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Analysis of Bioactive Compounds in Methanolic Leaf Extract of *Diospyros melanoxylon* Roxb. Challenged with AM Fungi by Liquid Chromatography Mass Spectrometry (LC-MS) Technique

S. Syam Prasad[1] and P.R. Sushama[2]*
[1]Research Scholar, Department of Botany, University College of Science, Osmania University, Hyderabad (Telangana), India.
[2]Assistant Professor, Department of Botany, University College for Women, Osmania University, Hyderabad (Telangana), India.

(Corresponding author: P.R. Sushama)*
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ABSTRACT: Plants are abundant sources of a variety of bioactive phytochemicals that have a variety of biological impacts. The protection of human health and benefit come from the screening of phytochemical substances. *Diospyros melanoxylon* (Roxb.), a member of the Ebenaceae family, is a significant commercial, multipurpose, and medical plant utilized in a variety of circumstances. The primary goal of the current experiment was to examine the phytochemical substances in the leaves of the *D. melanoxylon* challenged with AM fungi using liquid chromatography and mass spectrometry, (LC–MS). Plant challenged with AM fungi leaves samples were analyzed with LC-MS analysis and 27 different phytochemical substances with different molecular weight were found. The primary bioactive substances were gallic acid, ursolic acid, oleanolic acid and betulinic acid (Rt-1.07, 23.82, 23.70 and 24.44). Analysis of the mass spectra has allowed for the identification of the chemicals. The use of *D. melanoxylon* leaves for treating a number of ailments is confirmed by the presence of several bioactive chemicals with diverse chemical structures. Hence, it is claimed that plant is crucial for pharmaceuticals. Moreover, it might be necessary to isolate specific bioactive substances in order to discover a novel medicine.

Keywords: *Diospyros melanoxylon*, Leaves, LC–MS, Phytochemicals, Methanol.

INTRODUCTION

The most economically significant species of the *Diospyros* genus is *Diospyros melanoxylon* Roxb. (Ebenaceae), which is indigenous to India (Sastry, 1952). Thuniki, scientifically known as *Diospyros melanoxylon*, has been used to treat a variety of conditions including dyspepsia and diarrhoea (Ambastha, 1986). In Indian traditional medicine, *D. melanoxylon* leaves were used as a diuretic, styptic, laxative, carminative and to treat night blindness and improve eye sight (Chattarjee and Pakrashi 1995). Due to the using in beedi industries as wrapping, leaves are highly prized (Santapau and Henry 1994). For Indian tribal people, leaves were a crucial source of income (Rathore, 1972). Hostettmann *et al.* (1997) research on rapid detection and isolation of bioactive compounds in plant crude extracts. Moreover, the bark has diuretic, carminative, laxative, styptic, and ocular astringent properties (Mallavadhani *et al.,* 1998). Since plant materials are well known for producing new antimicrobial agents, researchers have been looking for new antibacterial medications with plant origins. Several substances originating from plants, including vincristine, quinine, salicyclic acid, eligitalis, morphine, and codeine, have significant medicinal promise (Paresh and Chanda 2007). There is an urgent need to identify lead chemicals that are active against resistant infections because the therapeutic qualities of many plants have not yet been thoroughly studied for phytochemistry and pharmacognosy (Recio *et al.,* 1995). Three groups (Gupta & Rao 1964; Rao *et al*, 1964, 1966; Row *et al*, 1969 and Choudhary, 1973) worked on *D. melanoxylon* leaves and reported the presence of ursolic acid and oleanolic acid. In this regard, *D. melanoxylon* leaves extracts have undergone a thorough chemical profiling. Vinaya Kumar *et al.* (2022) revealed that the *Diospyros melanoxylon* methanol leaf extracts have shown significant reduction of the super oxide anions and they inhibited the formation of formazan with high DPPH scavenging activity.

MATERIAL AND METHODS

Collection of Samples. *D. melanoxylon* Roxb. (Ebenaceae) leaves were collected from thefield site of germination studies in sathupally, Khammam, Telangana, India in the month of April to May 2022.

Preliminary phytochemical screening assay. First, we

screened the phytochemicals having in the leaves sample of *D. melanoxylon* by qualitative analysis with fresh extracts of leaves by following the standard methods (Harboene, 1998) to detect the presence of phytochemicals.

Phenolics:0.5 grams of leaves were boiled in 100 ml of water. 2 ml of $FeCl_3$ was added to the leaves extract and observed for formation of green or blue colour which indicates the presence of phenolics.

Flavonoids:0.5 grams of leaves were macerated in 100 ml of water and 5 ml of ethyl acetate was added to the leaves extract. The mixture was shaken and allowed to stand in the bottom. Production of yellow colour which indicates as positive for flavonoids.

Terpenoids: 50mg of leaves were macerated in 5 ml of ethanol. Extract was mixed with 2ml of chloroform, slightly heated and allowed to cool. 3 ml of H_2SO_4 was added slowly along the sides of the tubes. A reddish-brown coloured precipitate was formed at the interface indicates the presence of terpenoids (Indumathi *et al.,* 2014).

Alkaloids: 0.5 grams of leaves macerated in 100 ml of water. extract was dissolved in diluted HCL solution and filtered. The filtrate was tested with Drangendroff's and Mayer's reagent. Above treated solution observed for precipitates.

Tannins: 0.5 grams of leaves grinded in 100 ml of water and filtered. 10% $FeCl_3$solution was added to the clear supernatant and observed for change of colour to blue which indicates the presence of Tannins.

Saponins:0.5 grams of leaves were maceratedin 100 ml of distilled water and transferred to a test tube. The test tube was shakedfor 30 sec and allowed to stand and wait for 30 min. Above solution observed for the honey comb froth after 30 min, it indicates the presence of saponins.

Preparation of standard solution. Lupeol, betulinic acid, oleanolic acid, and ursolic acid stock solution. 1 mg of carefully weighed lupeol, betulinic acid, oleanolic acid, and ursolic acid were dissolved in 100 ml of methanol in a volumetric flask to create the solution (1 mg/ml). To create standard solutions comprising 2, 4, 6, 8, and 10 g/ml concentrations of lupeol, betulinic acid, oleanolic acid, and ursolic acid, the stock solutions of 2, 4, 6, 8, and 10 ml were transferred to 10 ml volumetric flasks and the volume of each vial was made up to 10 ml with methnol.

Preparation of sample solution. Diospyros melanoxylon leaves were initially kept in the shade, then dried at room temperature for a week before being milled into a fine powder. The following technique was carried out three times: 10ml of methanol was added to 1g of powdered material for 24 hours, and the solvents were filtered using Whatmann No. 1 filter paper. At a temperature of 40±2 °C, the extract was concentrated using a rotary evaporator under reduced pressure. The sample solution was created by dissolving precisely 1 mg of the extract in 1 mL of methanol and filtering the filtrate through a 0.45 m filter membrane.

LC-MS Equipment conditions. Instrumental settings for chromatography and mass spectrometry, phytochemicals were evaluated with an Acquity of H-Class UPLC system coupled online with a Xevo G2-XS QTOF-MS (Waters, Milford, USA) outfitted with a BEH C18 column (100 mm 2.1 mm id., 1.7 lm) at flow rate 0.4 ml/min. UPLC column was kept at 40°C with help of active pre heater. Each sample was subjected to a 31-minute (Mobile phase-B) gradient elution 5 - 95% acetonitrile with 0.1% formic acid and 0.1% formic acid with water in Millipore used as mobile phase A (0 min, 5% B; 5.0 min, 15% B; 9.0 min, 25% B; 14.0 min, 50% B; 20.0 min, 70% B; 23.0 min, 85% B; 25.0 min, 95% B; 30.0 min 95% B. At around 3 minutes, the UPLC column was re-equipped to its initial condition as the next injection was being prepared. The following parameters were used for mass spectrometric measurements: ESIMSE, range 50-2000m/z, capillary voltage 3.0/2.0 kV, sample cone 40 V, temperature 120°C, dissolved temperature 350°C, cone gas flow rate 50 l/hr and Mass fragmentation done at 20-50 eV collision energy in ramp mode. In order to carry out the instrumental error correction, leucine-enkephalin used as a lock mass solution in both positive and negative mode.

RESULT AND DISCUSSION

So many advance analytical methods have been reported for the identification of secondary metabolites from medicinal plants, like HPLC-MS, HPTLC-MS, DART-MS, 1H &13C NMR, Image mass spectroscopy, including UPLC-QTOF-MS/MS analysis. Among this, UPLC coupled with the mass spectrometry (MS) being widely used technique for the effective separation with less retention time and qualitative analysis of both known and unknown compounds from methanolic leaf extracts. UPLC-ESI-QTOF-MS/MS is a powerful tool for compounds identification. In this study, we are utilized UPLC-ESI-QTOF-MS/MS technique for chemical profiling of leaf of **Diospyros melanoxylon.** Firstly, we screened the phytochemicals by qualitative analysis for the different phytochemicals *i.e*., phenolics, flavonoids, alkaloids, saponins and tannins in methanolic leaf extracts of *D. melanoxylon.* All the tested compounds were showed the presence of all the tested phytochemicals. Within the tested phytochemicals phenolics, flavonoids and terpenoids were present strongly in great number (Table 1 and 2).

The LC-MS resulted for Thuniki leaves extract in Fig. 1, which showed the peaks obtained respect to retention time. The peaks indicated the concentration of substance, where the retention time indicated the type of compound which responsible for the peak. Secondary metabolites of *Diospyros melanoxylon* were analyzed by reverse-phase LC-MS eluted with gradient mobile phase containing the water and acetonitrile with 0.1% formic acid. In the optimized method, acquired LC - MS total chromatograms of ions in methanolic leaf extract of *D. melanoxylon* was analyzed in ESI negative ionization

mode and the results were presented on Fig. 1 and 2. The peak structural assignment was performed based on their mass spectral data (Mass, molecular formula& fragmentations pattern) retention time, isolated standard comparison with *D. melanoxylon* extract mass fragmentation and searched in online databases (Reaxys, SciFinder & DNP). Our UPLC-ESI(-)-QTOF-MS studies (Fig. 1 & Table 2) leads to the identified of several compounds from methanolic leaf extract of *D. melanoxylon.*

Table 1: Qualitative analysis of phytochemicals.

Sr. No.	Phytochemical	Presence (+) / Absence (-)
1	Phenols	++
2	Flavonoids	+++
3	Terpenoids	+++
4	Alkaloids	++
5	Saponins	+
6	Tannins	+

+++: Very strongly present; ++: strongly present; +: present: -: absent.

Table 2: LC-Q-TOF-MS analysis of bio active compounds in methanolic leaf extract of *D. melanoxylon*.

Rt (min)	Mass (M/Z)		Error (ppm)	Fragmentation	ion formula	Compound name
	Observed	Calculated				
1.07	169.0153	169.0137	9.5	$C_7H_5O_5$	$C_7H_6O_5$	Gallic acid
1.63	289.0715	289.0712	1.0	245.0801($C_{14}H_{13}O_4$) 203.0709($C_{12}H_{11}O_3$)	$C_{15}H_{13}O_6$	P-coumaroyltriacetate
3.33	300.9980	300.9984	-1.3	$C_{14}H_5O_8$	$C_{14}H_6O_8$	Ellagic acid
8.28	301.0346	301.03	-0.7	$C_{15}H_9O_7$	$C_{15}H_{10}O_7$	Quercetin
10.25	327.2174	327.2171	0.9	229.1445($C_{12}H_{21}O_4$) 211.1341($C_{12}HH_{19}O_3$)	$C_{18}H_{31}O_5$	9S,12R,13S -Trihydroxy10E, 15 zoctadecadienoic acid
10.67	331.2490	331.2484	1.8	313.2382($C_{18}H_{33}O_4$)	$C_{18}H_{35}O_5$	9,10,18-trihydroxystearate
11.11	329.2338	329.2328	3.0	233.1157($C_{14}H_{17}O_3$) 211.1347($C_{12}H_{19}O_3$)	$C_{18}H_{33}O_5$	9,12,13-trihydroxyoctadecenoate
15.30	487.3432	487.3423	1.8	469.3313($C_{30}H_{45}O_4$)	$C_{30}H_{47}O_5$	Holothurinogenin
17.12	457.3318	457.3318	0.0	297.1512($C_{19}H_{21}O_3$)	$C_{29}H_{45}O_4$	benzyl hydrogen 2-octadecenylsuccinate
17.53	667.3550	667.3541	1.3	487.3419($_{30}H_{47}O_5$) 577.3000($C_{31}H_{45}O_{10}$)	$C_{31}H_{55}O_{15}$	Vannilic ester
17.81	315.2535	315.2535	0.0	---	$C_{18}H_{35}O_4$	9,10-Dihydroxystearate
18.02	595.2900	595.2907	-1.2	279.2318($C_{18}H_{31}O_2$)	$C_{34}H_{43}O_9$	Unknown
18.63	533.3694	533.3690	0.7	275.1859($C_{14}H_{27}O_5$)	$C_{28}H_{53}O_9$	Triton X-100
19.21	295.2277	295.2273	1.4	277.2169($C_{18}H_{29}O_2$)	$C_{18}H_{31}O_3$	Hydroxylenoliate
18.77	471.3477	471.3474	0.6	311.1676($C_{20}H_{23}O_3$)	$C_{30}H_{47}O_4$	(3β,16β)-3,16-Dihydroxyolean-12-en-28-oatato
19.39	471.3481	471.3474	1.5	311.1676(C20H23O3)	$C_{30}H_{47}O_4$	(3β,16β)-3,16-Dihydroxyolean-12-en-28-oatato
19.70	471.3477	471.3474	0.6	311.1676($C_{20}H_{23}O_3$)	$C_{30}H_{47}O_4$	(3β,16β)-3,16-Dihydroxyolean-12-en-28-oatato
20.18	5973052	597.3064	-2.0	293.2119($C_{18}H_{29}O_3$) 281.2469($C_{18}H_{33}O_2$)	$C_{34}H_{45}O_9$	Salannin
20.30	633.3803	633.3791	1.9	571.3220($C_{33}H_{47}O_8$) 503.3362($C_{30}H_{47}O_6$) 469.3303($C_{30}H_{45}O_4$)	$C_{39}H_{53}O_7$	acylated triterpenoid
21.28	709.4019	7094010	1.3	663.3912($C_{40}H_{55}O_8$) 483.2699($C_{29}H_{39}O_6$) 397.2242($C_{21}H_{33}O_7$) 199.1698($C_{12}H_{23}O_2$)	$C_{34}H_{61}O_{15}$	Unknown
21.73	617.3858	617.3842	2.6	497.3238($C_{31}H_{45}O_5$) 453.3358($C_{30}H_{45}O_3$) 325.1831($C_{21}H_{25}$)	$C_{39}H_{53}O_6$	acylated triterpenoid
22.31	617.3861	617.3842	3.1	415.3197($C_{23}H_{43}O_3$) 453.3358($C_{30}H_{45}O_3$) 497.3238($C_{31}H_{45}O_5$)	$C_{39}H_{53}O_6$	acylated triterpenoid
22.93	511.3420	511.3423	-0.6	465.3369($C_{31}H_{45}O_3$) 339.1981($C_{22}H_{27}O_3$)	$C_{32}H_{47}O_5$	Unknown
23.41	619.4213	619.4210	0.5	417.3432($C_{30}H_{47}O_4$) 339.1995($C_{15}H_{31}O_8$)	$C_{36}H_{59}O_8$	Unknown

23.70	455.3532	455.3525	1.5	---	$C_{30}H_{47}O_3$	Oleanolic acid
24.30	277.2171	277.2168	1.1	---	$C_{18}H_{29}O_2$	Fatty acid
23.82	455.3525	455.3525	0.0	---	$C_{30}H_{47}O_3$	Ursolic acid
24.44	455.3526	455.3525	0.2	407.3308($C_{29}H_{43}O$) 377.2841($C_{27}H_{37}O$) 309.3156($C_{21}H_{41}O$) 269.2495($C_{17}H_{33}O$)	$C_{30}H_{47}O_3$	Betulinic acid
25.00	283.2636	283.2637	-0.4	227.1997($C_{14}H_{27}O_2$)	$C_{18}H_{35}O_2$	Fatty acid
25.63	603.4055	603.4049	1.0	427.3407($C_{25}H_{47}O_5$)	$C_{39}H_{55}O_5$	Unknown
25.76	453.3364	453.3369	-1.1	323.2932($C_{21}H_{39}O_2$) 307.2998($C_{21}H_{39}O$)	$C_{30}H_{45}O_3$	Moronate
26.22	279.2327	279.2324	1.1	261.2206($C_{18}H_{29}O$)	$C_{18}H_{31}O_2$	Linoleate
26.49	601.3893	601.3893	0	453.3352($C_{30}H_{45}O_3$) 311.2944($C_{20}H_{39}O_2$) 279.2296($C_{18}H_{31}O_2$) 255.2304($C_{16}H_{31}O_2$)	$C_{39}H_{53}O_5$	Betulinic acid
27.62	587.4296	587.4312	-2.7	---	$C_{36}H_{59}O_6$	Unknown

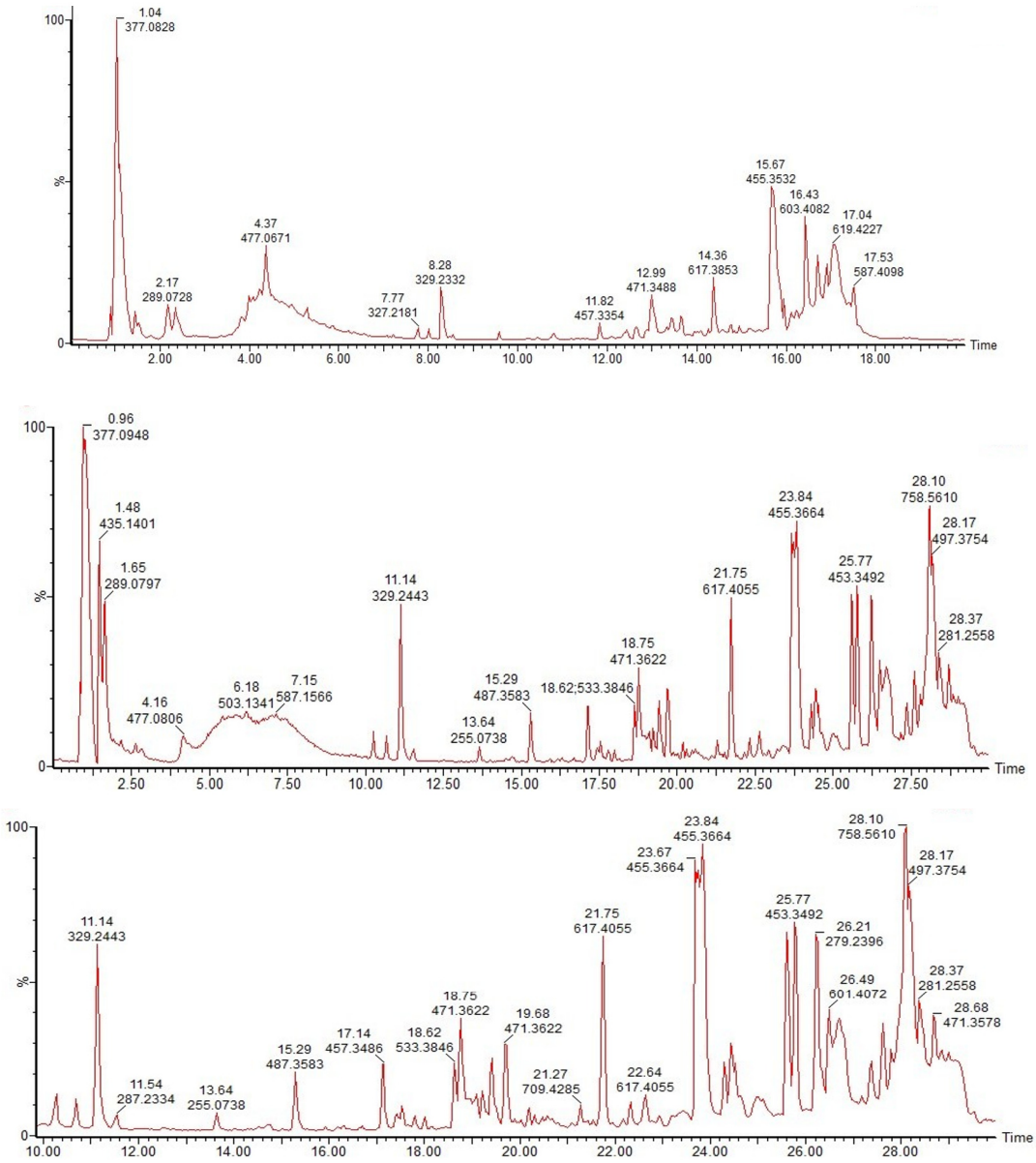
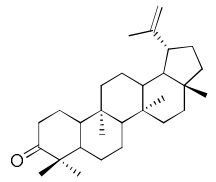
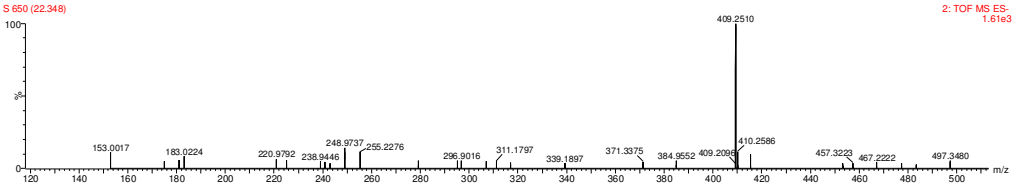
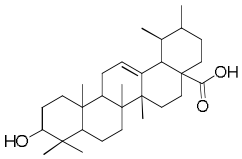


Fig. 1. A typical chromatogram of the bioactive compounds presents in methanolic leaf extract of *D. melanoxylon.*

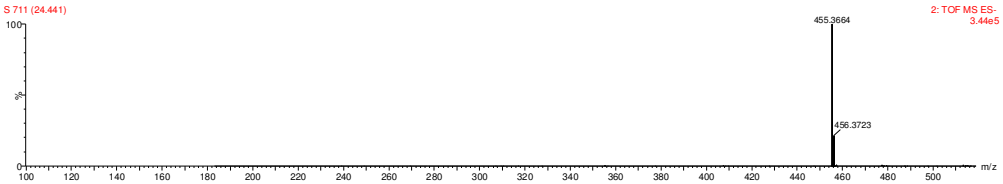


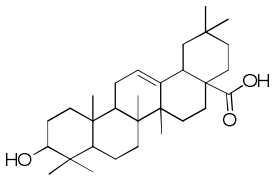
Chemical Formula: $C_{29}H_{46}O$
Exact Mass: 410.35



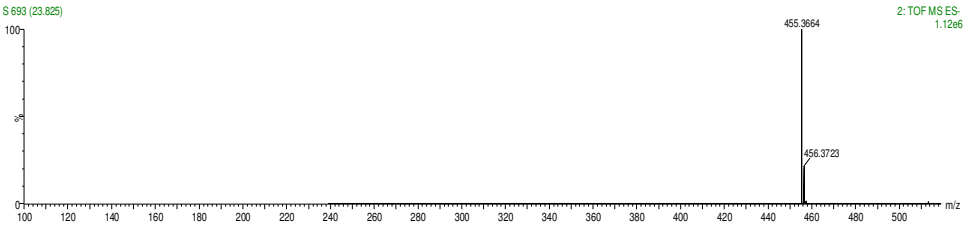


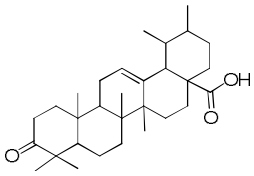
Chemical Formula: $C_{30}H_{48}O_3$
Exact Mass: 456.36





Chemical Formula: $C_{30}H_{46}O_3$
Exact Mass: 454.34





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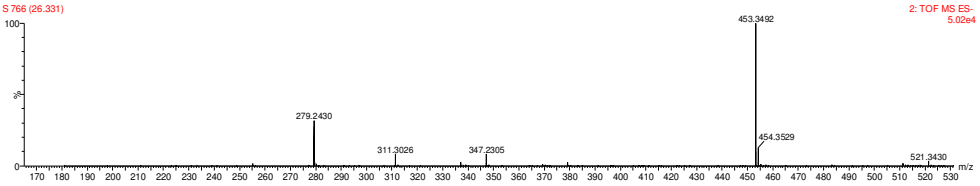

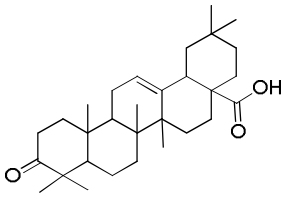

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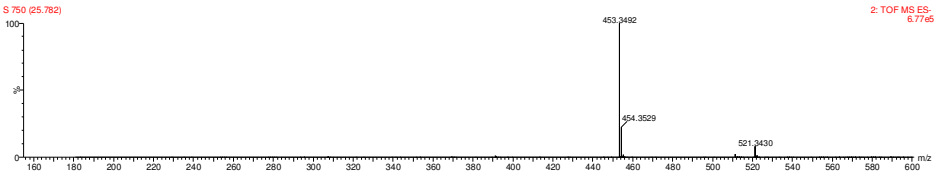

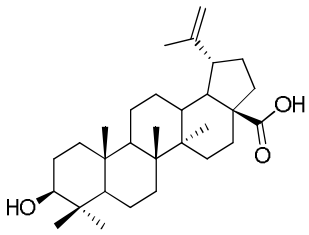

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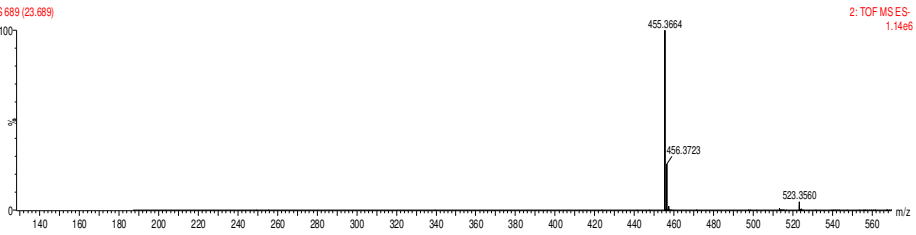

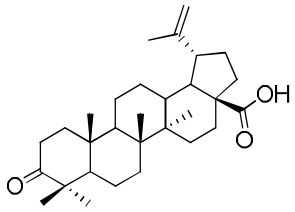

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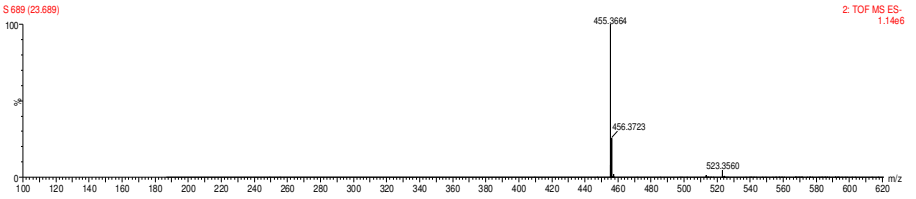
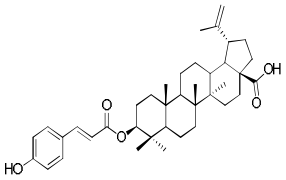
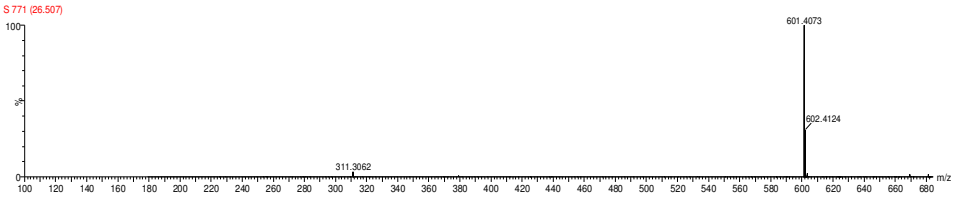
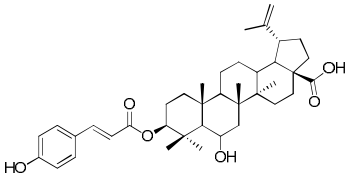
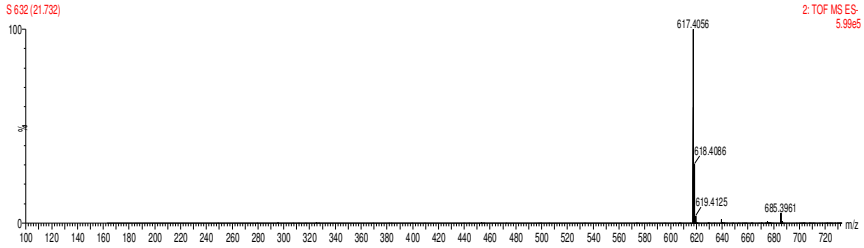


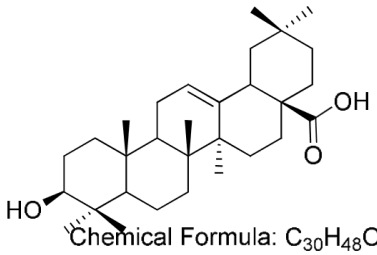


Chemical Formula: $C_{36}H_{54}O_5$
Exact Mass: 602.40



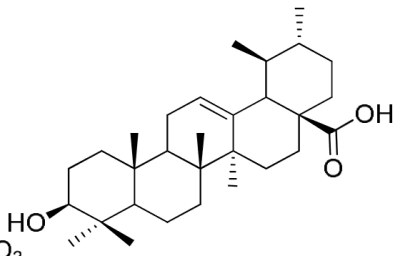






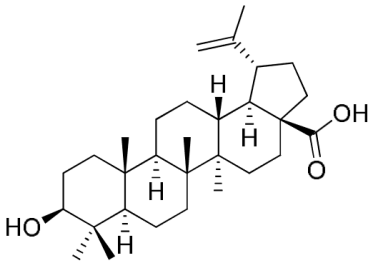
Chemical Formula: $C_{30}H_{48}O_3$
Exact Mass: 456.36

Oleanolic acid



Chemical Formula: $C_{30}H_{48}O_3$
Exact Mass: 456.36

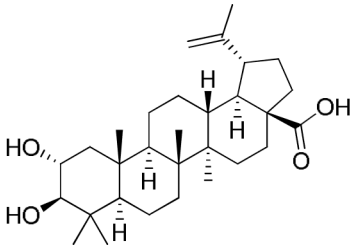
Ursolic acid



Chemical Formula: $C_{30}H_{48}O_3$

Exact Mass: 456.36

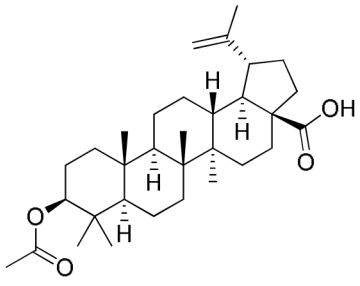
Betulinic acid



Chemical Formula: $C_{30}H_{48}O_4$

Exact Mass: 472.36

alphitolic acid



Chemical Formula: $C_{32}H_{50}O_4$
Exact Mass: 498.37

acetly- Betulinic acid

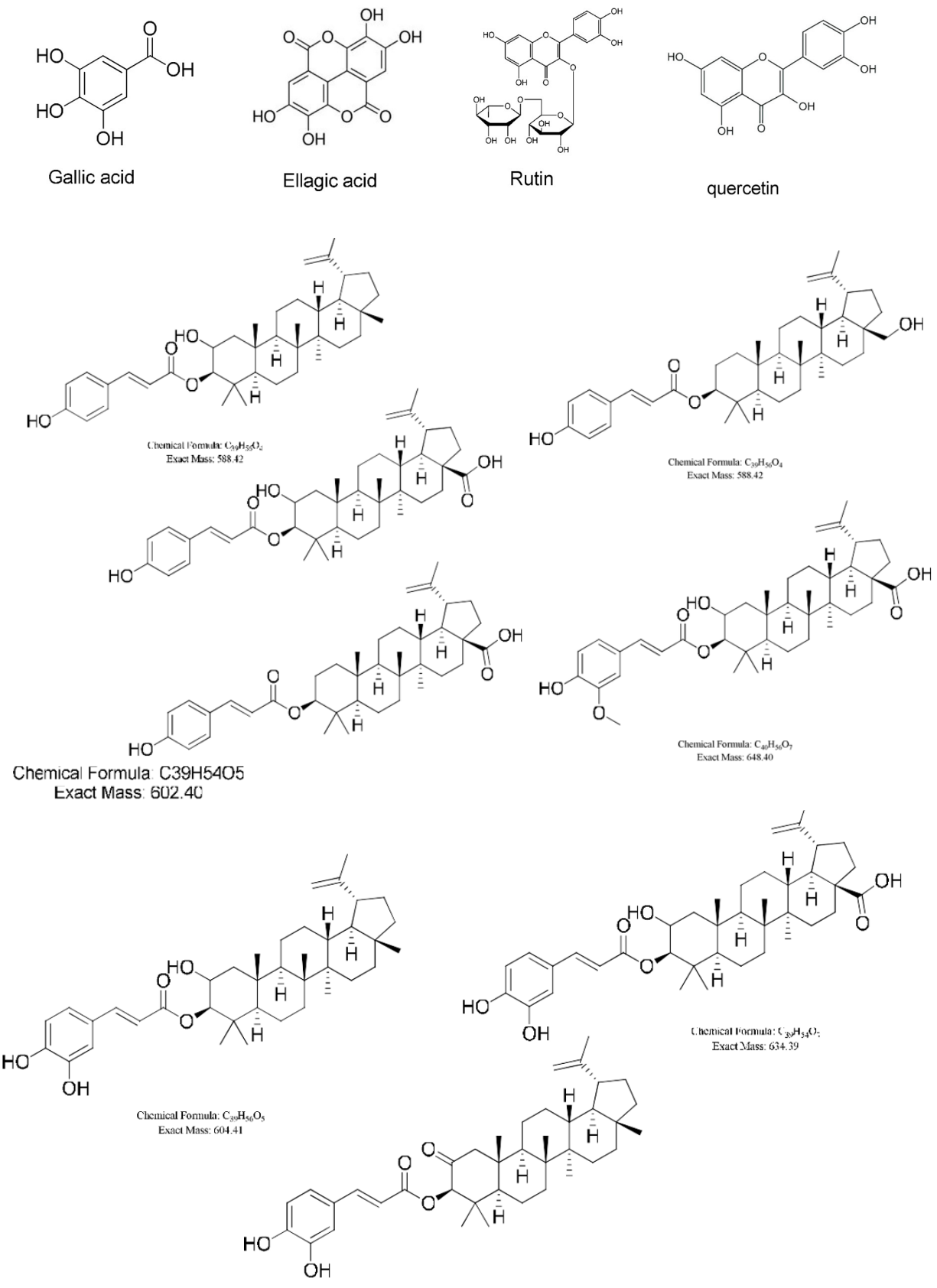


Fig. 2. Structures of some identified compounds in methanolic leaf extract of *Diospyros melanoxylon* using LC-MS.

Identified compounds belong to the Fatty acids, Tetra-cyclic triterpenoids, Penta-cyclic triterpenoids and phenolic compounds. Based on isolated stranded comparation, we have identified the Betulinic acid at Rt24.44 with m/z 455.3526[M-H]-, Oleanolic acid at Rt 23.70 with m/z 455.3532[M-H]-and ursolicacid at Rt 23.82 with m/z 455.352 [M-H]-. Based on identified mass coupled the DBE (double bond equivalence) and literate data, we have identified several betulinic acid derivatives were identified from the leaves of *Diospyros melanoxylon*. In these derivatives contains hydroxyl cinnamic acid coupled the betulinic acid. In addition to this we have identified few phenolic compounds that are gallic acid (m/z 169.0153), ellagic acid(m/z 300.9980) and two flavonoids are Quercetin(m/z 301.0346) and Rutin. Along with this we have identified several fatty acids shown in Fig. 2 and Table 2.

DISCUSSION

The present study of phytochemical investigation in *D. melanoxylon* leaves differs with earlier reports in so many aspects. In the present study, we have identified the unreported phenolics such as gallic acid and Ellagic acid and flavonoids quercetin and rutin and Triton × 100, Hydroxylenoleate, Vannilic acid, salannin, moronate and some fatty acids in added newly to the reported compounds (Mallavadhani *et al.,* 1998). More interestingly reported compounds were obtained in interestingly in large quantity and gallic acid, ellagic acid, quercetin and rutin were found in large quantities in *Diospyros* species associated with AM fungi screened so far. It is worthy that the present study was studied with young foliage leaves (Juvenile) treated with AM fungi, whereas earlier reports might be investigated in matured leaves without reporting AM fungi. The accumulation of phytochemicals Ursolic acid, Lupeol, Gallic acid, triterpenoids in huge quantity is relevant in view with the huge applications in biological and commercial. Khadzhieva *et al*. (1987) reported triterpenes were exhibit excellent active properties. Mezzetti *et al*. (1971) reported the ursolic acid used as an emulsifier in the industries of pharmaceutical, food and cosmetics. It has long been believed that pentacyclic triterpenes are pharmacologically inert, but their significance is growing in light of recently discovered intriguing actions like anti-cancer (Ishida *et al*., 1990), anti-HIV (Xu *et al*., 1996), and anti-inflammatory (Recio *et al*., 1995). In Japan, ursolic acid and oleanolic acid have been suggested as treatments for skin cancer (Muto *et al*., 1990). In Japan, ursolic acid/oleanolic acid-containing cosmetic products are patented for topical use in preventing skin cancer (Ishida *et al.,* 1990). Corsolic acid, a dihydroxy triterpenic acid, has recently been discovered to be an effective cytotoxic and protein kinase inhibitor. Ursolic acid was discovered to have strong antifeedant activity against the insects *Spilosoma obliqua* and *Spodoptera litura* on the biocidal front (Shukla *et al*., 1996). According to reports, the amyrin a-amyrin palmitate has high chemosterilant activity and insect growth inhibition capabilities (Shankaranarayana *et al.,* 1980). Patrakar and Ghiware (2017) reported that some pharmacological aspects of *Diospyros melanoxylon*. Usha Kumari *et al*, 2019 published a report on utilization of different parts of *D. melanoxylon*. The leaf extract also consists of ester group. Similar findings were observed in Junairiah *et al.* (2018) and the results showed that methanol extract of D. melanoxylon leaves contained of alkaloids, terpenoids, flavonoids, polyphenols and tannin compounds. The results of our study support the importance of *Diospyros melanoxylon* from its traditional medicinal value to advance pharmacognostic and pharmacological studies.

CONCLUSIONS

The compounds identified in the *D. melanoxylon* leaves extract most have medicinal properties and some have antimicrobial activities. Present study declared that the 27 different phytochemical compounds were identified as the phenolics, flavonoids, terpenoids, fatty acids and some unknown compounds having several enormous applications. Hence it can be used for further processing like encapsulation.

FUTURE SCOPE

The future of this research study is to provide a complete description of phytochemical constituents as well as a participatory scientific assessment of the important phytochemical compounds and their pharmacological action for the future creation of novel medicine to identify the exact applications in medicine to study and application of AM fungi to the plant and study the defense mechanism and sustainability at field level application with AM fungi.

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Abbreviations: LC-MS: Liquid Chromatography Mass Spectrometry; M/Z: Mass/charge number of ions; Rt: Retention time; ppm: Part per million; min-minutes; gm: gram; mg: milli gram; ml: microliter; µg: microgram; µm: micrometer; ºC: degree centigrade; ev: electronic volte; Acknowledgements: Not Applicable.

Author Contribution: SSP, KAK and PRS were involved in the conceptualization of research work and designing of experiments. SSP carried out the experiment and recorded the data. SSP, KAK and PRS were involved in the interpretation of data. SSP and KAK were first writing the manuscript. PRS revised the manuscript. All authors read and approved the final manuscript.

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