IJPSR (2021), Volume 12, Issue 11 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 03 January 2021; received in revised form, 10 April 2021; accepted, 27 May 2021; published 01 November 2021

ISOLATION, PURIFICATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM *STACHYTARPHETA URTICIFOLIA* (SALISB.) SIMS

Sreelatha Regalagadda [1] and Murali Mohan Challa [*2]

Department of Biochemistry [1], GITAM Institute of Science, Department of Biotechnology [2], GITAM Institute of Technology, GITAM Deemed to be University, Visakhapatnam - 530045, Andhra Pradesh, India.

Keywords:

Stachytarpheta urticifolia, TLC, Column chromatography, HPLC, FTIR, Mass spectra & NMR analysis

Correspondence to Author:
Prof. Murali Mohan Challa

Professor,
Department of Biotechnology,
GITAM Institute of Technology,
GITAM Deemed to be University,
Visakhapatnam - 530045, Andhra Pradesh, India.

E-mail: mmchalla@gmail.com

ABSTRACT: Natural products from various medicinal plants, in the form of pure compounds or standardized extracts, which provide unlimited opportunities for discovery of new drug leads, is due to the unmatched availability of chemical diversity. There is an increasing demand for chemical diversity in screening programs, seeking therapeutic drugs from natural products, interest, particularly in edible plants, has grown throughout the world. Botanicals and herbal preparations for medicinal usage contain various types of bioactive compounds. The focus of this paper is on the analytical methodologies, which include the extraction, isolation, and characterization of active ingredients of bioactive compounds from *Stachytarpheta urticifolia*. The common problems and key challenges in the extraction, isolation, and characterization of active ingredients of bioactive compounds are discussed. As extraction is the most important step in the analysis of constituents present in herbal preparations, the strengths and weaknesses of different extraction techniques are discussed. The analysis of bioactive compounds present in the plant extracts involving the applications of common phytochemical screening assays, chromatographic techniques such as HPLC and TLC as well as non-chromatographic techniques such as FTIR, Mass Spectroscopy and Structural elucidation are discussed. Purification of bioactive compounds was done using column chromatography. The structures of the compounds were elucidated on the basis of spectroscopic analysis, including Fourier transform infrared, electron spray ionization mass spectrophotometry, ^{1}HNMR and ^{13}C NMR. The bioactive compounds produced from *S. urtcifolia* were tested. Isolation and identification of bioactive compounds, namely pentacyclic triterpenoid as phenolics, are known to search free radicals along with various biological properties including antioxidant and anticancer activities.

INTRODUCTION: Among the thousands of components present in plants, but biological activity is shown by a few components, therefore it is difficult to screen and analyze the bioactive molecules among a large number of other components [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(11).6037-49

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(11).6037-49

Different parts of the plants were subjected to a suitable process of extraction and evaluated the biological activity of their constituents. Activity-guided isolation has been chosen for the process of isolation and purification of active compounds. The plant extracts were subjected to sequential fractionation, and a bioassay of each fraction was performed [2]. Isolation and separation of active components from the complex mixtures can be done using different chromatographic methods like Thin Layer Chromatography (TLC), high-performance liquid chromatography (HPLC), and Gas-liquid chromatography (GLC) [3].

Other techniques like Mass spectroscopy (MS) and Nuclear magnetic resonance spectroscopy (NMR) were further used to increase the sensitivity of detection and also to provide the structural information of active compounds [4]. Medicinal plants consist of chemical components like carbohydrates, terpenes, saponins, alkaloids, glycosides, *etc*. One of the prominent aspects is to identify the phytochemical composition of the plant. Important bioactive constituents of plants having medicinal values had sufficient attention on account of their economic importance. Therefore these studies on medicinal plants are helpful to develop small-scale industries that are involved in the isolation of crude herbal drugs [5]. The biological activity and phytochemical constituents of *S. urticifolia* have not yet been reported earlier. Based on the phytochemical screening and antioxidant properties exhibited by the different parts of the plant, the leaf was selected for isolation and purification of bioactive compounds. The present work is framed to extract the biologically active compound from *S. urticifolia* leaf using different solvents to investigate, isolate and purify the chemical components responsible for its antioxidant effects.

Phytochemical Extraction and its Importance: Active portions of plant tissues from cellular matrix having medicinal value are extracted using suitable solvents through the standard extraction procedures. Dried plant material is preferable over fresh plant material due to its reduced water content which makes it more suitable for solvent extraction [6] as the solvents diffuse into the tissue of solid plant and solubilize compound with similar polarity [7, 8, 9].

The most common steps involved during extraction includes, prewashing, drying of the plant material, freeze-drying, grinding to get the homogeneous sample and solvent extraction [2]. There are different methods used for extraction of bioactive compounds from medicinal plants like hot continuous extraction, percolation, decoction, counter-current extraction, soxhlet extraction, aqueous-alcoholic extraction by fermentation, ultrasound extraction (sonication), microwave-assisted extraction, liquid and phytonic extraction. Among all these extraction process, liquid extraction is effective and widely used. In order to isolate all the active compounds present in a medicinal plant, the solvents with polarity in increasing range are required. As the compound is heat labile, temperature of drying and extraction is generally desirable in mild range.

Separation Techniques used in Purification and Characterisation of Bioactive Compounds: The crude extract of medicinal plant usually has various types of metabolites bearing different polarities. Therefore it is a big challenge to separate the particular compound on the basis of its identification and characterization. Various techniques were implemented for separation and purification processes, these include chromatographic methods of separation such as TLC, Column chromatography as well as HPLC. The isolated pure compound is further used to determine the structure and its biological activity. Chromatogram is being generated through TLC and HPLC that is used to characterize the multi-component constituents as a fingerprint [10].

Thin Layer Chromatography (TLC): Thin-layer chromatography (TLC) is the separation technique where two or more compounds or ions are distributed between two phases, one which is moving and the other which is stationary. These two phases are mainly solid-liquid, liquid-liquid or gas-liquid. Among these, TLC is a chromatographic separation technique where solid-liquid phase is used. In this method, the components of a mixture are separated using a thin stationary phase supported by an inert backing (polar absorbent). It acts on adsorption principle where a compound has different affinities for the mobile and stationary phases, and this affects the speed at which it migrates and finally separated in various spots. Finally, the distance travelled by the spot is determined by retention factor (R_f) [11]. The other tests involve usage of phytochemical screening reagents, spraying these reagents on sample causes colour changes according to the constituents present in plant extract which can be observed under UV light [13]. To detect specific types of compounds, many spray reagents like chromogenic and fluorogenic reagents are available in literature [12, 14]. Spray reagents are specific to particular type of compound, or particular class of compounds. After applying to the plate, they react differently to different compounds, and produce different colour that indicate specific class of compounds [12]. This method is efficient, easy, reliable and reproducible [15].

Fourier Transform Infrared Spectrometry (FTIR): FTIR has proven to be a valuable tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plants extract [16, 17]. In addition, FTIR spectra of pure compounds are usually so unique that they are like a molecular "fingerprint". For most common plant compounds, the spectrum of an unknown compound can be identified by comparison to a library of known compounds. In FTIR, vibrations of bonds within chemical functional groups of a compound and generates a spectrum which is used for generation fingerprint of a biochemical metabolite. IR spectra of primary and secondary metabolites of plant samples can be used to detect the minor changes in them [18, 19]. In the current days particularly in phytochemistry, FTIR technique has been exercised to spot the structure of certain plant derived secondary metabolites [20, 21, 22]. FTIR has become one of the most important methods in identification of chemical constituents of the unknown sample and elucidate the structure of compounds, and has been made as a one of the requisite method of Pharmacopoeias of many countries to identify medicines [23].

Identification of the Structure of the active Constituents by Nuclear Magnetic Resonance (NMR) Spectroscopy: Natural products like, plant extracts can offer many opportunities for discovery of new drug because of their excellent chemical diversity. Medicinal herbs offer important constituents to food supplements, nutraceutical, modern medicines, folk medicines and lead compounds for synthetic drugs [24, 25]. Medicinal plants are screened for novel compounds with bioactivity are the sole remedy since, plant based drugs are biodegradable safe and have fewer side effects. Therefore the present study was undertaken to isolate and elucidate certain phenolic compounds from the plant leaf extract. Molecular structure of compound based on the chemical environment of the magnetic nuclei like ^{1}H, ^{13}C, 2D NMR, *etc*., are determined at minute concentration using NMR spectroscopy. It is the most important non-destructive technique in deciphering the molecular structure of biochemical and chemical compounds. NMR spectroscopy uses a strong radio frequency (R_f) pulse which excites the entire range of frequencies of a given nuclei whose time response is known as free induction decay (FID) containing every information. NMR spectrum is given by Fourier transformation of FID. This technique is used in JEOL GSX 400 NB FT-NMR spectrometer. The light spectra of samples containing less abundant nuclei like ^{1}H, ^{13}C, 2D NMR, etc., are thus easily obtained.

Liquid Chromatography Coupled Mass Spectrometry (MS): Because of the chemical nature of different constituents in medicinal plants only one an analytical technique does not have the capability in profiling all secondary metabolites [26]. Using chromatographic separation with mass spectrometry (MS detection). The chemical screening of plants crude extracts can be performed. Additional information can be obtained through MS, complementary to chromatographic process and DAD detection that helps in identification and specificity of detection [27]. MS is well known for its unique detection technique, revealing all the compounds present in plant extracts as particular molecule weight is obtained by natural compound [28]. MS coupling with high performance liquid chromatography offers, great selectivity and sensitivity, and helps in effective analysis of complex matrices. LC/UV/MS is commonly used as first step in chemical profiling of crude plant extracts. LC-MS is a well-established, powerful technique whereas, it has high range of ionization models for analysis of polar, nonvolatile semi polar and to an extent a polar compound. Ionized molecules in mass spectrometry and their fragmented ions are separated based on their mass by change ratio (M/Z) and the relative detector responses from each M/Z ratio is plotted in mass spectrum.

MATERIALS AND METHODS:

Collection of Plant Material: *Stachytarpheta urticifolia* leaves were collected from Aaraku Valley, Visakhapatnam, Andhra Pradesh, India. The plant identification was approved by Damaku rai village of Aaraku valley near Ananthagirimandal, Visakhapatnam. The *Stachytarpheta urticifolia* plant was taxonomically approved and authenticated by Dr. P. Venkaiah, Professor of the Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India. The *S. urticifolia* plant samples were deposited in the herbarium, Department of Botany, Andhra University. The

plant reference number assigned to *S. urticifolia* is 28083 is obtained from the department. The fresh leaves of *S. urticifolia* were dried on air at room temperature in shade approximately for 2 weeks. Once the leaves were well dried, they were powdered and stocked in glass bottle in the dark for further analyses.

Plant Extract Preparation: Leaf of the plant under study, *S. urticifolia* was taken to laboratory and washed using running tap water then blotted to dryness using filter paper and allowed to dry in shade on laboratory benches. The completely dried plant material was then grounded to powder using hand mill. Powdered plant material 100 g was extracted successively in hexane, ethyl acetate and methanol by using 250 ml of each solvent by a Soxhlet apparatus for 24 h. Each solvent extract was concentrated in vacuum at about 40 °C using a rotary evaporator (Superfit, PBU 6D model). The crude extraction thus obtained was stored in a refrigerator at -20 °C until use.

Reagents/Chemicals used in the Investigation: DPPH (1, 1-diphenyl-2-picrylhydrazyl) was bought from Sigma Chemical Company, India, Silica gel G for TLC (Qualigen chemicals, India) Silica gel for column chromatography (100-200 mesh) (Qualigen chemicals, India). All the other chemicals and reagents used are of analytical grade.

Preliminary Phytochemical Analysis: The plant extracts obtained in different solvents were analyzed for occurrence of alkaloids [29], tannins, phenols and flavonoids [30], steroids [31], saponins [32] and cardiac glycosides [33, 34].

Separation through Column Chromatography: The fractionation of methanol solvent extract (11.59 g) and ethyl acetate solvent extract (3.32 g) was done on in column using silica gel (100-200 mesh) separately by n-hexane was used in packing column. Column was eluted by increasing polarity first with hexane and then increasing the quantity of ethyl acetate in hexane, methanol in ethyl acetate and finally water in methanol. Each fraction of 10 ml volume was collected and the fractions were subjected to thin layer chromatography based on which similar fractions were pooled together. TLC chromatogram was visualized by spraying iodine vapors. The pooled fractions are six with ethyl acetate and were labeled as EaA to EaG. Similarly ten methanol fractions were labeled as MeA to MeJ. These fractions were then further analysed for phytochemicals using TLC method. The fractions those gave similar R_f value containing spots in TLC were again pooled into 5 major fractions labeled as F1 to F5.

Usage of Thin Layer Chromatography (TLC) for Separation of Secondary Metabolites: The above 5 major column fractions, F1 to F5 were subjected to thin layer chromatographic analysis, to identify the specific chemical constituents using chemical tests. Wide varieties of biochemicals are separated for qualitative, quantitative assay and preparative work using thin layer chromatography [35]. Based on differences in R_f values of compounds the chromatographic separation occurs by distributing compounds into mobile and stationary phase. Plates precoated with silica gel G were purchased from Qualigens, India and separated using appropriate solvent system.

Thin Layer Chromatographic Study for Phenols: Methanol and water at the ration of 6:3 solvent system was used for the separation of phenols in TLC. The R_f values and colour of phenols were noted under visible light which is sprayed with ferric chloride reagent [36].

Analysis for Saponins using Thin Layer Chromatography: Chloroform and methanol at the ration of 1.2:0.2 solvent system was used for the separation of saponins in TLC. The R_f values and colour of saponins were noted under visible light which is sprayed with vanillin hydrochloric acid reagent [37].

TLC Study of Alkaloids: Chloroform and methanol at the ratio of 3:1.3 solvent mixture is used to separate alkaloids in TLC. The R_f values and colour of the separated alkaloids were noted under visible light after spraying with Dragendorff's reagent [37].

Separation of Sterols through TLC: Chloroform, glacial acetic acid, methanol and water at a ratio of 64:34:12:8 solvent system is used for separation of sterols in TLC. The R_f values and colour of the separated spots were noted under visible light after spraying the plates with anisaldehyde - sulfuric acid reagent and heating at 100 °C for 6 min [38].

TLC Study of Glycosides: Ethyl acetate-Methanol-water (80:10:10) solvent mixture was used to separate glycosides. The R_f values and colour of separated glycosides were noted in ultraviolet light (UV 254 nm) by heating the developed plate at 110 to 120 °C for 5 minutes then spraying with Marquis reagent on to the plate in hot condition.

Assay of Free Radical Scavenging Activity using DPPH: Methanol, hexane and ethyl acetate fractions obtained from column chromatography were screened for radical scavenging activity with DPPH. The concentrated extracts were dissolved in 5% dimethyl sulfoxide (DMSO) at concentrations 10-1000 µg/ml to assay DPPH activity. DPPH radical scavenging activity was measured according to the method of [39]. Plant extract at various concentrations were mixed with aliquot of 3ml of 0.004% DPPH solution in ethanol and incubated at 37 °C for 30 min. and optical density of the test mixture was read at 517 nm. All tests were conducted in three replications, the results were then averaged.

The percentage of inhibition of DPPH radical was measured as

$$\text{Percentage of inhibition} = A_0 - A_1 \times 100 / A_0$$

Whereas A_0 is the optical density of the control and A_1 is the optical density with addition of plant extract/ ascorbic acid.

The fifty percentage inhibition concentration (IC_{50}) value i.e. the half maximal inhibition concentration of sample required to reduce DPPH free radical was obtained by linear regression analysis of dose-response curve plotting between percentage inhibition and concentrations by considering the lowest obtained inhibition value as 0% and maximum obtained inhibition value as 100%.

Purification of Active Column Fraction by Semi-Preparative HPLC: Chromatographic separation was carried out using Jasco semi-preparative HPLC system equipped with two pumps PU- 2087 *plus* connected to mixture, 2ml fixed loop (Rheodyne) injector and single wavelength UV-detector Jasco 2075*plus*. Preparative HPLC is very similar to analytical HPLC, instead of injecting a small amount of product stacked injections are given to maximize the resolution in order to maximize the purification *i.e.*, in particular to purify a single component from a complex mixture. Biologically active fifth fraction (F5) that was obtained from column pooled fractions was subjected to semi-preparative HPLC analysis on a Develosil, C18, 50 × 20mm, 5µ RP-HPLC column. Chromatographic separation was obtained by isocratic elution using acetonitrile and 5mM ammonium acetate (NH_4OAc) in the ratio of (90:10). Dry powdered sample extract (10 mg) was dissolve in 5ml of acetonitrile in 25 ml volume metric flask and diluted up to mark with same solvent. Buffer was prepared by dissolving 0.3854 g of NH_4OAc in 1 lit of Water, sonicated and filtered using 0.45µ filter paper. The flow rate was 18 ml/min. The eluting compounds were monitored continuously using a diode array detector at 205.00 ± 4.00 nm.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis of the Isolated Major Fraction of HPLC: The unique functional group using FTIR spectroscopy (The IRAffinity-1S FTIR spectro-photometer) was detected using purified compound. The functional group detections were recorded in the wavenumber range of 500–4000 cm^{-1} at a resolution of 4 cm^{-1}. Sample was mixed with KBr powder at thermo Nicolet nexus 670 spectro-photometer.

^{1}H NMR and ^{13}C NMR Spectra: The ^{1}H NMR and ^{13}C NMR techniques were carried at Laila Impex R & D center Vijayawada, Andhra Pradesh. These techniques were used to identify the bioactive compound present in the extracts responsible for the structural identification. Spectra were run on Bruker spectrometers operating at 10 MHz for 1H NMR and 200MHz for ^{13}C NMR using DMSO and D_2O (methanol) solvents. The chemical shifts were given in ppm (δ) and coupling constant was expressed in Hertz.

Mass Spectrometric Analysis of the Isolated Major Fraction of HPLC: The compound purified by HPLC was subjected to mass spectrometric analysis for structural determination. LC-MS analysis was carried out on a LC (Shimadzu-prominence) equipped with PDA detector using mobile phase, acetonitrile and water in the ratio of 90:10 with total flow of 0.30 mL/min whereas MS (Shimadzu-LCMS-2010 EV) was used with ESI

(Electrospray ionization) interface at CDL Temperature 250 °C, Heat Block temperature 200 °C and nebulising gas flow of 1.5 L/min. The detector Voltage was 1.5kV.

RESULTS:

Preliminary Phytochemical Assay: The phyto-chemical screening achieved on the crude extracts of *S. urticifolia* leaf revealed the occurrence of cardiac glycosides, saponins, tannins & phenolics and steroids **Table 1**. Glycosides present only for methanol extract absent in ethyl acetate and hexane extract. Alkaloids only present ethyl acetate extract and absent in methanol and hexane extracts. Phenolics, tannins, saponins and steroids were present in all three extracts.

TABLE 1: RESULTS OF PRELIMINARY PHYTO-CHEMICAL SCREENING OF THREE CRUDE PLANT EXTRACTS OF *S. URTICIFOLIA* LEAF

Phytochemical tests	HX	EA	ME
Tannins and phenols	+	+	++
Steroids	-	+	-
Alkaloids	-	+	-
Cardiac glycosides	-	-	+
Saponins	+	+	+

+ = presence; - = absence; HX = Hexane extract, EA = Ethyl acetate extract, ME = Methanol extract

Assay of Five Major Column Fractions for DPPH Radical Scavenging Activity: The five major fractions DPPH free radical scavenging activity was analysed **Fig. 1**. The IC_{50} values obtained for DPPH inhibition of five major column fractions are 1149.06 µg/ml, 1028.17 µg/ml, 922.93 µg/ml, 984.04 µg/ml, 520.72 µg/ml, and 450.04 µg/ml, for first fraction (F1), second fraction (F2), third fraction (F3), fourth fraction (F4), fifth fraction (F5) and ascorbic acid, respectively. The results revealed that fraction (F5) are having higher percentage inhibition of DPPH absorbance and lower IC_{50} value of 512.54 µg/ml. When compared to IC_{50} value of ascorbic acid 450.04 µg/ml.

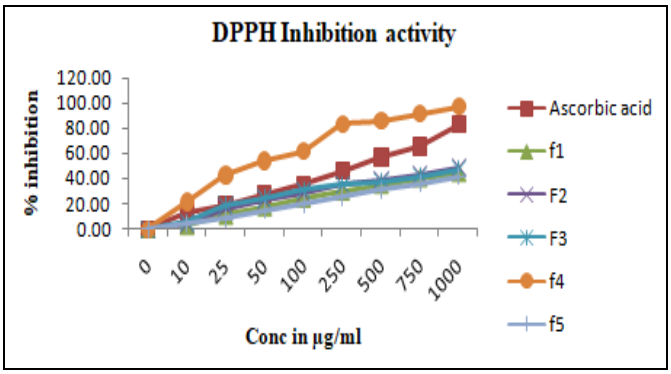

FIG. 1: PERCENT INHIBTIONOF DPPH RADICAL BY FIVE MAJOR COLUMN FRACTIONS OF *S. URTICIFOLIA* AND ASCORBIC ACID STANDARD

Phytochemical Screening of Five Major Column Fractions: The phytochemical screening of five major column fractions (F1 to F5) by TLC study confirmed the presence of phenols, saponins, steroids, and alkaloids in different fractions based on the difference in solvent elution mixtures which may be responsible for their antioxidant activities **Table 2.**

TABLE 2: DETECTION OF PHYTOCHEMICALS IN FIVE MAJOR COLUMN FRACTIONS BY THIN LAYER CHROMATOGRAPHY STUDY

Compounds	Column fractions									
	F1		F2		F3		F4		F5	
	Color	R_f	Color	R_f	Color	R_f	Color	R_f	Color	R_f
Phenols	No spot	-	Blue	0.48	Blue	0.70	Green	0.90	Blue	1.20
Saponins	Gray	0.67	No spot	-	Gray	0.78	Gray	0.81	Gray	0.94
Steroids	Pink	0.91	Pink	0.95	No spot	-	No spot	-	No spot	-
Alkaloids	Brown	0.52	Brown	0.80	Brown	0.92	No spot	-	No spot	-

R_f = Retardation Factor

Purification of Active Column Fraction by Semi-Preparative HPLC: Specific TLC methods of phytochemical screening revealed complex mixtures of plant constituents with different polarities **Table 2**. Which lead to purification of fraction by semi-preparative HPLC. Among the five-column fractions, fraction five (F5) obtained with solvent elution mixtures of ethylacetate and methanol possess higher percentage inhibition and lower IC_{50} value. Then the fraction five (F5) was selected for further purification through semi-preparative HPLC **Fig. 2**. HPLC chromatograms indicated that fraction (F5) got separated into two different fractions (such as fraction I & II). Based on the area of the percentage of peaks shown in **Fig. 2** and **Fig. 3**. Fraction-2 was selected for structural analysis. The description of HPLC fraction-I and fraction-II was shown in **Tables 3** & **4.**

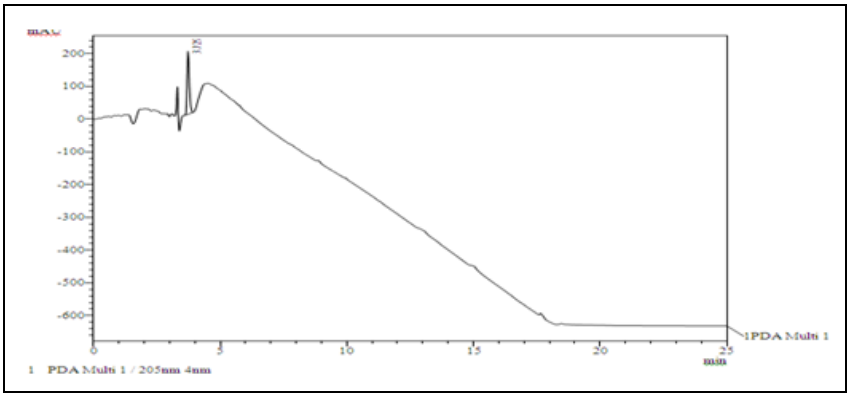

FIG. 2: SEMI-PREPARATIVE HPLC CHROMATOGRAM OF SEPARATED FRACTION-I AT 205nm ± 4.00nm

TABLE 3: CHROMATOGRAM REPORT OF HPLC FRACTION-I

Index	Retention Time [Min]	Peak Area [mV.Sec]	*Res. USP	**NTP USP	Peak Area [%]
1	3.729	1211910	6446.413	1.000	100.000
Total		1211910			100.000

* Res. USP- United States Pharmacopeia Resolution; **NTP USP- Number of Theoretical Plates, United States Pharmacopeia method

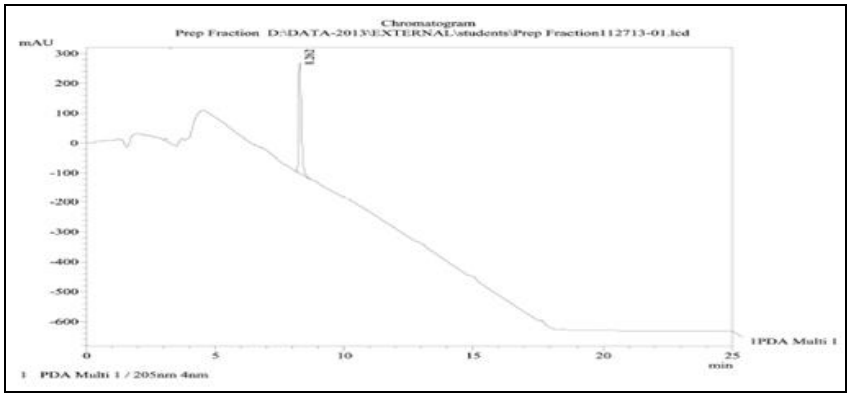

FIG. 3: SEMI-PREPARATIVE HPLC CHROMATOGRAM OF SEPARATED FRACTION –II AT 205 nm± 4.00 nm

TABLE 4: CHROMATOGRAM REPORT OFHPLC FRACTION-II

Index	Retention Time [Min]	Peak Area [mV.Sec]	*Res. USP	**NTP USP	Peak Area [%]
1	8.262	2814723	24697.479	1.000	94.185
Total		2814723			94.185

* Res. USP- United States Pharmacopeia Resolution; **NTP USP- Number of Theoretical Plates, United States Pharmacopeia method

DPPH assay of HPLC Fractions-I & II: The free radical scavenging activity of DPPH of the pure compound in fraction-I & II was assayed. The results were shown in the figure revealed that the purified compounds are having higher percentage inhibition of DPPH absorbance. Among fractions I and II, fraction -II exhibited the highest free radical scavenging activity **Fig. 4**. Hence fraction- II has been selected for further analysis.

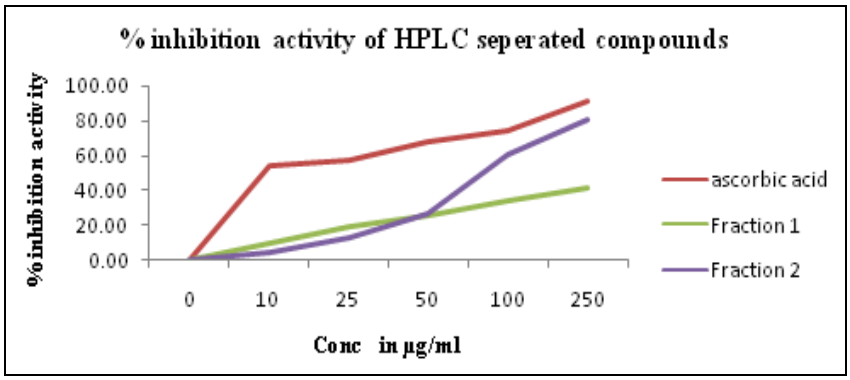

FIG. 4: RESULTS FOR PERCENTAGE INHIBITION ACTIVITY OF HPLC SEPARATED COMPOUNDS

Structural Elucidation of Purified Active Compound of HPLC Fraction-II: In comparison with the above two fractions, it was decided to continue with HPLC fraction-II for structural elucidation because of highest purity with peak area 94.184% and highest percentage inhibition of DPPH activity.

FTIR Analysis: The FTIR spectrum was used to identify the functional group of the active component based on the peak value in the region of infrared radiation. The methanolic leaf extract of *S. urticifolia* was passed into the FTIR and the functional groups of the component were separated based on its peak ratio. The results of *S. urticifolia*, FTIR analysis confirmed the presence of Alcohols or Phenols, Alkanes, and Alkenes, the compounds which show major peaks at 3444.14, 2925.92 and 1385.32 and minor peaks at 1204.36-C-C ring and 1034.30- C-OH bending respectively **Fig. 5; Table 5**.

TABLE 5: FT-IR PEAK VALUE AND FUNCTIONAL GROUPS OF METHANOL LEAF EXTRACT OF *S. URTICIFOLIA* (HPLC FRACTION-II)

S. no.	Peak Value cm^{-1}	Functional groups
1	3444.14	OH (Alcohols, Phenols)
2	2925.92	CH_3 Stretching (Alkanes)
3	1626.53	C-OH
4	1385.32	CH_2 bending
5	1204.36	C-C ring
6	1034.30	-C-OH- bending vibration

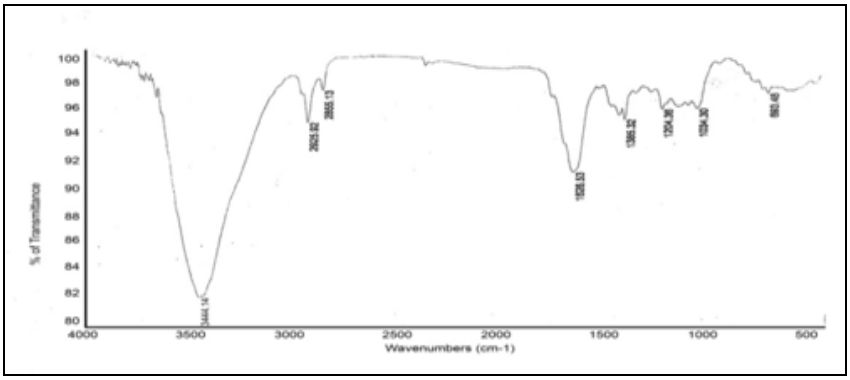

FIG. 5: FT-IR SPECTRUM OF S.URTICIFOLIA LEAF (HPLC FRACTION-II)

Proton NMR and D_2O Exchange: The proton NMR and D_2O Exchange spectrum of purified fraction-II from HPLC was obtained **Fig. 6**. The proton NMR exhibited single proton at 4.16 ppm is due to olifinic HC=CH Proton. D_2O Exchange exhibited, 4.218 ppm -OH group is disappeared, 2.512 ppm is due to CH2 protons, and 1.982 ppm is due to CH3 proton **Fig. 6a**.

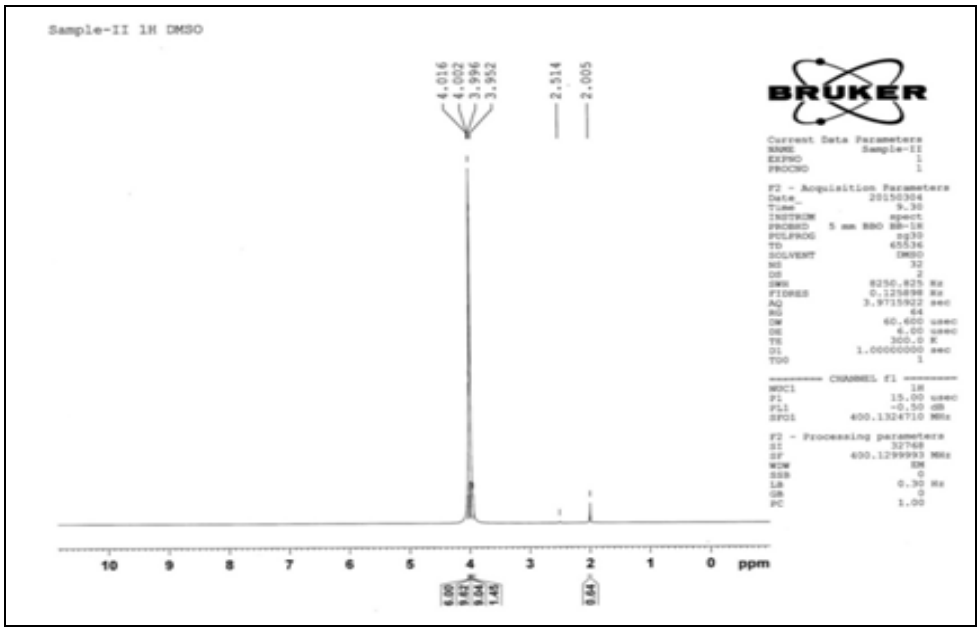

FIG. 6: ^{1}H-MR SPECTRA OF ISOLATED COMPOUND FROM *S. URTICIFOLIA* (HPLC FRACTION-II)

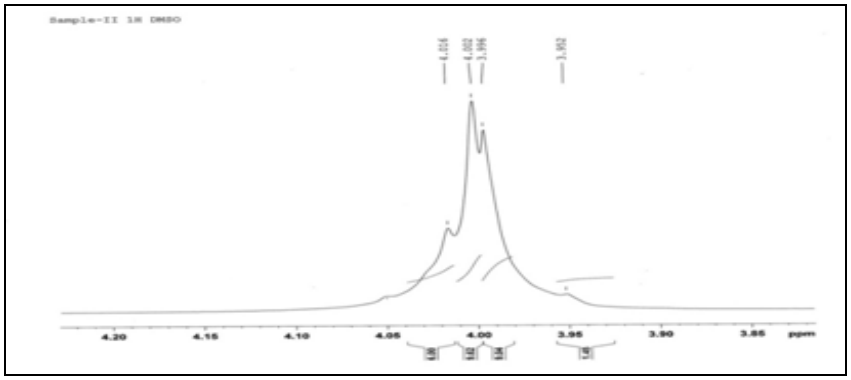

FIG. 6A: ^{1}H-MR SPECTRA OF ISOLATED COMPOUND FROM *S. URTICIFOLIA* (HPLC FRACTION-II)

^{13}C NMR: ^{13}C NMR Spectrum of purified HPLC fraction-II exhibited at 119.03 ppm is due to C=C carbon, 2.11ppm is due to CH_2 carbon, and 1.74ppm is due to CH_3 carbon respectively in **Fig. 7a** and **7b**.

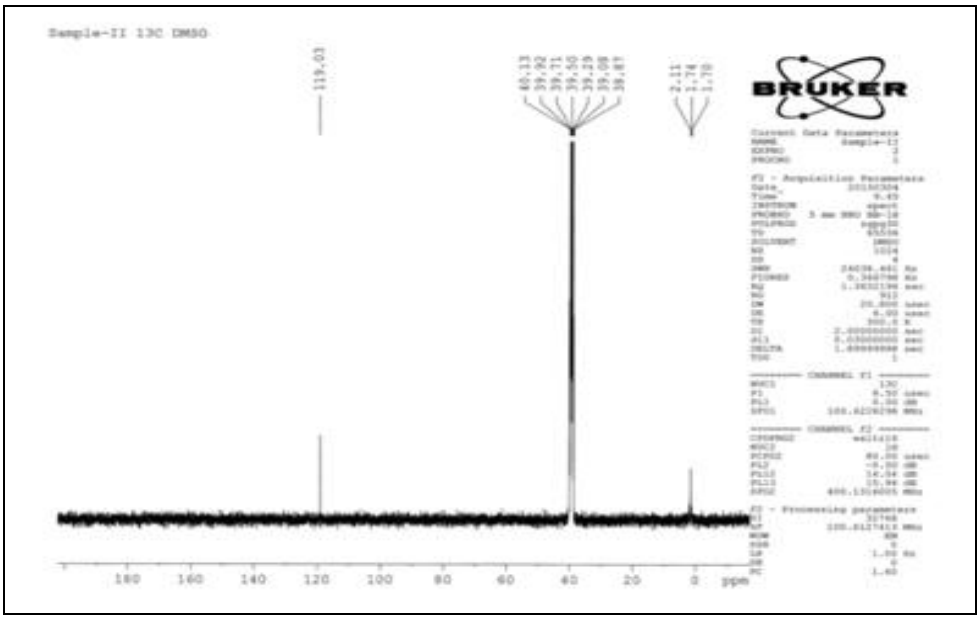

FIG. 7A: ^{13}C-NMR SPECTRA OF ISOLATED COMPOUND FROM *S. URTICIFOLIA* (HPLC FRACTION-II)

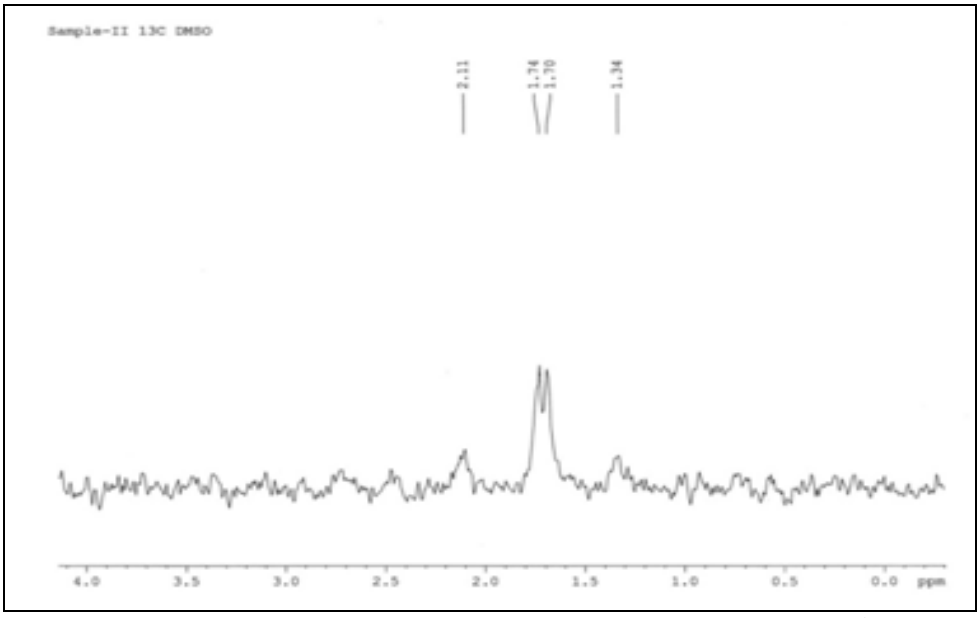

FIG. 7B: ^{13}C-NMR SPECTRA OF ISOLATED COMPOUND FROM S.URTICIFOLIA (HPLC FRACTION-II)

Mass Spectrum: The Liquid chromatography/ Mass spectrometry (LC/MS) chromatogram of the active pure compound of HPLC fraction-II was shown in **Fig. 8**. The compound was analyzed in positive ion mode. The MS fragmentation ions at m/z showed a main compound peak at 560.4 and high intensity (baseline) peak at 427.0.

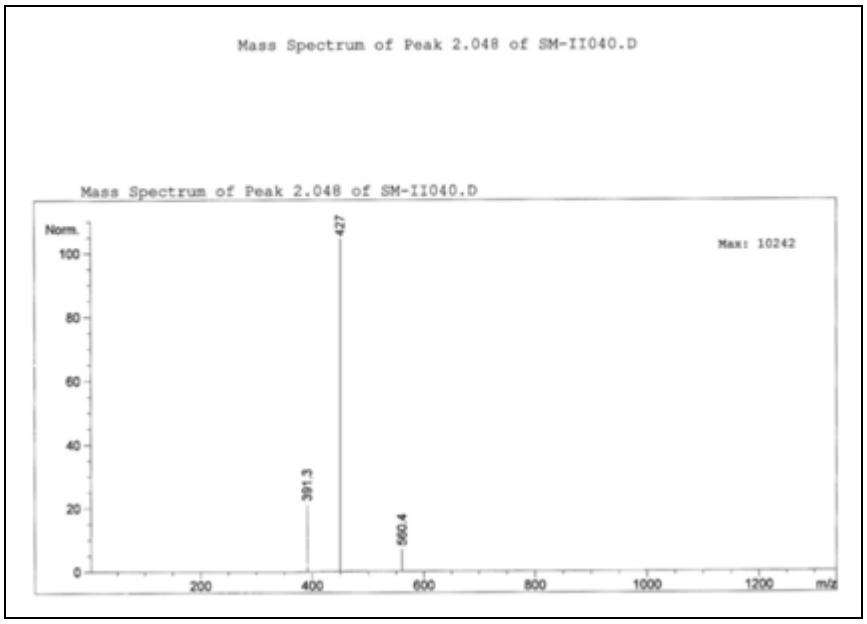

FIG. 8: MASS SPECTRUM (MS) OF HPLC FRACTION-II)

The structure was predicted and elucidated based on the MS fragmentation, ^{1}H proton and ^{13}C NMR. The stable compound with base peak m/z 427 was identified as having molecular formula $C_{30}H_{50}O$ and the structure was proposed as pentacyclic triterpenoid (Lupeol) **Fig. 9**. Some more spectral studies are required for more structural conformation. By the above spectral studies, we predict the presence of pentacyclic triterpenoid (Lupeol).

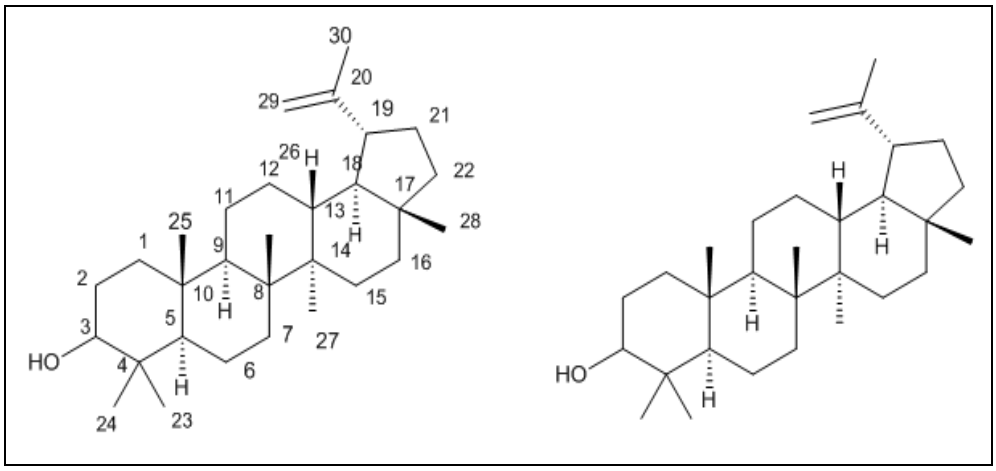

FIG. 9: THE STRUCTURE OF SECONDARY METABOLITES ISOLATED PENTACYCLIC TRITERPENOID (LUPEOL) Mol wt = 426.72 = 427; Mol formula = $C_{30}H_{50}O$

DISCUSSION: Plants are important source of a wide range of bioactive molecules which make them good source for different types of drugs. Biological activities of the biochemical compounds present in herbal drugs and their structures can be determined by various biochemical techniques. The process of isolation of pure bioactive components from medicinal plants is a long and tiresome process. The new useful compounds derived from plants and their screening for chemical structure are performed for isolation of new targets with potential activities. This leads to identifying known metabolites present in plant extracts at earlier stages during separation, which is an economically

very important step. Among biochemical techniques, chromatographic techniques are extensively used in the isolation and purification of bioactive compounds from plants. TLC is carried out by using solvent systems such as hexane, acetone, ethyl acetate, methanol, chloroform, and formic acid in various combinations and ratios such that they range from polar to non-polar. Except for ethylacetate and methanol solvent system gave successful results in chromatographic separation of the components. The ideal range of partition coefficient (K) achieved by using a suitable solvent system that suits for each target compound plays an important role in separation biomoleculae in chromatographic system [40]. Initial separation and detection of plant constituents are usually done by TLC which is a simple and cost-effective method with less equipment, easy to run, highly reproducible. HPLC technique is preferred as it is efficient in the separation of biochemicals with sensitivity of detection, good selectivity.

Chromatographic techniques play a vital role in the analysis of phytochemists for their efficient localization and quick characterization of natural compounds derived from plants. Toxicity of solvents, the reactivity of compounds, and solubilitiy are the important parameters that should be taken care of while selecting solvent systems in separation of biochemicals. The chromatographic techniques are easy sensitive and cost-efficient techniques in the separation zof secondary metabolites [41, 42]. In the current study, TLC chromatogram of *S. urticifolia* plant extracts exhibited the presence of phenolic compounds. Flavonoids and tannins belong to phenolic compounds that exhibit diverse biological properties like antibacterial and antioxidant activities [43]. Secondary metabolites like steroids, alkaloids, saponins, phenols present in plants might be responsible for bioactivity. Phenolic compounds are known to exhibit antioxidant activity. The methanol leaf extract of *S. urticifolia* exhibits strong antioxidant activity, which is attributed to the presence of phenolic compounds that possess free radical scavenging activity and multiple biological activities [44]. HPLC is one of the most important techniques used for the purification of bioactive compounds and their chemical characterization [45]. Most of the secondary metabolites present in plant cells perform specific roles in harmony in the defense mechanisms of the plants [46]. The methanol extract of *S. urticifolia* showed the occurrence of an olefin group of compounds using ^{1}H NMR analysis [47]. These compounds may be involved in antioxidant activity of the plant extract. A detailed structure of secondary metabolites and organic compounds in solutions can be determined by powerful spectroscopy, NMR [48]. The results of the current study showed the chemical basis for bioactive property and its therapeutic use of the plant by the tribal people can be ascribed to its adjustment in environmental stress and also the occurrence of phenolic compound (pentacyclic triterpenoid) as phenolics are known to scavenge free radicals along with various biological properties including antioxidant and anticancer activity.

CONCLUSION: The proposed structure is based on the information acquired by the LC/MS, FTIR, Proton NMR, and ^{13}C NMR analysis. So, it is therefore considered only as a Predicted structure of the active compound is pentacyclic triterpenoid (Lupeol).

ACKNOWLEDGEMENT: The author R. Sreelatha, Post-doctoral Fellow, is thankful to the University Grants Commission, New Delhi, India, for the financial support. The authors are also thankful to the Management of Gandhi Institute of Technology and Management (GITAM), (Deemed to be University) for their support.

CONFLICTS OF INTEREST: The authors declared no conflicts of interest.

REFERENCES:

1. Huang X, Kong L, Li X, Chen X, Guo M and Zou H: Strategy for analysis and screening of bioactive compounds in traditional Chinese medicines. Journal Chromatography B Analytical Technologies Biomedical Life Sciences 2004; B.812: 71-84.
2. Ong ES: Extraction methods and chemical standardization of botanicals and herbal preparations. Journal of Chromatography 2004; B.812: 23-33.
3. Hemavathy A, Shanthi P, Sowndharya C, ThiripuraSundari S and Priyadharshni K: Extraction and Isolation of Bioactive Compounds from a Therapeutic Medicinal Plant - *Wrightia tinctoria* (Roxb.) R. Br. International Journal of Pharmacognosy and Phytochemical Research 2019; 11(3): 199-204.
4. Ingle KP, Deshmukh AG, Padole DA, Dudhare MS, Moharil MP and Khelurkar VC: Phytochemicals: Extraction methods, identification and detection of bioactive compounds from plant extracts. JPP 2017; 6(1): 32-36.

5. Gurumurthy H, Krishna V, Ravikumar Patil H and Babu SP: A preliminary Phytochemical Studies on the seeds of *Celastrus paniculata*, Willd. The Internet Journal of Pharmacology 2008; 6(105): 580-703.
6. Eloff JN: Which extractant should be used for the screening and isolation of antimicrobial components from plants. Journal of Ethnopharmacology 1998; 60: 1-8.
7. Ncube NS, Afolayan AJ and Okoh AI: Assessment techniques of antimicrobial properties of natural compounds of plant origin, current methods and future trend. African Journal of Biotechnology 2008; 7: 1797-1806.
8. Dang and Raman: Role of antinutrient metabolites of plant on production of secondary metabolites and human health. Ann. Phytomed 2018; 7(1): 1-4.
9. Majeed M: Evidence-based medicinal plant products for the health care of world population. Ann Phytomed 2017; 6(1): 1-4.
10. Schulz V, Hansel R and Tyler VE: Rational Phytotherapy: A Physician's Guide to Herbal Medicine. 4th edition. Berlin .Springer-Verlag 2001; 172-73.
11. Hassan IA, Nasiru, Malut AM, Abdulkadir I and Ali AS: Phytochemical studies and thin layer chromatography of leaves and flower extracts of *Senna siamea* Lam. for possible biomedical applications. J Pharmacog Phytochem 2015; 7(3): 18-26.
12. Wagner R and Bladt S: Plant Drug Analysis: A Thin Layer Chromatography Atlas, 2nd edition. Newyork. Springer-Verlag Berlin Heidelberg 1996; 349-54.
13. Sasidharan S, Chen Y, Saravanan D, Sundram KM and Latha Y: Extraction, isolation and characterization of bioactive compounds from plants' extracts. The African Journal of Traditional, Complementary and Alternative Medicines 2011; 8: 1-10.
14. Sherma J: Thin-layer chromatography in food and agricultural analysis. Journal of Chromatography A 880 2000; 129-47.
15. Moffat CA: London: Pharmaceutical Press; Clarke's analysis of drugs and poisons 2001; 392.
16. Eberhardt TL, Li X, Shupe TF and Hse CY: Chinese Tallow Tree (*Sapium sebiferum*) utilization: Characterization of extractives and cell-wall chemistry. Wood Fiber Sci 2007; 39: 319-24.
17. Hazra KM, Roy RN, Sen SK and Laska S: Isolation of antibacterial pentahydroxy flavones from the seeds of Mimusopselengi Linn. Afr. J. Biotechnol 2007; 6(12): 1446-49.
18. McCann MC, Hammouri M, Wilson R, Belton P and Roberts K: Fourier transform infrared microspectroscopy is a new way to look at plant cell walls. Plant Physiol 1992; 100: 1940-47.
19. Surewicz WK, Mantsch HH and Chapman D: Determination of Protein Secondary Structure by Fourier Transform Infrared Spectroscopy. A Critical Assessment. Biochemistry 1993; 32(2): 389-93.
20. Yang J and Yen HCE: Early salt stress effects on the changes in chemical composition in leaves of ice plant and arabidopsis. A Fourier Transform Infrared Spectroscopy Study. Plant Physiology 2002; 130:1032-1042.
21. Stehfest K, Toepel J and Wilhelm C: The application of micro-FTIR spectroscopy to analyze nutrient stress-related changes in biomass composition of phytoplankton algae. Plant Physiology and Biochemistry 2005; 43: 717-26.
22. Ivanova DG and Singh BR: Nondestructive FTIR monitoring of leaf senescence and elicit in induced changes in plant leaves. Biopolymers 2003; 72(2): 79-85.
23. Liu H, Sun S, Lv G and Chan KKC: Study on Angelica and its different extracts by Fourier transform infrared spectroscopy and two-dimensional correlation IR spectroscopy. Spectrochimica Acta Part A 2006; 64: 321-26.
24. Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB and Kent J: Biodiversity hotspots for conservation priorities Nature 2000 403: 853-58.
25. Pavrez M, Mahboob H K, Zahuul I and Shek MH: Antimicrobial activities of the petroleum ether, methanol and acetone extracts of *Kaempferia galangal* rhizome. J Life Earth Sci 2005; 1: 25-29.
26. Wolfender JL, Ndjoko K and Hostettmann K: Liquid chromatography with ultraviolet absorbance-Mass spectrometric detection and with nuclear magnetic resonance spectroscopy: A powerful combination for the online structural investigation of plant metabolites. Journal of Chromatography A1000: 2003; 437-55.
27. Poole CF: The Essence of Chromatograph. Amsterdam. Elsevier 2003; 80-83.
28. Xian-Guo H: On-line identification of phytochemical constituents in botanical extracts by combined high-performance liquid chromatographic–diode array detection–mass spectrometric technique. Journal of Chromatography. A- 880, 2000; 203-32.
29. Trease GE and Evans WC: Phamacognos 13thEdBalliere-Tindal: London 1989; 176-90.
30. Sofowora LA: Medicinal plants and traditional medicine in Africa. Spectrum Books Ltd. Ibaban 1993; 55-71.
31. Kumar GS, Jayaveera KN, Kumar CKA, Sanjay UP, Swamy BMV and Kumar DVK: Antimicrobial effects of Indian medicinal plants against acne-inducing bacteria. Trop. J. Pharm. Res 2007; 6: 717-23.
32. Parekh J and Chanda SV: *In-vitro* antimicrobial activity and phytochemical analysis of some Indian medicinal plants. Turk J Biol 2007; 31: 53-58.
33. Ajiboye BO, Ibkun EO, Edobor G, Ojo AO and Onikanni SA: Qualitative and quantitative analysis of phyto-chemicals in *Senecion biafrae* leaf. International Journal of Invention and Pharmaceutical Sciences 2013; 1(5): 428-32.
34. Jaradat N, Hussen F and Ali AA: Preliminary phytochemical screening, quantitative estimation of total flavonoids, total phenols and antioxidant activity of *Ephedra alata* Dencne. Journal of Materials and Environmental Science 2015; 6(6): 1771-78.
35. Stahl E: In Thin-Layer Chromatography. New York. Academic Press 1965; 5-14.
36. Mehta S, Rana P and Pooja S: Phytochemical screening and TLC Profiling of various extracts of *Reinwardtia indica*. International Journal of Pharmacognosy and Phytochemical Research 2017; 9(4): 523-27.
37. Karthika K and Paulsamy S: TLC and HPTLC fingerprints of various secondary metabolites in the stem of the traditional medicinal climber, *Solena amplexicaulis*, Indian J Pharm Sci 2015; 77(1): 111-6.
38. Mallikharjuna PB, Rajanna LN, Seetharam YN and Sharanabasappa GK: Phytochemical studies of *Strychnos potatorum* L.f. a medicinal plant. EJC 2007; 4(4): 510-18.
39. Braca A, Fico G, Morelli I, De Simone F, Tome F and De Tommasi N: Antioxidant and free radical scavenging activity of flavonol glycosides from different *Aconitum* species. J Ethnopharmacol 2003; 86(1): 63-67.
40. Ito Y: Golden rules and pitfalls in selecting optimum conditions for high-speed counter current chromatography. J Chromatography A 1065. 2005; 145-68.

41. Saraf A: Phytochemical and antimicrobial studies of medicinal plant *Costus speciosus* (Koen.). E-J Chem 2010; 7(1): 405-13.
42. Sadasivam S and Manickum A: Biochemical methods, New Age Intern (P) Ltd Publication 2005; 284-88.
43. Nagavani V and Ragahava RT: Evaluation of antioxidant potential and identification of polyphenols by RP-HPLC in *Michelia champacai* flowers. Adv Biol Res 2010; 4: 159-68.
44. Rievere C, Van Nguyen TH, Pieters L, Dejaegher B, Heyden YV and Minh CV: Polyphenols isolated from antiradical extracts of *Mallotusmet calfianus*. Phytochem 2009; 70: 86-94.
45. Springfield EP, Eagles PKF and Scott G: Quality assessment of South African herbal medicines by means of HPLC fingerprinting. J Ethnopharmacol 2005; 101: 75-83.
46. Patil PH and Surana SJ: Gastroprotective effect of *Eranthemum roseum* R. Br. Linn root extracts in albino rats. Int J Pharmacol Biol Sci. 2009; 3(1): 81-93.
47. Silverstein RM, Webster FX and Kiemle DJ: Proton NMR spectrometry. Spectrometric identification of organic compounds. USA: John Wiley and Sons, Inc, 2005; 127-204.
48. Lim GK: Chemical Constituents and Biological Activity of *Clausena excavate* (Rutaceae); University Putra Malaysia: 2005.

How to cite this article:
Regalagadda S and Challa MM: Isolation, Purification and characterization of bioactive compounds from *Stachytarpheta urticifolia* (Salisb.) Sims. Int J Pharm Sci & Res 2021; 12(11): 6037-49. doi: 10.13040/IJPSR.0975-8232.12(11).6037-49.