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Terminalia arjuna Leaf Gall: The Possible Treatment for Sickle Cell Anaemia

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Authors' contributions

This work was carried out in collaboration among all authors. Author AS designed the study, performed the research work, the experimentation, prepared the manuscript. Author PL managed the analyses of the study and provided necessary support in the experimentation work. Author AS guidance and supervised the study. All authors read and approved the final manuscript.

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ABSTRACT

Objective: To check the presence of various secondary metabolites produced in the plant under study *Terminalia arjuna* (Roxb.) Wight and Arn. post insect attack and gall formation.
Study Design: *Terminalia arjuna* (Roxb.) Wight and Arn, a well known plant involved in treating heart ailments in the Ayurvedic system of medicine for centuries. The plant's astringent bark exhibits cardioprotective properties and is commonly used in the treatment of hypertension, angina and coronary artery disease. *T. arjuna* plant suffers from gall development in its leaves due to the attack of an hemipteran insect *Trioza fletcheri minor*. Insect attack acts as a stress stimulus for the plant to produce various bio-active compounds as a part of their defence mechanism. The present study was intended for the identification of various compounds present in the crude extract obtained from the healthy and galled leaves of *Terminalia arjuna*.
Place and Duration of Study: Experiments performed in the Plant pathology and tissue culture laboratory, Department of Botany University of Rajasthan, Jaipur, between April 2018 to March 2019.

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Methodology: Various methods are employed for the identification of possible bio-active compounds present in the plant crude extract, GC-MS (Gas Chromatography- Mass Spectroscopy) is one such method. To check the presence of phytochemicals produced post insect attack and infection in *T. arjuna* normal and galled leaves, Gas Chromatography-Mass Spectroscopy (GC-MS) of the methanolic extract was conducted. The normal healthy leaves and the galled leaves were collected, shade dried, pulverized and then soxhlet extracted for GC-MS analysis.
Results: GC-MS analysis of methanolic extract of *Terminalia arjuna* leaves and galls confess the presence of 57 compounds in the gall extract while 21 compounds in normal (uninfected) leaf extract. The gall extract revealed the presence of various novel and medicinally important bio-active compounds, one of them was 5-Hydroxy Methyl Furfural (5-HMF).
Conclusion: 5-HMF, an aromatic aldehyde is an economically important chemical compound and well-known for its wide application in various fields. It is an efficient anti-sickling agent and has undergone pre-clinical testing as a potential treatment for the fatal sickle cell disease. Qualitative phytochemical screening revealed the presence of various bio-active compounds both in leaf and gall methanolic extract of *Terminalia arjuna*, thus proving the plant's pharmaceutical importance. This study result will make a way for further research in plant's pharmacological research.

Keywords: Terminalia arjuna; leaf gall; Trioza fletcheri minor; GC-MS analysis; 5-hydroxy methyl furfural (5-HMF); sickle cell anaemia.

1. INTRODUCTION

Terminalia arjuna (Roxb.) Wight and Arn. commonly known as Arjuna, is a well-known plant with immense medicinal importance. The bark, leaves and fruits of *T. arjuna* have been used in the indigenous system of medicine for curing various ailments [1]. Indian physicians used the powdered tree bark for treating cardiovascular diseases. Its stem bark possesses glycosides, flavonoids, tannins and minerals. Flavonoids are associated with its antioxidant, anti-inflammatory and lipid lowering effects while glycosides are cardiotonic [2].

It has been used as a cardiotonic in heart failure, cardiomyopathy, atherosclerosis and also for treating various human diseases like anaemia, ulcers, liver disorders and showed antimicrobial, antitumoral, antioxidant, antifertility, antiallergic and anti-HIV properties [3-5].

A hemipteran insect, *Trioza fletcheri minor* attacks the plant *Terminalia arjuna* through leaves and induce gall formation in the leaves. The insect attack is primarily for nutrition and shelter, however plant galls also provide protection to the galling insects from their natural enemies [6].

Gall induction brings various morphological, anatomical, physiological and biochemical changes in the host plant. The insect attack acts as a stress stimulus for the host plant so the plant produces various bioactive compounds in response. It has been observed that the accumulation of secondary metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules.[7] In higher plants a wide variety of secondary metabolites are synthesized from primary metabolites (e.g., carbohydrates, lipids and amino acids) and are involved in the plant defense against herbivores and pathogens. Often they may confer protection against environmental stresses [8].

To find out the production of various bioactive compounds in *Terminalia arjuna* after the insect attack, Gas chromatography – Mass spectrometry (GC-MS) of the methanolic extract of the plant's normal healthy leaf and leaf galls was carried out, and the leaf extract showed the presence of 21 compounds whereas the gall extract was found with 57 compounds.

In the leaf gall, the most abundant compound observed was 2-Furancarboxaldehyde, 5-(hydroxymethyl)- representing 46.14% of the total area. This compound is commonly known as 5-Hydroxyl Methyl Furfural (HMF). It is an organic compound derived from dehydration of certain carbohydrates like fructose, glucose, sucrose, cellulose and inulin [9], found commonly in small amounts in foods such as coffee and prunes [10]. 5-HMF is also formed from reducing sugars in honey and other processed foods in acidic environments when they were heated through the Maillard reaction [11]. It has its wide application in various fields like in medical science. 5-HMF is considered to be an efficient anti-sickling agent and has undergone pre-clinical testing as a potential treatment for the fatal sickle cell disease [10].

Sickle cell disease is caused due to the polymerization of sickle haemoglobin because of the point mutation in the beta-globin gene (HBB). This sickle haemoglobin, under low oxygen saturation, assumes the tense (T-state) deoxygenated conformation which tends to form polymers. This further give rise to rigid erythrocytes with impaired blood vessel transit, initiated by adhesion of erythrocytes to endothelium, neutrophils and platelets. This process results in vessel occlusion and ischaemia with consequent acute pain, chronic organ damage, morbidity and mortality [12].

Sickle haemoglobin (Hb S) has significantly reduced oxygen affinity as compared to normal haemoglobin [13,14]. Some pharmacological agents stabilize the higher oxygen affinity relaxed state (R-state) of haemoglobin and destabilize the lower oxygen affinity T-state of haemoglobin, this leads to delay the sickling process of circulating red blood cells by slowing the polymerization kinetics. The classes of such agents include aromatic aldehydes, thiol derivatives, isothiocyanates and acyl salicylates derivatives. Among these, one such aromatic aldehyde, 5-hydroxymethylfurfural (5-HMF) increases oxygen affinity of sickle haemoglobin and reduces hypoxia-induced sickling in vitro [12]. It has also demonstrated a substantial anti-polymerization activity in concentration dependent manner at varied concentrations [15].

2. MATERIALS AND METHODS

2.1 Dry Powder Preparation

Normal and infected leaves of the plant were collected from various localities of Jaipur, Rajasthan in the months of April, May 2018. Normal and infected (galled) leaves were then shade dried separately. They were then pulverized to powder with the mechanical grinder.

2.2 Preparation of Extract

5.0 gm powder of normal and galled leaves was weighed and extracted with methanol (70-80ºC) by hot continuous percolation method in soxhlet apparatus for 24 hours. The extract was taken and filtered through whatmann filter paper. Then extract was concentrated by rotary evaporator to obtain extract.

2.3 GC-MS Analysis

The GC-MS analysis of methanolic extract of normal and galled leaves of *Terminalia arjuna* was carried out on Shimadzu QP-2010 plus with thermal desorption system TD 20. It includes auto sampler and a gas chromatograph which interfaced to a mass spectrophotometer. The column size of this system is 30 m × 0.25 mm i.d × 0.26 µm with a film thickness of 0.26mm, composed of 5MS (5% diphenyl/ 95% dimethyl poly siloxane). Helium gas (99.999%) was used as carrier gas at constant flow rate of 1ml/min. The 2 µl injection volume of sample was utilized with split ratio of 10:1. The injector temperature was programmed initially at 280°C, the ion-source temperature was 200°C, the oven temperature was programmed from 110 °C (for 4 min), with an increase of 10°C/min to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were analyzed using electron impact ionization at 70 eV. The total running time for each sample was 45 min.

2.4 Identification of Phytochemical

Interpretation of phytochemical present in the sample was conducted using NIST, having more than 62,000 patterns and Wiley8 Library. The comparison of unknown spectrum with known spectrum of various components was done by stored spectrum of NIST library and Wiley8 Library. The name, molecular weight and structure of the components were ascertained.

3. RESULTS

Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample. MS is wide ranging analytical technique, which identify the charged species according to their mass to charge ratio (M/Z). GC-MS is one of the best techniques to identify the constituents of volatile compounds. The GC-MS analysis of *T. arjuna* normal leaf showed the presence of twenty one compounds and galled leaf showed the presence of fifty seven compounds The identification of the phytochemical compounds was confirmed based on the peak area, retention time and molecular formula. The active principles with their retention time (RT), area %, name of the compounds present in the methanolic extracts of healthy and galled leaves of *Terminalia arjuna* are presented in the Figs. 1 and 2 and the Tables 1 and 2 respectively.

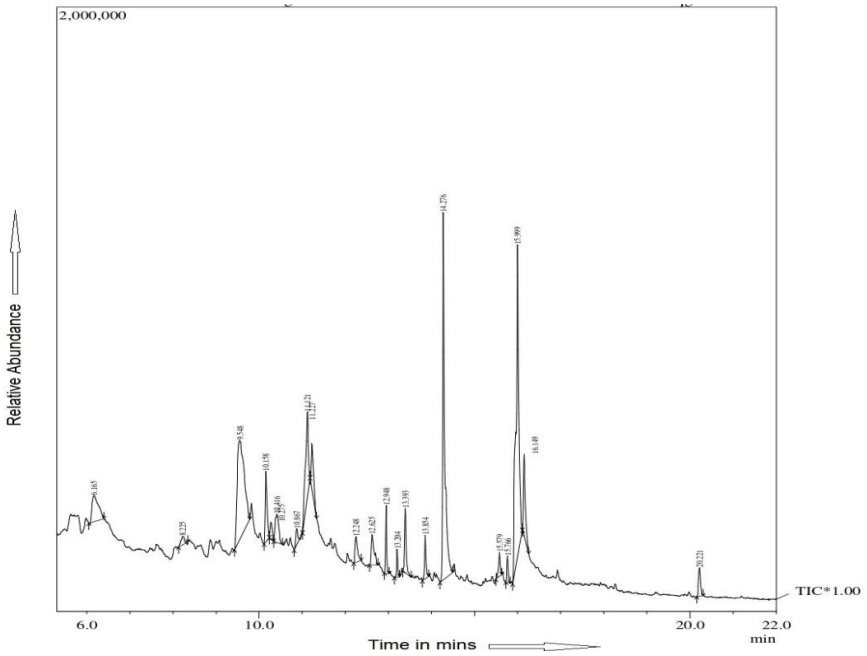
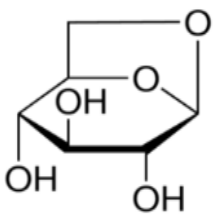
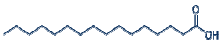
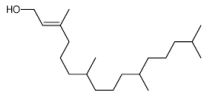
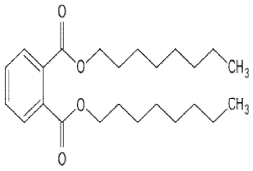
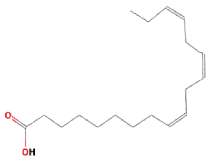


Fig. 1. Shows GC-MS Chromatogram of methanolic extract of the normal healthy leaves of *Terminalia arjuna*

Table 1. List of bio-active compounds identified from methanolic extract of normal leaf of *Terminalia arjuna* using GC-MS analysis

Peak	Retention time (min)	Area %	Name of the compound
1	6.165	4.21	
2	8.225	0.68	(3S)-(-)-3-Acetamidopyrrolidine
3	9.548	17.51	.beta.-D-Glucopyranose, 1,6-anhydro-
4	10.158	3.38	3,6-DIMETHYL-3-OCTENE-2,7-DIONE
5	10.275	0.84	1,5,5-Trimethyl-6-[3-acetoxybutyl]-3,6-epidioxycyclohexene
6	10.416	2.54	
7	10.867	0.87	cis-Z-.alpha.-Bisabolene epoxide
8	11.121	6.78	1,2,3-PROPANETRICARBOXYLIC ACID, 2-HYDROXY-,
9	11.227	2.61	3-BUTEN-2-OL, 4-(2,6,6-TRIMETHYL-1-CYCLOHEXEN-
10	12.248	1.60	TETRADECANOIC ACID
11	12.625	2.23	2(4H)-BENZOFURANONE, 5,6,7,7A-TETRAHYDRO-6-H
12	12.948	2.05	2,6,10-TRIMETHYL,14-ETHYLENE-14-PENTADECNE
13	13.204	0.92	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
14	13.393	2.45	Cyclopropanenonanoic acid, 2-[(2-butylcyclopropyl)methyl]-,
15	13.854	1.71	Pentadecanoic acid, 14-methyl-, methyl ester
16	14.276	19.45	n-Hexadecanoic acid
17	15.579	1.18	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-
18	15.766	0.87	OCTADECANOIC ACID, METHYL ESTER
19	15.999	22.83	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-
20	16.149	3.81	Octadecanoic acid
21	20.221	1.46	Di-n-octyl phthalate
		100.00	

Table 2. Activity of few phytochemicals identified in the methanolic leaf extract of *T. arjuna*

S.No.	Name of the compound	Molecular formula	Molecular weight	Chemical structure	Biological activity
1.	beta-D-Glucopyranose, 1,6-anhydro-	$C_6H_{10}O_5$	162		Used as a chemical tracer for biomass burning in atmospheric chemistry studies, esp airborne particulate matter. It is used as a marker for coal combustion as well as wood.
2.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256		Antioxidant, hypocholesterolemic nematicide, pesticide, lubricant, antiandrogenic, flavor, hemolytic, 5-alpha-reductase inhibitor.
3.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296		Antimicrobial, anti-inflammatory
4.	Octadecanoic acid	$C_{18}H_{36}O_2$	284		Anti-inflammatory and antiarthritic
5.	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390		Used as a Plasticizer.
6.	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	278		Anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, and antihistaminic
7.	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270		Antioxidant

4. DISCUSSION

The GC-MS chromatogram of methanolic extract of leaf of *T. arjuna* showed 21 peaks (Fig. 1) indicating the presence of 21 bio-active compounds while the methanolic extract of gall of *T. arjuna* showed 57 peaks (Fig. 2) indicating the presence of 57 phytochemicals. The methanolic extract of normal leaf had fewer compounds than the leaf gall extract of *Terminalia arjuna* (Table 1 and 3). In the leaf gall, the most abundant compound observed was 2-Furancarboxaldehyde, 5-(hydroxymethyl)- representing the peak area of 46.14% of the total area. This compound is commonly known as 5-Hydroxyl Methyl Furfural (5-HMF) and has its wide application as a potent anti-sickling agent and used for the treatment of sickle cell disease.

Table 3. List of bio-active compounds identified from methanolic extract of Galled leaf of *Terminalia arjuna* using GC-MS analysis

Peak	Retention time (min)	Area%	Name of the compound
1	6.030	46.14	2-Furancarboxaldehyde, 5-(hydroxymethyl)-
2	7.199	1.20	1-[N-Methylpiperazine]ethanol
3	8.247	0.57	1-Isopropoxy-2,2,3-trimethylaziridine (sin)
4	9.106	0.67	2-Cyclohexen-1-one, 2-hydroxy-3-methyl-6-(1-methylethyl)-
5	9.903	0.65	
6	10.012	0.38	N,N-BIS(2-HYDROXYETHYL)DODECANAMIDE
7	10.419	0.07	1,2-BENZENEDICARBOXYLIC ACID, DIETHYL ESTER
8	11.120	0.11	1,2,3-PROPANETRICARBOXYLIC ACID, 2-HYDROXY-,
9	11.254	0.09	Tricyclo[5.1.0.0(2,4)]octane-5-carboxylic acid, 3,3,8,8-tetram
10	11.750	0.44	1-Oxetan-2-one, 4,4-diethyl-3-methylene-
11	12.253	1.10	TETRADECANOIC ACID
12	12.448	0.23	1-HEPTADECENE
13	12.678	0.29	2,3-Dioxabicyclo[2.2.2]oct-5-ene, 1-methyl-4-(1-methylethyl)-
14	12.814	0.08	2-Cyclohexen-1-one, 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-bu
15	12.948	0.61	2,6,10-TRIMETHYL,14-ETHYLENE-14-PENTADECNE
16	13.202	0.17	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
17	13.269	0.09	Pentadecanoic acid
18	13.393	0.52	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
19	13.731	0.08	6,6,7-Trimethyl-octane-2,5-dione
20	13.845	0.76	Hexadecanoic acid, methyl ester
21	14.118	0.46	9-Hexadecenoic acid
22	14.347	13.15	n-Hexadecanoic acid
23	14.478	0.29	1-Nonadecene
24	14.821	0.07	EICOSANOIC ACID, METHYL ESTER
25	15.231	0.23	Heptadecanoic acid
26	15.394	0.15	1-Hexadecanol
27	15.499	1.06	9,12-Octadecadienoic acid, methyl ester, (E,E)-
28	15.678	0.11	Phytol
29	15.759	0.49	Octadecanoic acid, methyl ester
30	16.018	14.73	6-Octadecenoic acid, (Z)-
31	16.203	7.02	Octadecanoic acid
32	17.211	0.09	2,5-METHANO-1H-INDEN-7(4H)-ONE, HEXAHYDRO-
33	17.316	0.15	1-OCTADECANETHIOL
34	17.551	0.23	EICOSANOIC ACID, METHYL ESTER
35	17.973	1.05	EICOSANOIC ACID
36	19.067	0.08	NONADECANOIC ACID
37	19.354	0.16	Octadecyl trifluoroacetate
38	19.806	0.31	DOCOSANOIC ACID, METHYL ESTER
39	20.219	0.85	Di-n-octyl phthalate
40	20.428	0.37	Docosanoic acid
41	22.990	0.09	TETRACOSANOIC ACID, METHYL ESTER
42	23.426	0.05	1-Hexadecanol, 2-methyl-

Peak	Retention time (min)	Area%	Name of the compound
43	24.165	0.20	2,6,10,14,18,22-TETRACOSAHEXAENE, 2,6,10,15,19,23-
44	24.541	0.24	17-Pentatriacontene
45	24.784	0.10	FURAN, 4,5-DIETHYL-2,3-DIHYDRO-2,3-DIMETHYL-
46	25.456	0.08	9-Octadecenal, (Z)-
47	26.006	0.09	.beta.-Sitosterol
48	27.200	0.06	.beta.-Tocopherol
49	27.466	0.08	.beta.-Sitosterol
50	27.685	0.14	CHOLESTA-4,6-DIEN-3-OL, BENZOATE, (3.BETA.)-
51	28.024	0.37	.beta.-Sitosterol
52	28.558	0.22	dl-.alpha.-Tocopherol
53	31.234	0.27	Stigmasterol
54	32.642	1.90	.beta.-Sitosterol
55	34.105	0.18	d-Norandrostane (5.alpha.,14.alpha.)
56	36.292	0.36	4,4,6A,6B,8A,11,11,14B-OCTAMETHYL-1,4,4A,5,6,6A,6B
57	37.752	0.30	9,19-Cyclolanost-24-en-3-ol, acetate, (3.beta.)-
		100.00	

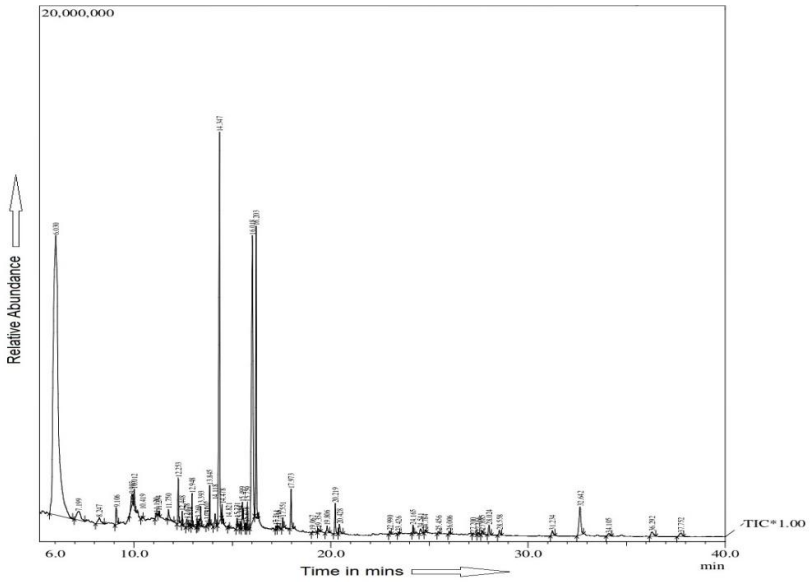
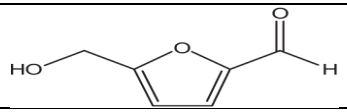
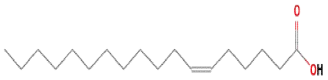
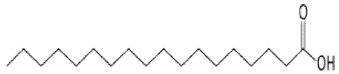
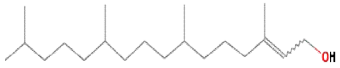
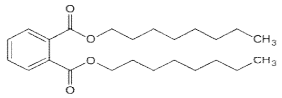
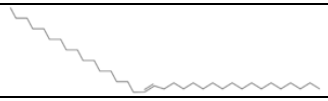


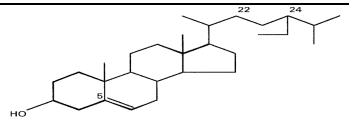
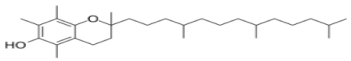
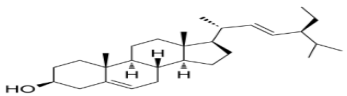
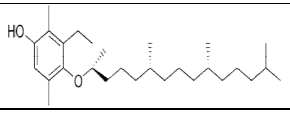
Fig. 2. Shows GC-MS Chromatogram of methanolic extract from the galled leaves of *Terminalia arjuna*

5-HMF has undergone pre-clinical testing as an efficient anti-sickling agent in the treatment of the deadly sickle cell disease [12]. It was found that 5-HMF increases oxygen affinity of sickle red blood corpuscles and inhibits hypoxia induced sickling in a concentration dependent manner [16], hydroxycarbamide addition enhanced this effect [17].

This is the only anti-sickling agent, undergoing clinical trials which directly modifies the Hb (haemoglobin) structure [18]. 5-Hydroxy Methyl Furfural (Aes-103), is an aldehyde therapeutic agent that forms a reversible Schiff base linkage primarily with the N-terminal amino group of alpha-globin resulting in a dose-dependent increase in oxygen affinity [12,16,19]. New add ref 5-HMF increased the oxygen affinity and delay time of HbS (Sickle haemoglobin), this makes this molecule an ideal anti-sickling therapeutic agent [20].

Table 4. Activity of few phytochemicals identified in the methanolic gall extract of *T. arjuna*

S.No.	Name of the compound	Molecular formula	Molecular weight	Chemical structure	Biological activity
1.	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	$C_6H_6O_3$	126		Anti-sickling agent to treat sickle cell anaemia.
2.	6-Octadecenoic acid, (Z)-	$C_{18}H_{34}O_2$	282		Used in cosmetic formulations, anti-aging agent.
3.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256		Antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic
4.	Octadecanoic acid	$C_{18}H_{36}O_2$	284		Anti-inflammatory and antiarthritic
5.	Phytol	$C_{20}H_{40}O$	128		Antinociceptive , antioxidant, antimicrobial, anti-inflammatory, antiasthamatic, anti cancer and anti-allergic activity
6.	Eicosanoic acid	$C_{20}H_{40}O_2$	312		Used to treat skin inflammation and reparation..
7.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296		Antimicrobial, anti-inflammatory
8.	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390		Used as a Plasticizer.
9.	17-Pentatriacontene	$C_{35}H_{70}$	491		Anti-septic property

S.No.	Name of the compound	Molecular formula	Molecular weight	Chemical structure	Biological activity
10	Beta-sitosterol	$C_{31}H_{52}O_2$	456		Hypocholesterolemic, also relieve symptoms of benign prostatic hyperplasia.
11.	dl-.alpha.-Tocopherol	$C_{31}H_{52}O_3$	472		dl-alpha-Tocopherol is a synthetic form of vitamin E, a fat-soluble vitamin with potent antioxidant properties.
12.	Stigmasterol	$C_{29}H_{48}O$	412		Anti-angiogenic and cancer effects.
13.	beta.-Tocopherol	$C_{28}H_{48}O_2$	416		Antioxidant activity

Another advantage of using 5-HMF as an anti-sickling agent is, it has no known side effects [11]; No adverse effects detected on the red blood corpuscles (RBCs); No signs of haemolysis , oxidation or denaturation observed when the sickled haemoglobin incubated with 5-HMF [16]. Infact 5-HMF was found inhibiting haemolysis under sheer stress *in vitro* [21]. Also, plasma and tissue proteins were not observed to inhibit binding to 5-HMF in HbS.

No binding of 5-HMF with serum albumin, myoglobin, or immunoglobulins was observed [16].

It was observed that when some healthy normal volunteers were given the single oral doses of 5-HMF, they very well-tolerated, rapidly absorbed and preferentially taken up into the RBCs relative to plasma [10,17,21].

Other benefits of using 5-HMF included, preventing dehydration of sickled RBCs during deoxygenation, inhibiting two of the main cation pathways that contribute to dehydration, the deoxygenation induced cation conductance (Psickle) and the Gardos channel [22].

Fens et al. [23] reported that 5-HMF increased the capacity of RBCs to generate nitric oxide (NO) to promote vasodilation and blood flow, as a hypothetical, added benefit to reducing the rate of Hb polymerization.

Additionally, 5-HMF had been shown to protect from oxidative stress and provide broad antioxidant effects, as evidenced by scavenging free-radical species, reduction of reactive oxidant species and membrane protein oxidation, as well as upregulation of genes implicated in enzymatic antioxidant defence and DNA repair [24,25].

Other important bio-active compounds with major biological activity observed in the methanolic leaf extract and methanolic gall extract of *T. arjuna* were listed in the Table 2 and Table 4 respectively. It was observed that most of the phytochemicals identified in the GC-MS analysis of both the samples i.e. leaf and gall of the selected plant showed pharmacological importance and thus can be further researched for their medicinal use.

5. CONCLUSION

GC-MS analysis of the methanolic extract of leaves and galls of *Terminalia arjuna* revealed the presence of several bioactive compounds which are medicinally valued. The present study showed a considerable increase in the number of phytochemicals post insect attack and gall development in the tested plant. An important phytochemical, 5-Hydroxy Methyl Furfural (5-HMF), an aromatic aldehyde, formed in the leaf galls, is of great economic value. It has been used as an anti-sickling agent to treat fatal sickle cell disease.

The present study also revealed the presence of various phytochemicals in the methanolicextract of *T. arjuna* leaves and galls which are medicinally used, thus proving the plant's pharmaceutical importance. Further work related to phytochemicals isolation will surely brighten the plant's pharmacological profile.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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