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Identification and Characterization of Bioactive Compounds of Leaves of *Justicia Gendarussa* Burm. F.

G. Shamili[1] and G. Santhi[2]*

[1,2] PG and Research Dept. of Botany, Kunthavai Naacchiyaar Govt. Arts College for Women, Thanjavur, India

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Abstract- The present investigation has been carried out to find the phytochemicals present in *Justicia gendarussa* leaves extract. The leaves of *Justicia gendarussa* were collected from Kadukaval in January 2018 Thanjavur, Tamil Nadu, India. The powder leaf was extracted with aqueous and 70% methanol for 24 hours and studied for FTIR, HPLC and NMR. A preliminary phytochemical testing of the leaves extract where than to identify the phytoconstiuents, which reveals that presence of tannin, saponin, flavonoids, steroids, terpenoids, triterpenoid, carbohydrate, anthroquinone, polyphenol, glycoside bioactive compound was isolated by column chromatography technique. The collected flavonoid fractions was purified by thin layer chromatography. FTIR, HPLC and NMR studies were carried out to find the structure of bioactive compound Quercetin. ^{1}H-NMR and ^{13}C – NMR that reveals the structure of flavonoids. The compound was identified as 3, 3', 4', 5, 7 – pentahydroxyflavanone by ^{1}H- NMR and ^{13}C – NMR. All these data obtained in the present investigation supported the rich source of phytochemicals preent in *J.gendarussa* leaves extract and therby traditional claim associated with *J. gendarussa* literature.

Keywords- *Justicia gendarussa*, phytochemical, FTIR, HPLC and NMR

I. INTRODUCTION

World Health Organization estimate over 80% of the people in developing countries depend on traditional medicines for their primary health care [1]. India is one of the largest producers of medicinal herbs and is rightly called the botanical garden of the world as it is sitting on a gold mine of well-recorded and traditionally well practiced knowledge of herbal medicine. Nearly 17,000 species of Indian flora, and 7500 species of higher plants are reported to possess medicinal value and in other countries it is projected about 7% and 13%. There are estimated to be around 25,000 effective plant-based formulations, used in folk medicine and known to rural communities in India[2].

The search for new molecules, nowadays, has taken a slightly different route where the science of ethno botany and ethno pharmacognosy are being used as guide to lead the chemistry towards different sources and classes of compounds[3]. Plant derived natural products hold great promise for discovery and development of new pharmaceuticals[4].

The search for biologically active compounds from natural sources has always been of great interests to researchers looking for new sources of drugs useful in various diseases. The indigenous population has developed vast knowledge on the uses of plant as traditional medicines to protect themselves and their crops, plants are known to contain numerous biologically active compounds which possess curative properties. Within a decade, there were a number of dramatic advances in analytical techniques including TLC, UV, NMR, FTIR, HPLC, HPTLC and GC-MS that were powerful tools for separation, identification and structure determination of phytochemicals[5]. Biological screening is necessary to provide a scientific basis for validating the traditional utilization of medicinal plants. A great number of screening programs are going on worldwide for new plant based bioactive molecules. NMR, HPLC and FTIR can be used to study traditional medicines and characterize the compound of interest. In the present study to investigate the bioactive compound in *J. gendarussa* leaves.

II. MATERIALS AND METHODS

Collection of Plant materials:

The leaves of *Justicia gendarussa* were collected from Kadukaval in January 2018 Thanjavur, Tamil Nadu, India. Identified with the help of Flora in Carnatic[6].

Preparation of alcoholic extract:

The leaves of *J. gendarussa* were first washed and dust was removed. The *leaves* were washed several times with

distilled water to remove the traces of impurities from the leaves. The leaves were dried at room temperature and coarsely powdered. The powder was extracted with aqueous and 70% methanol for 24 hours. The extract was stored in refrigerator until used.

Phytochemical screening:

Chemical tests were carried out on the alcoholic extract and on the powdered specimens using standard procedures to identify the constituents as described by Sofowara[7], Trease and Evans[8] and Harborne[9,10].

Quantitative assay:
Determination of total phenols by spectrophotometric method. Flavonoid determination by the method[11].

HPLC Analysis:

Flavonoids were analyzed by using HPLC method[12].

Column Chromatography:
Separation of flavonoid compound using in column chromatography adopted by the method[13].

Thin layer chromatography :

Thin layer Chromatography is based upon the principles of column and partition Chromatography. A thin layer of the stationary phase is formed on a suitable flat surface, such as glass. Separation of a mixture in this case is achieved over a thin layer of silica gel to which they are absorbed by different physical forces[9,10].

Fourier Transform Infrared (FTIR) spectroscopic analysis
FTIR spectrophotometer (Perkin Elmer Spectrophotometer system, USA) used to investigation of spectrum. A small amount of plant extract was respectively placed directly on sample holder of the infrared spectrometer with constant pressure applied and data of infrared absorbance, collected over the wave number ranged from 4000 cm^{-1} to 400 cm^{-1} and computerized for analyses by using the 21 CFR part 11 software. The reference spectra were acquired from the cleaned blank crystal prior to the presentation of each sample replicate. The peak values of FTIR were recorded.

NMR Spectroscopy
After the separation of plant extract to fractions using Column chromatography, Thin Layer chromatography was used for further purification of collected fraction. The NMR experiment was carried out in BRUKER-AMX400 MHz instrument with 5mg of purified compound in DMSO were used for ^{1}H NMR and ^{13}C NMR spectra recorded. Tetra Methyl Silane is used as the internal standard and chemical shifts are expressed in ppm.

III. RESULTS

Qualitative analysis

The phytochemical characters of the leaves of *J. gendarussa* investigated and summarized in Table 1 and Fig 2. The phytochemical screening aqueous extract of *J. gendarussa* leaves shows that the presence of saponin, flavonoids, steroids, terpenoids, triterpenoids, alkaloids, carbohydrate, anthroquinone, polyphenol, glycoside were absence of tannin, plobatannins and protein. Methanol extract of leaves *J. gendarussa* showed that the presence of tannin, saponin, flavonoids, steroids, terpenoids, triterpenoids, alkaloids, carbohydrate, anthroquinone, polyphenol, glycoside were absence of plobatannins and protein.

Column chromatography

Column chromatography of *J. gendarussa* leaves extract afforded 3 fractions. The result of chromatographic separation is given in Table 2, Fig 3 and Fig 4.

Thin Layer Chromatography

The presences of secondary metabolites in the extracts were detected by TLC using suitable reagents. The presence of flavonoid was detected by information of pale yellow colour spot in the positive reaction by exposure of ammonia. In the present study flavonoid was in the leaves detected of *J. gendarussa* (Table 3).

HPLC

HPLC study reveals the presence of quercetin in the leaves of *J. gendarussa* (Table 4 and Fig 6).

Fourier Transform Infra-Red Spectroscopy analysis

The FTIR spectrum of the *J. gendarussa* leaf extract source pronounced absorbance was recorded in the region between 4000 and 400 cm^{-1}. The peak indicates alcoholic and phenolic groups, alkenes (C-H strech stretch), alkenes (C-H stretch), carboxylic acids (O-H strech), alkenes (–C=C– stretch), alkynes (C=C– stretch), aromatics and alkenes (C-C stretch (in-ring) and C-H bend), aromatics (C-C stretch (in-ring), aromatic amines (C-N stretch), alkynes (C-O stretch and C-N stretch), 1049.33 indicates aliphatic amines (C-N stretch) Table 5, Fig 7.

NMR spectrum leaves of *J. gendarussa* extract

Nuclear magnetic resonance (NMR) spectroscopy has evolved as one of the most powerful analytical techniques. It allows the visualization of single atoms and molecules in various media in solution as well as in solid state. NMR is nondestructive and gives molar response that allows structural elucidation and quantification simultaneously. Magnetic interactions between NMR active nuclei along covalent bonds result in spin-spin couplings. Through space interactions can be detected using the

Nuclear Overhauser Effect (NOE). Both these interactions facilitate the three dimensional structure elucidations. One dimensional and two dimensional NMR data can be collected. The 1D NMR experiments are 1H, 13C, 31P, 19F, etc. The 1D NMR techniques will give information regarding the chemical shifts, spin-spin couplings and intensities. The chemical shifts will give the information regarding environment of the protons. Nuclei which are close to one another exert an influence on each other's effective magnetic field. This effect shows up in the NMR spectrum when the nuclei are non-equivalent. If the distance between non-equivalent nuclei is less than or equal to three bond lengths, this effect is observable. This effect is called indirect spin-spin coupling. The intensities will give the relative number of protons under the peak. The 2D NMR experiments are COSY, TOCSY, HSQC, HMBC, NOESY, ROESY, etc. These 2D experiments provide information regarding through bond or through space interactions.

The intensity or the integral of a signal is considered to be the area under that signal. The comparison of the signal intensities in a spectrum will give the ratios of the protons in the molecule. If there are multiples in a spectrum, the whole group of peaks should be integrated. Just like the chemical shifts and indirect spin-spin couplings, the signal intensities are also important for the structure determination. The signal intensities will help in the quantification of mixtures. In principle, the intensities of carbon-13 signals can also be used to infer the number of carbons responsible for the signal. Practically, the low abundance and sensitivity of the carbon-13 isotope will affect the quantification of number of carbons in a molecule. Due to this reason the carbon signals are generally not integrated in 13C NMR spectrum. The quantification of carbon-13 signal can be made possible with high digital resolution, suppression of NOE, a pulse repetition rate that is not too fast and small spectral width and high pulse power. The one dimensional NMR spectra have two dimensions, the abscissa and the ordinate. The abscissa corresponds to the frequency axis and the ordinate gives the signal intensities. But, in two dimensional (2D) NMR spectra, both the abscissa and the ordinate represent frequency axes; the third dimension gives the intensities. In the 2D J-resolved NMR spectrum, the chemical shifts will be plotted along one of the axes and the coupling constants along the other dimension. If both axes are chemical shifts, then it is called 2D (shift) correlated NMR spectrum. Most often the shift correlated 2D NMR data is used in structure elucidation. The correlations could be homo nuclear (H1-1H) or hetero nuclear (1H/13C) (Table 5).

^{13}C -NMR spectrum

Supporting evidence for the structure of the glycoside was provided by the analysis of ^{13}C-NMR data and a complete assignment. It was characterized as 2-(3, 4-Dihydroxyphenyl)-3, 5, 7-trihydroxy-4H-1-benzopyran-4-one (quercetin) (Fig 8).

^{1}H-NMR spectrum

In ^{1}H-NMR spectrum (500MHz, MeOD) the A ring protons at C-6 and C-8 appear as δ 6.28 and δ 6.43ppm respectively. A two proton singlet at δ 7.67 assigned to H-2′ and H-6′ (Mizuno *et al.,* 1992). The proton at C-5′ appears as δ 6.95ppm as doublet (Fig 9).

Table.1: Phytochemical screening leaves of *J. gendarussa*

S. No	Phytochemicals	Methanol extract	Aqueous Extract
1	Tannin	+	-
2	Phlobatannin	-	-
3	Saponin	+	+
4	Flavonoids	+	+
5	Steroids	+	+
6	Terpenoids	+	+
7	Triterponids	+	+
8	Alkaloids	+	+
9	Carbohydrate	+	+
10	Protein	-	-
11	Anthroquinone	+	+
12	Polyphenol	++	+
13	Glycoside	+	+

(+) Presence, (++) highly presence and (-) Absence

Table.2: Separation of fractions from column chromatography

S. No.	Eluents	Number of fraction(s)	Nature of fractions
1.	Hexane	01	Yellow
2.	Chloroform	01	White
3.	$CHCl_3$:MeOH (7:1)	01	Greenish Yellow

Table.3: Analysis of flavonoid by TLC in the leaves of *J .gendarussa*

Phytoconstituents	Rf Value	Results	Literature (Gordana, 2003)
Flavonoid	8.2/9.4	0.85	Flavonoid derivatives (Rf = 0.90)

Table.4: Compound identified HPLC in the leaves of *J.gendarussa*

Peak#	Ret. Time	Area	Height	Area%	Height %	Compounds identified by literature (Gupta Mradu et al., 2012)
1	3.063	126083	2000	93.549	90.992	Quercetin
2	14.752	8694	198	6.451	9.008	Unknown (impurity)
Total	-	134777	2198	100.000	100.000	-

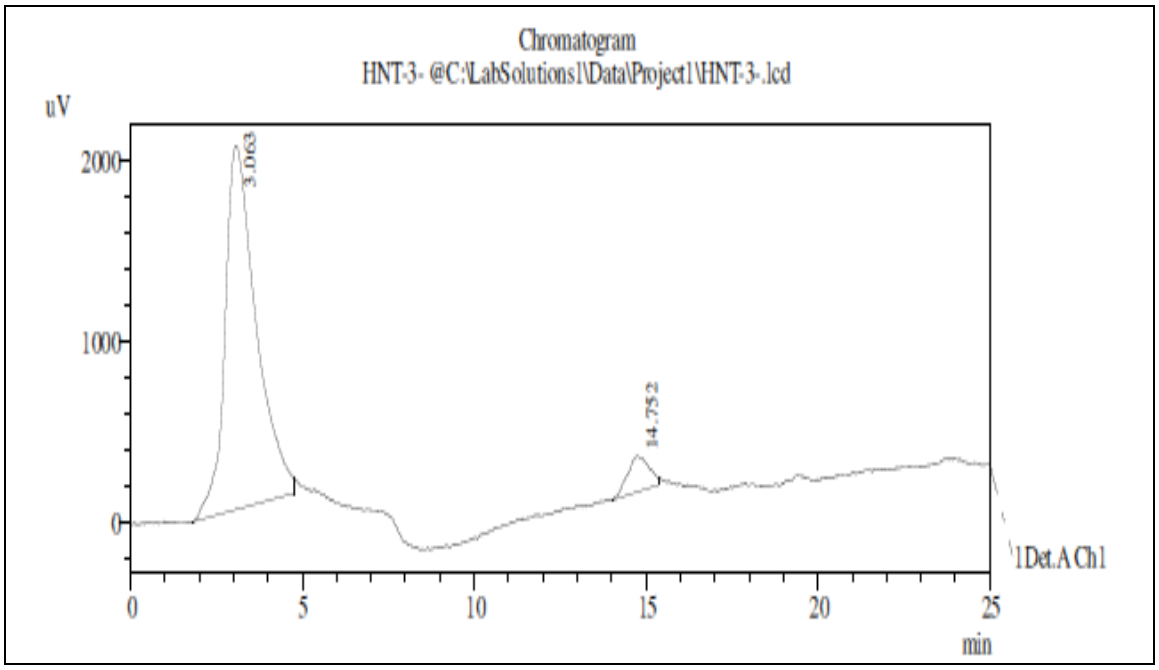

Fig.6: Analysis of HPLC in the leaves of *J. gendarussa*

Table.5: FTIR Peak Values of leaves of *J.gendarussa*

Peak Value	Bond	Functional group
3412.70	O–H stretch, H–bonded	Alcohols, phenols
2968.71	O–H stretch	Carboxylic acids
2951.19	O–H stretch	Carboxylic acids

2925.07	O–H stretch	Carboxylic acids
2843.59	O–H stretch	Carboxylic acids
2866.52	O–H stretch	Carboxylic acids
2524.13	O–H stretch	Carboxylic acids
1649.35	–C=C– stretch	Alkenes
1454.31	C–H bend	Alkanes
1412.59	C–C stretch (in–ring)	Aromatics
1112.14	C–H wag (–CH2X)	Alkyl halides
1053.29	C–N stretch	Aliphatic amines
1017.78	C–N stretch	Aliphatic amines
1031.31	C–N stretch	Aliphatic amines
691.97	C–H “oop”	Aromatics

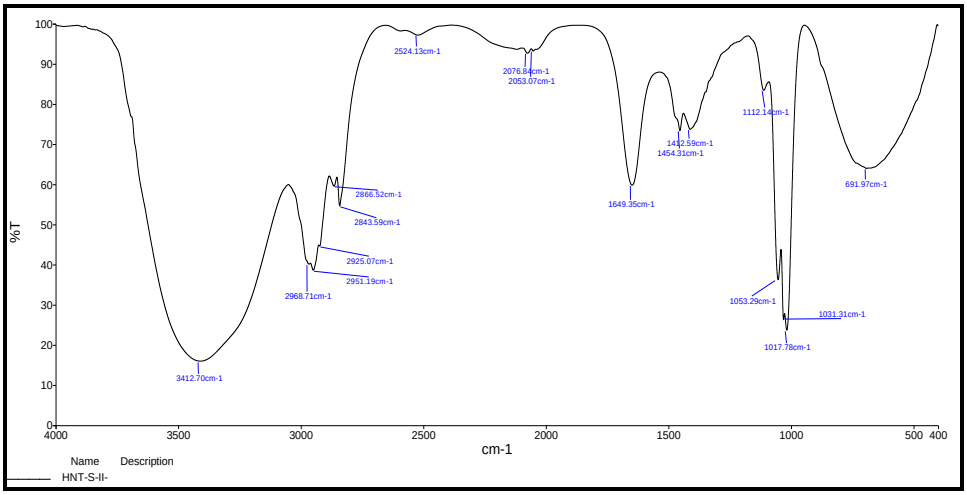

Fig.7: FTIR spectrum of *J. gendarussa* leaves

Table.6: ^{13}C-NMR DATA AND THEIR ASSIGNMENT OF QUERCETIN OBTAINED BY THE REFERENCES (Harborne and Williams, 1992; Guvenalp and Omur, 2005; Guvenalp and Nurcan, 2006).

Carbon	QUERCETIN		
	Literature	Reference Standard	Plant extract
2	147.90	147.65	146.10
3	137.20	135.68	137.18
4	177.30	175.79	177.24
5	162.50	160.67	162.03
6	99.30	98.12	99.26
7	165.70	163.83	165.98
8	94.40	93.29	94.30
9	158.20	156.08	157.11
10	104.40	102.96	103.02
1'	124.10	121.90	121.80
2'	116.00	115.55	116.11
3'	146.20	146.75	146.10
4'	148.70	147.65	148.50
5'	116.20	115.02	116.11
6'	121.60	121.90	121.80

Finding compound

Based on this the data has been characterized as Quercetin (Molecular Formula: $C_{15}H_{10}O_7$)

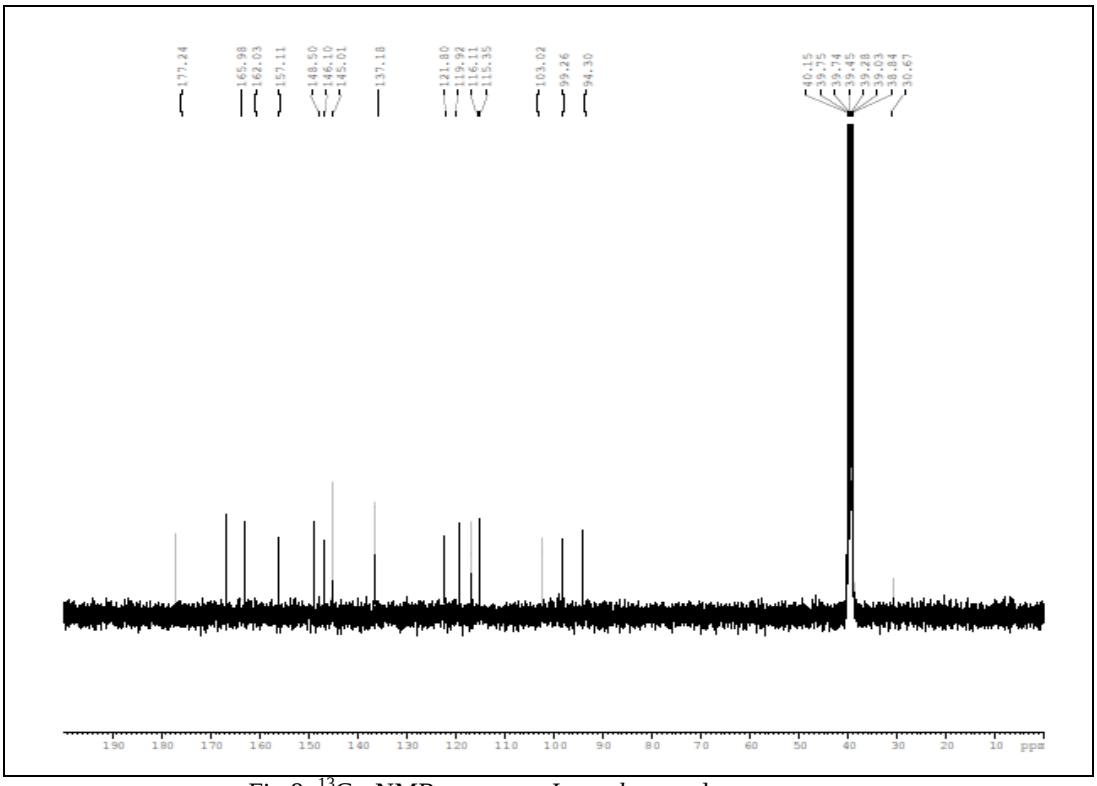

Fig 8: ^{13}C - NMR spectrum *J. gendarussa* leaves extract

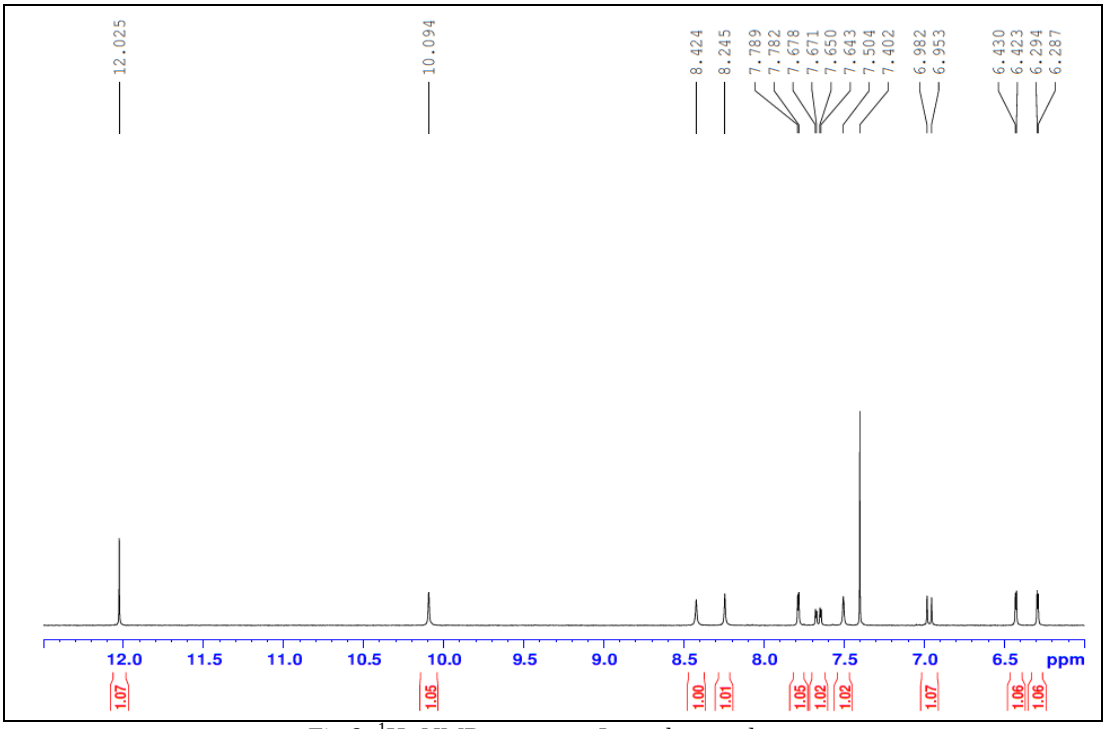

Fig 9: ^{1}H -NMR spectrum *J. gendarussa* leaves extract

IV. DISCUSSION

Phytochemicals in plant material have raised interest among scientists, food manufacturing and pharmaceutical industry, as well as consumers for their roles in the maintenance of human health. Phytochemicals are the bioactive, non-nutrient, and naturally occurring plant compounds found in fruits, vegetables, and whole grains. They can be categorized into various groups, i.e., polyphenols, organo sulfur compounds, carotenoids, alkaloids, and nitrogen-containing compounds. Many phytochemicals are potent effectors of biologic processes and have the capacity to influence disease risk via several complementary and overlapping mechanisms[14]. The phytochemical screening of aqueous extract of *J. gendarussa* leaves showed that the presence of flavonoids, terepenoids, steroids, saponins, triterpenoids, phenolics, alkaloids, carbohydrate, anthriquinone and glycosides while phlobatannins, tannin and protein were absent.

Abuzar *et al.*[15] reported the phytochemical analysis of *Heliotropium dasycarpum* and evaluating the presence of secondary metabolites like alkaloids and cardiac glycosides while the saponins, anthroquinone, glycoside and tannins were absent in the plant extract.

A simple, accurate, and reproducible high-performance liquid chromatography (HPLC) method has been developed and validated for the quantification of flavonoids[16]. Quercetin were confirmed in *Justicia gendarussa* using HPLC[17] were carried out to characterize the phenolic acids and flavonoids in methanolic extracts of *Withania somnifera* leaves by HPLC. Five phenolics (gallic, syringic, benzoic, p-coumaric and vanillic acids) and three flavonoids (catechin, kaempferol and naringenin) have been identified in *Withania somnifera* leaves. Similarly catechol, gallic acid, ellagic acid, and catechin of compounds were identified in *Cissus vitiginea* leaves among the four compounds of this present study.

Paranthaman *et al.*[18] Investigated the GC-MS analysis of phytochemicals and simultaneous determination of flavonoids in *Amaranthus caudatus* by RP-HPLC. A sensitive and selective high performance liquid chromatography method (HPLC) for simultaneous analysis of following five flavonoids like gallic acid (GA), caffeic acid (CA), rutin (RU), ferulic acid (FA) and quercetin (QU) in *Amaranthus caudatus* leaves. The results demonstrated that the *Cissus vitiginea* leaves were separately extracted and analyzed using HPLC method. The contents of gallic acid (0.083 µg/gm), Caffeic acid (0.004 µg/gm), Rutin (0.019 µg/gm), Quercetin (0.001 µg/gm) and Ferulic acid (0.001 µg/gm) in *Cissus vitiginea* leaves.

Fourier Transform Infrared (FTIR) Spectroscopy is a rapid, noninvasive, high resolution analytical tool for identifying types of chemical bonds in a molecule by producing an infrared absorption spectrum that is like a molecular fingerprint[19]. FTIR has been shown to be a valuable tool for differentiating, classifying and discriminating closely related microbial strains, plants and other organisms[20,21]. It is one of the most widely used methods to identify the chemical constituents and elucidate the structural compounds and has been used as a requisite method to identify medicines in pharmacopoeia of many countries.

It is well known that the medicinal materials comprise hundreds of components and produce their curative effects through mutual effects of many ingredients, so the limited numbers of specific components cannot availably reflect the real qualities of the herbal medicines. Therefore, an effective and inexpensive analysis method to entirely monitor the whole constituents of the medicinal materials and their corresponding extract products is required[22].

FTIR has played a vital role in pharmaceutical analysis in recent years[23]. FTIR spectroscopy is a physicochemical analytical technique that does not determine concentrations of individual metabolites but provides a snapshot of the metabolic composition of a tissue at a given time[19]. The FTIR method measures predominantly the vibrations of bonds within chemical functional groups and generates a spectrum that can be regarded as a biochemical or metabolic "fingerprint "of the sample[24].

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[25,26]. FTIR spectrum of the *J. gendarussa* leaf extract was pronounced absorbance was recorded in the region between 4000 and 400 cm^{-1}. The peak indicates alcoholic and phenolic groups, alkenes (C-H strech stretch), alkenes (C-H stretch), carboxylic acids (O-H strech), alkenes (–C=C– stretch), alkynes (C=C– stretch), aromatics and alkenes (C-C stretch (in-ring) and C-H bend), aromatics (C-C stretch (in-ring), aromatic amines (C-N stretch), alkynes C-O stretch and C-N stretch), 1049.33 indicates aliphatic amines C-N stretch).

Karpagasundari and Kulothungan[27] screened the bioactive components of *Physalisminima* leaves have been evaluated using UV-visible and FTIR. The UV-visible profile showed the peaks at 315.09 nm, 408.09 nm and 676.50 nm with the absorption 0.247, 0.106 and 0.003 respectively. The results of FTIR analysis confirmed the presence of phenol, alkanes, aldehyde, secondary alcohol, amino acid, aromatic amines and halogen compound. The results of this study offer a platform of using *Physalisminima* leaves as herbal alternative for various diseases.

Nuclear magnetic resonance (NMR) spectroscopy is usually the method of choice for natural product structure determination and it is not surprising that this powerful technique has come to the fore in plant metabolomics. The data requirements for metabolomics are the qualitative and quantitative analyses of the maximum number of metabolites in the highest achievable throughput. Most metabolomics laboratories deploy a range of spectroscopic technologies but use of NMR spectroscopy, particularly as a first pass screen, has a number of advantages over other analytical platforms currently being used. Sample preparation is relatively simple when compared to other analytical methods and a high sample throughput with little instrument drift is readily achieved. NMR is not discriminatory unlike certain mass spectrometry methods that rely on the prior derivatization of metabolites or the ability of them to ionize. Metabolite screening requires maximum sensitivity with a broad compound coverage. Based on ^{1}H and ^{13}C data has been characterized as Quercetin (Molecular Formula: $C_{15}H_{10}O_7$).

Sumit Arora and Prakash Itankar [28] showed the extraction, isolation and identification of flavonoid from *Chenopodium album* aerial parts. The flavonoids contained in *C. album* aerial parts were extracted, identified and characterized. Sequential soxhlet extraction was subjected to preliminary screening and flavonoid quantification. The results showed that maximum yield of the flavonoid (7.335 mg/g) were obtained from acetone extract. This acetone extract was subjected to flash chromatography for isolation of flavonoid. Characterization of isolated flavonoid was done by UV, IR, 1H & 13C NMR and MS. On the basis of chemical and spectral analysis structure was elucidated as 2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxy-4H-chromen-4-one, a flavonoid.

Ahmadu *et al*.[29] examined flavonoid glycosides from *Byrsocarpus coccineus* leaves. The bioactive ethyl acetate and N-butanol soluble parts of an ethanolic extract of *Byrsocarpus coccineus* leaves was subjected to column chromatography over silica gel G (60 - 120µ) and repeated purification of the flavonoid rich fraction over sephadex LH-20 eluted with methanol led to the isolation of three flavonoid glycosides identified as quercetin 3-O-α-arabinoside (I), quercetin(II) and quercetin 3-β-D-glucoside.

Present study concluded that FTIR, HPLC and NMR studies were carried out to find the structure of bioactive compound Quercetin. ^{1}H- NMR and ^{13}C – NMR that reveals the structure of flavonoids. The compound was identified as 3, 3', 4', 5, 7 – pentahydroxyflavanone by ^{1}H-NMR and ^{13}C – NMR. All these data obtained in the present investigation supported by traditional claim associated with *J. gendarussa* literature. Further biological studies along with chemical characterization of the separated compounds will have the way for a better understanding of the bioactive product and its subsequent application in disease.

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