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EXTRACTION AND IDENTIFICATION OF VARIOUS PHYTOPHARMACEUTICAL CONSTITUENT OF *CATHARANTHUS ROSEUS* L. (ALBA) LEAVES

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ABSTRACT

Catharanthus roseus, Madagascar periwinkle has an undaunted spirit with marvellous and manifold property to combat against the disease. In traditional and herbal medicine, the periwinkle has been used in prevention of diabetes, cancer, stomach-ache, malaria, sore-throat, eye-irritation, skin problem, kidney, liver, cardiovascular disease, relieving muscle pain, depression also used for applying to wasp stings and heal wounds. Root paste is used in septic wounds, root decoction is used in fever, leaves are used in menorrhagia, leaf juice is used in blood dysentery. Many drugs, flavouring agent, beauty and food products are sold today comprised of naturally produced compounds called secondary metabolites. Thousands of years, medicinal plants have been used as safe therapeutic in worldwide. Now a day's Pharmaceutical companies start processing of medicinal plants by using extraction of active components. In this study plant parts like leaves of *Catharanthus roseus* L. (Alba) were air dried and powdered separately. Varieties of flavonoids and phytosterols isolated by TLC. Dried plant leaves extract of flavonoids and phytosterols are dissolved in 10mg/ml ethanol and take 3 to 3.5ml sample for GCMS analysis. According to GCMS analysis of plant sample we have found some novel compound named Hentriacontane, 1-Heptacosanol, Ethyl 9 hexadecenoate, 2,6 octadien, Trimethylsilyl ester, Ethyl oleate, Ethyl nonadecanonoate, 9,10 Anthraquinone etc. having ant-inflammatory, anti-microbial and anti-diarrheal property which are useful in drug making for various disease.

Keywords **:** Flavonoids, Phytosterols, TLC,GCMS, Secondary metabolite etc.

Introduction

Apocynaceae (from Apocynum Greek word meaning is "dog-away") is a family of flowering plants that include trees, herbs, shrubs, herbs, stem succulents and vines commonly known as the dogbane or Milkweed family (Endress *et al*., 2000) because some taxa were used as dog poison (Simpson *et al*., 2010). Members of the family are native to the European, Asian, African, Australian and American topics or subtropics with some temperate members (Endress *et al*., 2000). Many of these plants have milky latex and rich in alkaloids and cardiac glycoides and many others secondary metabolites. Many species are poisonous if ingested. Medicinal plants have similar assets as conventional pharmaceutical drugs for fighting against fatal syndrome. Humans have used them throughout history to either cure or lessen symptoms from an illness. Free radical or ions that cause various human diseases and aging (Galati and Brien, 2004). Antioxidants are chemical compounds extremely useful to humans which are found in plants, inhibit the free radical ions. They are used as natural medicines directly and indirectly. These practices have existed since prehistoric times when there was no existence of pharmaceuticals. There are three ways in which plants have been used as medicine. First, they may be used directly as extracted forms for their natural chemical constituents. Second, they may be used as agents in the combination of drugs. Third one is the organic molecules found in plants may used as sculpt for man-made drugs. Chemical compounds in plants mediate their effects on the human body by binding to receptor molecules available in the body. Such processes are identical to those all, who are already well understood for conventional drugs and as such herbal medicines do not differ greatly from conventional drugs in terms of how they work. According to the World Health Organization (WHO), nearly 20,000 medicinal plants exist in 91countries including 12 mega biodiversity countries. The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation. Various phytochemical constituent is known as secondary metabolite. Secondary metabolites are organic compound often play an important role in plant defence against herbivory and other interspecies defences and not directly involved in the normal growth, development or reproduction of an organism. Secondary

metabolites, including antibiotics serve survival functions for the organisms. Secondary metabolites serve as competitive weapons used against other bacteria, fungi, amoebae, plants, insects, and large animals (Bell and Charlwood, 1981) metal transporting agents, as agents of symbiosis between microbes and plants, nematodes, insects, and higher animals, as sexual hormones, as differentiation effectors (Demain and Fang, 2000). Humans use secondary metabolites as medicines, flavourings and recreational drugs (Cragg and Newman, 2005, Eswaraiah, 2020) Secondary metabolism plays a pinnacle role in keeping all of the plants systems working properly. Secondary plant metabolites are also used in signaling and regulation of primary metabolic pathways. I have extracted secondary metabolites like Flavonoids and Phytosterols from *Catharanthus roseus* leaves. Flavonoids are polyphenolic molecules containing 15 carbon atoms and soluble in water. Some of the best known flavonoids include quercetin, kaempferol, rutin, catechins and anthocyanidins. They provide health benefits through cell signalling pathways and antioxidant effects (Biswas *et al*., 2005). Flavonoids are a group of plant metabolites with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action (Frankel *et al*., 1995; Karimi and Jaafar, 2011). Due to its antioxidant properties it can interfere with the oxidative process by reacting with free radicals chelating, catalytic metals and also by acting as oxygen scavengers (Karimi and Jaafar, 2011; Dreosti, 2002; Syeda, 2020). Phytosterols are plant sterols, belonging to the triterpene family. They are found in the cell membranes of plants, where they play important roles, just like cholesterol in humans. Phytosterols are pharmacologically important for human life (Hardman, 1980). The most common phytosterols in the human diet are campesterol, sitosterol and stigmasterol. Phytosterols are plant sterols structurally similar to cholesterol that act in the intestine to lower cholesterol absorption (Ostlund, 2004). Flavonoids having a number of medicinal benefits, including anticancer, antidiabetic, antioxidant, anti-inflammatory, anti-diarrheal and antiviral properties (Atta and Mouneir, 2005).They also have neuro protective and cardio-protective property. The discovery of lead compounds for use as therapeutic drugs, the active principals in medicinal plants needs to be identified (Vuorela *et al*., 2004).

Material and Methods

Extraction of Flavonoids

Different plant parts (leaves, stems, roots and flowers) were air dried and powdered, separately 20 gm powdered of each sample extracted separately with 80% methanol on water bath (Subramanian and Nagarajan, 1969) for 24h.The methanol soluble fractions were filtered, concentrated *in vacuo* and aqueous fractions were fractioned by sequential extraction with petroleum ether (Fraction I), diethyl ether (Fraction II) and ethyl acetate (Fraction III) separately. Each step was repeated thrice for complete extraction, fraction I (Fr I) was discarded in each case because it contained fatty substance, whereas fraction II (Fr II) free flavonoids and fraction III (Fr III) were concentrated were being used for determining flavonoids. After drying Fr III was further hydrolyzed by refluxing with 7% sulphuric acid ($10mLg^{-1}$ per plant material for 2 h), filtered and filtrate was extracted thrice with ethyl acetate. All ethyl acetate layers were pooled separately, neutralized by distilled water with repeated washings and concentrated *in vacuo* and bound flavonoids of Fr III were determined. All fractions of flavonoids II and III were taken up in small volume of ethanol (2-5mL) before chromatographic examination.

Isolation by Thin Layer Chromatography

TLC is used for majorly division, recognition, and quantification of components in a mixture. For isolation of flavonoids in *C. roseus* thin glass plates were coated with Silica gel G (8 gm silica gel in 40 mL DSW). The freshly prepared plates were air dried at room temperature thereafter these were kept at 100 ^{0}C for 30 minutes to activate and then cooled at room temperature. The freshly prepared and activated plates were used for analysis. Each of the extraction was co-chromatographed with authentic flavonoids as a marker (quercetin, luteolin, kaempferol and rutin). The FrII and FrIII plates sample dissolved in ethanol. In one wiles some quantity of quercetin with ethanol were used after shaking to make 20µL as a standard solution for TLC where as in another wiles each sample of leaf, stem, root and flower with ethanol used in order to make 40 µL solution for Fr II and Fr III through TLC. These plates were developed in an air tight chromatographic chamber saturated with solvent mixture (Benzene: Acetic Acid: Water: 125:72:3) (Wong and Francis, 1968). The developed plates were air dried and visualized under UV light by exposure of ammonia fumes. The mouth of a 100 mL containing concentrated 50% H_2SO_4 was held in contact with each spot for about 5-10 seconds and fluorescent spots corresponding to that of standard markers were marked. The developed plates were also sprayed with 0.1% alcoholic $AlCl_3$ and kept in I_2 chamber (Potassium Iodide, Iodine crystal and silica gel) separately. The developed colored spots were noted and against them RF value were calculated. It has been observed that the solvent system containing benzene, acetic acid, water (125:72:3) gave better results.

Identification

TLC Isolates were eluted with ethanol, crystallized by $CHCl_3$ and further confirmed by melting points, mass, UV maxima on spectrophotometer and infra red spectral studies. Quantitative estimation of the identified flavonoids was carried out calorimetrically following method of (Nagasaki *et al*., 1978) in case of quercetin and method of (Mabry *et al*., 1970) in case of luteolin and kaempferol. The RF value (retardation factor in chromatography) is being calculated by distance travelled by a given component divided by the distance travelled by the solvent front for a given system at a known temperature, gives the characteristic of the component

and that can be used to identify components. Identification of compound and Structure determination is carried out by various techniques like 1-D NMR, IR and GCMS.

Extraction of Phytosterols

Different plant parts (leaves, stems, roots and flowers) of *Catharanthus roseus* L. were air dried and powdered separately. Each of these 10 gm sample were put in incubator separately with 30% HCL (hydrochloric acid) for 24h and liquid obtained were discarded after these sample were filtered. The dry powder on filter paper put in incubator for 24-48 hrs then dry powder with distilled water is mixed for neutralization. After neutralization again the samples were filtered with filter paper and pH 6 were taken. Dry powder again was put in incubator after drying dry powder. These extractions were put in flask with Benzene and flasks were put in incubator till overnight for final extraction of phytosterols.

Isolation by Thin Layer Chromatography (TLC)

Thin glass plates were coated with Silica gel G (8gram silica gel in 40 ml DSW). The freshly prepared plates were air dried at room temperature thereafter these were kept at 100^0C for 30 minutes to activate and then cooled at room temperature. The freshly prepared and activated plates were used for further analysis. Each of the extract was co-chromatographed with authentic phytosterol as a marker (Stigmasterols and Sitosterols). In one vial some stigmasteroles were taken with 1 mL benzene used as a standard solution (20µL) after shaking and in another wiles each sample of leaf, stem, root and flower with benzene (40µL) used for TLC. These plates were developed in an air tight chromatographic chamber saturated with solvent mixture (Hexane: Acetone: 120mL: 30mL). After treating spray of 50% H_2SO_4 put plate in oven on 101^0 C. The developed plates were air dried and visualized under UV light by exposure to ammonia fumes. The developed colored spots were noted and against the RF value were calculated.

Identification

The identified of the isolated phytosterols were confirmed by melting points, mass,UV (Ultraviolet and visible- 5300 spectrophotometer) analysis along with their respective authentic samples stigmasteroles and by calculating RF value. Identification of compound and Structure determination is carried out through various techniques like 1-D NMR, IR spectroscopy and GCMS.

Gas Chromathography-Mass Spectrometry

The extraction method presented is simple, rapid and inexpensive, with reduced solvent consumption. GC-MS method used for the analysis of the obtained extracts can be an interesting tool for testing the amount of some active principles in herbs used in cosmetic, drugs, pharmaceutical or food industry, environmental and forensic applications (Uma *et al*. 2009). It combines two analytical techniques to a single method of analyzing mixtures of chemical compounds. Gas chromatography separates the components of the mixture and mass spectroscopy analyzes each of the components separately.

Compound Identification by GC-MS

The GC–MS chromatogram of flavonoids and phytosterols leaf extracts of *Catharanthus roseus* recorded a total of 16 peaks and 38 peaks corresponding to the bioactive compounds that were recognized by relating their peak retention time, Area, Area (%) and mass spectral fragmentation patterns to that of the known compounds described by the National Institute of Standards and Technology (NIST) library.

Results and Discussion

Medicinal plants are the resources of new drugs. Rutin, Luteolin, Quercetin and Kaempferol etc. varieties of flavonoids isolated by TLC which are identified by physical appearance, mass and Rf values 0.24, 0.56, 0.78 or 0.86 respectively. Identification of compound and structure determination is also carried out through various techniques like 1-D NMR, IR and GCMS. β-sitosterol and Stigmasterol etc. varieties of Phytosterols isolated by TLC which are identified by physical appearance, mass and Rf values 0.83 and 0.89 respectively.GCMS results as shown below in Table1 and Table 2. Among the identified phytocompounds Hentriacontane, 1-Heptacosanol, Ethyl 9 hexadecenoate, 2,6 octadien, Trimethylsilyl ester, Ethyl oleate,Ethyl nonadecanonoate,9,10 Anthraquinone etc. having ant-inflammatory,anti-microbial and anti-diarrheal property.Octadecadienoic acid (Z, Z) have the property of anti-inflammatory, hypocholesterolemic and antiarthritic activity which was reported by the earlier workers (Rani *et al*., 2009; Uma *et al*., 2009, Ponnamma and Manjunath 2012; Rani and Kapoor, 2019). Esters are important organic compounds with increasing number of commercial applications (Foresti *et al*., 2005). These compounds are largely used in fragrances, cosmetics detergents, flavors and pharmaceuticals. Esters (ethyl oleates) may also be used as plasticizers and lubrificants,biological additives and hydraulic fluids (Hazarika *et al*., 2002). The use of ethyl oleate in commercial applications has been hampered due to the low amounts that can be recovered from natural sources (Radzi *et al*., 2006; Martiinez-Ruiz *et al*., 2008) and 22,23-Dibromostigmasterol Acetate used as a raw material for the synthesis of steroid hormones.

Table 1: Phytochemical constituents (Flavonoids)identified within the leaf extract of *Catharanthus roseus* L. by GC-MS analysis.

Peak #	R. Time	Area	Area %	Name
1	16.251	115459	1.82	2,6-Octadien-1-Ol, 3,7-Dimethyl-, Acetate
2	17.723	89254	1.41	Oxacycloheptadec-8-En-2-One, (8z)
3	18.096	78934	1.24	Ethyl 9-Hexadecenoate
4	18.370	976976	15.40	Hexadecanoic Acid, Ethyl Ester (Thanwar, 2017; Gomathi, 2015)
5	19.080	131229	2.07	Hexadecanoic Acid, Trimethylsilyl Ester
6	20.617	706457	11.13	Linoleic Acid Ethyl Ester
7	20.682	2041687	32.18	Ethyl Oleate
8	20.996	636084	10.03	Octadecanoic Acid, Ethyl Ester
9	21.779	86118	1.36	Ethyl (9z,12z)-9,12-Octadecadienoate
10	22.050	72113	1.14	Ethyl 9-Hexadecanoate
11	23.410	82922	1.31	Ethyl Nonadecanoate
12	24.312	154287	2.43	1,2-Propanediol, 3-Benzyloxy-1,2 -Diacetyl-
13	25.560	156292	2.46	Bis (2-Ethylhexyl) Phthalate
14	25.727	72936	1.15	(2,3-Diphenylcyclopropyl) Methyl Phenyl Sulfoxide, Trans-
15	25.930	142902	2.25	(2,3-Diphenylcyclopropyl) Methyl Phenyl Sulfoxide, Trans-
16	31.378	801162	12.63	Pentacyclo[19.3.1.1(3,7).1(9,13).1(15,19)]Octacosa-1(25),3,5,7(28),9,11,13(27), 6344812 100.00
Total		6344812	100.0	

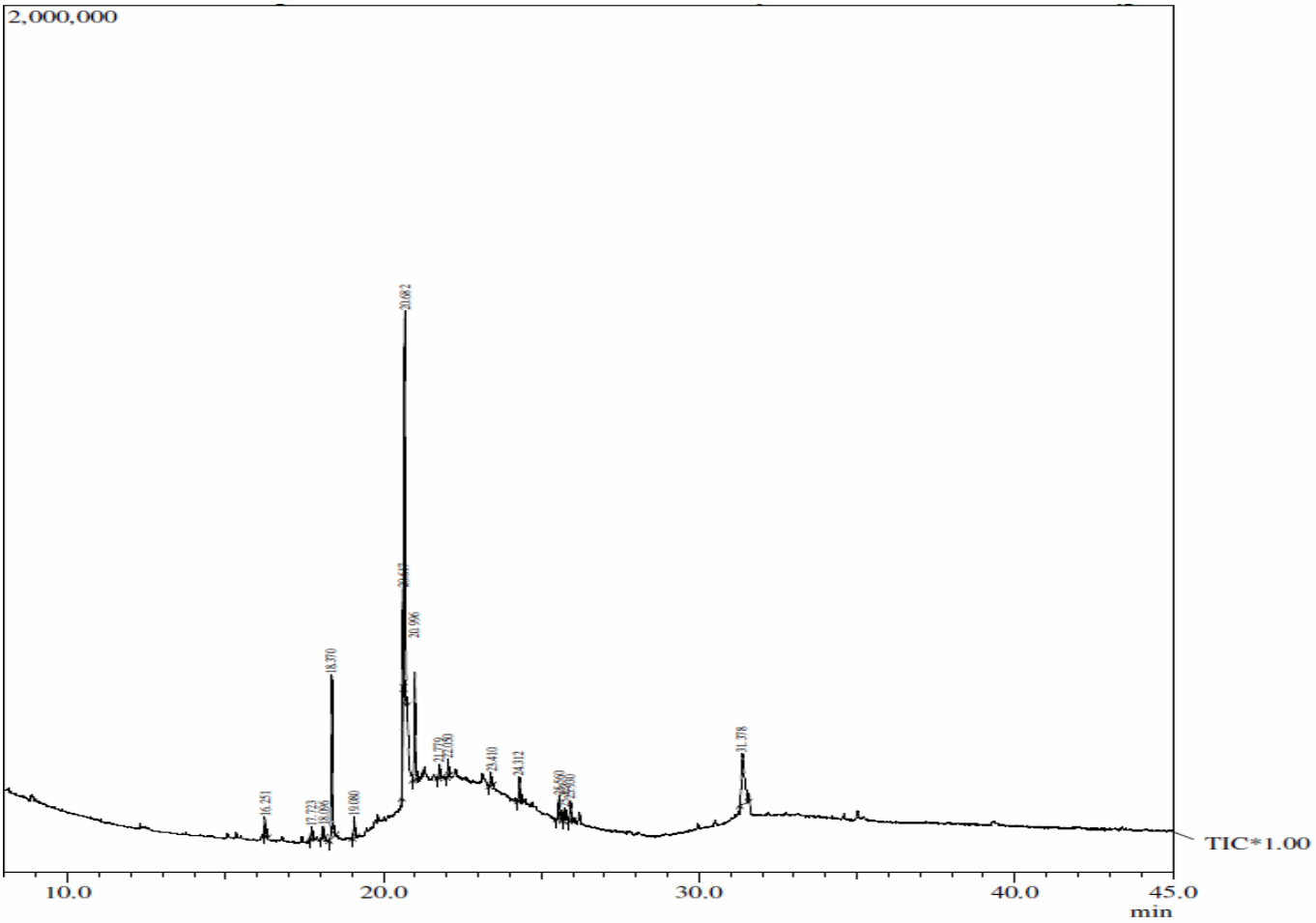

Fig. 1: GC-MS chromatogram of the flavonoids extract of the leaves of C*atharanthus roseus* L.(number on the chromatogram indicate the peak numbers of identified compounds)

Table 2: Phytochemical constituents (Phytosterols)identified within the leaf extract of *Catharanthus roseus* L. by GC-MS analysis.

Peak#	R. Time	Area%	Compound Name
1	8.87	0.54	1-Pentadecene
2	9.165	5.96	1,3-Butanedione
3	11.049	0.26	Phenol, 3,5-Di-Tert-Butyl-
4	12.309	0.53	1-Hexadecene
5	13.089	1.41	3,5-Dimethyl-4-Phenylpyridine
6	15.067	0.33	Furan-3-One, Tetrahydro-2,2-Dimethyl-5-Phenyl-
7	15.469	0.56	1-Nonadecene
8	15.821	0.16	Salicylic Acid, 2-Methylbutyl Ether, 2-Methylbutyl Ester
9	16.25	0.35	3,7,11-Trimethyl-2,6,10-Dodecatrienyl Acetate $$ Farnesylacetat, Alltrans-
10	17.722	0.4	Cyclododecene, (E)-
11	18.044	0.69	1,2-Benzenedicarboxylic Acid, Butyl 2-Methylpropyl Ester
12	18.368	7.68	Ethyl Pentadecanoate
13	19.081	1.32	Pentadecanoic Acid, Trimethylsilyl Ester
14	19.754	0.23	Z,Z-2,5-Pentadecadien-1-Ol
15	19.817	0.23	11-Octadecenoic Acid, Methyl Ester
16	19.885	0.54	2-Buten-1-One, 3-Amino-1-Phenyl-4,4,4-Trifluoro-
17	20.616	8.78	9,12-Octadecadienoic Acid, Ethyl Ester
18	20.682	22.72	Ethyl 9-Octadecenoate
19	20.995	4.64	Heptadecanoic Acid, Ethyl Ester
20	21.302	0.71	Oleic Acid, Trimethylsilyl Ester
21	22.05	0.26	Heptadecene-(8)-Carbonic Acid-(1)
22	23.412	0.36	Hexadecanoic Acid, Ethyl Ester
23	24.31	8.85	(2,3-Diphenylcyclopropyl) Methyl Phenyl Sulfoxide
24	24.702	0.42	Oxalic Acid, 3,5-Difluorophenyl Tetradecyl Ester
25	25.554	1.47	3-Nitrophthalic Acid
26	25.72	5.06	1-Propen, 3-(2-Cyclopentenyl)-2-Methyl-1,1-Diphenyl-
27	25.927	9.26	1-Propen, 3-(2-Cyclopentenyl)-2-Methyl-1,1-Diphenyl-
28	26.042	2.18	3,3-Diphenyl-4-Hexenoic Acid
29	26.211	3.89	1-Propene, 3-(2-Cyclopentenyl)-2-Methyl-1,1-Diphenyl-
30	27.106	2.93	5-(O-Benzamido-N-Bnezoylanilino) Tropolone
31	27.366	0.6	2,3-Dimethoxy-15-Methyl-6,7,9,11,12,15-Hexahydro-16-Oxa-8-Aza-Cyclopenta[A]Phenanthren-17-One
32	27.801	1.1	Benzene, 1,1'-[3-(2-Phenylethylidene)-1,5-Pentanediyl]Bis-
33	30.499	1.56	2,6,10,14,18-Pentamethyl-2,6,10,14,18-Eicosapentaene
34	31.386	0.4	Pentacosane
35	31.579	0.98	Cholesta-3,5-Diene
36	32.467	0.32	1-Heptatriacotanol
37	33.107	0.36	22,23-Dibromostigmasterol Acetate
38	34.57	1.97	3-Bromocholest-5-Ene

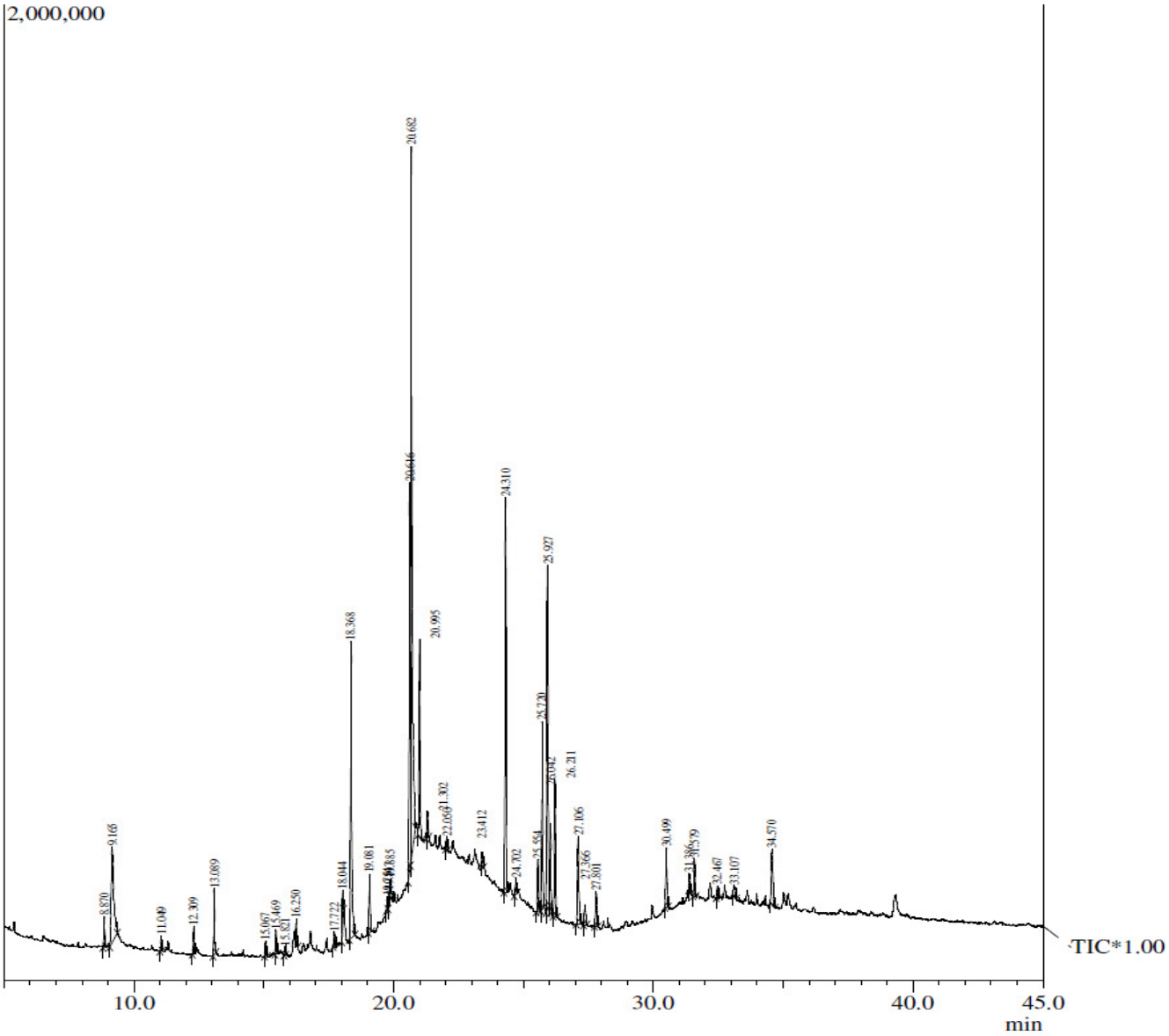

Fig. 2 : GC-MS chromatogram of the Phytosterols extract of the leaves of C*atharanthus roseus* L.(number on the chromatogram indicate the peak numbers of identified compounds)

Table 3: Some identified compounds, their Molecular weight, Formula, Medicinal and other uses

S.No.	Compound Name	Molecular Weight	Molecular Formula	Importance
1.	Hentriacontane	436.85 g/mol	$C_{31}H_{64}$	Urinary disease, diabetes, Skin disease, asthma, Dysentery, Gonorrhea, Leucorrhea, Piles, ulcers
2.	1-Heptacosanol	396.74 g/mol	$C_{27}H_{56}O$	Flavouring and fragrance agent, Cholesterol lowering, antimicrobial and Cytotoxicity, antithrombotic
3.	Ethyl 9 hexadecenoate	282.46 g/mol	$C_{18}H_{34}O_2$	Natural substances and extractives, Insoluble in water and neutral
4.	2,6 octadien-1-Ol	126.20 g/mol	$C_8H_{14}O$	Insect Repellent
5.	Trimethylsilyl ester	148.23 g/mol	$C_5H_{12}O_3Si$	Anti-inflammation, Anti-diabetic, Gastrointestinal disturbance, Allergy
6.	Ethyl oleate	310.51 g/mol	$C_{20}H_{38}O_2$	Pharmaceuticaldrug (lipo-philic substance-steroids, progesterone injection), lubricant, plasticizer
7.	Ethyl nonadecanonoate	326.56 g/mol	$C_{21}H_{42}O_2$	Flavouring agent, plasticizer
8.	9,10 Anthraquinone	256.28 g/mol	$C_{14}H_8O_3S$	Constipation, arthritis, Cancer, Antimicrobial, Anti-inflammatory
9.	Pentacosane	352.7 g/mol	$C_{25}H_{52}$	Pesticide
10.	Cholesta-3,5-Diene	368.63 g/mol	$C_{27}H_{44}$	Regulator of cholesterol homeostasis, To study of comparative index of microalgae

11.	5-(O-Benzamido-N-Bnezoylanilino) Tropolone	122.12 g/mol	$C_7H_6O_2$	Antibiotic, Reagent for sugar reduction
12.	Salicylic Acid	138.12 g/mol	$C_7H_6O_3$	Skin disorder, dendruff, psoriasis
13.	3-Nitrophthalic Acid	211.13 g/mol	$C_8H_5NO_6$	Corrosion inhibitors, medicines, agrochemical, dyes,crop protectiom
14.	Methyl Phenyl Sulfoxide	140.20 g/mol	C_7H_8OS	Nelfinavir HIV-protease inhibitor, flavoring agent
15.	Oxacycloheptadec-8-En-2-One	252.39 g/mol	$C_{16}H_{28}O_2$	Fruit flavors, perfume, fragrances

Conclusions

GC-MS plays an essential role in the phytochemical analysis of medicinal plants containing biologically active component which are useful in drug making, flavour in food, coloring agent, texturizing agents, preservative, cosmetic, forensic, agriculture and plastic industry.

Future Scope

This study can be exploited commercially to increase the synthesis of the medicinal drugs.

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Conflict of interest-The authors declare no conflict of interest.

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