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GAS CHROMATOGRAPHY-MASS SPECTROSCOPY PROFILE AND ANTI-OXIDANT ACTIVITY OF *MORUS ALBA* L. LEAF EXTRACTS

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ABSTRACT

Mulberry, belonging to the family Moraceae, is a mainstay of sericulture and is also found to be rich in bioactive phytoconstituents. It is well acclaimed for its antioxidant nature, therapeutic properties, and ethno-botanical desirability. In recent years, there has been a growing interest in exploring natural sources of antioxidants due to their potential health benefits. By analyzing the phytochemical constituents of these leaves using GC-MS, this study aims to provide valuable insights into their antioxidant properties and their potential role in preventing oxidative stress-related diseases. Understanding the antioxidant effects of *Morus alba* L. leaves can contribute to the development of new therapeutic interventions and dietary recommendations for maintaining optimal health. Methanol, ethyl acetate and n-hexane extracts of dried leaves were prepared by Soxhlet extraction method and analyzed for bioactive phytochemicals using GC-MS technique. The GC-MS analysis detected the presence of sixteen bioactive phytoconstituents in the leaf extracts of *Morus alba* L. mainly, alcohols, phytosterols, organic acids, terpenes, long-chain hydrocarbons, esters which were confirmed by comparing their retention time, peak area, peak height and mass spectra with known compounds identified by NIST library. All the three extracts analyzed by GC-MS analysis were found to be rich in bioactive compounds such as phenolics, esters, organic acids, volatile oils, aldehydes, alcohols and terpenes known to possess potent antioxidant activity along with antidiabetic, antibacterial,

anticancer, cardiovascular, hypolipidemic, antiatherogenic and anti-inflammatory properties. The present study reveals that mulberry leaves are a promising source of phytochemicals with established biological effects, the most important being the antioxidant effect.

Keywords: GC-MS, antioxidant potential, *Morus alba* L., Sericulture, phytochemicals, therapeutic potential, drug development

INTRODUCTION

Herbal medicinal plants have been distinctive in their potential to prevent, treat, and cure several human disorders since eternity. Natural, traditional herbs are considered to be safe, effective, and free of side effects as compared to their synthetic and chemical counterparts **[1]**. Phytomedicines are produced from the crude extracts of different parts of medicinal plants and consist of an intricate amalgamation of bioactive phytoconstituents exhibiting unique nutraceutical and therapeutic properties, which should be investigated for their application in the treatment of diseases **[2]**. A humongous reserve of bioactive phytochemicals resides in all the plant species, and there is a need to identify, isolate, quantify, and characterize them to obtain their full potential in the medical world **[3]**. To gain knowledge regarding the pharmacological properties and therapeutic potential of medicinal plants, initial screening by chromatographic and spectroscopic techniques plays a vital role, especially to identify new compounds and for quality control **[4]**.

The GC-MS technique is a quick and reliable method for the detection, identification, and characterization of the bioactive phytoconstituents like alcohols, phytosterols, organic acids, terpenes, long-chain hydrocarbons, esters, etc. found in medicinal herbs, and only a small amount of plant extract is required **[5] [6]**.

Morus species possess high environmental adaptivity and economic importance because of their foliage, which is used in sericulture and as fodder. Leaves, roots, and bark exhibit several health benefits and have been used to treat various diseases in Indian and Chinese traditional folk medicine since time immemorial **[7] [8]**.

This study aims to identify the bioactive compounds found in methanol, ethyl acetate, and n-hexane extracts of the leaves of *Morus alba* L. by GC-MS analysis to reveal the antioxidant effects and biological, therapeutic, and remedial potential of the identified and characterized phytochemicals and to discover new, unknown bioactive compounds and their medicinal properties.

In the present study, GC-MS analysis was used to profile the composition of antioxidants, while the hydrogen peroxide

method was used for antioxidant evaluation. The hydrogen peroxide method is a reliable technique for measuring antioxidant activity, resulting in a colored compound quantified spectrophotometrically. GC-MS analysis provides valuable information about antioxidants' chemical composition, allowing a better understanding of their molecular structure and potential health benefits **[9]**.

MATERIALS AND METHODS

Plant material and Chemicals: All compounds and reagents utilized in the study were of analytical quality. The plant material was prepared from fresh and healthy leaves of *Morus alba L.* obtained and authenticated by Central Seri cultural Research & Training Institute, Central Silk Board, Govt. Of India, Ministry of Textiles, Srinagar, Jammu and Kashmir, India.

Preparation of extract: Methanol, ethyl acetate, and n-hexane were used as solvents to prepare the crude extracts of the *Morus alba L.* leaf powder by using the Soxhlet apparatus. Each sample was extracted for 48 hours at a temperature not greater than the boiling point of the solvents, filtered by Whatman filter paper, and stored in glass vials for GC-MS analysis.

Gas chromatography-mass spectrometry analysis: The GC-MS analysis of bioactive phytoconstituents from the three different extracts of leaves of *Morus alba L.* was performed using an Agilent 7890 GC system coupled with an MS-5975 (Agilent technologies) and having a HP-5 MS fused silica column (5% phenyl methyl siloxane 30.0 m × 250 µm, film thickness 0.25 µm). Carrier gas used was pure Helium, with a flow rate of 1mL/min, sample injection 2 µL. The initial temperature was set at 50°C- 150°C with increasing rate of 3°C per min and held isothermally for 10 min. Total elution time was 40 min. GC-MS analysis used an electron ionization system with high energy electrons ionizing the sample constituents at 70 eV. Comparing the average peak area of each component to the total area yielded the proportionate quantity of each component.

Identification of bioactive constituents: The bioactive compounds extracted from the different leaf extracts were identified on the basis of retention times and matching mass spectra with already known compounds from different data software libraries. The mass spectra of the unknown components obtained from methanol, ethyl acetate, and n-hexane extracts of the leaves of *Morus alba L.* were compared with the standard mass spectra of known bioactive phytochemicals recorded in the National Institute of Standards (NIST) library. More than 62,000 patterns of known compounds can be found in the database of the NIST library **[10]**.

Test for antioxidant activity of *Morus alba L.* leaves extract by hydrogen

peroxide scavenging activity: Hydrogen peroxide scavenging activity was performed by using the method given by Ruch *et al* (1989). 40mM solution of hydrogen peroxide was made with phosphate buffer (pH 7.4). Methanolic leaf extract of *Morus alba L* (1mg/ml) was mixed with 0.6 ml of hydrogen peroxide. Absorbance value was measured at 230nm after 10 min incubation. Blank was prepared with plant extract and phosphate buffer. Baseline adjusted with phosphate buffer.

The percentage of hydrogen peroxide scavenging was determined by the given formula:

% Scavenged H_2O_2 = [(AC – AS)/AC] x 100

Where AC denotes the absorbance of control and AS denotes the absorbance in the presence of *Morus alba L.* leaf extract.

RESULTS AND DISCUSSION

Antioxidant activity

The percentage of H_2O_2 scavenging activity in the leaves of *Morus alba L.* was found to be:

$$\% \textbf{ Scavenged } \mathbf{H_2O_2} = \frac{(1.278 - 0.726)}{1.278} \times 100$$

% scavenged H_2O_2= 43.19%

The findings indicate that the *Morus alba L* leaf extracts have notable capacity to remove free radicals, indicating its potential as a natural antioxidant. Additionally, the extract's ability to reduce oxidative stress points to a potential role in the prevention of chronic illnesses associated with free radical damage.

The present study underscores the importance of investigating plant-derived antioxidants for natural health benefits, as they can reduce the risk of chronic diseases like cardiovascular disease and cancer, and may lead to the development of innovative treatments.

High value of antioxidant activity is due to the presence of Octadecanoic Acid, Didodecyl Phthalate, and Tetra tetracontane in the leaf extracts of *Morus alba L.*

Gas chromatography-mass spectroscopy profile

Gas chromatography-mass spectrometry (GC-MS) is a powerful analytical technique that combines gas chromatography and mass spectrometry to detect various bioactive components in a sample. It is widely used in forensic material identification due to its ability to focus on specific samples and demonstrating its effectiveness in identifying small quantities of chemicals in various substances **[11]**.

The GC-MS chromatogram of methanol, ethyl acetate and n-hexane extracts of leaves of *Morus alba L.* determined a total of sixteen peaks corresponding to the bioactive phytochemicals that were identified on the basis of comparison of their GC retention time, peak area percentage and mass spectra patterns with that of the known compounds

found in the National Institute of Standards (NIST) library,

The chromatograms of bioactive phytochemicals detected in the methanol, ethyl acetate and n-hexane extracts of leaves of *Morus alba L.* are given in **Figures 1, 2 and 3** respectively and the phytochemical compounds identified along with their molecular formula, molecular weight, retention time, peak area and structure for methanol, ethyl acetate and n-hexane leaf extracts of *Morus alba L*. are presented below in **Tables 3, 4 and 5** respectively.

A highest percentage composition of 77.04% in the GC-MS analysis indicates that palladium (0), bis(eta-2-butadiene)1,1,4,5,8 is the main compound identified in the methanolic leaf extract of *Morus alba L*. Its dominant presence suggests that palladium (0), bis(eta-2-butadiene)1,1,4,5,8 plays a crucial role in the overall properties and characteristics of the analysed sample and has been reported for the first time in *Morus alba L*, leaf extracts in the present investigation and seems to be a novel compound.

The second most prominent bioactive component was found to be 3-chloro-4-hydroxy-*N*-[2-[2-(2-methoxyphenyl) ethylcarbamoyl]-4-phenoxyphenyl] benzamide with the peak area percent of 42.12% found in the ethyl acetate leaf extract of *Morus alba L*. known for its antioxidant, anticancer and antifungal activity. Benzoic acid with the peak area percent of 26.88% found in the ethyl acetate leaf extract of *Morus alba L* known for its anticancer and antioxidant activity was the third most prominent phytochemical compound. 5H-Indeno[1,2-b] pyrazin-5-one, 6,7,8-tribromo-9-(ethoxycarbonyl)-N, N'-diethyl-1,2,3,4-tetrahydro-with a peak area percent of 21.61% was also found to be significant

The GC-MS analysis also indicated the presence of several minor compounds that could significantly affect the overall results and warrant further investigation, including Octadecanoic Acid (20.10%), Tripalmitin (17.94%), Didodecyl phthalate (13.75%), Tetra tetracontane (13.30%) and 4-O-Methylphorbol-12,13-didecanoate (8.97%).

All the bioactive phytoconstituents determined, identified and characterized by GCMS analysis have been previously reported to show antibacterial, antifungal, antiviral, antioxidant, hepatoprotective, anti-cancer, anti-inflammatory, antiulcer, antitumor, cytotoxic and anthelmintic activity. They can also be used as lipid biomarkers, skin conditioning, cosmetic and food flavoring ingredients.

Presence of phytol, Cholestane and palmitic acid in *Morus alba* leaves by GC-MS analysis has been recorded by Ki Kwang Oh *et al*. **[12]**. Presence of Hexadecanoic acid, octadecanoic acid/stearic acid, phytol has

been reported by Malai Satiraphan *et al*. in the ethanolic leaf extracts of *M. alba*. L **[13]**. Hexadecanoic acid and Phytol have been reported in the n-hexane leaf extracts of *Morus alba* and Hexadecanoic acid methyl ester has been reported in the chloroform extract of *Morus alba* L leaf by Yashvanth *et al* **[14]**. Niko S. Radulović *et al*. have also recorded the presence of alkanes, diterpenoids, carotenoid-derived compounds and fatty acid-related constituents by GC-MS analyses of essential-oil samples obtained by hydrodistillation of *Morus alba* L leaves like Decane, Benzoic acid, Octadecanoic acid, Hexadecanoic acid etc. **[15]**. Nuraniye Eruygur *et al* identified Hexadecanoic acid and palmitic acid in the ethanolic extract of *M. alba L.* leaves by GC-MS **[16]**.

GC-MS analysis identified three phytochemicals, Octadecanoic Acid, Didodecyl Phthalate, and Tetra tetracontane, as key contributors to the antioxidant properties of *Morus alba L.* leaves. Octadecanoic Acid, a popular ingredient in skincare, has potent antioxidant effects. Didodecyl Phthalate and Tetra tetracontane, have notable antioxidant properties, making them attractive for creating innovative antioxidants in various sectors. These components may be responsible for the antioxidant potential of *Morus alba L.* leaves. Further research could explore the mechanisms behind these components' antioxidant potential and their concentration and bioavailability.

This study provides a concise summary of the traditional and pharmacological activity of these GC-MS identified bioactive compounds, highlighting common characteristics and trends, allowing scientists to understand their unique features and applications through a comprehensive analysis of their traditional and medicinal actions.

Palladium (0), bis(eta-2-butadiene)1,1,4,5,8

Palladium is a catalyst used in hydrogenation and dehydrogenation processes, facilitating the addition of hydrogen atoms to unsaturated compounds, a crucial role in industries like pharmaceuticals and petrochemicals. known for its Antioxidant and Antimicrobial activity **[17]**

3-chloro-4-hydroxy-*N*-[2-[2-(2-methoxyphenyl) ethylcarbamoyl]-4-phenoxyphenyl] benzamide

Benzamide, an aromatic compound with a benzene ring and carboxamido ligand, is widely used in medicine and agrochemicals due to its adaptable reactivity and unique structure, allowing for the incorporation of various functional groups, making it a crucial component in organic chemistry. It is well-known for its antioxidant, antifungal activity **[18]** and treatment of prostate cancer and benign prostate hyperplasia.

Benzoic acid

Hydroxybenzoic acid belongs to a group of phenolic compounds. Hydroxybenzoic acids itself and its derivatives showed antioxidant properties against different type of free radicals and can prevent or decrease overproduction of reactive species **[19]**. Benzoic acid is also known for its Anticancer effects **[20]**. It is reported to show anticancer and antitumor activity **[21] [22]**.

5H-Indeno[1,2-b] pyrazin-5-one, 6,7,8-tribromo-9-(ethoxycarbonyl)-N, N'-diethyl-1,2,3,4-tetrahydro-

This compound is reported to exhibit Anti-inflammatory antimicrobial antioxidant and anticancer properties **[23]**.

Octadecanoic Acid

Octadecanoic acid can protect cortical neurons against oxidative stress by boosting the internal antioxidant enzymes. Its neuroprotective effect may be mainly mediated by the activation of PPAR gamma and new protein synthesis in cortical neurons **[24]**.

It is also reported to show Antibacterial and antioxidant activity **[25]**.

Tripalmitin

Tripalmitin is a triglyceride derived from the fatty acid palmitic acid. It shows antioxidant effect that helps protect cells from damage caused by free radicals. It has also been found to have potential anti-inflammatory properties, making it a promising compound for many therapeutic applications. A compound with antioxidant and anti-inflammatory properties, it has shown potential in therapeutic applications such as cancer prevention, hypocholesterolemia management, and preventing free radical damage in cells **[26]**. Tripalmitin acts as Lipid biomarker for fat components preserved in archeological matrices **[27]** and used as a skin conditioning agent **[28]**.

Didodecyl phthalate

Didodecyl phthalate is a phthalate ester and a di-ester. It is reported for Antioxidant, antitumor, antifungal and antibacterial activity **[29] [30]**

Tetra tetracontane

Tetra tetracontane is a long-chain alkane consisting of an unbranched chain of 44 carbon atoms. It has a role as a human metabolite and exhibits antioxidant and cytoprotective activities **[31]**. It possesses Anti-inflammatory, hypocholesterolemia, cancer preventive, hepatoprotective and antioxidant activity **[32][33]**. It has shown application in cosmetic industry **[34]**. It can act as a Lipid biomarker for Tuberculosis **[35]**.

This study is a unique GC-MS analysis of *Morus alba* L. leaves using three solvents: methanol, ethyl acetate, and n-hexane. The results provide a complete fingerprint of bioactive phytochemicals, exhibiting therapeutic and healing properties. These phytochemicals can be used for future drug

development as they show a good antioxidant activity. The growing need for identifying and characterizing phytochemical compounds and recording their biological activities is crucial for the production of new herbal medicines and justifying the use of mulberry leaves as a feed for silkworms and a valuable source of therapeutic compounds. Biomarkers could be explored further at various other diagnostic platform for detection of tuberculosis infection.

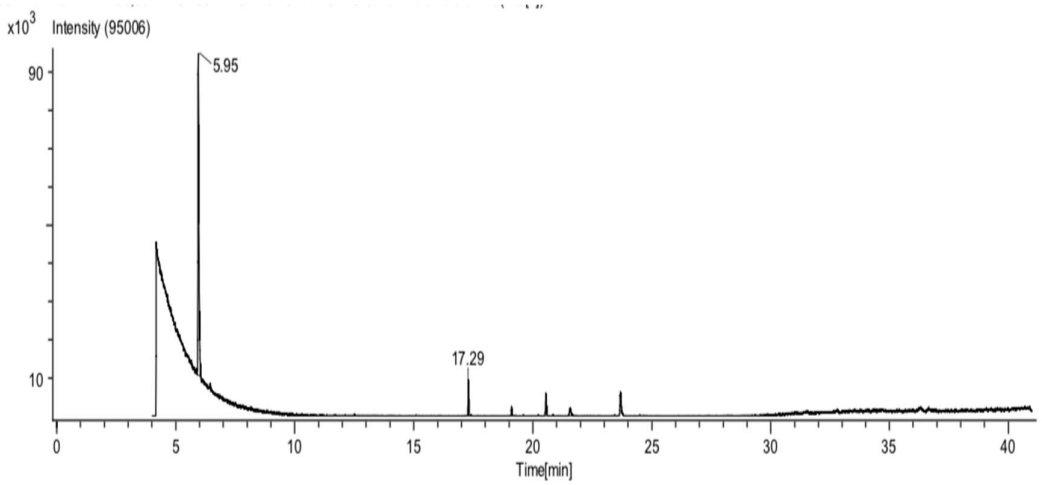
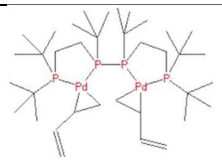
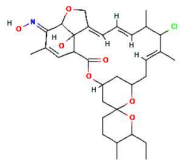
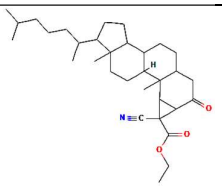
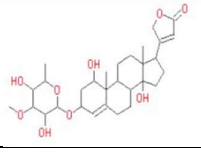
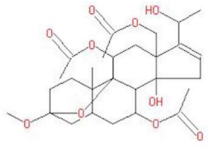
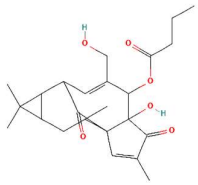


Figure 1: Chromatogram of bioactive phytochemicals detected in methanol leaf extract of *Morus alba L*

Table 3: Bioactive constituents identified in methanol leaf extract of *Morus alba L.*

S. No.	Name of the compound	Molecular Formula	Mol. weight	RT (min)	Peak Area %	Structure
1	Palladium (0), bis(eta-2-butadiene)1,1,4,5,8	$C_{36}H_{74}P_4Pd_2$	842	5.95	77.04	
2	Milbemycin B, 5-demethoxy-5-one-6,28-anhydro-25-ethyl-4-methyl-13-chloro-oxime	$C_{32}H_{44}ClNO_7$	589	17.29	6.02	
3	3'H-Cycloprop (1,2)-5-cholest-1-en-3-one,1'-carboethoxy-1'-cyano-1,2-dihydro	$C_{32}H_{49}NO_3$	495	19.11	1.38	
4	Carda-4,20(22)-dienolide,3-[(6-deoxy-3-O-methyl-àD-allopyranosyl) oxy]-1,14-dihydroxy-, (1á,3á)-	$C_{30}H_{44}O_9$	548	20.55	4.71	

S. No.	Name of the compound	Molecular Formula	Mol. weight	RT (min)	Peak Area %	Structure
5	7,11,18-triacetoxy-3-methoxy-3,9-Epoxypregn-16- ene-14,20-diol	$C_{29}H_{40}O_{10}$	536	21.57	3.06	
6	Butanoic acid, 1a,2,5,5a,6,9,10,10a-octahydro-5a-hydroxy-4-(hydroxymethyl)-1,1,7,9-tetramethyl-6,11-dioxo-1H-2,8a-Methanocyclopenta [a]cyclopropa[e]cyclodecen-5-yl ester,	$C_{24}H_{32}O_6$	416	23.69	7.79	

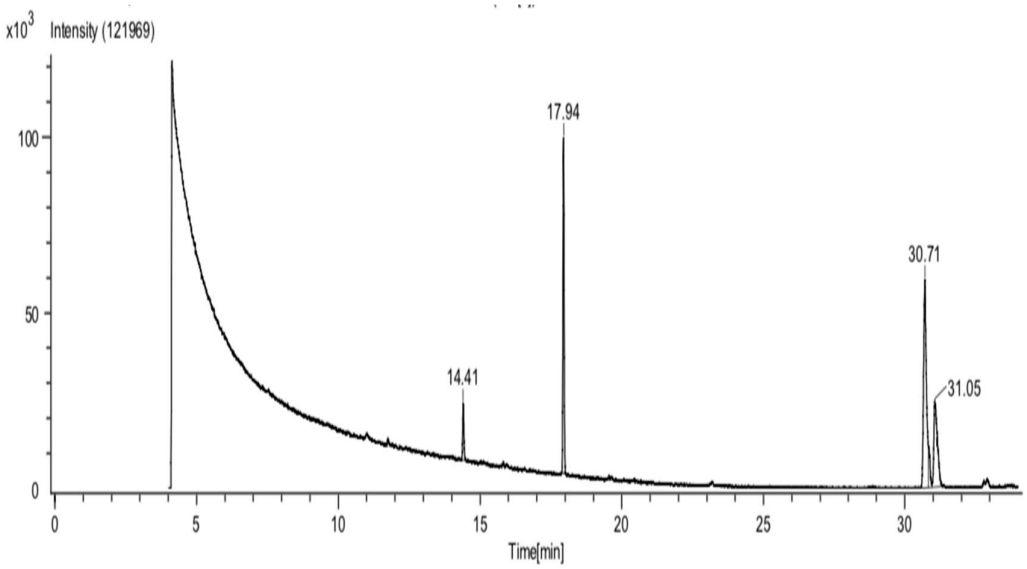
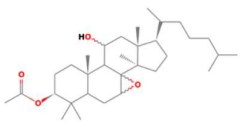
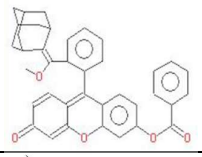
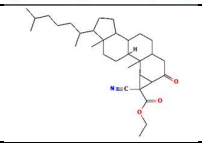
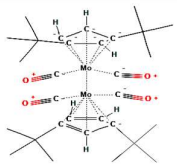
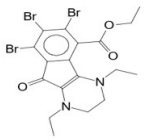


Figure 2: Chromatogram of bioactive phytochemicals detected in ethyl acetate leaf extract of *Morus alba L.*

Table 4: Bioactive constituents identified in ethyl acetate leaf extract of *Morus alba L.*

S. No	Name of the compound	Molecular Formula	Molecular weight	RT (min)	Peak Area %	Structure
1	3-acetoxy-7,8-Epoxylanostan-11-ol	$C_{32}H_{54}O_4$	502	14.41	4.80	
2	Benzoic acid, 9-[2-(adamantan-2-yliden-methoxymethyl)-phenyl]-6-oxo-6H-xanthen-3-yl ester	$C_{38}H_{32}O_5$	568	17.94	26.88	
3	3-chloro-4-hydroxy-*N*-[2-[2-(2-methoxyphenyl) ethylcarbamoyl]-4-phenoxyphenyl] benzamide	$C_{29}H_{25}ClN_2O_5$	517	30.71	42.12	

S. No	Name of the compound	Molecular Formula	Molecular weight	RT (min)	Peak Area %	Structure
4	Molybdenum, bis[(1,2,3,4,5.eta.)-1,3-bis(1,1-dimethylethyl)-2,4-cyclopentadien-1-yl]di-.mu.-carbonyldicarbonyldi-	$C_{30}H_{42}Mo_2O_4$	662	30.85	4.52	
5	5H-Indeno[1,2-b] pyrazin-5-one, 6,7,8-tribromo-9-(ethoxycarbonyl)-N, N'-diethyl-1,2,3,4-tetrahydro-	$C_{18}H_{19}Br_3N_2O_3$	548	31.05	21.61	

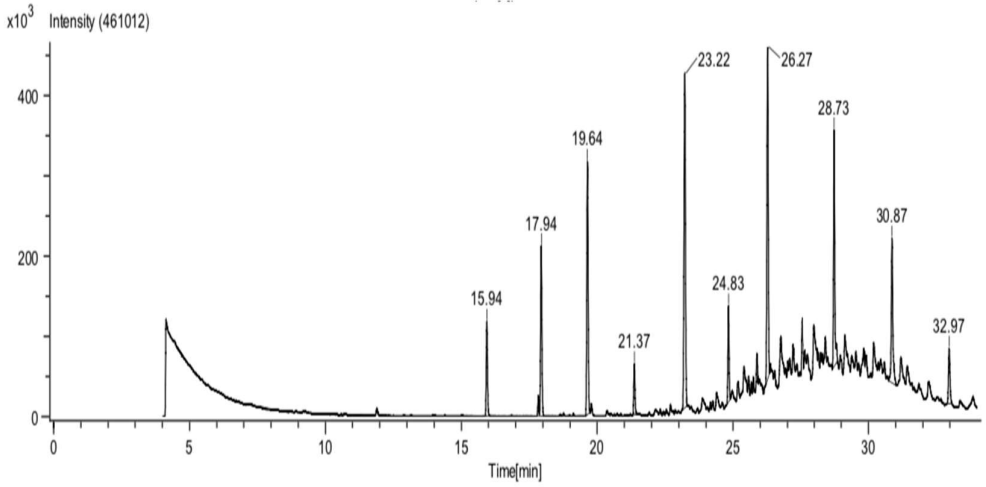
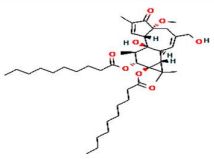
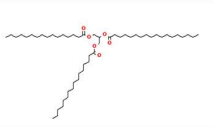
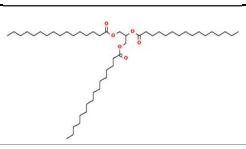
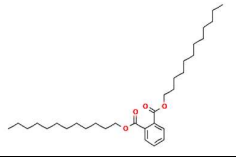


Figure 3: Chromatogram of bioactive phytochemicals detected in n-hexane leaf extract of *Morus alba L.*

Table 5: Bioactive constituents identified in n-hexane leaf extract of *Morus alba L.*

S. No	Name of the compound	Molecular Formula	Molecular weight	RT (min)	Peak Area %	Structure
1	4-O-Methylphorbol-12,13-didecanoate	$C_{41}H_{66}O_8$	686	17.94	8.97	
2	Octadecanoic Acid, 1-[[(1-Oxohexadecyl) Oxy] Methyl]-1,2-Ethanediyl Ester	$C_{55}H_{106}O_6$	862	23.22	20.10	
3	Didodecyl phthalate	$C_{32}H_{54}O_4$	502	19.64	13.75	
4	Tetratetracontane	$C_{44}H_{90}$	618	28.73	13.30	
5	Tripalmitin	$C_{51}H_{98}O_6$	806	26.27	17.94	

CONCLUSION

In today's research scenario, the main aim of undergoing research in the pharmaceutical field is the identification, isolation, characterization, quantification and bioengineering of novel bioactive phytochemicals from several medicinal herbs, that can act as remedial drugs for the treatment of different maladies. The Gas chromatography-mass spectroscopy technique detected the presence of a total of 16 bioactive phytochemicals in the methanol, ethyl acetate and n-hexane extracts, which portrays that mulberry plant is not only economically valuable as an only source of food for silkworms for silk production, but is also a therapeutic, wholesome, advantageous and beneficial medicinal herb which possess proven antioxidant, antidiabetic, antibacterial, anticancer, antiviral, cardiovascular, hypolipidemic, antiatherogenic and anti-inflammatory functions. This study exhibited the presence of valuable bioactive phytochemicals in all the extracts. These bioactive phytochemicals with therapeutic and nutraceutical potential show pharmacognostic and medicinal properties. The robust bioactive constituents confirm the promising ramifications of *Morus* species for the treatment of cancer and the diseases caused by free radicals.

This research aimed to explore the use of mulberry leaves, the primary food for silkworms, as an antioxidant source. Antioxidants are crucial for protecting the body against oxidative stress and diseases. The study aimed to develop sustainable alternatives for antioxidant-rich foods and understand the antioxidant properties of mulberry leaves, which could have significant implications for human health and the silk industry. Further investigations to determine the antioxidant and anticancer activity and general toxicity and more clinical studies are therefore essential for estimating the true medicinal potential of the plant species.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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