MATERIALS AND METHODS
3. MATERIAL AND METHODS

3.1. Location and geography of the study site

The Eastern Ghats are a discontinuous range of mountains, which, vivisected by the 4 major rivers of South India; Godavari, Mahanadi, Krishna and Kaveri. These mountains extend from West Bengal to Orissa, Andhra Pradesh and Tamilnadu along the coast and parallel to the Bay of Bengal. Though not as tall as Western Ghats, some of its peaks are over 1000m (3,281 ft) in height. The Nilgiri Hills in Tamilnadu lies at the junction of Eastern Ghats and Western Ghats. Jindagada peak (1690m) near Araku valley of Andhra Pradesh is the tallest peak in the Eastern Ghats. The Eastern Ghats in Andhra Pradesh are located between 77° 22’ and 85° 20’ East. In India, there are 29 states and one among the Southern states is Andhra Pradesh covering an area of 2,75,068sq km which lie between the latitudes 12° 37’ N and 19° 44’ N and longitudes 76° 46’E and 84° 46’E. It is situated in the middle portion of the eastern half of the Indian Peninsula. Nellore is one among 23 districts of Andhra Pradesh. Udayagiri town is one of the Taluk headquarters in Nellore District. Udayagiri hill (Sanjeevini Hill) is situated on the southern side of Udayagiri town, which forms the southernmost part of the Eastern Ghats. It is located at 14.8667°N 79.3167°E and has an average elevation of 3261 feet above the Mean Sea Level (MSL) (Fig.1). The climatic condition is semi arid with mixed type of vegetation consisting of semi-evergreen, dry deciduous, moist deciduous and scrub forest. Duragampalli, Durgampalli Road, Loddi vaagu (Loddi river), Salwapet, Kona canal, Kona, Tapka (Totti), Chinna masjid, Pedda masjid, Fort, Bijjampalli Road and Salivendra are the main places in Udayagiri Hills.
Fig.1 Location of the study site
Geographically, the Eastern Ghats occupy the 2,08,906 sq km in Andhra Pradesh. The Eastern Ghats are one of the richest floristic and phytogeographical regions of India. The Eastern Ghats along the Peninsular India is a long chain of broken hills and elevated plateaus (Narasimhan and Kumar, 2003). It is divisible into three zones, the northern eastern Ghats, the middle eastern Ghats and the southern eastern Ghats, extending over 1750 Km with an average width of about 100 km and covering an area under 76°51’ and 86°30’E longitudes and 11°30’ and 22° 00’N latitudes. The area covers parts of Odisha (South of river Mahanadi), Andhra Pradesh, Tamilnadu (North of river Vaigai) along the East Coast. Its northern boundary is marked by river Mahanadi basin while the southern boundary is the Cauvery and Tamilnadu uplands and passes through Visakhapatnam, Vijayanagaram, Srikakulam, East Godavari, West Godavari, Khammam, Krishna, Guntur, Mahaboobnagar, Prakasam, Kurnool, Kadapa, Nellore and Chittoor district. The altitudes range from 300-1500m above MSL. The highest peak in these Ghats is Sambari Konda with an elevation of 2527m near Gudem village in Visakhapatnam district. The vegetation varies from semi-evergreen forests to scrub jungles.

3.2. Survey, documentation and ethnobotanical studies

Field trips were made to survey, collect and document the natural flora during the study period July 2011 - March 2014. Among the chief aspects of Udayagiri Hills of research in ethnombotany, the field work forms an important part of the study besides the study of rural population. In collection of plants from the field, knowledgeable informants (Table 1) were taken to the field for identification of ethnomedicinal plants.
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name</th>
<th>Age(Yrs)</th>
<th>Gender</th>
<th>Profession</th>
<th>Address</th>
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<tbody>
<tr>
<td>1</td>
<td>Mr.S.K.Khajavali</td>
<td>55</td>
<td>Male</td>
<td>Senior Assistant</td>
<td>Forest Range office, Udayagiri.</td>
</tr>
<tr>
<td>2</td>
<td>Mr.G.Venkateswarlu</td>
<td>57</td>
<td>Male</td>
<td>Teacher&amp;Herbal practitioner</td>
<td>Durgam, Udayagiri Taluk</td>
</tr>
<tr>
<td>3</td>
<td>Mrs.G.Rama Subbamma</td>
<td>70</td>
<td>Female</td>
<td>Herbal practitioner</td>
<td>Durgam, Udayagiri Taluk</td>
</tr>
<tr>
<td>4</td>
<td>Mr. Ahmed Basha Khalid</td>
<td>64</td>
<td>Male</td>
<td>Teacher</td>
<td>Near Gov’t Junior college Udayagiri Taluk</td>
</tr>
<tr>
<td>5</td>
<td>Mr.Mohammed Khaja</td>
<td>54</td>
<td>Male</td>
<td>School Assistant</td>
<td>Near Gov’t Junior college Udayagiri Taluk</td>
</tr>
<tr>
<td>6</td>
<td>Mrs.Dilshad</td>
<td>59</td>
<td>Female</td>
<td>Teacher&amp;Herbal practitioner</td>
<td>Near Gov’t Junior college Udayagiri Taluk</td>
</tr>
<tr>
<td>7</td>
<td>Mr.Pedda Chennaiah</td>
<td>65</td>
<td>Male</td>
<td>Shepherd</td>
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</tr>
<tr>
<td>8</td>
<td>Mr.Chinna Chennaiah</td>
<td>60</td>
<td>Male</td>
<td>Shepherd</td>
<td>Durgam, Udayagiri Taluk</td>
</tr>
<tr>
<td>9</td>
<td>Mr.Khaja Masthan</td>
<td>32</td>
<td>Male</td>
<td>Teacher</td>
<td>Naagal baavi veedhi Udayagiri Taluk</td>
</tr>
<tr>
<td>10</td>
<td>Ms. Khaja Jameer</td>
<td>26</td>
<td>Female</td>
<td>Teacher</td>
<td>Naagal baavi veedhi Udayagiri Taluk</td>
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<tr>
<td>11</td>
<td>Dr. Rasool</td>
<td>30</td>
<td>Male</td>
<td>Regd. Medical Practitioner</td>
<td>Chirivella Atmakur Taluk</td>
</tr>
<tr>
<td></td>
<td>Name</td>
<td>Age</td>
<td>Gender</td>
<td>Occupation</td>
<td>Location</td>
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<tr>
<td>12</td>
<td>Mr. Upputuri Venkataiah</td>
<td>50</td>
<td>Male</td>
<td>Shepherd</td>
<td>Yadava veedhi, Udayagiri</td>
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<td>13</td>
<td>Mr. Poluboyina Kondaiah</td>
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<td>Male</td>
<td>Shepherd</td>
<td>Aavula veedhi, Udayagiri</td>
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<tr>
<td>14</td>
<td>Mr. Tinniboyina Venkataiah</td>
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<td>Male</td>
<td>Farmer/Shepherd</td>
<td>Yadava veedhi, Udayagiri</td>
</tr>
<tr>
<td>15</td>
<td>Mr. Panga Lakshmaiah</td>
<td>70</td>
<td>Male</td>
<td>Farmer/Shepherd</td>
<td>Yadava veedhi, Udayagiri</td>
</tr>
</tbody>
</table>

Table 1 concl.
Among the informants 12 males and 3 females of different age groups between 26-70 years were selected. The information given by them was recorded in the field book. For some of the specimens, they were brought from the field and shown to the informants to record the correct information. The data collected on a particular plant species was verified by discussing the same plant with other informants. This practice helped to bring out quite reliable information about the plant species. In the present study the non-tribals like forest officials, teachers, registered medical practitioners, local herbal healers, shepherds, farmers, men and women who had traditional knowledge about medicinal plants were interviewed based on information asked in the Questionnaire.

To determine the authenticity of information collected during field work, repeated verification of data from different informants in different areas at different times was done. For further authentication of information and future reference, voucher specimens were collected. During field work, special attention was paid to record local names of plants in order to make the work useful to the general public. Important morphological diagnostic characters of plants were also recorded.

Botanical names, local names, habit and locality is presented in Table-3. Herbarium was prepared for the collected plant specimens and Voucher number was assigned to them by following the standard procedure outlined by Jain and Rao (1977). The Herbarium prepared was deposited at the Department of Botany, RKM Vivekananda College, Mylapore, Chennai-600 004. Field photographs of all the plant species were taken and preserved digitally.

Ethnobotanical uses and details about treatment of different disorders and ailments, based on the information given by the informants and local inhabitants were recorded, using Questionnaire (pg. 53). The information pooled from the local inhabitants on ethnobotanical uses and vernacular names (Telugu) for the plant species were summarized in Table-4. The information on disease curing properties of the plants gathered from the local inhabitants was compared with the already existing literature of Kirtikar and Basu (1935 and 1995), Nadkarni (2010) and Bhikshapati (2011).

3.3. **Phytochemical, Antimicrobial, Antioxidant and GC-MS studies**

**Preparation of the plant extract.**

Phytochemical, Antimicrobial activity and Antioxidant potential studies were conducted on 18 plant parts of 14 plant species (Table 5), which were most commonly used to cure various diseases in the study area. The plant samples collected were washed in distilled water, shade dried and powdered. The extracts of the plant powder samples were prepared in a direct method by soaking one gram of samples in 20 ml of various extractants such as Ethanol, Methanol and Water for 48 hours. The filtrate was then concentrated under reduced pressure in vacuum at 40°C for 25min using a rotary evaporator and preserved for study of Phytoconstituents, Antimicrobial and Antioxidant activities.
3.3.1. Qualitative analysis of Phytochemicals.

Phytochemical screening (Qualitative analysis) was carried out on the Plant extracts in solvents like ethanol, methanol and water to assess the presence or absence of naturally occurring major chemical compounds such as tannins, saponins, flavonoids, phenols, terpenoids, alkaloids, glycosides, cardiac glycosides, coumarins, quinines, steroids and phytosteroids as per the standard methods as described by Harborne and Baxter (1995), Harborne (1998) and Ayoolal et al. (2008).

1). Test for Tannins

To 1ml of plant extract, 2ml of 5% Ferric chloride was added. Formation of dark blue or greenish black indicates the presence of Tannins.

2). Test for Saponins

To 2ml of plant extract, 2ml of distilled water was added and shaken in a graduated cylinder for 15minutes. Formation of 1cm layer of foam indicates the presence of saponins.

3). Test for Flavonoids

To 2ml of plant extract, 1ml of 2N NaOH was added. Presence of yellow color indicates the presence of flavonoids.

4). Test for Alkaloids

To 2ml of plant extract, 2ml of con. HCl was added. Then a few drops of Mayer’s reagent was added. Presence of green color or white precipitate indicates the presence of alkaloids.

5). Test for Quinones

To 1ml of extract, 1ml of con. Sulphuric acid was added. Formation of red color indicates presence of quinones.
6). **Test for Glycosides**

To 2ml of plant extract, 3ml of Chloroform and 10% ammonia solution was added. Formation of pink color indicates presence of glycosides.

7). **Test for Cardiac glycosides**

To 0.5ml of extract, 2ml of glacial Acetic acid and a few drops of 5% Ferric chloride were added. This was under layered with 1 ml of con. Sulphuric acid. Formation of brown ring at the interface indicates presence of cardiac glycosides.

8). **Test for Terpenoids**

To 0.5ml of extract, 2ml of Chloroform was added. Further a few drops of con. Sulphuric acid was added carefully. Formation of red brown color at the interface indicates presence of terpenoids.

9). **Test for Phenols**

To 1ml of the extract, 2ml of distilled water and few drops of 10% Ferric chloride were added. Formation of blue or green color indicates presence of phenols.

10). **Test for Coumarins**

To 1 ml of extract, 1ml of 10% NaOH was added. Formation of yellow colour indicates presence of coumarins.

11). **Test for Steroids and Phytosteroids**

To 1ml of plant extract, added an equal volume of Chloroform and a few drops of concentrated Sulphuric acid. Appearance of brown ring indicates the presence of steroids or appearance of bluish brown ring indicates the presence of phytosteroids.
3.3.2. Quantitative estimation of certain Phytochemicals.

The quantification of Saponins, Phenols, Tannins, and Flavonoids was carried out by following the methods as outlined below.

**Total Saponins**

The quantification of total saponin was done according to the method used by Obadoni and Ochuko (2001) with minor modifications. One gram of powdered plant material was added to 100 ml of 20% aqueous ethanol and kept in a flask on stirrer for half-an-hour and then heated for 4 hr at 45°C with mixing. The mixture was filtered and the residue was again extracted with another 100 ml of 25% aqueous ethanol. The combined extracts were concentrated by using rotary evaporator at 40°C to get 40 ml approximately. The concentrate was transferred into a separator funnel and extracted twice with 20 ml diethyl ether. The ether layer was discarded while the aqueous layer was kept and then re-extracted with 30 ml n-butanol. The n-butanol extract was washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was evaporated. After evaporation, the samples were dried in the oven at 40°C to a constant weight and the saponin content was calculated.

**Total Tannins**

Tannin content of the given sample was estimated by following the method as described by Fagbemi et al. (2005). The plant extract in ethanol (1ml) was mixed with Folin-Ciocalteau’s reagent (0.5 ml), followed by the addition of saturated Na₂CO₃ solution (1 ml) and distilled water (8 ml). The reaction mixture was allowed to stand for 30 min at room temperature. The supernatant was obtained by centrifugation and absorbance was recorded at 725 nm using UV-Visible Spectrophotometer. Serial concentrations of standard tannic acid was prepared and
the absorbance of various tannic acid concentrations was plotted on a standard graph. The tannin content was expressed as mg tannic acid equivalent per gram of the sample.

**Total Phenols**

Total phenolic content in the ethanol plant extracts was quantified by the Folin Ciocalteau colorimetric method (Slinkard and Singleton, 1984). For analysis to 0.5 ml of plant extract in ethanol was added to 0.1 ml of Folin-Ciocalteau reagent (0.5N) and the contents of the flask was mixed thoroughly. Later 2.5 ml of Sodium carbonate (Na$_2$CO$_3$) was added and the mixture was allowed to stand for 30 min after mixing. The absorbance was measured at 760 nm in a UV-Visible Spectrophotometer. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/g extract.

**Total Flavonoids**

Total flavonoids content in the ethanolic extracts was quantified by the aluminium chloride calorimetric method (Mervat et al., 2009). 0.5 ml of plant extracts at a concentration of 1mg/ ml was taken and the volume was made up to 3ml with methanol. Then 0.1ml AlCl$_3$ (10%), 0.1ml of potassium acetate and 2.8 ml distilled water were added sequentially. The test solution was vigorously shaken. After 30 minutes of incubation., the absorbance was recorded at 415 nm. A standard calibration curve was generated using known concentrations of quercetin. The concentration of flavonoid in the test samples was calculated from the calibration plot and expressed as mg quercetin equivalent /g of sample.
3.3.3. Study of Antimicrobial activity

The Aqueous, Ethanol and Methanol plant extracts were used for antibacterial study (Ozkan et al., 2004). Different concentrations (50mg, 100mg and 150mg /ml) of the concentrated aqueous, ethanol and methanol plant extracts were tested for their antimicrobial activity against two Gram-ve bacterial strains such as *E.coli*, *Pseudomonas aeruginosa* and three Gram +ve bacterial strains like *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis* and one Deuteromycetes fungus, *Candida albicans*. The bacterial cultures were grown in Muller Hinton Agar and Muller Hinton Broth (Himedia) (Lopez et al., 2001).

Antimicrobial activity assays

Antibacterial activity was measured using the standard diffusion disc method on agar plates (Erturk et al., 2006). For antimicrobial assay, all bacterial strains were grown in Mueller Hinton Broth Medium (Himedia) for 24 hours at 37°C and plated on Mueller Hinton Agar (Himedia) for agar diffusion experiments. Then 0.1ml of bacterial culture was spread on agar plate surfaces. Sterile discs (Hi Media, 6mm in diameter) were placed on the agar medium. 20µl each of different concentration (50mg, 100mg and 150mg /ml) of aqueous, ethanol and methanol extracts were tested against certain bacterial strains. Inhibition diameters were measured after incubation for 24 hours at 37°C. Blanks were also tested for antibacterial activity. In the case of fungal strain, the culture was maintained on Sabouraud Dextrose Agar (SDA) at 25°C for about 72 - 96 hours and then stored at 4°C as Stock. The medium used for antifungal activity was Sabouraud Dextrose Agar (SDA) and incubation period was 72 hours at 25°C. Rest of the method was same as that of antibacterial activity.
3.3.4. Study of Antioxidant potential

Qualitative analysis of antioxidant activity.

The antioxidant activity of plant extract was determined by following the method as described by George et al. (1996). 50µl of extract were taken in the microtiter plate. 100µl of 0.1% methanolic DPPH (2, 2'-Diphenyl - picrylhydrazyl) was added and the sample was incubated for 30 minutes in dark condition. The sample was then observed for discoloration; from purple to yellow and to pale pink. They were considered as significant, moderate and low activity respectively. The antioxidant positive samples were further subjected to quantitative analysis.

Quantitative analysis of Antioxidant activity by employing DPPH.

The antioxidant activity was assessed using DPPH (2, 2'-Diphenyl - picrylhydrazyl) as a free radical. 100µl of plant extract was mixed with 2.7ml of methanol and then 200µl of 0.1% methanolic DPPH was added. The suspension was incubated for 30 minutes in dark. Initially, absorption of blank containing the same amount of methanol and DPPH solution was prepared and absorbance was measured (Ayoolal et al., 2008). Subsequently, for every 5 min interval, the absorption maximum of the solutions was measured using a UV double beam spectrum (Chemito, India) at 517nm. The antioxidant activity of the plant sample was compared with 0.16% Butylated Hydroxy Toluene (BHT), a well known standard. The experiment was carried out in triplicates. Free radical scavenging activity was calculated using the following formula:-

\[
\% \text{ DPPH radical-scavenging} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of test Sample}}{\text{Absorbance of control}} \right) \times 100
\]
3.3.5. Gas Chromatography- Mass spectrometry studies

In the present study, among the 18 plant parts, based on the performance in antimicrobial and antioxidant assays, three plant parts (Mucuna atropurpurea - fruit, Dendrophthoe falcate - whole plant and Cassytha filiformis - whole plant) were subjected to Gas Chromatography - Mass Spectroscopic (GC – MS) analysis to study the molecular structure and profile of bioactive compounds present in them. GC-MS analysis was carried out on a GC-MS -5975C agilent system comprising an auto sampler and a gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument, employing the following conditions: column Elite-1 fused silica capillary column (30×0.25 mm ID × 1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70eV; helium (99.999%) was used as carrier gas at a constant flow of 1.51 ml/min. An injection volume of 1µl (split ratio of 10:1), injector temperature 240°C; ion-source temperature 200°C was maintained. The oven temperature was programmed from 70°C (isothermal for 2 min), with an increase of 100°C/min, to 300°C/min, ending with a 9 min isothermal at 300°C. Mass spectra were taken at 70eV; with a scan range 40-1000 m/z. Solvent cut time was 5 min; MS start time being 5 min; MS end time being 35 min; Ion source temperature was set at 200°C and interface temperature being 240°C.

Identification of Components

Interpretation of mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST), having more than 62000 patterns. The mass spectrum of the unknown component was compared with that of the spectrum of the known components stored in the NIST repository. The name, molecular weight and structure of the component of the test materials were characterized.
QUESTIONNAIRE – ETHNOBOTANICAL DOCUMENTATION

a. Information about the Traditional healer or informant

1. Name .................................. Age: ..............

2. Main occupation ..........................................................

3. Years of experience in the Medicinal Practice .................

4. Source of knowledge about the Medicinal plants and their curative properties

   From ancestors/ Experience/ books/ any other sources: .........................

b. Treatment / Informant's Knowledge on Herbal medicine

1. Types of diseases treated .................................

2. Do you charge for the Medicinal Practice services or is it offered free?
   YES/NO

   If Yes: How much: ..................................................

3. Name the list of plants which are used by you for the preparation of herbal
   medicine: ..........................................................

4. Locality in which the said plants are collected ........................

5. Number of patients treated till date. (Approximately) .................

6. Whether the herbal medicines suited well/ was there any side effect? YES/NO

   Details of side effects, If any ..................................................

7. Whether the disease was completely cured / temporarily relieved?

8. Any other comments ..........................................................

Signature of the Informant