MATERIAL AND METHODS

Plant material and authentication

Aganosma dichotoma roots were collected from Tumbura Kona Kshetram at Seshachalam hills and Tirumala hills, Chittoor District, Andhra Pradesh, South India in the month of April 2013. It was authenticated by Dr. K. Madhava Chetty, Taxonomist, S. V. University, Tirupati. A voucher specimen (COG/AD/17) has been retained in Department of Pharmaceutics, Indian Institute of Technology - BHU, Varanasi, India for further reference.

Macroscopic, microscopic and powder study

In the present investigation, the macroscopical evaluation of the root was done by observing them with reference to their color, shape, size, odor and taste etc. For microscopical examination, the root was cleaned and fixed in formalin, acetic acid and 70% ethanol mixture (Formalin 5 mL + Acetic acid 5mL + 70% Ethyl alcohol 90 mL) for 24 h prior to experimentation. Further, according to Sass (1940), the specimen was dehydrated with graded series of tertiary- butyl alcohol (TBA) followed by infiltration of the specimen by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super saturation. Then specimen was caste into paraffin blocks which were further sectioned with the help of Rotary Microtome (York Scientific Industries Pvt. Ltd.) at a thickness of 10-12 µm followed by dewaxing (Johansen, 1940). Finally, the sections were stained with toluidine blue, and phloroglucinol & HCl solution (1:1) and sections were photographed with a Nikon trinocular microscopic unit, Model E-200, Japan. For powder microscopy, fine powder of root was cleared with chloral hydrate and stained with phloroglucinol and

conc. HCl solution (1:1) and mounted with glycerin and for the study of individual cells, pieces of roots were macerated with the mixture of concentrated nitric acid and potassium chlorate which was later washed with distilled water and finally mounted in glycerin for observation. Isolated cell elements were measured and all the measured values were expressed in terms of µm (minimum–mean–maximum; length × width).

Physicochemical evaluations

The root of the plant was dried and powdered and various physicochemical constants were evaluated as described in WHO guidelines (WHO, 2002) and Indian Herbal Pharmacopoeia (IHP 2002).

Foreign matter

Dried root was spread evenly on a white paper and the presence of any foreign matter like sand, clay and other particles etc. was resoluted manually by using the magnifying lens. The foreign matter in plant material was calculated as percentage weight/weight (% w/w).

Total ash

For the determination of total ash about 2-4 *g* of the air-dried coarsely powdered root was taken in a tarred silica dish and incinerated at a temperature not exceeding 450°C until free from carbon by using Muffle Furnace (NSW-101). The silica dish bearing the total ash was cooled and weighed and percentage (w/w) of ash with reference to air dried drug was then calculated.

Acid-insoluble ash

Obtained amount of total ash by incinerating powdered drug material at a temperature, not beyond 450°C was weighed and boiled with 25 mL of hydrochloric acid (70 *g*/L) for 5 min. After that solution was filtered through an ash-less filter paper and later on washed with hot water to become neutral. Further, the filter paper bearing the insoluble matter was dried, ignited and later cooled in a desiccator and weighed. The percentage acid insoluble ash (w/w) with reference to air dried drug was calculated.

Water-soluble ash

Obtained amount of total ash by incinerating powdered drug material at a temperature not exceeding 450°C was boiled with 25 mL of water for 5 min. Followed by solution was then filtered and insoluble matter was collected on ash-less filter paper, washed with hot water and ignited for 15 min at a temperature not exceeding 450°C. The weight of the insoluble matter was subtracted from the weight of the total ash and the difference in weight represents the water soluble ash and the percentage (w/w) of water soluble ash was calculated.

Extractive values

About 5 *g* of the coarsely powdered plant material was taken in a glass stopper conical flask and macerated with 100 mL of different solvents (petroleum ether, chloroform, ethyl acetate, methanol, and water respectively) for 24h. The mixture was shaken frequently for the first 6h and then was allowed to stand for 18h. After 24h, the mixture was filtered and from the final volume, 25 mL of the filtrate was taken in a tarred flat bottom previously weighed glass dish. It was then evaporated to

dryness, at 105°C and was finally weighed and percentage (w/w) of extractive value with respect to respective solvents was then calculated.

Loss on Drying

About 1 *g* of air-dried powdered root drug was taken in a glass-Stoppard, shallow weighing bottle that was previously dried and weighed. The root powder was evenly distributed by gentle sidewise shaking to a depth not exceeding 10 mm and was dried to constant weight at a temperature not exceeding 105°C. After attaining constant weight, the bottle was cooled by keeping in the desiccator, weighed and the percentage (w/w) loss on drying was determined.

Foaming Index

Determination of foaming index was done as per method depicted in WHO guidelines. Accurately weighed root powder (1 *g*) was transferred into a 500 mL conical flask containing 100 mL of water which was boiled for 30 min. After boiling, the decoction was cooled and filtered into a 100 mL volumetric flask and the volume was made up to the mark. The filtrate was then transferred into 10 Stoppard test tubes (height 16 cm, diameter 16 mm) in successive portions of 1 mL, 2 mL, 3 mL up to 10 mL, and the volume was adjusted with water up to 10 mL. All the test tube was shaken in a lengthwise direction for 15 sec with two shakes per sec and was then allowed to stand for 15 min. The height of the foam was measured following the condition given below.

- If the height of the foam in each tube is less than 1 cm, the foaming index is taken to be less than 100.

- If in any of tube from 1 to 10, the height of foam of 1 cm is measured, then the volume of the decoction in this tube is used to determine the index and is marked as 'a' in the formulae given below.

- If this tube is the first or second tube in a series, then prepare an intermediate dilution in a similar manner to obtain a more precise result.

- If the height of the foam is more than 1 cm in every tube, the foaming index is over 1000. In this case, repeat the determination using a new series of dilutions of the decoction in order to obtain a result.

The foaming index was calculated using the following formula:

$$\text{Foaming Index} = 1000/a$$

Here 'a' is the volume in mL of the decoction used for preparing the dilutions in the tube where foaming to a height of 1 cm was observed.

Swelling Index

Accurately, 1 *g* of the root powder was taken in a 25 mL glass Stoppard measuring cylinder (16 mm internal diameter and 125 mm length) and 25 mL of water was added and the mixture was thoroughly shaken after every 10 min interval for 1h and was then allowed to stand for 3h at room temperature. The final volume (in mL) occupied by the plant material including any sticky mucilage, was measured and the mean value of the individual determinations, related to 1 *g* of plant material was calculated.

Hemolytic activity

The hemolytic activity of the plant material was done as per the method described in WHO guidelines, where diosgenin was taken as a positive control. A suspension of

erythrocytes obtained from ox blood was mixed with equal volumes of a serial dilution of the plant root extract. The lowest concentration producing complete hemolysis was taken as the hemolytic index.

Method:

The experimental method consists of two steps which include preliminary testing and the main hemolytic testing. The erythrocyte suspension was generated by mixing $1/10^{th}$ of its volume with sodium citrate (36.5 *g*/L) and 1 mL of this generated citrated blood was mixed with phosphate buffer (pH 7.4) in a 50 mL volumetric flask to obtain a 2% (v/v) solution. Further, reference solution was freshly prepared by dissolving 10 mg of diosgenin in phosphate buffer (pH 7.4) and the volume was made up to 100 mL with the buffer. After that serial dilution of the plant root extract was prepared in phosphate buffer pH 7.4. Followed by blood suspension and different dilutions of the extract and standard diosgenin were mixed appropriately and were allowed to stand for 6h at room temperature. All the tubes were vigilantly monitored and examined for hemolysis. The minimum concentration of plant root extract and diosgenin that produces hemolysis was noted and the hemolytic activity of the plant material was calculated by using the formula:

$$\text{Hemolytic index} = [(1000) \times (a/b)]$$

Where, 1000 is the defined hemolytic activity of diosgenin in relation to ox blood.

'a' is the quantity of diosgenin that produces total hemolysis (*g*).

'b' is the quantity of plant extract that produces total hemolysis (*g*).

Determination of crude fiber content (Dutch method)

Crude fiber is the residue of resistant tissue which can be obtained after giving treatment to the plant material with dilute acid and alkali. It was determined by treating 2 *g* of powdered root with 50 mL of 10% nitric acid and heated to boiling with constant stirring. The entire matter was strained through a fine muslin cloth on a Buckner funnel and obtained residue was washed with boiling water and it was further treated with 50 mL 2.5% w/v sodium hydroxide solution and heated to boiling. The content was again strained, dried and weighed, finally percentage (w/w) of crude fibers was calculated (Khandelwal, 2007).

Determination of starch grains content (Lycopodium spore method)

According to Wallis (1965), a total number of starch grains in plant material were determined by lycopodium spore method. The total amount of starch grains and lycopodium spore was counted in 25 fields by using a pre-calibrated microscope with 10× magnification. The amount of starch grain obtained was calculated and expressed in terms of a number of starch grains per milligram of powder using following formula:

$$P = \frac{N \times W \times 94{,}000}{S \times M}$$

Where

N = Weight of sample taken

M = No of characteristic structure (e.g. starch grains) in 25 fields.

W= Weight in mg of Lycopodium taken

S = No of Lycopodium spores in same 25 fields.

P = No. of starch grains per mg of drug.

Fluorescence powder drug analysis

Fluorescence analysis of powdered drug plays a key role in detection and differentiation of one powdered drug with another like for examples detection of ergot in flour, cocoa shells in powder cocoa and vice versa. This analysis was carried out according to the method of Kokoski (1958). The fluorescence pattern of the solution mixture was visualized under day light as well as under short and long ultra violet light (254 nm and 365 nm).

Pesticide residue

The pesticide residue of the roots was carried out according to WHO guideline. Pesticide content was determined by adding a mixture of 350 mL of acetonitrile: water (65: 35) to the 50 *g* of grinded root powder which was blended at high speed for 5 min followed by filtration. The filtrate (250 mL) was then transferred to a separating funnel to which further 100 mL light petroleum, 10 mL of sodium chloride (40%) and 600 mL of water were added with constant shaking up to 35-45 seconds. Solvent layer was separated and an aqueous layer was discarded from it then later was washed twice with 100 mL portions of water to which 15 *g* of anhydrous sodium sulfate was added with vigorous shaking. The extract was separated, and its volume was reduced to 5 to 10 mL, which was allowed to pass through a column packed with Florisil R grade 60/100 PR, activated at 650°C at a rate of not more than 5 mL/min. Three elutes were obtained after running the column with three different ratios of ether: light petroleum mixture as mobile phase i.e. Elute 1 contained 6% of ether

while elute 2 and 3 contained 15% and 50% of ether. The obtained elutes were evaporated to dryness, transferred to a sample holder, and burned in a suitable combustion flask flushed with oxygen. The gasses produced gets absorbed in a suitable solution in the combustion flask (water for chloride and H_2SO_4 in case of phosphate pesticides). For determining the chloride pesticides 15 mL of the solution obtained after combustion was mixed with 1 mL of ferric ammonium sulfate (0.25 mol/L) and 3 mL of mercuric thiocyanate followed by swirling it where absorbance was measured at 460 nm. In case of phosphate pesticides, 7 mL of the solution obtained after combustion was mixed with 2.2 mL of sulfuric acid (300 *g*/L), 0.4 mL of ammonium molybdate (40 *g*/L) and 0.4 mL of aminonaphtholsulfonic acid followed by swirling it and heating it at 100°C for 12 min which was then measured at 820 nm.

Preliminary phytochemical screening

Preliminary phytochemical screening of ethanolic root extract of *A. dichotoma* as well as its successive fractions was subjected to preliminary phytochemical tests to check the presence of various phytochemical classes (Trease and Evans, 2002; Khandelwal, 2007):

Test for alkaloids

Mayer's test

Test solution when treated with Mayer's reagent results in the formation of cream colored precipitate ensuring the presence of alkaloids.

Dragendroff's test

Required volume of the test solution when, treated with Dragendroff's reagent leads to the formation of orange to reddish colored precipitate confirming the presence of alkaloids.

Wagner's test

Test solution (extracts) when treated with Wagner's reagent, formation of reddish brown colored precipitate ensures the presence of alkaloids

Test for phytosterols/steroids

Libermann Burchard test

To the test solution (extract), 3 mL of acetic anhydride was added. To this solution, two drops of concentrated sulphuric acid were added slowly along the side of the test tube. Appearance of bluish green color confirms the presence of phytosterols/steroids.

Test for anthraquinones glycosides

Borntrager's test

In this test, the extract was first shaken gently with equal volume of chloroform and then the chloroform layer was separated. To this layer dilute ammonium solution was added and if ammonia layer acquires pink color, it confirms the presence of anthraquinone glycosides.

Modified Borntrager's test

To the extract, 5% ferric chloride solution and dilute hydrochloric acid was added, heated on boiling water bath for 5 min to bring about oxidative hydrolysis and was shaken gently by addition of benzene. The benzene layer was further separated and to this, dilute ammonia solution was added. Formation of pink color indicates the presence of anthraquinone glycosides.

Test for cardiac glycoside

Legal's test

To the test solution, 1 mL of pyridine and a few drops of sodium nitroprusside were added and were made alkaline with NaOH solution. Appearance of pink color is an indicative of presence of cardiac glycosides.

Test for coumarins glycoside

To the test solution (extract), 10% dilute NaOH was added and the mixture was monitored under UV light (366 nm). Appearance of blue color fluorescence confirms the presence of coumarin glycoside.

Test for cyanogenetic glycosides

Sodium picrate test

Sodium picrate paper turns brick red in color when comes in contact with the extract containing cyanogenetic glycosides. Sodium picrate paper is prepared by soaking filter paper first with 10% picric acid and then with 10% sodium carbonate.

Test for Phenols

To the test solution (extract dissolved in methanol), few drops of freshly prepared solution of 5% ferric chloride was added. Formation of a blue color indicates the presence of phenol.

Test for tannins

To the test solution (extract dissolved in methanol), 0.5 mL of 10% lead acetate was added. The appearance of white precipitate indicates the presence of tannins.

Test for flavonoids

To the test solution (extract dissolved in methanol), required amount of magnesium turnings and a few drops of concentrated hydrochloric acid were added. Formation of pink color indicates the presence of flavonoids.

Test for saponins

To the extract, 1 mL of distilled water was added and shaken vigorously. Formation of persistent foam/froth up to 10 min or more indicates the presence of saponins.

Test for carbohydrates

Molisch's test

In this test, the extract was made to react with 0.5 mL of α–naphthol solution. The mixture was vortexed and two drops of concentrated sulfuric acid was added from the side of the test tube. Formation of purple ring at the junction of two liquid showed the presence of carbohydrate.

Test for proteins

Biuret test

Test solution was treated with 40% sodium hydroxide and with dilute copper sulfate solution. Appearance of blue color indicates presence of proteins.

Xanthoproteic test

The test solution was treated with conc. HNO_3 and boiled for few minutes. Appearance of yellow precipitate indicates presence of proteins.

Test for amino acids

To the known volume of test solution, 2 mL of ninhydrin solution was added and the solution was heated. Formation of violet color indicates the presence of amino acids.

Quantification of phytoconstituents

Estimation of total phenolic content

Total phenolic content in ethanolic root extract of *A. dichotoma* was carried out by Folin–Ciocalteu method (Makkar, 2000). Accurately weigh 100 mg of plant extract and dissolved in 100 mL of methanol to make the stock solution. In a test tube aliquots of varying concentration of the extract were taken and volume was made up to 1 mL with distilled water. 0.5 mL of Folin-Ciocalteu reagent (1:1 with water) and 2.5 mL of sodium carbonate solution (20%) were added sequentially to each tube. Then the tubes were vortexed, placed in the dark for 40 min and the absorbance was recorded at 725 nm. The amount of total phenolics was calculated as tannic acid equivalents from the calibration curve.

Estimation of total tannin content

Tannins are separated from rest of the mixture by adsorption on insoluble matrix polyvinylpolypyrrolidone (PVPP), and the total tannin content was determined by Folin Ciocalteu procedure (mentioned as above). Insoluble, cross-linked PVPP (100 mg) was taken in test tubes and 1.0 mL distilled water and 1 mL tannin containing extract was added. Tubes were maintained at 4°C for 15 min, then subsequently vortexed and centrifuged for 10 min and the supernatant was collected. Aliquots of supernatant (0.2 mL) were transferred into test tubes, and then non-absorbed phenolics were determined. Finally observed values were subtracted from total polyphenol contents, and the total tannin content is expressed as; mg Tannic acid/100 *g* dry plant material. All measurements were done in triplicate (Hagerman *et al.*, 2000).

Estimation of total flavonoid content

The determination of the total flavonoid content was based on the aluminum chloride method by using rutin as the standard reference (Kumaran *et al*., 2006). The method for the estimation of flavonoid content is based on the formation of a flavonoid-aluminium complex (λmax 415 nm). In this method, 100 µL of extract in methanol (10 mg/mL) was mixed with 100 µL of 20 % aluminium trichloride in methanol, followed by addition of few drops of acetic acid, which was then diluted with methanol to 5 mL. The absorption at 415 nm was taken after 40 min. Blank samples were prepared by adding 100 µl of plant extracts and a drop of acetic acid, which was then diluted to 5 mL with methanol. The absorption of standard rutin solution (0.5 mg/mL) in methanol was measured under the same conditions. The amount of flavonoids in the sample was expressed as mg/*g* rutin equivalent, which was calculated by the following formula:

$$X = (A.\ m_o)/(A_o\ .\ m)$$

Where X is the flavonoid content, mg/mg sample in Rutin Equivalent, A is the absorbance of the sample solution, Ao is the absorbance of the standard rutin solution, m is the weight of sample in mg and m_a is the weight of rutin in the solution in mg.

Estimation of total flavonol contents

The estimation of total flavonol content was carried out according to Kumaran *et al*., 2006. 1 mL of plant extract (10 mg/mL) was mixed with 1 mL 20% aluminum trichloride and 3 mL 5% sodium acetate solution and absorbance of the solution was measured at 440 nm after 2.5h by using methanol as blank. The absorption of

standard rutin solution (0.5 mg/mL) in methanol was measured under the same conditions. The total flavonol content was expressed in mg rutin equivalent/mg plant extract.

Estimation of total alkaloid content

The total alkaloid content was estimated by gravimetric methods (Wagner and Bladt, 1996). About 5 *g* of the root powder was extracted repeatedly with 0.1N H_2SO_4 in an ultrasonic bath (3 x 50 mL). Then solution was filtered and acidic solution was washed with 4 successive quantities of 25 mL chloroform (washing each chloroform solution with 20 mL of acid). The chloroform washings was rejected, acid solution was basified with dilute ammonia solution and was further extracted with (5 x 20 mL) diethyl ether. The combined diethyl ether extracts were washed with 5 mL of distilled water and the ether was evaporated to dryness in a weighed beaker on a water bath, which was further dried to constant weight at 105°C and the % w/w of alkaloid was determined.

Estimation of total saponin content

The total saponin content was estimated as per the method described by Baccou *et al*., (1977). Accurately weigh 0.5 *g* of the defatted material and kept in plastic centrifuge tubes containing 10 mL of absolute methanol. The mixture was kept for overnight in a magnetic stirrer and the content was centrifuged and 25 mL of supernatant was collected in measuring flask. The rest out residue was again washed with 5 mL of fresh methanol twice and again centrifuged. The overall supernatant was collected and the final volume was made up to 25 mL with methanol. Standard diosgenin solution was freshly prepared by dissolving 10 mg diosgenin in 20 mL methanol and

its standard curve was prepared using different dilutions in the concentration ranging between 25-125 µg/mL. After that 0.25 mL extract solution was transferred to the test tubes placed in ice water bath and vanillin reagent (0.25 mL) was added to all the test tube and was kept on a magnetic stirrer. Then 2.5 mL (75 % v/v) of sulfuric acid with proper stirring was added slowly and the whole mixture was warmed in a water bath at 60°C for 10 min which was later cooled in ice-cold water. The absorbance of the solution mixture was measured at 544 nm after cooling and methanol was used as a blank. The results were calculated using the standard regression equation of saponins and the results were expressed as mg/*g* diosgenin equivalent.

Preparation of extract and fractions

After shade drying, the dried roots (1 Kg) were coarsely ground into homogenous powder using a mechanical grinder, passed through a 60 mesh sieve and exhaustively extracted with 95% ethanol (3 L) in a Soxhlet apparatus for 72h. Further, the extract was filtered and concentrated by evaporation using Rota evaporator (IKA Germany) to obtain the crude extract (% yield are 11.82% w/w) which were kept in a desiccator until use. For fractionation, the ethanol extract of *A. dichotoma* (EAD) was made hydro-alcoholic and then subjected to successive fractionation using solvents of increasing polarity such as petroleum ether (PF), chloroform (CF), ethyl acetate (EAF) and n-butanol fraction (BF). The fractions were concentrated under reduced pressure in a rotary evaporator and were then kept in a desiccator until use. The yield of the fractions reported were PF- 9.49% (w/w), CF- 3.05% (w/w), EAF- 1.17% (w/w) and BF- 16.13% (w/w).

Isolation and characterization

Based on maximum % yield about 9 *g* of petroleum ether fraction from *A. dichotoma* (EAD) was subjected to column chromatography on silica gel and eluted with 100% hexane then with increasing amount of ethyl acetate. Total 20 fractions with the volume of 500 mL were eluted with hexane: ethyl acetate (9:1) was collected and pooled and concentrated under vacuum evaporator to minimum volume a yellowish green oily residue was obtained and TLC chromatogram showed 6-7 spots. This oily residue was processed for esterification and subjected to Gas Chromatography-Flame ionization detection (GC-FID) analysis thereby yielding six compounds PEF-3, PEF-4, PEF-5, PEF-6, PEF-7, and PEF-8.

Isolation of ursolic acid and β-sitosterol

Fraction 24 to 35 of Hexane-Ethyl acetate (9:1) was again eluted with chloroform with increasing order of methanol. Fraction 1-12 from chloroform-methanol (8:2) (400 mL) were collected which showed similar TLC chromatogram (1 major spot with slight impurity) and were pooled together. The pooled fractions were concentrated under vacuum evaporator to minimum volume and were kept undisturbed for two days which later yielded the slightly yellowish powder i.e. compound PEF-1. Fraction 13-20 were also collected and pooled together which yielded a white powder of PEF-2. The compound was purified by multiple washing with chloroform to remove the impurity which was later subjected for re-crystallization in methanol. The final product obtained after re-crystallization was subjected for characterization by various sophisticated H^1NMR, C^{13}NMR and Mass spectrophotometry.

GC-FID analysis of the oil sample

Preparation of fatty acid methyl ester (FAME)

The fatty acid methyl ester was prepared according to the method anticipated by Griftin (1960). About 5 mg of oil (PEF 1-20) was taken in a reaction tube and 5 mL of the reagent mixture (BF_3: CH_3OH) was added and then boiled for 5 min. The mixture was again boiled for 1 min with 5 mL hexane and later cooled. A solution of saturated salt was added to the tubes and the mixture was vortexed. The upper layer containing methyl esters was transferred to a vial containing anhydrous sodium sulfate. The ester was filtered through a syringe filter and transferred to a small vial (2 mL). The fatty acid methyl esters were identified by gas chromatography (Chemito 8610) method using DB 23 Capillary Column (30m x 0.25 mm) and flame ionization detector. Nitrogen gas was used as the carrier gas at a flow rate of 1 mL/min. The detector and injector temperatures were kept constant at 245°C. The oven temperature was started from 75°C and heated up to 245°C with a heating rate of 10°C/min. Each fatty acid methyl ester (FAME) in the oil sample was identified by comparing retention times with the standard FAME available commercially.

Chemical standardization of Extract by *High Performance Thin Layer* Chromatography

HPTLC fingerprinting analysis of EAD, PF and CF

EAD, PF, and CF were dissolved in chromatographic grade methanol (1 mL) and solvent system was optimized to achieve good fingerprinting. A single solvent system consisting of Toluene: Ethyl acetae: Formic acid (7:3:0.5 v/v/v) has been used in this method to resolve and to quantitate all the characterized compounds. Sample

application was done and plate was run in above given solvent system then chromatogram was developed at 350 nm after that plate was derivatized by using anisaldehyde-sulfuric acid reagent followed by development of chromatogram which was scanned by densitometer and R_f value and fingerprint data were recorded by WINCATS software. Presence of kaempferol, quercetin, ursolic acid, β-sitosterol and lupeol in extract and fractions was confirmed with the help of R_f value and spectral comparison by using standards and were quantified in EAD, PF, and CF.

Quantification of kaempferol, quercetin, ursolic acid β-sitosterol and lupeol in EAD, PF and CF by HPTLC

The ethanolic root extract of *A. dichotoma* (EAD) was standardized with kaempferol, quercetin, ursolic acid, β-sitosterol, and lupeol by using high performance thin layer chromatography (HPTLC). A stock solution of EAD (10 mg/mL), kaempferol (1mg/mL), quercetin (1mg/mL), ursolic acid (1 mg/mL), β-sitosterol (1mg/mL), and lupeol (1 mg/mL) was prepared in methanol. The mobile phase for developing the chromatogram consisted of Toluene, ethyl acetate, and formic acid mixture in the ratio 7:3:0.1 (v/v/v). The study was carried out using Camag-HPTLC instrumentation (Camag, Mutten, Switzerland) equipped with Linomat V sample applicator, Camag TLC scanner 3, Camag TLC visualizer and WINCATS 4 software for data interpretation.

***In-vitro* antioxidant activity**

Free radical scavenging activity

The free radical scavenging activity of the plant root extract was measured by the DPPH (1, 1–diphenyl–2–picryl–hydrazil) assay method according to Blois (1958). In

this method, 100 µM/mL solution of DPPH in methanol was prepared and 5 mL of this solution was added to 1 mL of root extract solution in different concentrations (25–200 µg/ml). Then absorbance was measured after 30 min at 517 nm. The free radical scavenging activity was calculated by using the following equation:

DPPH scavenging activity (% Inhibition) = $[(1–A_1/A_0)] \times 100$……. (1)

Where, A_0 is the absorbance of the blank solution.

A_1 is the absorbance of the test sample.

Further, percentage inhibition was plotted against the respective concentrations and IC_{50} was calculated from the regression equation by using ascorbic acid as a positive control.

Total antioxidant capacity

The total antioxidant capacity of plant extract was done by Phosphomolybdenum method according to Prieto *et al*., (1999). This assay was based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. 0.3 mL of plant extract in methanol (1mg/mL) and aliquots of serial dilutions of ascorbic acid (25-300 µg/mL) were separately mixed with 3 mL of reagent mixture (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). A blank solution was prepared from 3 mL of reagent solution and 0.3 mL of methanol. All tubes were capped and incubated in a boiling water bath at 95ºC for 90 min. After cooling to room temperature, the absorbance of each sample was measured at 695 nm against the blank. The antioxidant activity was expressed as the number of equivalent of ascorbic acid.

Scavenging of hydroxyl radical by deoxyribose method

Hydroxyl radical scavenging activity of plant extract was measured by degradation of deoxyribose according to the method of Halliwell *et al*, (1987). 1.2 mL of the final reaction solution consisting the aliquots (500 µl) of various concentrations of the extract, ferric chloride (0.1 mM, 0.2 mL), EDTA (0.1 mM, 0.2 mL), and hydrogen peroxide (2 mM, 0.2 mL) in phosphate buffer (pH, 7.4, 20 mM) were added and incubated for 1h at 37ºC. After incubation, ice cold trichloroacetic acid (0.2 mL, 15%, w/v) and thiobarbituric acid (0.2 mL, 1%, w/v) in 0.25N HCl were added and heated in a boiling water bath for 15 min and cooled. Then color development was measured at 532 nm against a blank containing phosphate buffer. The butylated hydroxyl anisole (BHA) was used as a standard.

Nitric oxide scavenging assay

Nitric oxide scavenging assay was determined as described by Sreejayan and Rao, (1997). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated by the use of the Griess Illosvoy reaction. In this method, 0.5 mL of extract at various concentrations was taken in a test tube and 2 mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) was added then the mixture was incubated at 25ºC for 150 min. 0.5 mL solution was taken from the incubated mixture and added into 1.0 mL sulfanilic acid reagent (33% in 20% glacial acetic acid), which was incubated at room temperature for 5 min. Finally, 1.0 mL naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and again incubated at room temperature for 30 min. The absorbance was measured at 540 nm and the

nitric oxide radical scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = ((A_0\text{-}A_1) / A_0 \times 100)$$

Where, A_0 is the absorbance of the blank.

A_1 is the absorbance of the extract.

Further, % inhibition was plotted against the respective concentrations used and IC_{50} was calculated by using rutin, as a positive control.

Assay of reducing power

According to Yildrim *et al*., (2001) Potassium ferricyanide method was used for the estimation of reducing power of plant extract test sample. The plant extract of various concentration (1 mL) was added with 2.5 mL phosphate buffer (0.2M, pH 6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN_6)$] (10 *g*/l). Then the mixture was incubated at 50ºC for 20 minutes after that 2.5 mL of trichloroacetic acid (100 *g*/l) was added to the mixture. Then mixture was centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of the supernatant solution was taken and mixed with 2.5 mL of distilled water and 0.5 mL $Fecl_3$ (1 *g*/l) and absorbance was measured at 700 nm. Ascorbic acid was used as a standard and phosphate buffer was used as a blank solution. Higher absorbance of the reaction mixture indicates stronger reducing power.

Scavenging of hydrogen peroxide

Scavenging activity of plant extract for hydrogen peroxide radicals was evaluated by the method described by Jayaprakasha *et al*., (2004). A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Here, 1 mL of various concentrations of the extract and rutin standard in methanol were added to 2

mL of hydrogen peroxide solution in PBS. Finally, the absorbance was measured at 230 nm after 10 min and all estimation were performed in triplicates and the percentage inhibition was calculated.

RESULTS

Morphological characters

Dried roots are cylindrical with slightly tapered in shape, 6-10 cm long and 0.5-1.5 cm in width. Outer surface of root is dark reddish brown, much shriveled and wrinkled longitudinally while internally root is buff to light yellow in color. Outer layer is easily exfoliated, separating from the wood in large, papery flakes or strips (Figure 6). It has no perceptible odor and taste.

Figure 6: *Aganosma dichotoma* K. Schum root

Microscopical characters and Powder study

Transverse section of *A. dichotoma* root shows exfoliating wavy cork, consisting of 3-4 layered brick shaped cork cells filled with tannins (Phellem). Next to phellem, phelloderm is present, made up of 8-10 layered parenchymatous wide cells. Single layered pericyclic sclerenchymatous cells are situated in between the phellem and phelloderm. Periderm is followed by secondary cortex which is made up of 10-12 layered thin walled parenchymatous cells. A number of parenchymatous cells of secondary cortex contain starch grains, which are simple, round to oval in shape but the hilum and striation are indistinct. Solitary calcium oxalate crystals are also found

in cortex region. Anomalous structures are reported, the formation of xylem and phloem is irregular, which shows furrowed xylem and phloem is situated in furrows. The secondary xylem consists of narrow vessels and vasicentric tracheids, both having simple pits. The medullary rays become more deep and monoseriate in the section. Additional arcs of phloem more deeply seated in the pith known as intraxylary phloem which is the main characteristic of *Aganosma* (*Echites*) genus. Pith is frequently containing sclerosed elements (Figure 7).

The macerated powdered characteristics of *A. dichotoma* roots were expressed in terms of µm (minimum-meanmaximum; length x width) and showed the presence of a large number of fibres having slender shape and tapering ends with septa (131.4–158.45-183.55 x 3.6-5.4-7.2 µm), tracheids are pitted thickening with tapering ends, measuring (102.90-126.35-149.80 x 4.7-6.5-8.3 µm), xylem vessels of varying size and shape measuring (58.23-72.79- 97.85 x 17.90-24.67 28.90 µm) and have pitted thickenings. Group of cork cells and lignified sclerenchymatous cells are appeared in the powder microscopy. Parenchymatous cells were also visible which are round, oval and elongate in structure having size varying between (24.62-31.81-67.42 x 15.90-24.63-37.67). Solitary calcium oxalate crystals with varying size and shape (25.48-35.67-36.18) are also present (Figure 8).

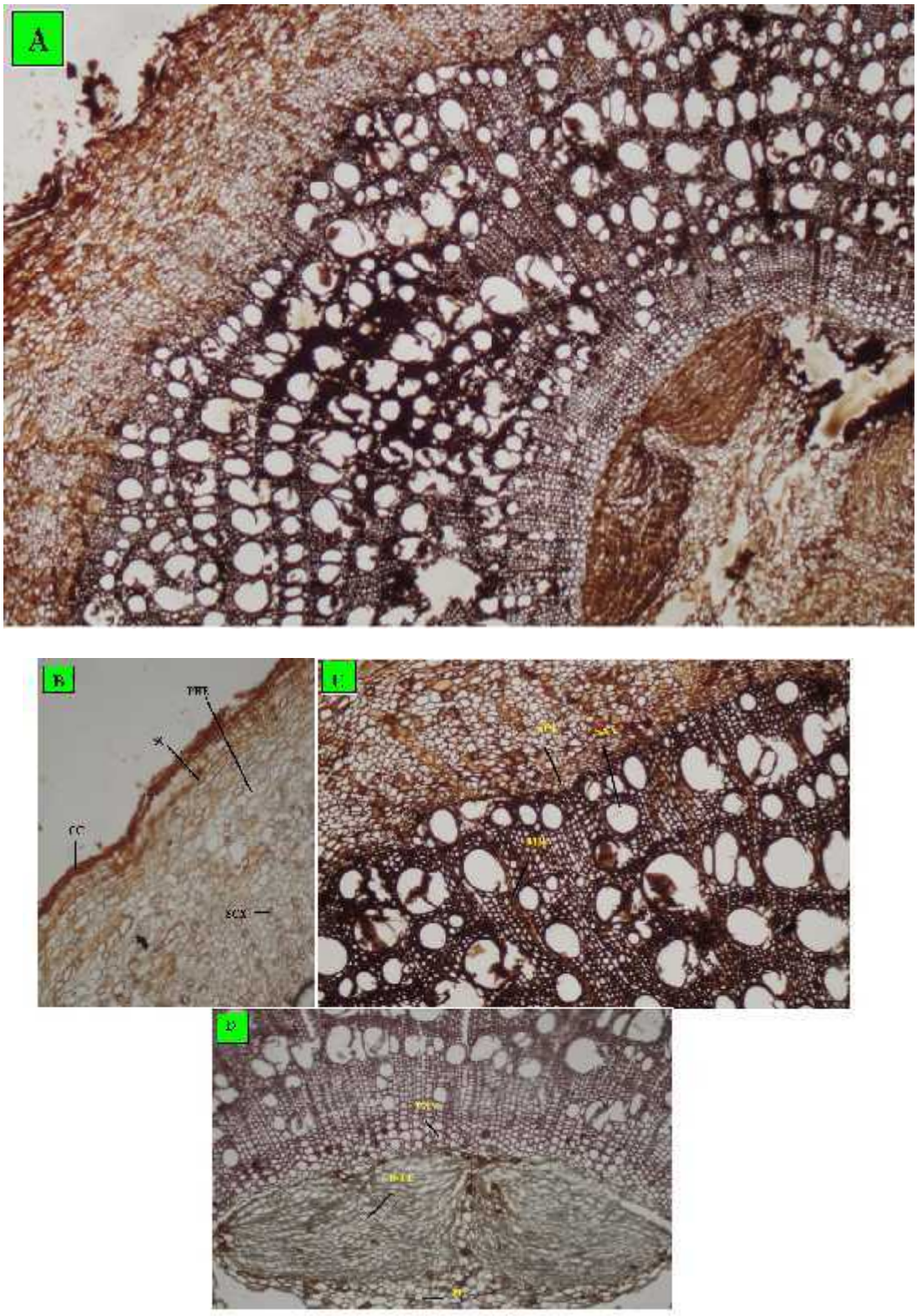


Figure 7: Microscopy of *A. dichotoma* root

[A]: Transverse section of root without staining, [B]: Outer region of root, [C]: Secondary growth structures, [D]: Central region of root [**CC** –cork cell layer, **SC**- Sclerenchymatous cell layer, **PHE**- Phelloderm, **SCX**- Secondary Cortex, **SPL**- Secondary Phloem, **MR**- Medullary Rays, **SXV**- Secondary Xylem Vessel, **PXV**- Primary Xylem Vessel, **INPL**- Intraxylary Phloem, **PI**- Pith

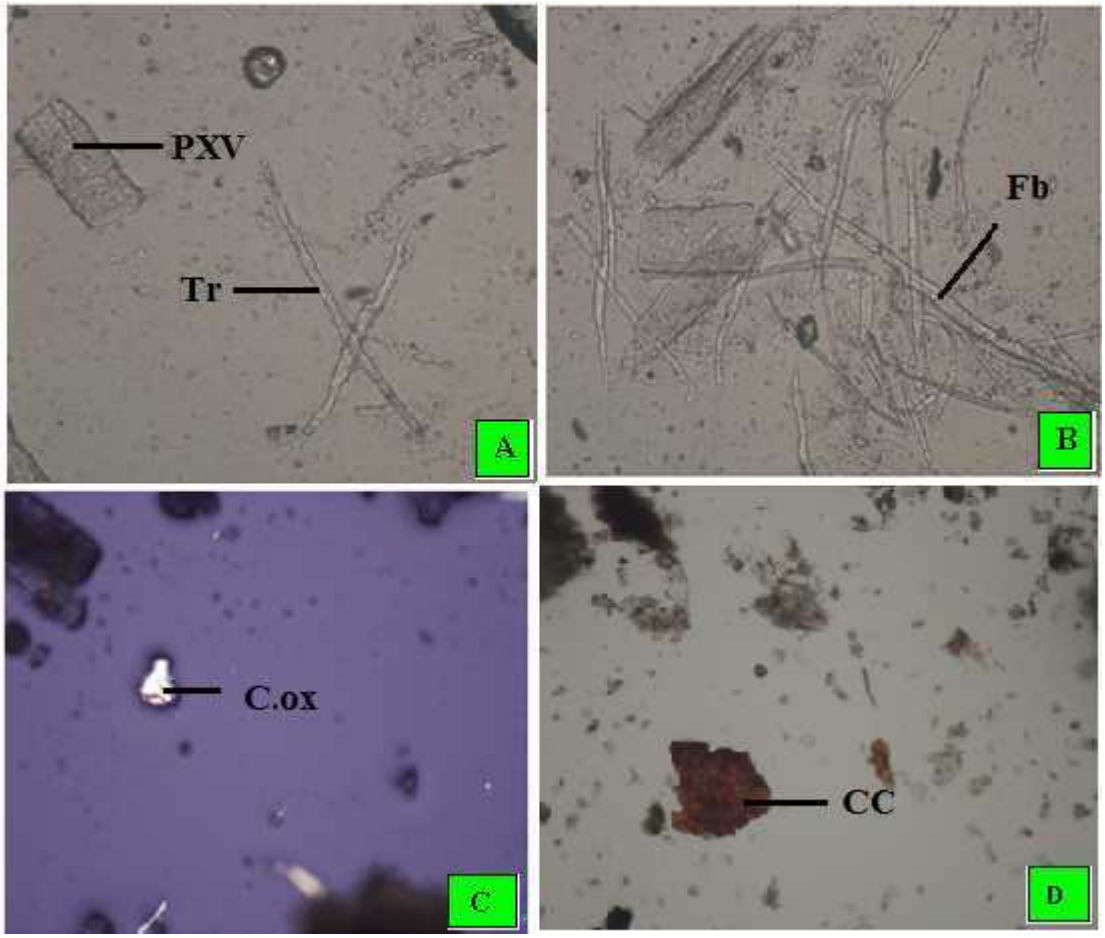


Figure 8: Powder characteristics of *A. dichotoma* root

[A]: Pitted Xylem Vessel **(PXV)** and Tracheids **(Tr)**, [B]: Fiber **(Fb)**, [C]: Solitary Calcium Oxalate Crystals **(C.ox)**, [D]: Cork Cells **(CC)**.

Physicochemical Evaluation

Table 8: Evaluation of Physicochemical parameters

S.No.	Parameters	Results
1.	Foreign matter*	0.837 % w/w
2.	Ash Value*	
	Total ash	13.75 % w/w
	Acid insoluble ash	5.75 % w/w
	Water soluble ash	3.60 % w/w
3.	Extractive value*	
	Petroleum ether soluble extractive value	3.16 % w/w
	Chloroform soluble extractive value	3.13 % w/w
	Ethyl acetate soluble extractive value	2.26 % w/w
	Ethanol soluble extractive value	11.82 % w/w
	Water soluble extractive value	12.75 % w/w
4.	Loss on drying*	6.70 % w/w
5.	Foaming index	181.81
6.	Swelling index	3.2 mL/g
7.	Hemolytic index	227.89 unit/gm of plant material
8.	Crude fiber content	19.4% w/w of plant material
9.	Starch grain content	2,49,981/mg of plant material
10.	**Chlorinated pesticide residue**	
	TS1 (First elute) TS2 (Second elute) **Phosphated pesticide residue** TS1 (First elute)	Not more than 0.1426 mg/kg Not more than 0.2794 mg/kg
	TS2 (Second elute)	Not more than 0.0032 mg/kg
	TS3 (Third elute)	Not more than 0.0215 mg/kg Not more than 0.0107 mg/kg

* All the values are performed in triplicate

Fluorescence powder drug analysis

Table 9: Fluorescence analysis of *A. dichotoma*

Test	Day light	Short UV	Long UV
Powder + 1 N NaOH in methanol	Peach Puff	Light green	Honey dew
Powder + 1 N NaOH in water	Dark golden rod	Green yellow	Lime green
Powder + 1 N HCL in methanol	Saddle Brown	Yellow green	Dark Salmon
Powder + 1 N HCL in water	Rosy Brown	Dark sea green	No Fluorescence
Powder + 1 N HNO_3 in methanol	Wheat color	Lawn green	Light green
Powder + 1N HNO_3 in water	Tan	Light green	No fluorescence
Powder + 5% iodine	Maroon	Dark red	No fluorescence
Powder + 5% $FeCl_3$	Sienna	Dark green	No fluorescence
Powder + 50% KOH	Khaki	Yellow green	Lime
Powder + 25% ammonia	Corn silk	Green yellow	Olive drab
Powder + picric acid saturated	Gold	Green yellow	No fluorescence
Powder + acetic acid	Antique white	Dark sea green	Dark olive green

Phytochemical screening

Table 10: Preliminary Phytochemical Screening of Ethanolic Extract of *A. dichotoma* and its Successive Fractions

S.No.	Phytoconstituents	EAD	PF	CF	EAF	BF	AF
1.	Flavanoids	+	-	+	-	+	-
2.	Phenolics & Tannins	+	-	-	-	+	+
3.	Steroids	+	+	+	+	-	-
4.	Triterpenoids	+	+	+	+	-	-
5.	Coumarins	-	+	+	+	-	-
6.	Cardiac Glycosides	+	-	-	-	-	+
7.	Anthraquinone Glycosides	+	-	-	-	-	-
8.	Alkaloids	-	-	+	-	+	+
9.	Saponin	+	+	-	-	-	+
10.	Carbohydrate	+	-	+	+	+	+
11.	Reducing Sugar	+	-	+	+	+	-

(+) indicate presence, (-) indicate absence.

Quantitative estimations

Table 11: Quantitative Estimation of Phytoconstituents of Ethanolic Extract of *Aganosma* Root

Phytoconstituents class	Total content in mg/gm of plant extract
Total Phenolic content	125.65 ± 2.58 (Equivalent to Tannic acid)
Total Tannin Content	104.96 ± 1.35 (Equivalent to Tannic acid)
Total Flavanoid Content	62.20 ± 2.01 (Equivalent to Rutin)
Total Flavanol Content	1.97 ± 0.06 (Equivalent to Rutin)
Total Saponin Content	49.2 ± 1.92 (Equivalent to Diosgenin)
Total Alkaloid Content	0.2% w/w

Isolation and Characterization

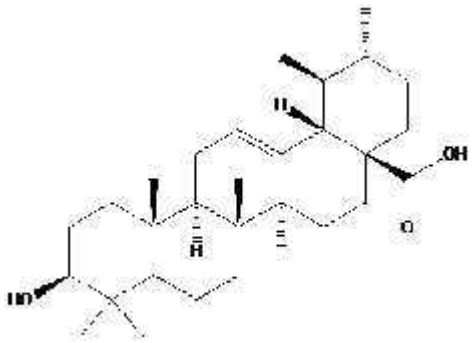
Isolation of ursolic acid from root of A. dichotoma

Column chromatography of the petroleum ether fraction (PF) from *A. dichotoma* was done and seven compounds were isolated. The physical and spectral characteristic of all the compounds were represented below.

Compound PEF-1 ($C_{30}H_{48}O_3$)

The characteristic signals (chemical-shifts) in ^{1}H-NMR and ^{13}C-NMR spectra's of the investigated compound (PEF-1) was recorded in $CDCl_3$. In ^{1}H-NMR spectral data (Figure 9), the Characteristic sharp singlet peak of an acidic proton was observed at 9.85 ppm (downfield chemical shift), whereas the (-OH) proton was observed as doublet at 1.634 ppm, This OH proton was further confirmed in D2O shake spectrum(this peak is missing). In ^{13}C-NMR spectrum (Figure 10) the characteristic peak of COOH carbon was found at 182.70 ppm while carbon atoms attached with double bond were found at 148.18, 120.47 ppm respectively. Carbon atom attached with OH group was found at 76.73 ppm. In mass spectrum (Figure 11) ESI-MS

methanol the characteristic M+ was observed at m/z 456.7 (9.7%), whereas the M-18, 439.2 (53.3%) and 438.2 (5.5%) M-19 peaks were also observed in the spectrum. The base peak was observed at 411.2 (100%). On the basis of spectral observations and melting point (288-290ºC), unknown compound was confirmed as ursolic acid.



Ursolic acid

^{1}H-NMR (PEF-1), [(CDCl3, 300 MHz) δ (ppm): 9.850(s, H, COOH), 4.727 (d,H, CH=), 2.372-2.322 (m, 18H, CH2,and 7H, CH), 1.634(d, H, OH), 0.880-0.857(s, 18H, CH3).

^{13}C-NMR (PEF-1), (75MHz, CDCl3) δ [ppm]: 182.70 [-COOH], 148.18, 120.47 [-C=C], 76.73[-C-OH], 40.06-38.66 [ring -CH2, -CH), 24.81-24.05[-CH3].

Mass [ESI] in CH3OH: m/z 456.7(9.7) M+, 439.2(53.3), 438.2(5.5), 411.2(100), 410.3(6.1), 392.7(18.2), 348.4(11.2), 346.6(20.8).

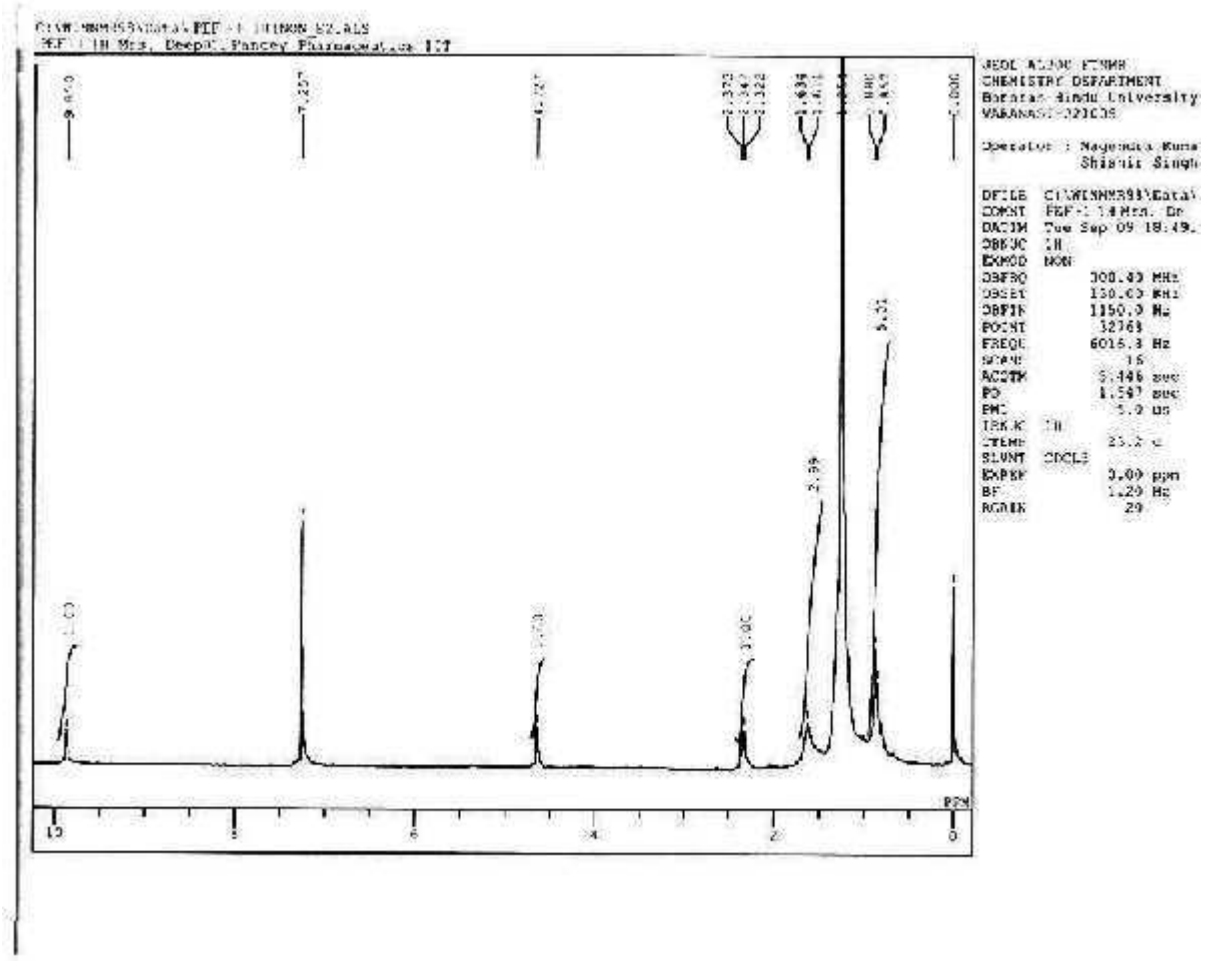


Figure 9: H^1 NMR spectra of ursolic acid isolated from *A. dichotoma* root

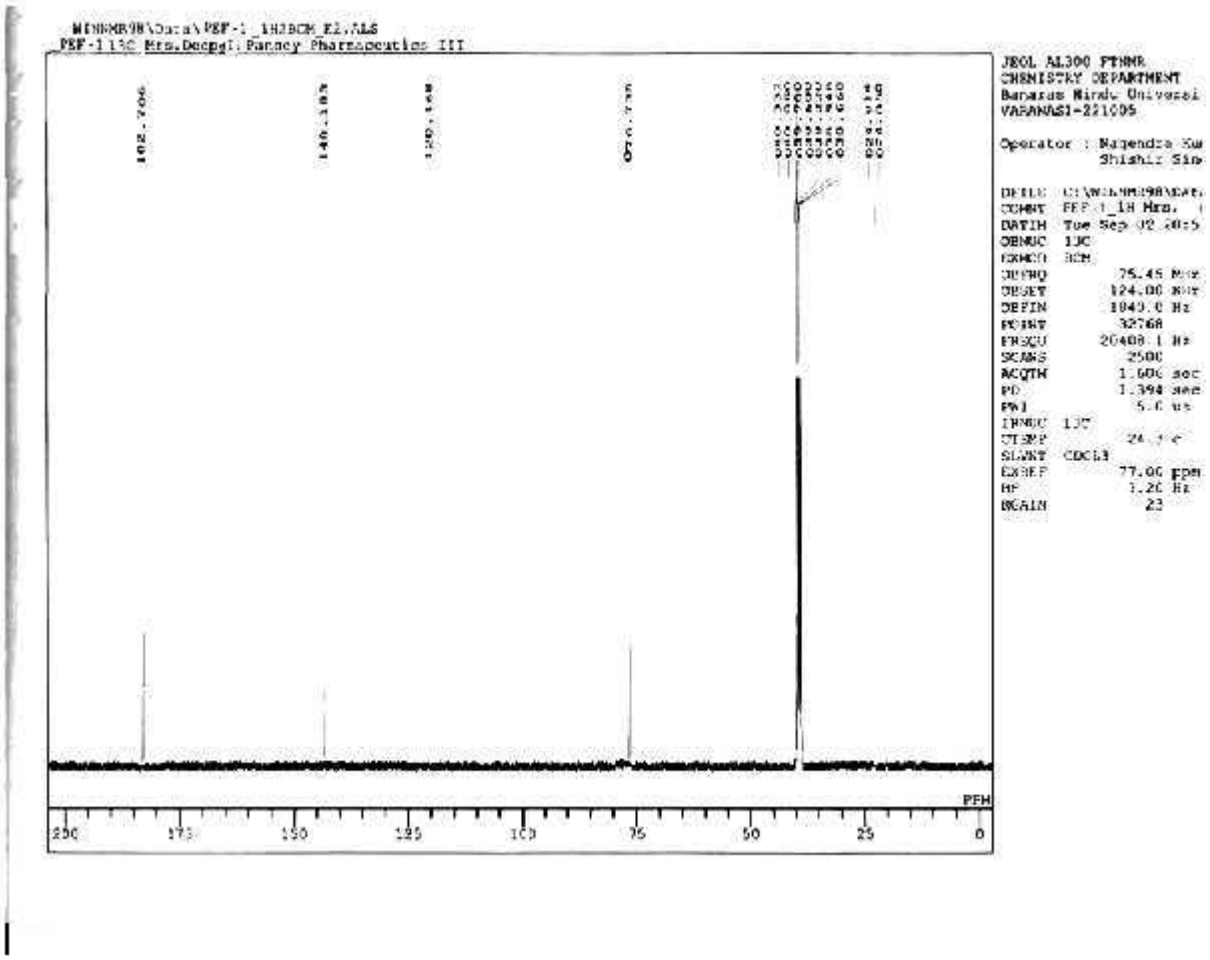


Figure 10: C^{13} NMR spectra of ursolic acid isolated from *A. dichotoma* root

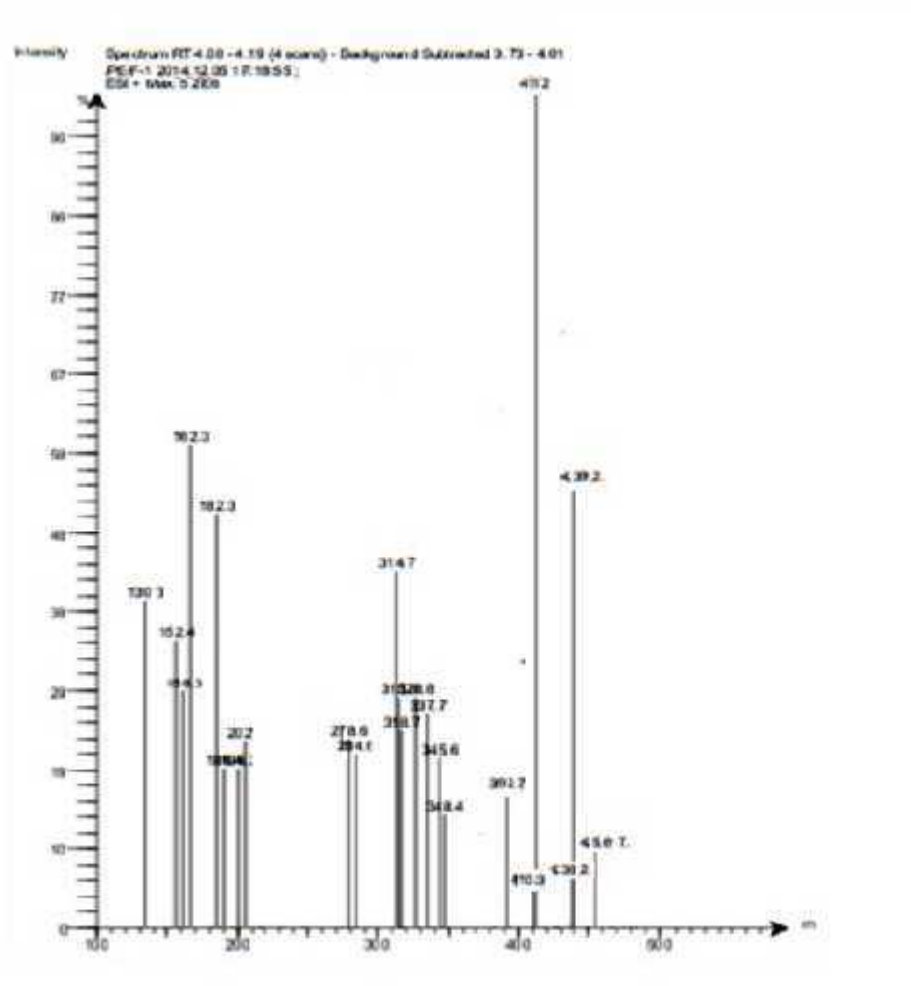


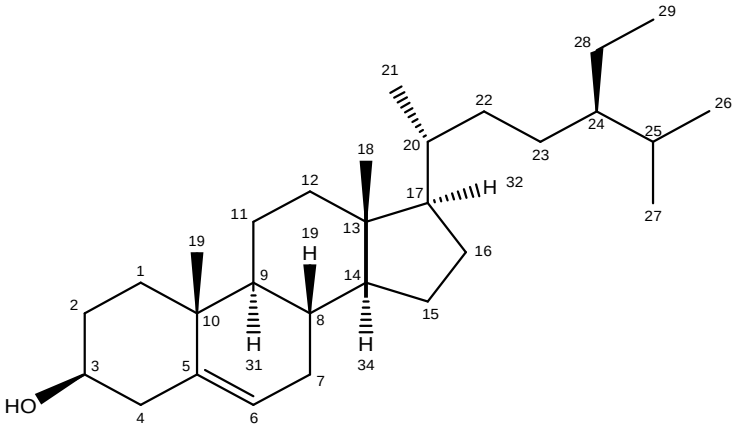
Figure 11: Mass spectra of ursolic acid isolated from *A. dichotoma* root

Compound PEF-2 ($C_{29}H_{50}O$)

- Nature: White amorphous compound soluble in chloroform.
- TLC analysis showed a single spot with R_f value of 0.65 (Chloroform: methanol; 9:1).
- Melting point of PEF-2 was observed at 137.5°C.
- IR bands (KBr, υ cm^{-1}): 3427 (–OH str), 2936 (C–H str), 1647 (tri–substituted double bond), 1463 (CH_2), 1377 (OH bend), 1059 (C–O str) (Figure 12).
- NMR spectra in CD_3OD: (H^1NMR: δ^H): 5.28 (1H, m, H–6), 3.25 (1H, m, H–3), 0.60–2.21 (48H, m). (Figure 13)

(C^{13}NMR: δ^C): 140.75 (C–5), 121.74 (C–6), 71.8 (C–3), 56.76 (C–17), 56.04 (C–14), 50.12 (C–9), 45.82 (C–24), 42.32 (C–13), 42.29 (C–4), 39.77 (C–12), 37.25 (C–22), 36.51 (C–10), 36.16 (C–20), 33.94 (C–2), 31.92 (C–1), 31.90 (C–25), 31.65 (C–7), 29.13 (C–8), 28.27 (C–23) , 26.04 (C–15), 24.32 (C–16), 23.06 (C–28), 21.09 (C–11), 19.84 (C–19), 19.42 (C–26, C–27), 19.04 (C–18), 18.79 (C–21), 12.00(C–29). (Figure 14)

From the overall spectral data the compound was characterized as β–sitosterol.



β–sitosterol

The compound PEF–2 (β-sitosterol) with a melting point of 137.5°C responds to Liebermann–Burchard test thereby indicating the steroidal nature. The Infrared spectra of the compound showed the appearance of a broad band at 3427 cm^{-1} indicating the presence of hydroxyl (–OH) group. The presence of hydroxyl group was further confirmed by the appearance of a singlet peak in H^1NMR spectra at δ 3.45. A band at 1647 cm^{-1} in infrared spectra, δ 5.27 in H^1NMR spectra (doublet) and δ 140.75 and δ 121.74 in C^{13}NMR indicate the presence of a double bond between a quaternary (C–5) and tertiary carbon (C–6) in the compound. Finally, on the basis of spectroscopic analysis available in literature and by direct comparison with an authentic sample (β–sitosterol) via. Melting point and TLC, the compound was identified as β–sitosterol.

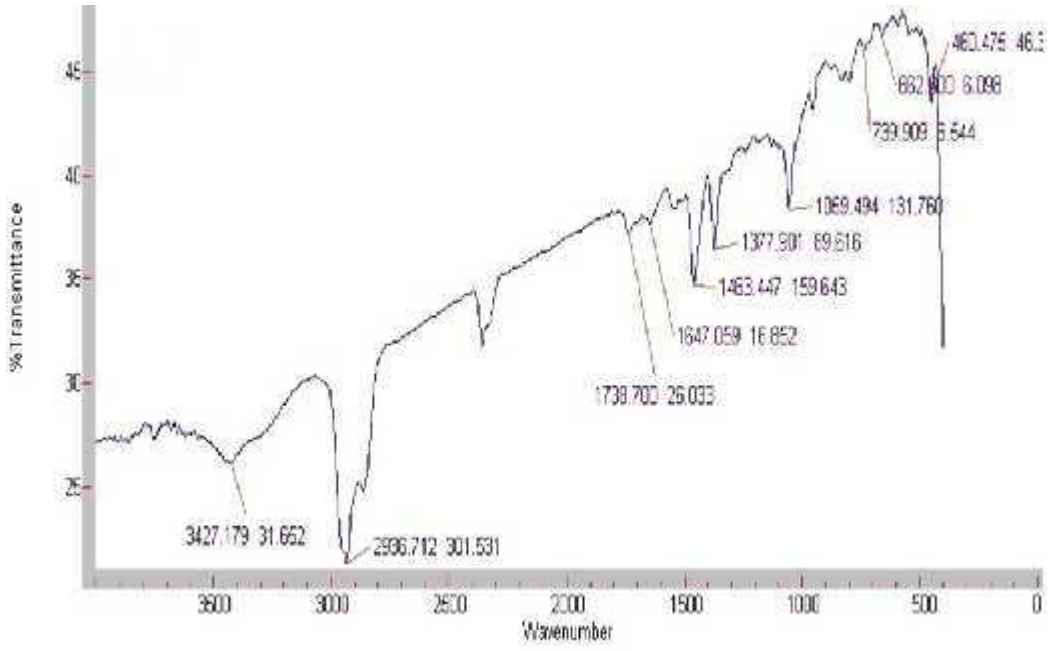


Figure 12: IR spectra of compound isolated β–sitosterol

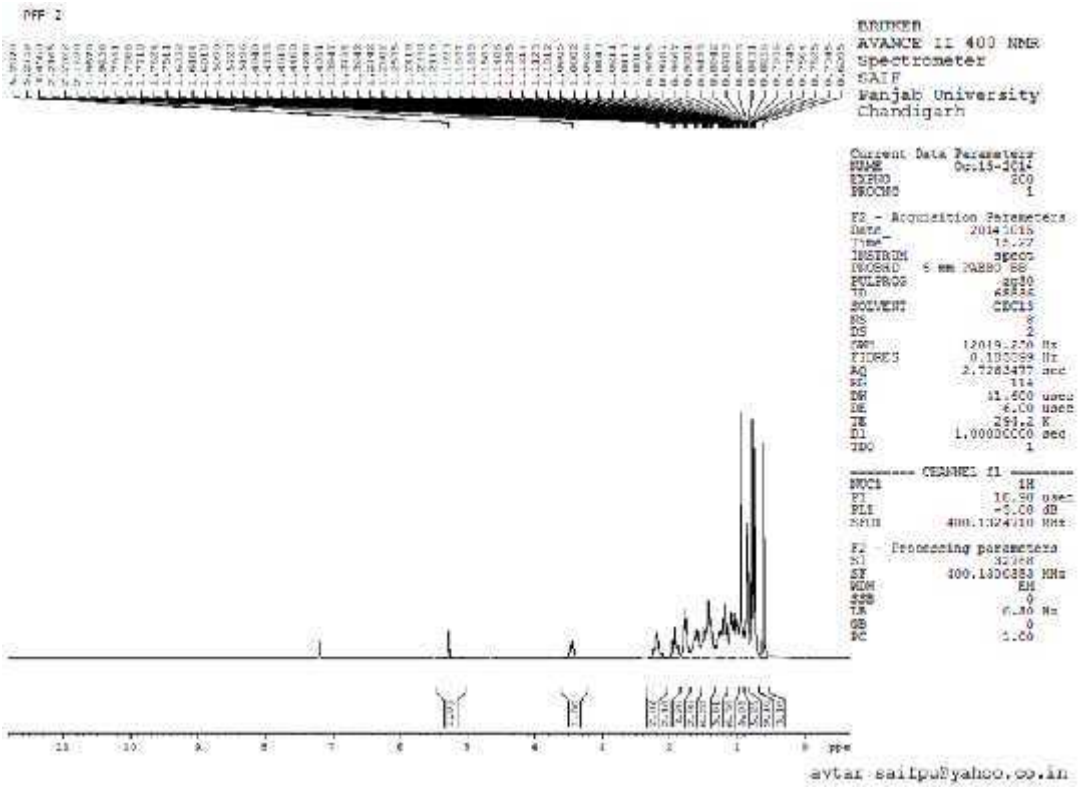


Figure 13: H^{1}NMR spectra of isolated β–sitosterol

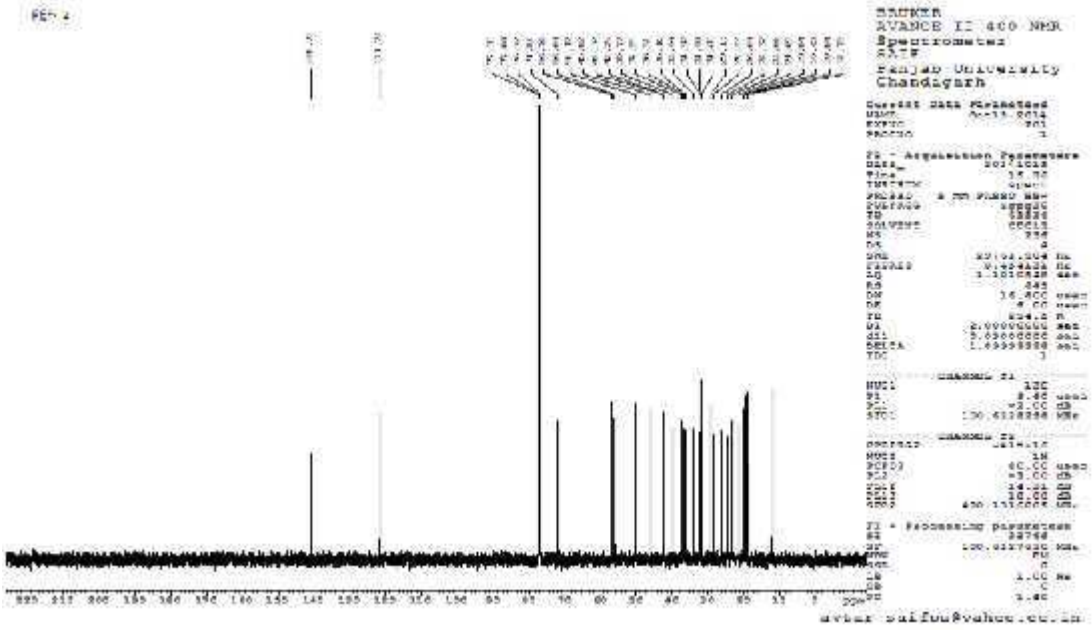


Figure 14: C^{13}NMR spectra of isolated β–sitosterol

Identification of compounds PEF-3, PEF-4, PEF-5, PEF-6, PEF-7 and PEF-8

Column chromatography of petroleum ether fraction was eluted with hexane: ethyl acetate (9:1) (1-20) performed and six fatty acid components were identified (Figure 15). The GC–FID results on the basis of retention time for compound PEF–3, PEF–4, PEF–5, PEF–6, PEF-7 and PEF–8 are given below. All these components were reported for the first time from *A.dichotoma* roots.

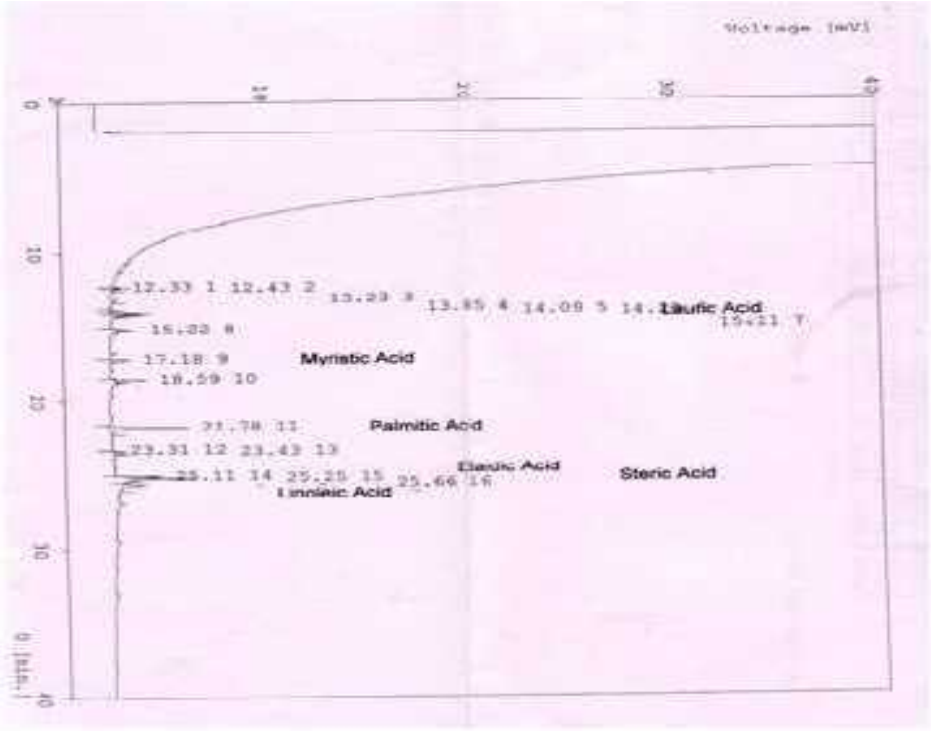
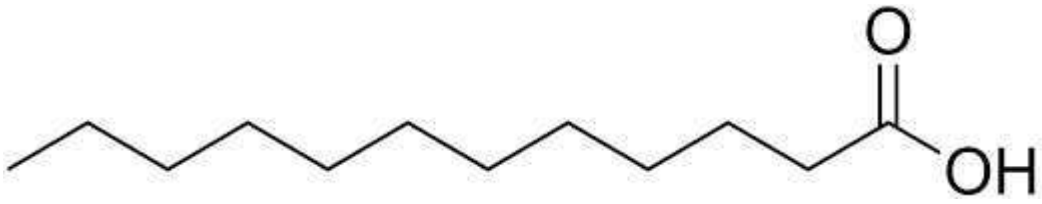


Figure 15: GC-FID spectra of compound PEF-3, PEF-4, PEF-5, PEF-6, PEF-7 and PEF-8

Compound (PEF–3)

- Retention time: 14.26 min.
- Percentage yield: 0.332 % w/w
- Class of phytoconstituents: Fatty acid

The compound PEF–3 was identified as lauric acid

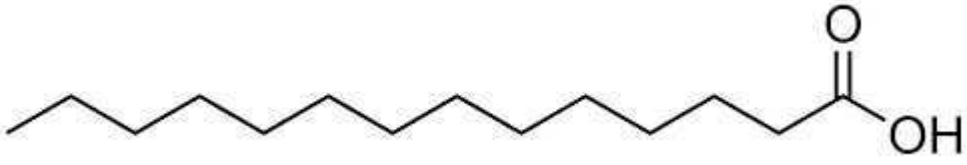


Lauric acid

Compound (PEF-4)

- Retention time: 17.25 min.
- Percentage yield: 3.49 % w/w
- Class of phytoconstituents: Fatty acid

The compound PEF–4 was identified as myristic acid

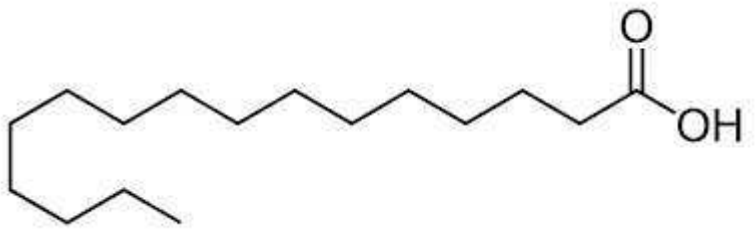


Myristic acid

Compound (PEF-5)

- Retention time: 21.92 min.
- Percentage yield: 1.22 % w/w
- Class of phytoconstituents: Fatty acid

The compound PEF–5 was identified as palmitic acid

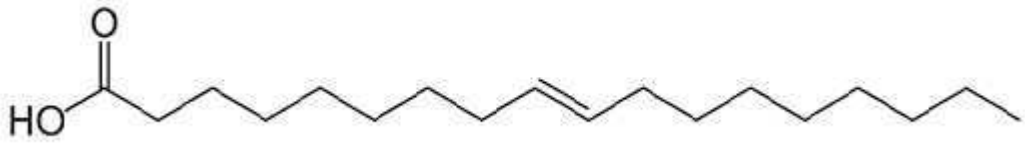


Palmitic acid

Compound (PEF-6)

- Retention time: 25.22 min.
- Percentage yield: 0.487 % w/w
- Class of phytoconstituents: Fatty acid

The compound PEF-6 was identified as elaidic acid

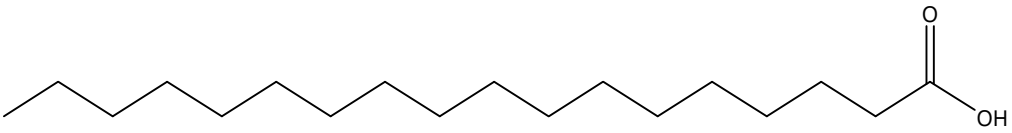


Elaidic acid

Compound (PEF-7)

- Retention time: 25.36 min.
- Percentage yield: 0.917 % w/w
- Class of phytoconstituents: Fatty acid

The compound PEF-7 was identified as stearic acid

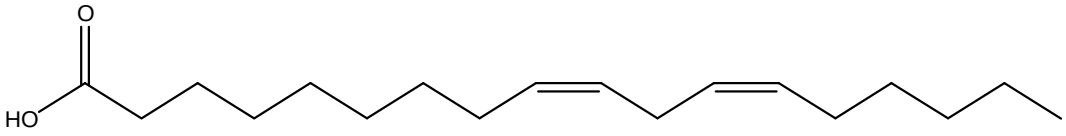


Stearic acid

Compound (PEF-8)

- Retention time: 25.77 min.
- Percentage yield: 0.392 % w/w
- Class of phytoconstituents: Fatty acid

The compound PEF-8 was identified as linoleic acid



Linoleic acid

Chemical standardization of *A. dichotoma* by HPTLC

HPTLC fingerprinting analysis of EAD

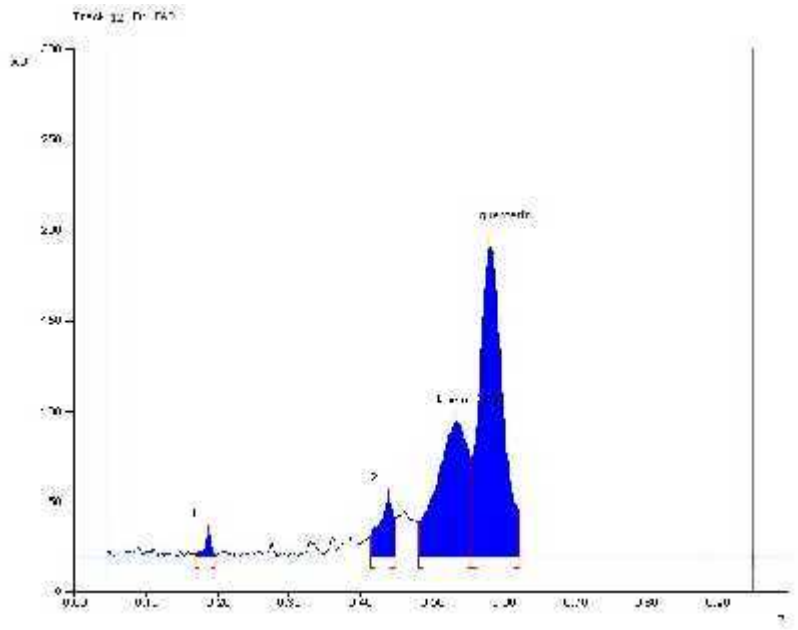


Figure 16: HPTLC fingerprinting of EAD at 350 nm

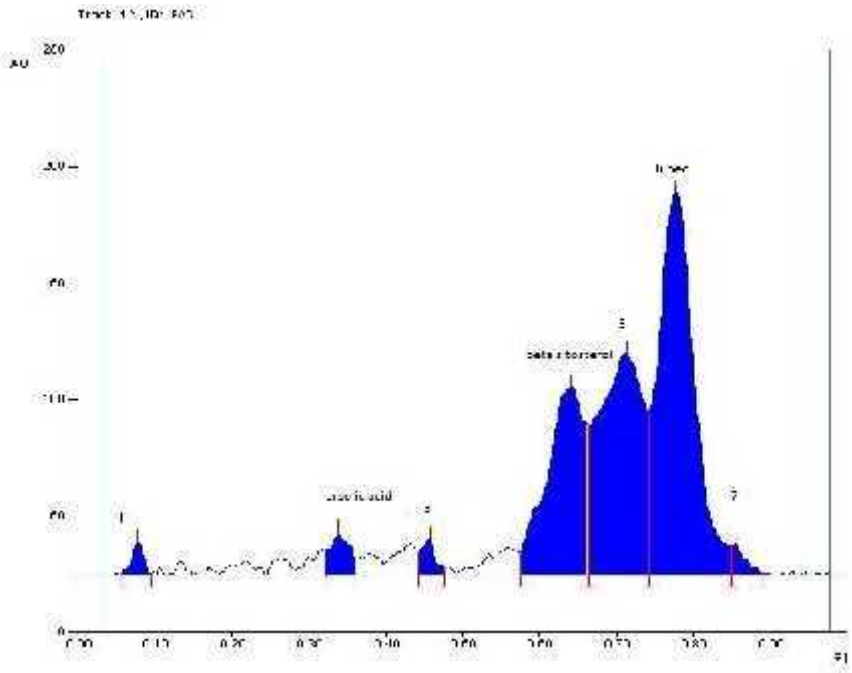


Figure 17: HPTLC fingerprinting of EAD at 650 nm

HPTLC fingerprinting analysis of CF

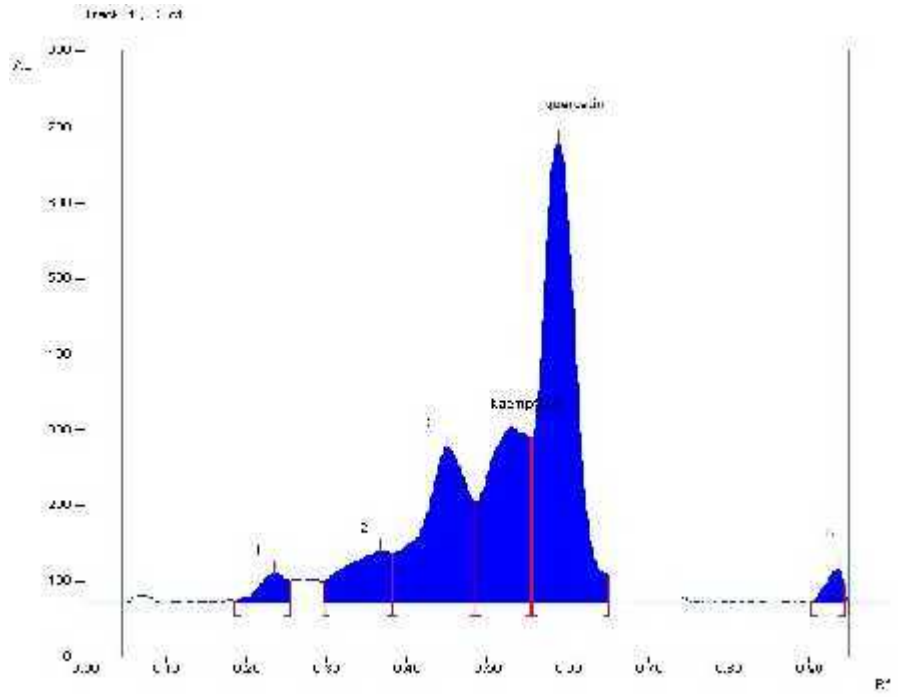


Figure 18: HPTLC fingerprinting of CF at 350 nm

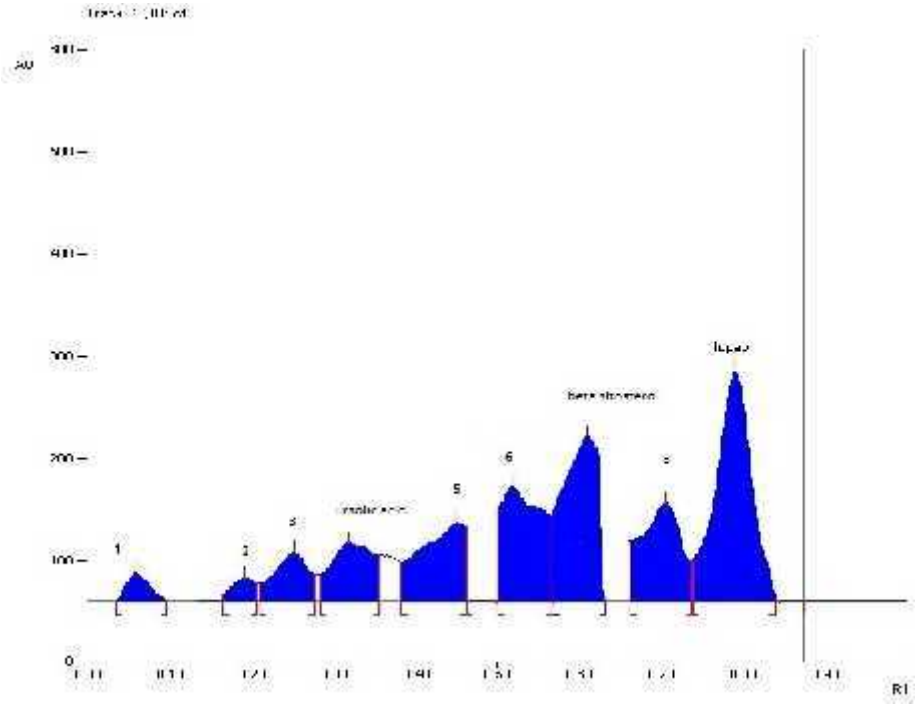


Figure 19: HPTLC fingerprinting of CF at 650 nm

HPTLC fingerprinting analysis of PF

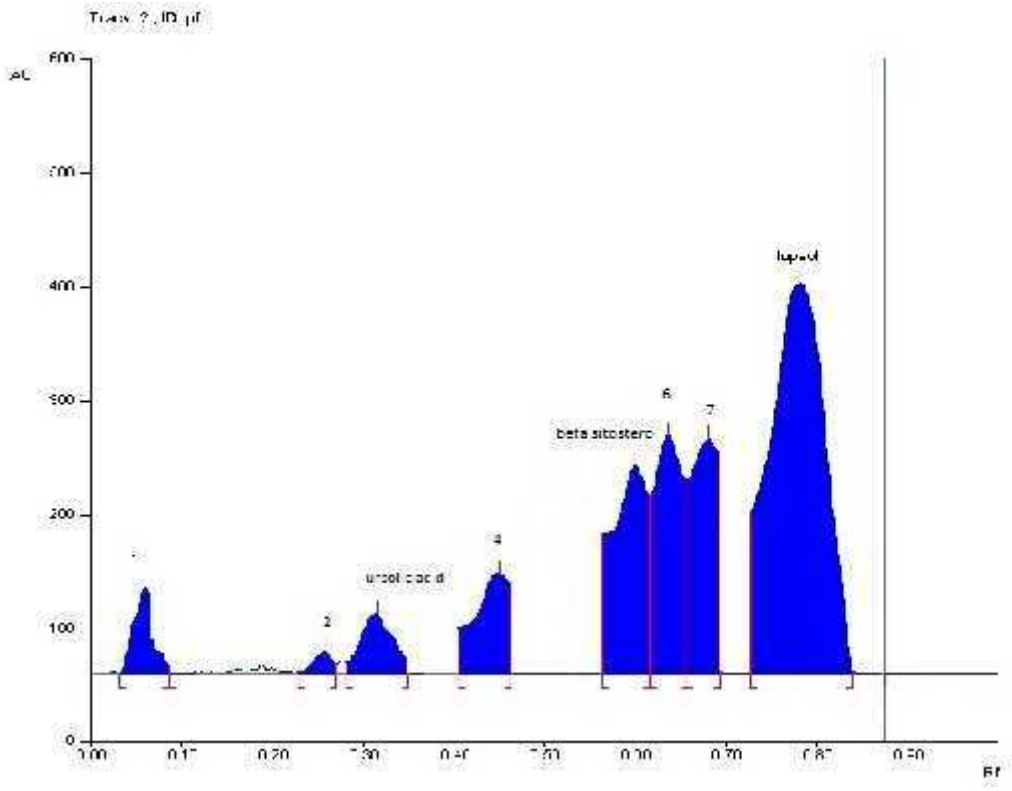


Figure 20: HPTLC fingerprinting of PF at 650 nm

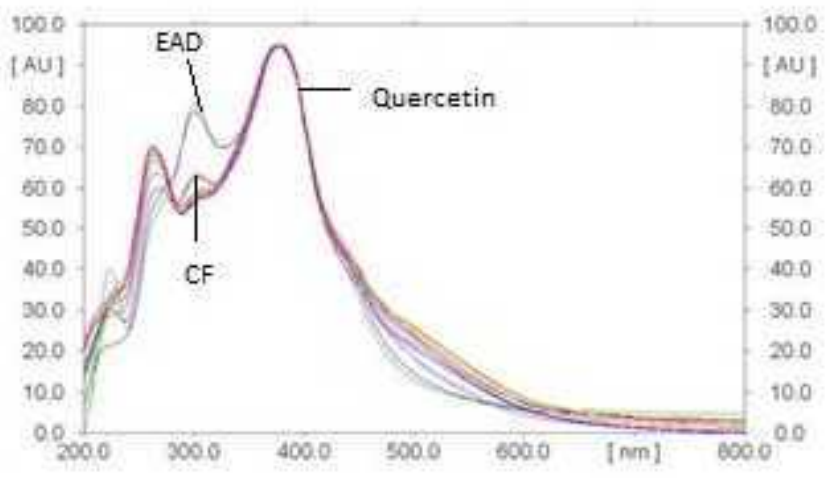


Figure 21: Overlay spectra of EAD and CF with standard quercetin at 350 nm.

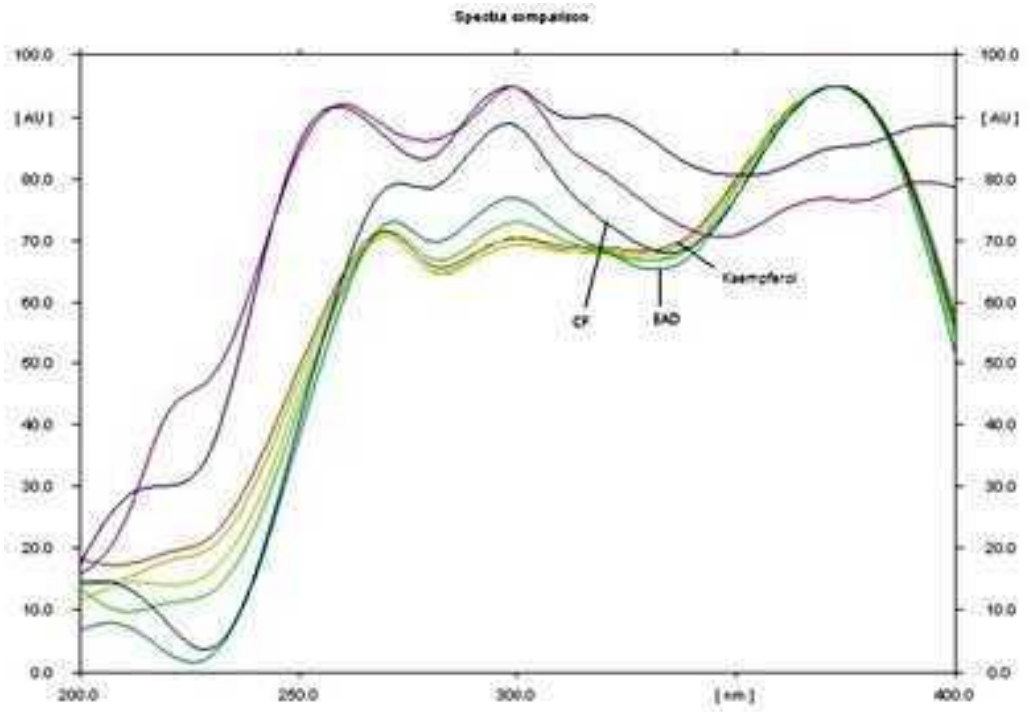


Figure 22: Overlay spectra of EAD and CF with standard kaempferol at 300 nm.

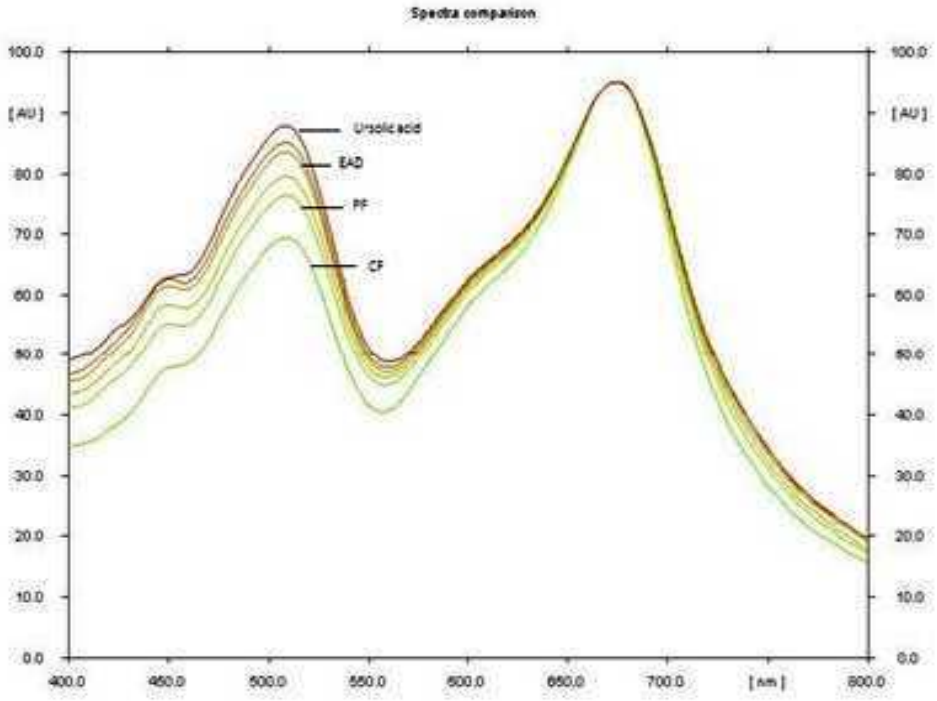


Figure 23: Overlay spectra of EAD, CF and PF with standard ursolic acid at 650 nm.

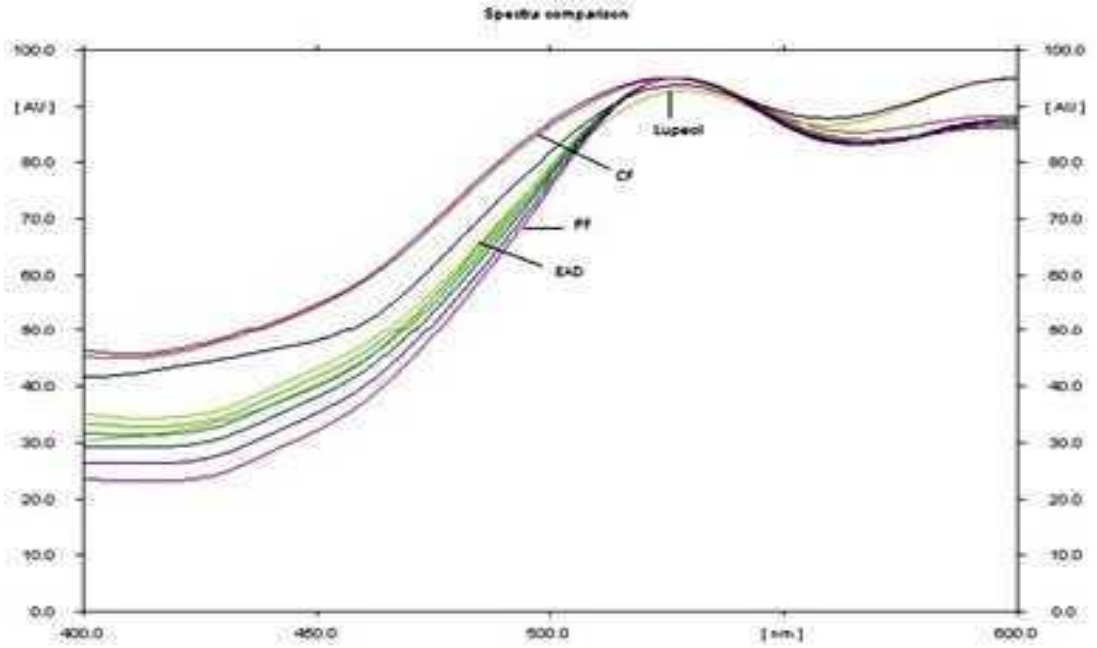


Figure 24: Overlay spectra of EAD, CF and PF with standard lupeol at 550 nm.

HPTLC fingerprinting of EAD was depicted in figure 16 which shows 4 peaks and 7 peaks in figure 17. The presence of kaempferol (R_f 0.56) and quercetin (R_f 0.59) was observed at 350 nm which was confirmed by R_f value and spectral comparison by using marker compounds (Figure 21-24). Triterpenoids were observed after derivatization of the plate which indicates the presence of ursolic acid (R_f 0.34) and lupeol (R_f 0.78). Figure 18 and 19 represents the fingerprinting data of CF while Figure 20 represent the fingerprinting of PF. PF consists of only triterpenoids i.e. ursolic acid and lupeol while kaempferol and quercetin were absent. Quantification study of Kaempferol, quercetin, ursolic acid, β-sitosterol and lupeol were carried out and found to be EAD (0.523% w/w, 2.07% w/w, 1.26% w/w, 2.23% w/w and 5.81% w/w), CF (1.06% w/w, 3.86% w/w, 1.59% w/w, 1.84% w/w and 2.61% w/w) PF (1.63% w/w, 1.06% w/w and 4.93% w/w) respectively.

***In-vitro* antioxidant studies**

The total antioxidant capacity of ethanolic extract of root of *A.dichotoma* showed potential antioxidant activity compared with the ascorbic acid used as a standard. The absorbance of ethanolic extract of plant (100µg/ml) was 0.356 at 695 λmax. Thus the result of Total antioxidant capacity was found to be 57.75 µg/ml of ethanolic extract of *A.dichotoma* which was equivalent to 100 µg/ml of standard ascorbic acid. Ethanolic extract in DPPH scavenging activity showed IC_{50} value (104.41±0.2) while standard ascorbic acid showed IC_{50} value (98.20±4.11). Nitric oxide scavenging activity was estimated by using Griess reagent, which showed a very moderate scavenging activity of plant extract (IC_{50} 137.07±0.72) in comparison to rutin (IC_{50} 71.32±1.91). The scavenging potential of hydrogen peroxide by ethanolic extract was also found to be considerably moderate with IC_{50} value of 143.80±0.81 compared to rutin (IC_{50} 99.92±1.18). Hydroxyl radical production was assessed by the iron (II) – dependent deoxyribose damage assay following the fenton reaction. The result demonstrated that EAD showed an IC_{50} value of 127.63±0.56 compared to positive control BHA (IC_{50} 103.14±2.53). Assay of reducing power is a concentration-dependent reaction. The assay of reducing power depicted a very moderate reducing capacity of the extract (0.198±0.01 µg/mL) as compared to standard ascorbic acid (0.419±0.006 µg/mL) (Figure 25).

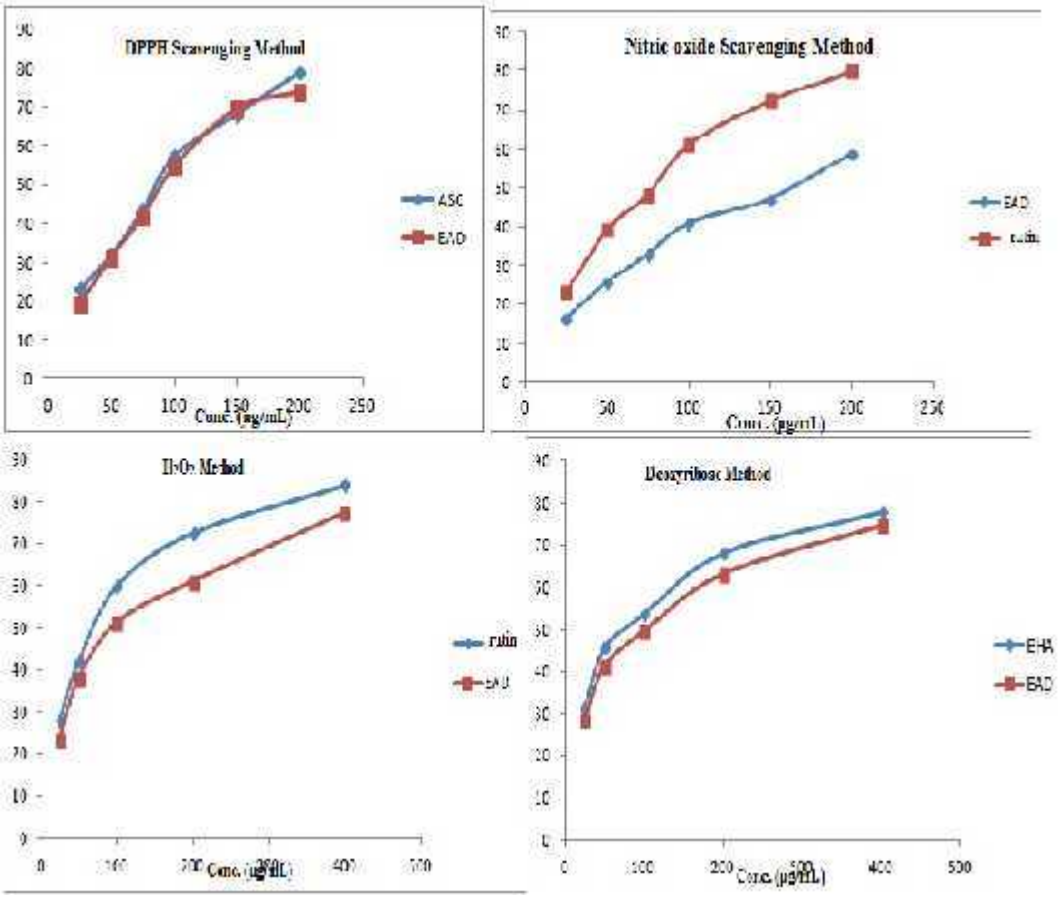


Figure 25: *In-vitro* antioxidant activity of *A. dichotoma*. Where Asc: Ascorbic acid, BHA: Butylated Hydroxy Anisole and EAD: Ethanolic root extract of *A. dichotoma*.

DISCUSSION

Quality control standardization of herbal drugs provides the essential information regarding correct identification and authentication of the plant material. Currently, there is a great demand on the quality control profile and standardization of medicinal plant drug/plant parts for their therapeutic potentials. According to the World Health Organization, modern pharmacognostic techniques are available for the identification and evaluation of crude drugs which are more reliable, accurate and inexpensive (Pandey *et al*., 2015). Pharmacognostical evaluation of a particular drug of a plant/plant parts provides valuable information in terms of its morphological, microscopical, and physical characteristics and therefore, it is preferred as a primary step in standardization of a plant (Sindhu *et al*., 2010). Macroscopical examination of a plant/plant parts represents detailed information regarding the qualitative estimation of plant based on its morphological and sensory characters such as size, shape color, taste, odor etc while microscopical evaluation provides us widespread knowledge about the cellular arrangement of tissues (Nagani *et al*., 2012). Therefore some diagnostic features have been evolved to identify and to differentiate *A. dichotoma* root from the other crude drugs and its adulterants.

From the microscopical studies, it was observed that the T.S. of root showed the presence of various unique indicative characters such as intraxylary phloem (anamolus secondary growth), solitary calcium oxalate crystals etc. The physicochemical evaluation is an imperative parameter which is useful for the detection of adulteration or improper handling of the drug. The results showed the presence of low moisture content in the roots of *A. dichotoma* but were within the

prescribed limits. It is very crucial to control the moisture content since higher moisture content in plant material may escort to its deterioration and may therefore, result in percentage discrepancy of active constituents. The significance of ash values are quantitative standards that correspond to the presence of various impurities like carbonate, oxalate, and silicate which may be naturally occurring or intentionally added to the crude drug as a form of adulterant. Total ash includes both physiological as well as non–physiological ash, while acid insoluble ash consist mainly silica and indicate contamination with the earthy material. Water soluble ash is used to estimate the amount of inorganic elements present in drugs (Kokate, 2006). Extractive values are valuable to evaluate the amount of active chemical constituents present in the plant/plant parts using different solvents. The foaming index parameter shows the ability of plant material and their extracts to form importunate foam. The swelling index is carried out due to therapeutic or pharmaceutical value of many medicinal plants may be attributed to its swelling property, which is due to the presence of gums, mucilage, pectin, and hemicelluloses. Hemolytic activity is seen in plants containing mainly saponins that diffuse hemoglobin into the surrounding medium (Anonymous, 2002). Fluorescence powder drug analysis is a vital parameter in qualitative determination of crude drug since many compounds in plant material can be judged by their property of exhibiting fluorescence in day light or in ultra violet range (e.g. alkaloids like berberine) (Prasad *et al*., 2012).

Contamination of medicinal plant/plant parts with pesticides may occur due to the spraying and treatment of soils occurring throughout the process of cultivation, and administration of fumigants during storage. Therefore, WHO suggested that every

nation trading with production of medicinal plants/plant parts should have at least one central laboratory which will provide information regarding standard limits of pesticides (Anonymous, 2002). All the plant material showed the presence of chlorinated and phosphated pesticides but the levels were present in accordance with the standard limits of these pesticides. Furthermore, pesticide residue in the root of *A. dichotoma* estimated was found to be within the limits as prescribed by the WHO guidelines.

The chemical nature of the active constituents present in the plant material can be identified by performing preliminary phytochemical screening of that plant material (Khandelwal, 2007). The result of preliminary phytochemical screening revealed the presence of anthraquinone and steroidal glycosides, alkaloids, flavonoids, tannins, steroids, saponin, and coumarins in different fractions of *A. dichotoma*. Such phytochemical screening is helpful in the prediction of natural phytoconstituents present in the tested drugs, which further leads to the isolation of compounds since phytochemicals are proven to be responsible for the activity of the drugs (Pandey *et al*., 2015). Quantitative estimation of phytoconstituent is efficient parameters to set up standards for crude drugs and result showed the higher amount of phenol, tannin, and flavanoid.

The results obtained from the *in-vitro* antioxidant studies showed that ethanolic extract of *A. dichotoma*, possess potent antioxidant activity, which may be attributed to the presence of phenols, tannins, and flavonoids. The total antioxidant capacity and reducing power of a plant play a dominant role in depicting its antioxidant activity.

The study also included the isolation of ursolic acid, β-sitosterol, and some fatty acids first time from the roots of *A. dichotoma*. Ursolic acid is a pentacyclic triterpenoid exhibiting various pharmacological properties. The content of ursolic acid in root extract was found to be 1.26 %w/w and it varies in different medicinal plants and species due to the presence and activity of enzyme responsible for their synthesis. Some plants with high content of ursolic acid (% w/w) are given below.

Rosemarinus officinalis (2.95), *Salvia officinalis* (1.80), *Mallus domestica* (1.43), *Lavandula angustifolia* (1.59), *Coffea arabica* (1.80), *Arctostaphylos uva-ursi* (1.24), *Nerium oleander* (1.27), *Origanum majorana* (0.66), *Thymus vulgaris* (0.94) (Sebastian *et al*., 2009).

Chemical standardization of EAD and their fractions were also ascertained with the help of HPTLC fingerprinting and the amount of ursolic acid and lupeol was quantified as a chemical marker. All these plant based constituents should be well standardized and documented and their limits present in the plants should be estimated. Hence, to avoid the misuse of harmful plant material it is necessary to scientifically develop a pharmacognostical and physicochemical standards of a particular plant material which may ensure and maintain its quality, efficacy and safety profile. The parameters, which are being reported first time in this work, could be useful in the preparation of the herbal monograph for proper identification and authentication of *A. dichotoma*.