CHAPTER - 4

Materials and Methods

Materials and Methods

4.1. FLORISTIC SURVEY

4.1.1. Collection and Preservation of Materials

The Laurel flora of Terai-Duars region has been prepared through random sampling in three different season *viz.* winter, pre-monsoon and post-monsoon for five consecutive years, 2008 to 2012. Several places in Terai-Duars region, mainly Wildlife Sanctuaries and National Parks like Mahananda Wildlife Sanctuary, Gorumara National Park, Buxa Tiger Reserve, Chapramari Wildlife Sanctuary, Raja Bhatkhawa Reserve Forests and Jaldapara National Park were visited regularly for the collection of all relevant field data including local uses, time of flowering, fruiting etc.

During numerous field trips, round the year, mainly healthy twigs with flowers and /or fruits and generally with leaves were collected in triplicates. In case, flowering and/or fruiting twigs were not available, specimens with healthy and mature leaves were collected. The specimens were tagged and recorded in the *Field Note Book*, and temporarily kept in air tight polythene pouches. On return to the field camp or to the laboratory specimens were cleaned, trimmed and 10% formalin were added at nodes and other soft joints to save the specimens from shattering. Then, the specimens were transferred to a heavy wooden plant press between blotting papers or old newsprints for drying. After proper drying specimens were poisoned by soaking with saturated solution of Mercuric Chloride [generally 4 – 6%] in rectified ethanol and then dried again using blotters. After proper drying and poisoning, specimens were mounted on standard herbarium sheets, labeled and temporarily stored in the Taxonomy & Environmental Biology Laboratory in the Department of Botany of the University of North Bengal. After completion of the work, the main set of herbarium specimens will be deposited at NBU-Herbarium and the duplicates will be deposited CAL. However, all these works were guided by the methodology provided by Jain & Rao (1977).

4.1.2. Field Note Book

The records in the Field Note Book covers specific locations, altitude, dates of collections, availability, habit, habitat, flower colour and such other characters of plants which are not available with dry and mounted herbarium specimens. The field notes were transferred to herbarium labels for ready reference. After completion of the work, the *Field Note Book* was deposited at the NBU-Herbarium.

4.1.3. Identification

The specimens were primarily identified in the Taxonomy & Environmental Biology Laboratory of the Department of Botany, North Bengal University using available literature including Hooker (1886), Brandis (1906), Kanjilal *et al.* (1940), Momiyama (1966), Long (1984), Ara *et al.* (2007), Li *et al.* (2008a) and matching with the available predetermined specimens at the NBU-Herbarium. Finally, for confirmation, specimens were matched at CAL.

4.1.4. Flowering and fruiting calendar

The flowering calendar were prepared by regular monitoring of the vegetation and recording the flowering and fruiting time of different species. All data were derived from direct observation only as the flowering period varies in different species with the change of physiography, altitude, longitude, latitude etc. For detailed methodology Das & Chanda (1987) and Panda *et al.* (1992) have been followed in general.

4.1.5. Enumeration and Description

Different recorded species of Laurels are enumerated below with genera and species under different genera in alphabetic order. For each species the correct name is followed by basionym, if any, and other available or important synonyms. Proper author citation, protologue references and references to the record in other relevant floras of nearby regions are also cited. This is followed by the local name(s). A short description of each species has been given. To cite the voucher specimen, the place of collection with altitude, collector's names, field number and date of collection have been provided separately for the specimens collected from different places. Ranges of flowering and fruiting time have been given. Local distribution provided in the enumeration are based both on collected specimens and observation made during the field trips and is not fully based upon available literature. On the other hand, information related to the general distribution have been determined from published literature and deposited specimen from the different herbaria visited. At the end, a *note* has been provided which mainly covers the local uses and any other interesting observation made during the field study.

4.2. ANATOMICAL STUDIES

4.2.1. Plant samples

For the present study eight species representing two genera of Lauraceae were chosen. Plants were collected from different places in Terai-Duars region of West Bengal. The collected materials and voucher number are given in table no 4.1.

Table 4.1. Laurels with voucher number collected for the anatomical study

Species	**Voucher no. [***Dibakar Choudhury & AP Das]*
Cinnamomum bejolghota (Buchanan–Hamilton) Sweet	064
Cinnamomum camphora (Linnaeus) J. Presl	175
Cinnamomum tamala ((Buchanan–Hamilton) Nees & Ebermaier	006
Cinnamomum verum J. Presl	003
Litsea assamica Hooker *f.*	095
Litsea glutinosa (Loureiro) Robinson	026
Litsea laeta (Nees) Hooker *f.*	090
Litsea monopetala (Roxburgh) Persoon	109

4.2.2. Anatomical work

The materials for anatomical study were fixed in FAA (Formaldehyde: Acetic Acid: Alcohol, 1:1:18 v/v) for 24 hours and then preserved in 70% ethanol (Johansen 1940).All observations were performed on

transverse sections of well-developed stem, petiole and lamina with mid-vain taken by hand. Double staining method was used for this study (Santra *et al*. 1989; Maji 2004). The fine thin sections were dehydrated and double-stained with safranin and light-green through ethanol grades. The cell wall (cutinized / lignified wall) took the colour of safranin; whereas soft tissues were stained with light green and were mounted in Canada balsam (Santra *et al.* 1989; Maji 2004). The sections were studied and photographed from permanent slides with a digital camera attached to an Olympus BX51 light microscope. All measurements and observations were made three times and expressed in micrometer.

All permanent slides were deposited in the Taxonomy and Environmental Biology Laboratory of the Department of Botany, University of North Bengal.

4.3. LEAF ARCHITECTURE

4.3.1. Plant samples

Leaf architectural study has been carried out with same species as selected for anatomical study i.e. *Cinnamomum bejolghota* (Buchanan–Hamilton) Sweet, *Cinnamomum camphora* (Linnaeus) J. Presl, *Cinnamomum tamala* ((Buchanan–Hamilton) Nees & Ebermaier, *Cinnamomum verum* J. Presl, *Litsea assamica* Hooker *f., Litsea glutinosa* (Loureiro) Robinson, *Litsea laeta* (Nees) Hooker *f.* and *Litsea monopetala* (Roxburgh) Persoon.

4.3.2. Clearing of leaves

Entire mature leaves were immersed in 10% NaOH at room temperature until soft tissue was discolored as well as dissolved and leaves become fully transparent. Most of the leaves were cleaned within 25 – 30 days, depending upon their thickness. After putting out from the solution, the leaves were washed thoroughly with distilled water to remove the sodium hydroxide and then cleared with a brush. In case the leaves remained opaque after clearing it was then boiled with lactic acid to achieve the desired level of clearing (Lama 2004). After clearing specimens were stained with 2% saffranin prepared in 70% ethanol. The excess stain was washed out with 70% ethanol and finally mounted between two glass plates with DPX mountant (Foster 1952).

4.3.3. Study of Venation

4.3.3.1. Major Venation pattern

The first step in describing venation is to recognize the first two orders of veins. In general the primary and secondary veins are the major structural veins of the lamina which can be easily recognized with naked eye or through a simple magnifying lens. The mid-vein or primary vein is the thickest vein of the lamina and thickness decreases gradually toward the apex. This also includes the study of 2^o vein spacing, 2^o vein angle, inter 2^o veins (veins similar to 2^o s and do not reach the margin), and agrophic veins (comb like complex comprising of lateral 1^o or 2^o veins).

4.3.3.2. Minor venation pattern

The highest orders of veins were identified up to 5° in all cases. Minor venation patterns included several microscopic studies like 3^o vein category, 3^o vein course, 3^o vein angle variability, 3^o vein angle to 1^o, 4^o and 5^o vein categories, marginal ultimate venation etc. Lamina with a vein that form high number of discrete orders or that has regular courses, is considered to be more organized or 'higher rank' leaves. Leaf rank is a semi-quantitative description of the leaf venation system.

For minor venation pattern study the stained segments were observed under 5X objective of a compound microscope using a 10X eye piece. Camera Lucida drawings were made from apical, middle and basal portion including the mid-vein and other tertiary veins. Marginal venations were also studied in this manner. Descriptions were made following the scheme of Hickey (1973) and Leaf architecture working Group (1999).

4.3.4. Study of Areole (vein-islet) & F.E.Vs frequency

Areoles or vein islets are smallest areas of the leaf tissue bounded by veins. These may be of different shape and considered as a significant tool in recognizing a species. Any order of venation can form one or more sides of an areole. F.E.Vs is the freely ending ultimate veins of the lamina. Both, areoles and F.E.Vs can provide important data of taxonomic significance.

Using a stage micrometer and Camera Lucida a rectangular area was made within which areoles along with the mid-vein were drawn. The exact area of the rectangle was measured through proper calculation of magnification. For each species three such drawing were made comparing of one from each of the apex, median and basal portion of the lamina. Frequencies were determined through the following formulas (Chatrath 1992):

Areolar frequency = no. of areoles/mm^2 area

F.E.Vs frequency = no. of F.E.Vs/mm^2 area

In calculation, two incomplete areoles were considered as a complete areole.

4.3.5. Study of Indumentum

For the determination of the location and type of indumentums, fresh lamina and petioles were observed under the low and then high power objectives of the compound microscope. The measurements were made to utilizing the ocular micrometer after proper standardization of the microscope.

4.3.6. Study of Stomata

Several techniques were followed for stomatal study, *viz*-

1. Peeling of lamina mainly from dorsal surface (as stomatal density is much higher in lower surface) with the help of forceps and mounted in 10% glycerin for observation.
2. In impression technique, colorless nail polish was used as an impression material. Impressions of foliar epidermal cells were taken by smearing the nail-polish on the dorsal surface of the leaf and it was allowed to dry completely. Then the thin impression layers were taken out from leaves and placed on glass slides. In this way temporary slides were prepared and studied under compound microscope.
3. In case of thick leaves (such as *C. tamala*, *C. verum*, *C. bejolghota*) where peeling method and impression technique were not producing appreciable results, there the scrapping technique was used. The upper epidermis and mesophylls were scrapped out with the help of a scalpel or a sharp blade. Then the scrapped pieces were dipped into the FAA solution (formalene, glacial acetic acid and 50% ethanol in 1:1:18 ratio) for few minutes. After that, it was placed on clean glass slide and mounted with 10% glycerin. Slides were then studied under the compound microscope.

4. In some cases, leaf samples were boiled approximately for 15 minutes in 15 ml of 10% aqueous HNO_3. After that samples were dipped in lactic acid for 2 minutes to become transparent and finally with the help of forceps small portion was taken out and mounted with 10% glycerin on glass-slide for observation.
5. Boiling technique was also attempted with absolute alcohol till the sample becomes colorless followed by lactic acid treatment for becoming transparent and observed under compound microscope.

Determination of stomatal type was made following the scheme of Leaf architecture working Group (1999). Finally Camera Lucida drawings were made using high power objective. Stomatal Index and Stomatal Frequency were determined through the following formula along with proper measurements (Salisbury 1927):

$$\textbf{Stomatal index (SI)} = \frac{\text{Total number of stomata in each field}}{\text{Number of stomata + Number of Epidermal cells in the field}} \times 100$$

$$\textbf{Stomatal frequency} = \frac{\text{Total number of stomata in each field}}{\text{Area of the field in mm}}$$

4.4. ANTIOXIDANT BASED CHEMOTAXONOMIC APPROACH

4.4.1. Plant samples

Antioxidant activities has been carried out with same species i.e. *Cinnamomum bejolghota* (Buchanan–Hamilton) Sweet, *Cinnamomum camphora* (Linnaeus) J. Presl, *Cinnamomum tamala* ((Buchanan–Hamilton) Nees & Ebermaier, *Cinnamomum verum* J. Presl, *Litsea assamica* Hooker *f.*, *Litsea glutinosa* (Loureiro) Robinson, *Litsea laeta* (Nees) Hooker *f.* and *Litsea monopetala* (Roxburgh) Persoon.

4.4.2. Preparation of methanolic plant extracts

Different fresh leaves and barks were surgically separated and were separately crushed with mortar and pestle. Under a soxhlet extractor, crushed samples were individually extracted with methanol for 8h. The methanol was completely removed by vacuum rotary evaporator at 50°C. These crude extracts were freeze-dried. The powder was stored at 4°C and used for further investigation. The extractive value of the plant materials were calculated on dry weight basis from the formula given below:

$$\text{Percent extractive value (yield \%)} = \frac{\text{Weight of dry extract}}{\text{Weight taken for extraction}} \times 100$$

4.4.3. Animal material

Goat liver, used for anti-lipid peroxidation assay, were collected from slaughter house immediately after slay and experiment was conducted within one hour after collection.

4.4.4. Determination of Antioxidant

4.4.4.1. DPPH radical scavenging assay

Radical scavenging activity of plant extracts against stable DPPH (2,2-diphenyl-1- picrylhydrazyl) was determined spectrophotometrically. The changes in colour of DPPH free-radical (from deep-violet to light-yellow) were measured at 517 nm wavelength in presence of antioxidants. Radical scavenging activity of extracts was measured by standard method proposed by Blois (1958). Two microliters of each sample, prepared at various concentrations were added to 2 ml of 0.2 mM DPPH solution. The mixture was shaken and allowed to stand for 30 min at 20ºC in dark condition and then the absorbance was measured at 517 nm with UV-VIS spectrophotometer (Systronics, 2201). The percentage inhibition activity was calculated by the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_{control} - A_{sample}) / A_{control} \times 100]$$

Where, $A_{control}$ is the initial concentration of the stable DPPH radical without the test compound and A_{sample} is the absorbance of the remaining concentration of DPPH in the presence of methanol. IC_{50} values (mg/ml) were determined from a plotted graph of scavenging activity against the concentrations of the extracts, where IC_{50} is defined as the total amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%.

4.4.4.2. Superoxide anions scavenging activity

The superoxide anions generated by phenazine methosulphate (PMN) and reduced nicotinamide-adanine dinucleotide phosphate (NADPH), were detected by the reaction with 2,2´-di-*p*-nitrophenyl-5,5´-diphenyl-(3,3´-dimethoxy-4,4´-diphenylene)di-tetrazoliumchloride, nitroblue tetrazolium (NBT) (Nishikimi *et al*. 1972). Reaction mixture contained 1 ml samples (different concentration), 1 ml of NBT solution (312 µM prepared in phosphate buffer, pH-7.4) and 1 ml of NADH solution (936 µM prepared in phosphate buffer, pH-7.4). Finally, the reaction was accelerated by adding 100 µl PMS solution (120 µM prepared in phosphate buffer, pH -7.4) to the mixture. The reaction mixture was incubated at 25ºC for 5 minutes and absorbance at 560 nm was measured against methanol as control. Percentage inhibition and IC_{50} value was calculated using the same formula mentioned above.

4.4.4.3. Nitric oxide activity

Nitric oxide was generated from sodium nitroprusside and was measured by the Greiss reaction (Marcocci *et al*. 1994). In reaction 320 µl methanolic plant extract, 360µl (5mM) sodium nitroprusside-PBS solution, 216 µl Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% napthylethylene diamine dihydrochloride) was sequentially mixed and incubated at 25ºC for one hour. Lastly 2 ml water was added and absorbance was taken at 546 nm. The IC_{50} value was calculated by the same procedure mentioned above.

4.4.4.4. Metal chelating activity

The chelating activity of the extracts for ferrous ions (Fe^{2+}) was measured according to the method of Dinis *et al.* (1994) with slight modification. To 0.4 ml of methanolic extract, 1.6 ml of methanol was diluted and mixed with 0.04 ml of $FeCl_2$ (2 mM). After 30 seconds, 0.8 ml ferrozine (5 mM) was added. Then, after 10 min at room temperature, the absorbance of the Fe^{2+}-Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated by using the same formula mentioned above.

4.4.4.5. Reducing power

One millilitre of plant extract, 2.5 ml sodium phosphate buffer (0.2 M, pH 6.6), and 2.5 ml potassium ferricyanide (1% w/v) were incubated at 50º C for 20 minutes. The tube was cooled on ice and 2.5 ml 10% trichloroacetic acid was added. The mixture was centrifuged at 3000 rpm for 10 minutes to collect the upper layer of solution (2.5 ml) and mixed with distilled water (2.5 ml) and 0.25 ml of $FeCl_3$ (0.1% w/v). Finally, the absorbance was measured at 700 nm against blank sample (Aiyegoro & Okoh 2009).

4.4.4.6. Anti-lipid peroxidation (ALP) assay

The anti-lipid peroxidation activity of the extracts of plants was determined by the standard method followed by slight modification with the goat liver homogenate (Bauchet & Barrier 1998). 2.8 ml of 10% goat liver homogenate, 0.1ml of 50 mM hydrated ferrous sulphate and 0.1 ml extract was mixed. This mixture was incubated for 30 minutes at 37ºC. 1 ml of reaction mixture was taken with 2 ml 10% trichloroacetic acid (TCA) -0.67% thiobarbituric acid (TBA) in acetic acid (50%) for blocking the reaction. Then the mixture was boiled for 1hour at 100ºC and centrifuged at 10,000 rpm for 5 minutes. Supernatant was taken for absorbance at 535 nm. BHT was used for standard. ALP % was calculated by using the following formula:

$$\text{ALP percent} = \frac{\text{Abs. of } Fe^{2+} \text{ induced peroxidation} - \text{abs. of sample}}{\text{Abs. of } Fe^{2+} \text{ induced peroxidation} - \text{abs. of control}} \times 100$$

4.4.5. Estimation of Phytochemicals

4.4.5.1. Total phenol estimation

Total phenolic compounds of plant extracts were determined by Folin-Ciocalteu method (Folin & Ciocalteu 1927). For the preparation of the calibration curve, 1 ml aliquot of 0.025, 0.05, 0.075, 0.1, 0.2 and 0.3 mg/ml methanolic gallic acid solution was mixed with 5 ml of Folin-Ciocalteu reagent (10 times diluted) and 4 ml sodium carbonate (75 g/L). The absorbance at 765 nm was measured after 1 hour at 20º C and the calibration curve was drawn. 1 ml methanolic fruit extracts (50 mg/ml FWT) was mixed to the same reagent and the mixture was incubated for one hour in room temperature. After 1 hour the absorbance was measured at 765nm.

4.4.5.2. Total flavonoids determination

Spectrophotometric aluminium chloride method was used for flavonoids determination (Sultana *et al*. 2009). Each methanolic leaf and bark extracts (0.5 ml of 100 mg/ml FW) were separately diluted with 4 ml double distilled water. Then the diluted extracts were mixed with 5% (0.3 ml) $NaNO_2$ and 10% aluminium chloride were then added with reaction mixture. After 6 minute 2 ml (1.0 M) NaOH and 2.4 ml double distilled water was added and mixed well. Thereafter, absorbance of the reaction mixture was measured at 510 nm in spectrophotometer. Standard solution of quercetin (0-500 mg L^{-1}) was used as calibration curve.

4.4.6. Phytochemical evaluation of the crude extracts:

The methanolic crude extract (200 mg/ml) of the plant was subjected to various chemical tests in order to determine the secondary metabolites present by employing the use of various methods as follows:

4.4.6.1. Test for resins

0.5ml of extract was evaporated and dissolved in 2ml of petroleum ether; 2ml of 2% cupper acetate solution was then added and the mixture was shaken vigorously and allowed to separate; a green colour indicated the presence of resin (Trease & Evans 1983).

4.4.6.2. Test for amino acid

0.5 ml methanolic plant extracts were treated with few drops of ninhydrin reagent, heated in water bath, a purple colour indicated the presence of amino acids (Kumar *et al*. 2009).

4.4.6.3. Test for anthraquinones

1ml methanolic plant extracts were evaporated and dissolved in 2ml chloroform. 2ml of ammonia was then added. Occurrence of red colour suggested the presence of anthraquinones (Kumar *et al*. 2009).

4.4.6.4. Test for tannin

0.5 ml methanolic extract of each plant part was added with 0.5 ml 1% lead acetate; a yellow colour precipitation indicated the presence of tannin (Kumar *et al*. 2009).

4.4.6.5. Test for triterpenoids

0.5 ml of methanolic plant extracts were evaporated and dissolved in 1ml chloroform. 1ml acetic anhydride was then added and chilled. After cooling, conc. H_2SO_4 was added. If reddish violet colour appeared, the existence of triterpenoids was confirmed (Kumar *et al*. 2009).

4.4.6.6. Test for alkaloids

0.5 ml of each plant extract was added with 0.2 ml of 36.5% hydrochloric acid and 0.2 ml Dragendorff's reagent. Production of orange precipitation denoted the presence of alkaloids (Kumar *et al*. 2009).

4.4.6.7. Test for glycosides

0.5 ml methanolic extracts of plant were added with 2 ml of 50% hydrochloric acid. The mixtures were hydrolyzed for 2 hrs on a water bath. After that, 1 ml pyridine, few drops of 1% sodium nitroprusside solution, and 5% sodium hydroxide solution were added. Pink to red colour designated the presence of glycosides (Kumar *et al*. 2009).

4.4.6.8. Test for steroid

0.5 ml methanolic extracts were evaporated and dissolved in 2 ml chloroform. 2ml of conc. H_2SO_4 was introduced carefully by the side wall of the test tube. Formation of red colour ring confirmed the presence of steroid (Kumar *et al*. 2009).

4.4.6.9. Test for cardiac glycoside

0.5 ml of methanolic plant extracts were evaporated and dissolved in 1 ml glacial acetic acid. One drop of 10% ferric chloride was then added. 1 ml of conc. H_2SO_4 was added by the side of the test tube. Appearance of brown colour ring at the interface indicated of presence of cardiac glycosides (Ngbede *et al*. 2008).

4.5. CHEMOTAXONOMY THROUGH ANTIOXIDAT ACTIVITY OF ESSENTIAL OIL

4.5.1. Plant samples

Antioxidant activities of the essential oil has been carried out with same species i.e. *Cinnamomum bejolghota* (Buchanan–Hamilton) Sweet, *Cinnamomum camphora* (Linnaeus) J. Presl, *Cinnamomum tamala* ((Buchanan–Hamilton) Nees & Ebermaier, *Cinnamomum verum* J. Presl, *Litsea assamica* Hooker *f.*, *Litsea glutinosa* (Loureiro) Robinson, *Litsea laeta* (Nees) Hooker *f.* and *Litsea monopetala* (Roxburgh) Persoon.

4.5.2. Extraction of essential oils

The barks of each Laurals (200 g) were placed in a round-bottom flask with 1 litre of deionised water. The solution was steam distilled at 55°C for 3 hrs under reduced pressure. The distillate (900 ml) was extracted with 100 ml of dichloromethane for 6 hrs. After that the extract was dried with anhydrous sodium sulphate. The distillation was stopped when the volume of extract was reduced to approximately 1 ml, and then the solvent was further removed under a purified nitrogen stream until the volume was reduced to 0.2 ml (Lee & Shibamoto 2000)

The extractive value of the plant materials were calculated on dry weight basis from the formula given below:

$$\text{Percent extractive value (yield \%)} = \frac{\text{Weight of dry extract}}{\text{Weight taken for extraction}} \times 100$$

4.6. TLC BASED CHEMOTAXONOMIC APPROACH

4.6.1. Plant samples

Thin layer chromatography has been carried out with same species i.e. *Cinnamomum bejolghota* (Buchanan–Hamilton) Sweet, *Cinnamomum camphora* (Linnaeus) J. Presl, *Cinnamomum tamala* ((Buchanan–Hamilton) Nees & Ebermaier, *Cinnamomum verum* J. Presl, *Litsea assamica* Hooker *f.*, *Litsea glutinosa* (Loureiro) Robinson, *Litsea laeta* (Nees) Hooker *f.* and *Litsea monopetala* (Roxburgh) Persoon.

4.6.2. Extraction of the plant samples

For extraction of different secondary metabolites bark of eight Laurels were used. Bark specimens were surgically separated from plants, washed thoroughly and one gm of each bark material was weighted separately and extracted with 5ml of methanol for 10 min. Methanolic filtrate was concentrated through vacuum rotary evaporator for application on TLC plates.

4.6.3. Extraction of essential oil

Essential oil was extracted by using the method proposed by Lee and Shibamoto (2000). Bark of eight Laurels (200 g) was placed in a 3 litre round-bottom flask with 1 litre of distilled water. The solution was steam distilled at 55 ºC for 8 hrs. Then, 900 ml distillate was fractionated with 100 ml of dichloromethane for 6 h. After the dichloromethane extract was dried over anhydrous sodium sulphate, the solvent was removed until the volume was reduced to 2 ml.

4.6.4. Thin layer chromatography (TLC)

Methanolic bark extracts of each Laurals were subjected to qualitative phytochemical detection as well as DPPH based antioxidant fingerprint through TLC (Wagner *et al.* 1984). It was performed by using silica gel-60 F_{254} chromatographic plates of 8cm x 2cm with 3mm thickness to confirm the presence of secondary metabolites. For the separation of phytochemical compounds, the methanolic bark extracts were spotted manually using micro-pipette. 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 specific for detection of special class of secondary metabolites were used to identify the compounds (Wagner & Bladt 1996). The colour of the spots was noted and hR_f values were calculated by using the following formula:

$$\text{Retention factor } (hR_f) = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled the solvent}} \times 100$$

4.7. STATISTICAL ANALYSIS

The data were pooled in triplicate and subjected to analysis of correlation co-efficient matrix using SPSS (Version 12.00, SPSS Inc., Chicago, IL, USA) for drawing the relation between different types of antioxidant attributes and MS Excel 2007 (Microsoft, Redmond, WA, USA) was used for comparing the antioxidant attributes. Different group means were compared by Duncan's Multiple Range Test (DMRT) through DSAASTAT software (version 1.002; DSAASTAT, Peruglia, Italy); $p < 0.05$ was considered significant in all cases. The software package Statistica (Statsoft Inc., Tulsa, OK, USA) was used for analysis of other data. Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) was used for determining the IC_{50} values of antioxidants and their standard error of estimates (SEE). Thin layer chromatography hR_f values were done through MS-Excel. In order to examine and visualize relationships between different plants in accordance with the presence of phytochemicals and antioxidant capacity, dendrogram were drawn by XLSTAT 2009 software (Levei *et al.* 2013).