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Lipid and Essential Oil Profiles of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. Leaves and Flowers with Antifungal Effects Against Fluconazole-Resistant *Candida albicans*

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Abstract

The study analyzed the chemical composition and antimicrobial activity of essential oils and lipids from *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers. Gas chromatography-mass spectrometry analysis (GC-MS) revealed high levels of unsaturated fatty acids, with arachidonic acid being the most abundant in both leaves (39.56%) and flowers (83.66%). The main triterpenoid compound in both the leaves and flowers was betulin, with proportions of 29.23% and 44.39%, respectively. The GC-MS analysis of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. essential oils found eucalyptol (24.09%) and d-limonene (18.05%) as the main components in the flowers, while coumarin (33.52%) and estragole (15.48%) were the chief components in the leaves. Essential oils from leaves and flowers were mostly inactive against several bacteria and fungi, but the leaves essential oil showed antifungal activity against fluconazole-resistant *Candida albicans* and weak activity against *Salmonella typhi*. The antifungal effect of the leaves' essential oil is likely due to coumarin, estragole, and betulin.

Keywords: Lipid profile**;** Essential oils; *Gliricidia sepium* (Jacq.) Kunth. ex. Walp; Antifungal activity; Fluconazole-resistant *Candida albicans*

1. Introduction

Antimicrobial drug resistance (AMR) is a serious global health concern that affects both humans and animals. It is distinguished by the ability of microorganisms such as bacteria, viruses, fungi, and parasites to withstand the effects of medications that were previously effective against them**[1]**. In 2019, Egypt faced a severe burden from antimicrobial resistance (AMR), with 16,100 fatalities directly attributable to the disease and 56,600 deaths linked with it. Out of 204 nations, Egypt has the 58th highest age-standardized death rate per 100,000 population due to AMR. Among 21 nations in North Africa and the Middle East, Egypt has the second-highest age-standardized AMR mortality rate**[2]**. The World Health Organization (WHO) has recognized the serious threat posed by AMR. If no action is taken, AMR might become the world's leading cause of death by 2050, with yearly expected deaths to increase from over 1.2 million in 2019 to about 10 million**[3]**. World Bank Report predicted that AMR in animals might lead to a 3-8% decline in cattle production by 2050, which would seriously impair global food security**[4]**.

Antimicrobial resistance develops as a result of a variety of circumstances, including abuse and misuse in human medicine, agriculture, and veterinary medicine, poor infection control strategies in humans and animals, global travel, and genetic factors related to the spread and development of resistant pathogen**[5]**. Methicillin-resistant *Staphylococcus aureus* (MRSA) and triazole-resistant *Candida albicans* are major examples of AMR affecting both humans and animals.

MRSA is a *staphylococcus aureus* bacterial strain that is resistant to methicillin and other beta-lactam antibiotics. It causes infections such as pneumonia, bloodstream infections, and surgical site infections in humans and animals. It spreads throughout hospital systems and the population[6]. According to WHO and the Centers for Disease Control and Prevention (CDC), MRSA

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is a significant and serious threat on the pathogenic bacteria target list, respectively. MRSA alone was responsible for more than 100,000 fatalities in 2019, according to a recent detailed study published in the Lancet**[6]**.

Fluconazole is an antifungal medicine used to treat fungal infections, notably those caused by the *Candida* species. It works by inhibiting the production of ergosterol, a necessary component of fungal cell membranes, which disrupts their integrity and causes cell death**[7]**. *Candida* is a type of fungi that is a major concern in both immunocompetent and immunocompromised people because it can cause a variety of infections, ranging from superficial mucosal infections like oral thrush and vaginal candidiasis to more serious systemic infections like candidemia and invasive candidiasis**[8,9]**. The emergence of fluconazole-resistant *Candida* strains is a significant challenge, limiting treatment options and increasing morbidity and mortality. Prior antifungal exposure, genetic alterations, and biofilm formation all contribute to fungus resistance to both the host's immune response and antifungal therapy**[10]**.

Triazole-resistant *Candida albicans* is a fungal pathogen that has evolved resistance to popular antifungals like fluconazole**[11]**. It can cause systemic candidiasis in the mouth and genital organs and candidemia in the bloodstream of humans and animals**[12]**. Candidemia is a bloodstream infection affecting hospitals and communities. It is the fourth leading cause of nosocomial bloodstream infections in the United States and seventh in Europe. The overall mortality rate for candidemia ranges between 22% and 75%**[13]**.

It is important to find alternative antifungals, particularly in the context of increasing resistance to pathogens like MRSA and triazole-resistant *Candida albicans*. Plant-based treatments, particularly essential oils obtained from diverse plants, represent intriguing options. Essential oils have antifungal characteristics and may be helpful against resistant *Candida albicans* strains and other infections.

Essential oils are known for their anti-bacterial, anti-fungal, anti-viral, and antiparasitic which means their broad spectrum activity**[14]**. The anti-microbial activity of essential oils is related to the diversity of their chemical composition, especially sterols, triterpenoids, hydrocarbons, saturated and unsaturated fatty acids**[15]**. These compounds exhibit their antimicrobial activity by disrupting microbial cell membranes, inhibiting enzyme activity, and interfering with nutrient uptake which affects the growth and survival of microorganisms**[16]**.

Many plants are known for their lipid contents, essential oils, and their antifungal activities**[17,18]** *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. is one of these plants. *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. is multipurpose with a variety of uses. These include providing wood fuel, animal feed, green manure, and plant support. Additionally, it is used in traditional medicine for various applications such as antibacterial, antifungal, wound healing, and insect repellent**[19]**. It has been reported that the leaves and flowers of this plant are rich in lipid content and essential oils with antifungal activity**[20]**.

2. Materials and methods

2.1. Plant material and collections

Leaves and flowers of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. were obtained from the Mazhar Botanical Garden in July 2021. The plant was identified by Therease Labib, a plant taxonomy consultant at El-Orman Botanical Garden in Giza, Egypt. A specific sample of the leaves and flowers was preserved in the herbarium of the Department of Pharmacognosy at Cairo University's Faculty of Pharmacy, with the voucher specimen number 15-8-2021. These leaves and flowers underwent air-drying, were crushed into coarse powder, and then carefully stored in amber-colored glass containers to shield them from light exposure. Storage took place at room temperature. The study strictly followed the occupational health and safety guidelines set by the Scientific Research and Ethics Committee of the Faculty of Pharmacy, Cairo University, Giza, Egypt**[21]**.

2.2. Preparation of the plant extract samples for lipid content analysis

Fifty grams of fresh leaves and flowers of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. were immersed in 70% ethanol for constituent extraction each separately. They were then blended with the solvent and the ethanol was removed under vacuum conditions, maintaining a temperature below 50°C**[22]**. With some modification for lipid content analysis the resulting dry extracts were subjected to fractionation with hexane, mixed with the solvent, and the hexane was evaporated under vacuum conditions, keeping the temperature below 50°C. The resulting dry extracts were stored at -20°C for further analysis**[23]**.

2.3. Preparation of essential oils for in-vitro antimicrobial analysis

Fresh *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers, weighing 1 kg each, were mixed with 5 L of 95% ethanol. After blending, each filtrate received 0.5 L of water. The resultant solutions appeared turbid, so they were partitioned with petroleum ether (40-60) using the procedure indicated in**[24]**. The petroleum ether fractions were then treated with activated charcoal to remove chlorophyll before being evaporated under vacuum at 30°C using a rotary evaporator. This technique produced 1 ml and 1.5 ml of essential oils from *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers, respectively.

2.4. Fatty acid extraction (saponified portion extraction)

Folch method is the most well-known fatty acid extraction method proposed by Jordi Folch and the most reliable method for the extraction of lipids**[25]**. A mixture of chloroform and methanol at a ratio of 2:1 (v/v) was used as the extraction solvent. After that, water or a salt solution was added to cause the phase separation. The lower phase was used in analyzing fatty acids**[26]**. This protocol can be used to extract lipids from common lipid-rich foods such as avocados, eggs, and mayonnaise**[27–30]**.

2.3. Fatty acid methylation

The sulfuric acid derivatization method has also been widely used for the analysis of fatty acids in biological samples. Sulfuric acid -methanol 2% (v/v) was added into a vial containing previously weighed 10 mg of fat. The vial was heated at 80 °C with occasional shaking. Afterward, 0.25 ml of the neutralized aqueous solution (sodium hydroxide 1 M) was added, and it was smoothly shaken. Before the analysis of the n-hexane phase, the sample was allowed to rest for 5 min. The reaction procedure is similar to that of other derivatization methods because sulfuric acid is a strong oxidizing agent**[31,32]**.

2.4. Preparation of the unsaponifiable matter

Accurately weighing 5.0 gm of the leaves and flowers was individually saponified by refluxing with 50 ml of 30% alcoholic potassium hydroxide for 3 hours followed by distillation of the alcohol under reduced pressure and dilution with 100 ml distilled water. The aqueous solution was extracted with diethyl ether (100 ml) in a separating funnel several times till complete exhaustion and then, washed several times with distilled water till completely free of alkalinity, anhydrous sodium sulfate was used for dehydration. The extract was concentrated under reduced pressure. the unsaponifiable matter was kept in sealed containers for further investigation**[33,34**].

2.5. Gas chromatography-mass spectrometry (GC-MS) analysis of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers lipid contents

The chemical composition of your samples was performed using a Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was initially held at 50 °C and then increased by 5°C /min to 230°C held for 2 min. increased to the final temperature of 290°C by 30°C /min and hold for 2 min. The injector and MS transfer line temperatures were kept at 250°C, and 260°C respectively; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 3 min and diluted samples of 1 µl were injected automatically using Autosampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 40–1000 in full scan mode. The ion source temperature was set at 200 °C. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 14 mass spectral databases**[35]**.

2.6. Headspace solid-phase microextraction (HS-SPME) for *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers essential oils analysis

For HS-SPME extraction, 1 g of ground dried leaves and flowers were put in a 20 ml headspace vial closed with a magnetic crimp cap with septum (Gerstel, Mülheim a/d Ruhr, Germany). Volatiles were directly sampled using headspace SPME for 60 min at 30°C with a 50/30 µm DVB/CAR/PDMS divinylbenzene/carboxen/polydimethylsiloxane) fiber (Supelco, Bornem, Belgium) and desorbed for 2 minutes at 250°C in the GC-MS inlet. SPME extraction and desorption were performed automatically utilizing a Multipurpose Sampler (MPS-2, Gerstel)**[36]**.

2.7. Gas chromatography-mass spectrometry (GC – MS) analysis of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers essential oils prepared by headspace solid-phase microextraction

Analysis of the samples was conducted by using gas chromatography (Agilent 8890 GC System), coupled to a mass spectrometer (Agilent 5977B GC/MSD) and equipped with HP-5MS fused silica capillary column (30 m, 0.25 mm i.d., 0.25 mm film thickness). The oven temperature was maintained initially at 50 °C, then programmed from 50 to 220°C at a rate of 5°C/min and from 220°C to 280°C at a rate of 10°C/min. Helium was used as the carrier gas, at a flow rate of 1 ml/ min. The temperature of the injection was 250 °C. Mass spectra in the electron impact mode (EI) were obtained at 70 eV and scan m/z range from 39 to 500 amu. The isolated peaks were identified by matching them with data from the library of mass spectra (National Institute of Standards and Technology, NIST)**[36]**.

2.8. *In-vitro* antimicrobial activity of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers essential oils

The antimicrobial activity of the *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers essential oils were examined against gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, and *Bacillus subtilis* ATCC 6633), gram-negative bacteria (*Escherichia coli* ATCC 8739 and *Salmonella typhi* ATCC 19430), and fungi (*Aspergillus niger* NRRL-A326 and fluconazole-resistant *Candida albicans* ATCC 10231) via the application of well diffusion method**[37]**. For the bacterial strains, 200 µL of each nutrient broth cell suspension (corresponding to 0.5 McFarland standard solution) was uniformly spread over nutrient agar plates. After that, wells of 7 mm diameter were formed in the agar plates in which 100 µL of each sample dissolved in dimethyl sulfoxide (DMSO) was placed in the wells and then incubated at 37 °C for 24 h. For the antifungal activity, 200 µL of *Aspergillus niger* and *Candida albicans* sabouraud dextrose broth suspension (corresponded to McFarland standard solution) was uniformly spread over sabouraud dextrose agar plates and after processing as previously, the plates were incubated for 24h at 30°C. The experiment was carried out in triplets in which the average diameter of the inhibition zone in millimeters was represented. In addition, the minimal inhibitory concentration (MIC), the least growth-preventable concentration, of the positive sample was estimated via the examination of different concentrations in separated wells**[9,38]**.

2.9. Chemicals and reagents

70% ethanol (Sigma-Aldrich, USA), hexane (Thermo Fisher Scientific, USA), potassium hydroxide (KOH) (Merck, Germany), diethyl ether (Acros Organics, USA), 95% ethanol (Sigma-Aldrich, USA), petroleum ether (Fisher Scientific, UK), methanol (BDH Chemicals, UK), sulfuric acid (Merck, Germany), sodium hydroxide (NaOH) (Alfa Aesar, USA), divinyl benzene/carboxen/poly dimethyl siloxane (DVB/CAR/PDMS) fiber (Supelco, USA), dimethyl sulfoxide (DMSO) (Sigma-

Aldrich, USA), nutrient broth (Oxoid, UK), sabouraud dextrose broth (Merck, Germany), and sabouraud dextrose agar plates (BD Biosciences, USA).

2.10. Microbial strains

Staphylococcus aureus (gram-positive) was obtained from the American Type Culture Collection (ATCC 6538), originally isolated from a clinical sample. The methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591), also gram-positive, was sourced from ATCC as a reference strain known for its resistance to β-lactam antibiotics. Additionally, *Bacillus subtilis* (gram-positive, ATCC 6633) was acquired from the American Type Culture Collection (ATCC), where it was isolated from soil samples. The gram-negative bacteria *Escherichia coli* (ATCC 8739) and *Salmonella typhi* (ATCC 19430) were sourced from ATCC, with *Escherichia coli* being a common intestinal pathogen and *Salmonella typhi* known for causing typhoid fever. Furthermore, fluconazole-resistant *Candida albicans* (ATCC 10231) was isolated from a clinical patient sample, highlighting its significance in opportunistic infections, while *Aspergillus niger* (NRRL-A326), a fungus commonly found in decaying vegetation and soil, was sourced from the Northern Regional Research Laboratory (NRRL).

2.11. Statistical analysis

The data analysis was done using GraphPad Prism software (version 8.00), with statistical significance set at a p-value of less than 0.05. A one-way analysis of variance (ANOVA). Following the ANOVA, we conducted a Tukey post-hoc analysis to provide more detailed results. The data was evaluated through determining several metrics such as percentages, numerical counts, means, and standard deviations (SD).

3. Results

3.1. Identification of lipid contents of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers by GC-MS analysis

The lipid analysis of the fatty acids in the leaves (Table 1& figs. 1& 3) and flowers (Table 1 & figs. 2&3) of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. revealed distinct profiles with significant variations. In the leaves, the total identified compounds constituted 99.99%, with unsaturated fatty acids accounting for 72.17% and saturated fatty acids making up 27.82%. The predominant unsaturated fatty acid in the leaves was arachidonic acid (39.56%).

In contrast, the flowers exhibited a markedly different composition, with the total identified compounds at 99.96%, and a higher proportion of unsaturated fatty acids (99.6%) compared to saturated fatty acids (0.36%). Arachidonic acid dominated the flowers lipid profile, comprising 83.66% of the total fatty acids. Saturated fatty acids were present in minor quantities in both leaves and flowers.

Table 1: Fatty acid fractions of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers.

	Rt (min)	Compound name	Fatty acid type	Molecular Formula	Leaves %	Flowers %
1.	2.875	Enanthic acid	Sat	$C_7H_{14}O_2$	1.69	0.02
2.	3.161	Butyric acid	Sat	$C_4H_8O_2$	2.25	0.03
3.	4.607	Caproic acid	Sat	$C_6H_{12}O_2$	1.76	0.01
4.	5.108	Caprylic acid	Sat	$C_8H_{16}O_2$	2.36	0.01
5.	5.601	Capric acid	Sat	$C_{10}H_{20}O_2$	1.37	0.02
6.	6.714	Undecanoic acid	Sat	$C_{11}H_{22}O_2$	1.62	0.01
7.	7.042	Lauric acid	Sat	$C_{12}H_{24}O_2$	1.87	0.01
8.	7.998	Tridecanoic acid	Sat	$C_{13}H_{26}O_2$	1.51	0.01
9.	9.696	Myristic acid	Sat	$C_{14}H_{28}O_2$	1.69	0.01
10.	9.815	Myristoleic acid	Unsat	$C_{14}H_{26}O_2$	2.32	0.03
11.	9.974	Pentadecanoic acid	Sat	$C_{15}H_{30}O_2$	2.11	0.02
12.	11.076	Cis-10-pentadecenoic acid	Unsat	$C_{15}H_{28}O_2$	2.82	0.01
13.	11.289	Palmitic acid	Sat	$C_{16}H_{32}O_2$	2.08	0.01
14.	11.624	Palmitoleic acid	Unsat	$C_{16}H_{30}O_2$	1.62	0.01
15.	12.338	Heptadecanoic acid	Sat	$C_{17}H_{34}O_2$	1.94	0.01
16.	13.303	Cis-10- heptadecenoic acid	Unsat	$C_{17}H_{32}O_2$	1.41	0.02
17.	13.591	Stearic acid	Sat	$C_{18}H_{36}O_2$	1.27	0.15
18.	14.213	Oleic acid	Unsat	$C_{18}H_{34}O_2$	1.94	0.01
19.	14.654	Elaidic acid	Unsat	$C_{18}H_{34}O_2$	2.25	0.02
20.	14.849	Linoleic acid	Unsat	$C_{18}H_{32}O_2$	2.99	0.01
21.	14.961	Linolelaidic acid	Unsat	$C_{18}H_{32}O_2$	1.27	0.02

22.	15.776	Linolenic acid	Unsat	$C_{18}H_{30}O_2$	2.57	0.01
23.	15.899	γ -linolenic acid	Unsat	$C_{18}H_{30}O_2$	1.23	0.01
24.	16.085	Arachidic acid	Sat	$C_{20}H_{40}O_2$	1.37	0.01
25.	16.221	Cis-11-eicosenoic acid	Unsat	$C_{20}H_{38}O_2$	1.59	0.77
26.	16.474	Cis-11,14-eicosadienoic acid	Unsat	$C_{21}H_{38}O_2$	1.76	0.01
27.	16.482	Cis-11,14,17-eicosatrienoic acid	Unsat	$C_{20}H_{34}O_2$	1.41	0.01
28.	16.586	Cis-8,11,14-eicosatrienoic acid	Unsat	$C_{21}H_{36}O_2$	1.30	0.01
29.	16.992	Arachidonic acid	Unsat	$C_{20}H_{32}O_2$	39.56	83.66
30.	17.036	Cis-5,8,11,14,17-eicosapentaenoic acid	Unsat	$C_{21}H_{32}O_2$	1.87	14.79
31.	17.375	Heneicosanoic acid	Sat	$C_{21}H_{42}O_2$	1.59	0.01
32.	19.066	Behenic acid	Sat	$C_{22}H_{44}O_2$	1.34	0.02
33.	19.302	Erucic acid	Unsat	$C_{22}H_{42}O_2$	1.41	0.01
34.	19.504	Cis-13,16-docosadienoic acid	Unsat	$C_{22}H_{40}O_2$	1.37	0.01
35.	19.774	Cis-4,7,10,13,16,19-hexaenoic acid	Unsat	$C_{22}H_{32}O_2$	1.48	0.18
		% Total identified compounds			99.99	99.96
		% Identified unsaturated fatty acids			72.17	99.6
		% Identified saturated fatty acids			27.82	0.36

*sat: saturated *unsat: unsaturated * Rt: retention time.

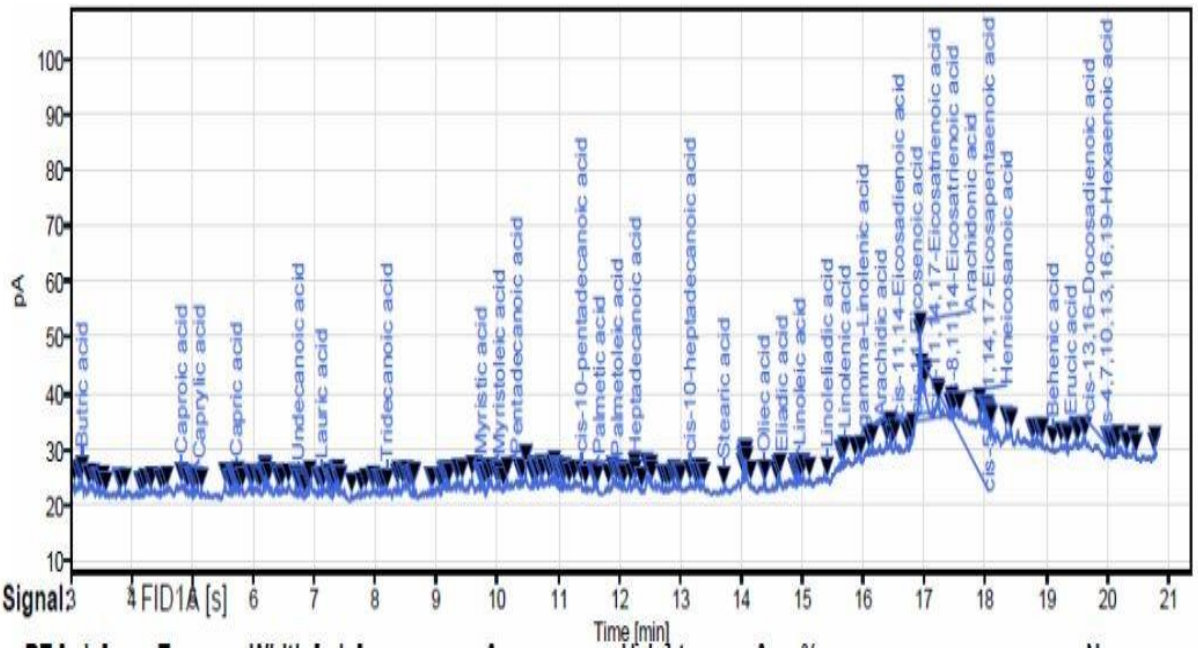

Fig. 1. GC-MS total ion chromatogram for a fatty acid fraction of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves.

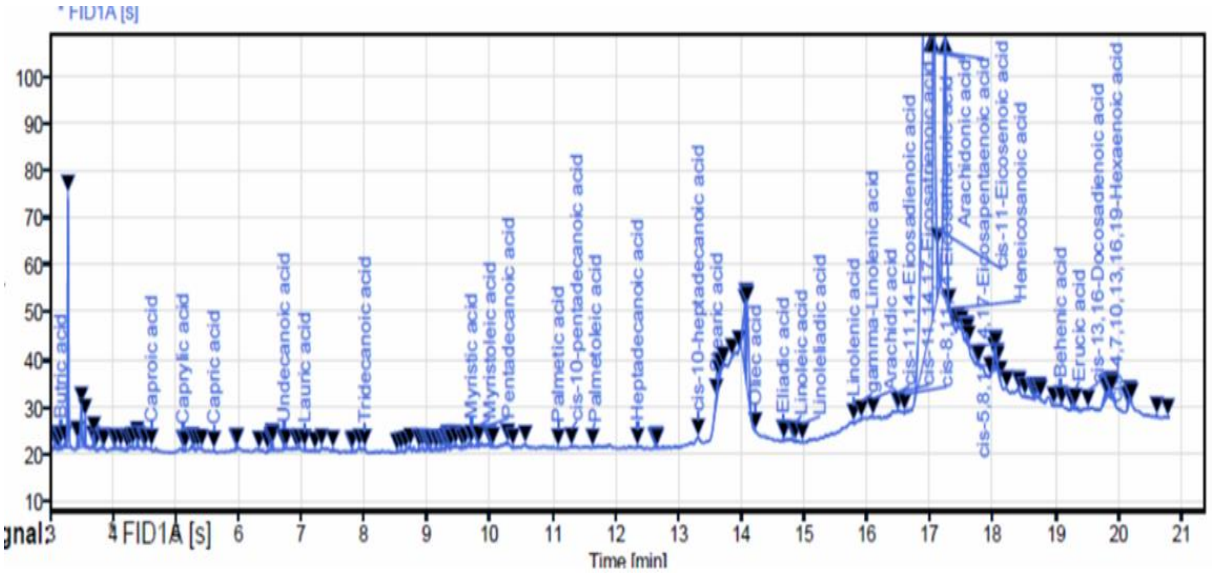

Fig. 2. GC-MS total ion chromatogram for a fatty acid fraction of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. flowers.

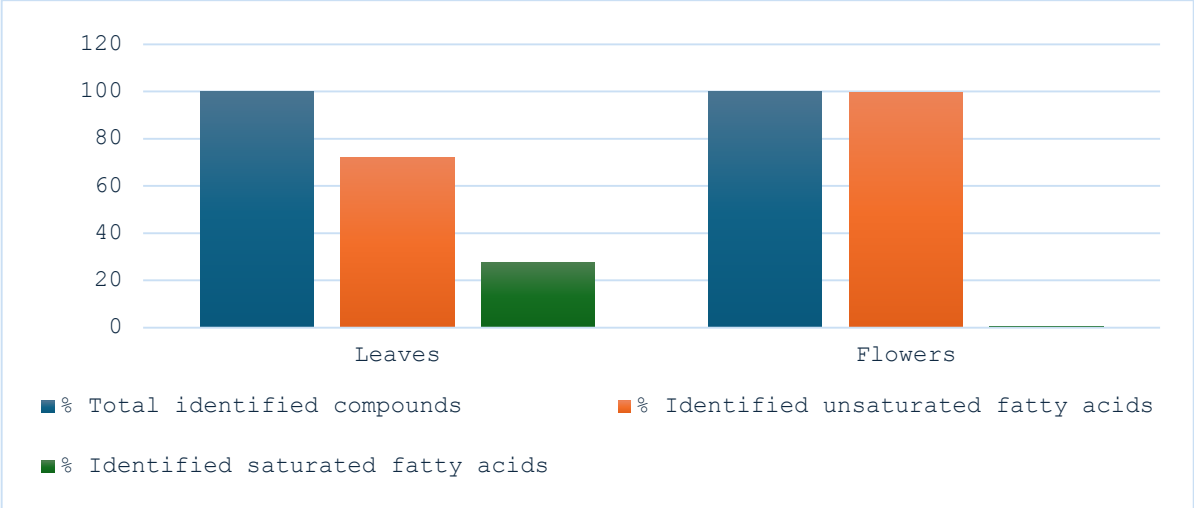

Fig. 3. A chart represents the percentages of the fatty acids in the leaves and flowers of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp.

The analysis of the unsaponifiable matter in the leaves (Table 2 & figs. 4&6) and flowers (Table 2 & figs. 5&6) of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. reveals a diverse composition of hydrocarbons, sterols, and triterpenoids. In the leaves exhibited a total identified compounds percentage of 99.87%, with a higher proportion of hydrocarbons at 68.26%, compared to 2.38% for sterols and 29.23% for triterpenoids. In contrast, in the flowers, the total identified compounds accounted for 99.98%, with hydrocarbons constituting 42.07%, sterols 2.38%, and triterpenoids 55.53%. Betulin was the most abundant triterpenoid in both leaves and flowers, with 29.23% and 44.39% respectively.

Table 2: The unsaponifiable matter of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers.

	Rt (min)	Compound name	Molecular formula	Leaves %	Flowers %
1.	4.49	Dodecane	$C_{12}H_{26}$	2.47	3.50
2.	7.65	Tetradecane	$C_{14}H_{30}$	3.50	5.41

3.	7.81	Hexadecane	$C_{16}H_{34}$	3.50	0.95
4.	14.26	Nonadecane	$C_{19}H_{40}$	5.97	4.22
5.	15.20	Eicosane	$C_{20}H_{40}$	2.14	2.86
6.	16.58	Heneicosane	$C_{21}H_{44}$	31.80	11.38
7.	16.85	Docosane	$C_{22}H_{46}$	2.14	3.18
8.	19.08	Tricosane	$C_{23}H_{48}$	2.38	3.26
9.	20.01	Tetracosane	$C_{24}H_{50}$	1.44	0.79
10.	21.40	Pentacosane	$C_{25}H_{52}$	11.48	5.09
11.	21.82	Dotriacontane	$C_{32}H_{66}$	1.44	1.43
12.	31.17	β -Sitosterol	$C_{29}H_{50}O$	-	1.51
13.	31.56	β-Amyrin	$C_{30}H_{50}O$	-	9.39
14.	31.90	Lupeol	$C_{30}H_{50}O$	-	1.75
215.	32.33	3 β-methoxy Stigmast-5-ene	$C_{30}H_{52}O$	2.38	0.87
16.	32.48	Betulin	$C_{30}H_{50}O_2$	29.23	44.39
		% Total identified compounds		99.87	99.98
		% Identified hydrocarbons		68.26	42.07
		% Identified triterpenoids		29.23	55.53
		% Identified sterols		2.38	2.38

*Rt: retention time.

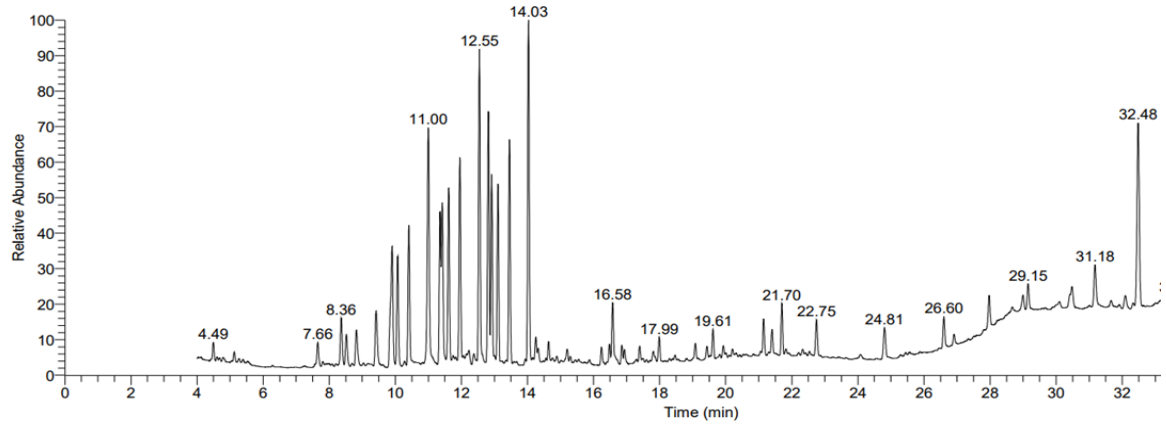

Fig. 4. GC-MS total ion chromatogram for an unsaponifiable matter of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves.

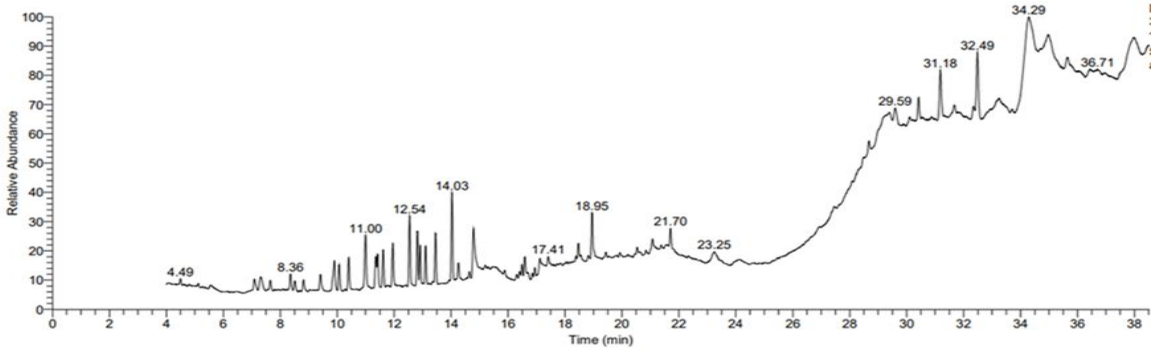

Fig. 5. GC-MS total ion chromatogram for an unsaponifiable matter of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. flowers.

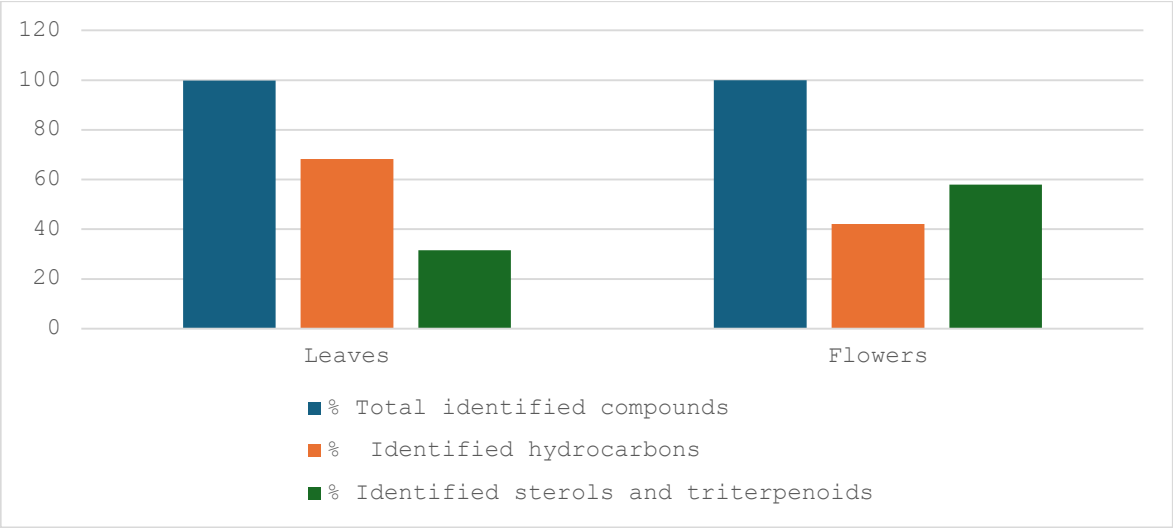

Fig. 6. A chart represents the percentages of the unsaponifiable matter in the leaves and flowers of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp.

3.2. Gas chromatography-mass spectrometry (GC – MS) analysis of Gliricidia sepium (Jacq.) Kunth. ex. Walp. flowers essential oil by headspace solid-phase microextraction

The GC-MS analysis of essential oils extracted from *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. flowers after being subjected to headspace solid phase microextraction (Table 3 &figs. 7&8) (SUPP 1-16) identified a complex mixture of compounds, totaling 99.93% of the sample composition. The identified compounds are categorized into hydrocarbons and oxygenated hydrocarbons, with the hydrocarbons constituting 48.67% and the oxygenated hydrocarbons making up 51.26% of the total identified compounds. Prominent hydrocarbons include d-limonene (18.05%), *β*-pinene (13.77%), and *α*-pinene (5.16%), indicating a significant presence of monoterpenes. Oxygenated hydrocarbons, such as eucalyptol (24.09%) and carvone (15.41%)dominate the composition, suggesting a rich profile of oxygen-containing terpenes and other functional groups. Notably, eucalyptol is the major compound essential oil.

Table 3: The composition of essential oil in *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. flowers.

	Rt(min)	Compound name	Area %	Molecular formula
1.	4.586	2-trans-Hexenal	1.86	$C_6H_{10}O$
2.	6.348	*α*-Pinene	5.16	$C_{10}H_{16}$
3.	6.714	Camphene	0.91	$C_{10}H_{16}$
4.	7.309	Sabinene	2.58	$C_{10}H_{16}$
5.	7.406	*β*-Pinene	13.77	$C_{10}H_{16}$
6.	7.727	*β*-Myrcene	2.02	$C_{10}H_{16}$
7.	8.751	D-Limonene	18.05	$C_{10}H_{16}$
8.	8.837	Eucalyptol	24.09	$C_{10}H_{18}O$
9.	9.569	*γ*-Terpinene	3.35	$C_{10}H_{16}$
10.	10.393	Terpinolene	1.23	$C_{10}H_{16}$
11.	12.545	Rhodinal	1.18	$C_{10}H_{18}O$
12.	14.759	Carvone	15.41	$C_{10}H_{14}O$
13.	19.463	*β*-Caryophyllene	0.8	$C_{15}H_{24}$
14.	20.985	*β*-Copaene	0.8	$C_{15}H_{24}$
15.	21.540	2,4-Di-tert-butyl phenol	5.74	$C_{14}H_{22}O$
16.	31.061	n-Hexadecanoic acid	2.98	$C_{16}H_{32}O_2$
		% Total identified compounds		99.93

% Identified hydrocarbons	48.67
% Identified oxygenated hydrocarbons	51.26

*Rt: retention time.

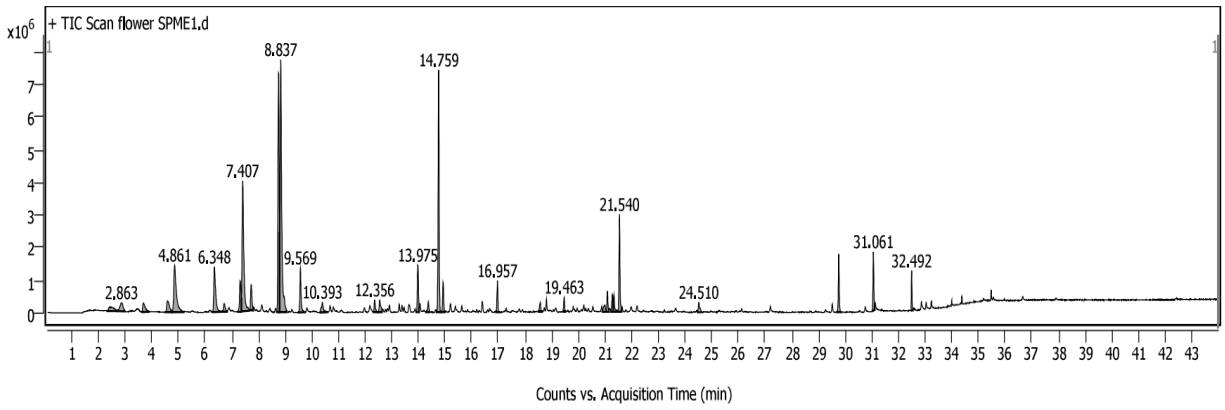

Fig. 7. GC-MS total ion chromatogram for essential oil in *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. flowers.

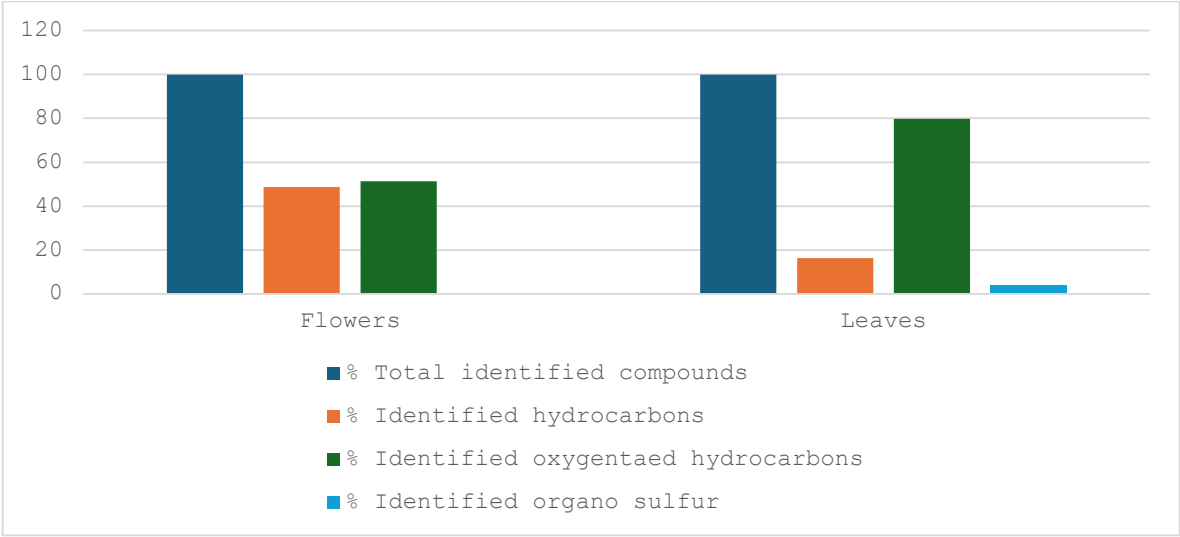

Fig. 8. A chart represents the percentages of the essential oils of leaves and flowers of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp.

3.3. Gas chromatography-mass spectrometry (GC – MS) analysis of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves essential oil by headspace solid-phase microextraction

The GC-MS analysis of the *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves essential oil following exposure to headspace solid-phase microextraction (Table 4 &figs. 8&9) (SUPP 17-34) revealed a complex mixture of compounds with a total of 99.92% of the sample composition. The identified compounds have been classified into three categories: hydrocarbons, oxygenated hydrocarbons, and organosulfur compounds. Hydrocarbons constitute 16.29% of the total identified compounds, while oxygenated hydrocarbons make up the majority at 79.74%. Organosulfur compounds represent 3.89% of the total identified compounds. Coumarin was the most abundant component, making up 33.52% of the oil. Other significant major compounds included estragole (15.48%), followed by d-limonene (7.63%), 2,4-di-tert-butylphenol (6.51%), and cuminaldehyde(5.23%). The essential oil also contains minor organosulfur compounds like diallyl disulfide (2.42%) and diallyl trisulfide (1.47%).

Table 4: The composition of essential oil in Gliricidia sepium (Jacq.) Kunth. ex. Walp. leaves.

	Rt(min)	Compound name	Area %	Molecular formula
1.	4.524	2-trans-Hexenal	2.48	$C_6H_{10}O$
2.	7.651	*β*-Myrcene	2.11	$C_{10}H_{16}$
3.	8.573	m-Cymene	2.84	$C_{10}H_{14}$
4.	8.686	D-Limonene	7.63	$C_{10}H_{16}$
5.	9.411	Dodecane	1.38	$C_{12}H_{26}$
6.	9.5	*γ*-Terpinene	2.33	$C_{10}H_{16}$
7.	9.833	Trans-3,trans-5-Octadien-2-one	1.39	$C_8H_{12}O$
8.	10.077	Diallyl disulfide	2.42	$C_6H_{10}S_2$
9.	12.194	Isomenthone	2.87	$C_{10}H_{18}O$
10.	13.449	Estragole	15.48	$C_{10}H_{12}O$
11.	14.596	Cuminaldehyde	5.23	$C_{10}H_{12}O$
12.	14.709	(-)-Carvone	3.87	$C_{10}H_{14}O$
13.	15.821	Anethole	2.44	$C_{10}H_{12}O$
14.	16.22	diallyl trisulfide	1.47	$C_6H_{10}S_3$
15.	17.563	(E)-Methyl cinnamate	4.23	$C_{10}H_{10}O_2$
16.	19.966	Coumarin	33.52	$C_9H_6O_2$
17.	21.41	2,4-Di-tert-butyl phenol	6.51	$C_{14}H_{22}O$
18.	31.043	n-Hexadecanoic acid	1.72	$C_{16}H_{32}O_2$
	% Total identified compounds		99.92	
	%Identified hydrocarbons		16.29	
	% Identified oxygenated hydrocarbons		79.74	
	% Identified organ sulfur		3.89	

*Rt: retention time.

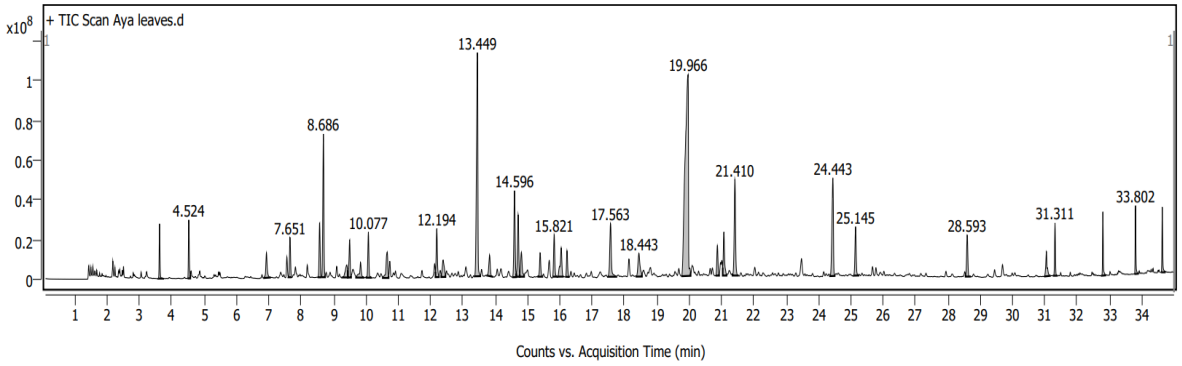

Fig. 9. GC-MS total ion chromatogram for essential oil in *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves.

3.4. *In-vitro* antimicrobial activity of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers essential oils

The essential oils extracted from the leaves and flowers of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. exhibited no antibacterial activity against the tested gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, and *Bacillus subtilis* ATCC 6633), as well as the gram-negative bacterium *Escherichia coli* ATCC 8739. Furthermore, both the leaves and flowers essential oils showed no antifungal activity against *Aspergillus niger* NRRL-A326. Additionally, the flowers essential oil exhibited no antibacterial activity against the tested gram-

negative bacterium *Salmonella typhi* ATCC 19430, while the leaves essential oil showed weak antibacterial activity against *Salmonella typhi* ATCC 19430 (fig.10), with an inhibition zone of 11±0.2 mm. The results are represented in Table 5.
However, the essential oil from the leaves demonstrated antifungal activity against the fluconazole-resistant *Candida albicans* ATCC 10231 strain(Table 5 &fig.10), with a minimum inhibitory concentration (MIC) of 3.125 mg/ml. In contrast, the essential oil from the flowers exhibited no antifungal action against the fluconazole-resistant *Candida albicans* ATCC 10231 strain(Table 5&fig.10).

The antifungal potential of the leaves essential oil was further evaluated at different concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 mg/ml yielding inhibition zones (mm) of 11±0.2, 12±0.2, 13±0.1, 14±0.1, 15±0.1, and 16±0.1, respectively. These results are summarized in Table 6 as mean ± S.D and figs.10&11.

Table 5: Zones of inhibition (mm) for *in-vitro* antimicrobial activity of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers essential oils against different test microbes representing gram-positive bacteria (*Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*, and *Bacillus subtilis*), gram-negative bacteria (*Escherichia coli* and *Salmonella typhi*), and fungi (fluconazole-resistant *Candida albicans* and *Aspergillus niger*).

Test microbes	Zones of inhibition (mm)	
	Samples	
	Leaves	Flowers
Staphylococcus aureus (gram +ve)	0±0	0±0
Methicillin-resistant *Staphylococcus aureus* (gram +ve)	0±0	0±0
Bacillus subtilis (gram +ve)	0±0	0±0
Escherichia coli (gram -ve)	0±0	0±0
Salmonella typhi (gram -ve)	11±0.2	0±0
fluconazole-resistant *Candida albicans* (fungi)	13.5±0.1	0±0
Aspergillus niger (fungi)	0±0	0±0

The results are represented in the form of mean ± S.D., *gram +ve: gram-positive bacteria, *gram -ve: gram-negative bacteria.

Table 6: Zones of Inhibition (mm) observed in the antifungal activity of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves against fluconazole-resistant *Candida albicans* ATCC 10231 with minimum inhibitory concentration at 3.125 mg/ml.

Test microbe	Fluconazole-resistant *Candida albicans*						
Concentrations (mg/ml)	3.125 mg/ml	6.25 mg/ml	12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml	Control is fluconazole(5 mg/ml)
Zones of inhibition (mm)	11 ±0.2	12±0.2	13±0.1	14±0.1	15±0.1	16±0.1	0±0
P values	*	*	*	**	**	**	

The results are represented in the form of mean ± S.D. Statistical analysis was performed using the one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical Significance: *p ≤ 0.05 and **p ≤ 0.01 compared to control fluconazole (5 mg/ml).

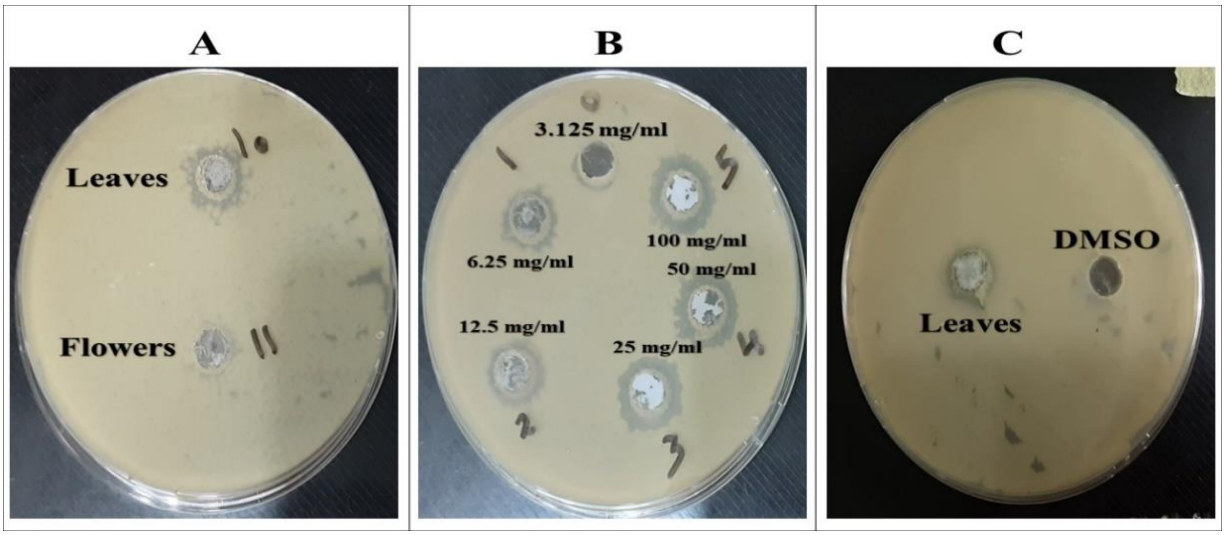

Fig. 10. (A): The antifungal activity of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers essential oils against fluconazole-resistant *Candida albicans* ATCC 10231, (B): antifungal activity of different concentrations of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves essential oil against fluconazole-resistant *Candida albicans* ATCC 10231 (3.125,6.25, 12.5, 25, 50, and 100 mg/ml), and (C): the weak antibacterial activity of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves against *Salmonella typhi* ATCC 19430. DMSO: Dimethyl sulfoxide solvent which the sample is dissolved in it.

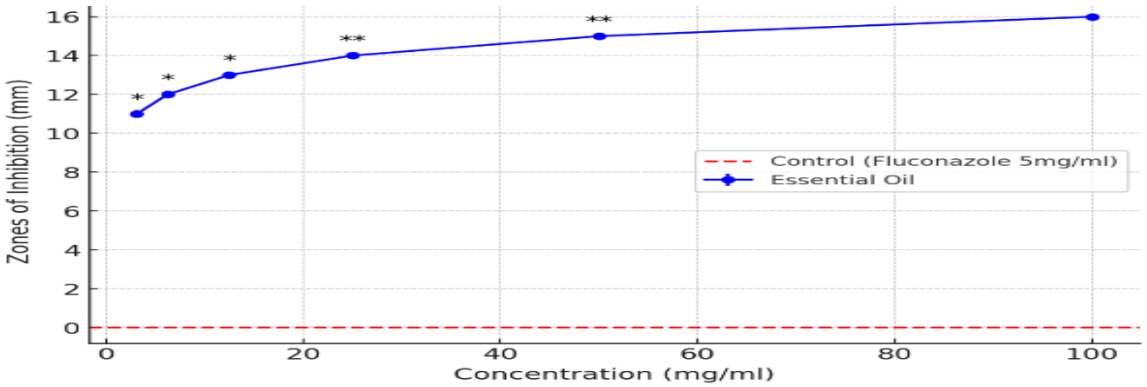

Fig. 11. A graph for the zones of Inhibition (mm) observed in the antifungal activity of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves against fluconazole-resistant *Candida albicans* ATCC 10231 at various concentrations (3.125,6.25, 12.5, 25, 50, and 100 mg/ml) with minimum inhibitory concentration at 3.125 mg/ml. Statistical analysis was performed using the one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. P values for all concentrations are * $p \leq 0.05$ and ** $p \leq 0.01$ which are statistically significant from the control fluconazole (5mg/ml).

4. Discussion

Antimicrobial resistance is becoming a major concern in the treatment of many illnesses, with triazole-resistant *Candida albicans* emerging as a particularly difficult issue**[39]**. Triazole antifungals, which are routinely used to treat *Candida* infections, have decreased efficacy due to resistance mechanisms within the pathogen, complicating therapy and leading to more severe and persistent infections[**40]**. As resistance rates rise, it becomes critical to investigate alternate treatment options. One intriguing avenue is the use of plant-derived compounds, particularly essential oils, which have shown strong antifungal activity**[41]**. Essential oils derived from different plants contain bioactive chemicals that can suppress fungal development and alter the pathogen's cellular activities. Research into these natural alternatives provides a viable way to reduce the impact of resistant strains and improve treatment outcomes**[9,42]**.

In the current study, the GC-MS study of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers essential oils revealed diverse chemical profiles, with each oil dominated by a different component. The prominent compounds in the flowers essential oil are eucalyptol and d-limonene, indicating a high concentration of oxygenated terpenes and monoterpenes. In contrast, in the leaves essential oil, coumarin and estragole are the major components. Hexanal, *γ*-terpinene, limonene, and hexadecanoic acid are previously reported in the essential oil of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves**[43,44]**.

Coumarin has considerable antifungal action, especially against *Candida albicans*. It limits cell growth and strain viability by causing apoptosis, which is characterized by phosphatidylserine externalization, DNA fragmentation, cytochrome C release, and metacaspase activation. Coumarin causes reactive oxygen species (ROS) buildup, mitochondrial malfunction, and increased cytosolic and mitochondrial calcium levels, all of which contribute to apoptosis. Notably, ROS removal did not inhibit apoptosis, implying that coumarin-induced apoptosis is caused by mitochondrial calcium rather than oxidative stress. Furthermore, coumarin has been demonstrated to be efficacious in vivo, with a dose of 40 mg/kg improving survival in *Candida*-infected mice without considerable toxicity, indicating its promise as a therapeutic agent against fungal infections**[45**]. Estragole exhibits significant antifungal activity against *Aspergillus niger*. It effectively inhibits spore germination with a minimum inhibitory concentration of 0.5 µL/ml**[46]**.

In the current study, the lipid analysis of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers highlight notable differences in their fatty acid and unsaponifiable matter compositions. Arachidonic acid is the most abundant unsaturated fatty acid in both leaves and flowers. Furthermore, the unsaponifiable matter differs between the two plant parts: leaves have a higher proportion of hydrocarbons, while flowers feature a more diverse array of sterols and triterpenoids. Betulin is the predominant triterpenoid in both leaves and flowers.

Betulin belongs to the pentacyclic triterpene family and has a variety of biological functions, including antifungal characteristics. Betulin has been shown in studies to be particularly efficient against dermatophytes such as *Fusarium solani*, *Microsporum canis*, *Aspergillus niger*, and *Candida albicans* **[47]**.

Many studies have been conducted recently to support the beneficial effects of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. on health[20]. Various studies have reported on the antibacterial, anti-fungal, and anti-microbial activities of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves essential oil**[48,49]**.

In the current study, the antibacterial activity of essential oils derived from *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers vary significantly. Both oils had no antibacterial action against the tested gram-positive and gram-negative bacteria, and no antifungal activity against the tested fungi. On the other hand, the leaves essential oil possesses weak antibacterial activity against *Salmonella typhi* and antifungal activity against fluconazole-resistant *Candida albicans* with minimum inhibitory concentrations of 3.125 mg/ml. It was noticed from measuring the zones of inhibition of the various concentrations that the antifungal activity is dose-dependent as the concentration increases the zone of inhibition increases.

We could conclude that the antifungal activity of the leaves essential oil are due to the major compounds identified by the GC-MS analysis, especially coumarin and estragole, which are known for their antifungal activity as mentioned before.

Additionally, the lipid content of the leaves may contribute to their antifungal activity, particularly due to the presence of betulin, a major triterpenoid identified in both the GC-MS analysis of the lipid content of the leaves, which also possesses antifungal properties as stated previously.

The antifungal activity of the *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves essential oil against fluconazole-resistant *Candida albicans* represents a significant advancement in healthcare. Identifying natural sources with efficacy against resistant fungal strains is crucial for developing alternative therapeutic strategies. The observed antifungal effectiveness underscores the potential of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves essential oil as a valuable natural resource in combating resistant fungal infections and highlights its role in the search for new, effective antifungal agents.

5. Conclusion

The GC-MS analysis of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. leaves and flowers reveal significant changes in lipid and essential oil composition. The flowers essential oil has significant levels of eucalyptol and d-limonene, whilst the leaves essential oil includes large amounts of coumarin and estragole. Betulin predominates in both the leaves and flowers. Notably, the essential oil from the leaves has weak antibacterial activity against *Salmonella typhi* and antifungal action against fluconazole-resistant *Candida albicans*, with coumarin and estragole most likely contributing to this impact. These findings suggest the therapeutic potential of *Gliricidia sepium* (Jacq.) Kunth. ex. Walp. essential oils, notably in the treatment of resistant fungal infections, and call for additional research into their mechanisms and broader applications in healthcare.

6. Funding

This study didn't receive any funding support.

7. Conflict of Interest

There are no conflicts to declare.

8. Author`s contribution

Aya A. Wafaey contributed to data curation; formal analysis; investigation; methodology; software; and roles in writing- original draft and writing- review and editing. **Seham S. El-Hawary** provided conceptualization; project administration; supervision; validation and

visualization. **Shaymaa A. Ismail** contributed to data curation, formal analysis, investigation; methodology; and software. **Sherif S. Mohamed** contributed to data curation and formal analysis**. Mohamed F. Abdelhameed** contributed to data curation; formal analysis; investigation; methodology; investigation; validation; visualization; software and writing- review and editing. **Farid N. Kirollos** contributed to validation; and visualization.

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