MATERIALS AND METHODS
3. Materials and Methods

3.1 Cultivation of *Rosa bourboniana* under open field condition

Cultivation of *Rosa bourboniana* was done based on the methods described by Nagaraja (1997). Cultivation study was done from January 2012 to February 2014, during the period plant parameters were evaluated by the application of organic and inorganic amendments. Plant parameters like height, number of branches, number of flowers, bud diameter, bud length, flower size, length of pedicle, flower petal count and flower weight were recorded.

3.1.1 Area selected for the study

The study areas selected for the present study are Natham, Athupakkam and Rettembedu villages located in the Gummidipoondi (National Highway NH5) Panchayat union of Thiruvallur District in Tamilnadu (Fig. 1).

3.1.2 Geographical location of the study area

The site selected for study is a small agricultural town located at 13.41° North latitude, 80.12° East longitude and 17 meters elevation above the Mean Sea Level (MSL) located at a distance of about 42 Km from Chennai, Tamilnadu, India (Fig. 1).

3.1.3 Climatic conditions of the study area

The average temperature in the study area was 35º C - 40º C in summer and 27º C - 30º C in winter. April, May, June and July are the months with maximum temperatures while November, December and January are the months with minimum temperatures. The study area received rainfall in two seasons namely South West Monsoon prevailing from June to September (451.6 mm) and North East Monsoon (589.3 mm) prevailing from October to December every year.

3.1.4 Preparation of land for cultivation of *Rosa bourboniana*

The land selected for cultivation was well prepared and leveled prior to cultivation. The field selected for cultivation was such that the plants can get proper sunlight for at least 6 hours in a day in every season avoiding shade beneath trees and root competition with those of rose plants for available nutrients and moisture. Double-dig bed raise method was adopted for the cultivation of *Rosa bourboniana*. The top soil was removed, mixed and tiled
to a depth of about 24 hours facilitating aeration to roots, good drain and easy penetration of roots deeper into the soil. This was done to remove rocks and debris that could obstruct root growth. Double-digging provided a reservoir of steady nutrients and sufficient water which can be accessed by rose plants deeper roots. A tractor was employed for leveling the cultivation bed. Both clockwise and anti-clockwise mode of leveling was done during the preparation of cultivation bed. Pebbles and rocks were handpicked manually. Weeds, grasses and other herbs were removed that colonized the field. The cultivation bed was leveled in such a way to ensure proper drain during rainy season (Plate. 1).

3.1.5 Plot preparation: Cultivation bed and Field experimental design and treatment

The experimental plot was a completely randomized block design (CRBD). A plot was designed in the prepared cultivation bed. The size of the study plot was in an area of 10,000 sq.ft with equal length and width. Thirteen rows were partitioned so as to plant rose seedlings in single line pattern (Plate. 2). Each row was planted with ten siblings of *Rosa bourboniana*. Plants were planted at a spacing of 60 cm × 30 cm (Plate. 3). The reason for maintaining space is to avoid collision of plants and to ensure proper aeration when it attains a bushy nature. The plot was designed in such a way to prevent nutritional mixing with the adjacent rows. Each row is designated for the application of its respective amendments and easy application of vermicompost and chemical fertilizer to the study plot (Plate 6), and the experiments were done in triplicates.

3.1.6 Irrigation method

The cultivation bed of *Rosa bourboniana* was irrigated daily during peak summers and as per requirements during rainy season. Irrigation was done by canals constructed from bore wells to the study plot (Plate. 3).

3.1.7 Soil fertility analysis

In order to fulfill the main objective of the study i.e., monitoring the soil fertility status, the initial fertility level of the study plot was assessed by studying the physical, chemical and biological properties.
Figure 1. Geographical location of the study area
Plate 1. Land preparation for the cultivation of *Rosa bourboniana*

A & B - Initial eradication of weeds in the study plot; C - Clockwise tilling of the study plot; D - Anticlockwise tilling of study plot; E - Tilting of soil bed for uniform mixing of soil; F - Prepared field bed for cultivation.
Plate 2. Land preparation for the cultivation of *Rosa bourboniana*

A & B - Bullock drawn plough for designing rows for planting seedlings of *Rosa bourboniana*; C & D - Rows depthened to hold fertilizers and to ensure proper nutrition; E - Manual digging of pits to plant seedlings; F - Wet rows ready for planting seedlings
Plate 3. Planting of *Rosa bourboniana* in the study plot

A - Healthy disease free mother plant seedlings of *Rosa bourboniana*;  
B - Planting of seedling; C & D - Plantation at proper depth and spacing;  
E - Young seedlings of *Rosa bourboniana*; F - Canal irrigation to the study plot
3.1.8 Fertility status (physico-chemical analysis) of the study plot following the amendments of organic and inorganic fertilizers

The physico-chemical analysis of the study plot was characterized prior to study and also during the course of cultivation. Analysis was done during the 0\textsuperscript{th} day, 60\textsuperscript{th} day, 150\textsuperscript{th} day, 240\textsuperscript{th} day and 330\textsuperscript{th} day respectively.

Soil sampling

Soil sampling was carried out following a standard method (Jackson, 1973). For this, samples from the cultivation bed was collected by digging pits between each plants at three different depths i.e. from 0 to 15 cm (surface), 15 to 30 cm (middle) and 30 to 60 cm (lower) and scrapping the walls from all sides at each depth. The process was repeated at 4-5 locations for each treatment and the samples were thoroughly mixed. After dividing the samples into four equal parts, the two opposite parts were rejected and the other two parts were mixed again. By repeating the process, 250 g of soil samples were taken for analysis. The top soil (0 - 22 cm) was considered for analyzing the physical properties and 15 to 30 cm depth soil was assessed for soil micro flora. The soil was analyzed for pH, Electrical conductivity, organic matter, total Kjeldahl nitrogen, phosphorous, potassium, calcium, magnesium, sulphur, zinc and micronutrients like manganese, iron, copper, Boron and saturations of potassium, calcium, magnesium and sodium.

Determination of soil pH

The pH of the soil was tested following standard protocols of Jackson (1973).

Determination of electrical conductivity

The electrical conductivity was measured as per the protocols of Jackson (1973).

Estimation of organic matter

The total organic matter content of the soil samples were analyzed by through the protocols of Walkley and Black (1934).

Estimation of total Kjeldahl nitrogen

Total Kjeldahl nitrogen content of the soil sample was estimated by Kjeldahl method as described by Tandon (1993).
**Estimation of cation exchange capacity**

Estimation of cation exchange capacity of soil is done following the protocols of Toth and Prince (1949).

**Estimation of available phosphorous**

Available phosphorous was estimated by the methods of Olsen *et al.* (1954).

**Estimation of available potassium**

Level of potassium in the soil sample was estimated by the methods of Tandon (1993).

**Estimation of exchangeable calcium and magnesium**

Level of exchangeable calcium and magnesium in the soil sample is determined by the protocols stated by Chang and Bray (1951).

**Estimation of exchangeable sulphur**

The level of exchangeable sulphur is determined following the protocols of Chesnin *et al.* (1950).

**Estimation of zinc, iron, manganese and copper**

Level of zinc, iron, manganese and copper was determined following the protocols advocated by Lindsay and Norvell (1978).

**Determination of boron level**

Determination of available boron is done following the protocol stated by Berger and Truog (1939).

**Saturation levels of potassium, calcium, magnesium and sodium**

Saturation levels in the soil samples were analyzed by the protocol of Tandon (2005).

**3.1.9 Estimation of bacterial and fungal loads of the cultivation bed amended with various organic and inorganic fertilizers**

The record for the populations of bacteria and fungi in the soil samples in the cultivation bed exposed to organic and inorganic amendments during the course of cultivation and prior to study was carried at regular intervals from the beginning of the study (whole plot) and on 60th day, 150th day, 240th day, 300th day and 330th day till the end of the experiment (treatments T1 - T13) following the protocol stated by Baruah and Barthakur (1997) for bacteria and Aneja (1996) for fungi. Soil samples for analysis were randomly
collected from 4-5 locations which are in close proximity of root zones at 15-30 cm depth and carried in a sterile polythene bag for further analysis. Record of bacterial and fungal population is expressed as the colony forming units (CFUs) per gram of soil sample. The number of bacterial and fungal colonies appeared over the medium in the petridishes were counted and recorded. The colony morphology for bacteria and fungi were studied using high power binocular microscope (Canon). The observations were compared and studied with the monographs by Gilman (1957), Subramanian (1971), Domsch *et al.* (1980) and Ellis (1985).

### 3.1.10 Analysis of enzyme activity of the soil in the cultivation bed amended with various organic and inorganic fertilizers

Analysis of the soil enzymatic activities were done prior to start of the cultivation study and also during the course of cultivation at regular intervals at 60th day, 150th day, 240th day, 300th day and 330th day till the end of the experiment (treatments T1 - T13). Activity was measured for Indole acetic acid (Woheler, 1997), Dehydrogenase (Garcia *et al.*, 1997), Acid and Alkaline Phosphatases (Garcia *et al.*, 1997), Urease (Kandeler and Gerber, 1988) and Catalase (Rodriguez-Kabana and Truelove (1982) enzyme activity. The analysis was done as per the protocol stated in the upcoming sections (3.3.8 - 3.3.11).

### 3.1.11 Organic and Inorganic amendments treatment details

- **T1** - Control (C)
- **T2** - Cow dung (CD)
- **T3** - Press mud Vermicompost (PMVC)
- **T4** - Vegetable waste Vermicompost (VWVC)
- **T5** - Tea dust Vermicompost (TDVC)
- **T6** - Cow dung Vermicompost (CDVC)
- **T7** - Chemical Fertilizer (CF)
- **T8** - Pressmud Vermicompost + Vegetable waste Vermicompost + Tea dust Vermicompost + Cow Dung Vermicompost (MIX1)
- **T9** - Pressmud Vermicompost + Vegetable waste Vermicompost + Tea dust Vermicompost + Cow Dung Vermicompost + Chemical Fertilizer (MIX2)
- **T10** - Pressmud Vermicompost + Vegetable waste Vermicompost (PMVW)
- **T11** - Pressmud Vermicompost + Tea dust Vermicompost (PMTD)
T12 - Pressmud Vermicompost + Cow dung Vermicompost (PMCD)
T13 - Pressmud Vermicompost + Chemical Fertilizer (PMCF)

3.1.12 Overall inputs of organic and inorganic amendments used in the present study

The various organic and inorganic amendments were given in split doses. All the necessary cultural practices and protective measures were followed uniformly for all the respective treatments during the entire period of experimentation. Organic amendments either in single amendment or in mixed amendment was applied at the rate of 6 tons ha\(^{-1}\), individual inorganic amendment was applied at the rate of 14 tons ha\(^{-1}\) (recommended dosage for the cultivation of *Rosa bourboniana* as practiced by the local farmers in the study area). Integrated fertigation of organic (vermicompost) and inorganic amendment (chemical fertilizer) was applied at the rate of 6 tons ha\(^{-1}\) during the overall period of cultivation. The different proportions and ratios of dosage imposed during the cultivation are as follows:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proportions / ratio</th>
<th>Quantitative extrapolation per Hectare area</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 - control</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T2 - CD</td>
<td>-</td>
<td>6 tons ha(^{-1})</td>
</tr>
<tr>
<td>T3 - PMVC</td>
<td>100 %</td>
<td>6 tons ha(^{-1})</td>
</tr>
<tr>
<td>T4 - VWVC</td>
<td>100 %</td>
<td>6 tons ha(^{-1})</td>
</tr>
<tr>
<td>T5 - TDVC</td>
<td>100 %</td>
<td>6 tons ha(^{-1})</td>
</tr>
<tr>
<td>T6 - CDVC</td>
<td>100 %</td>
<td>6 tons ha(^{-1})</td>
</tr>
<tr>
<td>T7 - CF</td>
<td>100 %</td>
<td>6 tons ha(^{-1})</td>
</tr>
<tr>
<td>T8 - MIX 1</td>
<td>100 %</td>
<td>14 tons ha(^{-1})</td>
</tr>
<tr>
<td>T9 - MIX 2</td>
<td>1:1:1:1</td>
<td>6 tons ha(^{-1})</td>
</tr>
<tr>
<td>T10 - PMVC + VWVC</td>
<td>1:1:1:1</td>
<td>6 tons ha(^{-1})</td>
</tr>
<tr>
<td>T11 - PMVC + TDVC</td>
<td>1:1</td>
<td>6 tons ha(^{-1})</td>
</tr>
<tr>
<td>T12 - PMVC + CDVC</td>
<td>1:1</td>
<td>6 tons ha(^{-1})</td>
</tr>
<tr>
<td>T13 - PMVC + CF</td>
<td>1:1</td>
<td>6 tons ha(^{-1})</td>
</tr>
</tbody>
</table>

3.2 Morphological observations of *Rosa bourboniana* grown under organic and inorganic amendments

3.2.1 Measurement of plant height
Height changes observed in the plants of *Rosa bourboniana* were measured for all the plants in each plot (T1 - T13) and the average was taken and recorded. Height was measured from the base of the plant to the apex of the main stem and expressed in centimeter. Height was measured at the end of every 30 days till the end of the cultivation period.

### 3.2.2 Count for the number of branches

The number of branches was counted from each plant in the plot and the average was taken and recorded. The record was done at 30 days interval upto the end of cultivation study.

### 3.2.3 Measurement of bud diameter

Fully formed buds were measured lateral axially in all the plants of the respective plots and the average in centimeter was taken as bud diameter for the plot. The bud diameter was recorded periodically in all the plots.

### 3.2.4 Measurement of bud length

Bud length was calculated by measuring the longitudinal axis of the fully formed buds (yet to bloom) from each plant from their respective treatments was calculated and the average values were recorded and expressed in centimeters. The record was made at regular intervals till the end of the cultivation period.

### 3.2.5 Count for the number of flowers

The number of flowers bloomed from all the plants in their respective treatments from each harvest in each month till the end of the cultivation study were counted, added up and the average values were recorded.

### 3.2.6 Measurement of pedicle length

Length of the stalk was measured from the point of attachment of flower to the stem and to the point below the base of the flower for all the flowers and the average values were recorded for every flower in a plant in the plot from the beginning till the end of the cultivation study. The values were expressed in centimeters.

### 3.2.7 Measurement of the flower size

The flower size was measured by calculating the longitudinal distance of the completely bloomed flower and the average values were recorded for every flower in each plant in the plot during every harvest till the end of the cultivation study.
3.2.8 Analysis of floral weight

Fully bloomed flowers from every harvest were weighed individually in a weighing balance and the average values for every plant in the plot was recorded from the first formed bloom till the end of the cultivation study.

3.2.9 Count for the number of petals

Petals from the fully bloomed flower were withered and counted for the number of petals. The average count for every flower in all the plants in the plot was recorded during every harvest from the first phase of flowering till the end of the cultivation study.

3.3 Collection and authentication of local worm for vermicomposting

*Perionyx excavatus* - Indian Blue worm

A garden soil in a nearby shade of tree was selected for collecting worms since the population is high below tree shades. Worm collection was also done from the banks of ponds in and around Elavur village located near Gummidipoondi. For collecting worms fresh cow dung was scattered over one meter square area. This is covered with hay and leaf litter. The entire set up was finally covered by an old jute bag or jute cloth. The set up was maintained for moisture and left for some time. In about a fortnight of time worms were found to be observed beneath the jute bags. These worm populations were collected along with the native soil and were carefully taken for further use. Another method we adopted to collect worms was the application of 1 kg of jaggery and 1 kg of fresh cow dung dissolved in 20 litres of non saline water to that area once or twice a week (Ismail, 1997). This protocol was very effective since, large number of worms obtained by this method.

The worm thus obtained was *Perionyx excavatus* commonly known as blue worm used for the present study of Vermicomposting (Plate 5C). The collected species was identified and authenticated by the Post Graduate and Research Department of Zoology, The New College, Chennai 600014, Tamilnadu, India.

3.3.1 Maintenance of worm stock culture

The authenticated worms were cultured in large plastic bins amended with 50 % cow dung and 50 % of native soil with an optimum temperature of 28±5°C and 60 - 70 % moisture in vermicomposting unit maintained at the PG and Research Department of Botany,
Pachaiyappas College, Chennai, India. From this stock culture, healthy adult clitellate worms were picked and utilized for vermicomposting (Plate 5).

3.3.2 Construction of vermicomposting unit

Vermicomposting was carried out in cement tanks with dimensions of 5 meters length, 2 meters breadth and 2 meters height and were constructed above the ground level beneath the shade of a tree with thatched shed to prevent sunlight and rain water entering the unit (Plate 5A).

3.3.3 Preparation of vermibed

Vermibed refers to the composting unit of the constructed pit. It was carried out by first placing a basal layer of broken bricks and pebbles followed by coarse sand to a thickness of 6 cm for ensuring proper drainage. This is topped by a layer of coir waste, paddy and coconut husk facilitating retention of water during composting. Further, it is topped by a layer of native garden soil to a height of about 15 cm. The vermibed thus prepared was maintained for moisture by spraying water with a sprayer at regular interval of time.

3.3.4 Collection of organic wastes for vermicomposting

a. Cow dung (CD)

Fresh urine-free Cow dung was collected from local municipal territory and agricultural lands of Gummidipoondi panchayat union of Tiruvallur district in Tamilnadu, and it was heaped for 1 week in open air condition for initial aerobic decomposition (Plate 4).

b. Press mud (PM)

Press mud was collected from Sausan Sugar mills located in Kallakurichi of Tamilnadu about 150 km from Chennai. The collected press mud was transported to the study site. It was cured to remove sulphur residues by mixing with water and dried in open air conditions for 1 week to remove heat and foul smelling odor (Plate 4).
Protocol for the Preparation of Vermicompost

Collection of Organic waste materials for vermicomposting (cow dung, press mud, tea dust, vegetable waste)

Preparation of vermicells in the vermpit using the lower layer with bricks and pebbles, middle layer with coarse sand and the upper layer with garden soil

Primary decomposition of the composting material in open air

Loading of the vermpit with the processed composting material and cow dung in 1:1 ratio (Maintaining the moisture at 60-70% with 20 ± 5°C)

Secondary decomposition for 15 days by sprinkling water and giving turn

Introduction of worm culture (Eisenia fetida) to the vermpit

Composting period

45-60 days

Harvesting of the compost

Compost sieved through 2mm sieve and packed in poly bags to retain moisture

Ready to apply in the field
c. Vegetable waste (VW)
Vegetable wastes were collected from Koyambedu market located in the heart of Chennai, India. The wastes were transported to the study site (Plate 4) for further usage.

d. Tea dust (TD)
Tea dust waste was collected from the restaurants and cafeteria located in and around Gummidipoondi area in Tiruvallur district of Tamilnadu. It was heaped in open sunlight for 1 week to reduce heat during Vermicomposting (Plate 4).

3.3.5 Initial physico-chemical properties of selected organic wastes

Cowdung (CD)
The initial physico-chemical properties of cowdung waste were,
1. pH (1:10) ratio - 7.73
2. Electrical conductivity - 0.46
3. Total organic carbon (TOC) - 10.23
4. Total Kjeldhal nitrogen (TKN) - 1.08
5. Total Phosphorous (TP) - 0.02
6. Total Potassium (TP) - 0.17
7. C:N ratio - 9.46

Pressmud (PM)
The initial physico-chemical properties of pressmud waste were,
1. pH (1:10) ratio - 7.36
2. Electrical conductivity - 3.49
3. Total organic carbon (TOC) - 30.21
4. Total Kjeldhal nitrogen (TKN) - 2.34
5. Total Phosphorous (TP) - 2.83
6. Total Potassium (TP) - 1.53
7. C:N ratio - 12.90

Vegetable waste (VW)
The initial physico-chemical properties of vegetable waste were,
1. pH (1:10) ratio - 8.51
2. Electrical conductivity - 2.81
3. Total organic carbon (TOC) - 25.36
4. Total Kjeldhal nitrogen (TKN) - 3.21
5. Total Phosphorous (TP) - 0.11
6. Total Potassium (TP) - 0.76
7. C:N ratio - 7.89

Tea dust (TD)

The initial physico-chemical parameters of tea dust waste were,

1. pH (1:10) ratio - 8.27
2. Electrical conductivity - 3.27
3. Total organic carbon (TOC) - 19.55
4. Total Kjeldhal nitrogen (TKN) - 2.15
5. Total Phosphorous (TP) - 0.36
6. Total Potassium (TP) - 0.26
7. C:N ratio - 9.09

3.3.6 Processing of the organic waste material

The organic waste thus collected cannot be used for vermicomposting directly as it generates lot of heat. The waste material was heaped in sunlight and exposed to temperatures ranging from 29°C to 32°C for two weeks to get rid of toxic gases generated (Plate 5B). To prepare a uniform feed mixture, the waste was individually mixed with fresh cowdung (urine free) in the ratio of 1:1 on dry weight basis and maintained for 45 – 50 % moisture to initiate microbial activity and break down of complex organic biomolecules into tiny particles. Turning of mixture was given at alternate days manually for uniform decomposition.

3.3.7 Physico-chemical analysis of the prepared vermicompost

Physico-chemical analysis was done at regular intervals. Analysis was done from the beginning till the end of the experiment. Samples were collected in a sterile polythene bags from the ongoing vermicomposts and were taken to the laboratory for complete analysis. Sampling was done at 0th, 30th and 60th day. Analytical grade chemicals supplied by Himedia, Chennai were used for the overall study. All the experiments were done in triplicates and the average values were taken.
**pH (Hanna, 1968)**

pH of the sample was measured using an ‘Elico’ digital meter. The electrode of the pH meter was rinsed in double distilled water and it was standardized using buffer solutions. The samples to be tested were suspended in double distilled water in the ratio of 5:1 (w/v). The suspension was mechanically agitated for 15 minutes and filtered through Whatman No.1 filter paper. The filtrate was measured for pH using the calibrated electrode.

**Electrical conductivity (Hanna, 1968)**

For measuring electrical conductivity (EC) the sample was mixed in the ratio of one gram of sample in 5 ml of sterile double distilled water. The suspension was agitated mechanically in a shaker for 4 hours and filtered using Whatman No.1 filter paper. The filtrate was measured for EC using ‘Elico’ digital electrical conductivity meters.

**Organic carbon estimation (Jackson, 1958)**

Organic carbon in the sample was estimated by using the modified calorimetrical method of Walkley-Black partial oxidation protocol of Jackson (1958). One gram of sample from the pit was air dried in a blower and it was powdered and sieved through sieve plate (0.5 mm). The sample was taken in a 500 ml conical flask. 10 ml of N-Potassium dichromate, 20 ml of distilled water and 10 ml of 85% phosphoric acid was added and mixed. Diphenylamine was used as the indicator. The solution was titrated against ferrous sulphate. Appearance of permanent pale pink color is the end point. Again 0.5 ml of N-Potassium dichromate was added with continuous stirring and it was further titrated against more amount of ferrous sulphate until the color of the solution disappeared. Since 1 ml of N-Potassium dichromate corresponds to 3 mg of carbon, the percentage of organic carbon was calculated using the equation,

\[
\% \text{ of Organic carbon} = \frac{V_1 - V_2 \times 0.003 \times 100}{W}
\]

Where,

\( V_1 = \text{ml of N-Potassium dichromate} \)

\( V_2 = \text{ml of N-Ferrous sulphate} \)

\( W = \text{Weight of the sample} \)
Analysis of Total Kjeldahl Nitrogen - TKN % (Tandon, 1993)

Total Kjeldahl Nitrogen content in the sample was analyzed by Kjeldahl method stated by Tandon (1993). About 0.5 g of dried sample was transferred to 100 ml Kjeldahl flask add 20 ml of sulphuric salicylic acid mixture was added allowed to react overnight. After incubation 5 g of sodium thiosulphate was added and heated gently for 5 minutes. After cooling of the mixture 10 g of sulphate mixture was added and digested over Kjeldahl apparatus at high temperature for 1 hr. when the digestion was completed the mixture was cooled. About 10 ml of the cooled digest mixture was transferred into the vacuum jacket of micro-Kjeldahl distillation apparatus. About 10 ml of 4% boric acid solution was taken in a conical flask along with few drops of bromicresol green and methyl red indicators. The condenser outlet of the micro-Kjeldahl apparatus is dipped into this conical flask. The funnel of the micro-Kjeldahl apparatus was washed with 2-3 ml of deionized water followed by 10 ml of 40 % NaOH solution. Aliquot of 5 ml was distilled to the flask containing the boric acid solution. The boric acid solution is further titrated against N/200 H$_2$SO$_4$. Blank was also titrated till the same end point as that of the sample.

Total phosphorous (TP %)

Colorimetric method (Tandon, 1993)

Preliminary Diacid digestion of vermicompost sample:

Diacid digestion of vermicompost sample was done using HNO$_3$ and HClO$_4$ acid in the ratio of 9:4. About 10 ml of the prepared diacid was added to the vermicompost sample taken in the flask and mixed properly by gentle swirling. The flask was placed over hot plate at low temperature initially and at high temperatures till the red colored NO$_2$ fumes ceases. The end point of digestion was confirmed when the liquid becomes colorless. After cooling of the digest 20 ml of deionized distilled water was added in drops and the solution was made up to the mark. The solution was filtered through Whatmann No.1 filter paper.

Procedure:

About 5 ml of aliquot was pipette out to a volumetric flask and 10 ml of vanadomolybdic reagent was added to the flask. The volume was made up with deionized water by complete mixing. The solution was allowed to settle for 30 minutes. On appearance
of yellow color absorbance was read at 420 nm with spectrophotometer and the readings were recorded. Total phosphorous content in the sample was determined using the equation,

\[
P_2O_5 \% = \frac{\text{Concentration of sample (ppm)}}{\text{Weight of sample}} \times \frac{100}{\text{aliquot of sample}}
\]

Quantification of Total Potassium (TK)

Total potassium content of the vermicompost sample was estimated by Flame Photometric method (Tandon, 1993).

Preparation of Standard Stock solution of Total potassium

One gram of analytical grade KCl was weighed and dissolved in double distilled water in a 1000 ml standard flask and made up to 1 litre. The stock thus prepared contains 1000 ppm of dissolved potassium. From this stock, 10, 20, 30, 40 and 50 ppm standard concentrations of potassium solutions were prepared by appropriate dilutions.

Preparation of Standard Curve

The galvanometer reading was adjusted to 0 in the flame photometer using distilled water. Then, 10, 20, 30, 40 and 50 ppm standard potassium solution was fed into the flame photometer and the corresponding galvanometer reading was recorded. A standard graph was drawn by plotting flame photometer readings on Y-axis and potassium concentrations on X-axis.

Analysis of Potassium content of vermicompost sample

The vermicompost sample was atomized in the flame photometer and the galvanometer readings were recorded. The potassium concentration in the sample was determined referring the standard graph and multiplying it with dilution factor.

Estimation of Zinc and Copper

Zinc and Copper content in the vermicompost sample were estimated using Atomic Absorption Spectrophotometer (AAS) as per the protocol stated by Tandon (1993). The AAS readings were read at 213 nm for the estimation of zinc and 324 nm for the estimation of copper in the vermicompost samples. A standard graph was prepared using standard concentrations of zinc and copper (1000 mg/l).
The zinc/copper was determined referring to the standard curve and multiplied with dilution factor.

\[ M \times 1000 \text{ ppm.} \]

Where, \( M \) is the dilution factor.

**Estimation of Calcium in the vermicompost sample**

Calcium content in the prepared vermicompost sample was estimated by titration method with EDTA. About 50 grams of dried and sieved sample was taken in a 500 ml conical flask. To this 40 % ethyl alcohol was added. The suspension was shaken for 15 minutes in a rotatory shaker. The aliquot was filtered using a Whatmann No.2 filter paper and the filtrate was washed for 4 - 5 minutes by 40 % ethyl alcohol. The suspension was mixed with 100 ml of ammonium sulphate and it is incubated overnight. It was filtered again and was used as the extract for Ca estimation. The filtrate was taken in a volumetric flask and was made upto 250 ml with sterile distilled water. From this 10 ml was taken along with 2 ml of NaOH solution and titrated against 0.01 M EDTA solution. Murexide (100 mg) was used as the indicator. Color change from Pink to purple was taken as the end point (Chang and Bray, 1951).

Calculations:

\[ \% \text{ of Calcium} = A \times 400.8 \times V/v \times 1000 \times S \]

Where,

- \( A \) = Volume of EDTA used (ml)
- \( V \) = Total volume of extract (500 ml)
- \( S \) = Weight of the sample taken (50 gm)
- \( V \) = Volume of extract (ml).

**Estimation of Magnesium in the vermicompost sample**

About 50 grams of dried sample was mixed with 100 ml of ammonium acetate in a conical flask. The solution was allowed to leach overnight at room temperature. Further the mixture was filtered using a Whatmann No. 42 filter paper. The filtrate was taken in a volumetric flask and made up to 250 ml with distilled water from which 10 ml was taken in a beaker. To this 2 ml of buffer solution was added and titrated against 0.01 M EDTA. 100 mg
of Erichrome black T was used as an indicator. Endpoint is the change in wine red color to blue color (Chang and Bray, 1951).

Calculations:
% of Magnesium = \((B - A) \times 400.8 \times V/v \times 1.645 \times 1000 \times S\)

Where,
\(V\) = Volume of extract (ml)
\(S\) = weight of sample taken (gm)

**Estimation of Iron (Fe) in the vermicompost sample**

The iron content in the prepared vermicompost sample was estimated using a spectrophotometer as per the protocol stated by Jackson (1973).

**Preparation of Standard Iron stock solution**

About 0.10 gm of ferrous ammonium sulphate was dissolved in 500 ml of double distilled water in a 1000 ml volumetric flask. 20 ml of 0.6 N HCl was added and made up to 1 litre with double distilled water. The concentration of the solution thus prepared is 100 ppm of iron.

**Preparation of Standard Curve**

A standard curve was drawn using a spectrophotometer reading of the working standard iron solution prepared from the standard stock solution.

**Protocol for the estimation of Iron concentration in the vermicompost**

An aliquot of vermicompost sample was digested by dry ashing method. 5 to 60 mg of this content was transferred to 25 ml of volumetric flask. The pH of the test solution was adjusted in the range of 1.5 - 2.7 with dilute Hydrochloric acid followed by the addition of 2 ml of 10 % Hydroxylamine Hydrochloric acid and 1 ml of 1.5 % Orthophanonthroline solution. The solution was made up to 25 ml with distilled water. Solution was measured for absorbance. From the absorbance reading the concentration of Iron in the vermicompost sample were recorded by comparing with Standard Curve.

**Calculation**

Concentration of Iron in the vermicompost sample (ppm) = \(M \times \text{ppm read on the graph}\). Where, \(M\) is the dilution factor.
Estimation of Manganese in the vermicompost sample (Mn)

The Manganese concentration was estimated using spectrophotometer as per the protocol stated by Tandon (1993).

The sample solution (digested by dry ashing method) was taken in a beaker and evaporated at low temperature over a hot plate till it was dried. To this 5 ml of HNO₃ and 2 ml of Hydrogen Peroxide was added and dried again. On cooling, 2 ml of HNO₃ followed by 5 ml of Phosphoric acid was added and gently heated till the boiling point. The solution was further cooled and diluted with 10 ml of distilled water.

Further 0.3 g of Potassium Periodate was added. The beaker was covered with watch glass and heated again till the appearance of pink color. To this 20 ml of distilled water was added with continuous heating for 10 to 15 minutes. The hot solution was cooled and transferred into a 50 ml volumetric flask. The volume was made to 50 ml with proper mixing. The solution was read for absorbance at 540 nm in the spectrophotometer. From the readings the concentration of Manganese in the vermicompost sample was calculated from the Standard Curve.

Calculation

Concentration of Manganese in vermicompost sample = M × ppm read on graph

Where, M is the dilution factor.

Estimation of Nitrate Nitrogen in the sample

Nitrate content was estimated by following the protocol stated by Stewart et al. (1973).

Reagents required

Preparation of Phenol disulphonic acid stock

Concentrated sulphuric acid of 150 ml was mixed with 25 grams weighed volumes of Phenol. After mixing of Phenol it the solution was made upto 225 ml using fuming H₂SO₄ with continuous stirring. It was gently heated for 2 hours in a water bath and stored in amber bottles in refrigerator.

Preparation of Ammonium hydroxide solution

Ammonium hydroxide is mixed with water in the ratio of 1:1 in a beaker. 10 mg of sample is mixed with 2 ml of Phenol disulphonic acid. The mixture was further diluted with
10 ml of distilled water. To this mixture ammonium hydroxide solution was added in drops until the pH shoots to 11. The mixture was now filtered through Whatmann No: 1 filter paper and the filtrate were measured for optical density at 420nm. A graph was drawn by using different concentrations of nitrate nitrogen with the same protocol followed for the sample.

**Estimation of Nitrate Nitrogen in the sample Barnes and Folkhard (1951).**

Nitrite nitrogen estimation in the sample was estimated following the protocol stated by Barnes and Folkhard (1951).

**Reagents required**

**Preparation of Sulfanilic stock**

Sulfanilic acid (Merck) of 500 mg was weighed and taken in a conical flask. To this 100 ml of 70 % 0.12 M HCL was added and mixed until crystals of sulfanilic acid dissolves. The solution was stored in amber bottles under cold conditions.

**Preparation of N - (1 naphthyl) - ethylene diamine hydrochloride**

N - (1 naphthyl) - ethylene diamine hydrochloride of 500 mg was weighed and dissolved in 100 ml of HCL in a conical flask and was stored in amber bottles under cold condition.

About 10 mg of sample was extracted with 5 ml of distilled water. The extract was centrifuged at 3000 rpm for 15 minutes to get a clearer supernatant. The supernatant was mixed with 1 ml sulfanilic acid followed by 1 ml of N - (1 naphthyl) - ethylene diamine hydrochloride and shaken well for proper mixing. The solution was diluted to 25 ml with distilled water and left for 10 minutes. The solution was read for optical density at 520 nm. A standard graph was plotted by using different concentrations of nitrite nitrogen with the same protocol followed for the sample.

**Analysis of Carbon: Nitrogen Ratio in the vermicompost sample**

The ratio of Carbon to that of Nitrogen in the vermicompost sample was calculated by dividing the percentage of carbon estimated for the vermicompost sample with the percentage of nitrogen estimated for the vermicompost sample (MASAH, 2001).

\[
C: N \text{ Ratio} = \frac{\text{Percentage of carbon estimated for the vermicompost sample}}{\text{Percentage of nitrogen estimated for the vermicompost sample}}
\]

**Estimation of Total organic matter content in the vermicompost sample**
Total organic matter content (TOMC) estimation in the prepared vermicompost sample was done by heating the sample at 540°C in an electric oven for a duration of 16 hours. The loss in weight of the sample gives the TOMC value (Navarro et al., 1990).

**Estimation of Total carbon content and Oxidisable carbon content**

Total carbon content Oxidisable carbon content in the vermicompost sample was estimated by the expression stated by Navarro et al., (1990). Which states,

Percentage of Total carbon content (% TCC) = (TOMC - 9.33)/1.745


3.3.8 Analysis of the enzyme activity in the prepared vermicompost- Indole-3-acetic acid (IAA)

It was studied by using the protocol stated by Woheler (1997). About 2 grams of fresh sample were scooped from the vermicomposting pit and was collected in a 50 ml centrifuge flask. To this mixture 6 ml of 1% glucose prepared in phosphate buffer (pH 7.5) and 4ml of 1% L-tryptophan were added and mixed. The mixture was incubated at 37 ºc for 24 hours in dark condition. After incubation 2 ml of L-tryptophan was added to the controls. 2 ml of 5% trichloroacetic acid solution followed by 1 ml of 0.5 M CaCl₂ solution was added to inactivate the active enzymes. The aliquot was centrifuged at 5000 rpm for 5 minutes. About 3 ml of the supernatant was taken in a test tube. To this 2 ml of salpers’s solution (2 ml of 0.5 M FeCl₃ and 98 ml of 35 % perchloric acid) were added. The mixture was incubated at 25°C for 30 minutes in dark. After incubation the red colored solution was measured for absorbance in a spectrophotometer at 535 nm. The same test was done for the control pit.

3.3.9 Analysis of Dehydrogenase activity in the prepared vermicompost sample (Garcia et al., 1997)

**Reagents**

**Preparation of 3% (w/v) 2,3,5-triphenyl tetrazolium chloride (TTC) Stock solution :**

About 300 mg of of TTC and 0.6057 grams tris- (hydroxymethyl) methylamine were weighed and taken in a 50 ml conical flask. It was dissolved in 10 ml deionized water. The solution was prepared fresh before use and kept in dark until use.

**Methanol stock solution**
About 10 mg of 2, 3, 5-triphenyl tetrazolium formazan (TPF) was dissolved in 1 ml methanol. The solution is stored below 4°C until further use. Working standards were prepared by diluting the stock solution with methanol to give TPF concentrations in the range 2 to 8µg TPF/ml.

About 1g of Sample from the vermicomposting and control pits was weighed in weighing balance and was taken in screw cap bottles. To the sample 0.2ml TTC solution and 0.5 ml deionized water was added with mild agitation. The mixture was well mixed and was incubated under dark condition at 30°C for 8 hours duration. The incubated mixture was further added with 10 ml of methanol and shaked by placing over a shaker for 30 minutes. The aliquot was further centrifuged at 2000 rpm for 30 minutes. The supernatant was collected in a separate volumetric flask and made up to 25 ml with methanol. The solutions were allowed to stand and the optical densities were read at 485 nm in a spectrophotometer. Methanol was used as the blank.

**Calculation**

Results were obtained in volumes of hydrogen transferred during the reduction of 2, 3, 5-triphenyl tetrazolium chloride (TTC) to 2, 3, 5-triphenyl tetrazolium formazan (TPF) in 1 gram of dry sample. The dehydrogenase acticity was calculated by the formulae,

\[
\text{Dehydrogenase activity} = \frac{(S-B) \times F \times 25 \times 0.15035}{W}
\]

Where,

- S - Height of the Peak
- B - Baseline Height
- F - Slope of the calibration graph
- W - Weight of the sample (g)

Analysis of Dehydrogenase activity was done at every 10 days of duration.

**3.3.10 Analysis of Acid and Alkaline Phosphatases activity in the prepared vermicompost** (Garcia et al., 1997)

Acid and alkaline phosphatases were assayed by picking 1 g of fresh sample from the composting pit. The sample was taken in a clean test tube. To this sample 4 ml of 0.1 M maleate buffer (pH 6.5) was added followed by 1 ml of 25 mM p-nitro phenyl phosphate solution (chromogenic substrate). The mixture was allowed to react for 1 hour at 37 °C in a
water bath. The reaction was terminated by keeping on ice and adding 4 ml of 0.5 M NaOH and 1 ml of 0.5 M CaCl₂. The mixture was centrifuged at 2000 rpm for 30 minutes. The supernatant was collected and the absorbance was read at 405 nm. Enzyme activity was expressed as 1 gram p-nitro phenol released g⁻¹ soil h⁻¹. Phosphatases activity was done at every 10 days of duration.

3.3.11 Analysis of Urease activity in the prepared vermicompost sample (Kandeler and Gerber, 1988).

About 2.5 ml of urea (80mM) solution and 20 ml of 75 mM borate buffer (pH 10.0) was prepared and taken in a conical flask. Vermicompost sample of 5g was added to the flask and the mixture was made to react for 4 hours placing over a mechanical shaker. Controls were prepared by adding 2.5 ml of sterile double distilled water and 20 ml of borate buffer. After 4 hours of incubation time 2.5 ml of sterile double distilled water was added to the mixture. 2.5 ml of urea was added to the controls. It is then extracted with 30 ml acidified 2 M KCL. The flask was again placed over the mechanical shaker for 30 minutes. From this 1.5 ml aliquots were taken in a centrifuge tube and were centrifuged at 5000 rpm for 5 minutes. 1 ml supernatant was collected and mixed with 9 ml of sterile double distilled water, 5 ml of sodium salicylate and 2 ml of dichloroisocyanuric acid (Na⁺ salt). The suspension was incubated at 20 ± 2 °C for 1 hour. The color intensity was measured at 690 nm using a spectrophotometer. Ammonium concentrations were calculated using a calibration curve of standard ammonium chloride solutions from 0 to 2.5 µg ml⁻¹. Urease activity was studied every 10 days of duration.

3.3.12 Analysis of Catalase activity in the vermicompost sample (Rodriguez-Kabana and Truelove, 1982)

About 0.5 g of sample was added to 40 ml of distilled water and 5 ml of 30 % H₂O₂ in a conical flask and shaken well for 15 minutes for proper mixing. To this solution 5 ml of 3 N H₂SO₄ was added in drops. The solution was filtered through Whatmann No:1 filter paper and the filtrate was titrated against 0.05 N KMNO₄. Samples without the addition of H₂O₂ are treated as the controls. Catalase activity analysis was done once in 10 days.

3.3.13 Analysis of microbial load in the prepared vermicompost
Estimation of total population of bacteria and fungi

Microbial load in the prepared vermicompost was carried at regular intervals from the beginning till the end of the experiment following the protocol stated by Baruah and Barthakur (1997) for bacteria and Aneja (1996) for fungi.

Nutrient agar medium for the estimation of total bacteria

Preparation of Nutrient agar medium (Baruah and Barthakur, 1997)

1. Beef extract (water soluble substances including carbohydrates, vitamins, organic nitrogen compounds and salts) - 3.0g
2. Peptone (amino acids and long-chained peptides) - 5.0 g
3. Sodium chloride - 5.0 g
4. Agar (solidifying agent) - 15.0 g

The components mentioned above were weighed and dissolved in 1000 ml of sterile double distilled water in a conical flask. Then the pH was adjusted to 7.2. The conical flask was autoclaved at 121°C, 15 lb pressure for 15 minutes and cooled to 50°C. 20 ml of this medium was poured to autoclaved petridishes and allowed for solidification. The plates are again autoclaved at 121°C, 15 lb pressure for 15 minutes prior to study.

Growth medium for the estimation of total fungi in the prepared vermicompost

Martin’s Rose Bengal – Streptomycin agar medium (RBA medium) (Aneja, 1996)

Composition

1. Soy peptone (carbon and nitrogen source) - 5.0 g
2. Dextrose (energy source) - 10.0 g
3. Potassium dihydrogen phosphate (buffer) - 1.0 g
4. Magnesium sulphate (trace elements) - 0.5 g
5. Rose Bengal (bacterial growth regulator) - 0.05 g
6. Streptomycin (antibiotic) - 0.30 g
7. Agar (solidifying agent) - 15.0 g

About 32.0 g of medium was dissolved in 1000 ml of sterile distilled water in a conical flask and the pH was adjusted to 7.2. It was autoclaved at 121°C, 15 lb pressure for 15 minutes. The medium was cooled to 45°C. The antibiotic streptomycin was sterilized separately and was added to the sterile medium. Pore 20 ml of medium in each sterile petri
dishes and allowed to solidify. The Petri dishes were again autoclaved at 121°C, 15 lb pressure for 15 minutes prior to study.

**Protocol**

About 1 g of sample was taken from each pit and was suspended in 10 ml of sterile double distilled water. The mixture was shaken 5 minutes for proper mixing. Soil particles in the mixture were allowed to settle at the bottom of the container. From this 1 ml of the solution was drawn aseptically into a test tube containing 9 ml of sterile double distilled water to give a 10 fold dilution. Serial dilutions were prepared from this suspension to obtain $10^2$, $10^3$, $10^4$, $10^5$, $10^6$ and $10^7$ suspensions.

About 1 ml of aliquot was pipetted out from each dilution and was aseptically poured onto the sterile petridishes containing Nutrient Agar and Martin’s Rose Bengal medium. The aliquot was dispersed over the medium by gently rotating the petridish. The inoculated plates were incubated for 24 to 72 hours at 37°C for Nutrient Agar and at 25°C for Martin’s Rose Bengal agar. All the experiments were carried out in triplicates. Plates without inoculums were taken as the control.

After incubation the petriplates were studied for colony counts. The number of bacterial and fungal colonies appeared over the medium in the petridishes were counted and recorded. The colony morphology for bacteria and fungi were studied using high power binocular microscope (Canon). The observations were compared and studied with the monographs by Gilman (1957), Subramanian (1971), Domsch et al. (1980) and Ellis (1985).

**Plot selection according to their flower yield for further phytochemical studies**

Based on the quality and yield of flowers of *Rosa bourboniana* cultivated under various organic and inorganic amendments, the flowers harvested from three plots were selected for further phytochemical and biological efficacy analysis. They are, T1 - control plot (CP), T8 - the plot treated with a mixture of all vermicompost (MIX 1 plot) and the T7 - plot treated with chemical fertilizer (CF). In all the studies flowers harvested from these three plots were used and compared for their potentialities.

3.4. Preliminary phytochemical screening of flowers of *Rosa bourboniana*

3.4.1 Qualitative Phytochemical analysis of flowers of *Rosa bourboniana*

Collection of flower material
For phytochemical analysis fully opened floral blooms were plucked in the early hours of dawn and was collected in wet cloth bags and carried to the PG & Research Department of Botany, Pachaiyappas college, Chennai for further studies.

**Processing of floral material for solvent extraction**

The plucked flowers were rinsed in distilled water to get rid of contaminants adhering to the petals. The petals were drawn and air-dried under shade for a week at room temperature. The dried petals were segregated and pulverized to fine coarse powder in a blender and sieved through 1 mm sieve.

**Preparation of crude extracts**

Floral extracts were prepared by serial extraction involving successive extraction with organic solvents of increasing polarity starting from a non polar to a polar solvent (Hexane, Chloroform, Ethyl acetate and Ethanol). About 100 grams of weighed sieved petal powder was extracted with 1000 ml of each solvent twice with overnight incubation at room temperature for 48 hours. The individual extracts were filtered using Whatmann filter paper No. 1. The filtrate was processed in a vacuum evaporator under reduced pressure to recover the excess solvents for further use. The sticky extract obtained was dried in an oven at 32°C and stored in vials at 4°C for further analysis.

**3.4.2 Qualitative phytochemical analysis of the floral extracts**

Qualitative phytochemical screening of the prepared flower extracts with Hexane, Chloroform, Ethyl acetate and Ethanol solvents were performed as per the protocols stated by Sofowora (1993), Trease (1989) Evans (1997), Mace (1963), Kokate (1999) and Harborne (1973).

**Test for Carbohydrates**

Exactly 5 ml of distilled water was added to individual extracts and dissolved. The vortexed mixture was filtered and the filtrate was used to screen for the presence of Carbohydrates.
Fehling’s test

The filtrate was hydrolysed with dilute HCl acid and neutralised with alkali. It is heated in water bath with the addition of 6-7 drops of Fehlings A & B solution. Appearance of red coloured precipitate indicates the presence of Carbohydrates.

Test for Glycosides

About 50 mg of extract was hydrolysed with concentrated HCl for a duration of 2 hours by keeping in a water bath. The solution was filtered and the filtrate was subjected to further tests.

Borntrager’s test

About 3 ml of chloroform was added to 2 ml of filtrate and shaken well. On separation of the two phase, chloroform layer was pipetted out and 10 % of ammonia solution was added in drops. Appearance of pink colour indicates the presence of Glycosides.

Test for Cardiac Glycosides

Keller-Kiliani test

About 0.5 g of extract was treated with 2 ml of glacial acetic acid and 2 drops of 5 % ferric chloride solution. The mixture was underlayered with 1 ml of concentrated H2SO4. Brown ring formation at the interface confirms the presence of Cardiac Glycosides.

Test for Anthraquinones

About 0.5 g of extract was boiled with 10 % of HCl in a water bath for few minutes. the mixture was filtered and cooled. equal volume of CHCl3 was added in drops. It is further heated gently with the addition of 10 % NH3. Appearance of rose pink colour indicates the presence of anthraquinones.

Test for Flavonoids

Alkaline reagent test

About 0.5 g of extract was treated with few drops of NaOH solution. Appearance of intense yellow colour which disappears on addition of dilute acid confirms the presence of flavonoids.

Test for Alkaloids
Mayer’s test

Extracts were dissolved in dilute HCl individually and filtered. The filtrate was treated with 2-3 drops of Mayer’s reagent (Potassium Iodide). Formation of yellowish green or creamy white precipitate indicates the presence of alkaloids.

Test for Tannins

About 1 g of extract was treated with 2 ml of 5 % ferric chloride solution. Formation of dark blue or greenish black colour confirms the presence of Tannin.

Test for Steroids

Salkowski’s test

About 1 gm of extract was treated with 2 ml of Chloroform and 2 ml of Concentrated H$_2$SO$_4$ and shaken well. Formation of red colour at the chloroform layer and greenish fluorescent yellow colour at the acid layer indicated the presence of steroid (Terpenoid).

Liebermann Burchard test

To 2 g of extract, 1 ml of Liebermann-Burchard reagent was added and shaken well. Formation of blue-green colour indicates the presence of triterpenoids.

Test for Phenols

About 1 g of extract was treated with 2 ml of distilled water followed by the addition of few drops of 10 % ferric chloride solution. The formation of blue or green colour confirms the presence of phenol.

Test for Amino acid

Ninhydrin test

To 2 g of plant extract was treated with 2-3 drops of 0.2% Ninhydrin reagent and heated for 5 minutes in a water bath. Formation of blue colour indicates the presence of protein.

3.4.3 Quantitative phytochemical analysis of the floral extracts

Quantitative phytochemical screening of flowers was carried out by air drying the petals of *Rosa bourboniana* for 5 days in shade at 37°C. About 100 gms of dried petals were powdered in a blender, sieved and were subjected to analysis. The tests were performed following the protocols of Herin Sheeba *et al.* (2013).

3.4.4 Antioxidant assays
**DPPH assay**

**Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH)**

DPPH is a stable free radical with red colour (absorbed at 517 nm). On scavenging of free radicals DPPH changes its colour to yellow. The intensity of colour change indicates the amount of antioxidant substances present in the plant extract.

The free radical scavenging activity of hexane, chloroform, ethyl acetate and ethanolic floral extracts of *Rosa bourboniana* was analysed with stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) (Sigma - Aldrich) photo spectrometrically as stated by Blois (1958). Stock solution was prepared with Methanol solution for each plant extract at a concentration of 1 mg/ml. From this stock solution 100 µg was taken and mixed with equal volume of Methanolic solution of DPPH (0.1 mM). 0.5 ml of each sample in Methanol solution was mixed with 2.5 ml of 0.5 mM of Methanolic DPPH solution. The mixture was vortexed vigourously and incubated for 30 minutes in a dark room under room temperature. The absorbance was measured at 517 nm against a blank using a UV Spectrophotometer (DU 800; Beckman Coulter, Fullerton, CA, USA). Ascorbic acid was used as the positive control. The experiments were conducted in triplicates. The annihilation activity of free radicals was measured as a decrease in the absorbance of DPPH in the extracts which was calculated using the formula,

\[
\% \text{ of inhibition} = \frac{\text{Abs control}_{517} - \text{Abs of test sample}_{517}}{\text{Abs of control}_{517}} \times 100
\]

Where,

\[
\text{Abs control} = \text{Absorbance of DPPH solution.}
\]

\[
\text{Abs sample} = \text{Absorbance of floral extracts and Ascorbic acid solution (Control)}
\]

**3.4.5 Iron chelating activity (FRAP - Ferric Reducing Antioxidant Power Assay)**

FRAP assay of various floral extracts of *Rosa bourboniana* was performed as per the protocol sated by Benzie and Strain (1996). The principle is based on the formation of O-Phenanthroline - Fe\(^{2+}\) complex and its disruption with a colour change in the presence of a chelator. Measurement of this colour reduction allows the estimation of the chelating activity of the test plant sample. A reaction mixtures containing 1 ml of 0.05 % of
O-Phenanthroline in Methanol solution and 2 ml of Ferric chloride solution (200µm) was added and mixed. To this 2 ml of various concentrations of floral sample extracts ranging from 100 - 1000 µg was added and incubated at room temperature for 10 minutes. After incubation absorbance was measured at 510 nm using a UV Spectrophotometer (DU 800; Beckman Coulter, Fullerton, CA, USA). EDTA (Ethylene diamine tetra acetic acid) was used as the standard classical metal chelator. All the experiments were performed in triplicates and the mean average values were recorded.

Iron chelating activity was calculated using the formula,

\[
\% \text{ of iron chelating activity} = 100 \times \left[ \frac{(\text{Abs}_C - \text{Abs}_S)}{\text{Abs}_C} \right]
\]

Where,

\( \text{Abs}_C \) is the absorbance of control
\( \text{Abs}_S \) is the absorbance of sample (plant extract/standard).

### 3.4.6 Antibacterial assay of floral extracts of *Rosa bourboniana*

#### Selection of test organisms for antibacterial activity

**Microbial test suspensions**

Bacterial test organisms included in the study were 5 clinical isolates. The bacterial strains were *Staphylococcus aureus* MTCC No: 3160, *Bacillus subtilis*: MTCC No: 2756, *Vibrio cholerae*: MTCC No: 3904, *Escherichia coli*: MTCC: 1576 and *Staphylococcus epidermidis*: MTCC No: 3382. The authenticated strains were collected from the division of Clinical Microbiology Pondicherry Centre for Biological Sciences, India.

**Growth and maintenance of test organisms for antibacterial studies**

The bacterial isolates were sub cultured and established in Muller Hinton Nutrient broth (Himedia, Bombay) at pH 7.4 and incubated at 37ºC in an incubator for 24 hours prior to study.

**Antibacterial susceptibility testing using Disc Diffusion Method**

Inoculum suspensions containing \(10^8\)CFU/ml of bacteria equivalent to 0.5 McFarland standard were used for the study. The sterile Mueller Hinton Nutrient Agar (HiMedia, Bombay) was poured onto the sterile petriplates and on solidification it is streaked with the suspensions of each test organisms and a lawn culture is established. Sterile discs (0.5 cm) obtained from HiMedia (Bombay) was loaded with 25µl of each extract dissolved
independently in DMSO at a concentration of 1mg/ml (Romero, 2005). The loaded discs were impregnated over the established lawn culture aseptically and the plates were incubated at 37°C in an incubator for 24 hours. Zone of inhibition (mm in diameter) were measured with three repeated experiments and the average of readings was taken as the antimicrobial activity against the test pathogen for the particular extract. Ciprofloxacin (30µg/disc) were used as the positive control. Each assays were repeated thrice and the average values were recorded (Bauer et al., 1996).

**Minimum inhibitory concentration for the selected bacterial strains (MIC)**

The minimum inhibitory concentration (MIC) is defined as the lowest concentration at which the flower extract inhibits the growth of the microorganism after 24 hours incubation at 37°C in an incubator. It was determined by a serial dilution technique using 96 - well microtitre plates (Wiegand et al., 2008). 12 wells in each row of the plate were filled with sterilized 0.1 ml of Mueller Hinton Agar (HiMedia, Bombay). Column wells 1 - 7 were loaded with a mixture of Nutrient Agar (HiMedia, Bombay) and the floral extracts diluted serially in a concentration range from 50 µl to 1000 µl/well. Well 8 served as the growth control. The loaded titer plates were incubated in an incubator at 37°C for 24 hours and observed for turbidity formation using optical readings at 600 nm using 0.1 % alamar blue staining using Beckman DU-70 UV-Vis Spectrophotometer. The results were analyzed in triplicates. The wells in which no turbidity was observed was taken as the MIC of the particular floral extract against that bacterial strain. The activity at a concentration of < 50 µg/well of extract was considered as the strongest MIC activity for the particular extract.

**3.4.7 Selection of test organisms for anti - dermatophytic activity**

Fungal test organisms included in the study were five dermatophytic strains which includes *Trichophyton rubrum* (MTCC NO: 3272), *Trichophyton mentagrophytes* (MTCC NO: 8476), *Candida albicans* (MTCC NO: 7315), *Epidermophyton floccosum* (MTCC NO: 7880) and *Trichophyton tonsurans* (MTCC NO: 8475). The highly pathogenic microorganisms were procured from the Microbial Type Culture Collection and Gene bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India.

**Growth and maintenance of fungal test organisms for study**
The procured fungal strains was established by culturing on Potato dextrose agar (PDA - HIMEDIA, Mumbai, India) slants and incubated at 37°C in an incubator for 48 hours. The viability is further maintained by frequent culturing in fresh medium once in a week and stored at 4ºC until used in experiments.

Testing of Anti-dermatophytic activity by disc diffusion method

Sterile PDA plates were seeded with respective inoculum at 1×10⁷ cells/ml and a lawn culture was established in the petri dishes. Sterile discs were loaded with 25µl of each extract dissolved independently in DMSO at a concentration of 1 mg/ml (Romero, 2005). The loaded discs were impregnated over the established lawn culture aseptically and the plates were incubated at 37°C in an incubator for 48 - 72 hours. Zone of inhibition (mm in diameter) were measured and the readings were taken as the antimicrobial activity against the test pathogen for the particular extract. Clotrimazole at concentrations of 30µg/disc were used as the positive control drug. Each assay was repeated thrice and the average values for inhibition zones was recorded and compared with the standard reference antibiotic used (Bauer et al., 1996).

Minimum inhibitory concentration for the selected dermatophytic strains (MIC)

The minimum inhibitory concentration (MIC) for the fungal strains was determined by using the same serial dilution technique using 96 - well microtitre plates. 12 wells in each row of the plate were filled with sterilized 0.1 ml of Potato dextrose broth (HiMedia, Bombay). Column wells 1 - 7 were loaded with a mixture of Nutrient broth (HiMedia, Bombay) and the respective floral extracts diluted serially in a concentration range from 0.25 µl to 5 µl/well. Well 8 served as the growth control. The loaded titre plates were incubated at 37°C for 48 - 72 hours and observed for turbidity formation using optical readings at 600 nm using 0.1 % alamar blue staining using Beckman DU-70 UV-Vis Spectrophotometer. The results were analyzed in triplicates. The wells in which no turbidity was observed (no growth of colonies) was taken as the MIC of the particular floral extract for the particular strain. The activity at a concentration of < 50 µg/well of extract was considered as the strongest MIC activity for the particular extract.

3.4.8 Macrophage scavenging assay
**Effect of solvent fractions on Macrophage cells**

The effect of the solvent fractions on the phagocytic activity of RAW 264.7 macrophages was studied according to the method of Manosroi *et al.* (2003). RAW 264.7 macrophages cell lines obtained from Tysel biotech park, Chennai were suspended in RPMI-1640 medium supplemented with 10% FBS. The cell number was adjusted to $1 \times 10^6$ cell/ml. The tryphan-blue dye exclusion techniques were used to determine the viability of macrophages. From the suspension 20 µL of cells $(1 \times 10^6$ cells/ mL) were added with 5, 10 and 15 µL (50, 100 and 150 µg/ml) of the sample with 140 µL of RPMI1640. The plates were incubated at 37ºC in humidified 5% CO₂ atmosphere. After 24 hours, 20 µL of yeast suspension $(5 \times 10^7$ particles/ mL) and 20 µL of NBT (1.5 mg/mL in PBS) were added and incubated for 10 minutes. PBS and dimethylsulfoxide (DMSO) which was adjusted to 0.1 % (v/v) were used as the control. The medium was removed aseptically and the cells were rinsed again with the fresh medium. Then the cells were washed with 200 µL of methanol to remove unreduced NBT. Finally, 120 µL of 2M KOH and 140 µL of DMSO were added consecutively to each well and the absorbance was read at 570 nm by a well reader (Seikagaku SK601, Japan). The percentage of NBT reduction was calculated by the following formula.

$$\text{BT reduction} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{control}}}{\text{OD}_{\text{control}}} \times 100$$

**3.4.9 MTT Assay (MCF-7 cell line study) - Percentage of viability analysis**

**Dulbecco’s Modified Eagles Medium (DMEM) (pH 7.4)**

DMEM medium was added to 900 ml of sterile double distilled water. To this solution, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate and 10 ml of antibiotic-antimycotic (50 U/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml amphoterecin B) solution were added; the pH was adjusted to 7.4 using 1 N NaOH and the final volume was made up to one liter with distilled water. The medium was then filtered through 0.22µm filter membrane and dispensed into a sterile container and stored at 4°C.

**Preparation of growth medium (10% FBS)**
About 100 ml of growth medium was prepared by adding 10 ml of Fetal Bovine Serum in 90 ml of DMEM, and was stored in a sterile container.

**Preparation of Phosphate buffered saline (PBS) (pH 7.4)**

About 0.63 g of sodium phosphate monobasic (NaH$_2$PO$_4$), 0.17 g of sodium phosphate dibasic (Na$_2$HPO$_4$) and 4.5 g of sodium chloride (NaCl) were dissolved in 500 ml of sterile double distilled water. The pH was adjusted to 7.4 with 0.1 N NaOH and stored in a refrigerator.

**Culture medium**

Human Breast cancer cell lines (MCF-7) were purchased from National Center for Cell Science (NCCS, Pune). The cells were cultured aseptically in a 75-cm$^2$ flask containing Dulbecco’s modified Eagle’s medium (DMEM; Sigma) the medium was supplemented with 10% Fetal bovine Serum (FBS; invitrogen), 1.5 g/L of sodium bicarbonate (Gibco), 10,000 U/ml of penicillin (Gibco), 10 mg/ml of streptomycin (Gibco), and 25µg/ml Ampotericin B (Gibco). Cell cultures were established as monolayers in culture flasks at 37°C under a humidified atmosphere of 5% CO$_2$ in air. All experiments were performed using cells from passage 20 or less. During the experiment time, the serum containing medium was replaced by serum free medium containing 20 - 2.5µg/ml of the crude, which were dissolved in DMSO and the stock was maintained in at -20°C. The final working concentration of DMSO was less than 1.0%.

**Passaging the cells**

The cells after reaching 80-90% of confluence were trypsinized and used for subculture. The medium from the culture flask was aspirated; cells were rinsed with 2 ml of PBS and aspirated quickly and 0.5 ml of trypsin-EDTA (0.5% trypsin, 5.3 mM EDTA sodium salt) solution was added and incubated at room temperature (in the laminar hood) for 30 - 60 sec. Then the trypsin-EDTA solution was aspirated quickly and the flask was incubated in CO$_2$ incubator for 2 min and tapped gently at the bottom for complete detachment of cells from the surface of the flask. The cells were then gently re-suspended in fresh growth medium and transferred to sterile 75 cm$^2$ flasks and the volume of medium was made up to 20 ml with growth medium.

**Drug preparation**
The extract was dissolved in Dimethyl Sulfoxide (DMSO) to give a final concentration of DMSO not more than 5% and did not affect cell survival.

**Cell viability test**

The viability of cells was assessed by MTT assay (Mosmann, 1983) using Human Breast cancer cell line (MCF-7)

**Principle**

The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Only live cells are able to take up the tetrazolium salt. The enzyme (mitochondrial dehydrogenase) present in the mitochondria of the live cells is able to convert internalized tetrazolium salt to formazan crystals, which are purple in colour. Then the cells are lysed and dissolved in DMSO solution. The colour developed is then determined in an ELISA reader at 570 nm.

**Reagents**

1. **MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide):** 0.5 mg MTT/ml of serum-free DMEM medium.
2. **Solubilizing solution:** Dimethyl sulfoxide
3. **Phosphate buffered saline (PBS - pH 7.4):** As described under cell culture reagents.

**Procedure**

The cells were plated separately in 96 well plates at a concentration of $1 \times 10^5$ cells/well. After 24 h, cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37°C. After starvation, cells were treated with different concentrations of test compound (50-150 µg/ml) for 24 h. At the end of the treatment period, the medium was aspirated and serum free medium containing MTT (0.5 mg/ml) was added and incubated for 4 h at 37°C in a CO₂ incubator.

The MTT containing medium was then discarded and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of DMSO and this was mixed properly by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity percentage was determined using Graph pad prism software.
3.4.10 Volatile constituents study of flowers of *Rosa bourboniana*

Volatile analysis of the flowers grown under the influence of various organic (vermicompost treated) and inorganic amendments was done for the floral samples harvested from the plots T1, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12 and T13. Cow dung plot (T2) was excluded and a total of 12 samples were used for the volatile study.

**Harvest of flowers for volatile study**

Fully bloomed flowers of *Rosa bourboniana* was handpicked in the early hours of dawn with dews over the bloom and was collected in wet cloth bags and carried to laboratory for analysis. It was processed for volatile analysis immediately.

**Processing of blooms for volatile analysis**

Organic solvent extraction protocol was adopted for the extraction of fragrance volatiles (Georgive *et al.*, 1981), The petals were separated and was agitated in a cold percolation chamber with n-Hexane in 1:3 ratio for 24 hours. The extract was distilled at 78°C in rotary evaporator under low pressure to remove the solvent and waxy concrete. The waxy concrete contains essential oils, waxes resins etc which was further extracted twice with ethanol in the ratio of 1:1.5 and the resulting slurry was freezed. The enriched rose oil which is a concentrate was directly used for GC-MS analysis.

**Analysis of volatile concentrate by GC-MS**

The crude concentrate thus obtained was analyzed by GC-MS for the identification of essential compounds. Due to the low temperatures applied in this process, the absolute may be more faithful as the scent of the fresh rose. This study revealed about the variations in volatile scented compounds in flowers of *Rosa bourboniana* grown under various organic and inorganic amendments.

**Analysis of the volatile constituents**

Gas Chromatography and Mass Spectroscopy study was carried out at Bureau Veritas laboratories, Chennai. GC-MS analysis of the volatile extract was performed using GC shiimadzu QP2010 model system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with elite I fused silica capillary column (length : 30.0 mm, Diameter : 0.25mm, Film thickness : 0.25 composed of 100 % Dimethyl poly siloxane). An electron ionization energy system with ionization energy of 70 eV was used. Helium gas (99.999%)
was used as the carrier gas at a constant flow rate of 1.51 ml/min and an injection volume of 2µl was employed with a split ratio of 20. Injector temperature and ion-source temperature were maintained at 200°C; the oven temperature was programmed from 70°C (isothermal for 2 minutes), with an increase of 300°C for 10 minutes mass spectra were taken at 70 eV at a scan interval of 0.5 seconds with scan range of 40 - 1000 m/z. Total GC running time was 35 minutes. The relative percentage amount of each component was calculated by comparing its average peak area with the total peak areas. Mass spectra patterns were prepared by using GC-MS solution software version of 2.53.

Identification and interpretation of compounds

Interpretation of spectral patterns of GC-MS was conducted by accessing the database of National Institute Standard and Technique (NIST08s), WILEY8 and FAME having standard patterns. The spectrum of unknown component was compared with the spectrum of the known components stored in the NIST08s, WILEY8 and FAME library. The Name, Molecular weight, Molecular formula and structure of the component of the test material were correlated and the results were recorded.

The field cultivation, flower morphology and its yield were photographed with Nikon Camera. The result were tabulated and statistically interpreted and presented as per the standard procedure.