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Leucaena leucocephala: Novel Mutants with New Chemical Compositions through MMS-Induced Mutagenesis

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ABSTRACT

This work presents the first detailed characterization of the phytochemicals in leaf extracts of *Leucaena leucocephala* seedlings subjected to MMS mutagenesis using GC–MS. The analysis revealed 45, 70, 72, 65, and 65 phytochemical compounds in the control (0% MMS), mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2% MMS), and mutant 4 (1.5% MMS), respectively. The major components identified were oleic acid (48.00%) in the control, and 3-O-methyl-D-glucose at varying concentrations in the mutants: 71.84% in mutant 1, 38.88% in mutant 2, 40.02% in mutant 3, and 60.98% in mutant 4. Notably, 27 novel phytocompounds were identified exclusively in the mutagenized seedlings, including hexadecanoic acid ethyl ester, E,E,Z-1,3,12-nonadecatriene-5,14-diol, octadecanoic acid ethyl ester, 17-pentatriacontene, tetracontane-1,40-diol, 5-nonanone, 2,2,8,8-tetramethyl-, dodecanoic acid methyl ester, kolavelool, 11,14-eicosadienoic acid methyl ester, 2-methylhexacosane, pentaethylene glycol monododecyl ether, 1,3-dioxolane derivatives, and several other complex hydrocarbons, esters, alcohols, and terpenoids. Many of these identified compounds are known to possess notable biological activities, such as antibacterial, anti-inflammatory, anticancer, anti-arthritic, antioxidant, and antidiabetic effects. Statistical analysis confirmed that the phytochemical profiles of the control and mutant lines were significantly different. Additionally, the phytocomponents were found across five different solvents used for extraction. The results highlight substantial differences between the control and mutants, revealing novel compounds that could serve as valuable sources for pharmaceutical and therapeutic development.

Keywords: *Leucaena leucocephala*- Mutagenized seedlings- Chemical components- Bioresources- Phytopharmaceutics.

INTRODUCTION

Mutations are the primary drivers of genetic diversity. Induced mutations, which occur more frequently than spontaneous ones, have been extensively applied to accelerate crop improvement, increase variability, and boost yield traits in several plant species (Oladosu et al., 2016). In addition to their relevance in plant breeding, induced mutations also contribute to the release of gene silencing in transgenic plants (Anwar and Kim, 2020). Numerous crops and tree species, including *Leucaena leucocephala* (Zayed et al., 2014), *Oryza sativa* (Shoba et al., 2017), *Hordeum vulgare* (Ramesh et al., 2001), *Triticum durum* (Elyadini et al., 2021) and *Sesamum indicum* (Jayaramachandran, 2020), have successfully utilized induced mutagenesis to enhance genetic variability and yield components.

Among chemical mutagens, methyl methane sulfonate (MMS), a compound belonging to the alkyl sulfonate group is widely recognized for its high mutagenic efficiency in higher plants. MMS typically induces a high frequency of gene mutations while causing a low frequency of chromosomal abnormalities (Amin, 2016). However, cases of chromosome loss or deletion have also been reported (Binodh et al., 2024).

L. leucocephala (Lam.) de Wit (Leguminosae), previously referred to as *L. glauca* and locally known as leucaena in Egypt (Zayed et al., 2019), is often described as a "miracle tree" due to its versatility and high nutritional value as forage (Sharma et al., 2022), in addition to its utility in medicine and agroforestry systems (Renner and Müller,

2021) and (Brewbaker and Hutton, 2019). Although native to southern Mexico and northern Central America, it is now widely naturalized across tropical and subtropical regions (Brewbaker and Hutton, 2019 and Patzelt and Lupton, 2021). Traditionally, it has been used to treat gastrointestinal issues and as a contraceptive agent (Odekanyin et al., 2024).

Phytochemical studies have revealed the presence of a diverse range of secondary metabolites, including alkaloids, cardiac glycosides, tannins, flavonoids, saponins, and other glycosides (Septina et al., 2020). Chemical profiling of *L. leucocephala* leaves from Malaysia identified compounds such as squalene, phytol, oxalic acid, and various methyl esters (Ogunniyi et al., 2023), whereas Egyptian samples contained mono (2-ethylhexyl) phthalate (17.7%), betulin (15.7%), lupeol (14.7%), and β-sitosterol (9.1%) (Zayed et al., 2019). Chinese whole-plant extracts were found to include ficaprenol-11, squalene, pheophytin-a, and coumaric acid derivatives (Ogunniyi et al., 2023).

The species demonstrates a wide spectrum of biological activities, including antimicrobial, antioxidant, anticancer, anti-inflammatory, antihistaminic, nematicidal, and hepatoprotective effects (Zayed et al., 2019 and Odekanyin et al., 2024).

Despite extensive pharmacological research, the phytochemical composition of EMS-mutagenized *L. leucocephala* seedlings remains uncharacterized. This study presents the first gas chromatography–mass spectrometry (GC-MS) analysis of four mutant lines of *L. leucocephala* generated through ethyl methanesulfonate (EMS)-induced mutagenesis, aiming to elucidate mutation-induced changes in phytochemical profiles.

MATERIALS AND METHODS

2.1. Plant materials and experimental design:

L. leucocephala seeds were obtained from the nursery of Forestry and Wood Technology Department, Faculty of Agriculture, Alexandria University, Egypt. The completely randomized design (CRD) containing five replicates was used in the MMS study. Each replicate contained five treatments and 250 seeds.

2.2. The Mutagenic treatments:

Seeds were pre-soaked in distilled water for one hour and then air dried before soaked in different MMS concentrations in petri dishes for three hours. Laboratory temperature during the treatment was 21°C, whereas the relative humidity was about 59%. Four different concentrations of MMS were used, i.e. 0% (distilled water) (Control), 0.6% (Mutant 1), 0.9% (Mutant 2), 1.2% (Mutant 3) and 1.5% (Mutant 4). After the treatment, seeds were washed in distilled water for 15 minutes; air dried and then soaked in hot water for 24 hours to 48 hours. The treated seeds were then sown in seed trays for recording the germination behavior such as germination percentage, survival after germination and maturation, and lethality over control (LOC). The germination percentage per treatment with five replicates was counted and recorded on 21st day after seed sowing. Percentage of inhibition or stimulation over control (lethality over control, LOC) was calculated as [Control – Treated / Control] x 100.

2.3. Sample extraction:

A total of 100 grams of powdered leaves from *L. leucocephala* mutants and the control were weighed, transferred to a flask, and extracted using a sequential solvent extraction method. The solvents used were hexane, petroleum ether, chloroform, ethyl acetate, and methanol. The plant material was fully immersed in the solvents and incubated overnight. The resulting extracts were filtered through Whatman No. 1 filter paper along with 2 grams of sodium sulfate to remove sediments and water traces. Before use, the filter paper and sodium sulfate were pre-wetted with 95% ethanol.

The filtrates were then air-dried and subjected to phytochemical screening and gas chromatography–mass spectrometry (GC–MS) analysis.

2.3. Gas Chromatograph-mass Spectroscopy (GC-MS):

GC-MS (Gas Chromatography-Mass Spectrometry) analysis of the chemical composition investigation of methanol extract of the mutants and the control of *L. leucocephala*leaves grown in Egypt were performed on a GC-MS equipment Thermo Scientific Trace GC 1300 - TSQ 8000 evo equipped with TG-5MS ; carrier gas was He with flow 1 ml/min. Experimental conditions of GC-MS system were as follows: DB-5 cross-linked column (30 m long x 0.25 mm ID x 0.25 µm film thickness composed of 5% phenyl methyl polysiloxane). The initial temperature was programmed at 50 °C and held for two minutes, and then it was increased to 300 °C with the rate of 6.5 °C/min. The final temperature was held for ten minutes. The temperature of the injector and detector were set up to 280 °C and 300 °C, respectively. 1 µl of the fractions was diluted in 100 µl hexane and then injected into the GC-MS. Interpretation of mass-spectrum was conducted using the database of National Institute Standard and Technology (NIST). The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular mass and structure of the components of the test materials were ascertained.

2.4. Multivariate analysis:

Principal Component Analysis (PCA) based on the correlation matrix was conducted to investigate correlated genetic variation, mimosine content, and phytochemical composition in the leaves of *L. leucocephala* mutants and the control. Further analysis was performed using hierarchical cluster analysis (HCA) via the pvclust function in R software. A heatmap was generated in R based on the proximity score matrix.

RESULTS AND DISCUSSION

A total of 165 one-year-old *L. leucocephala* mutagenized seedlings survived after a year of germination, 70, 30, 30 ,22 and 13 seedlings for the control (0% MMS), mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2% MMS), and mutant 4 (1.5% MMS), respectively. The components present in the hexane, petroleum ether, chloroform, ethyl acetate, and methanol extracts of the five *L. leucocephala* mutagenized seedlings from each treatment were identified by GC- MS. The active principles with their retention time (RT), molecular formula, molecular weight (MW), peak area in percentage, and similarity index (SI) are presented, and the identified compounds are enlisted in supplementary Tables 1 through 5.

3.1. Phytochemical *constituents of leaves of* L. leucocephala *(0% MMS):*

GC-MS analysis of *L. leucocephala* leaves (0% MMS) identified 45 compounds. Major constituents included oleic acid (48%), betulin (23.68%), 9-octadecenoic acid (19.93%), mono (2-ethylhexyl) phthalate (19.67%), β-sitosterol (19.08%), lupeol (15.68%), and squalene (12.56%) (Supplementary **Table 1**). These compounds were also reported in earlier studies on *L. leucocephala* (Zayed et al., 2019 and Ogunniyi et al., 2023), Andrographis paniculata (Bakewell-Stone, 2023), Gracilaria dura (Bassey et al., 2023), Orthosiphon stamineus (Natarajan et al., 2024) and Broussonetia luzonica (Zayed and Samling, 2016 and Krishnamoorthy and

Kalaiselvan, 2016). Findings confirm consistency with reported phytochemicals from different extracts, including petroleum ether and methanol, supporting their potential pharmacological relevance (Zayed et al., 2019 and Ogunniyi et al., 2023).

3.2. Phytochemical study of the Mutant 1 (0.6% MMS) of L. *leucocephala* leaves:

Approximately 70 phytochemical compounds were identified in the mutant 1 of *L. leucocephala* leaves using five different solvents (Supplementary **Table 2**). GC-MS analysis of leaves extracts of *L. leucocephala* mutant 1 (0.6% MMS) revealed that the major compounds were 3-o-methyl-d-glucose (71.84%), betulin (20.80%), octadecanoic acid, ethyl ester (19.54%), lupeol (17.98%), β-sitosterol (17.43%), linoleic acid ethyl ester (16%), 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester (15.51%), and squalene (14.78%). There was no previous report on the phytochemical screening of hexane, petroleum ether, chloroform, ethyl acetate, and methanol extracts of *L. leucocephala* leaves mutant 1 (0.6% MMS) by GC-MS. The major compounds were also reported elsewhere in different species, such as β-sitosterol in *Triphaladi rasayana* (Cyriac and Eswaran, 2023), 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester in Andrographis paniculatas (Natarajan et al., 2024), lupeol in *Albizia adianthifolia* (Hossain and Ismail, 2013), betulin in *Orthosiphon stamineus* Gracilaria dura (Bassey et al., 2023). Squalene in Broussonetia luzonica leaves (Casuga et al., 2016). These major compounds had important biological activities for medical application. β-sitosterol is known to reduce cholesterol and control benign prostrate hypertrophy as well as inflammation (Zayed et al., 2019). Lupeol has anti-inflammatory and anti-cancer activities (Zayed et al., 2019). Betulin has anticancer and apoptosis activities Zayed et al., 2019).

3.3. Phytochemical composition of leaves of L. leucocephala Mutant 2 (0.9% MMS):

GC-MS analysis of L. leucocephala mutant 2 (0.9% MMS) leaves using five solvents identified 72 phytochemicals. Major constituents included 3-O-methyl-D-glucose (38.88%), 1,5-Hexadien-3-ol, 3-methyl (33.88%), octadecanoic acid ethyl ester (21.57%), betulin (20.56%), linoleic acid ethyl ester (19.30%), lupeol (18.22%), squalene (17.65%), and vitamin E (13.88%) (Supplementary **Table 3**). These compounds were identified based on peak area, molecular weight, formula, and similarity index. Similar compounds were found in other species: 1,2-benzenedicarboxylic acid in Pleiospermium alatum (Kalaivani et al., 2012), Oleic Acid in *Triphla Rasayana* (Muthiah et al., 2017), lupeol in *Albizia adianthifolia* (Abubakar and Majinda, 2016) and in *Pterocarpus marsupium Roxb* (Maruthupandian and Mohan, 2011), betulin in *Pleiospermium alatum* (Parthipan et al., 2015), β-sitosterol in Hugonia mystax L (Rajeswari et al., 2012) and Dolichandrone atrovirens (Reddy et al., 2017) and squalene in Aquilaria malaccensis (Santhanam et al., 2016). 3-O-methyl-D-glucose was reported in Alnus glutinosa (Felföldi-Gáva et al., 2012) and Taxus baccata (Mohan and Sudha, 2013) and vitamin E in Dolichandrone atrovirens (Deepa and Murugesh, 2013). Linoleic and palmitic acid esters were found in Azadirachta indica and Aquilaria malaccensis (Ramli, 2019). These bioactive compounds demonstrate significant pharmacological potential and confirm the diverse phytochemical profile of this mutant line.

3.4. Phytochemical composition of leaves of *L. leucocephala* Mutant 3 (1.2% MMS):

GC-MS analysis of hexane, petroleum ether, chloroform, ethyl acetate, and methanol extracts of *L. leucocephala* mutant 3 (1.2% MMS) leaves were presented in Supplementary **Table (4).** There were 65 compounds revealed in the hexane, petroleum ether, chloroform, ethyl acetate and, methanol extracts *L. leucocephala* mutant 3 (1.2% MMS) leaves with the presence of 3-o-methyl-d-glucose (40.02), 1,5-hexadien-3-ol, 3-methyl-6-(methylthio)-1-(2,6,6-trimethyl-1-decanoic acid, 3-methyl- (29.02%), octadecanoic acid, ethyl ester (22.00%), lupeol (21.34%), linoleic acid ethyl ester (20.34%), betulin (19.43%), and squalene (18.24%), and vitamin E (16.55%) (Supplementary Table, 4).

This was the first study for the chemical composition of *L. leucocephala* mutant 3 (1.2% MMS) leaves. The major compounds of the hexane, petroleum ether, chloroform, ethyl acetate, and methanol extracts of leaves of L. leucocephala mutant 3 (1.2% MMS) were 3-o-methyl-d-glucose, 1,5-hexadien-3-ol, 3-methyl-6-(methylthio)-1-(2,6,6-trimethyl-1-decanoic acid, 3-methyl-, octadecanoic acid, ethyl ester, lupeol, linoleic acid ethyl ester, and squalene. These major compounds were also found in species other than L. leucocephala such as *Dolichandrone atrovirens* (Reddy et al., 2017), *Pleiospermium alatum* (Kalaivani et al., 2012), *Dolichandrone atrovirens* (Reddy et al., 2017), *Robinia pseudoacacia* (Sousa et al., 2023) and *Kirganelia reticulate* (Rajeswari et al., 2012), respectively.

3.5. Phytochemical composition of leaves of *L. leucocephala* Mutant 4 (1.5% MMS):

This was the first study for the chemical composition of *L. leucocephala* mutant 4 (1.5% MMS) leaves (Supplementary Table 5). 65 compounds revealed in the hexane, petroleum ether, chloroform, ethyl acetate and, methanol extracts *L. leucocephala* mutant 4 (1.5% MMS) leaves with the presence of 3-o-methyl-d-glucose (60.98%), octadecanoic acid, ethyl ester (22.55%), linoleic acid ethyl ester (21.56%), squalene (19.45%), vitamin E (16.77%), and phytol (15.00%). 3-O-methyl-d-glucose was identified in the extracts of *Taxus baccata L.* leaf (Mohan et al., 2013). Squalene compound was extracted from the *Aquilaria malaccensis* leaves (Shirmohammadli et al., 2020). Octadecanoic acid ethyl ester was extracted from *Dipteryx punctata* leaves (Ayoola et al., 2020); linoleic acid ethyl ester, and Octadecanoic acid ethyl ester were identified from the extract of *Echium angustifolium Mil* aerial parts (Sneha et al., 2020). Vitamin E identified as the major compound of the extraction of Pithecellobium Jiringan (Hosseinihashemi and Kanani, 2012). Squalene and phytol have been reported in the leaf extract of the same species from Malaysia (Zayed et al., 2019 and Ogunniyi et al., 2023).

3.6. Phytochemical constituents of leaves of L. leucocephala mutagenized seedlings:

Five *L. leucocephala* seedlings from each treatment were analyzed using GC-MS across hexane, petroleum ether, chloroform, ethyl acetate, and methanol extracts. A total of 45, 70, 72, 65, and 65 phytochemicals were identified in the control (0% MMS), mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2% MMS), and mutant 4 (1.5% MMS), respectively **(Table 1).** Seventeen compounds present in the control disappeared post-treatment, including oleanolic acid, rhodoxanthin, and 1,2-benzenedicarboxylic acid mono(2-ethylhexyl) ester. Conversely, 27 novel

compounds appeared, such as hexadecanoic acid ethyl ester, kolavelool, and phytol. These changes suggest that MMS-induced mutation may alter biosynthetic pathways, leading to either the loss or formation of specific phytochemicals (Zayed et al., 2014). Decreased levels of oleic acid, methyl 10-trans,12-cis-octadecadienoate, and 9-octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester were associated with increasing MMS concentration, likely due to DNA damage, methylation, and chromosomal alterations impacting compound production.

Table (1). The percentage for identified components of five solvent extracts from the leaves of the *L. leucocephala* mutagenized seedlings analyzed by gas chromatography–mass spectrometry (GC–MS)

Solvent	No.	Chemical Compounds	(0% MMS)	Mutant 1 (0.6% MMS)	Mutant 2 (0.9% MMS)	Mutant 3 (1.2% MMS)	Mutant 4 (1.5% MMS)
HE	1	Benzene, (1-butylhexyl)-	6.67	0	0	0	0
	2	Benzene, (1-pentylhexyl)-	6.46	0	0	3.45	6.00
	3	Hexadecanoic acid, ethyl ester	0	2.50	2.80	3.30	3.60
	4	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	0	1.20	0	0	1.30
	5	9,12,15-Octadecatrienoic acid, ethyl ester, (Z	7.32	5.64	4.44	3.56	0
	6	Linoleic acid ethyl ester	17.98	16.00	19.30	20.34	21.56
	7	Octadecanoic acid, ethyl ester	0	19.54	21.57	22.00	22.55
	8	2,2-Dimethyl-3-[3,7,12-trimethyl-14-(1,4,4-trimethylcyclohex-2-enyl)tetradeca-3,7,11-trienyl]oxirane	9.45	7.67	7.56	5.76	4.88
	9	Heneicosane, 11-phenyl-	10.58	0	0	0	0
	10	Squalene	12.56	14.78	17.65	18.24	19.45
	11	β-sitosterol	18.37	17.43	16.54	15.65	13.45
	12	Lupeol	10.16	8.88	8.57	7.56	5.67
	13	17-Pentatriacontene	0	3.40	1.30	0	1.50
	14	Tetracontane-1,40-diol	0	2.77	0	0	0
PE	1	5-Nonanone, 2,2,8,8-tetramethyl-	0	1.89	2.20	2.30	2.67
	2	Dodecanoic acid	0	3.00	3.13	4.17	6.23
	3	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	19.67	15.51	13.43	11.43	10.34
	4	Methyl 14-methyl-hexadecanoate	0	1.98	2.30	3.40	4.99
	5	Kolavelool	0	5.32	6.89	7.98	9.78
	6	Octadecanoic acid, ethyl ester	0	6.00	7.20	8.30	10.43
	7	Androstan-17-one,3-ethyl-3- hydroxy-, (5à)-	13.34	11.65	10.76	10.89	5.98
	8	11,14-Eicosadienoic acid, methyl ester	0	1.67	1.80	2.00	2.23
	9	2-Methylhexacosane	0	2.00	2.20	2.30	2.43
	10	Pentaethylene glycol monododecyl ether	0	2.66	2.87	2.91	3.2
	11	1,3-Dioxolane, 4-[[(2-methoxy-4-octadeceny	0	2.77	2.89	2.95	3.4
	12	Cyclohexane,1,3,5-trimethyl- 2-octadecyl-	5.44	0	0	0	0
	13	β-sitosterol	19.08	17.43	15.76	13.45	11.54
	14	1,1,6-trimethyl-3-methylene-2-(3,6,9,13-tetra	0	3.36	3.70	5.90	9.78
	15	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11, 15-tetraenyl)-cyclohexanol	8.50	0	0	0	0
	16	Oleanolic acid	14.43	12.22	11.34	10.43	6.00
	18	Astaxanthin	15.37	12.34	11.23	10.45	8.53
	19	Tetratetracontane	3.95	0	2.00	0	0
CH	1	Ethane, 1,1-diethoxy	9.02	0	0	0	0

	2	3-O-Methyl-d-glucose	0	8.98	11.15	14.45	16.67
	3	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	0	2.00	2.45	0	7.43
	4	9,10 Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol	0	1.34	1.65	4.88	5.00
	5	1-Octadecyne	0	1.50	1.23	0	0
	6	2-Pentadecanone, 6,10,14-trimethyl-	0	1.40	1.30	1.20	1.10
	7	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	10.81	9.23	9.00	8.77	6.65
	8	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	0	1.34	1.56	7.75	10.40
	9	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl- heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	9.96	0	0	0	0
	10	Octadecanoic acid, methyl ester	0	2.50	2.89	10.00	0
	11	9-Octadecenoic acid, methyl ester, (E)-	0	9.34	14.00	0	0
	12	Phytol	0	4.00	5.76	6.98	15.00
	13	Betulin	23.68	20.80	20.56	19.43	17.34
	14	Oleanolic acid	4.42	0	0	0	0
	15	Rhodoxanthin	9.60	8.50	0	0	0
	16	Hexadecanoic acid, 1-(hydroxymethyl)-1,2- ethanediyl ester	11.37	10.11	9.85	8.89	7.77
	17	Stearic acid, 3-(octadecyloxy)propyl ester	9.88	8.67	8.56	7.45	5.45
	18	Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester	10.71	9.56	9.33	9.23	6.33
EA	1	Ethan 1,1-diethoxy-	4.65	0	0	0	0
	2	3-O-Methyl-d-glucose	0	13.24	16.65	17.68	17.98
	3	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	0	0	4.79	0	2.54
	4	9,10 Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol	0	1.34	1.56	2.98	3.00
	5	1-Octadecyne	0	1.98	1.87	0	0
	6	2-Pentadecanone, 6,10,14-trimethyl-	0	1.50	1.67	2.80	3.00
	7	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	4.07	0	0	0	0
	8	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	0	1.45	1.67	2.78	2.90
	9	Octadecanoic acid, methyl ester	0	1.50	1.69	2.98	3.30
	10	9-Octadecenoic acid, methyl ester, (E)-	0	10.00	3.32	0	0
	11	Phytol	0	1.23	1.45	2.65	2.90
	12	Betamethasone	9.73	8.87	7.78	6.87	4.87
	13	β-sitosterol	14.97	13.33	12.54	11.67	10.98
	14	Lupeol	15.68	17.98	18.22	21.34	22.89
	15	Propanoic acid, 2-(3-acetoxy-4,4,14- trimethylandrost-8-en-17-yl)-	7.67	6.87	5.56	4.73	3.67
	16	Vitamin E	11.09	12.78	13.88	16.55	16.77
	17	2-Butenoic acid, 2-methyl-, 2-(acetyloxy)	3.05	0	0	0	0
	18	Oleic acid, 3-(octadecyloxy)propyl ester	11.56	0	0	0	0
	19	Astaxanthin	8.55	7.68	6.87	5.98	4.99
	20	psi., psi.,- Carotene, 1,1'2,2'-tetrahydro-1,1'- dimethoxy-	8.68	0	0	0	0
ME	1	1,5-Hexadien-3-ol, 3-methyl-6-(methylthio)-1-(2,6,6-trimethyl-1-decanoic acid, 3-methyl-	0	0	33.88	29.02	0

2	1-Butanol, 3-methyl-, acetate	0	3.47	4.67	5.80	6.89
3	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	0	0.98	0	0	0
4	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2-Trimethylsilyl-1,3-dithiane Oxirane, [(dodecyloxy)methyl]-	0	0.42	0.59	0.77	0.94
5	3-O-Methyl-d-glucose	0	71.84	38.88	40.02	60.98
6	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	0	0	0.76	0	0.82
7	9,10 Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol	0	0.39	0.49	0.59	0.97
8	Fumaric acid, ethyl 2-methylallyl ester	0	0	0	0.98	1.88
9	1-Dodecanol, 3,7,11-trimethyl-	0	0	0	1.99	0
10	1-Octadecyne	0	0.38	0.58	0	0
11	n-Hexadecanoic acid	13.04	1.70	2.22	2.25	3.70
12	1-Hexadecanol, 2-methyl-	0	0	1.43	0	0
13	2-Pentadecanone, 6,10,14-trimethyl-	0	0.67	1.74	1.92	2.63
14	Hexadecanoic acid, methyl ester	2.80	2.90	3.30	3.60	3.77
15	Oleic Acid	48.00	2.58	3.13	3.22	3.26
16	Octadecanoic acid	4.91	0.63	0.69	0.75	0.84
17	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	0	0.67	0.89	1.55	3.34
18	Methyl 10-trans,12-cis-octadecadienoate	2.32	0	0	0	0
19	11-Octadecenoic acid, methyl ester, (Z)	2.48	0	0	0	0
20	9-Octadecenoic acid, methyl ester, (E)-	0	9.76	1.39	1.29	1.18
21	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0	0	0	0.51	0.76
22	Phytol	0	2.56	2.77	2.86	5.67
23	Octadecanoic acid, methyl ester	0	0.58	0.68	0.87	0
24	9,12,15-Octadecatrienoic acid, ethyl ester, (Z	0	0	1.19	1.27	2.35
25	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	6.38	0	0	0	0
26	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	19.93	0	0	0	0

3.7. Multivariate statistical analysis of the phytochemical constituents of leaves of L. *leucocephala* Mutagenized seedlings.

Multivariate statistical analysis revealed interconnected correlation patterns among the phytochemical components of the four mutants of L. leucocephala **(Fig. 1).**

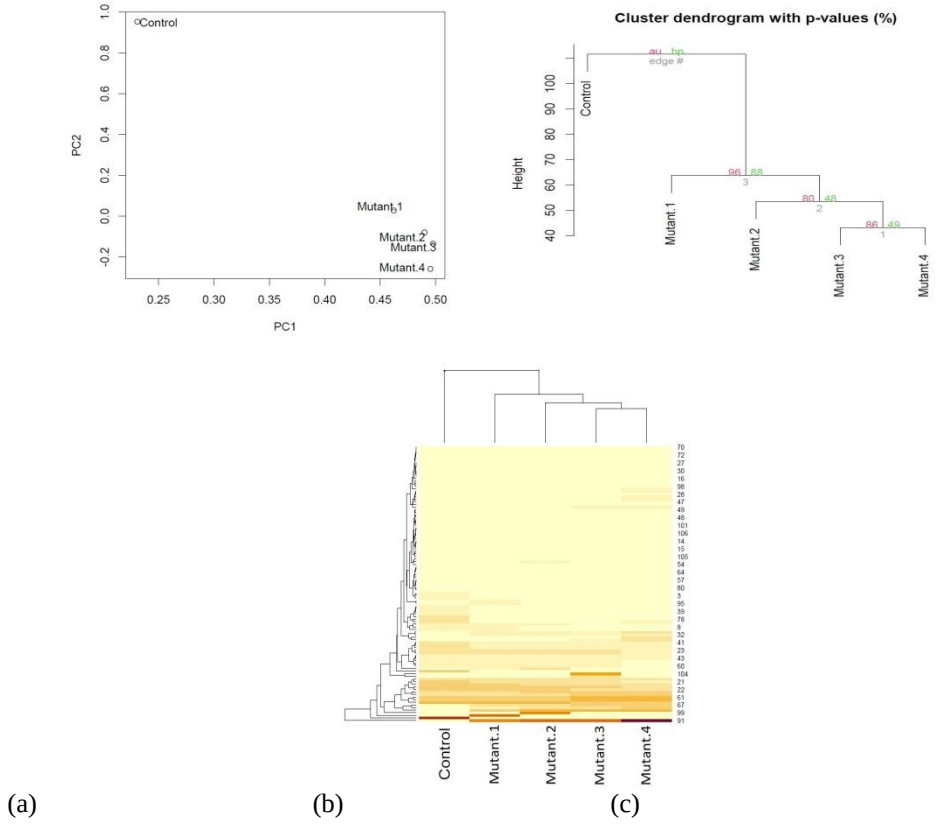

Fig. (1). Multivariate statistical analysis of the phytochemical constituents of leaves of L. *leucocephala* mutagenized seedlings. (a) Principal component analysis (PCA) of four mutants (Mutant 1, Mutant 2, Mutant 3, and Mutant 4) and the control of L. leucocephala. A PCA was based on the correlation matrix of phytochemical composition in control, mutant 1, mutant 2, mutant 3, and mutant 4. The first and second principal components accounted for 66.8% and 17.8% of variation, respectively; (b) A consensus tree of the relationships among control, mutant 1, mutant 2, mutant 3, and mutant 4 of L. leucocephala. Pvclust package in R was used to cluster these traits according to the Euclidean distance matrix. The numbers at the forks were the percentages of approximately unbiased (AU; in red) p-values and bootstrap probabilities (BP, in green) estimated from 1000 bootstrapping samples; (c) A heatmap showing cluster groups in both phytochemical composition and different mutants of L. leucocephala (control, mutant 1, mutant 2, mutant 3, and mutant 4). The numbers on the right side represent the codes for 106 phytochemical components within five solvents that showed a mixed pattern.

Partial convergence in phytochemical composition among *L. leucocephala* mutants and the control was attributed to shared primary and secondary metabolic pathways, resulting in common or derivative compounds across samples. Several metabolites were identified in multiple mutants, indicating overlapping biosynthetic routes. The spatial distribution of phytochemicals in PCA **(Fig. 1a)** showed that both mutants and control clustered within the double-positive quadrant, suggesting interrelated chemical profiles. Principal components 1 and 2 accounted for 66.8% and 17.8% of the total variance, respectively. While clustering patterns were mixed, distinct chemical compositions were evident in the separated locations of the control and mutants in both PCA and the correlation matrix **(Table 2).** Hierarchical clustering **(Fig. 1b),** based on Euclidean distance, aligned with PCA results, forming

coherent groupings. The heatmap **(Fig. 1c)** further supported these findings, illustrating proximity-based clustering and partial overlap of phytochemical constituents among the five groups, confirming both diversity and similarity in metabolic profiles.

Table (2). Correlation Coefficient Matrix of Phytochemical Composition of *L. leucocephala* in control, mutant 1, mutant 2, mutant 3, and mutant 4.

	Control	Mutant 1	Mutant 2	Mutant 3	Mutant 4
Control	1.0000				
Mutant 1	0.331 (0.001)	1.0000			
Mutant 2	0.305 (0.001)	0.683 (6.7×10^{-16})	1.0000		
Mutant 3	0.285 (0.003)	0.675 (2.1×10^{-15})	0.751 (2.2×10^{-16})	1.0000	
Mutant 4	0.192 (0.048)	0.675 (2.1×10^{-15})	0.779 (2.2×10^{-16})	0.845 (2.2×10^{-16})	1.000

Note: p-values for statistical tests are in parentheses

From the GC-MS analysis of all solvent extracts of *L. leucocephala* mutagenized leaves, 45, 70, 72, 65 and 65 phytocomponents were identified from *L.* leucocephala leaves of control (0% MMS), mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2% MMS), and mutant 4 (1.5% MMS) extracts, respectively. Some of the identified compounds contribute to various biological effects such as anti-microbial, anti-cancer, antimutagenic, antipeptic, antiseptic, antispasmodic, antiadrenogenic, and hypocholester- olemic activities, as summarized in **Table (3)** (Duke, 2007) [43].

3-O-methyl-d-glucose was the major compounds in the four mutants of *L.* leucocephala. Meanwhile, oleic acid was the major compounds of *L. leucocephala* leaves of control (0% MMS). β-sitosterol, lupeol, and betulin were also identified as the major compounds of these four mutants extracts of *L. leucocephala*.

Vitamin E, hexadecanoic acid, methyl ester, n-hexadecanoic acid, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester and squalene have been shown in *L. leucocephala* leaves extracts of control (0% MMS), mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2% MMS), and mutant 4 (1.5% MMS). Phytol and 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- were found in the L. leucocephala leaves extracts of mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2% MMS), and mutant 4 (1.5% MMS).

Table (3). Summary of Chemical Compounds Identified from the Extracts of *L. leucocephala* mutagenized leaves, and their General Biological Activities (Modified from Dr. Duke's: Phytochemical and Ethnobotanical Databases 2007)

Compound	Treatments	Secondary Metabolite	Biological Activities
9,12,15-Octadecatrienoic acid, ethyl ester, (Z	0% MMS, mutant 1(0.6% MMS), mutant 2(0.9% MMS)), mutant 4 (1.2% MMS)	omega-3 fatty acids ester	Antiinflammatory, Insectifuge Hypocholesterolemic, Cancer preventive, Nematicide, Hepatoprotective, Insectifuge, Antihistaminic, Antieczemic, Antiacne, 5-Alpha reductase
Linolenic acid, ethyl ester	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Fatty acids ester	inhibitor, Antiandrogenic, Antiarthritic and Anticoronary Hypocholesterolemic, Nematicide, Antiarthritic, Hepatoprotective Anti-androgenic, Hypocholesterolemic, 5-Alpha reductaseinhibitor, Antihistaminic, Anticoronary, Insectifuge, Anti-eczemic, Anti-acne
Squalene	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Triterpene	Antibacterial, antioxidant, antitumor, cancer-preventive, chemopreventive, immunostimulant, lipoxygenase-inhibitor, perfumery, pesticide,sunscreen
β-sitosterol	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Steroids	Antimicrobial, anticancer, anti-inflammatory, anti-asthma, diuretic, antiarthritic
Lupeol	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Pentacyclic triterpene	Anti-inflammatory, antiarthritic, antimicrobial, antitumor, antiprotozoal,chemopreventive, antibacterial, antioxidant, cancer preventive, immunostimulant, lipoxygenase inhibitor, pesticide
1,2-Benzenedicarboxylic acid,mono(2-ethylhexyl) ester	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Plasticizer compound	Antifouling, antimicrobial, antifungal, anti-retroviral, anti-tumor, anti-diabetic, anti-cancer, antioxtioxidant, anti-scabies, anti-inflammatory, potent antimicrobial agent
Androstan-17-one,3-ethyl-3-hydroxy-, (5à)-	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Steroid	Neuroactive, analgesic, anesthetic
Cyclohexane,1,3,5-trimethyl- 2-octadecyl-	0% MMS	Alcohol	Anticancer
Oleanolic acid	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Pentacyclic triterpene	Antioxidant, antimicrobial, anti-inflammatory, antibacterial
Astaxanthin	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Tetraterpen	Antioxidant, anti-inflammatory
Tetratetracontane	0% MMS, mutant 2 (0.9% MMS)	Alkane	Antioxidant, anti-inflammatory, antibacterial, antiulcerogenic
Ethane, 1,1-diethoxy	0% MMS	Ether	Flavoring agent
Betulin	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Pentacyclic triterpene	Anticancer, anti-HIV, anti-bacterial, antimalarial, anthelmintic, antifeedant, antimicrobial
Hexadecanoic acid, 1- (hydroxymethyl)-1,2-ethanediyl Ester	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Fatty acid	Antioxidant, hypocholesterolemic, antiandrogenic, hemolytic

Stearic acid, 3-(octadecyloxy)propyl ester	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Fatty acid methyl ester	5-alpha-reductase-inhibitor, cosmetic, flavor, hypocholesterolemic, lubricant, perfumery, propecic, suppository
Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Fatty acid	Hypocholesterolemic, antiarthritic, nematicide, 5-alpha reductase inhibitor, antiacne, hepatoprotective
Betamethasone	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Steroid	Anti-inflammatory
Vitamin E	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Vitamin	Antiageing, analgesic, antidiabatic, antiinflammatory, antioxidant, antidermatitic, antileukemic, antitumor, anticancer, hepatoprotective, hypocholesterolemic, antiulcerogenic, vasodilator, antispasmodic, antibronchitic, anticoronary
n- Hexadecanoic acid	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Palmitic acid ester	Antioxidant, Hypocholesterolemic, Nematicide, Anti-androgenic, Flavor, Hemolytic
Hexadecanoic acid, methyl ester	0% MMS, mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Palmitic acid , methyl ester	Antioxidant
Octadecanoic acid, ethyl ester	Mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Stearic acid	Anti-microbial activity, hypocholesterolemic, nematicide antiarthritic, hepatoprotective anti-androgenic, hypocholesterolemic nematicide, 5-alpha reductase inhibitor, antihistaminic, anti-coronary insectifuge, anti-eczemic, anticancer
Hexadecanoic acid, ethyl ester	Mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Palmitic acid	Antioxidant, Hemolytic, Hypocholesterolemic, Flavor, Nematicide, Anti-androgenic.anticancer
3-O-methyl-d-glucose	Mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Glycosides	protective agent against the alloxan toxicity to pancreatic beta cells
9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	Mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Fatty acid	Hypocholesterolemic, Nematicide,Antiarthritic, Hepatoprotective, Anti androgenic, Hypocholesterolemic,Nematicide, 5-Alpha reductase inhibitor, Antihistaminic, Anticoronary, Insectifuge,Antieczemic , Antiacne
9-octadecenoic acid, methyl ester, (E)-	Mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Fatty acid, ester	Antibacterial, antitumor, antiinflammatory,5-α reductase inhibitor, allergenic, anemiagenic,antialopecic, choleretic.
Phytol	Mutant 1 (0.6% MMS), mutant 2 (0.9% MMS), mutant 3 (1.2%MMS), mutant 4 (1.5% MMS)	Palmitic acid	Cytotoxic, anti-inflammatory, anti-diabetic, anti-hyperalgesic, antibiotic chemotherapy, Antimicrobial, anti-tumor, Antifungal, anti-mutagenic, anti-teratoenic, anticonvulsant, anti schistosomal, lipid restriction, antispasmodic, anti-scratching behavioural effects, anxiolytic, hair growth facilitator, antidepressant

The founding compounds from the GC-MS analysis of all solvent extracts of L. leucocephala mutagenized seedlings had important biological activities for medical applications, such as plasticizer compounds that were reported to have antifouling and antimicrobial activities (Bruna et al., 2023). β-sitosterol has the properties of anticancer, antimicrobial, antiasthma, diuretic, antiarthritic, and anti- inflammatory (Rajeswari et al., 2012). Lupeol is one of the triterpenoids that is used as anti-inflammatory activity and anti-cancer (Hanan et al., 2022). Betulin has been reported to have antimalarial, anticancer, anti-HIV, anthelmintic, anti- bacterial, antimicrobial, and antifeedant activities (Younes et al., 2020).

CONCLUSIONS

Mutation induction and detection are the key element for developing improved new varieties through improving character of direct importance, early maturity and tolerance to biotic and abiotic stresses. Further, A variety of mutant cultivars have evolved because of the widespread application of MMS in many crops to obtain high frequency gene mutation and chromosomal alteration. To the best of our knowledge, this is the first report on the use of MMS-induced mutations to obtain novel chemical compositions in *L. leucocephala* leaves in Egypt. The current study's findings show that as MMS doses increased, novel chemical compositions were considerably obtained. In comparison to the control, the effects of MMS treatments were much more efficient in increasing the content of chemical compounds. Four novel mutants with different chemical compositions have been obtained in this study. Therefore, we recommend using these mutants of *L. leucocephala* as bioresource for phytopharmaceutical importance. However, further studies need to be undertaken to ascertain fully its bioactivity.

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الملخص العربي

الليوسينا ليوكوسيفالا: طفرات جديدة بتركيبات كيميائية مبتكرة من خلال الطفرات المُحفّزة بمادة (MMS)

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يستعرض هذا البحث أول تحليل شامل للمكونات الكيميائية لأوراق نبات الليوسينا ليوكوسيفالابعد تعريض بذوره لمادة كيميائية مطفّرة تُدعى" ميثيل ميثان سلفونات ، باستخدام تقنية التحليل الكروماتوجرافي الغازي المرتبط بالكتلة.

تم تحليل خمس مجموعات من النباتات واحدة ضابطة وأربع مجموعات تعرضت لتركيزات متزايدة من مادة الميثيل ميثان سلفونات (0%و 0.6% و 0.9% و 1.2% و 1.5%) و أظهرت النتائج وجود اختلافات ملحوظة في التركيب الكيميائي للأوراق بين النباتات المعالجة وغير المعالجة .عدد المركبات الكيميائية تراوح بين 45 في المجموعة الضابطة و 72مركبًا في بعض المعالجات. و أهم المركبات في العينة الضابطة كان حمض الأوليك، بينما ظهرت مركبات جديدة بشكل ملحوظ في الطفرات، أبرزها أورثو ميثايل جلوكوز وأحماض دهنية استيرية، وهيدروكربونات طويلة السلسلة، واسترات، وتربينويدات. هذه المركبات تمتلك خصائص طبية متنوعة تشمل مضادات للبكتيريا، والالتهابات، والأكسدة، والسرطان، والسكري، وغيرها. أكد التحليل الإحصائي وجود اختلافات جوهرية بين المجموعات، وأُثبت أن الطفرات الكيميائية باستخدام ميثيل ميثان سلفونات أسفرت عن إنتاج مكونات كيميائية جديدة ذات قيمة علاجية محتملة، مما يفتح الباب أمام استخدامها في الصناعات الدوائية والطبية.