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Exploring the untargeted metabolites of *Moringa oleifera* Lam seed oil using two-dimensional gas chromatography with time of flight mass spectrometry for therapeutic application

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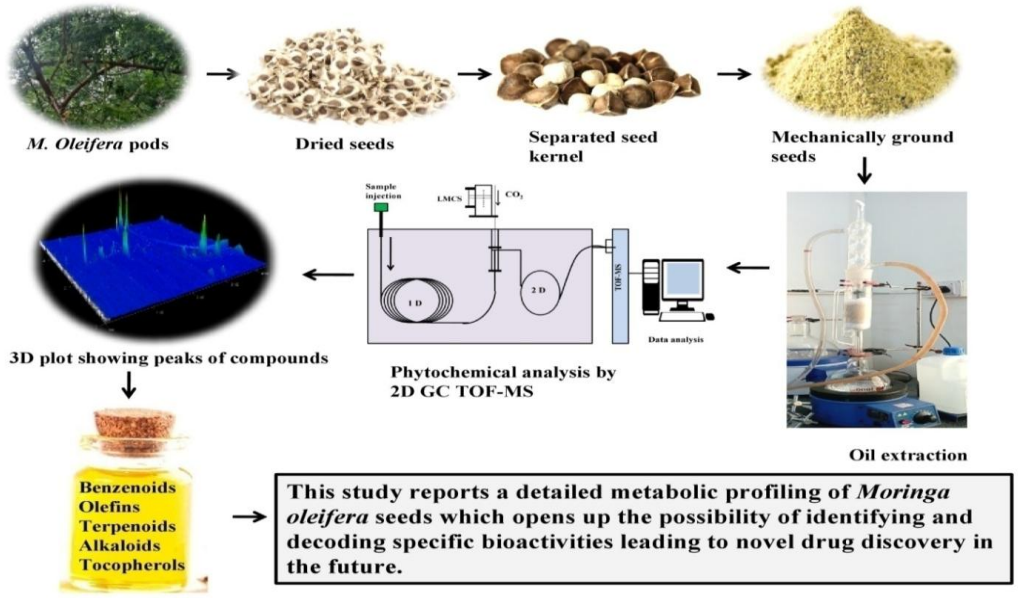
GRAPHICAL ABSTRACT



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KEYWORDS

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ABSTRACT

Moringa oleifera Lam is an economically and medicinally important plant. However, its essential oil characterization has been limited to one-dimensional gas chromatography and mass spectrometry. This study identified secondary metabolite composition and variation in *M. oleifera* seed oil through two-dimensional gas chromatography with time of flight mass spectrometry and their associated bioactivity. GC×GC TOF MS analysis of *M. oleifera* seed oil was performed on an Agilent 7890 Gas chromatograph equipped with Pegasus 2D GC-TOFMS. About 1µl of the sample (dissolved in n-Hexane) was injected into the system, and the carrier gas was Helium. Identification was made using ChromaTOF software with reference to the NIST library. A total of 2000 phytoconstituents were obtained, of which 236 were identified using the NIST mass spectral values**.** Total constituents were classified into alkanes (64), alkenes (11), aldehydes (7), alcohol (10), acids (18), acid esters (70), Ketones (10), benzenoids (10), Monoterpenoids (1), olefins (6), Phenols (1), an alkaloid (1), triterpenoid (4), diterpenoid (1), sesquiterpenoid (2), tocopherol (2), and Others (18). Based on area percentage, fatty acids and their derivatives were predominant. The major constituents were Erucic acid (9.10%), trans-13-Octadecenoic acid (6.06%), Triethyl citrate (5.15%), Bis-(3,5,5-trimethylhexyl) phthalate (4.94%). This study reports a detailed metabolic profiling of *M. oleifera* seeds, which opens up the possibility of identifying and decoding specific bioactivities leading to novel drug discovery in the future.

1 Introduction

Moringa oleifera (MO) is the best-known species in the Moringaceae family, which consists of only 14 species. It is also known as a drumstick tree, with a height ranging from 5 to 10 meters (Liu et al. 2022). The plant has three pinnate compound-structured leaves and yellowish or white flowers without red streaks. The three-valved and elongated fruits contain winged seeds (Patil et al. 2022). This plant grows best at temperatures between 25 and 35 °C and can withstand 48°C. It is susceptible to weather conditions and poor soil varieties and has even been known to withstand draughts, high temperatures, and light frosts (Trigo et al. 2020). Further, it is indigenous to the sub-Himalayan regions of North-western India and is now cultivated in many other countries (Mashamaite et al. 2021).

It is also known as the Miracle Tree because of its tremendous medicinal value with no harmful side effects. Various parts of *M. oleifera* are used for the treatment of various ailments, such as stress, hypertension, depression, diabetes, anemia, blindness, malnutrition, arthritis, and kidney stone disorders, and also help in the regulation of the blood glucose levels, cardiovascular health, urinary tract infection, as well as provides anti-inflammatory, antioxidant, and anticancer activity (Meireles et al. 2020; Mohanty et al. 2021). According to research, most of the studies have been conducted on *M. oleifera* leaves compared to other parts of the plant, and they have reported beneficial effects on various chronic conditions (Islam et al. 2021). Its seeds constitute a high proportion of oil, making it an excellent source of edible and non-edible oil (Özcan 2020). Its seed oil is popularly known in the cosmetic industry for its use in perfume, hair care, and cream due to its efficacy in enhancing skin hydration, reducing skin erythema and has no side effects like skin irritation (Athikomkulchai et al. 2021). Plant seed extracts are also used for tertiary wastewater treatment through sedimentation, flocculation, coagulation, and rapid granular filtration (Andrade et al. 2021). A recent surge of interest has resulted from the discovery of potent antimicrobial, diabetic, hypertensive, antioxidant, anti-inflammatory, anticancer, and cardio-protective properties **(**Gu et al. 2020; Wang et al. 2022; Aldakheel et al. 2020; Das et al. 2023**).** The extent of effects obtained from the seeds is attributed to variability in metabolite composition. According to the previous estimation, over a hundred and thousands of phytochemicals are present in this plant (Kashyap et al. 2022). Detecting and identifying the complete phytochemical profile in a given extract is a serious challenge for plant biologists because the available platforms have limited sensitivity (i.e., limited metabolic coverage) (Fiehn 2002).

Chromatography with mass spectrometry is the preferred method for analyzing secondary metabolites from plant extracts. Using a one-dimensional GC with MS to analyze complex seed oils leads to poorly resolved metabolites and consequently limits metabolic coverage. This study identifies secondary metabolite composition and variation in *M. oleifera* seed oil through systematic untargeted chemical profiling by two-dimensional gas chromatography with time-of-flight mass spectrometry and their associated bioactivity.

2 Materials and methods

2.1 Plant materials

Fresh and matured fruits of *M. oleifera* were harvested from the Malkangiri district of Odisha, India, in December. The plant

sample was validated by Prof. Pratap Chandra Panda, Taxonomist, and a voucher specimen (2023/CBT dated 27.12.2021) was deposited at the herbarium of Centre for Biotechnology, Siksha O Anusandhan University, Odisha, India.

2.2 Preparation of seed oil

The fruits were cleaned with tap water and dried under the sun for three days. After three days, the seeds were separated from the fruits and shade-dried for two weeks until they were entirely dried. The seed kernels were separated and dried under shade for another two weeks. After the seed kernels were utterly dried, they were ground into a fine powder and stored in airtight bags. Oil extraction was done in a soxhlet apparatus by solvent distillation (Palafox et al. 2012). 100g of ground sample was put into the thimble of the soxhlet apparatus, and 250 ml of 100% ethanol was used as a solvent for oil extraction. The process continued for 8 hrs at a temperature of 30℃. The oil and solvent mixture was collected from the round bottom flask in a beaker and left in a water bath for solvent evaporation. The oil was collected in sterile bottles, appropriately sealed, and stored at 4℃ for further use.

2.3 2D GC×GC TOF MS analysis of *M. oleifera* seed oil

The phytochemical profiling of *M. oleifera* seed oil was performed on an Agilent 7890 gas chromatograph attached to a Pegasus 2D GC-TOFMS. A non-polar Rxi-5 MS column (30 m × 0.25 mm × 0.25 µm) was separated. Rxi-17Sil MS (2 m × 0.25 mm × 0.25 µm) was used as a secondary column. A volume of 1µl of the sample (dissolved in n-Hexane) was injected into the system in split mode (1:100). The carrier gas was high-purity Helium at a flow rate of 1ml/min. The initial temperature was set at 60℃, heated at a rate of 3℃ per minute gradually; the temperature was increased to 280℃, then held for 6 mins isothermally. This study used a 200℃ ion source temperature, a 250℃ interface temperature, a 70ev solvent cut time, and a linear velocity of 36.8 cm/sec for the column.

2.4 Identification of phytochemicals in the seed oil of *M.oleifera*

Data were processed using ChromaTOF software (LECO, version: 4.51.6.0 Optimized for Pegasus®). Compounds were identified by comparing their mass spectra to the mass spectral database (NIST 11). Based on PubChem and the Human Metabolome Database, compounds were classified into different chemical groups (Figure 1).

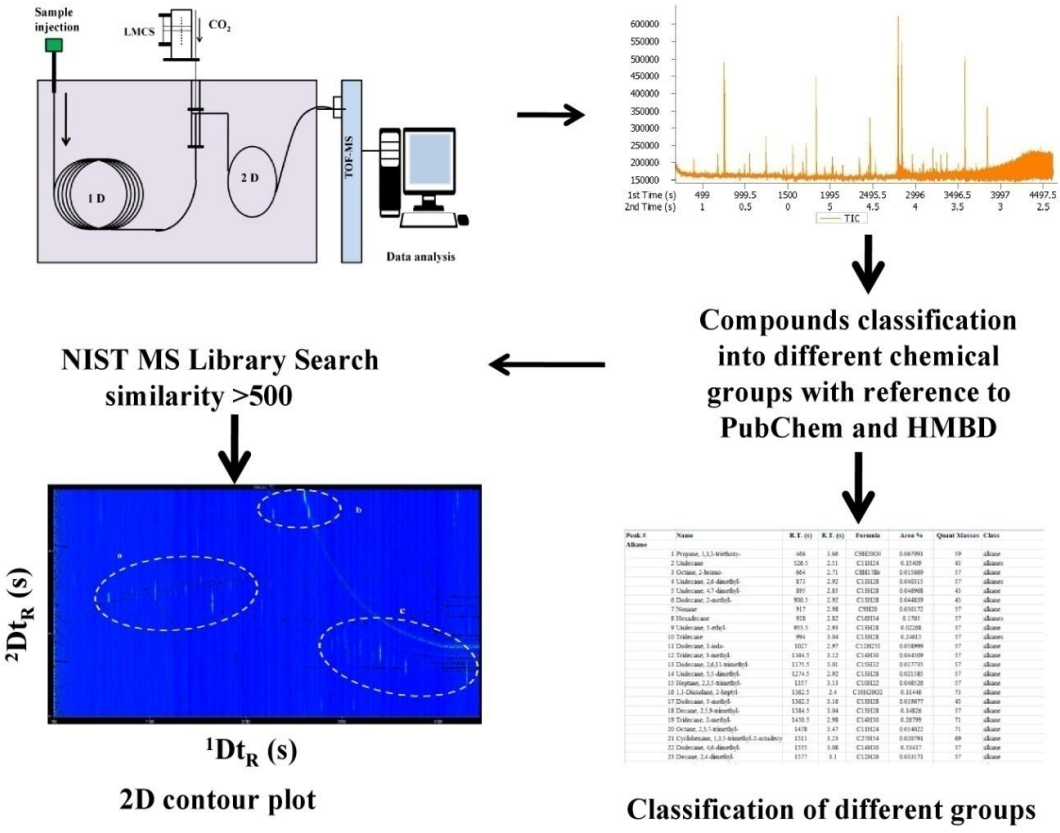

Figure 1 Schematic representation of phytochemical analysis by GC×CG-TOFMS

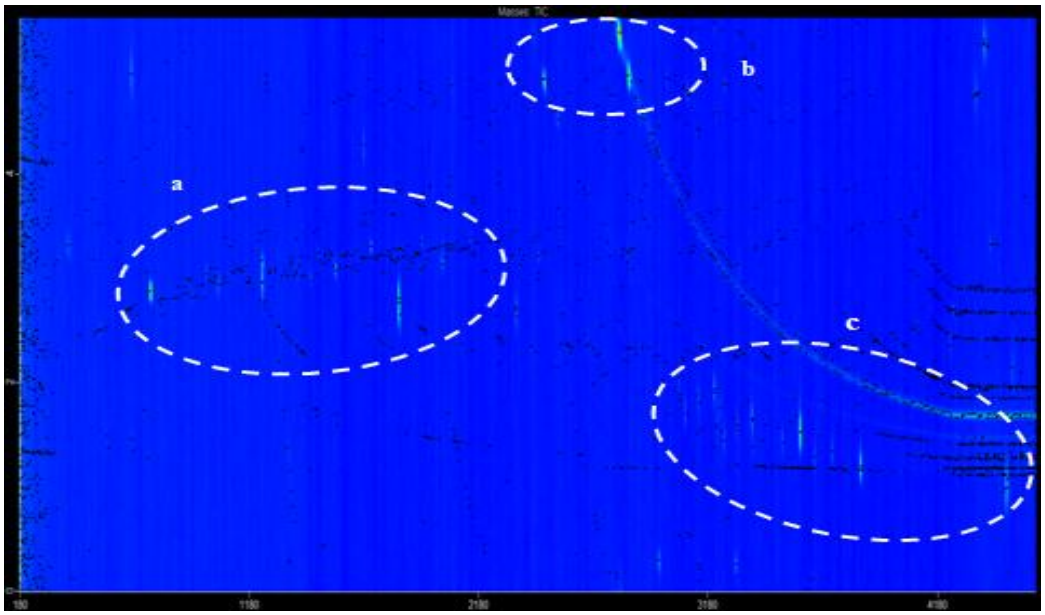

Figure 2 Contour plot showing the area acquired by the identified secondary metabolites (a) alkanes and benzenoids (b) fatty acids and esters (c) terpenes and tocopherols

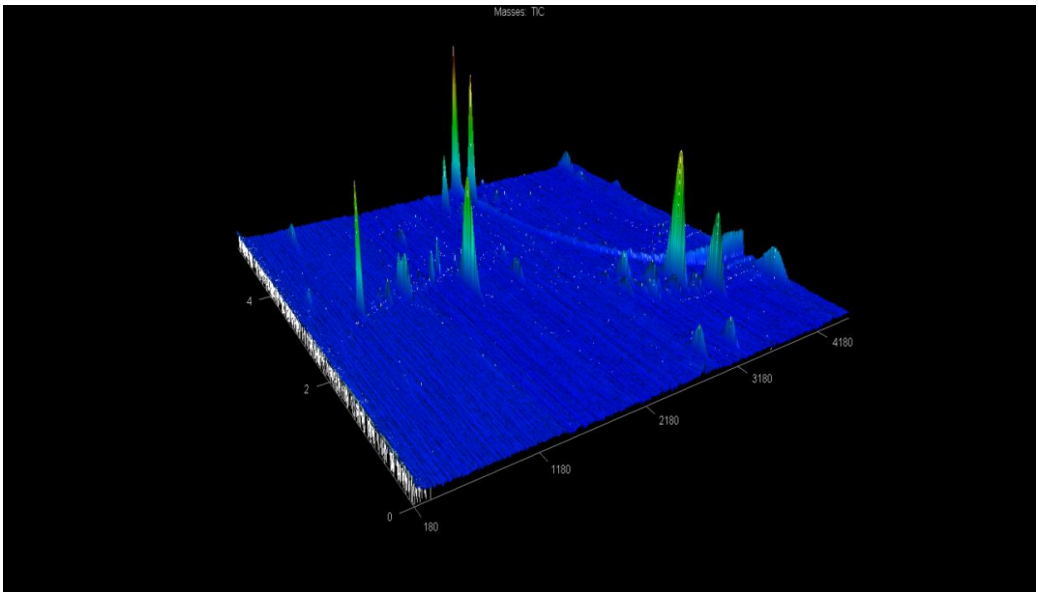

Figure 3 3-dimensional plot of phytochemicals from MOSO analyzed by GC×GC- TOF- MS

3 Results

The oil obtained from *M. oleifera* seeds was light brown, with a total oil yield of 7.2% (v/w). A detailed phytochemical characterization of the *M. oleifera* seed oil (MOSO) was obtained by the GC×CG-TOFMS approach. The GCGC-TOF-MS system achieved higher peak capacities and improved sensitivity through thermal modulation. To detect the peaks in the GC×GC chromatogram, automated peak screening of ChromaTOF software was applied (Figure 2 & 3). The only peaks considered were those with minimum signal noise (S/N) ratio thresholds more significant than 100. A total of 2000 phytoconstituents were obtained with total area percentage of 99.967, out of which 236 (total area % of 94.07) were analyzed using the NIST mass spectral values with a similarity of more than 500 considered. Based on PubChem and the Human Metabolome Database, compounds were classified into different chemical groups.: Alkanes, alkenes, Aldehydes, Alcohol, Acids, acid Esters, Ketones, benzenoids, Monoterpenoids, olefins, Phenols, alkaloids, triterpenoid, diterpenoids, sesquiterpenoid, tocopherol, and Others (Table 1) The major components found were:

Table 1 Compounds identified in *M.oleifera* seed oil analyzed using GC×CG-TOFMS

S. N.	Name	RT (s)	RT (s)2	Formula	Area %	Quant Masses
	Alkane					
1.	Tridecane	994	3.04	$C_{13}H_{28}$	0.24615	57
2.	Tridecane, 2-methyl-	1450.5	2.98	$C_{14}H_{30}$	0.26799	71
3.	Dodecane, 4,6-dimethyl-	1555	3.08	$C_{14}H_{30}$	0.53437	57
4.	Heptacosane	2022.5	3.14	$C_{27}H_{56}$	0.56746	43
5.	Isooctadecane	2143.5	3.4	$C_{18}H_{38}$	0.24582	57
6.	Heptacosane	2022.5	3.14	$C_{27}H_{56}$	0.56746	43
7.	Tetracosane	2721	3.52	$C_{24}H_{50}$	0.38289	43
8.	2,8,9-Trioxa-5-aza-1-silabicyclo(3.3.3)undecane, 1-methoxy-	2770.5	4.75	$C_7H_{15}NO_4Si$	0.19657	174
	Alkenes					
9.	4,5-Nonadiene	4294 ,	1.7	C_9H_{16}	0.54436	67
10.	5,7-Dodecadiene, (Z, Z)-	4426	1.7	$C_{12}H_{22}$	0.15768	81
	Alcohols					
11.	2-Ethyl-1-hexanol	389	3.32	$C_8H_{18}O$	1.0724	57
12.	(-)-Etafedrine	2308.5	3.65	$C_{12}H_{19}NO$	0.24139	86
	Acids					
13.	Hexadecenoic acid, Z-11-	2418.5	5.34	$C_{16}H_{30}O_2$	0.16225	55
14.	Palmitic Acid	2462.5	4.91	$C_{16}H_{32}O_2$	1.0783	60
15.	trans-13-Octadecenoic acid	2792.5	5.35	$C_{18}H_{34}O_2$	6.0679	55
16.	Oleic Acid	3089.5	4.75	$C_{18}H_{34}O_2$	0.30975	57
17.	Erucic acid	4283	1.68	$C_{22}H_{42}O_2$	9.1027	43
18.	linoleic acid	4602	1.69	$C1_8H_{32}O_2$	0.23508	67
	Acid esters					
19.	Triethyl citrate	1830	2.77	$C_{12}H_{20}O_7$	5.1576	157
20.	ethyl palmitate	2523	4.52	$C_{18}H_{36}O_2$	0.37497	88
21.	Phthalic acid, 2-methyl butyl pentyl ester	2655	2.37	$C_{18}H_{26}O_4$	0.3287	149
22.	Ethyl elaidate	2831	4.94	$C_{20}H_{38}O_2$	2.1677	55
23.	Glycol palmitate	2963	0.31	$C_{18}H_{36}O_3$	0.44578	43
24.	Phthalic acid, cyclohexyl 2-pentyl ester	3078.5	1.71	$C_{19}H_{26}O_4$	0.73616	149
25.	1,2-Benzenedicarboxylic acid, butyl octyl ester	3227	1.76	$C_{20}H_{30}O_4$	0.55539	149
26.	Phthalic acid, di(2-methyl butyl) ester	3304	1.6	$C_{18}H_{26}O_4$	0.57039	149
27.	1,2-Benzenedicarboxylic acid, butyl octyl ester	3227	1.76	$C_{20}H_{30}O_4$	0.55539	149
28.	Phthalic acid, di(2-methyl butyl) ester	3304	1.6	$C_{18}H_{26}O_4$	0.57039	149

S. N.	Name	RT (s)	RT (s)2	Formula	Area %	Quant Masses
29.	Phthalic acid, dodecyl pentyl ester	3370	1.66	$C_{25}H_{40}O_4$	1.5529	149
30.	Phthalic acid, 4-methyl pent-2-yl nonyl ester	3375.5	1.39	$C_{23}H3_6O_4$	0.32399	149
31.	Phthalic acid, bis(7-methyl octyl) ester	3502	1.43	$C_{26}H_{42}O_4$	0.61359	149
32.	Bis-(3,5,5-trimethylhexyl) phthalate	3579	1.53	$C_{26}H_{42}O_4$	4.9429	57
33.	Phthalic acid, hex-3-yl undecyl ester	3639.5	1.29	$C_{25}H_{40}O_4$	0.52037	149
34.	Didecan-2-yl phthalate	3843	1.2	$C_{28}H_{46}O_4$	2.0879	149
35.	Z-10-Tetradecen-1-ol acetate	4365.5	1.68	$C_{16}H_{30}O_2$	2.2428	55
	Ketone					
36.	3-Hexanone, 2,5-dimethyl-4-nitro-	1676	5.13	$C_8H_{15}NO_3$	0.37313	71
37.	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	2341.5	2.7	$C_{17}H_{24}O_3$	0.4964	57
38.	Diazoprogesterone	4470	0.96	$C_{21}H_{30}N_4$	0.12988	43
	Benzenoids					
39.	Diphenyl ether	1236	2.97	$C_{12}H_{10}O$	1.3847	51
40.	Diethyl Phthalate	1676	4.28	$C_{12}H_{14}O_4$	1.0292	149
	Monoterpenoids					
41.	Isoborneol	664	4.96	$C_{10}H_{18}O$	1.0472	95
	Phenol					
42.	2,4-Di-tert-butyl-phenol	1500	0.46	$C_{14}H_{22}O$	0.05232	191
	Alkaloid					
43.	Neronine, 4á,5-dihydro-	2957.5	4.21	$C_{18}H_{21}NO_6$	0.00866	73
	Titerpenoid					
44.	Squalene	3826.5	5.44	$C_{30}H_{50}$	0.075712	69
45.	Stigmastan-6,22-dien, 3,5-dedihydro-	4376.5	5.25	$C_{29}H_{46}$	0.29268	55
46.	Stigmasterol	4382	5.29	$C_{29}H_{48}O$	0.027612	131
	Diterpenoid					
47.	Andrographolide	4343.5	4.76	$C_{20}H_{30}O_5$	0.29971	43
	Sesquiterpenoids					
48.	(Z, E)-alpha-Farnesene	4497.5	1.27	$C_{15}H_{24}$	0.02049	81
	Tocopherols					
49.	ç-Tocopherol	4134.5	2.15	$C_{28}H_{48}O_2$	0.19886	151
50.	dl-à-Tocopherol	4233.5	2.36	$C_{29}H_{50}O_2$	0.4854	165

2-Ethyl-1-hexanol (1.07%), Palmitic Acid (1.07%), trans-13-Octadecenoic acid (6.06%), Erucic acid (9.10%), Triethyl citrate (5.15%), Ethyl elaidate (2.16%), Phthalic acid, dodecyl pentyl ester (1.55%), Bis-(3,5,5-trimethylhexyl) phthalate (4.94%), Didecan-2-yl phthalate (2.08%), Z-10-Tetradecen-1-ol acetate (2.24%), Diphenyl ether (1.38%), Diethyl Phthalate (1.02%), Isoborneol (1.04%), Silane, tetramethyl- (2.14). The percentage area covered by different groups is depicted in the figure 4.

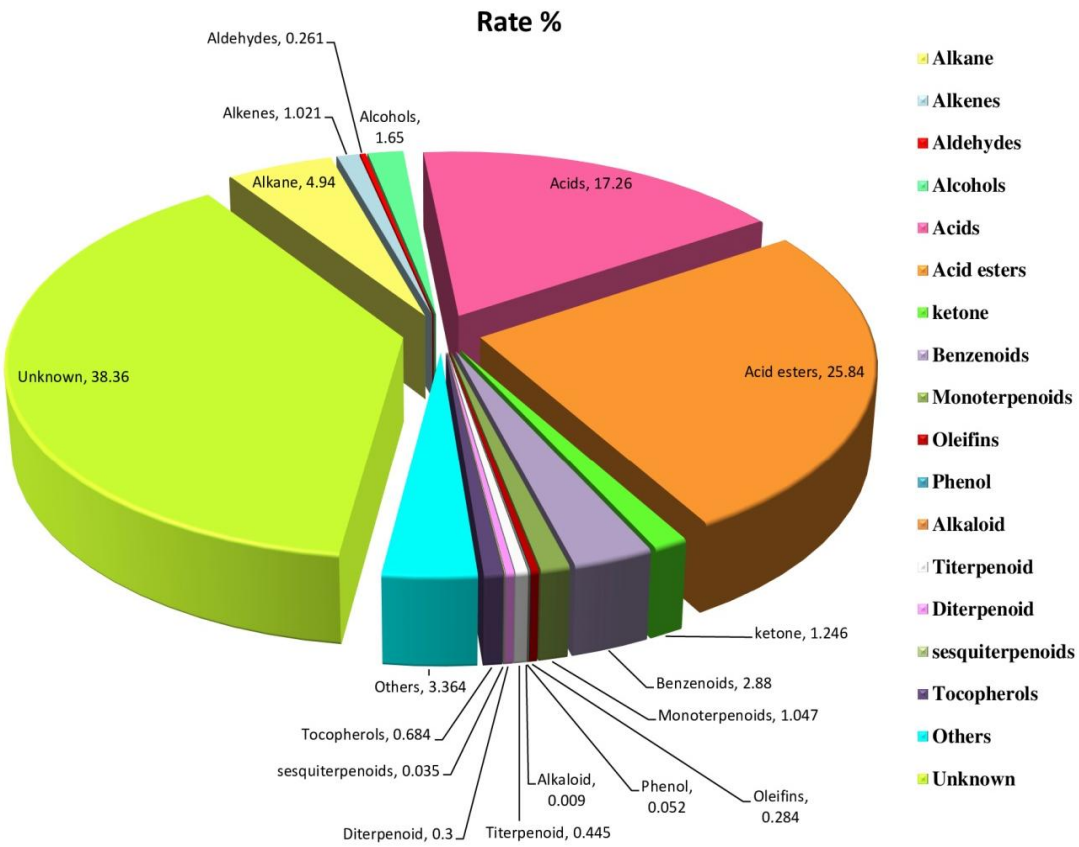

Figure 4 Pie chart depicting the areas covered by different groups of secondary metabolites

4 Discussion

A plant metabolomics analysis seeks to identify and quantify plants simultaneously and unbiasedly (Salem et al. 2020). The plant kingdom contains more than thousands of secondary metabolites (Wang et al. 2019). However, the oils require identification of the entire suite terminal and intermediate metabolites present for the associated pathways to be further interpreted for various bioactivities and potential biomarkers to study the metabolism process. Accurately and reliably identifying phyto-components in plant essential oils and achieving reasonable separation is a significant challenge. Co-eluting components contribute differently to an unresolved peak, identifying fewer compounds (Wong et al. 2015).

The most widely used method in metabolomics is one-dimensional gas chromatography-mass spectrometry, with nearly 50 years of established protocols. Being the most cost-effective one, it is the most commonly used method (Liu et al.2021). Various reports describe the chemotype of MOSO using GC-MS across the globe. According to a study conducted in Egypt, Phytochemical analysis of *M. oleifera* seed extract by GC-MS reported 50 identified phytochemical and major components were 2,5-Di-tert-butyl-1,4-benzoquinone (15.43%) and 4',6-dimethoxyisoflavone-7-O-β-D-glucopyranoside (9.25%) (Atta et al. 2019). An analysis with multidimensional gas chromatography and gas chromatography-mass spectrometer of Nigerian *M. oleifera* seed oil revealed twenty-four components, and among these Oleic acid was identified in its most concentrated form (Adegbe et al. 2016). The seed extract of *M. oleifera* from Iraq resulted in 41 bioactive compounds by GC-MS analysis, and Pentadecanoic acid 34.43% being the major one (AL-Obaidi et al. 2021). GC-MS analyzed thirty-six phyto-compounds in a study carried out in Tunisia, Cis, 6-octadecenoic acid showed the highest peak area (70.68%) (Zhou et al. 2023).

Prior work should have addressed the complexity and limitations of 1DGC, particularly when components with low abundance overlap with the major ones (Abdulhussain et al. 2021). There is an increase in demand for comprehensive metabolomics approaches for measuring plant metabolism, improving detection, global compound identification, and gaining a deeper understanding of how plants regulate biochemical processes (Raza 2020). The only existing study reported on phytochemical analysis using 2D GC-TOF-MS chemical profiling of MOSO; 250 compounds were obtained with cis-octadecenoic acid (78.62%) as the major compounds (Bassey et al.

2022). In this study, a total of 2000 phytoconstituents were obtained, out of which 236 were identified due to 2-dimensional TOF-MS analysis with Erucic acid (9.10%) as the major component. According to previous records, the total number of phytochemicals analyzed is more than double that identified in GC-MS.

The bioactivity of a plant extract is generally determined by its major phytoconstituents, but a synergistic effect between corresponding mixtures results in more significant bioactivity than individual constituents alone (Vaou et al. 2022). For instance, some studies have reported that Isoborneol (monoterpenoid) has antioxidant and antiviral properties and is a potent inhibitor of the herpes simplex virus (Kazi et al. 2023). The *M. oleifera* oil contains a high level of monounsaturated fatty acids like Palmitic Acid, Oleic Acid, Capric acid, Erucic acid, Linoelaidic acid, Hexanoic acid, Capric acid, Laevulinic acid, linoleic acid. It is related to reducing all-cause mortality, stroke, cardiovascular events, and cardiovascular mortality (Leone et al.2016). The tocopherol group is a higher source of essential vitamin E than other oils. Alfa-tocopherol has the most significant vitamin E potency (Delgado et al. 2020). Monoterpenes, diterpenes, tetraterpenes, triterpenes, sesquiterpenes,and glycoside compounds have substantial roles as anti-inflammatory, anticancer agents, antiallergic, antimicrobial, neuroprotective, antioxidant, anti-coagulation, sedative and analgesic activity (Masyita et al.2022).

This study reports a detailed metabolic profiling of secondary metabolites in the seed oil of *M.oleifera* using high-resolution GC×GC TOF-MS analysis. It provides a deeper characterization of the metabolic composition of *Moringa* when compared with conventional 1-dimensional GC-MS. Oil metabolism can be discriminated against using the metabolic profile on a contour plot. This can be further extended to chemo-taxonomical applications like metabolite fingerprinting and characterization of seed oil of *M. oleifera*. In addition to the high-resolution platform and identification procedures proposed, a wide range of metabolite profiles can be determined using untargeted phytochemical profiling of other parts of plant-derived extracts, resulting in a substantial increase in coverage of secondary metabolites. This opens up the possibility of identifying and decoding specific bioactivities in the future.

Conclusion

Many pharmaceutical uses have been reported for *Moringa* in India, mainly for its leaves. However, a recent upsurge in interest has risen to explore the pharmaceutical potential of the seeds to combat various health conditions**.** The extent of effects obtained from the seeds is attributed to variability in metabolite composition. GC×GC TOF-MS is considered over 1D GC-MS to unfold the metabolic coverage in this work. The experimental conditions were optimized to achieve high metabolic coverage, and as a result, some untargeted metabolites were discovered. The oil's fatty acid content was more similar to that of Olive oil. In addition, other groups of compounds like benzenoids, monoterpenoids, olefins, phenols, alkaloids, triterpenoids, diterpenoids, sesquiterpenoids, tocopherol are obtained, which is attributed to various pharmaceutical potency. These new untargeted metabolites can be helpful for future research after proper in-vitro and in-vivo validations.

Abbreviations

MOSO: Moringa oleifera seed oil; GC-MS: Gas chromatography Mass spectrometry; GC×GC TOF-MS: Two dimensional Gas chromatography Time of Flight Mass spectrometry

Ethics approval and consent to participate

Not applicable

Consent for publication

The authors declare no conflict of interest and have approved for publication.

Availability of data and material

This article includes all the data generated or analyzed during this study.

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Author’s contributions

Conceptualization, RB, and SKB; Validation, RB, and JNM; Original draft preparation, MD; Review and editing, RB and JNM; Supervision, RB, and SKB.

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