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(RESEARCH ARTICLE)

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Phytochemical composition and GC-MS analysis of *Hymenodictyon orixense* Mabb. leaves

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Abstract

Hymenodictyon orixense Mabb. (Rubiaceae); commonly known as Bridal Couch Tree, is used to treat ulcer, fever, inflammation, malaria, menstrual disorders and other ailments by ethnic communities of India. The present study aimed to document phytocompounds present in methanolic leaf extract using qualitative phytochemical screening and Gas Chromatography-Mass Spectrometry (GC-MS) analysis for the first time. Phytochemical screening revealed the presence of protein, carbohydrate, tannin, phlobatannin, alkaloid, cardiac glycoside, phytosterol, terpenoid, saponin, phenol and flavonoid. In quantitative tests, the total phenolic and flavonoid contents were found to be 62.57±4.28 mg GAE/g and 14.89±0.95 mg QE/g, respectively. GC-MS approach exhibited presence of 46 phytocompounds as determined through fragmentation pattern, molecular weight, molecular formula, and phytocomponent structure. The major compounds identified were, 1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid (14.34%), Squalene (14.25%), β-sitosterol (11.37%), (z)-9-octadecenamide (9.25%), Phytol (6.92%) and Ergost-5-en-3-ol (4.59%) etc. The results exposed the rich phytochemical profile of *H. orixense* leaves suggesting its further *in vitro*, *in vivo* and *in silico* exploration for assessment of pharmacological activities and structural characterization of bioactive molecules.

Keywords: Flavanoids; Quinic Acid; Rubiaceae; Squalene; Terpenoids; Vitamin E

1. Introduction

Medicinal plants have been fundamental to traditional healthcare systems for ages and remain crucial in modern pharmacotherapy [1]. They play a major role in drug development research due to their medicinal potential and as scientific support for ethnobotanical assertions. Despite the breakthroughs in synthetic drug discovery, natural products are preferred due to their therapeutic chemical structures and biological properties. Their growing acceptance is attributed to established roles in traditional medicine and benefits including safety, cost-effectiveness, and versatility [2].

Phytochemical analysis is fundamental to pharmacognostic investigations, focusing on the separation, identification, and quantification of bioactive molecules in medicinal plants. These substances, termed as secondary metabolites, exhibit diverse pharmacological actions and are categorised into several categories, for example, alkaloids, flavonoids, terpenoids, glycosides, phenolic compounds, essential oils etc. [3]. Phytochemicals have also been demonstrated to exhibit various biological activities, for example, antioxidant, antidiabetic, anti-inflammatory, antimicrobial, anticancer etc. [4]. The qualitative and quantitative investigation of these phyto-constituents provides insights that are crucial for substantiating traditional knowledge in a scientific manner and overall valuable for novel drug discovery [5].

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Gas Chromatography-Mass Spectrometry (GC-MS) is a very effective analytical technique utilized for the identification and characterization of volatile and semi-volatile chemicals in complex plant matrices. Gas chromatography separates mixtures into distinct components utilizing a temperature-regulated capillary column [6,7]. In addition to volatile compounds, GC-MS is also utilized to elucidate structure of a wide range of compounds, such as steroids, glycosides, essential oils, flavonoids, phenols, alkaloids, saponins, and their derivatives [8]. The preliminary phytochemical screening with GC-MS profiling offers a more thorough comprehension of the bioactive compounds present in the plant extracts, facilitating the discovery of essential therapeutic phyto-constituents [9]. Usually, methanolic extracts are preferred in these investigations as methanol effectively extracts a broad range of polar and somewhat non-polar phytochemicals [10].

Hymenodictyon orixense Mabb. (Figure 1.); a member of the Rubiaceae family, is called as Bridal Couch Tree, *Bhorsal*, *Kadambu*, *Bhurkund*, *Kusan*, *Bhorkud*, *Kukurkat, Diddi Mara*, *Kala Bachnag*, etc. in different languages [11]. It is used for treatment of skin ailments, inflammation, fever, atrophy, ulcerous wounds, sores, cholera, malaria, liver complaints, body swelling, diarrhoea, gout, menstrual disorders, snake bite, smallpox etc. by ethnic communities of India [12,13]. It has also revealed presence of various phytochemicals and several pharmacological activities, such as antioxidant, antimicrobial, antimalarial, antipyretic, anti-atherothrombotic, central nervous system depressant, cytotoxic, and acetylcholinesterase inhibition [11,14]. Despite the medicinal value, there exists a paucity of scientific studies regarding the phytochemical composition of *H. orixense* leaves, especially through utilising advanced techniques such as GC-MS. Therefore, the present research work has been envisaged to determine its phyto-constituents profile that will be helpful to validate the traditional medicinal applications of the plant as well as provide a basis for subsequent phyto-pharmaceutical researches.

2. Materials and Methods

2.1. Plant collection and authentication

The leaves of *H. orixense* were collected from Amberi, Udaipur, Rajasthan, India in June 2023. The voucher specimen was prepared (Field no. 08) and authentication of the plant was carried out at Arid Zone Regional Centre, Botanical Survey of India (BSI), Jodhpur (No. BSI/AZRC/l.12012/2023-24/Tech. (Pl.Id.)/317 dated 03.08.2023).

Figure 1 *Hymenodictyon orixense* Mabb

2.2. Preparation of plant extract

The leaves were air dried under shade then powdered and kept in an air-tight container. The extraction was done using the cold extraction method. Ten-gram leaves powder was dissolved in 100 ml of methanol, and was kept on a shaker at 30°C at 90 rpm for 72 hours. The extract was filtered and kept for drying in pre-weighed petri dish at room temperature till it gained constant weight, labelled, and stored until use. The percent yield was calculated as follows:

$$\text{Extraction yield (\%)} = \text{Weight of crude extract} / \text{Weight of dried powder} \times 100$$

2.3. Preliminary qualitative phytochemical screening

Preliminary phytochemical screening of methanolic leaf extract was conducted to determine the presence or absence of various phyto-constituents such as carbohydrates, proteins, alkaloids, flavonoids, terpenoids, tannins, phytosterols, cardiac glycosides and saponins using standard methods with slight modifications [15].

2.4. Assessment of Total Phenolic Content (TPC)

The total phenolic content in plant extract was quantified using a spectrophotometric technique. The Folin-Ciocalteu method was employed to ascertain the total phenol content. The reaction mixture comprises 1 ml of extract and 9 ml of distilled water, contained within a 25 ml volumetric flask. 1 ml of Folin-Ciocalteu phenol reagent was added to the mixture and thoroughly agitated. After 5 minutes, 10 ml of a 7% sodium carbonate (Na_2CO_3) solution was added to the mixture and the volume was made up to 25 ml. A series of standard solutions of gallic acid (50, 100, 150, 200, 250, 300, 350, 400, 450 µg/ml) were also made in the same manner. After incubation for 90 minutes at an ambient temperature, the absorbance of test and standard solutions was measured against the blank at 550 nm using a UV/Visible spectrophotometer. The total phenolic content was quantified as mg of GAE/g of extract [16].

2.5. Assessment of Total Flavonoid Content (TFC)

Total flavonoid content was estimated using a colorimetric assay with aluminium chloride. The reaction mixture contains 1 ml sample and 4 ml of distilled water in a 10 ml flask. To the mixture, 0.3 mL of 5% sodium nitrite was mixed and 5 minutes later, 0.3 ml of 10 % aluminium chloride was added. After five minutes, 2 ml of 1M Sodium hydroxide (NaOH) was mixed and made up to 10 ml using distilled water. A set of standard solutions (positive control) of quercetin (50, 100, 150, 200 and 250 µg/ml) were prepared similarly as explained earlier. The absorbance for sample and the standard was taken against the blank reagent at 510 nm using an UV/Visible spectrophotometer. The total flavonoid content was demonstrated as mg of QE/g of extract [16].

2.6. GC-MS Analysis

The GC-MS analysis was performed at the Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, Delhi. GC-MS analysis was conducted using a GC-MS-QP-2010 Plus Ultra. The GC/MS equipment utilised a Rxi-5 SIL MS column of 30 m × 0.25 mm × 0.25 µm in film thickness, consisting of 5% diphenyl and 95% dimethyl polysiloxane, and operated in electron impact mode at 70 eV. The carrier gas utilised was helium, maintaining a constant flow rate of 1.21 ml/min, with an injection volume of 1 µl and a split ratio of 10:1. The oven temperature was originally set to 60 °C for 2 minutes, and thereafter scheduled to rise to 300 °C at a rate of 10 °C/min for 19 minutes. The operational conditions for the MS were as follows: Interface Temperature: 270 °C, Ion Source Temperature: 220 °C, Solvent Cut Time: 2.50 minutes, Scan Speed: 3333, Mass Scan Range (m/z): 40-600, Threshold: 1000. GC-MS was examined via electron impact ionization at 70 eV, and the data was assessed using Total Ion Count (TIC) for the identification and quantification of compounds. The relative percentage of each component in the GC-MS spectrum was determined by comparing the average peak area of individual compounds to their total areas. The identification of compounds was determined by comparing their retention times and mass spectra with reference compounds from the NIST/Wiley spectral database. The biological activities of the compounds identified through GC-MS are also given in Table 3 after thorough search of online scientific databases such as Google Scholar, Science Direct, ResearchGate, Wiley etc.

3. Results

3.1. Extraction yield

In the present study, the extraction yield obtained for methanolic leaves extract of *H. orixense* was 9.66%. The extract exhibited a dark green colour.

3.2. Preliminary phytochemical screening

A qualitative analysis of the methanolic extract of *H. orixense* leaves was conducted to detect the presence of various phytochemicals (Table 1). The result revealed the presence of primary and secondary metabolites, including carbohydrates, proteins, saponins, tannins, alkaloids, cardiac glycosides, phytosterols, phlobatannins, phenols, flavonoids and terpenoids.

Table 1 Preliminary qualitative phytochemical screening of methanolic leaves extract of *H. orixense*

Phytochemical test and method	Result
Carbohydrate (Fehling's test)	+
Protein (Ninhydrin test)	+
Tannin (Gelatin solution+ NaCl test)	+
Alkaloid (Mayer's test)	+
Cardiac glycoside (NaOH test)	+
Saponin (Honey comb test)	+
Phytosterol (Libermann-Burchard's test)	+
Phlobatannin (HCl test)	+
Phenol ($FeCl_3$ test)	+
Flavonoid (Lead acetate test)	+
Terpenoid test (Salkowski test)	+

+ = present

3.3. Total Phenolic Content (TPC)

Total phenolic content was determined utilizing the Folin–Ciocalteu method. The result was obtained from a calibration curve ($y = 0.0007x + 0.1072$, $R^2 = 0.9893$) of the standard compound gallic acid (50–450 µg/ml) as shown in Figure 2. The concentration of total phenol is expressed as mg/g gallic acid equivalent (GAE). The total phenolic content was found to be 62.57 mg GA/g in the methanolic leaves extract of *H. orixense* (Table 2).

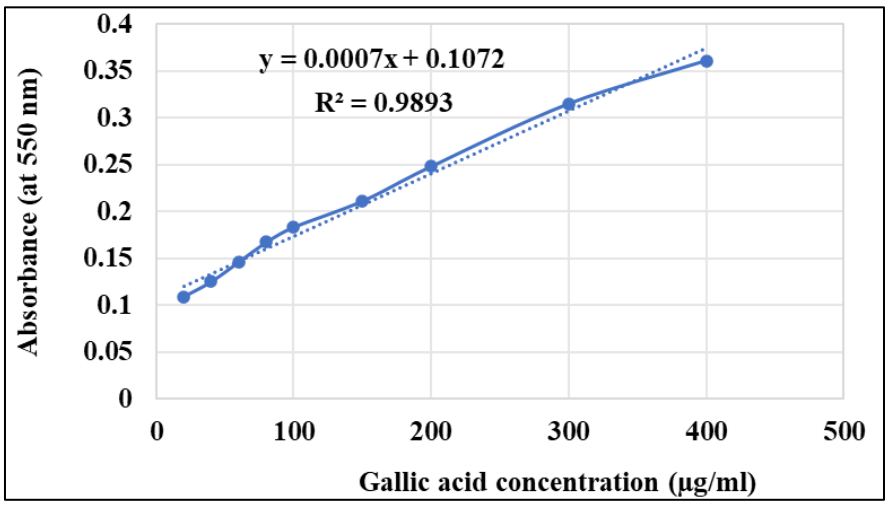


Figure 2 Standard curve of Gallic acid at 550 nm for calculating Total Phenolic Content

3.4. Total Flavonoid Content (TFC)

The flavonoid concentration was quantified as quercetin equivalents (mg of Q/g of extract). The concentration of flavonoids was derived from a standard calibration curve ($y = 0.0016x + 0.0955$, $R^2 = 0.9982$) of quercetin (50–250 µg/mL) as shown in Figure 3. In the methanol extract, TFC was found to be 14.89 mg QE/g (Table 2).

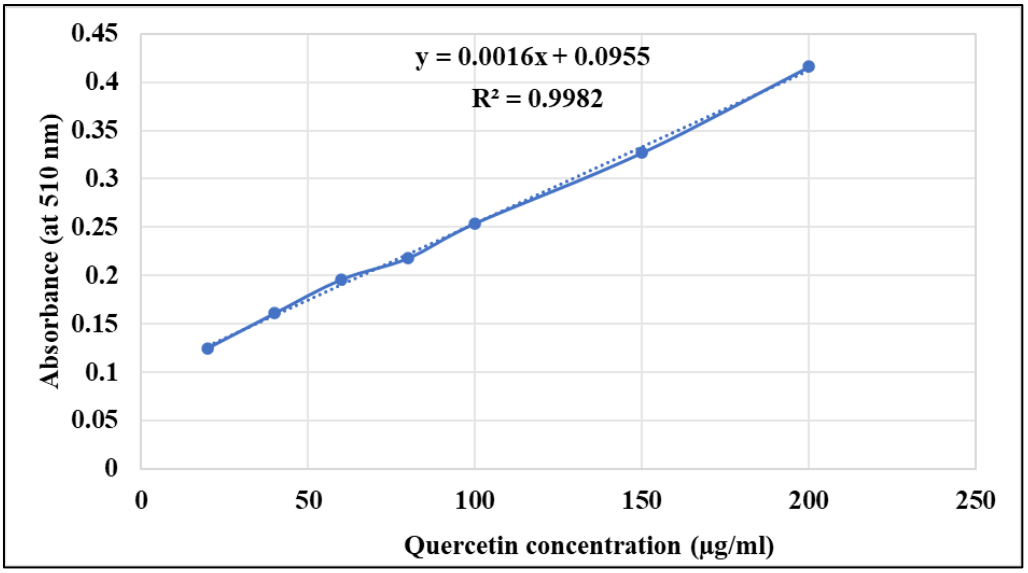


Figure 3 Standard curve of Quercetin at 510 nm for calculating Total Flavonoid Content

Table 2 Total phenolic and flavonoid contents in the methanolic leaf extract of *H. orixense*

Total phenolic content (mg of GAE/g of extract)	62.57 ± 4.28
Total flavonoid content (mg of QE/g of extract)	14.89 ± 0.95

Results are expressed in terms of gallic acid equivalent (mg of GAE/g of extract) and quercetin equivalent (mg of QE/g of extract), respectively as mean ± standard deviation.

3.5. GC-MS Analysis

GC-MS is a method that integrates the separation of phytochemicals using gas chromatography with their detection via mass spectrometry [17]. The methanolic extract of *H. orixense* leaves was analysed using GC-MS, revealing 50 peaks in the chromatogram (Figure 4). A total of 46 compounds were identified, among which 1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid (14.34%), squalene (14.25%), β-sitosterol (11.37%), (z)-9-octadecenamide (9.25%), phytol (6.92%), and ergost-5-en-3-ol (4.59%) were the most abundant (Table 3).

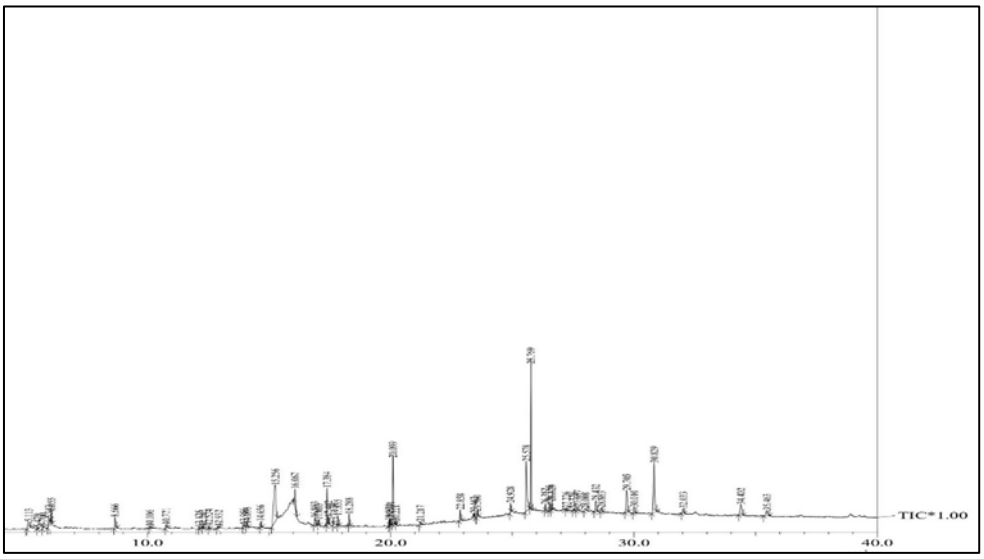
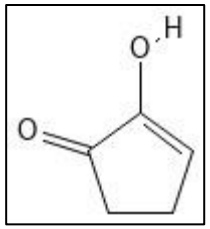
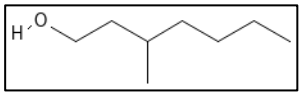
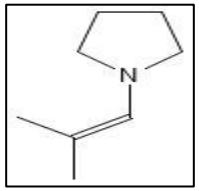
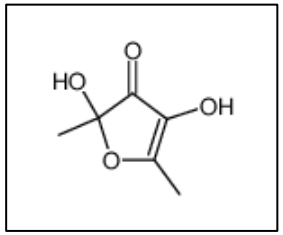
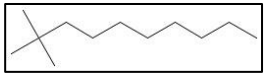
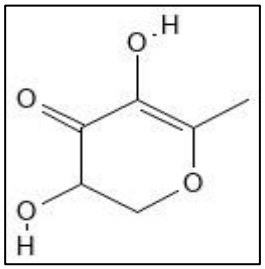
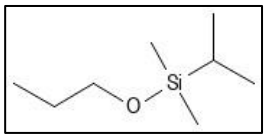
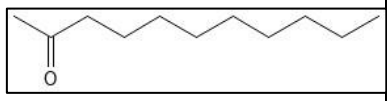
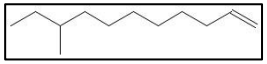
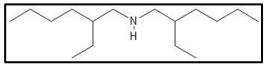
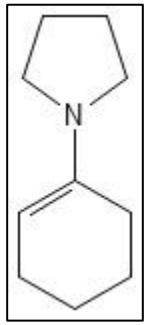
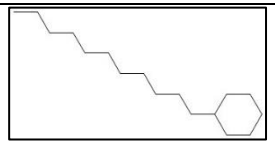
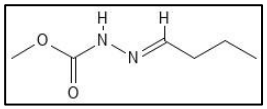
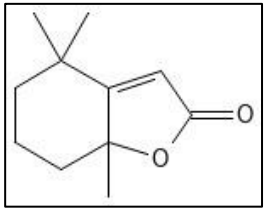
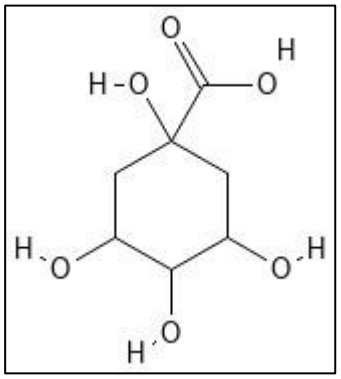


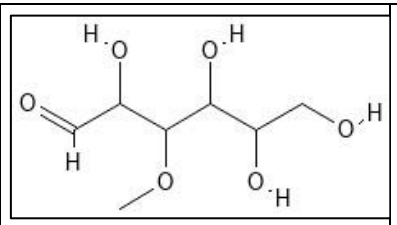
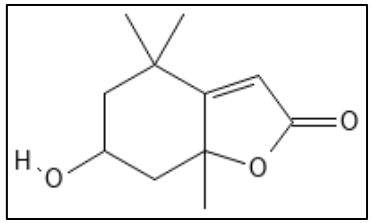
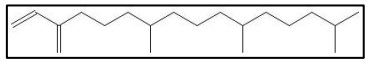
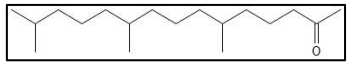
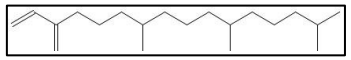
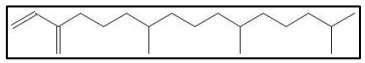
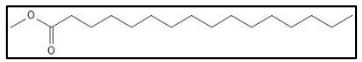
Figure 4 GC-MS Chromatogram of methanolic leaf extract of *H. orixense*

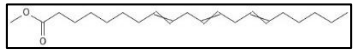
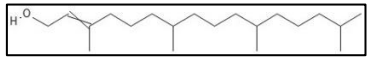
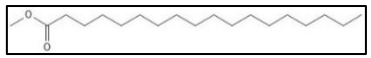
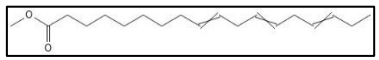
Table 3 Bioactive compounds identified in methanolic extract of *H. orixense* leaves using GC-MS technique

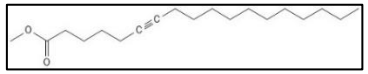
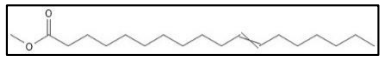
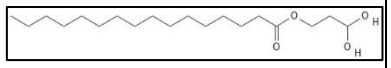
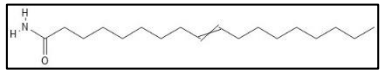
Peak	R. Time	Area%	Name	Molecular formula	Structure	Mol. Weight (g/mol)	Nature of compound	Biological activities	References
1.	5.113	1.88	2-Hydroxycyclopent-2-en-1-one	$C_5H_6O_2$		98	Cyclic hydroxy ketone	NR	-
2.	5.479	0.16	3-Methyl-1-heptanol	$C_8H_{18}O$		130	Alcohol	NR	-
3.	5.700	0.26	1-(2-Methyl-1-propenyl) pyrrolidine	$C_8H_{15}N$		125	Pyrrolidine alkaloid derivative- (Specifically heterocyclic amine)	NR	-
4.	5.943	1.33	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	$C_6H_8O_4$		144	Furanone derivative	NR	-
5.	6.055	0.98	2,2-Dimethyldecane	$C_{12}H_{26}$		170	Hydrocarbon (Alkane)	NR	-

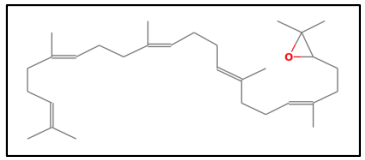
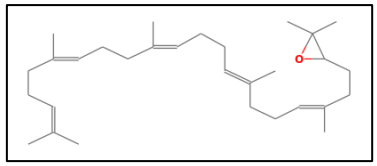
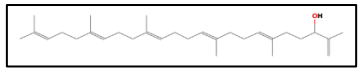
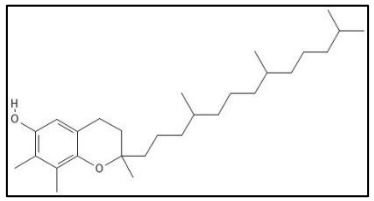
6.	8.666	1.63	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	$C_6H_8O_4$		144	Oxygenated heterocyclic compound – Pyranone derivative	Anti-diabetic, Antioxidant, Antimicrobial, Anti-inflammatory	[6,18-20]
7.	10.106	0.21	1-Dimethyl(isopropyl)silyl oxypropane	$C_8H_{20}OSi$		160	Silyl ether	NR	-
8.	10.771	0.59	Undecan-2-one	$C_{11}H_{22}O$		170	Ketone (Fatty acid derivative/Aliphatic ketone)	Antioxidant, Antimicrobial	[21,22]
9.	12.125	0.15	9-Methyl-1-undecene	$C_{12}H_{24}$		168	Hydrocarbon (unsaturated alkene)	NR	-
10.	12.282	0.15	Bis-2-ethylhexylamine	$C_{16}H_{35}N$		241	Alkyl amine	NR	-
11.	12.524	0.31	1-Pyrrolidinocyclohexene	$C_{10}H_{17}N$		151	Alkaloid (cyclic amine)	Cytotoxic, Antimicrobial	[23]
12.	12.932	0.33	Undecylcyclohexane	$C_{17}H_{34}$		238	Hydrocarbon (Alkane)	NR	-

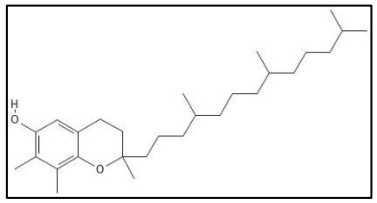
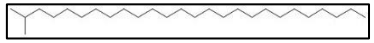
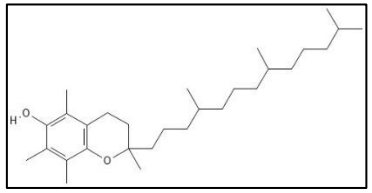
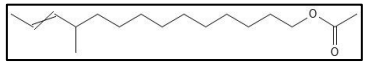
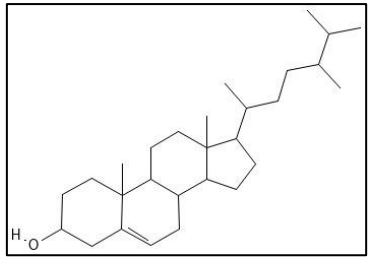
									
13.	13.956	0.31	Hydrazinecarboxylic acid, butylidene-, methyl ester	$C_6H_{12}N_2O_2$		144	hydrazone ester / Hydrazine derivative	NR	-
14.	14.098	0.34	4,4,7a-Trimethyl-5,6,7,7a-tetrahydro-1-benzofuran-2(4H)-one (Dihydroactinidiolide)	$C_{11}H_{16}O_2$		180	Benzofuranone (lactone) derivative	Antioxidant, Acetylcholinesterase inhibition, Neuroprotective, Anticancer, Antibacterial	[24]
15.	14.656	0.57	Hexadecanol (n-Cetyl alcohol)	$C_{16}H_{34}O$		242	Fatty alcohol (Fatty acid derivative)	Antimicrobial, Anti-inflammatory	[25]
16.	15.256	14.34	Quinic acid (1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid)	$C_7H_{12}O_6$		192	Phenolic acid derivative / Cyclitol derivative	Antioxidant, Antidiabetic, Antibacterial, Antimicrobial, Anti-nociceptive, Anticancer, Antiviral, Analgesic,	[26]

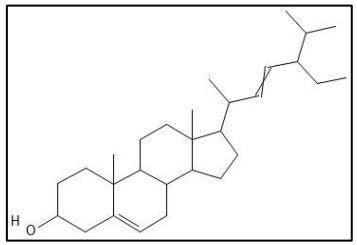
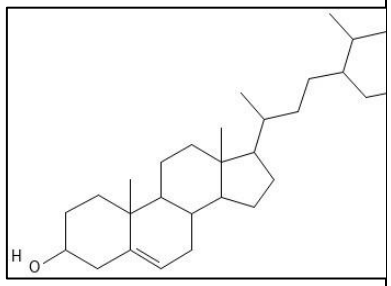
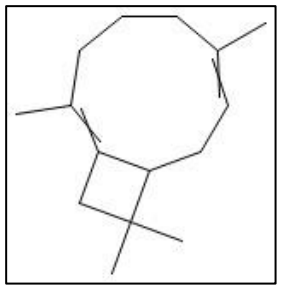
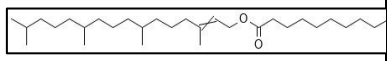
17.	16.067	2.85	MOME INOSITOL (3-O-Methyl-D-glucose)	$C_7H_{14}O_6$		194	Monosaccharide derivative	Anti-inflammatory, Antitumor	[27]
18.	16.883	1.52	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one	$C_{11}H_{16}O_3$		196	Monoterpene lactone	Anti-inflammatory	[28]
19.	17.033	0.49	Pluchidiol	$C_{13}H_{20}O_2$	-	208	Triterpene	Anti-inflammatory, Antioxidant	[29]
20.	17.384	3.38	Neophytadiene (7,11,15-Trimethyl-3-methylenehexadec-1-ene)	$C_{20}H_{38}$		278	Terpenoid (Diterpenoid)	Antioxidant, Antipyretic, Anti-microbial, Anti-inflammatory, Vermifugic, Analgesic, Antibacterial	[30,31]
21.	17.442	0.48	6,10,14-Trimethyl-2-pentadecanone (Hexahydrofarnesyl acetone)	$C_{18}H_{36}O$		268	Terpenoid (Acyclic Sesquiterpenoid Ketone)	Anti-inflammatory, Antimicrobial, Cytotoxic	[32]
22.	17.636	0.41	Neophytadiene	$C_{20}H_{38}$		278	Terpenoid	As reported in S. No. 20	As reported in S. No. 20
23.	17.835	1.05	Neophytadiene	$C_{20}H_{38}$		278	Terpenoid	As reported in S. No. 20	As reported in S. No. 20
24.	18.288	1.15	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$		270	Fatty acid ester	Antioxidant,	[6,27,33,34]

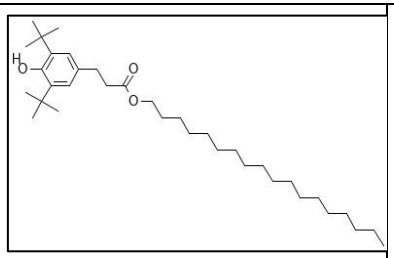
			(Palmitic acid methyl ester)					Anti-diabetic, Nematicide, Hypocholesterolemic, Hemolytic, Pesticidal, Antiandrogenic, Antimicrobial	
25.	19.930	0.44	9,12-octadecadien-1-ol (Linoleyl alcohol)	$C_{18}H_{34}O$		266	Fatty alcohol	Antimutagenic, Antioxidant	[35]
26.	19.991	0.69	8,11,14-Eicosatrienoic acid, methyl ester	$C_{21}H_{36}O2$		320	Fatty acid ester	Antimicrobial	[36]
27.	20.099	6.92	Phytol (3,7,11,15-Tetramethyl-2-hexadecen-1-ol)	$C_{20}H_{40}O$		296	Terpenoid (Diterpenoid alcohol) (acyclic diterpene)	Antioxidant, Anticancer, Anti-diabetic, Anticancer, Antimicrobial, Anti-inflammatory, Anti-diuretic	[6,33,34,37,39]
28.	20.221	0.25	Octadecanoic acid, methyl ester (stearic acid methyl ester) (Methyl stearate)	$C_{19}H_{38}O_2$		298	Fatty acid ester	Antimicrobial, Cytotoxic, Anti-diarrheal, Antiproliferative	[34,37,40]
29.	21.217	0.30	9,12,15-Octadecatrienoic acid, methyl ester (Linolenic acid, methyl ester)	$C_{19}H_{32}O_2$		292	Fatty acid ester	Antibacterial, Anti-inflammatory, Anticancer, Hypocholesterolemic, Antiarthritic, Antihistaminic,	[20]

								Hepatoprotective	
30.	22.858	1.23	6-Octadecenoic acid, methyl ester	$C_{19}H_{34}O_2$		294	Fatty acid Ester	NR	-
31.	23.442	0.16	9-octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$		296	Fatty acid Ester	Antioxidant, Anticancer, Antibacterial, Anti-inflammatory	[40-42]
32.	23.560	0.67	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (Glycerol, 2-palmitate)	$C_{19}H_{38}O_4$		330	Fatty acid Ester	Antioxidant, Hypocholesterolemic, Pesticide, Antiandrogenic 5-Alpha reductase inhibitor, Hemolytic	[43]
33.	24.928	1.02	Heneicosane	$C_{21}H_{44}$		296	Hydrocarbon (Alkane)	Antibacterial	[43]
34.	25.578	9.25	(z)-9-octadecenamide	$C_{18}H_{35}NO$		281	Fatty acid derivative	Antibacterial, Anti-inflammatory	[44]
35.	25.769	14.25	Squalene	$C_{30}H_{50}$		410	Triterpenoid	Antioxidant Anti-diabetic, Antiageing, Analgesic, Anti-inflammatory, Antileukemic, Hepatoprotective,	[31,33-34, 45-48]

								Hypocholesterolemic Antimicrobial Antitumor Anticancer	
36.	26.352	0.50	Heneicosane	$C_{21}H_{44}$		296	Hydrocarbon (Alkane)	As reported in S. No. 34	As reported in S. No. 34
37.	26.535	1.00	Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-heneicosapentaenyl)-, (all-E)-	$C_{30}H_{50}O$		426	Triterpenoid	Anti-diarrhoeal	[39]
38.	26.629	1.07	Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-heneicosapentaenyl)-, (all-E)-	$C_{30}H_{50}O$		426	Triterpenoid	As reported in S. No. 37	As reported in S. No. 37
39.	27.226	0.30	1,6,10,14,18,22-Tetracosahexaen-3-Ol, 2,6,10,15,19,23-Hexam Ethyl-, (All-E)-	$C_{30}H_{50}O$		426	Terpenoid (Triterpenoid alcohol)	Anti-inflammatory, Antimicrobial, Antiarthritic, Cytotoxic	[49]
40.	27.538	0.25	δ-Tocopherol methyl ether	$C_{28}H_{48}O_2$		416	Terpenoid	NR	-

41.	27.687	0.59	Gamma-Tocopherol (Vitamin E)	$C_{28}H_{48}O_2$		416	Phenolic compound	Antioxidant, Anti-inflammatory, Antitumor, Hypocholesterolemic, Cardioprotective, Anticancer	[39,43,50]
42.	28.008	0.46	2-Methylhexacosane	$C_{27}H_{56}$		380	Hydrocarbon (branched alkane)	Insect pheromone	[51]
43.	28.432	1.88	Alpha -Tocopherol (Vitamin E)	$C_{29}H_{50}O_2$		430	Phenolic compound	Antioxidant, Anti-diabetic, Anticancer, Antiageing, Antimicrobial, Anti-inflammatory	[20,33,39-40,52-53]
44.	28.663	0.64	E-11-Methyl-12-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$		268	Ester (a fatty acid ester derivative)	NR	-
45.	29.705	4.59	Ergost-5-en-3-ol (Campesterol)	$C_{28}H_{48}O$		400	Phytosterol	Hypocholesterolemic, Antioxidant, Anticancer	[43,54,55]

46.	30.010	1.25	Stigmasterol	$C_{29}H_{48}O$		412	Phytosterol	Cholesterol-lowering, Antioxidant, Anticancer, Anti-inflammatory, Anti-osteoarthritis, Anti-diabetic, Neuroprotective, Antiparasitic, Immunomodulatory, Antimicrobial	[56,57]
47.	30.829	11.37	β-Sitosterol	$C_{29}H_{50}O$		414	Phytosterol	Antioxidant, Anti-diabetic, Anti-inflammatory, Antimicrobial, Anticancer, Antifertility, Antiobesity	[27,40,58-61]
48.	32.033	1.14	2,6,10,10-tetra methyl bicyclo [7.2.0] undeca-1,6-diene (Caryophyllen-(I1))	$C_{15}H_{24}$		204	Terpenoid (Sesquiterpene hydrocarbon)	NR	-
49.	34.402	3.34	Phytyl tetradecanoate	$C_{34}H_{66}O_2$		506	Phytyl ester	Antioxidant, Antimicrobial, Anticancer, Anti-inflammatory	[62]

50.	35.463	1.58	Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate	$C_{35}H_{62}O_3$		530	Phenolic ester	Antioxidant, Antifungal	[63-65]
		100.00							

NR = Not reported

Table 5 Distribution of phytochemicals analyzed through GC-MS

S. No.	Phytochemical group	Total % Area
1.	Terpenes and terpenoid derivatives	32.26
2.	Fatty acid derivatives	18.69
3.	Phenolic compounds and their derivatives	18.39
4.	Phytosterols	17.21
5.	Hydrocarbons	3.44
6.	Miscellaneous compounds	10.01

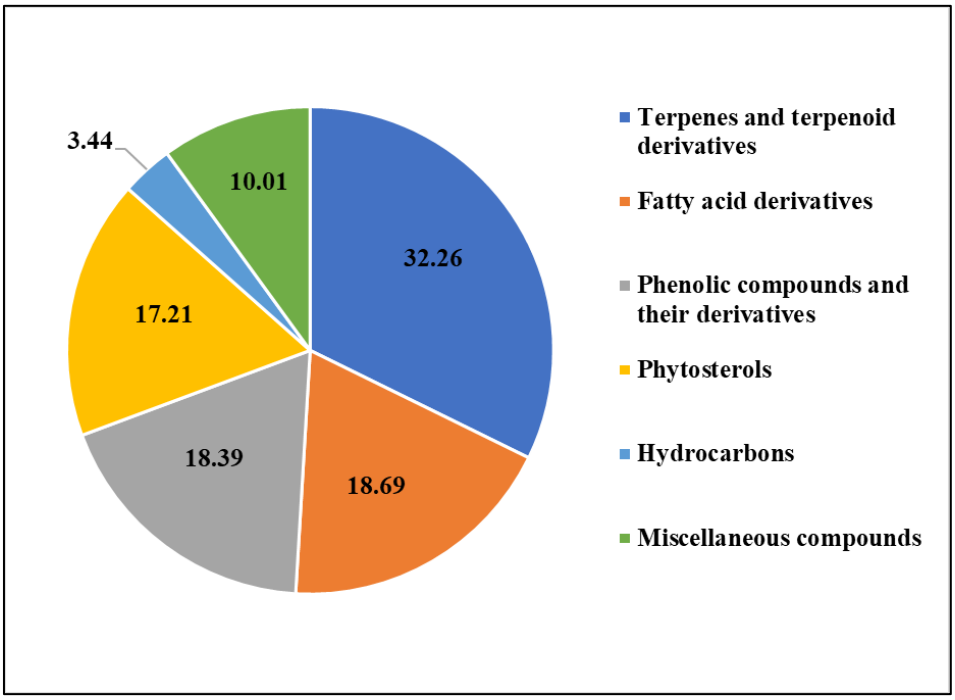


Figure 4 Composition of phytocompounds in methanolic leaves extract of *H. orixense* after GC-MS analysis

4. Discussion

Herbal medicines embody a combination of historic traditional knowledge and modern pharmacology, reflecting centuries of empirical learning from various civilizations [66]. However, a comprehensive scientific investigation of phytochemical composition of plants is necessary to assess their therapeutic efficacy. Bioactive molecules obtained from plants, play an important role in modern medicine owing to their varied therapeutic potential [67,68]. The qualitative screening of methanolic extract of *H. orixense* leaves exhibited a variety of phytochemicals, comprising carbohydrates, proteins, saponins, tannins, alkaloids, cardiac glycosides, phytosterols, terpenoids, phlobatannins, phenols, and flavonoids. The presence of these valuable secondary metabolites suggests that the plant holds significant potential for treating a range of human diseases and could serve as a promising source for the development of new therapeutic drugs.

The constituents in the methanolic extract of *H. orixense* are categorised into major phytochemical groups based on their structural class as analyzed through the GC-MS data (Table 3 & 4, Figure 5). The GC-MS spectrum revealed 50 peaks, corresponding to 46 phytocompounds (Figure 4). Among these compounds, many possess pharmaceutical significance, with several earlier studies documenting their therapeutic potential, for example, antioxidant, anti-diabetic, anticancer, antimicrobial, anti-inflammatory and other activities (Table 3).

Phenolic compounds and their derivatives comprised 18.39% of the total area, mostly dominated by quinic acid (14.34%), in addition to tocopherols and phenolic esters (Figure 5). The quantitative assessment of total phenolic content and total flavonoid content yielded values of 62.57 ± 4.28 mg GAE/g and 14.89 ± 0.95 mg QE/g, respectively (Table 2), reflecting a significant abundance of antioxidant compounds in the extract [69].

Flavonoids are among the most notable bioactive groups identified, distinguished by their broad pharmacological properties. These chemicals are recognised for their significant antioxidant, anti-carcinogenic and anti-inflammatory properties [70]. Their capacity to regulate essential cellular enzymes and signalling pathways renders them vital in the prevention and control of chronic diseases, including diabetes, cardiovascular disorders, and neurodegenerative problems [71]. Likewise, phenolic compounds have shown to enhance the antioxidant capacity of plant extracts and demonstrated anti-aging, antibacterial, anticancer, anti-diabetic, anti-inflammatory and antiviral effects [72,73].

The GC-MS profile revealed the presence of two tocopherol isomers, α-tocopherol and γ-tocopherol, which are effective phenolic antioxidants. γ-Tocopherol exhibits potent antioxidant, anti-inflammatory, and anticancer activities by neutralising reactive nitrogen species and regulating COX-2 and TNF-α pathways [50]. Similarly, α-tocopherol has also demonstrated antioxidant, anti-diabetic, anticancer, antibacterial, anti-inflammatory, antitumor, and anti-aging properties [33,53]. Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoate is a sterically hindered phenolic compound that was previously isolated from *Alcaligenes faecalis* MT332429, demonstrates significant antifungal activity [65]. It is also extensively utilized in industrial applications due to its antioxidant properties [64]. Quinic acid is another powerful antioxidant linked to antidiabetic, antibacterial, and antiviral properties [26].

Tannins, prevalent in numerous medicinal plants, enhance gastrointestinal health and exhibit significant antioxidant, antimicrobial, anti-mutagenic, anti-inflammatory, anticancer, antidiabetic, cardioprotective, hypolipidemic and anthelmintic properties [74,75].

Terpenes and terpenoid derivatives constituted the predominant class, with 32.26% of the overall makeup (Table 4). This dominance was mostly attributable to elevated concentrations of squalene (14.25%), phytol (6.92%), neophytadiene, triterpenoid epoxides, and 6,10,14-trimethyl-2-pentadecanone (hexahydrofarnesyl acetone). Phytol (3,7,11,15-Tetramethyl-2-hexadecen-1-ol), a diterpenoid alcohol, is recognised for its diverse biological activities, including antioxidant, antidiabetic, anticancer, anti-inflammatory, antidiuretic, and antimicrobial effects [6]. Its derivative, phytyl tetradecanoate, similarly exhibits antioxidant, anticancer, anti-inflammatory, and antimicrobial properties and serves as precursors in the synthesis of vitamins E and K1 [62]. The occurrence of neophytadiene, a diterpene detected in methanolic extract of *Abutilon pannosum* leaf and ethanolic extract of *Glochidion ellipticum* leaf, indicates its potential functions in antioxidant, antimicrobial, antipyretic, analgesic, and anti-inflammatory activities, as well as its application in alleviating headache, rheumatism, and certain dermatological conditions [30,31]. The ethanolic extract of *Polygonum chinense* previously demonstrated that squalene, a triterpene, possesses anti-cancer, antioxidant, anti-tumor, chemo-preventive, pesticidal and sunscreen effects [47]. Squalene has also shown to possess anti-diabetic, anti-aging, analgesic, anti-inflammatory, antimicrobial, antileukemic, hepatoprotective, hypocholesterolemic activities [34,48].

A sesquiterpene, hexahydrofarnesyl acetone (6,10,14-trimethyl-2-pentadecanone), earlier identified in essential oil of *Albizia zygia*, essential oil of *Lindera nacusu* leaves and from essential oil of *Graptophyllum pictum* leaves, is distinguished by its antibacterial, anti-inflammatory, antioxidant and anticancer activities [32,76,77]. Likewise, 6-hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one, a monoterpene lactone derived from the ethanolic extract of *Sargassum horneri*, also demonstrated anti-inflammatory efficacy by mitigating LPS-induced inflammation via the inhibition of pro-inflammatory mediators and the modulation of NF-κB and MAPK signalling pathways [28].

On the other hand, fatty acid derivatives constituted the second largest group with 18.69%, comprising fatty acid esters, alcohols, and amides (Table 3 & 4). Notably, z-9-octadecenamide (9.25%) a fatty acid amide, was the predominant compound in this group, which has exhibited significant antibacterial and anti-inflammatory properties [44] suggesting its potential role in the therapeutic efficacy of the extract. Palmitic acid methyl ester is recognised for its antioxidant [6,27,33], anti-diabetic, nematicidal, hypocholesterolemic [27], and antibacterial characteristics [6,34]. Methyl stearate has demonstrated antibacterial [34,40], antiproliferative, cytotoxic, and anti-diarrheal properties [37]. Linolenic acid methyl ester had also shown various pharmacological activities such as antibacterial, anti-inflammatory, hypocholesterolemic, nematicidal, anticancer, and hepatoprotective effects [20]. Another compound observed in the methanolic extract was hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (Glycerol 2-palmitate), which is a fatty ester well-known for its antioxidant, nematicide, hypocholesterolemic, and antiandrogenic activities [43].

Phytosterols (17.21%) identified in the extract (Table 3 & 4) have structural similarities to cholesterol, facilitating cholesterol reduction and enhancing lipid metabolism. These chemicals exhibit anti-inflammatory, antioxidant, and anti-cancer properties, and are associated with a decreased risk of cardiovascular illnesses and Alzheimer's disease [78]. For example, β-sitosterol, stigmasterol, and campesterol (ergost-5-en-3-ol) were detected in substantial amounts in leaves of *H. orixense* (Table 2). Previous studies have demonstrated that β-sitosterol exhibits various activities, such as antioxidant, anti-diabetic, anti-inflammatory, antimicrobial, anti-fertility, anti-obesity and anticancer [27,40,61]. The significant relative abundance of β-sitosterol in this study (11.37%) indicates a substantial contribution to the extract's bioactivity.

Stigmasterol is also reported for its diverse activities, including cholesterol-lowering [56], anti-diabetic, neuroprotective, antiparasitic, immunomodulatory, antimicrobial, antioxidant, anticancer, anti-inflammatory, and anti-osteoarthritis properties [57]. It has also demonstrated disruption in carcinogenic signalling pathways, including Akt/mTOR and JAK/STAT, and inhibition of angiogenesis via suppression of VEGFR-2 [57]. Campesterol has shown to diminish cholesterol absorption and also possesses anticancer and antioxidant properties [43,54,55].

Saponins, a significant category included in the extract, are recognised for their diverse biological actions. They possess immunostimulatory, hypocholesterolemic, anticancer, antibacterial, antiviral, antifungal, and anti-inflammatory activities [79]. Moreover, saponins have also demonstrated antihypertensive, hypoglycemic, and antioxidant properties, highlighting their potential in the management of metabolic syndromes and associated illnesses [80]. Cardiac glycosides, traditionally employed in the treatment of heart failure, have also been linked to antibacterial, anti-inflammatory, anticancer, and neuroprotective properties [81].

Aliphatic hydrocarbons represented 3.44% (Fig. 5), primarily comprising long-chain alkanes such as heneicosane and branched alkanes such 2-methylhexacosane, which may serve in plant defense or as pheromone analogues [51] whereas heneicosane has shown antimicrobial properties [43]. 4,4,7a-Trimethyl-5,6,7,7a-tetrahydro-1-benzofuran-2(4H)-one (Dihydroactinidiolide), derived from β-ionone, demonstrates significant multi-target efficacy against Alzheimer's disease, characterized by robust acetylcholinesterase inhibition (IC_{50} = 34.03 nM), antioxidant properties, metal-chelating abilities, and anti-amyloid aggregation effects. It demonstrates remarkable neuroprotective potential without cytotoxicity, reinforcing its promise as a principal drug for Alzheimer's disease [24].

The miscellaneous compounds, comprising 10.01%, covered monosaccharide derivatives such as MOME inositol (2.85%), alkaloids and their derivatives (1.05%), lactones, non-alkaloidal nitrogen heterocyclic, pyranones, silyl ethers, and other minor compounds. Although individually present in small quantities, these compounds may produce specific pharmacological effects or function synergistically with the primary components. For example, alkaloids as nitrogenous compounds exhibit pharmacological versatility demonstrating analgesic, antihypertensive, anticancer, antibacterial, and anti-hyperglycemic properties. Additionally, several alkaloids demonstrate psychotropic and neuro-protective properties, rendering them potential candidates for the management of inflammatory and neurodegenerative disorders [82,83]. The alkaloids identified through GC-MS technique in the present study were, 1-(2-methyl-1-propenyl) pyrrolidine, 1-pyrrolidinocyclohexene, and 2,3-bis(1-methylallyl) pyrrolidine. The pyrrolidine nucleus is recognized as a favoured scaffold in drug discovery owing to its antioxidant, antibacterial, anticancer, and anti-inflammatory activities [84]. Although any specific biological activities of these individual alkaloids have not been demonstrated yet, but their structural characteristics imply therapeutic significance.

Interestingly, several phytochemicals detected in the current study have been previously reported in some other plant species of Rubiaceae family, though their relative abundance was variable. The presence of 7.90% heneicosane was reported in the leaves of *Morinda citrifolia* [85]. Haruna identified squalene at 15.13% [86], whereas Danial *et al*. documented elevated squalene levels at 41.50%, in addition to phytol (13.06%), neophytadiene (10.06%), α-tocopherol (5.94%), stigmasterol (2.74%) and 9-octadecenamide (1.03%) in *M. citrifolia* [87]. Jeon *et al*. identified quinic acid (7.87%), 9-octadecenamide (3.84%), phytol (2.67%), neophytadiene (2.38%), stigmasterol (1.74%), γ-tocopherol (1.01%) and palmitic acid methyl ester (0.48%) in Arabica coffee leaves [88]. Likewise, 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (29.87%), squalene (14.58%), α-tocopherol (5.78%) and phytol (5.66%) were reported from leaves of *Spermadictyon suaveolens* [89].

In the present study, methanolic extract of *H. orixense* exhibited several of these compounds, but with varying concentrations. Quinic acid was present in higher concentration 14.34% in comparison to 7.87% in Arabica coffee. Squalene was found at 14.25%, nearly aligning with the 14.58% as documented for *S. suaveolens*; however, far lower than the remarkably high concentration of 41.50% in *M. citrifolia.* The fatty acid amide 9-octadecenamide constituted 9.25%, far above the 1.03% found in *M. citrifolia* and 3.84% in Arabica coffee, suggesting it may serve as a chemotaxonomic identifier for *H. orixense.* The concentration of phytol was higher (6.92%) than that found in Arabica

coffee (2.67%) and *S. suaveolens* (5.66%) but lower to *M. citrifolia* (13.06%). Similarly, Neophytadiene (3.38%) exceeded that of Arabica coffee (2.38%) but was lower than *M. citrifolia* (10.06%). α-Tocopherol was measured at 1.88%, which is lower than that found in *M. citrifolia* (5.94%) and *S. suaveolens* (5.78%). Stigmasterol was present at 1.25%, marginally lower than in Arabica coffee (1.74%) and *M. citrifolia* (2.74%). The quantity of palmitic acid methyl ester in *H. orixense* was more (1.15%) than that in Arabica coffee (0.48%). Heneicosane was identified at 1.02%, in contrast to 7.90% in *M. citrifolia*, indicating that this hydrocarbon is less prevalent in *H. orixense*. γ-Tocopherol levels were lower in *H. orixense* (0.59%) as compared to Arabica coffee (1.01%).

These variations can be assigned to species-specific metabolic pathways, environmental factors, the maturity stage of leaves upon harvest, extraction techniques etc. Squalene, a triterpene possessing antioxidant, antimicrobial, anti-inflammatory, antidiabetic and anticancer activities, is a significant component in several members of the Rubiaceae family, with concentrations ranging from modest (~14% in *H. orixense* and *S. suaveolens*) to exceptionally high (~41% in *M. citrifolia*). Quinic acid, a prominent phenolic acid derivative known for its antioxidant and antidiabetic properties, is prevalent in *H. orixense*, indicating its potential contribution to the plant's bioactivity profile. Likewise, the high concentration of 9-octadecenamide in *H. orixense* may indicate distinctive pharmacological potential, particularly in antibacterial and anti-inflammatory applications.

These comparative data suggest that *H. orixense* possesses a fundamental set of bioactive compounds which is common with other Rubiaceae species, such as phytol, squalene, tocopherols, terpenes, and various fatty acid derivatives; however, the relative abundances vary significantly, influencing potential biological activities and chemotaxonomic characteristics.

Overall, the wide range of pharmacological activities attributed to the identified compounds—including antioxidant, anti-diabetic, antimicrobial, anticancer, anti-inflammatory, hypocholesterolemic, etc. provide strong scientific backing to the ethnomedicinal use of *H. orixense* leaves in traditional medicine. The richness in terpenes, fatty acid esters, phenolic compounds, and phytosterols suggests a multifaceted therapeutic potential and supports further pharmacological investigations and drug development from this plant. Subsequent research should concentrate on isolating these phytocompounds and validating their activity via targeted *in vitro* and *in vivo* investigations.

5. Conclusion

The present study provides comprehensive insight into the phytochemical composition of the methanolic extract of *H. orixense* leaves through preliminary screening and GC-MS analysis for the first time. The qualitative phytochemical screening confirmed the presence of key secondary metabolites, including phenolics, flavonoids, terpenoids, saponins, and alkaloids, indicating the reason behind plant's therapeutic potential. The GC-MS analysis revealed the presence of a wide spectrum of bioactive compounds, notably, terpenes, fatty acid derivatives, phenolic derivatives, and phytosterols. Many of these identified constituents such as phytol, squalene, α-tocopherol, β-sitosterol, and various methyl esters of fatty acids are known for their strong antioxidant, anti-inflammatory, antimicrobial, and anti-diabetic properties, as reported in previous scientific literature.

The present findings are indirectly supporting the scientific validation of the traditional medicinal applications of *H. orixense* and establishing a phytochemical basis for its diverse biological activities. The chemical diversity observed in the methanolic leaf extract could be further investigated for pharmacological potential and natural product-based drug development. Future studies, including isolation, structural characterization, and *in vivo* evaluations are therefore, recommended to fully understand the therapeutic implications of the chemical compounds identified in the present study.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors declare no conflict of interest in publication of this manuscript.

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