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Analysis of phytochemical constituents in leaves of Bhumyamalaki (*Phyllanthus debilis* Klein ex Willd.) from Servaroy hills, Tamil Nadu, India

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Abstract
Phyllanthus debilis is one of the folkloric medicinal plant which belongs to the family Euphorbiaceae. The whole plant is reported to have many pharmacological properties such as an antihepatotoxic, laxative, tonic, antibacterial, antioxidative, immunomodulatory, Analgesic, anti-inflammatory, antipyretic and antiviral. The main aim of the present study was to evaluate the phytochemical properties of *Phyllanthus debilis.* Qualitative phytochemical analysis of leaf extract revealed the presence of steroids/sterols, cardiac glycosides, saponins, alkaloids, tannins, phenols, flavonoids and terpenoids. The FTIR spectrum of a methanolic leaf extract of *Phyllanthus debilis* indicates the presence of different functional groups. GC-MS chromatogram analysis of the methanolic leaf extract revealed the phytochemical constituents. Based on the results this study can validated rich phytocomponds in *P. debilis*, may be with the presence these bioactive metabolites it is used to treat various ailments.

Keywords: *Phyllanthus debilis*, phytoconstituents, FTIR and GC-MS

1. Introduction
Medicinal plants and plant extracts as most virtually used in all over 80% human populations around the world as a source of harmless and effective medicine for human health care. Ancient people used plants as purgatives, sedatives, fever, insanity and wide range of ailments [1]. Many medicinal plants were utilized by traditional healers across the world and also the most widely used medicinal plants are *Phyllanthus* genus. The usage of *Phyllanthus* plants has its origin in the Siddha system of South India and used as a common folk remedy for the treatment of jaundice and hepatitis [2, 3]. Plants of genus *Phyllanthus* have been used in ancient medicine for a variety of uses. Scientific studies have found that the plants of this genus possess pharmacological properties such as an antihepatotoxic, laxative, tonic, antibacterial, antioxidative, immunomodulatory, analgesic, anti-inflammatory, antipyretic, antiviral, antiatherosclerotic, and antineoplasic [3, 4, 5].
Among the herbs of *Phyllanthus* species in India *P. amarus, P. debilis, P. fraternus, P. rheedi, P. urinaria, P. kozhikodianus, P. maderaspatensis, P. emblica,* and *P. indofischeri* are widely used as herbal medicines, and a few of those species are also cultivated in southern India. The cluster of similar herbs, *P. amarus*, *P. fraternus*, *P. debilis* and *P. urinaria* is known as '*Bhumyamalaki*' in Indian ancient literature [6]. The genus *Phyllanthus* has potential and beneficial therapeutic action in the management of hepatitis B, nefrolitiase, painful disorders and very effective hepatoprotective agents in the Indian indigenous system of medicine.
Phytochemical investigation is one of the main steps in the plant therapeutic drug discovery. Plants have a wide variety of chemical constituents which are mainly classified on the basis of their role in plant metabolism into primary and secondary metabolites. The primary metabolites comprise the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's. The important secondary metabolites are organic compounds that are alkaloids, phenolics, terpenes, flavonoids, tannins, lignans, coumarins, steroids, curcumines, saponins and glycosides [7]. Natural products from microbial sources have been used as the primary source of antibiotics, but with the increasing recognition of plant-based herbal medicines as an alternative form of human health care industry [8].

2. Materials and Methods
2.1. Collection of Plant Material
The *Phyllanthus debilis* Klein ex Willd was collected from Yercaud located on Shevaroy hills, part of the Eastern Ghats, Salem district, Tamil Nadu, India. The collected plant was identified by Dr. S. John Britto S.J at The Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph's College (Campus), Tiruchirappallai, Tamil Nadu, India.

2.2. Preparation of extracts

25 g of crushed fine powders of *Phyllanthus debilis* leaves was weighed separately extracted with 250 ml of organic solvent of hexane, chloroform, acetone, ethyl acetate, ethanol and methanol. The solutions were stirred in a rotator shaker with temperature control (of 28 ± 2ºC) at constant stirring rate at 180 rpm for seven days to allow the extraction of phytocompounds from leaf sample. After 7 days of incubation period the solution was filtered through Whatman No-1 filter paper. The extract was allowed to evaporate the solvent at room temperature and sticky substances obtained were preserved in the refrigerator in airtight container for future use and redissolved with respective solvents prior to use.

2.3. Qualitative phytochemical tests

In the present study, preliminary qualitative phytochemical tests were carried out by using hexane, chloroform, acetone, ethyl acetate, ethanol and methanol solvent extract of *P. debilis* leaves using standardized procedures to identify the phytoconstituents as described by Harborne (1973), Sofowara (1993), Trease and Evans (2002) [9, 10, 11].

a. Steroids/sterols test (Salkowski's test): The extracts were dissolved in 1ml of chloroform and an equal volume of concentrated sulfuric acid at the side of the test tube wall. The upper Chloroform layer turning red color and acid layer shows greenish yellow florescence color reveal the presence of Steroids/sterols in the test sample.

b. Cardiac Glycosides test (Keller-Killani test): Five ml of extract was treated with 2 ml of glacial acetic acid and a few drops of 10% ferric chloride solution were added. This was under layer with 1 ml of concentrated sulfuric acid.
A brown ring on interface formed between the layers indicated presence of cardiac glycosides.

c. Anthraquinones test (Borntrager's test): 200mg of the extract (Past form) was shaken with 2 ml benzene solution the layer is separated and half of its own volume of 10% ammonia solution was added to the extract and shaken vigorously for 30 seconds. A pink, red, or violet coloration indicated the presence of anthraquinone.

d. Saponins test (Foam test): One ml of extract was shaken vigorously for 30 seconds with little quantity of distilled water, it forms persistent foam which indicates the presence of saponins.

e. Alkaloids test (Mayer's reagent test): Two ml of extract treated with a few drops of Mayer's reagents, which produces white, yellowish precipitation or turbidity which indicates the presence of alkaloids.

f. Tannins test (Lead acetate test): Five ml of extract was treated with a few drops of 1% lead acetate solution, produces yellow or red precipitation to show the presence of tannins.

g. Phenolic compound test (Ferric chloride test): One ml distilled water and a few drops of 10% ferric chloride solution were added in 0.5 ml of extract. A dark blue or bluish black color shows the presence of phenolic compounds in the test sample.

h. Flavonoids test (Alkaline Reagent test): Plant extract was treated with a few drops of 2% sodium hydroxide solution. The Formation of intense yellow color was produced, which became colorless, when the addition of a few drops of diluted hydrochloric acid to the mixture. These results showed the presence of flavonoids.

i. Terpenoids test (Liberman test): Three ml of acetic acid and a few drops of concentrated sulfuric acid were added to 1 ml of extract, the color change from red to blue indicates the presence of terpenoids.

2.4. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR investigations for methanolic leaf extract of *P. debilis* was carried out using FTIR Shimadzu-8400S at St. Joseph's College, Tiruchirappalli, Tamil Nadu, India. The prepared disc was then scanned over a wavelength range of 4,000 cm^{-1} to 400 cm^{-1} using an FTIR spectrometer and the corresponding spectra were recorded in the transmittance mode from 4,000 cm^{-1} to 400 cm^{-1} using FTIR software. Various modes of stretches were identified and wave numbers are assigned to know the different functional groups in the extract**.**

2.5. Gas Chromatography-Mass Spectrometry (GC- MS)

The methanolic leaf extract was further used for the identification of bioactive chemical compounds by GC-MS analysis, at The South India Textile Research Association (SITRA), Coimbatore, Tamil Nadu, and India. In this experiment, 20 µl aliquots were injected into GC8000 series GC coupled with MD800 Mass Spectrum with quadrapole mass analyzer. The chromatography was performed by using the DB5-MS column. The injection temperature was 230°C and Helium flow was 1ml/min.

2.6. Identification of Components by NIST Libraries

Interpretation of GC-MS spectrum of the unknown compounds was compared with the spectrum of known compounds stored database of National Institute Standard and Technology (NIST) and Wiley spectra Libraries. The molecular weight, molecular formula, structure of the compound and the number of hits were used to identify the name of the compound from the recorded NIST and Wiley spectra Libraries. The Identification of Phyto compounds was accomplished by searching plant compound library.

3. Results and Discussion

3.1. Qualitative analysis of bioactive metabolites

The preliminary qualitative phytochemical analysis is used to understand the presence of some active secondary metabolites; steroids/sterols, cardiac glycosides, anthraquinones, tannins, Saponins, Flavanoids, Steroids, terpenoids and so on. The hexane, chloroform, acetone, ethyl acetate, ethanol and methanolic leaves extract of *Phyllanthus debilis* Klein ex Willd. Were subjected to qualitative analysis of phytochemicals and the results of various phytocompounds are depicted in table-1.
The result observed in methanolic extract of *P. debilis* leaves was found to contain steroids/sterols, cardiac glycosides, saponins, alkaloids, tannins, phenols, flavonoids and terpenoids. Anthraquinones was not observed in methanolic extract. Ethanolic extract of plant leaves showed presence of active constituents like steroids/sterols, saponins, alkaloids, tannins, phenols, flavonoids and terpenoids, whereas cardiac glycosides, anthraquinones were not present in ethonol extract. The observation reveals the ethyl acetate extracts of leaves possessed the steroids/sterols, cardiac glycosides,

flavonoids and terpenoids. Anthraquinones, saponins, alkaloids, tannins and phenols were absent in ethyl acetate extracts. Acetone extracts of plant leaves exhibit only saponins, alkaloids, tannins and phenols. Steroids/sterols, cardiac glycosides, anthraquinones, flavonoids and terpenoids were absent in acetone extracts.

Chloroform extracts of plant leaves were indicated the occurrence of cardiac glycosides, terpenoids and all other phytocompounds such as steroids/sterols, anthraquinones, saponins, alkaloids, tannins, phenols, flavonoids were absent in chloroform extracts. Hexane extracts of plant leaves have determined the presence of cardiac glycosides, flavonoids, terpenoids and steroids/sterols, anthraquinones, saponins, alkaloids, tannins and phenols did not present in hexane extracts of *P. debilis.* Thus, the preliminary qualitative phytochemical analysis has revealed that the methanolic leaves extract *P. debilis* possesses a vast number of phytoconstituents compare to other solvents like ethanol, ethyl acetate, acetone, chloroform and hexane. Anthraquinones were completely absent in all the other solvents attempted for the leaves extract. The present investigation is in line with the earlier findings. The results of the qualitative phytochemical investigation, appears to validate the ethnomedicinal uses of plants for the traditional treatment of different ailments.

The qualitative phytochemical observation was supported by Poongani *et al.* [12] who have reported the phytochemicals present in the *Phyllanthus debilis* Klein ex Willd. and *Phyllanthus virgatus* G. Tanins, saponins, flavonoids, quinines, cardio glycosides, terpenoids, phenols, coumarins, steroids and alkaloids are present in all the test samples. Glycosides are present in *P. virgatus* were as absent in *P. debilis*. Among the five organic solvents; ethanol extracts possessed a maximum number of phytoconstituents, followed by acetone > aqueous > chloroform > petroleum ether. Senthilkumar *et al.*, observed the phyto compounds like alkaloids, flavonoids, phenols, steroids, terpenoids and cardio glycosides in the *Sida acuta* leaf extract [13]. Chandrashekar *et al.*, also reported that the phytoconstituents such as lignan, phytosterol in methanol, petroleum ether extract and followed by glycoside was present in ethyl acetate, methanol extract. Alkaloids, saponins, flavonoids, tannins and triterpinoids were not found in all five extracts [14].

The study of Awasthi *et al.*, indicated the presence of alkaloids in ethyl acetate extracts of *Phyllanthus amarus* leaf. Flavonoids were screened in whole plant parts. Tannins were present in all extracts of whole plant parts except ethyl acetate extract of leaves. Cardiac glucosides were absent in all the four plant parts of *P. amarus* were as triterpenes were present in all plant parts [15]. The similar study of Sree Devi *et al.*, reported in *Phyllanthus retusa* leaf extracts on preliminary phytochemical evaluation. The steroids were present in hexane, chloroform and methanolic extract. The alkaloids were present in ethyl acetate, chloroform and hexane extracts except the methanolic extract. Cardio glycoside was present in methanol and chloroform extract. The other metabolites such as tannins, resins, flavonoids, coumarins were not found in all the extracts [16].

Table 1: Qualitative analysis of bioactive metabolites in *Phyllanthus debilis* leaves using different solvents

S. No	Phytoconstituent	Hexane	Chloroform	Acetone	Ethyl Acetate	Ethanol	Methanol
1	Steroids/sterols	-	-	-	+	+	+
2	Cardiac glycosides	+	+	-	+	-	+
3	Anthraquinones	-	-	-	-	-	-
4	Saponins	-	-	+	-	+	+
5	Alkaloids	-	-	+	-	+	+
6	Tannins	-	-	+	-	+	+
7	Phenols	-	-	+	-	+	+
8	Flavonoids	+	-	-	+	+	+
9	Terpenoids	+	+	-	+	+	+

"+" indicated the presence of bioactive metabolites in leaves; "-" indicate the absence of bioactive metabolites in leaves.

3.2. FTIR spectrum of methanol leaves extract of *Phyllanthus debilis*

The FTIR spectrum analysis was performed to identify the presence of functional groups in the methanol extract of *P.debilis* leaves, based on the peak intensity in the region infrared radiation. The methanol extract of *Phyllanthus debilis* leaves extract absorption peaks is represented in Fig. 1. The major band peaks were observed at 3404.64 cm^{-1} 2927.97 cm^{-1}, 2855.74 cm^{-1}, 2089.29 cm^{-1}, 1733.42 cm^{-1}, 1631.63 cm^{-1}, 1609.56 cm^{-1}, 1515.15 cm^{-1}, 1443.34 cm^{-1}, 1401.53 cm^{-1}, 1245.54 cm^{-1}, 1167.88 cm^{-1}, 1079.31 cm^{-1}, 1054.23 cm^{-1}, 916.03 cm^{-1}, 819.25 cm^{-1}, 776.91 cm^{-1} and 603.07 cm^{-1}.

The strong, broad band at 3404.64 cm^{-1} can be assigned to O-H stretching of the hydroxyl group. Similar to our report, Senthil kumar *et al.*, also reported the O-H stretching vibration around 3410 cm^{-1} which is originated from physical absorbed H_2O or surface OH clusters [17]. The band located at 2927.97 and 2853.04 cm^{-1} were attributed Alkanes C-H stretching. The peak at 2089.29 cm^{-1} was recognized to alkanes C≡C stretch [18].

The band 1733.42 cm^{-1} corresponds to Aldehydes C=O stretch. Alkenes, Aromatic compound was stretching frequencies at 1631.63, and 1609.56 cm^{-1}, $cm^{-1}$and 1515.15 cm^{-1} C=C stretch respectively. The peak represents at 1443.34 cm^{-1}1401.53 cm^{-1}, 916.03 cm^{-1}, 819.25 cm^{-1} (Alkanes C-H bending) and 1245.54 cm^{-1} (Alkanes C-C stretch). The stretching frequencies at 1167.88 cm^{-1} (Amines C-N stretch), 1079.31 cm^{-1} and 1054.23 cm^{-1} (Ethers C-O stretch). Aliphatic Bromo compounds (C-Br) and Aliphatic Chloro compounds (C-Cl) found stretching frequencies of 603.07 cm^{-1} and 776.91 cm^{-1} were observed [19]. In the present study, FTIR spectrum of a methanol extract of *Phyllanthus debilis* leaves revealed that it contains different phytocompound functional groups such as alkanes, ethers, amines, aldehydes, aliphatic bromo and aliphatic chloro compounds and aromatic compounds, which may be lead to pharmaceutical applications.

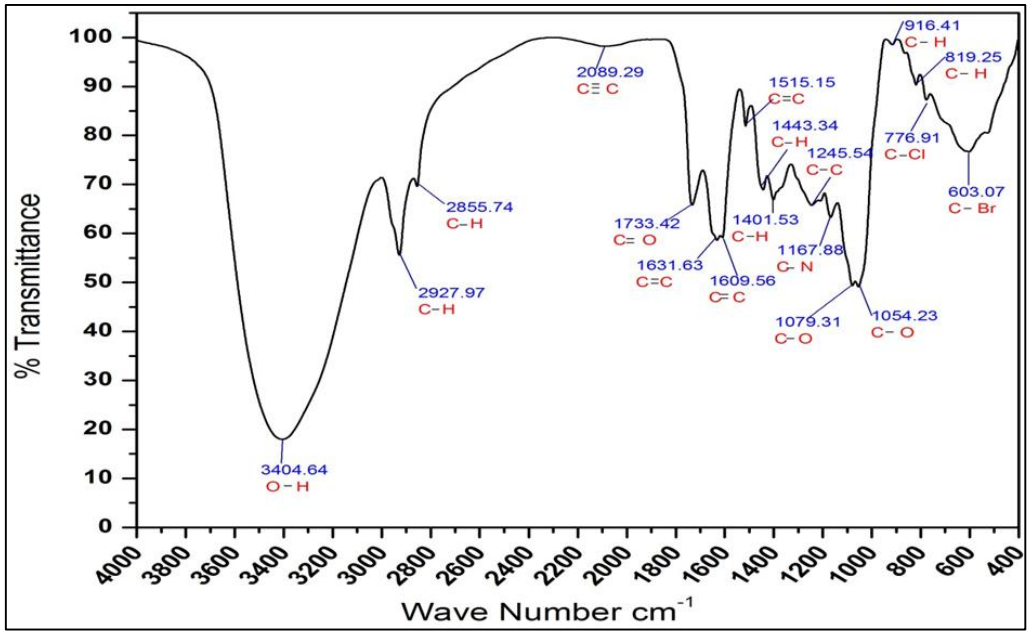

Fig 1: FTIR spectra of methanol leaf extract of *Phyllanthus debilis*

3.3. Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis of the methanol leaf extract of *Phyllanthus debilis* showed the presence of 9 various major compounds which were identified based on the retention time (RT), molecular formula (MF) and molecular weight (MW) (Figure-2 and Table-2). The biological activity of phytocomponents identified in *P. debilis* leaves by GC-MS is based on Dr. Duke's (2017) phytochemical and ethnobotanical databases [20] and PubChem online database [21]. Methanolic extract of *P. debilis* has 1, 2, Benzenedicarboxylic acid, Mono (2-ethylhexyl) ester acting as a major compound with a retention time 26.32 which may be lead to its antimicrobial and other biological applications followed by Phyto l was observed with retention time of 21.08 min which lead to its anticancer, antioxidant, anti-inflammatory, diuretic, antitumor, chemo preventive, antimicrobial, used in vaccine formulations.

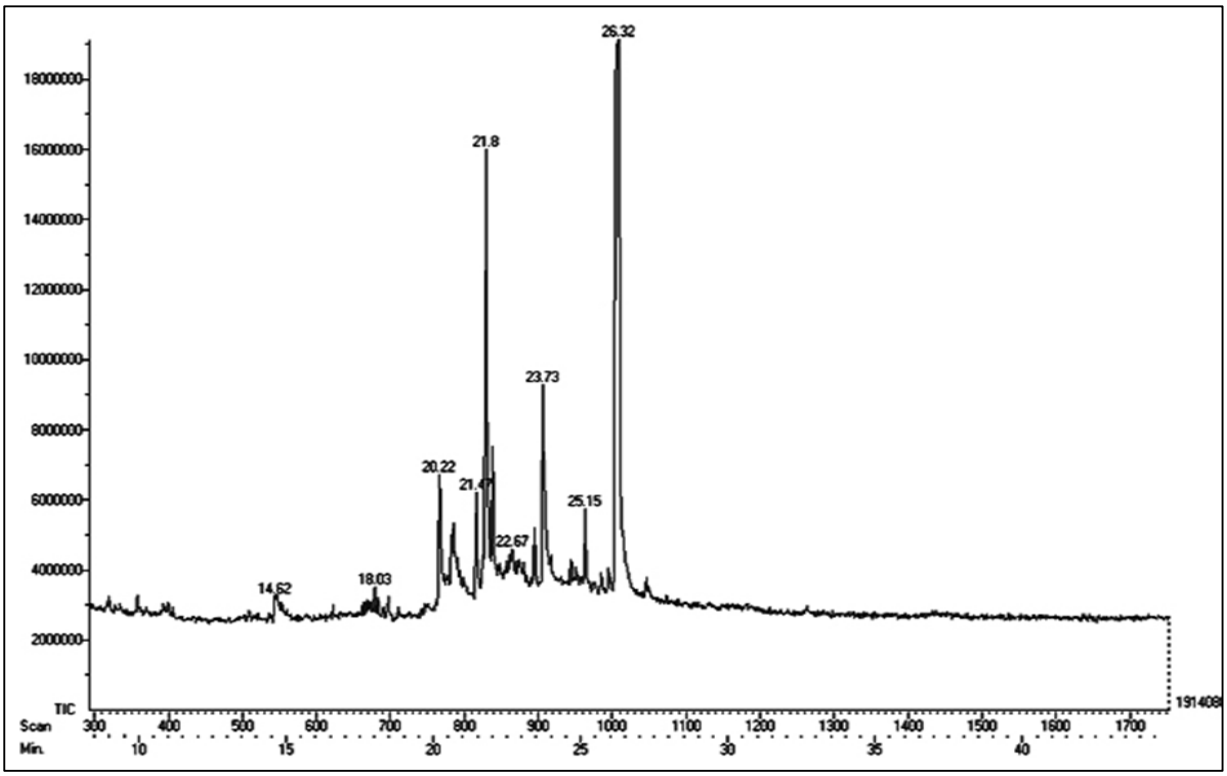

Fig 2: Gas chromatography-mass spectrometry chromatogram of methanol leaf extract of *Phyllanthus debilis*

The variety of compounds, such as [Phenol, 2,4-bis(1,1)-dimethylethyl], [Flavone, Tridecanoic acid Methyl ester], [13-Hexyloxacyclotridec-10-en-2-one], [Phytol], [Oleic acid], [2H-Naphthalen-1-one, 3, 4-dihydro-6-methoxy–2-(4-methoxybenzylidene)-], [Benzonaphth-1-one, 6-acetate-9-pheny l], [1, 2, Benzenedicarboxylic acid], [Mono (2-ethylhexyl)ester] with different retention time. Similar secondary compound Phenol, 2,4-bis (1,1)-dimethylethyl was present in *Phyllanthus wightianus* [22], *Phyllanthus emblica* [23] and followed by 1,2,Benzenedicarboxylic acid Mono(2-

ethylhexyl)ester compound were present in the methanolic extract of *Phyllanthus acidus* [24]. Several fatty acids and phenolic compounds were known in GC-MS analysis of methanol extract which may be responsible for the antimicrobial activity [25]. The previous reports showed that 1,2-Benzenedicarboxylic acid, butyl 2- compounds obtained from plant extract showed various biological activities like antibacterial, antifungal, antiviral and anticancer [19] and also reported methanolic extract of *P. amarus* was screened for the presence of several bioactive compounds [26].

4. Conclusions

In the present study, phytochemical components such as steroids/sterols, cardiac glycosides, saponins, alkaloids, tannins, phenols, flavonoids and terpenoids were present in the methanolic leaf extract of *Phyllanthus debilis*. FTIR spectrum analysis of a methanolic leaf extract of *Phyllanthus debilis* indicated the presence of different phytofunctional groups such as alkanes, ethers, amines, aldehydes, aliphatic bromo and aliphatic chloro compounds and aromatic compounds. GC-MS chromatogram analysis of the methanolic leaf extract of *Phyllanthus debilis* revealed the 9 major bioactive compounds, which have antioxidant, antimicrobial and antifungal activity. Usually medicinal plants contain numerous secondary metabolites, which are plays a pivotal role to control the growth of the microorganisms. Therefore, based on the results it can be concluded that the methanolic extract of *Phyllanthus debilis* may hold enormous resource of pharmaceutical properties which might be used for natural therapy.

Table 2: Biochemical Compounds detected in methanol leaf extract of *Phyllanthus debilis* by GC-MS analysis, and its Biological activity

S. No	R.T	Name of the compounds	M. F	M.W	Category of compound	Biologocal activity
1.	14.62	Phenol, 2,4-bis(1,1)-dimethylethyl	$C_{14}H_{22}O$	***206.32***	Phenolic compound	Antibacterial, antifugal, antimalarial and antioxidant activity
2.	18.03	Flavone	$C_{15}H_{10}O_2$	222.24	Flavonoid compound	Anticancer, antioxidant, anti-inflammatory, Antibacterial, Antiviral, Hepatoprotetive activity
3.	20.22	Tridecanoic acid, Methyl ester	$C_{14}H_{28}O_2$	228.37	Fatty acid	Cancer preventive
4.	21.47	13-Hexyloxacyclotridec-10-en-2-one	$C_{18}H_{32}O_2$	280.45	Fatty acid	Antimicrobial activity
5.	21.8	Phytol	$C_{20}H_{40}O$	296.53	Diterpene	Anticancer, antioxidant, anti-inflammatory, diuretic, antitumor, Chem Opreventive, antimicrobial, use in vaccine formulations
6	22.67	Oleic acid	$C_{18}H_{34}O_2$	282	Fatty acid	Antibacterial
7.	23.73	2H-Naphthalen-1-one, 3,4-dihydro-6-methoxy-2-(4-methoxybenzylidene)-	$C_{19}H_{18}O_3$	294.12	Keto compounds	Antiaggregant and antifungal
8.	25.15	Benzonaphth-1-one, 6-acetate-9-phenyl	$C_{21}H_{14}O_3$	314.34	Keto compounds	Anticancer
9.	26.32	1,2,Benzenedicarboxylic acid, Mono(2-ethylhexyl)ester	$C_{16}H_{22}O_4$	278.34	Plastizer compound	Antimicrobial activity

R.T: Retention Time, M.F: Molecular Formula, M.W: Molecular Weight, ∗Source: Dr. Duke's Phytochemical and Ethno botanical Databases (online database), https://pubchem.ncbi.nlm.nih.gov

5. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

6. Acknowledgments

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