ANALYTICAL PROFILING OF *AZADIRACHTA INDICA* LEAF EXTRACTS USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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By

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Faculty of Veterinary Medicine

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2025

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ANALYTICAL PROFILING OF *AZADIRACHTA INDICA* LEAF EXTRACTS USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY

ABSTRACT

An abstract of the research paper was presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement for the course DVT 55204 – Research Project.

All parts of the tree, from seeds, flowers, twigs, barks, roots, and leaves have their medicinal potential to humans. The efficacy of *Azadirachta indica* must be explored in order to determine its potential benefits. This study investigated the analytical profiling of methanol and hexane extracts of *A. indica*. Based on the gas chromatography-mass spectrometry (GS-MS), the methanol extract showed phosphoric acid, trimethyl ester, the most abundant compound, constituting 54.41% of the extract, followed by gamma-sitosterol (4.47%), hexadecanoic acid (3.11%), phytol (2.89%), and octadecanoic acid (2.39%). In hexane extract, phosphoric acid, trimethyl ester, also dominates with a slightly higher percentage of 61.36%. Hexadecanoic acid is the second most prevalent compound in hexane extracts at 5.14%, whereas gamma-sitosterol is present in a much lower proportion (0.45%). Octadecanoic acid and phytol were not detected in hexane extracts. These results provide valuable insights for future research focused on isolating and evaluating these compounds for their preservative properties.

Keywords: *Azadirachta indica*, analytical profiling, methanol, hexane

PROFIL ANALITIK EKSTRAK DAUN *AZADIRACHTA INDICA* MENGGUNAKAN KROMATOGRAFI GAS-SPEKTROMETRI MASSA

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 55204 – Projek Penyelidikan.

Semua bahagian pokok, dari biji, bunga, ranting, kulit batang, akar, hingga daun mempunyai potensi perubatannya untuk manusia. Keberkesanan *Azadirachta Indica* perlu diteroka untuk menentukan potensi manfaatnya. Kajian ini menyiasat profil analitik ekstrak metanol dan heksana dari *Azadirachta Indica*. Dalam pengekstrakan metanol, asid fosforik, trimetil ester merupakan sebatian paling banyak, merangkumi 54.41% daripada ekstrak, diikuti oleh gamma-sitosterol (4.47%), asid heksadekanoik (3.11%), fitol (2.89%), dan asid oktadekanoik (2.39%). Dalam pengekstrakan heksana, asid fosforik, trimetil ester juga mendominasi dengan peratusan yang sedikit lebih tinggi iaitu 61.36%. Asid heksadekanoik adalah sebatian kedua paling banyak dalam ekstrak heksana dengan 5.14%, manakala gamma-sitosterol hadir dalam kadar yang jauh lebih rendah (0.45%). Asid oktadekanoik dan fitol tidak dikesan dalam ekstrak heksana. Hasil ini memberikan pandangan yang berharga untuk kajian masa depan yang memberi tumpuan kepada pengasingan dan penilaian sebatian ini bagi sifat pengawetnya.

Kata kunci: *Azadirachta indica*, profil analitik, metanol, heksana

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Table Content

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List of abbreviations

kPa: Kilopascal

mL: Milliliter

Min: Minute

Cm: Centimeter

GC-MS: Gas Chromatography-Mass Spectrometry

List of symbols

%: Percent

°C: Degrees Celsius

B: Beta

1.0 INTRODUCTION

1.1 Research Background

According to Maithaniet et al. (2011), the neem, belonging to the mahogany family Meliaceae, is one of two species in the genus *Azadirachta*. It is indigenous to India and Myanmar, thriving in tropical and subtropical regions. This fast-growing tree can attain a height of 15 to 20 meters, occasionally reaching 35 to 40 meters. Although evergreen, during severe drought conditions, it may shed most or nearly all of its leaves. The branches have a widespread. The beneficial properties of neem have been recognized in the Indian tradition for thousands of years. Every part of the neem tree possesses medicinal properties. As proven scientifically, it has a wide spectrum of biological activity and is classified as one of the most versatile plants. All parts of the tree, from seeds, flowers, twigs, barks, roots, and leaves have their medicinal potential to humans (Mondal et al., 2016).

Part of it, the leaves of the neem tree are traditionally used in medicinal preparations purposely for anti-inflammatory, antibacterial, antiviral, antioxidant, hepatoprotective and others. Neem seed has the most oil content among the parts of the neem tree. It contains around 45% oil including oleic acid, linoleic acid, palmitic acid, stearic acid and arachidic acid. This seed is functional as anti-malarial, antipyretic, and antifungal (Mongkholkharjornsilp et al., 2005)

According to Sharma et al. (2012), the neem flower also shows a medicinal effect. Neem flower contains aromatics, fatty acids, steroids, hydrocarbon and sesquiterpenes. These active ingredients can be used in treating intestinal worm, removal of phlegm and bile suspension. All parts of the selected plant are used as medicine for the treatment of many diseases and illnesses. Traditionally, the leaves and their paste are used for curing allergic skin reactions and antivirally treating smallpox and chicken pox (Khine et al., 2013)

1.2 Problem Statement

There is a need to determine the most effective solvent for extracting compounds from *A. indica* to maximize their extraction level and potential therapeutic benefits. The choice of solvent significantly influences the efficiency, and it is essential to identify the optimal solvent for veterinary applications.

1.3 Research Questions

What is the most effective solvent for sample extracting from *A. indica* to maximize their potential therapeutic benefits in the context of veterinary medicine?

1.4 Research Hypothesis

There is a significant difference in the sample extraction of *A. indica* using different solvents.

1.5 Significance of the Study

This study is being conducted due to limited awareness of this plant among the general populace. Furthermore, this research can contribute significantly to the scientific community by addressing critical gaps in our understanding of the sample extraction potential of *A. indica*. The exploration of various solvents provides valuable insights, enabling researchers to employ more accurate and reliable methods in future studies.

1.6 Research Objectives

The objectives of the study:

a. To determine different compound constituents of *A. indica* using different solvents for extraction

2.0 LITERATURE REVIEW

2.1 *Azadirachta indica*

The development of medicines from natural products is garnering significant global attention today. These natural products possess unique chemical structures, exhibit a wide range of biological activities, and have properties similar to conventional drugs (Yuan et al., 2016). The use of natural products in medicine has been practiced since prehistoric times for the prevention and treatment of various diseases. Natural products offer numerous advantages, including high efficiency and selectivity of molecular targets, enabling their repeated use to effectively meet the urgent need for medicines (Velu et al., 2018). Natural products contain active compounds that exhibit a range of pharmacological activities against various diseases, often with minimal or no side effects (Rout et al., 2009).

Neem or scientifically known as *Azadirachta indica*, belonging to the Meliaceae family, is native to India and is widespread across the globe. It thrives in most tropical and subtropical regions, including Indonesia, where it is locally known as semambu or mencepu (Isdadiyanto et al., 2018). Neem offers significant medical benefits due to its diverse biological properties (El-Hawary et al., 2013). Every part of the neem plant can be utilized to treat a variety of diseases (Zhu et al., 2018). Different parts of the neem plant have been successfully isolated, revealing over 140 chemical compounds (Subapriya & Nagini, 2005). According to Saleem et al (2018), these compounds have been used in herbal medicine for thousands of years.

Neem contains a variety of primary compounds, including fat derivatives, carbohydrates, and proteins, as well as secondary compounds such as flavonoids, steroids, saponins, terpenoids, alkaloids, glycosides, and tannins (Gupta et al., 2017). Neem plants have attracted global attention due to their effectiveness without apparent side effects. Traditional use of the neem plant is considered safe, with over 75% of traditional medicine incorporating neem leaf extract. Historical accounts of neem leaf usage do not suggest any adverse effects. Nevertheless, there has never been a comprehensive safety assessment of neem leaf formulations (Kumar et al., 2016).

More than 150 compounds have been isolated from different parts of neem. The compounds have been divided into two major classes which are isoprenoid (Chaerjee and Pakrashi, 1991) such as diterpenoids and triterpenoids. *A. indica* also possess compounds acts as anti-inflammatory, antiarthritic, antipyretic, hypoglycaemic, antigastric ulcer, spermicidal, antifungal, antibacterial, diurec, antimalarial, antitumour and immunomodulatory (Joshi et al., 2010; Saleem et al., 2018).

A. indica has major ectoparasiticides in its various parts, especially in seeds and leaves. Herbal combinations containing *A. indica* has been found quite useful and edacious against ectoparasites such as nymphal ticks, fleas, lice and mites (Abdel-Ghaar et al., 2008; Habluet-zel, et al., 2007; Kilonzo, et al., 2001; Lima de Sou-za et al., 2017). The aqueous extract of *A. indica* has been shown to have activity against the maturation of parasite oocytes, the oviposition, the reproductive potential and embryonic development of an ectoparasite (Banerjee et al., 2014).

2.2 Application of mass spectrometry in compound profiling

According to Khan and Javaid (2021), the GC-MS analysis of extract from *Azadirachta indica* and five column chromatography fractions was performed using an Agilent Technologies 7890A GC system. The detector utilized was an Agilent Technologies 5975C inert MSD with a Triple-Axis Detector, equipped with a 30 m × 0.25 mm fused silica capillary column coated with Polysiloxanes. The software used for processing mass spectra and chromatograms was the National Institute of Standards and Technology (NIST) MS 2005 Library. The following temperature protocol was applied for GC-MS detection: the injection port temperature was set to 200°C, and the helium flow rate was maintained at 1 mL/min. The system performs five pre- and

post-solvent rinses for cleaning, with a column oven temperature of 60°C and an injection temperature of 300°C in splitless mode for 1 minute. Flow control operates at linear velocity with a pressure of 53.2 kPa, total flow of 4.9 mL/min, and column flow of 0.95 mL/min. A purge flow of 3.0 mL/min is maintained, with a split ratio of 1.0. High-pressure injection is set at 150 kPa for 1 minute, with the carrier gas saver off. The oven program starts at 60°C (3 min), increases to 160°C at 10°C/min, then to 280°C at 5°C/min, holding for 5 minutes.

3.0 RESEARCH METHODOLOGY

3.1 Study Design

The study design was conducted using cross-sectional study design with samples within the period of time

3.2 Sample Collection and Preparation

Azadirachta indica was acquired from a local farm in Kota Bharu. Then, the samples were washed and rinsed using tap water to remove contaminants. The sample were dried by using oven at 46°C for three days. The dried samples were ground into powder form using a mechanical grinder. The ground sample were kept in a seal plastic bag with silica gel desiccant (Figure 3.1).

Figure 3.1 Dried *A. indica* at room temperature before grinding process.

3.3 Sample Extraction

According to Balasubramanian et al. (2014), The dried neem leaves were soaked in hexane at a ratio of 200 g of neem leaves to 1000 mL of hexane and left overnight under a fume hood. The soaked leaves were then strained and filtered using a filter funnel and filter paper. The filtered sample was poured into a rotary evaporation flask, where the rotary evaporator water bath was set

at 50°C (below hexane's boiling point of 68.7°C), and the evaporation process was initiated. After 1 hour and 45 minutes, the solvent (waste hexane) was separated into a receiving flask. The crude extracted compounds were collected in a universal bottle, which was covered with parafilm (with holes) and left to dry under a fume hood. The collected hexane waste was reused to re-soak the neem leaves. This process (steps 5–12) was repeated using methanol instead of hexane. The re-soaking step was repeated three times for both hexane and methanol.

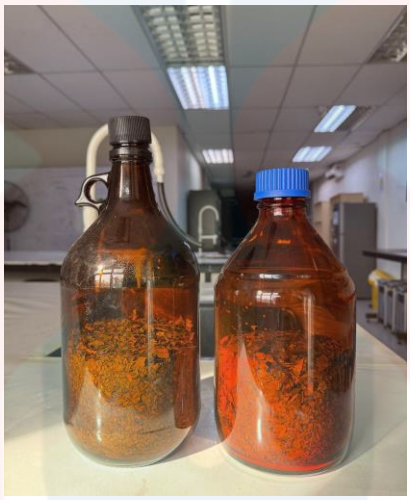

Figure 3.2 Soaked sample in hexane and methanol.

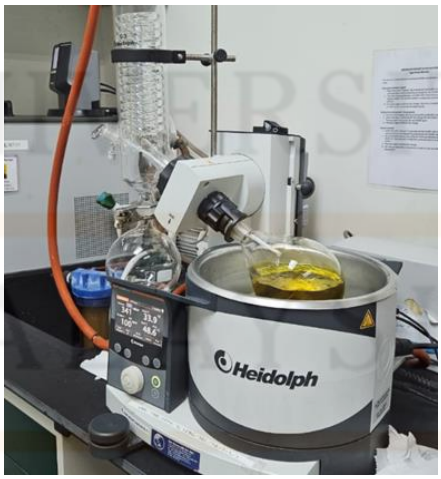

Figure 3.3 Concentrating sample using a rotary evaporator.

3.4 Compound profiling

Compound profilings were conducted using heliumgas chromatography-mass spectrometry. Single sample was used in this study. The system performs five rinses with a pre-solvent before handling the sample and five rinses with a post-solvent for cleaning after sample handling. The system was configured with a column oven temperature set to 60.0°C and an injection temperature of 300.0°C. The injection mode was set to splitless, with a sampling time of 1.00 minute. The flow control mode operates at linear velocity, with a system pressure of 53.2 kPa. The total flow is 4.9 mL/min, and the column flow is 0.95 mL/min, with a linear velocity of 35.5 cm/sec. A purge flow of 3.0 mL/min is maintained, and the split ratio is set to 1.0.

High pressure injection is enabled, with a pressure of 150.0 kPa applied for 1.00 minute. The carrier gas saver function is turned off. The oven temperature program begins at 60.0°C, holding for 3.00 minutes. The temperature is then increased at a rate of 10.00°C/min to reach 160.0°C, with no hold time, and further increased at 5.00°C/ min to reach 280.0°C, where it holds for 5.00 minutes.

The software used to compute and analyze the graph results from the GC-MS machine is LabSolutions. This software provides advanced tools for processing and interpreting chromatographic data, ensuring accurate and reliable analysis.

4.0 RESULTS

4.1 Compound profiling of methanol and hexane extract

Table 4.1 showed a list of compounds from methanol extract after profiling using gas chromatography-mass spectrometry (GC-MS). GC-MS analysis reveals that 50 compounds in methanol extract of *A. indica*.

Table 4.1 List of compounds in. methanol extract after profiling using GC-MS.

Peak#	Area%	Name
1	54.41	Phosphoric acid, trimethyl ester
2	0.17	1-Pyrrolidinylacetonitrile
3	0.12	6,6,8,8,10,10-Hexamethyl-2,5,7,9,11,14-hexaoxa-6,8,10-trisilapentadecane
4	0.59	Undecane
5	1.11	Undecane
6	0.50	Cyclopropane, nonyl-
7	0.76	Dodecane
8	0.15	2,2'-Bis-trimethylsilylbenzhydryl methyl ether
9	1.43	1-Tridecene
10	1.14	Tetradecane
11	0.36	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-
12	0.42	Phenol, 3,5-bis(1,1-dimethylethyl)-
13	0.80	1-Pentadecene
14	0.56	Hexadecane
15	0.28	Fumaric acid, 4-octyl undecyl ester
16	1.56	1-Octadecene
17	0.48	Octadecane
18	0.21	Dodecanoic acid, 2,3-bis(acetyloxy)propyl ester
19	1.33	Neophytadiene
20	0.16	Neophytadiene
21	0.18	2,6-Dihydroxybenzoic acid, 3TMS derivative
22	0.60	Neophytadiene
23	3.11	Hexadecanoic acid, methyl ester
24	0.53	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester

25	0.57	Heptadecanoic acid, heptadecyl ester
26	0.16	7-Tetradecanol
27	1.37	1-Heneicosanol
28	0.33	Octadecane
29	0.18	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane
30	0.27	8,11-Octadecadienoic acid, methyl ester
31	2.89	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-
32	2.39	Phytol
33	1.19	Methyl stearate
34	0.29	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate
35	0.82	Octacosanol
36	0.33	1-Nonadecene
37	0.19	Cyclononasiloxane, octadecamethyl-
38	0.21	Bis(2-ethylhexyl) phthalate
39	0.19	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate
40	1.28	Eicosyl isopropyl ether
41	0.96	Decanedioic acid, bis(2-ethylhexyl) ester
42	4.47	.gamma.-Sitosterol
43	0.66	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3.beta.,4.alpha.,5.alpha.)-
44	0.98	phenol, 4-[4,5-bis[4-(dimethylamino)phenyl]-4H-imidazol-2-yl]-
45	1.50	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-, TMS derivative
46	1.42	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, (3.beta.,4.alpha.,5.alpha.)-
47	3.76	Lanosterol
48	1.60	Spiro[bicyclo[3.1.1]heptane-2,2'-oxirane], 6,6-dimethyl-
49	0.63	17a-Methyl-3.beta.-methoxy-17a-aza-D-homoandrost-5-ene-17-one
50	0.39	Nonanedioic acid, dimethyl ester
	100.00	

Table 4.2 showed a list of compounds from hexane extract after profiling using GC-MS.

Table 4.2 List of compounds in. hexane extract after profiling using GC-MS.

Peak#	Area%	Name
1	0.10	Benzamide, 3-methoxy-N-methyl-
2	61.36	Phosphoric acid, trimethyl ester
3	0.21	6,6,8,8,10,10-Hexamethyl-2,5,7,9,11,14-hexaoxa-6,8,10-trisilapentadecane
4	0.12	1-Nonene
5	0.47	Undecane
6	1.15	Undecane
7	0.47	1-Dodecene
8	0.76	Dodecane
9	0.25	2,2'-Bis-trimethylsilylbenzhydryl methyl ether
10	0.09	1-Octanol, 2-butyl-
11	1.17	1-Dodecanol
12	1.03	Tetradecane
13	0.14	Tridecane, 3-methylene-
14	0.13	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane
15	0.20	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-
16	0.67	E-14-Hexadecenal
17	0.51	Hexadecane
18	0.09	Cyclooctasiloxane, hexadecamethyl-
19	0.18	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate
20	0.19	3H-Thieno[3,2-d]pyrimidin-4-one, 3-ethyl-2-[2-(2-methylpiperidin-1-yl)-2-oxoethylsulfanyl]-
21	1.26	1-Heptadecene
22	0.42	Octadecane
23	0.24	2,6-Dihydroxybenzoic acid, 3TMS derivative
24	0.45	Hexadecanoic acid, methyl ester
25	0.33	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester
26	0.19	8-Heptadecene, 8-methyl-, (E)-
27	0.99	1-Heneicosanol
28	0.28	Hexadecane, 1-iodo-
29	0.26	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane
30	0.28	1-Dodecanol, 2-octyl-

31	0.28	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate
32	0.43	9-Tricosene, (Z)-
33	0.30	2,6-Dihydroxybenzoic acid, 3TMS derivative
34	0.27	1-Heptacosanol
35	0.38	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate
36	0.21	Bis(2-ethylhexyl) phthalate
37	0.17	10-Heneicosene (c,t)
38	0.32	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate
39	0.33	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate
40	0.62	Decanedioic acid, bis(2-ethylhexyl) ester
41	5.14	.gamma.-Sitosterol
42	0.29	1-Methyl-1-n-hexyloxy-1-silacyclobutane
43	0.23	Trimethylsilyl 3-methyl-4-[(trimethylsilyl)oxy]benzoate
44	1.42	Cyclononasiloxane, octadecamethyl-
45	2.66	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-
46	3.64	Lanosterol
47	0.38	.alpha.-D-Galactopyranoside, methyl 2,3-bis-O-(trimethylsilyl)-, cyclic phenylboronate
48	7.41	2-tert-Butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl)phenol
49	0.59	Spiro[3,5-dioxatricyclo[6.3.0.0(2,7)]undecan-6-one-4,2'-cyclohexane], 1'-isopropyl-2,4'-dimethyl-, Z-
50	0.94	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-

Table 4.3 Percentage extracts and dominant chemical compounds in methanol and hexane extraction.

Name of Compound	Methanol (%)	Hexane (%)
Gamma - sitosterol	4.47	0.45
Hexadecanoic acid	3.11	5.14
Octadecanoic acid	2.39	-
Phytol	2.89	-

Phosphoric acid, trimethyl ester	54.41	61.36

Table 4.3 showed the percentage composition of dominant chemical compounds extracted using methanol and hexane solvents. In methanol extract, phosphoric acid, trimethyl ester, is the most abundant compound, constituting 54.41% of the extract, followed by gamma-sitosterol (4.47%), hexadecanoic acid (3.11%), phytol (2.89%), and octadecanoic acid (2.39%). In hexane extract, phosphoric acid, trimethyl ester, also dominates with a slightly higher percentage of 61.36%. Hexadecanoic acid is the second most prevalent compound in hexane extracts at 5.14%, whereas gamma-sitosterol is present in a much lower proportion (0.45%). Octadecanoic acid and phytol were not detected in hexane extracts.

5.0 DISCUSSION

Major compound isolated from hexane and methanol extracts are phosphoric acid trimethyl esther. From previous study, this compound showed wide range of activities (Table 5.1)

Table 5.1 Common compounds isolated from *A. indica* with its activities

Name of Compound	Molecular Formula	Activity
Gamma - sitosterol	$C_{29}H_{50}O$	• Antidiabetic drug • Antioxidant • Antibacterial • Anti-angiogenic • Anticholesterolemic • Anticancer (Tripathi et al., 2013)
Hexadecanoic acid	$C_{16}H_{32}O_2$	• Antifungal • Antioxidant • Hypercholesterolemia • Nematicide • Pesticide • Anti-androgenic flavor (Wang et al., 2002)
Octadecanoic acid	$C_{18}H_{36}O_2$	• Anti inflammatory • Hypocholesterolemic • Cancer preventive • Hepatoprotective • Nematicide • Insectifuge • Antihistaminic • Antieczemic • Antiacne • 5-Alpha reductase inhibitor • Antiandrogenic • Antiarthritic • Anti Coronary (Mujeeb et al., 2014)
Phytol	$C_{20}H_{40}O$	• Antioxidant • Antiallergic • Antinociceptive • Anti-inflammatory

		(Santos et al., 2013)
Phosphoric acid, trimethyl ester	$C_3H_9O_4P$	Active. New Compound (Bomhard et al., 1997)

Gamma-sitosterol, also known as Stigmast-5-en-3-ol, is a phytosterol with significant therapeutic potential. It is chemically related to palmitic acid, a saturated fatty acid, and has the molecular formula $C_{29}H_{50}O$. As an epimer of β-sitosterol, gamma-sitosterol has been studied for its potential in developing effective antidiabetic treatments (Tripathi et al., 2012). Research shows that gamma-sitosterol exhibits anticancer activity by inhibiting cell growth, inducing cell cycle arrest, and promoting apoptosis in cancer cells (Balamurugan et al., 2011).

Gamma-sitosterol also demonstrates notable antioxidant, antibacterial, and prophylactic properties. Widely found in many plants, this phytosterol has been used in traditional medicine for its antifungal, antibacterial, and anti-angiogenic effects. It has been traditionally employed to treat conditions such as ulcers, bronchitis, diabetes, and heart disease (Venkata et al., 2012). In some analyses, gamma-sitosterol was identified as one of the most abundant compounds (9.10%) and is recognized for its diverse biological activities, including anticholesterolemic, anti-inflammatory, and anticancer effects, among others.

Hexadecanoic acid, commonly known as palmitic acid, is a saturated fatty acid with the chemical formula $C_{16}H_{32}O_2$. It is one of the most prevalent saturated fatty acids found in animals, plants, and microorganisms (Smart, 2024).

The methyl esters form of hexadecanoic acid is noted for its antifungal, antioxidant, hypocholesterolemic, nematicidal, pesticidal, anti-androgenic, flavor-enhancing, haemolytic, and 5-alpha reductase inhibitory properties. It also exhibits potent antimicrobial activity (Snehal and Anjali, 2022). Palmitic acid has been reported to possess antibacterial and cholesterolemic effects (Barbara et al., 2002). Additionally, it demonstrates significant cytotoxicity against various cancer cell lines, including MCF-7, WRL-68, CaCo2 and Colo-320 DM. Furthermore, palmitic acid has shown hepatoprotective effects, particularly against galactosamine-induced liver damage (Samad et al., 2022).

Octadecanoic acid, commonly known as stearic acid, is a saturated fatty acid with the chemical formula $C_{18}H_{36}O_2$. Stearic acid is classified as a fatty acid methyl ester and exhibits several biological activities, including anti-inflammatory, hypocholesterolemic, cancer-preventive, hepatoprotective, nematicidal, and insect-repelling properties. Additionally, it has been shown to have antihistaminic, antieczemic, anti-acne, 5-alpha reductase inhibitory, anti-androgenic, anti-arthritic, and anti-coronary effects (Farina et al., 2014).

Phytol has been reported to possess antioxidant, antiallergic, antinociceptive, and anti-inflammatory activities (Santos et al., 2013). Recent studies have shown that phytol is an excellent immunostimulant, outperforming many commercial adjuvants by promoting long-term memory induction and activating both innate and acquired immunity (Lim et al., 2012). Additionally, phytol has demonstrated antimicrobial activity against *Mycobacterium tuberculosis* and *Staphylococcus aureu*s (Saikia et al., 2010).

According to Bomhard (1997), a study on repeated of trimethyl ester dose toxicity, significant effects were observed across different dose levels. At the highest dose of 250 mg/kg, twelve male rats and one female died during weeks 4 to 6 of treatment, showing symptoms like difficulty walking and reduced movement before death. Males and females in this group also had reduced weight gain, and males consumed significantly less food. At 100 mg/kg, male rats exhibited reduced red blood cell counts, hemoglobin levels, and hematocrit, along with changes in blood protein ratios. They also showed higher platelet counts, neutrophil percentages, cholinesterase activity, total cholesterol, and calcium levels. Similar effects were seen in the one surviving male from the 250 mg/kg group. Pregnant females given 40 mg/kg gained significantly less weight during mid to late pregnancy, and there was an increase in embryo deaths, though pup weights were higher than in the control group. Histopathological examination at doses of 100 mg/kg or more revealed organ damage, including kidney disease, thymus atrophy, liver and testis damage, and nerve degeneration in the spinal cord and peripheral nerves. These effects worsened with higher doses and were more severe in males. In terms of reproductive and developmental toxicity, the ability of rats to mate (copulation rate) was significantly reduced at 250 mg/kg, while fertility and implantation rates were significantly decreased at 100 mg/kg. At 40 mg/kg, there was a significant increase in embryo mortality. The "No Observed Effect Level" (NOEL) for both repeated toxicity and reproductive/developmental effects was determined to be less than 40 mg/kg/day for both males and females.

6.0 CONCLUSION

The findings of this study demonstrate that methanol extraction yielded several chemical compounds with potential applications as natural preservatives. These compounds include gamma-sitosterol, hexadecanoic acid, octadecanoic acid, phytol, and phosphoric acid, trimethyl ester. Similarly, hexane extract resulted in the isolation of gamma-sitosterol, hexadecanoic acid, and phosphoric acid, trimethyl ester. These results provide valuable insights for future research focused on isolating and evaluating these compounds for their preservative properties.

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