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ANALYSIS OF BIOACTIVE COMPOUNDS IN METHANOLIC LEAF EXTRACT OF FOREST TREE *DIOSPYROS MELANOXYLON* ROXB. ASSOCIATED WITH AM FUNGI BY LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LC-MS) TECHNIQUE.

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ABSTRACT
Plants are rich sources of several diverse bioactive phytochemical compounds with a wide range of biological effects. The screening of phytochemical compounds plays a vital role in the protection of human health and benefit. *Diospyros melanoxylon* (Roxb.) belonging to the family Ebenaceae is an important medicinal, commercial and multi-purpose useful plant used in several conditions. The main aim of the current investigation was to study the phytochemical compounds in the leaves of *D. melanoxylon* plant associated with AM fungi by using liquid chromatography and mass spectrometry (LC–MS). The LC–MS study revealed that the presence of 27 phytochemical compounds with high and low molecular weight chemical entities. The main bioactive compounds were Betulinic acid (Rt- 24.44), Oleanolic acid (Rt-23.70), Ursolic acid (Rt-23.82) and Gallic acid (Rt-1.07). The compounds have been identified by interpretation of the mass spectra. The presence of various bioactive compounds with diverse chemical structures confirms the application of leaves of *D. melanoxylon* for several diseases. Hence, that plant is suggested as pharmaceutically important. Further, isolation of individual bioactive compounds may be necessary to find a novel drug.

Key Words
Diospyros melanoxylon; Leaves; LC–MS; Phytochemicals; Methanol.

INTRODUCTION
Diospyros melanoxylon Roxb. (Ebenaveae) plant is endemic to India (Sastry, 1952) and the most economically important species in *Diospyros*. *Diospyros melanoxylon* is commonly known as Thuniki, has been used for treatment of various ailments like diarrhea and dyspepsia (Ambastha, 1986). *D. melanoxylon* leaves have been used in Indian traditional medicine as diuretic, styptic, laxative, carminative and to cure night blindness and improves the eye sight (Chattarjee & Pakrashi, 1995). Leaves are highly valued for their use as wrappers in beedi industries (Santapau & Henry, 1994). Leaves are very important economic source for tribals in India (Rathore 1972). The bark is also diuretic, carminative, laxative, styptic and used as an astringent lotion for eyes (Mallavadhani et al.,1998). Plant materials are known as source of new antimicrobial agents, as a result search has been to discover new antibacterial drugs of plant origin. A number of compounds like vincristine, quinine, salicyclic acid, eligitalis, morphine and codeine have been derived from plants which are having enormous therapeutic potential (Parekhand Chanda, 2007). Still medicinal properties of many plants are yet to be investigated for phytochemistry and pharmacognosy and there is an urgent need to identify lead substances that are active towards resistant pathogens (Recio, 1989). In this connection, a detailed chemical profiling of *D. melanoxylon* leaf extracts has been performed.

MATERIAL AND METHODS

Collection of Samples

D. melanoxylon Roxb. (Ebenaceae) leaves were collected from the studied area in forests of Sathupalli, Khammam district, Telangana, India in the month of March to April 2022. The plant was identified by Department of Botany, Osmania University.

Preliminary phytochemical screening assay

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

Test for Phenolics: The leaf extract was taken in a test tube (0.5 gm of leaves in 100 ml of water) and boiled. 2 ml of ferric chloride was added to the above solution and observed for formation of green or blue colour which indicates the presence of phenolics.

Test for flavonoids: Ethyl acetate (5 ml) was added to the leaf extract (0.5 gm of leaves in 100 ml of water). The mixture was shaken and allowed to settle. Production of yellow colour which indicates as positive for flavonoids.

Test for Terpenoids: 50mg of leaf extract was taken and added in 5 ml of ethanol. Extract was mixed in 2ml of chloroform, it was slightly heated and allowed to cool. 3 ml of conc. 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. (Indumati et. al., 2014)

Test for Alkaloids: The leaf extract was prepared (0.5 gm of leaves in 100 ml of water). It was dissolved in diluted Hcl solution and clarified by filtration. The filtrate was tested with Drangendroff's and Mayer's reagent. The treated solution was observed for precipitation.

Test for Tannins: The leaf extract was prepared (0.5 gm of leaves in 100 ml of water) and clarified by filtration. 10% ferric chloride solution was added to the clear filtrate, and it was observed for a change in colour to blue indicates the presence of Tannins.

Test for Saponins: Leaves (0.5 gm) were kept in 100 ml of distilled water and transferred to a test tube. The test tube was shaken vigorously for about 30 sec and allowed to stand in vertical position and wait for 30 min. If the observe honey comb froth above the surface of the liquid persists after 30 min, then it indicates the presence of saponins.

Preparation of standard solution

The stock solution of standards lupeol, Betulinic acid, Oleanolic acid, Ursolic Acid. (1 mg/ml) was prepared by dissolving 1 mg of accurately weighed lupeol, Betulinic acid, Oleanolic acid, Ursolic Acid in methanol and making up the volume of the solutions to 100 ml with methanol in a volumetric flask. The stock solution 2, 4, 6, 8 and 10 ml were transferred to 10 ml volumetric flasks and the volume of each vial was made upto 10 ml with methnol to get standard solutions containing 2, 4, 6, 8 and 10 µg/ml concentration of lupeol, Betulinic acid, Oleanolic acid, Ursolic Acid.

Preparation of sample solution

The leaves of *Diospyros melanoxylon* were first kept in shaded and then dried at room temperature for one week and the dried leaves were grounded to fine powder. 1gm powdered sample was added in 10ml of methanol for 24 hours and the solvents was filtered through whatmann No. 1 filter paper, this procedure were repeated trice (Triplicates). The extract was concentrated using rotary evaporator under reduced pressure at a temperature of 40±2 °C. Precisely 1 mg of the extract was dissolved in 1 mL of methanol and filtered through a 0.45 μm filter membrane and the filtrate was used as the sample solution.

LC-MS conditions

Instrumental settings for chromatography and mass spectrometry, Secondary metabolites were evaluated with an Acquity 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 a flow rate of 0.4 mL/min. With an active pre-heater, the UPLC column was kept at 40°C. Each sample was subjected to a 31-minute gradient elution from 5% to 95% acetonitrile with 0.1% formic acid (mobile phase B) and 0.1% formic acid in Millipore water 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-equilibrated to its initial condition as the next injection was being prepared. The following parameters were used for mass spectrometric measurements: ESIMSE mode, scan range 50-2000m/z, capillary voltage 3.0/2.0 kV (ve/-ve), sample cone 40 V, source temperature 120°C, desolvation temperature 350°C, cone gas flow rate 50 L/h, and MS fragmentation done at 20-50 eV collision energy in ramp mode. In order to carry out the instrumental error correction in both positive and negative mode, leucine-enkephalin was utilized as a lock mass solution.

RESULT

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.

Secondary metabolites of *Diospyros melanoxylon* were analyzed by reverse-phase UPLC-MS eluted with gradient mobile phase containing the acetonitrile and water with 0.1% formic acid. In the optimized method, acquired UPLC and MS total ion chromatograms of methanolic leaf extract of *D. melanoxylon* was analyzed in ESI negative ionization mode and these results are presented (fig. 1-2). The peak structural assignment was performed based on their MS spectral data (Mass, molecular formula & fragmentations pattern) retention time, isolated standard comparison with *D. melanoxylon* extract mass fragmentation and search in public online databases (Reaxys, SciFinder & DNP).

Our UPLC-ESI (-) -QTOF-MS studies (Figute-1 & Table-2) leads to the identified of several compounds from methanolic leaf extract of *D. melanoxylon*. 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 Rt 24.44 with m/z 455.3526[M-H]-, Oleanolic acid at Rt 23.70 with m/z 455.3532[M-H] - and Ursolic acid 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 table-2.

DISCUSSION

The present study of phytochemical investigation in *D. melanoxylon* leaves differs significantly with earlier reports in various aspects. In the present investigation, we have identified the unreported phenolics gallic acid and Ellagic acid and flavonoids quercetin and rutin and Triton X 100, Hydroxylenoleate, Vannilic acid, salannin, moronate and some fatty acids in addition to the reported compounds (Mallavadhani et al., 1998). More interestingly reported compounds were obtained in significantly large quantities 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 noteworthy that the present work was done with young foliage leaf (Juvenile) material treated with AM fungi, whereas earlier workers might have investigated mature *D. melanoxylon* leaves without reporting the AM fungi. The accumulation of compounds Ursolic acid, Lupeol, Gallic acid, triterpenoids in large quantities is relevant in view of their interesting commercial and biological applications. The triterpenes are reported to exhibit excellent active properties (Khadzhieva et al., 1987). Ursolic acid is being used as an emulsifier in the pharmaceutical, cosmetics and food industries (Mezzetti et al., 1971). The pentacyclic triterpenes have long been thought of pharmacologically inactive but gaining importance in view of their recently attributed interesting activities like anti-cancer (Ishida et al., 1990), anti HIV (Xu et al., 1996) and anti-inflammatory (Recioet al., 1995). Ursolic acid and oleanolic acid have been recommended for skin cancer therapy in Japan (Muto et al., 1990). Cosmetic preparations containing ursolic acid/oleanolic acid are patented in Japan for the prevention of skin cancer for topical use (Ishida et al., 1990). Recently the dihydroxy triterpenic acid, corsolic acid was found to be active as protein kinase inhibitor and cytotoxic agent. In the biocidal front ursolic acid was found to exhibit potent antifeedant activity against Spilosoma obliqua and Spodoptera litura insects (Shukla et al., 1996). In the case of amyrins, a-amyrin palmitate was reported to exhibit excellent insect growth inhibiting properties and chemosterilant activity (Shankaranarayana et al., 1980).

CONCLUSION: Results of the 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.

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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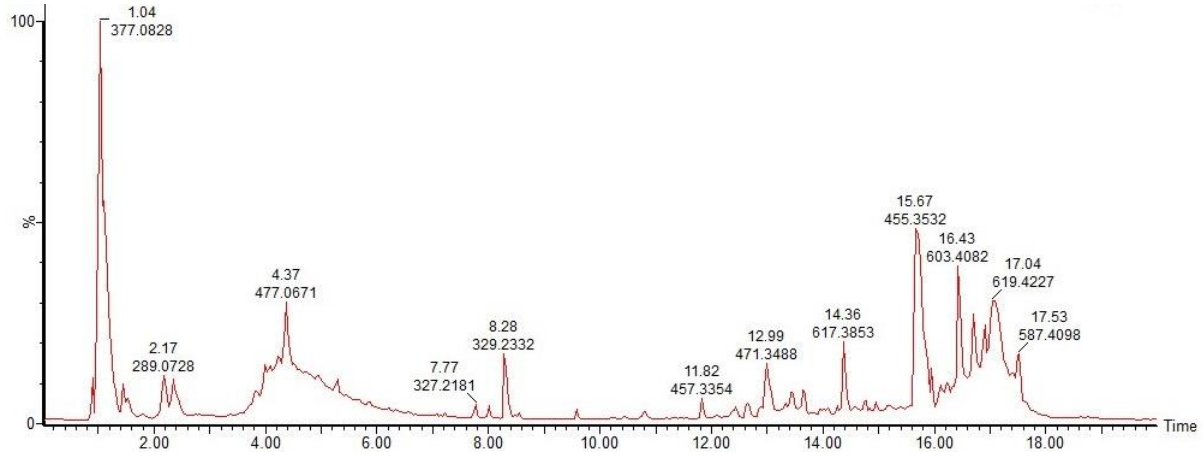
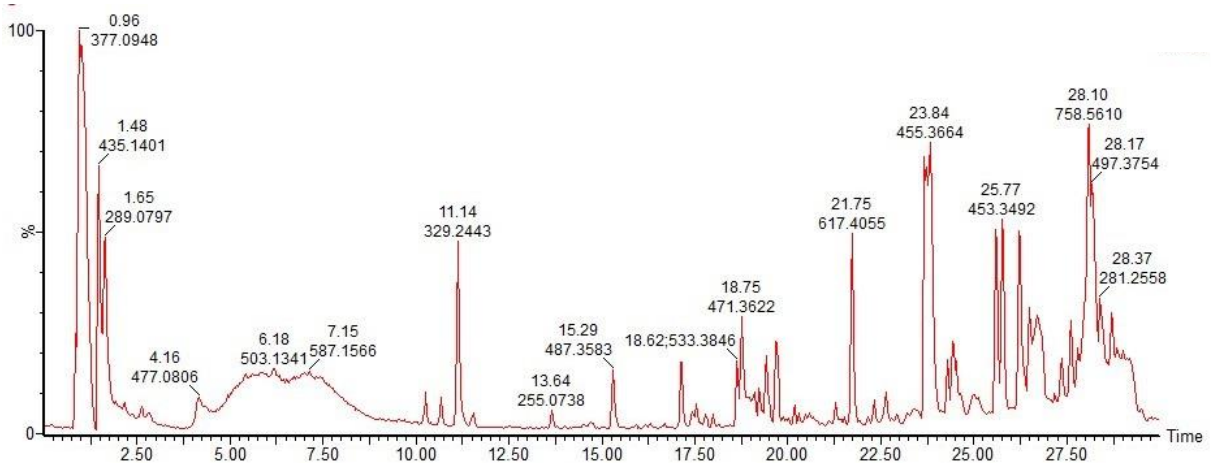
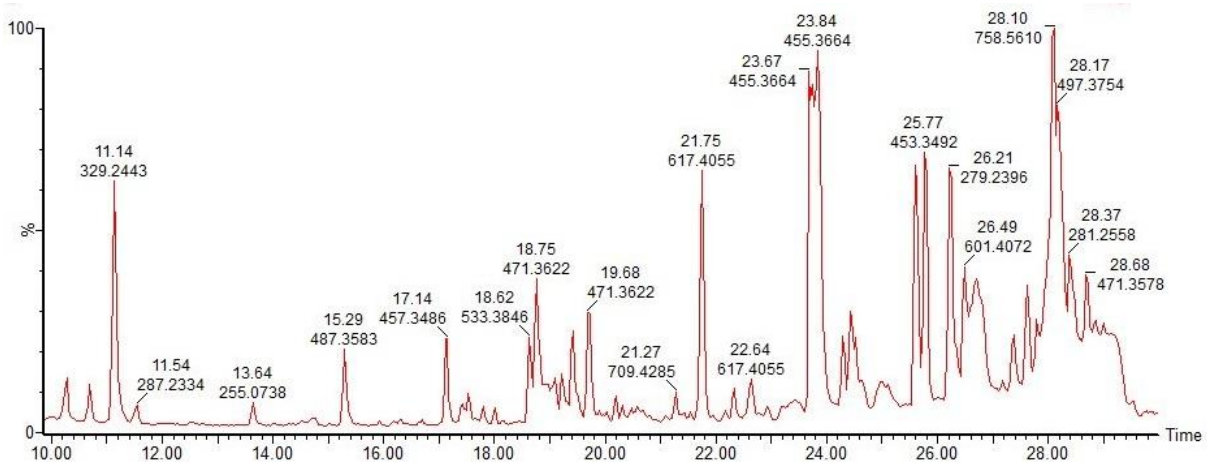
Figure.1. A typical chromatogram of the bio active compounds presents in methanolic leaf extract of *D. melanoxylon.*



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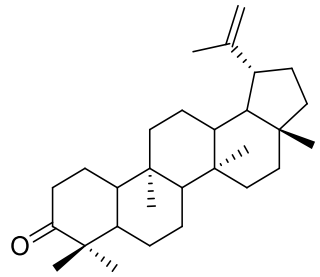

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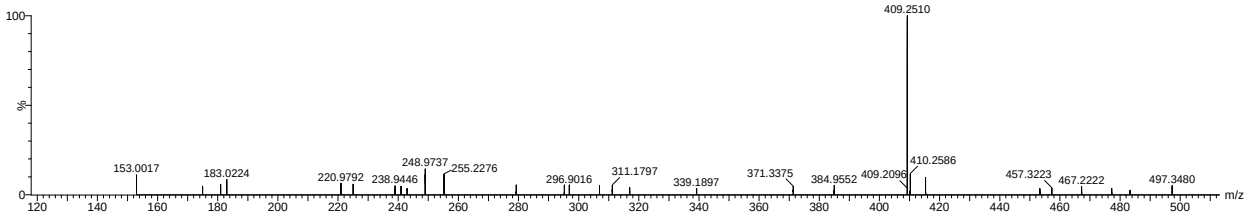

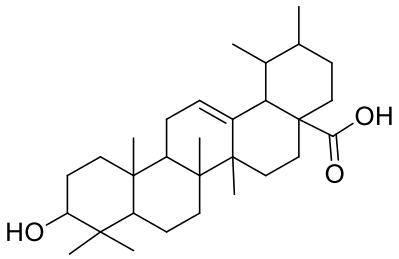

Chemical Formula: $C_{30}H_{48}O_3$
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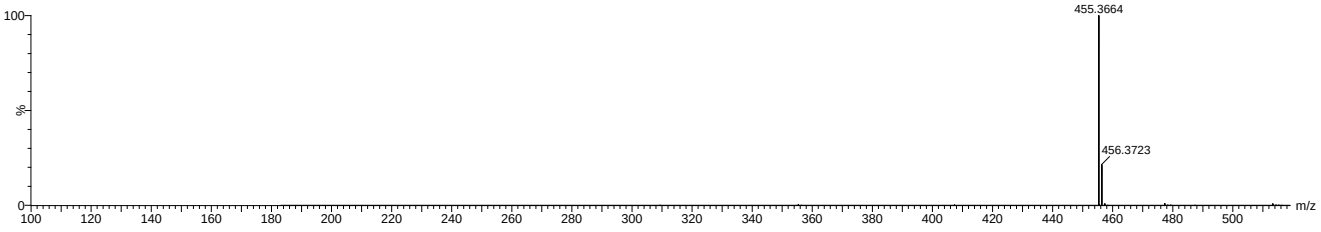

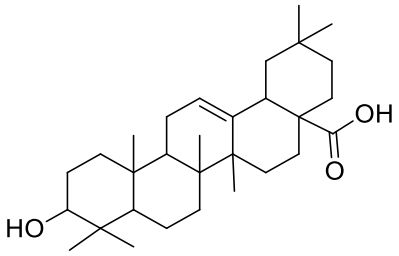

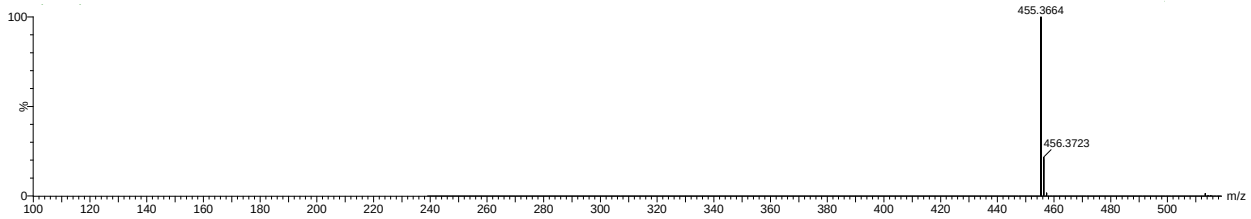

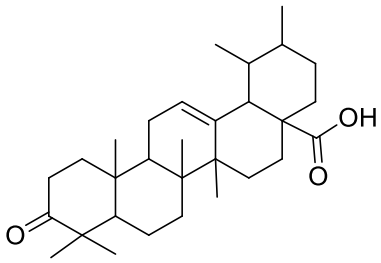

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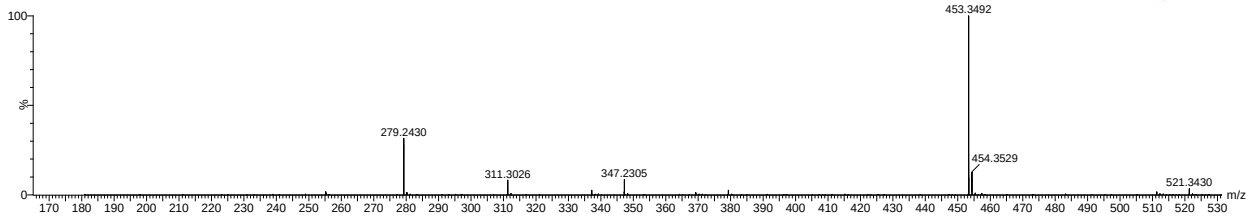

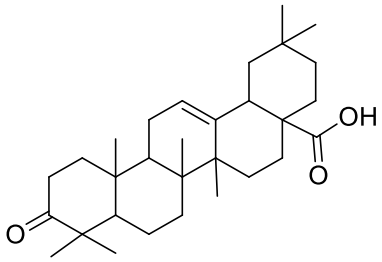

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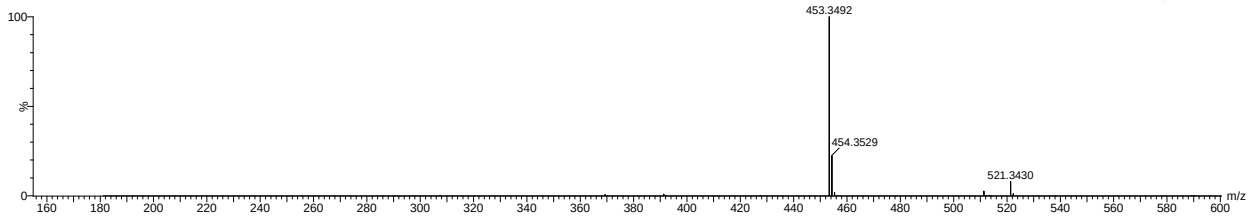

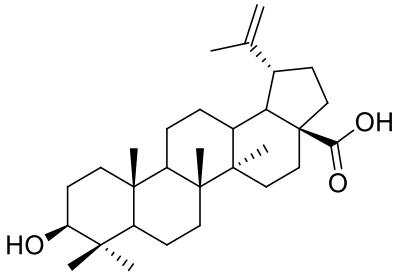

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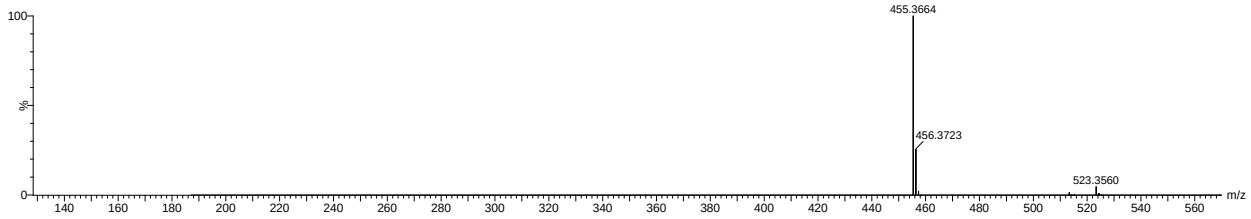

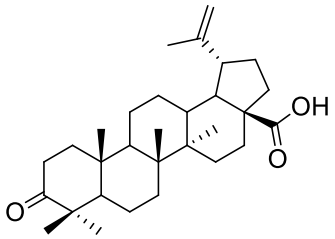

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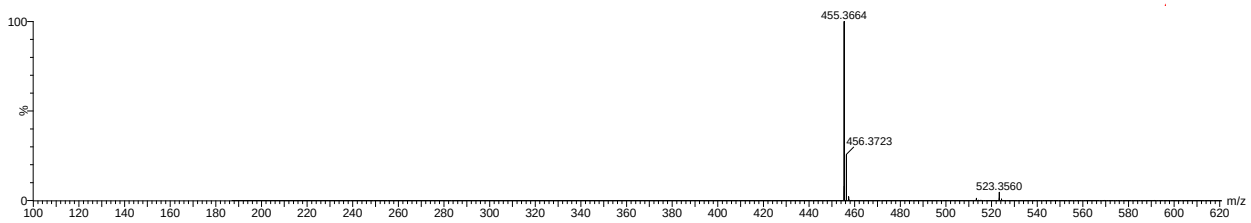

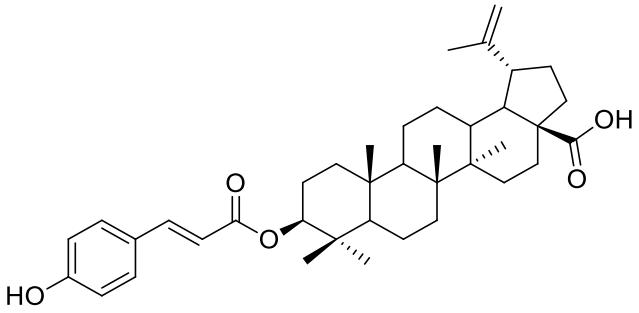

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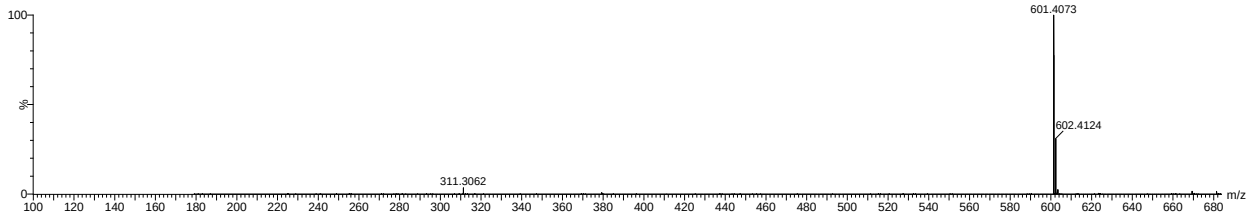

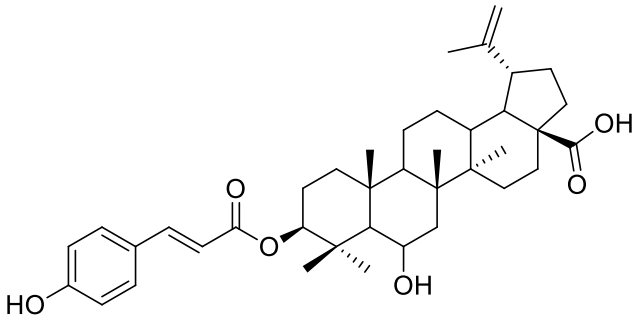
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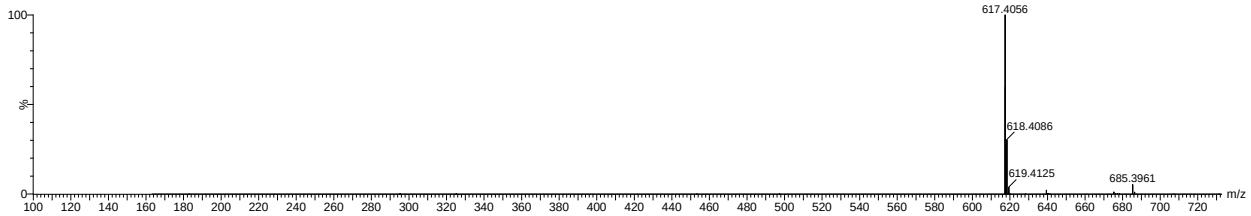
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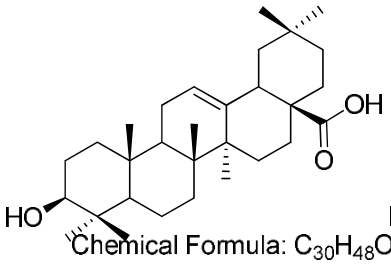

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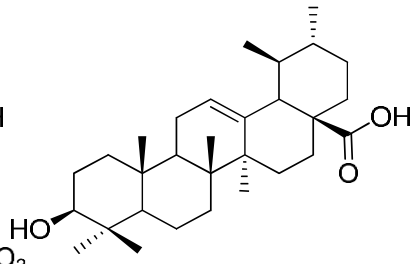
Oleanolic acid



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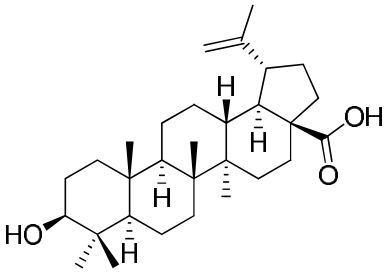
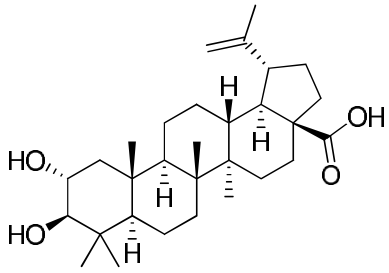
Exact Mass: 456.36

Ursolic acid



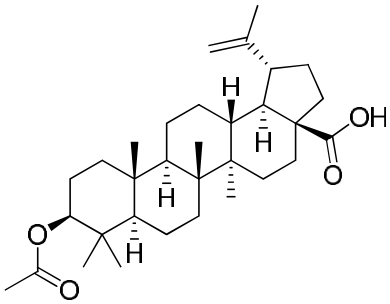
Chemical Formula: $C_{30}H_{48}O_3$
Exact Mass: 456.36

Betulinic acid



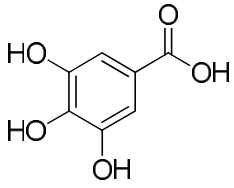
Chemical Formula: $C_{30}H_{48}O_4$
Exact Mass: 472.36

alphitolic acid

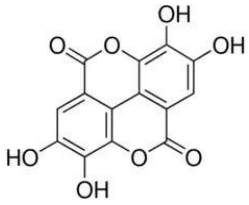


Chemical Formula: $C_{32}H_{50}O_4$
Exact Mass: 498.37

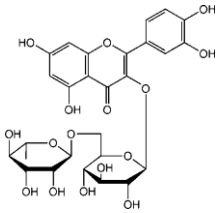
acetly- Betulinic acid



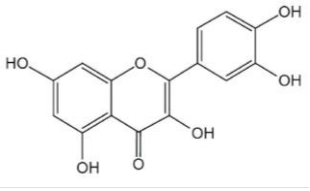
Gallic acid



Ellagic acid



Rutin



quercetin

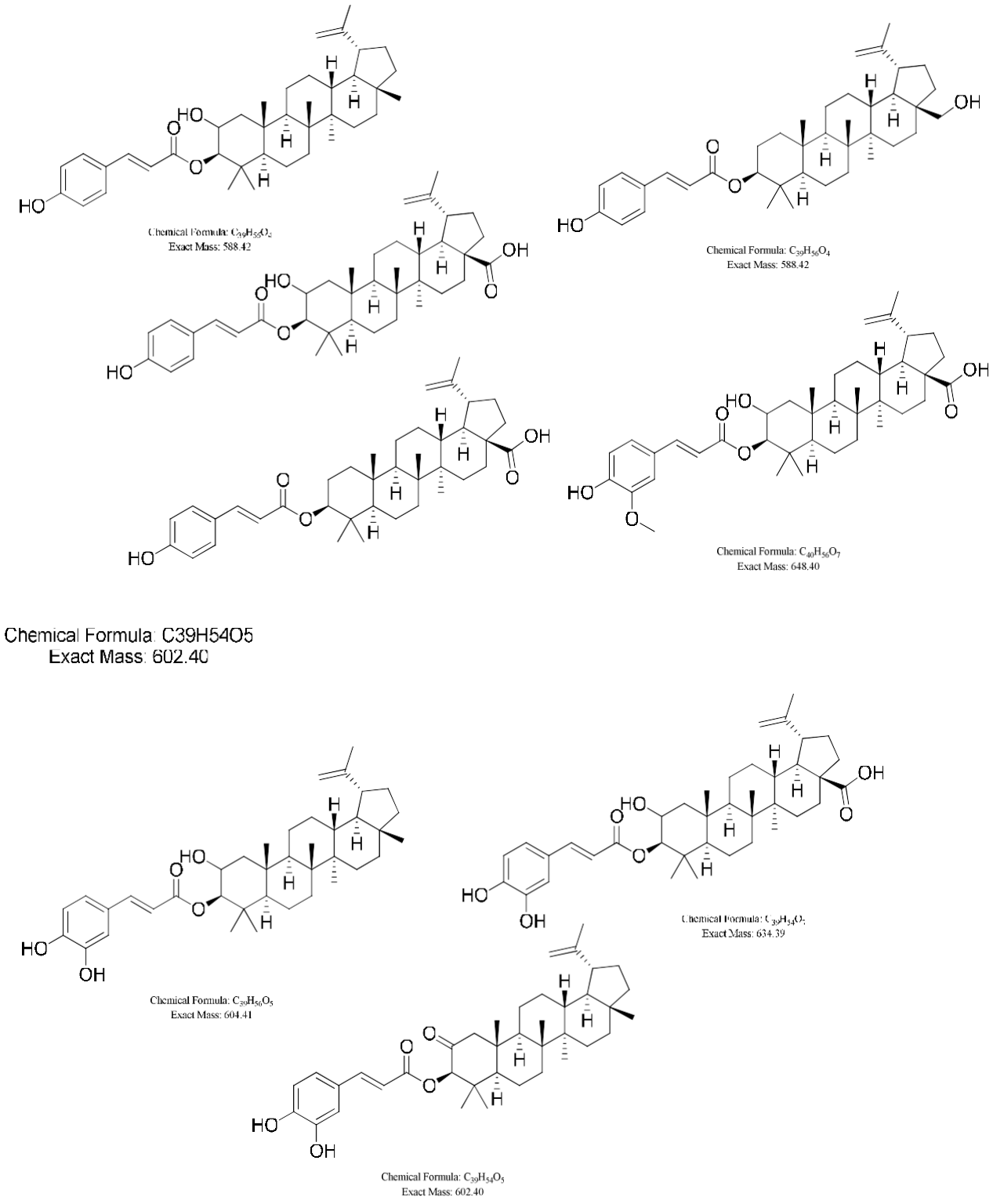


Figure 2. Structures of some identified compounds in methanolic leaf extract of *Diospyros melanoxylon* using LC-MS.

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Table 1. Qualitative analysis of phytochemicals

Sl. 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	C7H5O5	C7H6O5	Gallic acid
1.63	289.0715	289.0712	1.0	245.0801 (C14H13O4) 203.0709 (C12H11O3)	C15H13O6	P-coumaroyltriacetate
3.33	300.9980	300.9984	-1.3	C14H5O8	C14H6O8	Ellagic acid
8.28	301.0346	301.03	-0.7	C15H9O7	C15H10O7	Quercetin
10.25	327.2174	327.2171	0.9	229.1445 (C12H21O4) 211.1341(C12HH19O3)	C18H31O5	9S,12R,13S - Trihydroxy10E, 15 zoctadecadienoic acid
10.67	331.2490	331.2484	1.8	313.2382 (C18H33O4)	C18H35O5	9,10,18-trihydroxystearate
11.11	329.2338	329.2328	3.0	233.1157 (C14H17O3) 211.1347 (C12H19O3)	C18H33O5	9,12,13-trihydroxyoctadecenoate
15.30	487.3432	487.3423	1.8	469.3313 (C30H45O4)	C30H47O5	Holothurinogenin
17.12	457.3318	457.3318	0.0	297.1512 (C19H21O3)	C29H45O4	benzyl hydrogen 2-octadecenylsuccinate
17.53	667.3550	667.3541	1.3	487.3419 (30H47O5) 577.3000(C31H45O10)	C31H55O15	Vannilic ester

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17.81	315.2535	315.2535	0.0	---	C18H35O4	9,10-Dihydroxystearate
18.02	595.2900	595.2907	-1.2	279.2318 (C18H31O2)	C34H43O9	Unknown
18.63	533.3694	533.3690	0.7	275.1859 (C14H27O5)	C28H53O9	Triton X-100
19.21	295.2277	295.2273	1.4	277.2169 (C18H29O2)	C18H31O3	Hydroxylenoliate
18.77	471.3477	471.3474	0.6	311.1676 (C20H23O3)	C30H47O4	(3β,16β)-3,16-Dihydroxyolean-12-en-28-oatato
19.39	471.3481	471.3474	1.5	311.1676 (C20H23O3)	C30H47O4	(3β,16β)-3,16-Dihydroxyolean-12-en-28-oatato
19.70	471.3477	471.3474	0.6	311.1676 (C20H23O3)	C30H47O4	(3β,16β)-3,16-Dihydroxyolean-12-en-28-oatato
20.18	5973052	597.3064	-2.0	293.2119 (C18H29O3) 281.2469 (C18H33O2)	C34H45O9	Salannin
20.30	633.3803	633.3791	1.9	571.3220 (C33H47O8) 503.3362 (C30H47O6) 469.3303 (C30H45O4)	C39H53O7	acylated triterpenoid
21.28	709.4019	7094010	1.3	663.3912 (C40H55O8) 483.2699 (C29H39O6) 397.2242 (C21H33O7) 199.1698 (C12H23O2)	C34H61O15	Unknown
21.73	617.3858	617.3842	2.6	497.3238 (C31H45O5) 453.3358 (C30H45O3) 325.1831 C21H25	C39H53O6	acylated triterpenoid
22.31	617.3861	617.3842	3.1	415.3197 (C23H43O3) 453.3358 (C30H45O3) 497.3238 (C31H45O5)	C39H53O6	acylated triterpenoid
22.93	511.3420	511.3423	-0.6	465.3369(C31H45O3) 339.1981 (C22H27O3)	C32H47O5	Unknown
23.41	619.4213	619.4210	0.5	417.3432 (C30H47O4) 339.1995 (C15H31O8)	C36H59O8	Unknown

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23.70	455.3532	455.3525	1.5	---	C30H47O3	Oleanolic acid
24.30	277.2171	277.2168	1.1	---	C18H29O2	Fatty acid
23.82	455.3525	455.3525	0.0	---	C30H47O3	Ursolic acid
24.44	455.3526	455.3525	0.2	407.3308 (C29H43O) 377.2841 (C27H37O) 309.3156 (C21H41O) 269.2495 (C17H33O)	C30H47O3	Betulinic acid
25.00	283.2636	283.2637	-0.4	227.1997 (C14H27O2)	C18H35O2	Fatty acid
25.63	603.4055	603.4049	1.0	427.3407 (C25H47O5)	C39H55O5	Unknown
25.76	453.3364	453.3369	-1.1	323.2932 (C21H39O2) 307.2998 (C21H39O)	C30H45O3	Moronate
26.22	279.2327	279.2324	1.1	261.2206 (C18H29O)	C18H31O2	Linoleate
26.49	601.3893	601.3893	0	453.3352 (C30H45O3) 311.2944 (C20H39O2) 279.2296 (C18H31O2) 255.2304 (C16H31O2)	C39H53O5	Betulinic acid
27.62	587.4296	587.4312	-2.7	---	C36H59O6	Unknown