5.1. Introduction

Phytochemicals are present in a variety of plants, and are utilized as important components of both human and animal diets. These chemical compounds formed during the plants normal metabolic processes. These chemicals are often referred to as ‘secondary metabolites’. Plant-based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc (Gordon and David, 2001). The medicinal actions of plants unique to particular plant species or groups are consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Wink et al., 1999). There is growing awareness in correlating the phytochemical constituents of medicinal plants with their pharmacological activity (Byung-Hak et al., 2014; Martins et al., 2014). Screening of active compounds from plants has lead to the invention of new medicinal drugs which have efficient protection and treatment roles against various diseases (Susana et al., 2010; Mansour et al., 2014; Mariam et al., 2014). Therefore, isolation of phytochemicals of medicinal importance is most important for value addition of species.

In this line, no major works have been carried out in the study species, Hypochaeris radicata on the aspect of phytochemical compounds. To address this lacuna, the present study was carried out for qualitative phytochemical analysis through TLC, HPTLC and GC-MS techniques and quantitative determination of secondary metabolites present in the leaf and root parts of H. radicata. Further, isolation, purification and identification of medicinally important active compounds by column chromatography and determination of their structures were established by spectroscopic data (1HNMR, 13CNMR, IR and MS) along with biological activities of the isolated compounds which were predicted by computer programme through PASS software.

5.2. Materials and methods

5.2.1. Chemicals

For the present study all the chemicals are purchased from HiMedia Pvt. Ltd., Bombay. The chemicals used were of analytical grade.
5.2.2. Collection and authentication of the plant material

The fresh plant material of *H. radicata* was collected from Kattabettu, Nilgiris, the Western Ghats, Tamil Nadu, India, during February, 2012 (Plate I). The authenticity of the plant was confirmed in Botanical Survey of India, Southern Regional Circle, Coimbatore by referring the deposited specimen. The voucher number of the specimen is BSI/SRC/5/23/2010-11/Tech.153. The fresh leaf and root parts of this species were washed under running tap water to remove unwanted materials, shade dried at room temperature and powdered.

5.2.3. Preparation of the plant extracts

The powdered plant samples (50g/250mL) were extracted successively with petroleum ether, chloroform, ethylacetate, methanol and aqueous using soxhlet apparatus at 55-85°C for 8-10hrs in order to extract the polar and non-polar compounds (Elgorashi and Van Staden, 2004). For each solvent extraction, the powdered pack material was air dried and then used. The solvents of the respective extract were reduced under room temperature and stored at 4°C for further use.

5.2.4. Preliminary qualitative phytochemical analysis

Preliminary qualitative phytochemical analysis was carried out to identify the secondary metabolites present in the various alcoholic and aqueous extracts of leaf and root parts of *H. radicata*.

5.2.4.1. Test for alkaloids

i) Dragendorff Test: 1mL of each sample extract was taken in two separate test tubes. Then 2-3 drops of Dragendorff reagent was added. Appearance of orange red precipitate indicated the presence of alkaloids (Ciulci, 1994).

ii) Meyer’s Test: To a few mL of extract, one or two drops of Mayer’s reagent were added by the side of the test tube. A white creamy precipitate indicated the test as positive (Ciulci, 1994).

5.2.4.2. Test for cardiac glycosides

*Keller Killiani test*: 100mg of each extract was mixed with 1mL of glacial acetic acid containing one drop of ferric chloride solution. Then Con. H₂SO₄ (concentrated sulphuric acid) was added to the side of the test tubes. Formation of brown ring obtained
at the interface indicates the presence of a de-oxysugar, characteristic of cardenolides (Trease and Evans, 2002).

5.2.4.3. Test for flavonoids

i) Shinoda test (Magnesium hydrochloride reduction test): A piece of magnesium ribbon was added to 4mL of each extract followed by concentrated HCl (hydrochloric acid) drop wise. A colour ranging from crimson to magenta indicated the presence of flavonoids (Sofowora, 1993).

ii) Lead acetate test: 1mL of the extract was mixed with few drops of 10% lead acetate. Formation of yellow precipitate indicates the presence of flavonoids (Sofowora, 1993).

5.2.4.4. Test for glycosides

i) Keller Kiliani test: 2mL of each extract was taken and 1mL of glacial acetic acid was added. Then con. H$_2$SO$_4$ was added side of the test tubes. Appearance of blue colour indicates the presence of glycosides (Gohale et al., 2008).

ii) Legal’s test: To the extract, few drops of 10% NaOH (sodium hydroxide) were added to make it alkaline. Then freshly prepared sodium nitroprusside was added to the solution. Presence of blue colouration indicated the presence of glycosides in the extract (Gohale et al., 2008).

5.2.4.5. Test for phenols

Ferric chloride test: To 2mL of each extract, 2mL of neutral ferric chloride solution was added. Appearance of dark violet colour indicates the presence of phenols (Krishnamoorthy, 1988).

5.2.4.6. Test for resins

To 2 mL of extract, 2-3mL of acetic anhydride was added, dissolved by gently heating cooling and then 0.5mL of H$_2$SO$_4$ was added. Bright pruple colour was produced. It indicates the presence of resins (Ciulci, 1994).

5.2.4.7. Test for saponins

Frothing/foam test: 1mL of extract was taken in a test tube. To this 5mL of distilled water was added. Then this mixture was shaken vigorously. A persistent froth
that lasted for at least 15min indicated the presence of saponins (Brain and Turner, 1975).

5.2.4.8. Test for steroids

**Libermann-Burchard’s test:** 2 mL of the sample extracts were taken in the test tubes and it was evaporated. Then the residues were dissolved in acetic anhydride. To this mixture chloroform and conc. H$_2$SO$_4$ were added by the side of the test tube. Formation of brown ring at the interphase of the two supernatant layers indicated the presence of steroids (Ciulci, 1994).

5.2.4.9. Test for tannins

**Braemer’s test:** 2mL of each extract was diluted with distilled water and 2-3 drops of ferric chloride solution (5%). Indication of green-black or blue-black coloration showed the presence of tannins (Mace Gorbach, 1963; Ciulci, 1994).

5.2.4.10. Test for terpenoids

**Salkowski test:** 2mL of chloroform and concentrated sulphuric acid were added to 1mL of extract. Appearance of reddish brown colour indicates the presence of terpenoids (Harborne, 1973).

5.2.4.11. Test for triterpenoids

**Salkowski test:** The extracts were treated in chloroform with few drops of con. H$_2$SO$_4$, shaken well and allow standing for some time. Formation of yellow coloured lower layer indicates the presence of triterpenoids (Harborne, 1984).

5.2.5. Quantitative estimation of phytochemical constituents

The powdered and crude extract of leaf and root parts of *H. radicata* were used for the gravimetric and spectrometric (Visible spectrophotometer, Elico, SL-177) analysis respectively.

5.2.5.1. Determination of total alkaloids

200mL of 20% acetic acid was added to 5g of leaf and root powders taken in a separate 250mL beaker and covered to stand for 4hrs. This mixture containing solution was filtered and the volume was reduced to one quarter using water bath. To this sample, concentrated ammonium hydroxide was added drop-wise until the precipitate was complete. The whole solution was allowed to settle and the precipitate was collected by
filtration and weighed (Obadoni and Ochuko, 2001). The percentage of total alkaloid content was calculated as,

\[
\text{Percentage of total alkaloids} = \frac{\text{Weight of residue}}{\text{Weight of sample taken}} \times 100
\]

5.2.5.2. Determination of total phenolics content

The total phenolics content of *H. radicata* was estimated using Folin-Ciocalteau reagent by the method of Siddhuraju and Becker (2003). 20µg of leaf and root extracts were taken separately and it was made up to 1mL with distilled water. Then 500µL of diluted Folins-phenol reagent (1:1 ratio with water) and 2.5mL of sodium carbonate \( \text{Na}_2\text{CO}_3 \) (20%) were added. This mixture was shaken well and incubated in dark condition for 40min for the development of colour. After incubation the absorbance was measured at 725nm. A calibration curve of gallic acid was constructed and linearity was obtained in the range of 10-50µg/mL. The total phenolics content in the plant extract was expressed as mg of gallic acid equivalent (GAE)/g extract by using the standard curve.

5.2.5.3. Determination of total flavonoids content

The total flavonoids content was estimated using the procedure described by Zhishen *et al.* (1999). 1mL of plant extract was diluted with 200µL of distilled water separately followed by the addition of 150µL of sodium nitrite (5%). This mixture was incubated for 5min and then 150µL of aluminium chloride (10%) solution was added and allowed to stand for 6min. Then 2.0mL of sodium hydroxide (4%) solution was added and made up to 5mL with distilled water. The mixture was shaken well and leaves it for 15min at room temperature. The absorbance was measured at 510nm. Appearance of pink colour shows the presence of flavonoids content. The total flavonoids content was expressed as mg quercetin equivalent (QE)/g extract on a dry weight basis using the standard curve.

5.2.5.4. Determination of total tannins content

Total tannins content of *H. radicata* was estimated by the method of Siddhuraju and Manian (2007). 500µL of the extracts were taken in test tube separately and treated with 100mg of polyvinyl polypyrrolidone (PVPP) and 500µL of distilled water. This solution was incubated at 4°C for 4hrs. Then the sample was centrifuged at 5000rpm for 5min. 20µL of the supernatant was taken which has only simple phenolics free of tannins
(the tannins would have been precipitated along with the PVPP). The phenolics content of the supernatant was measured at 725nm and expressed as the content of free phenolics on a dry matter basis. From the above results, the tannins content of the extract was expressed as mg gallic acid equivalent (GAE)/g extract and calculated as follows:

\[
\text{Total tannins} = \text{Total phenolics} - \text{Free phenolics}
\]

5.2.5.5. **Determination of total saponins content**

Total saponins content was determined by the method described by Makkar et al. (2007) based on vanillin-sulphuric acid colorimetric reaction with some modifications. 50μL of plant extract was added with 250μL of distilled water. To this, about 250μL of vanillin reagent (800mg of vanillin in 10mL of 99.5% ethanol) was added. Then 2.5mL of 72% sulphuric acid was added and it was mixed well. This solution was kept in a water bath at 60°C for 10min. After 10min it was cooled in ice cold water and the absorbance was read at 544nm. The values were expressed as mg diosgenin equivalent (DE)/g extract derived from a standard curve.

5.2.5.6. **Determination of ascorbic acid (Vitamin C)**

Ascorbic acid determination was done according to the method of Klein and Perry (1982). 10mg of dried plant powder was re-extracted with 10mL of 1% metaphosphoric acid. They were allowed to stand for 45min at laboratory temperature and filtered through Whatman No. 4 filter paper. 1.0mL of filtrate was taken and it was mixed with 9.0mL of 50µM 2, 6-dichloroindophenol sodium salt hydrate and the absorbance was measured with 30min at 515nm. Ascorbic acid content was calculated on the basis of calibration curve of authentic L-ascorbic acid and the results were expressed as mg of ascorbic acid equivalent (AE)/g of extract.

5.2.5.7. **Statistical analysis**

Analysis was carried out in triplicates and mean±SD (Standard Deviation) using Duncan’s Multiple Range Test (DMRT) (Duncan, 1955). Statistical significance (p<0.05) were subjected to one way analysis of variance (ANOVA) by using a statistical Package for Social Science (SPSS) (Version 9, SPSS, Inc., Chicago, USA).

The qualitative and quantitative phytochemical analysis revealed that the methanol extract had higher content of phytoconstituents than other solvent extracts.
Therefore, further studies (TLC, HPTLC and GC-MS) were carried out in this solvent extract on both parts of the species, *H. radicata*.

**5.2.6. Extraction of the plant samples**

50g of shade dried leaf and root powders were extracted with methanol (250mL) using soxhlet apparatus at 60-80°C independently. The solvent present in the extract was reduced by rotary vacuum evaporator (Rotary vacuum evaporator “superfit”, PBV 7D vertical R/170, Mumbai, India) to obtain viscous semisolid masses. For analysis of TLC, HPTLC and GC-MS, the crude methanolic extract was redissolved in the same solvent.

**5.2.7. Thin Layer Chromatography (TLC)**

TLC is the first step to identify the phytochemical compounds present in the samples. Different retention factor (Rf) value of various phytochemicals provide valuable clue regarding their polarity and selection of solvents for separation of phytochemicals. It has many advantages such as cost effective, short time analysis, the possibility of multiple detection and specific derivatization on the same plate.

To support preliminary phytochemical analysis, methanolic leaf and root extracts were subjected to qualitative phytochemical analysis of TLC (Wagner *et al.*, 1984). It was performed by using silica gel-G as stationary phase in the chromatographic plates of 15x5cm with 3mm thickness to confirm the presence of secondary metabolites. For the separation of phytochemical compounds, the methanolic leaf and root extracts were spotted manually using capillary tube. The spotted plates were put in a solvent chamber which contained various solvent systems to detect the suitable mobile phase. After the separation of phytochemicals, various spray reagents such as 10% ethanolic sulphuric acid, Dragendorff reagent, 5% ferric chloride solution, Kedde reagent, vanillin sulphuric acid reagent and vanillin phosphoric acid reagent were used to identify the compounds. The colour of the spots was noted and Rf values were calculated by using the following formula:

\[
\text{Retention factor (Rf)} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}
\]

**5.2.7.1. Preparation of spray reagents**

**5.2.7.1.1. Alkaloids (10% Ethanolic sulphuric acid reagent)**

10mL of ethanol was added with 90mL of sulphuric acid.
Inference

The appearance of brown coloured spots confirms the presence of alkaloids.

5.2.7.1.2. Alkaloids (Dragendorff reagent)

100g tartaric acid was dissolved in 400mL of water. To this, 8.5g basic bismuth nitrate was added and the solution was shaken for 2hrs. 200mL of 40% potassium iodide was then added and the solution was shaken vigorously. After standing 24hrs, the solution was filtered.

Inference

Brown or orange zones appear immediately on spraying and the colours noted were not stable for longer periods.

5.2.7.1.3. Flavonoids (5% Ferric chloride)

5g of ferric chloride was dissolved in 100mL of distilled water.

Inference

The appearance of gray or black coloured spots confirms the presence of flavonoids.

5.2.7.1.4. Glycosides (Kedde reagent)

5mL of freshly prepared 3% ethanolic 3, 5-dinitrobenzoic acid was mixed with 5mL of 2M NaOH. The plate was sprayed with 5-8mL of freshly prepared mixture to evaluate the presence of glycoside compounds.

Inference

Immediately on spraying, glycosides form a pink or blue violet colour. The colour fades after few minutes, but can be regained by repeated spraying even after several days.

5.2.7.1.5. Saponins (Vanillin-sulphuric acid reagent)

5% ethanolic sulphuric acid (solution I)

1% ethanolic vanillin (solution II).

The plate was sprayed vigorously with 10mL solution I followed immediately by 5-10mL solution II. After heating at 110°C for 5-10min, the plates were observed for colouring.
Inference

With this reagent, saponins form mainly blue or pinkish-violet and sometimes yellowish zones.

5.2.7.1.6. Terpenoids (Vanillin-phosphoric acid reagent)

1g vanillin dissolved in 100mL of 50% phosphoric acid.

Inference

After spraying this solution, the plate was heated for 10min at 100°C. Vanillin-phosphoric acid reagent shows blue colour when viewed under visible light.

5.2.8. High Performance Thin Layer Chromatography (HPTLC)

HPTLC is a simple, rapid and accurate method for analyzing plant material. It has better resolution, and estimation of active constituents is done with reasonable accuracy in a shorter time. It can provide chromatographic fingerprints that can be visualized and stored as electronic images (Yamunadevi et al., 2011b). Qualitative densitometric HPTLC analysis was performed to resolve the phytochemical compounds such as alkaloids, flavonoids, glycosides, saponins and terpenoids as marker compound in the methanolic leaf and root extracts of H. radicata.

5.2.8.1. HPTLC fingerprinting analysis

HPTLC studies were carried out by following the method of Wagner and Baldt (1996), Harborne (1998) and Eike and Anne (2007).

5.2.8.2. Sample preparation

The methanolic leaf and root extracts of 100mg each was dissolved in 1mL HPTLC grade methanol and centrifuged at 3000rpm for 5min. These solutions were used as test solution for HPTLC analysis.

5.2.8.3. Developing solvent system

Different solvent systems were used to develop HPTLC fingerprint profile for different secondary metabolite groups separately viz., alkaloids, flavonoids, glycosides, saponins and terpenoids.
5.2.8.4. Sample application

2µL of test solution and 2µL of standard solution were spotted in the form of bands of 5mm length using Hamilton syringe on silica gel 60F254 (precoated on aluminum plate 4x10cm) with the help of CAMAG LINOMAT 5 applicator, which was programmed through WIN CATS software.

5.2.8.5. Development of chromatogram

After the application of spots, the chromatogram developed in ascending order with a CAMAG twin trough glass chamber (20x10cm) was pre-saturated with respective mobile phase for 15min; the length of each run was 8cm (90mm). The TLC runs were performed under laboratory conditions (temperature: 25±2°C and relative humidity: 60±5). The plates were then air dried by hot air to evaporate solvents.

5.2.8.6. Photo-documentation

The air dried plates were kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at visible light, UV 366nm and UV 254nm.

5.2.8.7. Derivatization

The developed plate was sprayed with respective spray reagent for alkaloids, flavonoids, glycosides, saponins and terpenoids and dried at 100°C in hot air oven.

5.2.8.8. Scanning

After derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 254nm. The peak numbers with their height and area, peak display and peak densitogram and Rf values were programmed through WIN CATS software (1.3.4 version).

5.2.9. Gas Chromatography - Mass Spectrometry (GC-MS)

GC-MS is an analytical method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation and identification of unknown samples. GC-MS can also be used in airport security to detect substances in luggage or on human beings. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification.
GC-MS is one of the key techniques generally used for screening/identification of many groups of plant phytochemicals. The GC-MS study includes the important optimization process viz., i) introduction of sample extract onto the GC column, ii) separation of its components on an analytical column and iii) detection of target analysis by using mass spectrometric (MS) detector.

5.2.9.1. Preparation of extract

1µL of the methanolic leaf and root extracts of *H. radicata* was employed separately for GC-MS analysis.

5.2.9.2. Instruments and chromatographic conditions

GC-MS analysis was carried out on a GC-MS 5975C (AGILENT) instrument employing the following conditions: - column DB-5ms Agilent (30m X 0.25mm, 0.25µm film thickness), operating in electron impact mode at 70eV. Helium (99.9995%) was used as carrier gas at a constant flow of 1.51mL/min in the split mode (split ratio-10:1), injector temperature 240°C and ion source temperature 200°C. The oven temperature was programmed from 70°C (hold time for 2min) with an increase of 10°C/min to 300°C/min ending with a 9 min isothermal. Mass spectrum was taken at 70eV, scan range of 40-1000m/z (mass/charge) and a scan interval of 5min.

GC analysis separates all of the components in a sample and provides a representative spectral output. The sample was injected into the injection port of the GC device. The GC instrument vaporizes the sample and then separates the compounds. The separated compounds were eluted from the column and entered a detector which was capable of creating an electronic signal. Each component ideally produces a specific spectral peak that may be recorded on a paper chart or electronically. The time elapsed between injection and elution is called the ‘retention time’. The size of the peaks is proportional to the quantity of the corresponding substances in the specimen analyzed. The peak is measured from the baseline to the tip of the peak. The x-axis showed the RT (retention time) and the y-axis measured the intensity of the signal to quantify the components in the sample injected. As individual compounds eluted from the gas chromatography column, they entered the electron ionization (mass spectroscopy) detector, where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments obtained were actually charged ions with certain
mass. The m/z (mass/charge) ratio obtained was calibrated from the graph obtained, which was called as the mass spectrum graph which is the fingerprint of a molecule.

5.2.9.3. Data analysis

Identification of bioactive compounds from methanolic extracts of leaf and root parts of *H. radicata* was based on the molecular structure, molecular mass and calculated fragmentations. The mass spectrum of the unknown compound was compared with the spectrum of the known components stored in the National Institute Standard and Technology [NIST-11.LIB (Stein, 1990)] library, having more than 62,000 patterns. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The name, retention time (min), molecular formula, molecular weight (Dalton), peak area (%), nature of the compound, structure and activity were ascertained. The biological activities of constituents are based on data obtained from Pubchem an online database and published literature.

5.2.10. Isolation, purification, identification and structural elucidation of phytochemical compounds

5.2.10.1. General experimental procedure

Mass spectra (MS) were measured on a QP-1000 Shimadzu (Japan) mass spectrometer. Infra-red (IR) spectral data were recorded using a Shimadzu FTIR-8400S spectrophotometer (Tokyo, Japan). \(^1\)H and \(^{13}\)C-NMR data were acquired with a Bruker Avance DRX 500 NMR (Nuclear Magnetic Resonance) spectrometer, using TMS (Tetramethylsilane) as an internal standard. Chemical shifts (δ) are expressed in parts per million (ppm). Column chromatography was performed on silica gel (60-120 mesh, Merck). Thin layer chromatography (TLC) was conducted on silicagel 60 F\(_{254}\) plates (Merck) and the compounds were visualized by UV light, iodine chamber and spraying reagent. PASS prediction was carried out to know the biological activities of the isolated compounds.

5.2.10.2. Extraction

The shade dried leaf and root parts of *H. radicata* were powdered and extracted with methanol (200g for leaf and 100g for root powder/500mL methanol) using soxhlet apparatus. The solvent present in the extract was removed by using rotary vacuum evaporator (Rotary vacuum evaporator “superfit”, PBV 7D vertical R/170, Mumbai,
India). The crude extract (20 and 15g for leaf and root extracts respectively) was subjected for further analysis.

5.2.10.3. Isolation, purification and identification

Isolation was carried out by column chromatography (Reid and Sarker, 1998). The crude methanolic leaf (20g) and root extracts (15g) were dissolved in minimum quantity of methanol. To this, 20g of silica gel was mixed and then evaporated to dryness. The column was packed with silica gel by wet packing method. Firstly the absorbent bed was prepared by gradually adding silica gel up to approximately two thirds of the column height; hexane (100%) was run through the column for proper packing of the column. The sample was loaded as dried slurry of silica gel to the top of the column and eluted with a stepwise gradient of hexane: ethyl acetate (100:0, 95:5, 90:10, 85:15 etc. and 100:0, 99:1, 98:2, 97:3, 96:4, 95:5 etc. for leaf and root extracts respectively) in increasing order of polarity.

5.2.10.4. Leaf part

A total of 130 fractions of 100mL each were collected and analyzed by TLC. The TLC analysis were carried out by using hexane and ethylacetate (3:0.1) as the mobile phase and the separated bands were visualized using iodine vapors, UV light and vanillin phosphoric acid reagent to check the presence of terpenoids. Fractions of 71 to 83 (eluted with hexane: ethyl acetate in the ratio of 95:5) showed the same Rf value (RF=0.57), it was combined to afford white compound (200mg). It was also further, detected and confirmed through preliminary phytochemical test (terpenoids).

5.2.10.5. Root part

A total of 230 fractions (100mL each) were eluted. Fractions 104-125 (eluted with hexane: ethylacetate in the ratio of 97:3) yielded a residue of about 400mg. This residue was further purified by TLC using the solvent system hexane (100%) to afford white compound. The eluted spots were seen under iodine vapors and UV lamp to confirm the presence of single compound. It was also detected and confirmed by preliminary test (coumarin).

5.2.10.5.1. Test for coumarin

3 mL of 10% NaOH was added to 1mL of column purified fraction, formation of yellow colour indicates the presence of coumarins (El-Tawil, 1983).
5.2.10.6. Prediction Activity Spectra for Substances (PASS)

The computer system can predict biological activity based on structural formula of a chemical compound. The PASS approach is based on the suggestion, Activity=Function (Structure). Thus, comparing structure of a new substance with that of the standard biologically active substances, it is possible to find out whether a new substance has a particular effect or not. PASS estimates the probabilities of a particular substances belonging to the active and inactive sub-sets from the SAR Base (Structure-Activity Relationships Base) (Gloriozova et al., 1998; Bently et al., 2000).

PASS provides simultaneous predictions of many types of biological activity based on the structure of compounds. Thus, PASS can be used to estimate the biological activity profiles for virtual molecules, prior to their chemical synthesis and biological testing. If Pa > 0.7 the substance is very likely to exhibit the activity in experiment, but the chance of the substance being the analogue of a known pharmaceutical agent is also high. If 0.5 < Pa<0.7 the substance is likely to exhibit the activity in experiment, but the probability is less, and the substance is unlike known pharmaceutical agent.

The result of prediction is returned in the form of a table containing the list of biological activity with the appropriate probability values (i.e) the values defining the likelihood for a given activity type to be either revealed (Pa) or not revealed (Pi) for each activity type from the predicted biological activity spectrum. Their values vary from 0.000 to 1.000. Only those activity types for which Pa > Pi are considered possible (Poroikov et al., 2001).

5.3. Results

5.3.1. Extraction yield

Table 4 shows the percentage of yield of crude successive extracts (petroleum ether, chloroform, ethyl acetate, methanol and aqueous) of leaf and root parts of *H. radicata*. Methanolic extracts of root exhibited higher yield (25.6%) followed by methanolic leaf extract (18.4%). The ethyl acetate leaf extract showed the lowest yield of 0.4%. Aqueous extracts of root and leaf also showed lower yields (3.58 and 1.72% respectively) followed by petroleum ether leaf extracts (5%). Petroleum ether and chloroform root extracts were determined to have the same quantity of yield of 2.8% each (Fig. 10).
5.3.2. Preliminary qualitative phytochemical analysis

The present study revealed that the various alcoholic and aqueous extracts of leaf and root parts of *H. radicata* contained alkaloids, cardiac glycosides, flavonoids, glycosides, phenols, resins, saponins, steroids, tannins, terpenoids and triterpenoids (Table 4). However, phenols were detected only in methanolic extracts of both parts and the cardiac glycosides were found in root extracts of the solvents, chloroform, ethyl acetate and methanol. Next to methanol extract, ethyl acetate extracts of both parts showed the presence of rich variety of secondary metabolites. Petroleum ether, chloroform and aqueous extracts showed less variety of these secondary metabolites. Compared to all other solvent extracts, methanolic leaf and root extracts had higher number of secondary metabolites with high degree of precipitation (++++). Triterpenoids and resins were determined to be present with lesser amount (+) only in all extracts.

5.3.3. Quantitative estimation of phytochemical constituents

5.3.3.1 Determination of total alkaloids

The gravimetric analysis for total alkaloid contents in leaf and root parts of *H. radicata* exhibited that higher alkaloid contents were present in leaf powder (45.62mg/g dry powder) than that of the root powder (38.83mg/g dry powder) (Table 5 and Fig. 11).

5.3.3.2 Determination of total phenolics content

Total phenolics content of various extracts of leaf and root parts of *H. radicata* was varying widely between 1.08 to 50.42mg GAE/g extract (Table 6 and Fig. 12). Methanolic extract of leaf and root parts were demonstrating higher total phenolics content (37.53 and 50.42mg GAE/g extracts respectively) than that of the other solvent extracts.

5.3.3.3 Determination of total flavonoids content

Total flavonoids content was high in ethyl acetate leaf extract (177.93mg RE/g extract) followed by root chloroform and ethyl acetate extracts (143.10 and 142.75mg RE/g extract respectively) (Table 6 and Fig. 13). In addition, it has noted that the flavonoids content was not detected in petroleum ether leaf and root extracts and chloroform leaf extracts.
5.3.3.4 Determination of total tannins content

The total tannins content of the various extracts of leaf and root parts of *H. radicata* was determined to be ranged between 0.34 and 16.11mg GAE/g extract (Table 6 and Fig. 14). Among the solvents used, the methanolic leaf and root extracts registered high amount of tannins, 10.29 and 16.11mg GAE/g extract respectively.

5.3.3.5 Determination of total saponins content

The total saponins contents of both leaf and root parts of *H. radicata* were ranging between 119.55 and 166.93mg DE/g extract across the various solvent extracts studied (Table 6 and Fig. 15). The ethylacetate extract of leaf and root parts depicted high content of saponins 166.67 and 166.93mg DE/g extract respectively.

5.3.3.6 Determination of ascorbic acid (Vitamin C)

The ascorbic acid content of both parts of *H. radicata* was found to be present between 0.03 and 2.13mg AE/g extract (Table 6 and Fig. 16). Among the solvents used, the methanol has drawn high amount of ascorbic acid content, 1.62 and 2.13mg AE/g extracts respectively from leaf and root parts. On the other hand, ethyl acetate leaf extract contained lower amount of ascorbic acid (0.03mg AE/g extract).

5.3.4. Thin Layer Chromatography (TLC)

The TLC studies proved the presence of secondary metabolites such as alkaloids, flavonoids, glycosides, saponins and terpenoids present in the methanolic leaf and root extracts of the study species, *H. radicata* (Tables 7 and 8).

5.3.4.1. Alkaloids

For the detection of alkaloids present in the methanolic leaf extract, two different combinations of chloroform, methanol and glacial acetic acid (83:17:10) and chloroform and methanol (30:15) have been used as mobile phases. Similarly, two different combinations of chloroform and methanol in the ratio of 83:17 and 30:15 were used for the detection of alkaloids in methanolic root extract. The Rf values of methanolic leaf and root extracts were 0.69, 0.23 and 0.71, 0.21 respectively. For this study, 10% ethanolic sulphuric acid and dragendorff reagents were used to detect the presence of alkaloids (Plate V).
5.3.4.2. Flavonoids

The TLC studies were performed for the detection of flavonoids using the mobile phase of ethyl acetate, glacial acetic acid and water (80:10:10, 90:10:10 and 100:10:10) for methanolic leaf extract, and ethyl acetate, methanol and water (100:20:12) for methanolic root extracts. Their Rf values were seen at 0.64, 0.62, 0.77 and 0.45. After derivatization the spots were gray in colour with the spraying reagent 5% ferric chloride (Plate V).

5.3.4.3. Glycosides

The TLC was well resolved for methanolic leaf and root extracts of the species, *H. radicata*. For the detection of glycosides, the solvent system ethyl acetate and methanol were used. For leaf extract, two different combinations 13:4 and 12:4 and for root extract one combination 13:4 were used. Rf value of methanolic leaf extract was 0.79 and 0.43 and root extract was 0.89. Pink colour was obtained by spraying the Kedde reagent (Plate VI).

5.3.4.4. Saponins

In the present study, saponins in methanolic leaf and root extracts were determined by using the solvent system chloroform and methanol (30:5 and 15:1 respectively). Three spots detected in leaf extracts were seen at Rf 0.79, 0.56 and 0.92 and in root extract the spots were seen at Rf 0.85. They were violet in colour after spraying the vanillin sulphuric acid reagent (Plate VI).

5.3.4.5. Terpenoids

In the TLC fingerprinting analysis, three different compositions of solvent systems were used for detecting terpenoids for the study species, *H. radicata*. The mobile phases identified for both leaf and root extracts were benzene and ethylacetate (20:1), petroleum ether and ethyl acetate (40:10) and, hexane and ethylacetate (30:10). The separated peaks were identified at Rf values for leaf extract 0.48, 0.74, 0.79 and 0.18. In methanolic root extract, three spots were detected in benzene and ethyl acetate (Plate VI) and their Rf values were seen at 0.49, 0.69 and 0.85, two spots were detected in petroleum ether and ethyl acetate (Plate VII); their Rf values were seen at 0.77 and 0.86 and also three spots were detected in hexane and ethylacetate (Plate VII) with Rf values were seen at 0.26, 0.67 and 0.86. After spraying vanillin phosphoric acid reagent, spots turned blue in colour.
5.3.5. High Performance Thin Layer Chromatography (HPTLC)

In the present study, methanolic leaf and root extracts of *H. radicata* were subjected to HPTLC analysis to prepare the fingerprint profiles of secondary metabolites. It reveals the occurrence of the secondary metabolites *viz.*, alkaloids, flavonoids, glycosides, saponins and terpenoids. The mobile phase and spraying reagents used and the colour change according to secondary metabolite are given in Table 9. The densitogram and 3D display were exhibited in Figs. 17 and 18 respectively. HPTLC profile under UV 254 and 366nm was presented in Plates VIII and IX. The sample extracts were run along with the standards and it was observed to confirm the presence of phytochemical compounds from chromatogram after derivatization.

5.3.5.1. Alkaloids

The results from HPTLC chromatogram for alkaloids can be observed at UV 254nm before derivatization. The methanolic leaf extract showed 10 spots with their corresponding ascending order of Rf values, 0.02, 0.09, 0.22, 0.31, 0.46, 0.62, 0.75, 0.83, 0.88 and 0.91. The methanolic root extract showed the presence of 12 spots with the Rf values in the ascending order of 0.01, 0.07, 0.22, 0.30, 0.35, 0.38, 0.40, 0.45, 0.49, 0.63, 0.67 and 0.83. Among the separated compounds, one compound from each part was detected as alkaloid at the Rf 0.46 (yellow) and 0.49 (yellow) for the leaf and root extract respectively. The desired profile was achieved in the mobile phase of ethylacetate: methanol: water [10:1.35:1] (Table 10).

5.3.5.2. Flavonoids

HPTLC finger prints of *H. radicata* was done by using selected solvent system toluene: acetone: formic acid [4.5:4.5:1] for both leaf and root extracts, visualized under UV 254nm before derivatization. The leaf extract showed 10 prominent bands, of which 3 were flavonoids at the Rf 0.19, 0.37 and 0.64 and in root extract exhibited 14 bands, of which 7 were flavonoids at the Rf 0.06, 0.21, 0.31, 0.38, 0.64, 0.70 and 0.97. The flavonoids with Rf value 0.64 was present commonly in leaf and root extracts. Maximum number of flavonoids (7 peaks) has been observed in root part followed by leaf part (3 peaks) (Table 11).

5.3.5.3. Glycosides

The HPTLC fingerprinting for methanolic leaf and root extracts of *H. radicata* revealed the existence of 15 peaks. Pinkish violet zones at day light mode present in the
given standard and sample track observed in the chromatogram after derivatization, which confirmed the presence of glycosides in the given standard and in the samples. The mobile phase ethylacetate: ethanol: water [8:2:1.2] gave good resolution with Rf value. The compounds were seen at the Rf 0.01 to 0.93 in the methanolic leaf extract. The root extract also showed the presence of 15 peaks and they were seen at Rf 0.01 to 0.90. When compared to root part (3 peaks), leaf part (1 peak) contained lesser number of compounds (Table 12).

5.3.5.4. Saponins

Blue, yellow, green and violet colours, after derivatization at visible light confirmed the presence of saponins. They were best observed at the wavelength, UV 366nm after derivatization. Mobile phase consisting of chloroform: glacial acetic acid: methanol: water [6.4:3.2:1.2:0.8] gave a sharp and well defined peak. The methanolic leaf extract exhibits 17 peaks which were seen at the Rf 0.03 to 0.96. The methanolic root extract also showed the presence of 17 peaks which were seen at Rf 0.01 to 0.97. The root extract attained more numbers of saponins (15 peaks) than that of the leaf extract (12 peaks). The Rf value of standard was found to be 0.34 and peak area 3126.9AU (absorbance unit). In root extract, the Rf value of 8th peak (Rf=0.34) was coinciding with standard Rf value and its area calculated was 26992.7AU at 100μg/mL of standard and sample concentration (Table 13).

5.3.5.5. Terpenoids

The chromatographic fingerprinting for terpenoids was well resolved at UV 366nm after derivatization. The plates were sprayed with anisaldehyde sulphuric acid reagent followed by heating and then visualized in day light shows 15 and 10 prominent peaks in methanolic leaf and root extracts respectively. Among them, the peaks of 7, 8 and 10-15 (leaf extract) and 1-4 and 7-10 (root extract) were confirmed to be terpenoids. The terpenoids with the Rf value, 0.77 is expressed jointly in both extracts. Well defined spots were obtained in the mobile phase of n-hexane: ethylacetate [7.2:2.9] (Table 14).

5.3.6. Gas Chromatography - Mass Spectrometry (GC-MS)

GC-MS chromatogram of the methanolic leaf and root extracts of H. radicata was presented in Tables 15 and 16 which showed 11 and 9 peaks respectively indicating the presence of phytochemical compounds. These compounds were identified through mass spectrometry attached with GC. The identification of phytochemical compounds is
based on the active principles with their retention time (RT), molecular formula, molecular weight, peak area (%), nature of the compound, structure and activities. The compound prediction is based on Dr. Duke's Phytochemical and Ethnobotanical Databases by Dr. Jim Duke of the Agricultural Research Service/USDA. In the present investigation, the methanolic leaf extract showed the presence of 11 compounds namely, docosanoic acid 1-methyl-butyl ester (2.0%), N-methyl-N-acetyl-3,4-methylendioxy benzylamine (3.89%), triazabicyclodecene (2.39%), phytol,acetate (19.22%), 2-hexadecene,3,7,11,15-tetramethyl-,(R- (R*,R*-(E))- (6.64%), 1,13-tetradecadiene (6.15%), 1,4-eicosadiene (8.32%), hexadecanoic acid, methyl ester (17.37%), 9,12-octadecadienoic acid, methyl ester (3.68%), 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)-(16.74%) and phytol (13.60%), and the prevailing 9 compounds in the methanolic root extract are, undecane (1.23%), hexadecanoic acid, methyl ester (1.51%), 9,12-octadecadienoic acid (Z,Z), methyl ester (0.71%), 5-acetamido-4,7-dioxo-4,7-dihydrobenzofurazan (0.86%), methyl(5-hydroxy-1H-benzimidazol-2-yl)carbamate (0.50%), hexahydropyridine,1-methyl-4-(4,5-dihydroxyphenyl)- (1.78%), 2(1H) naphthalenone,3,5,6,7,8,8a-hexahydro-4,8a-dimethyl-6-(1-methylethenyl)- (19.23%), 1-benzazirene-1-carboxylic acid, 2, 2, 5a-trimethyl-1a-(3-oxo-1-butenyl)perhydro-.,methyl ester (30.31%) and (3β)-11-oxolanosta-8,24-dien-3-ylacetate (43.86%). The GC-MS spectrum confirmed the presence of various compounds with different retention times. The individual fragmentation pattern of the components was illustrated in Figs. 19 - 25.

**5.3.7. Structural elucidation of isolated compounds**

**5.3.7.1. Compound 1**

The purified white powder was visualized by spraying with vanillin phosphoric acid reagent followed by heating at 100°C and displayed as purplish black spots on TLC plates. It also displayed positive responses against the terpenoid test. The molecular formula of compound 1 was established as C₁₅H₂₀O₃ which coincide with [M+H]+ ion peak (249) in Mass spectrum (Fig. 27b). Its IR spectrum displayed absorption peaks attributable to ketone (1624.12cm⁻¹) and olefinic carbon (2926.11cm⁻¹) functional groups (Fig. 27a). In the ¹H NMR spectrum of compound 1, the signals appeared in between δ 1.0 to 4.0 ppm corresponds to aliphatic protons. The signal at δ 5.7 indicates the existence of the olefinic protons (Fig. 26b). The ¹³C NMR spectrum of compound 1 showed carbonyl carbon signal at 162ppm and aliphatic carbon signal at 60-80ppm (Fig. 26a). The complete assignments of ¹H and ¹³C NMR signals of compound 1 were
furnished from the spectral analysis. On the basis of spectral analysis, the structure of compound 1 was assigned as confertin (Fig. 27c) which was isolated from methanolic leaf extract of *H. radicata*.

### 5.3.7.2. Compound 2

Compound 2 was isolated as a colourless needles, which exhibited molecular ion peak [M+H]$^+$ at $m/z$ 192 corresponds to molecular formula C$_{10}$H$_8$O$_4$, (Fig. 29b). It also displayed positive responses against the coumarin test. These results suggested that compound 2 to be a coumarin. The IR spectrum of compound 2 showed the presence of functional groups such as hydroxyl (3410.26 cm$^{-1}$) and carbonyl (1639.55 cm$^{-1}$) (Fig. 29a). Further analysis of the $^1$H NMR spectrum revealed the occurrence of methoxyl group (δ 4.4) and aromatic portion (δ 7-8.2) (Fig. 28b). The $^{13}$C NMR spectrum of compound 2 (Fig. 28a), indicates the presence of aromatic carbon atoms (104-127ppm) and carbonyl carbons (160-162ppm). On the basis of the physical data and spectral data compound 2 is identified as scopoletin (Fig. 29c) which was isolated from methanolic root extract of *H. radicata*.

### 5.3.7.3. Prediction Activity Spectra for Substances (PASS)

In order to find out the specific activity of the isolated compounds, it is undergone for prediction of activity by using PASS software. Pa (probability to be active) and Pi (probability to be inactive) were estimates of probability for the compound to be active and inactive respectively for each type of activity from the biological activity spectrum. Pa and Pi values of the isolated compounds such as confertin and scopoletin were presented in Tables 17 and 18 respectively. The Pa and Pi values of confertin were ranging between 0.942 and 0.704 (Pa), and 0.004 and 0.016 (Pi). The predicted values exhibited the activities like antineoplast (0.942Pa-0.004Pi), antiprotozoal (Lieshmania) (0.908Pa-0.003Pi), antieczematic (0.842Pa-0.011Pi) and cardiovascular analeptic (0.825Pa-0.004Pi) for the compound confertin. The prediction of PASS for scopoletin showed the activities like antimutagenic (0.890Pa-0.002Pi), spasmyolytic and urinary (0.872Pa-0.004Pi), antiseptic (0.853Pa-0.004Pi), cardiovascular analeptic (0.784Pa-0.005Pi) and antiseborrheic (0.791Pa-0.021Pi).
5.4. Discussion

5.4.1. Extraction yield

The extraction yield calculated for petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts of both parts of H. radicata showed that methanol extract registered higher percentage of yield. It may be due to high polarity of methanolic solvent which can draw high variety of plant constituents than the other solvents did (Paulsamy and Jeeshna, 2011).

5.4.2. Preliminary qualitative phytochemical analysis

Preliminary qualitative phytochemical analysis made for the leaf and root parts of H. radicata revealed the presence of alkaloids, cardiac glycosides, flavonoids, glycosides, phenols, resins, saponins, steroids, tannins, terpenoids and triterpenoids. These secondary metabolites are reported to have many biological and therapeutic properties (Rathbone and Bruce, 2002; Sandhyarani and Portssangham, 2011; Narender et al., 2012; Vishnu et al., 2013) and so this species is expected to have many medicinal uses.

5.4.3. Quantitative estimation of phytochemical constituents

Generally majority of the secondary metabolites studied and ascorbic acid in leaf and root parts of H. radicata have present with higher amount in methanolic extract than that of the other alcoholic and aqueous solvents. However, flavonoids and saponins were rich in ethyl acetate extracts. It is explained that the polarity level and species nature are playing major role in extracting the secondary metabolites (Ali and Asmah, 2011).

5.4.4. Thin Layer Chromatography (TLC)

The TLC analysis is an important technique that has been used in many cases, such as agricultural products, foods, beverages, and plant constituents for many years (Sherma, 2000). TLC is the earliest of chromatographic techniques to perform and require simple apparatus (Smith and Seakins, 1976). It is a valuable tool for preliminary exploration of many kinds. Similar to paper chromatography, TLC can be used to determine characteristic component patterns of plant extracts and biochemical preparations (Touchstone, 1992). They readily provide qualitative information and it separate small amounts of compounds. Compared to paper chromatography, the special advantages of TLC are the versatility (using different adsorbents), speed (more compact
nature of the adsorbent) and sensitivity (only μg amounts of materials can be achieved). The separation and identification of the phytochemical compounds can be made by scanning these chromo-strips with/without detecting reagents, under visible or UV-light. The resulting degree of difference in chromatographic “fingerprints” can essentially be used as “markers” in standardization of all extracts in particular solvent system separating compounds at specific Rf value which differ to other plant extracts. These Rf values are simple, reproducible and thus reliable marker to verify the purity of the crude drugs. TLC has many advantages such as lower cost, short time analysis, the possibility of multiple detection and specific derivatization on the same plate. The results of the study revealed the presence of phytochemical constituents such as alkaloids, flavonoids, glycosides, saponins and terpenoids in the leaf and root extracts of this species. Further, the thin layer chromatographic profiles may serve as characteristic fingerprint for leaf and root extracts of *H. radicata*. It could be concluded that as the species, *H. radicata* contained rich variety of phytochemicals, it can be used for drug preparations in pharmaceutical industries.

### 5.4.5. High Performance Thin Layer Chromatography (HPTLC)

HPTLC is a planer chromatography where separation of the sample components is achieved on high performance layers with detection and data acquisition using an advanced workstation. These high performance layers are pre-coated plates with a sorbent of particle size 5-7 microns and a layer thickness of 150-200 microns. The reduction in the thickness of the layer and the particle size results in increasing the plate efficiency as well as nature of the separation. Separations on high performance thin layer plates give sharper and more compact bands with shorter distances of migration. HPTLC is suitable for qualitative and quantitative analyze and micropreparative techniques.

The evaluation of crude extract is an integral part of correct identity. HPTLC is useful as a phytochemical marker and also a good estimator of genetic variability in populations. In the field of plant taxonomy for the identification of plants through secondary metabolites, it is more effective (Salim *et al.*, 2011). HPTLC fingerprinting is proved to be a linear, precise and accurate method for herbal identification (Marcello and Chiara, 2012; Mona *et al.*, 2014).

The qualitative analysis through HPTLC for methanolic leaf and root extracts of *H. radicata* confirmed the presence of many secondary metabolites like alkaloids,
flavonoids, glycosides, saponins and terpenoids. The well resolved HPTLC profiles also showed the occurrence of these metabolites of medicinal importance which support the traditional therapeutic uses of this species.

Alkaloids are the N-sources found in plants that are the amino acids such as lysine, ornithine, phenyl alanine, tyrosine, tryptophan and histidine. They have been a particularly rich source of medicinal uses as analgesic, antiinflammatory and adaptogenic activities which help to alleviate pains, developed resistance against diseases and endurance against stress and also to cure many diseases (Yan et al., 2008). In the present study, methanolic extract of *H. radicata* showed that two different types of alkaloids which were identified with different Rf levels.

Out of 24, 10 spots were identified as flavonoids present in methanolic extract of *H. radicata*. Flavonoids are most importantly present in fruits, teas and soybeans. The flavonoids are reported to have the medicinal properties like antiallergic, anticancer, antioxidant, antiinflammatory and antiviral activities (Ammar et al., 2009; Vijayalakshmi et al., 2012; Bashir et al., 2013; Manases et al., 2013; Tebekeme and Diepreye, 2013; Tunde et al., 2013). Glycosides are getting hydrolysed and released phenolics that are toxic to microbial pathogens (Nakamura et al., 2008; Zhongliu et al., 2013). In the present study, 4 different types of glycosides were noted to be present in methanolic extract of *H. radicata* which confirms the therapeutic values of this species.

A good separation of saponins has been observed in methanolic leaf (12 spots) and root (15 spots) extracts of *H. radicata*. Saponins are glycosidic triterpenoids widely found in plants. They are soluble in water, giving stable foams. Three major classes are found as steroid glycosides, steroid alkaloid glycosides and the largest group, triterpene glycosides. They contain polycyclic aglycones attached to one or more sugar side chains. They have bitter principles and found in most vegetables, beans and herbs. They have the properties like leishmanicidal, nematicidal and antimicrobial (Venkatesan et al., 2009; Amita et al., 2013; Geethalakshmi and Sarada, 2013). Terpenoids are defined as secondary metabolites with molecular structures containing carbon backbones made up of isoprene units. They have properties like antimicrobial, antiinflammatory and hepatoprotective properties (Ji-Feng et al., 2013; Shi-Yie et al., 2013). Rich varieties of terpenoids were detected in methanolic leaf and root extracts of *H. radicata* as 8 peaks each were obtained by HPTLC technique.
5.4.6. Gas Chromatography - Mass Spectrometry (GC-MS)

In the present study, the GC-MS analysis of the methanolic leaf extract of *H. radicata* showed the presence of 11 compounds. The mass spectra identified for four major compounds are presented in methanolic leaf extract (Figs. 20-22). They are phytol, acetate (19.22%) (Fig. 20d), hexadecanoic acid, methyl ester (17.37%) (Fig. 21d), 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)- (16.74%) (Fig. 22b) and phytol (13.60%) (Fig. 22c). The compounds phytol, acetate is reported to have antitubercular activity. Hexadecanoic acid, methyl ester has the property of antioxidant, hypercholesterolemic and pesticide (Antara and Amla, 2012; Jagadeeswari et al., 2012). 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)- found to have antiinflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic, antieczemic, antiacne, antiarthritic and anticoronary properties (Ravi et al., 2012). The compound, phytol which was identified in the leaf extract of *H. radicata* having anticancer, antioxidant and antiinflammatory properties. It also has the ability of curing the arthritis (Ravi et al., 2012). The methanolic root extract of *H. radicata* showed the presence of 9 compounds. Of them, the two compounds viz., hexadecanoic acid, methyl ester (1.51%) (Fig. 23b) and 9,12-octadecadienoic acid, (Z,Z)-methyl ester (0.71%) (Fig. 23c) were determined to be present in leaf extract. The other major compounds reported to be present in the root extract were (3β)-11-oxolanosta-8,24-dien-3ly acetate (43.86%) (Fig. 25c) and 1-benzazirene-1-carboxylic acid,2,2,5a-trimethyl-1a-(3-oxo-1-butenyl) perhydro-, methyl ester (30.31%) (Fig. 25b). For these compounds, the therapeutic properties could not be reported.

5.4.7. Structural elucidation of compounds

In continuation of medicinal plant research, more recently, drug discovery techniques have been applied to the standardization of herbal medicines, to elucidate analytical marker compounds. Drugs derived from medicinal plants can serve not only at new drugs themselves but also as drug leads suitable for optimization by medicinal and synthetic chemists. Several known compounds from traditionally used medicinal plants have already been reported for Asteraceae members (Johann et al., 2012). The present study was aimed to explore the potential bioactive substances from methanolic leaf and root extracts of *H. radicata*. Through the analysis of spectral data two compounds were isolated from leaf and root parts of this species such as confertin and scopoletin respectively. The medicinal properties of these two isolated compounds were confirmed
through literature (Taslima et al., 2006; Bina et al., 2007; Demerdash et al., 2009; Yi et al., 2010; Xiaobo et al., 2011; Mogana et al., 2013; Soodabeh et al., 2013).

The compound confertin is a sesquiterpenoid generally synthesized by the mevalonate pathway and they are formed from three C5 units (Dewick, 2009). The sesquiterpenoids are generally being less volatile than monoterpenoids (Robbers et al., 1996; Heinrich et al., 2004; Dewick, 2009). α-Bisabolol, a major component of matricaria (Matricaria chamomilla); γ-bisabolene which contributes to the aroma of ginger (Zingiber officinale); costunolide a bitter principle found in the roots of chicory (Cichorium intybus); parthenolide, an antimigraine agent in feverfew are some of the naturally occurring sesquiterpenoids (Dewick, 2009). Sesquiterpene lactones are a group of secondary metabolites found across the plant kingdom comprising a large group of over 5000 known compounds (Wedge et al., 2000), being most common in families like Cactaceae, Solanaceae, Araceae, Euphorbiaceae, etc (Canales et al., 2005). However, they are most prevalent in the family Asteraceae, where they can be found almost ubiquitously (Rodriguez et al., 1976). Asteraceous plants are in turn are the most diverse and prolific plant family in the world. Many sesquiterpenoids were isolated from different types of plants, artemisinin (Artemisia annua) (Klayman et al., 1984), daucuside (Tanacetum parthenium), valerenane (Valeriana officinalis) and zedoarol, germacrone, curdione, β-elemene and curzeone (Curcuma zedoaria) (Shiobara et al., 1986). Sesquiterpenoids are typically located in laticifers, which are specialized secretary cells in most of the Asteraceae, but can also be found within the vacuoles of other cell types in the plant, specifically when produced in response to biotic stresses. They are one of the main constituents of latex in latex producing plants, and they are frequently potent antimicrobial agents as well as antifeedants to chewing insects and birds. To humans, lettuce and chicory (Lactuca sativa and Chicorium intybus respectively) represent the main dietary source of sesquiterpene lactones on the basis of the levels of their global consumption. Sesquiterpene lactones are used in the treatment of cardiovascular diseases (Rodriguez et al., 1976; Wong and Menendez, 1999), antimalarial and antimicrobial (Luna-Herrera et al., 2007; Duraipandiyan et al., 2012), antioxidant (Chakraborty and Paulraj, 2010; Zhao et al., 2010), cytotoxic (Jing et al., 2008), antiinflammatory (Li-Jun et al., 2014), anticancer (Zhang et al., 2005), antitumor (Wang et al., 2014) and antiacetylcholinesterase activity (Chen et al., 2014) and are responsible for a range of other effects such as prevention of neurodegeneration, antimigraine activity, analgesic
and sedative activities and treatment of ailments such as diarrhoea, flu and burns (Prehn and Krieglstein, 1993; Heinrich et al., 1998; Ahlemeyer et al., 1999; Canales et al., 2005; Wesolowska et al., 2006). In addition, some sesquiterpene lactones protect the gastric lining during ulcer development (Giordano et al., 1990). Sesquiterpenes have also been used as chemo systematic markers in the subtribe Hypochaeridinae (Lactuceae tribe) of the Asteraceae family (Zidorn, 2006).

The compound scopoletin (6-methoxy-7-hydroxycomarin) is a phenolic coumarin and an important member of the group of phytoalexins isolated from many plants (Tal and Robeson, 1985) and also a prominent coumarin derivative occurring in various plants (Hiroki et al., 1984; Mizuo et al., 1992; Cristina et al., 2007; Soad et al., 2008; Simoes et al., 2009; Yang et al., 2009; Mirunalini and Krishnaveni, 2011; Raju et al., 2012). More than 1300 coumarins have been identified as secondary metabolites from plants, bacteria and fungi (Iranshahi et al., 2009). A variety of biological activities viz., antiinflammatory, antiallergy (Cheng et al., 2012) and antiangiogenesis have been reported for this compound (Pan et al., 2011a, b). Accumulation of scopoletin has been correlated with resistance to microbial attack and other stress such as mechanical injury and dehydration (Tanaka et al., 1983). Scopoletin often seems to be the most important product rising in concentration in the infected plant as compared to other related coumarins and coumarin glycosides such as scopolin (the glycoside of scopoletin), esculetin and esculin (Uritani, 1999; Buschmann et al., 2000; Giesemann et al., 2008). The present study also revealed the presence of scopoletin as one of the major constituent of the genus, Hypochaeris. Scopoletin, as an antitumour agent has been reported by many authors. Darmawan et al. (2012) reported the cytotoxic activity of scopoletin against P-388 murine leukemia cells showed strong cytotoxic activity with IC$_{50}$ 17.42 μg/mL. In-vitro and in-vivo antioncogenic potential of the same compound was reported by Bhattacharyya et al. (2010). Also Liu et al. (2012) developed twenty derivatives of scopoletin and compared its anti tumour activity against mammary (MCF-7 and MDA-MB 231) and colon (HT-29) carcinoma cells. The compound is of great pharmacological importance mainly used as an antiinflammatory (Witaicenis et al., 2010; Kwon et al., 2011; Yasser and Ouf, 2012), anticoagulant (Poole and Poole, 1994; Hirsh et al., 2001), antibacterial (Raja et al., 2011), antifungal (Wang et al., 2009), antiviral (Kashman et al., 1992; Patil et al., 1993; Newman et al., 1998; Sancho et al., 2004), anticancer (Lee et al., 2011; Yun et al., 2011; Sadia et al., 2013), antihypertensive
(Mead et al., 1958; Nguelefack-Mbuy et al., 2008), antitubercular (Cohen, 1979), anticonvulsant (Lusczki et al., 2009), multiple sclerosis (Chen et al., 2010), antiadipogenic (Shin et al., 2010), antihyperglycemic (Fort et al., 2000), antioxidant (Kim et al., 2008) and neuroprotective (Wang et al., 2012) activities.

In the present investigation, isolated known compounds viz., confertin and scopoletin from leaf and root extracts of Hypochaeris radicata proved the traditional usage. From the results, this species could be treated as potential medicinal herb for treating various diseases. In addition, research activities on isolation of new bio-active compounds from this species are necessary for the developing pharmaceutical aids.

5.4.7.1. Prediction Activity Spectra for Substances (PASS)

The computer system, PASS provides simultaneous predication of several hundreds of biological activities of any drug like compounds. The software, PASS estimates the probabilities of 900 types of biological activity on the basis of structural formulae of compounds with the accuracy of 85%. PASS predications are based on the analysis of structure-activity relationship (SAR) for the training set of about 46,000 biologically active compounds (Anzali et al., 2001; Sadym et al., 2003). Therefore, PASS once trained is able to predict simultaneously all biological activities which are included in the training set. To provide the best quality of predication, new information about biologically active compounds is collected permanently from papers and electronic sources and after the expert’s evaluation, is regularly added to the training set (John et al., 2001; Maridass et al., 2008; Maridass, 2008). Jeeshna and Paulsamy (2011b) and Mallikadevi et al. (2012a) determined the biological activity of GC-MS compounds using PASS programme in the medicinal species, Exacum bicolor and Mukia maderaspatana respectively. Farkaad et al. (2014) predicted the medicinal uses by employing PASS in Caesalpinia sappan. These prediction activity spectra guided for selecting a promising pharmaceutical lead with high accuracy and required antioxidant and hepatoprotective properties.

PASS can be a possible approach to determine the novel possible activities of the existing phytoconstituents. In the present study on examination of the PASS spectrum of the isolated phytoconstituents of Hypochaeris radicata, it was found that there were a significant number of unexplored pharmacological activities in each constituent. As the PASS predicted pharmacological activities with a score of Pa>0.7 have very high
chances to be obtained experimentally, therefore only the unexplored pharmacological activities with a score of Pa>0.7 have been summarized and listed as hidden pharmacological potential of these compounds.

The report on phytochemicals present in this plant extract of *H. radicata* justifies the traditional medicinal usage of this species by the local healers of Nilgiris, the Western Ghats and it could be recommended as a plant of phytopharmaceutical importance. However, further studies are needed to ascertain its bioactivity and toxicity profile for various diseases.