 DATA INTERPRETATION

The samples (SB1, SB2 and SB3) were subjected to GC-MS. Extracted ion chromatograms were obtained from all the major peaks. The mass of the compounds and fragments recorded were matched with NIST database for identification of probable compounds present in the sample.

3.11 QUERCETIN QUANTIFICATION

3.11.1 PREPARATION OF PLANT MATERIAL

The fresh leaf samples of plant B. montanum, D. roxburghii and C. variegatum were collected the Department of Molecular Biology, Bangalore University, Bangalore. The leaf samples were washed individually under running tap water to remove any traces of soil particles and other dirt. The samples were air dried at room temperature for 6 to 8 d. They were then macerated to powder form with a mixer grinder.

3.11.2 SAMPLE PREPARATION

The dried sample was mixed with 1% of extract was prepared in HPLC grade methanol. Then the sample was sonicated using ultrasonicator for 10 min. 1 ml of this solution was injected into the HPLC column using mobile phase of 3664 (acetonitrile
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and 0.1 %phosphoric acid). Quercetin in the samples was identified by comparison of their retention times (tR) with the standard Quercetin.

3.11.3 STANDARD PREPARATION

Standard preparation for quercetin Standard (100 mg) were transferred to 100 ml volumetric flask and dissolved in mobile phase. The flask was shaken for 10 minute and the volume was made up to the mark with methanol to obtain stock solution of Quercetin (1000 µg/mL), stock solution was filtered through a 0.2 µm membrane filter. The working standard solution of quercetin was prepared from suitable aliquots of stock solution were pipetted out and volumes were made up to the mark with mobile phase as diluents.

3.11.4 HPLC ANALYSIS

The quercetin quantification was done by HPLC Shimadzu LC-20A. The Column Phenomenox Luna C18 column, 250mm x 4.6 mm Particle size 5 micro meter was used with Mobile Phase Acetonitrile Water (7030). The Flow rate was maintained at 1ml/min 5. Sample Volume injected 20 ul sample volume was injected and it was run for 45min. The The HPLC equipment comprised Hewlett-Packard (HP) 1050 ChemStation Software, an HP model 35900 interface unit, an HP 9000 Series 300 computer, and an HP DeskJet 500 Printer. A Waters 486 tuna-ble absorbance detector was operated at 254 nm; detector sensitivity was 0.05 AUFS and the column oven temperature was 30°C.

3.12 SILVER NANOPARTICLES SYNTHESIS

3.12.1 PREPARATION OF THE LEAF EXTRACT

Fresh and healthy leaves were collected from B. montanum, D. roxburghii and C. variegatum. Firstly leaves were rinsed with tap water and followed by distilled water to remove all the dust and unwanted visible particles. It was cut into small pieces and dried at room temperature. About 10 g of these finely incised leaves of all plants were weighed separately and transferred into 250 mL beakers containing 100 mL distilled water and boiled for about 20 min. The extracts were then filtered thrice through Whatman No. 1 filter paper to remove particulate matter and to get clear solutions which were then refrigerated (4°C) in 250 mL Erlenmeyer flasks for further
experiments. In each and every steps of the experiment, sterility conditions were maintained for the effectiveness and accuracy in results without contamination.

3.12.2 SYNTHESIS OF NANOPARTICLES
1 mM aqueous solution of silver nitrate was prepared for synthesis for silver nanoparticles. 1 mL of this solution was added to 5 mL extract of the plant material to obtain silver nanoparticles. The plant extract with the substrate (i.e. silver nitrate) were kept at 25°C on a shaker at 150 rpm in dark. Different concentrations of silver nitrate were used to standardize the optimum concentration of silver nitrate for synthesis of silver nanoparticles. The concentrations ranged from 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM and 0.5 mM of silver nitrate (Prathna et al., 2011).

3.12.3 CHARACTERIZATION OF SILVER NANOPARTICLES

UV- VIS SPECTRA ANALYSIS
The reduction of metallic Ag+ ions was monitored by measuring the UV-Vis spectrum after about 16 h of reaction. A small aliquot was drawn from the reaction mixture and a spectrum was taken on a wavelength from 200 nm to 720 nm on UV-Vis spectrophotometer.

3.12.4 ANTIBACTERIAL ACTIVITY
Antibacterial activity was assayed by turbidometric method. The antimicrobial activity was tested against E. coli. fresh colonies were inoculated from agar media into 10 mL of Luria Bertani broth media. The media was supplemented with 10-30 μg/mL silver nanoparticles synthesized from silver and plant extract (B. montanum, D. roxburghii and C. variegatum). The bacterial cultures with silver nanoparticles were incubated at 37° C with continuous shaking at 150 rpm. The growth of E. coli in broth media was indexed by measuring the optical density (at λ=600 nm) at regular intervals using UV-Vis spectrometer (Swarnali et al., 2014).