International Research Journal of Natural and Applied Sciences
Vol. 2, Issue 10, Oct 2015 IF- 2.818 ISSN: (2349-4077)
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Website: www.aarf.asia Email : editor@aarf.asia , editoraarf@gmail.com

PHYTOCHEMICAL INVESTIGATION AND IN VITRO ANTIBACTERIAL EVALUATION ON STEM EXTRACTS OF HELINUS MYSTACINUS

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ABSTRACT

The aim of this study was to identify and characterize compounds from the stem extracts of Helinus mystacinus (Rhamanceae) and evaluating its antibacterial activity. The plant material was collected from Jimma area around Aba Jifar Palace south Western Ethiopia. For isolation of the compound, the dried stem parts powder of Helinus mystacinus was subjected to cold extraction with petroleum ether, chloroform, acetone and methanol. Antibacterial evaluations of crude extracts of stem of the plant were screened using in vitro method against four standard bacterial species. Staphylococcus aureus (ATCC25903), Escherichia coli (ATCC 25722), Pseudomonas aeruginosa DSMZ (1117) and Salmonella thyphimurium (ATCC 13311). Out of the four extracts chloroform extract showed relatively good antibacterial activity and hence were chosen for isolation of compounds. The chloroform extract was subjected to column over silicagel with PE: EA as eluting solvents. Four compounds ***HMS-1****,* ***HMS-2****,* ***HMS-3*** *and* ***HMS-4*** *isolated. The IR,* ^{1}*H-NMR,* ^{13}*C-NMRand DEPT-135 spectra of all the compounds were recorded.* ***HMS-1*** *and* ***HMS-2*** *were concluded as β-Sitosterol and betulinic acid, respectively, after comparing spectral data of compounds with literature reported.* ***HMS-3*** *and* ***HMS-4*** *were not fully characterized. Anti bacterial evaluation of pure isolates was done and* ***HMS-1*** *and* ***HMS-4*** *have less activity towards the four strains where* ***HMS-2*** *and* ***HMS-3*** *almost has no activity against the strains.*

1.0 Introduction

1.1 Natural Products

The interaction between man and plants is as old as the history of mankind. Since antiquity man has studied plants and animals, particularly as a source of food and to ward off ailments. As time has gone man was able to distinguish between harmful or poisonous and useful plants. Some plants were found useful as food while others showed beneficial effect against various types of diseases. Different living organisms produce substances which has a pharmacological or biological activity in nature. Those compounds or substances which are obtained from these different living organisms are known as natural products. Plants synthesize two classes of chemical compounds which are classified as: primary metabolites and secondary metabolites, Primary metabolites are those which are common to all species and can be subdivided into proteins, carbohydrates, lipids and nucleic acids. These four groups of materials are defined according to the chemical structures of their members. The secondary metabolites are often referred to as "natural products".

1.1.2 Classification of Natural Products

Natural products further are classified into three main groups: terpenes (such as plant volatiles, cardiac glycosides, carotenoids and sterols), phenolics (such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignin) and nitrogen containing compounds (such as alkaloids and glucosinolates). The taxonomy is based on the way by which the materials were prepared. The reaction path leading to a particular natural product is called the *biosynthetic pathway*, and the parallel event is known as the *biogenesis* [1]. The most important areas of organic chemistry on natural products consist of the isolation, their structure elucidation, their chemistry, synthesis, biosynthesis, and its uses [1, 2].

1.1.3. Medicinal use of Natural Products

The use of medicinal plants as a source of medicine is based on the experience of many generations of traditional physicians and herbal practitioners found in different ethnic societies. Plants which have active constituents, commercially needed is investigated throughout the world for its active constituents. A lot of these active constituents are natural products. Secondary metabolites and their derivatives are usually used as drugs or drug leads [3].Natural products have played a very important role in health care and prevention of

diseases for thousands of years. The ancient civilizations of the Chinese, Indians and North Africans provide written evidence for the use of natural sources for curing various diseases. The natural products are screening out by different programs for the biological active constituents, which lead to a new drug, such as taxol which is used for the treatment of cancer [4]. The natural products can be protective agents, anti-oxidant, pheromones, anti-feedants attractants and also show various ecological effects in regulating the interaction between the different living organisms. Phytochemical survey shows that the natural products also act as botanical and evolutionary "markers". A lot of countries in Africa, Asia and Latin America use traditional medicine (TM) to meet some of their primary health care needs. In Africa, up to 80% of the population uses traditional medicine for primary health care. Traditional medicine has maintained its popularity in all regions of the developing world and its use is rapidly spreading in the industrialized countries. Over one-third of the population in developing countries lack access to essential medicines [5]. In Ethiopia up to 80% of the population uses traditional medicine due to the cultural acceptability of healers and local pharmacopeias, the relatively low cost of traditional medicine and difficult access to modern health facilities [6].

Use of medicinal plants as a source of medicine is based on the experience of many generations of traditional physicians and practitioners found in different ethnic societies. The utilization of medicinal plants in modern medicine suffers from the fact although plants are used at the time to prevent or cure disease, but scientific evidence in terms of modern medicine is lacking in many cases. However, it is with development of scientific knowledge in pharmacology has become necessary to provide scientific bases as to whether or not it is justified to use a plant and its active principles for scientists and has been subjected to scientific investigations. There are different systems of treatment by medicinal plants in different regions of the world, including Ethiopia.

Ethiopia has a flora that is extremely rich in its diversity. It is therefore not surprising that some of these plants have chemical compounds of therapeutic value that may be used in the treatment of major diseases such as HIV/AIDS, malaria, cancer, etc [7]. The use of medicinal plants as medicine is still wide spread in Ethiopia, and its acceptability, availability and popularity is no doubt as about 90% of the populations use it for health care needs [8]. There

are a lot of medicinal plants in Ethiopia that have potential natural products, but not investigated thoroughly.

The *Helinus mystacinus* is the species under the family of Rhamnaceae. It is a climber with pubescent coiled tendrils. Branches reddish, pubescent. Leaves alternate, ovate; margins entire. Flowers with a distinct pentagonal disk, in auxiliary umbels, greenish white. Sepals ovate-deltate, pubescent. Petals obovate, rolled around the stamens. Fruit obovoid, pubescent, tuberculate. *Helinus mystacinus* is one of the plants grows in Ethiopia and it is used by Shinasha people for curing malaria and abdominal pain [9]. The phytochemical screeninig of the plant recently done by Getahun *et al* indicated that the plant contains different types of natural products (alkaloids, tannins, saponins, terpenes, flavonoids Anthraquinones and glycosides).

Apart from this report there are no other reports on biological activity and phytochemical studies on this plant. Hence the present study was conducted to study the antibacterial activity of the crude extracts of *Helinus mystacinus* and to isolate and characterize compounds from the extracts.

2.0 Review of literature

There has been extensive study on the biological activity and phytochemicals from the plant family Rhamnaceae. Some of the phytochemical studied are reviewed below. Rhamnaceae is a family of about 55 genera and 900 species, cosmopolitan in distribution, especially warm temperate regions. *Rhamnus, Ceanothus* and *Ziziphus* are the chief genera of this family [10]. Many of this family are species of *Ceanothus* are cultivated as ornamentals. Plants mostly shrubs and trees, sometimes lianoid, rarely herb. The Rhamnaceae have a worldwide distribution, but are more common in the subtropical and tropical regions.

2.2. Review of natural products isolated from plant family Rhamanceae

Phytochemical study on *Rhamnus prinoides* **(**Amharic name 'Gesho') has led to isolation and characterization of different classes of compounds such as flavonoids and anthraquinones. Cohumlone(**1**), humulone (**2**), adhumulone **(3**), colupulone **(4**), lupulone (**5**), adhupulone (**6**), quercetin (**7**), kaempferol (**8**) and one anthraquinones compound (**9**) were isolated and characterized from the leaves of *R. prinoide* (Figure.2) [11] .

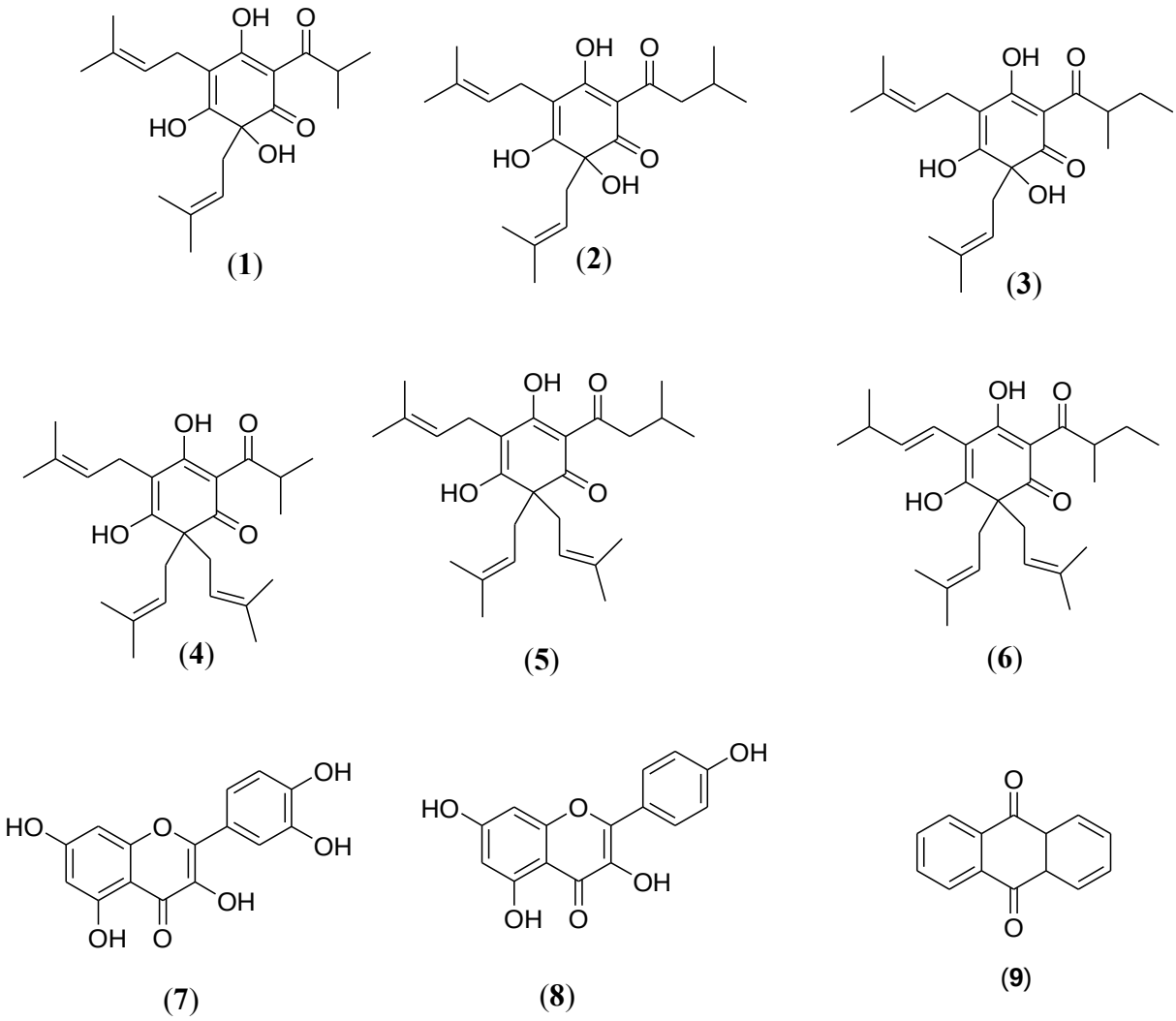


Figure 1. Isolation and characterization of different classes of compounds from *Rhamnus prinoides* [11].

A study on extracts of *Rhamnus alaternus leaves* (Rhamnaceae**)** has resulted in three flavonoids namely :kaempferol 3-o-isorhamnoside (**10**), rhamnocitrin-3-o-isorhamninoside (**11**) and rhamnetin-3-o –isorhamninoside (**12**) , and kaempferol (**8**) [12].

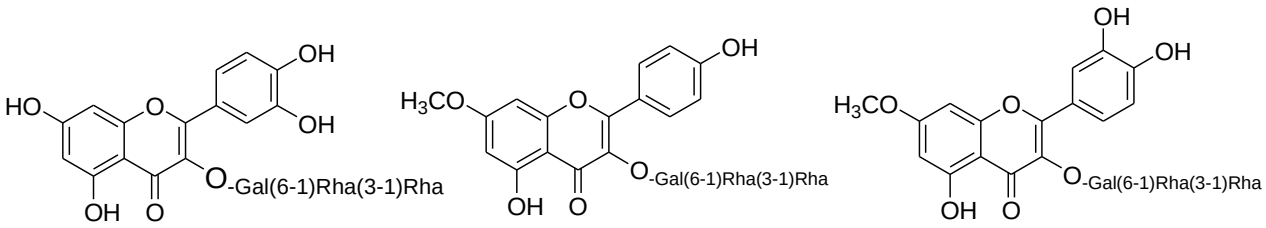


Figure 2. Flavonoids isolated from extracts of *Rhamnus alaternus* leaves [12].

Another study on *Rhamnus alaternus* indicated that, one new ceanothane- type triterpene and one new sesquiterpene, together with two known triterpenes were isolated from the fruits of *Rhamnus alaternus*. The structures of two new compounds were elucidated as 2α-aldehydo-A (1)-norlup-20(29)-en-27, 28-dioic acid (zizyberanalic acid) (**14**) and zizbernone (**15**), while the two known triterpenes were identified as zizyberanalic acid (**16**) and Ursolic acid (**17**) [13].

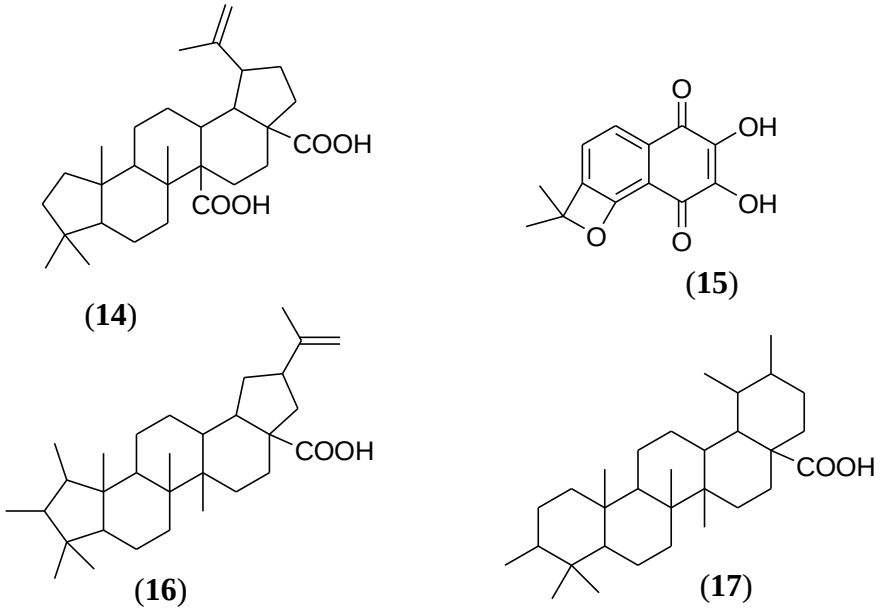


Figure 3. Triterpenoid isolated from the fruits of *Rhamnus alaternus* [13].

Phytochemical study on *Ziziphus jujube species* (Rhamanceae) lead to isolation and characterization of different class of compounds such as alkaloids, flavonoids, terpenoids and some phenolic compounds. Several pentacyclic triterpenoic acids like betulinic acid (**18**), betulonic acid (**19**) and oleanolic acid (**20**) have been isolated from the fruit of *Ziziphus jujube* [14].

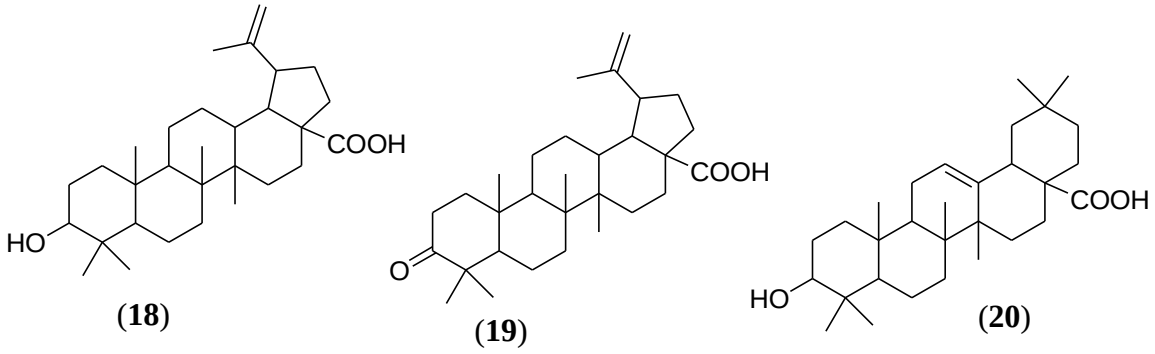


Figure 4. Pentacyclic triterpenoid extracts from *Ziziphus jujube species* [14].

2.3. Review of natural products isolated from *Helinus mystacinus*

The genus *Helinus* is in the family *Rhamnaceae* in the major group *Angiosperms* (Flowering plants). *Helinus mystacinus* is one of the plants grows in Ethiopia and it is used by Shinasha people for anti malaria and abdominal pain. A recent study on this plant conducted by Getahun *et al* from our lab also indicated presence of betulinic acid (**18**) and benzoic acid (**21**) from *Helinus mystacinus* root part. Also triterpenoid (**22**) has been isolated which was not fully characterized (Figure,**1**) [15].

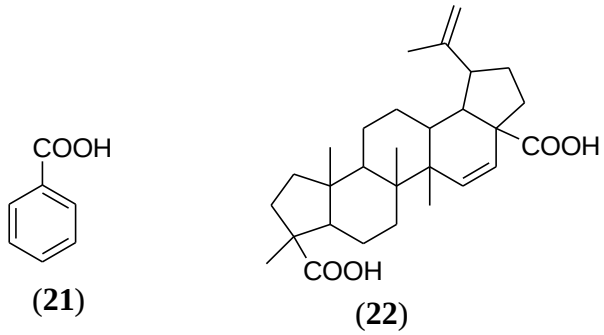


Figure 5. Compounds isolated from *Helinus mystacinus* [15].

2.4 Statement of the problem

Since ancient time, traditional healers long have used plants to prevent or cure infectious disease. Many of these plants have been investigated scientifically for antimicrobial activity, and a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms. A number of these agents appear to have structures and modes of action that are distinct from those of the antibiotics in current use, suggesting that cross-resistance with agents already in use may be minimal. So, it is worthwhile to study plants and plant products for activity against resistant bacteria. Traditionally people in Ethiopia have been used medicinal plants to treat different disease and also it has great contribution in primary health care systems, however, most of the existing medicinal plants are not well investigated to identify the chemical principles for their claims. *Helinus mystachinus* is one of the Ethno medicinal plant used in some parts of Ethiopia, however there is no systematic phytochemical investigation was made. Therefore the present study is focused on the isolation and identification of phytochemical from the plant and give information on the chemical constitutes including their biological importance.

3.0 Objectives

3.1 General objective

- The main objective of this study is to isolate and characterize the antibacterial active fractions of *Helinus mystacinus*.

3.2 Specific objectives

- To obtain gradient extracts of petroleum ether, chloroform, acetone, and methanol of *Helinus mystacinus* using maceration technique.
- To assess antibacterial activities of the crude extracts of *Helinus mystacinus* against four bacterial species namely *Staphylococcus aureus (ATCC25903)*, *Escherichia coli (ATCC 25722)*, *Pseudomonas aeruginosa DSMZ (1117)* and *Salmonella thyphimurium (ATCC 13311)* using *in vitro* tests.
- To isolate compounds from the crude extracts that showed relatively better antibacterial activity against the test bacterial species.
- To characterize the isolated compounds using spectroscopic techniques (IR and NMR), and to evaluate their antibacterial activities.

3.3. Significance of the study

Helinus mystacinus is a medicinal plant used by Shinasha people. There is no much information regarding the phytochemical and biological activity studies from this plant. So this present study will give us some idea:-

- About the antibacterial activity of the various extract
- The chemical constituents of this plant.
- To get some data that can be used for future pharmacological and bioprospecting studies.

4.0 Methods and Materials

4.1. Materials and Chemicals

All chemicals used during this work were products of Aldrich and Sigma Chemical Companies of Analytical grade. The solvents used were petroleum ether, chloroform, acetone, ethyl acetate, and methanol. Melting point was determined in capillary tube with a digital

electro thermal melting point apparatus (Griffin). TLC was run on a 0.25 mm thick layer of silica gel GF254 (Merck) on aluminum plate. Spots were detected by observation under UV light (254 nm and 365 nm) and then under iodine vapour. Column chromatography was performed using silica gel (60-120 mm mesh size) Merck. Samples were applied on the top of the column by adsorbing on silica gel. ^{1}H and ^{13}C- NMR were obtained on Bruker-Avance instrument at 400MZ and 100MZrespectively, with TMS as internal standards and $CDCl_3$ as a solvent. IR spectra were recorded with a Perkin-Elmer BX Spectrometer (400-4000 cm^{-1}) in KBr. All spectroscopic analysis were carried out at Department of chemistry, Addis Ababa University.

4.2 Collection and preparation of plant material

The plant material of *Helinus mystacinus* was collected from Jimma area around Aba Jifar Palace in South West Ethiopia in November, 2012. The plant material was identified by Dr. M.Remesh, Botanist of Department of Biology, Jimma University and voucher number 00191 was given and specimen of *Helinus mystacinus* was deposited in the herbarium. The collected plant material (2kg) was cut into small pieces, and air dried in laboratory at room temperature. The dried plant material was then powdered in to coarse powder.

4.3 Extraction of plant material for preliminary activity

100 gm of plant material from stem of *Helinus mystacinus* was soaked in 500ml of various solvents such as petroleum ether, chloroform, acetone and methanol respectively using maceration technique for 72 hrs with constant and continuous shaking using HY-5A Manoeuvre style vibrator shaker in each solvent [17]. TLC of each extract was examined and antibacterial activity of was checked. The resulting crude extract of each solvent was weighed and stored in refrigerator below 4 ^{0}C ,until used for microbial assay, TLC fractionation and isolation using column chromatography [18-19].

4.4 Antibacterial activity test

Bacterial strains used for evaluation of antibacterial activities of the crude extracts and fractions were Gram-positive strain *Staphylococcus aureus (ATCC25903)* Gram-negative strains *Escherichia coli (ATCC 25722)*, *Pseudomonas aeruginosa (DSMZ 1117)* and

Salmonella thyphimurium (ATCC 13311). These standard bacterial strains were obtained from the Department of Biology, Jimma University. For bioassays, suspension of approximately 1.5 × 108 bacterial cells/ml in sterile normal saline were prepared and about 1.5 ml of it was uniformly seeded on Mueller-Hinton-Agar medium with 3–4 mm thickness in 12 cm × 1.2 cm glass petridishes, left aside for 15 min and excess of suspension was then drained and discarded properly. Each of the discs which are approximately 6 mm in diameter was cut from Whatman No.1 filter paper [20-22]. The discs were put into a petri dish and then sterilized in the oven at 120 ^{0}C for 1hr. The discs were then impregnated with the extract by soaking in the extract for 24 hours. Bioactivity was determined by measuring Diameter of Inhibition Zones (DIZ) in mm [23-26].

4.5. Bulk extraction

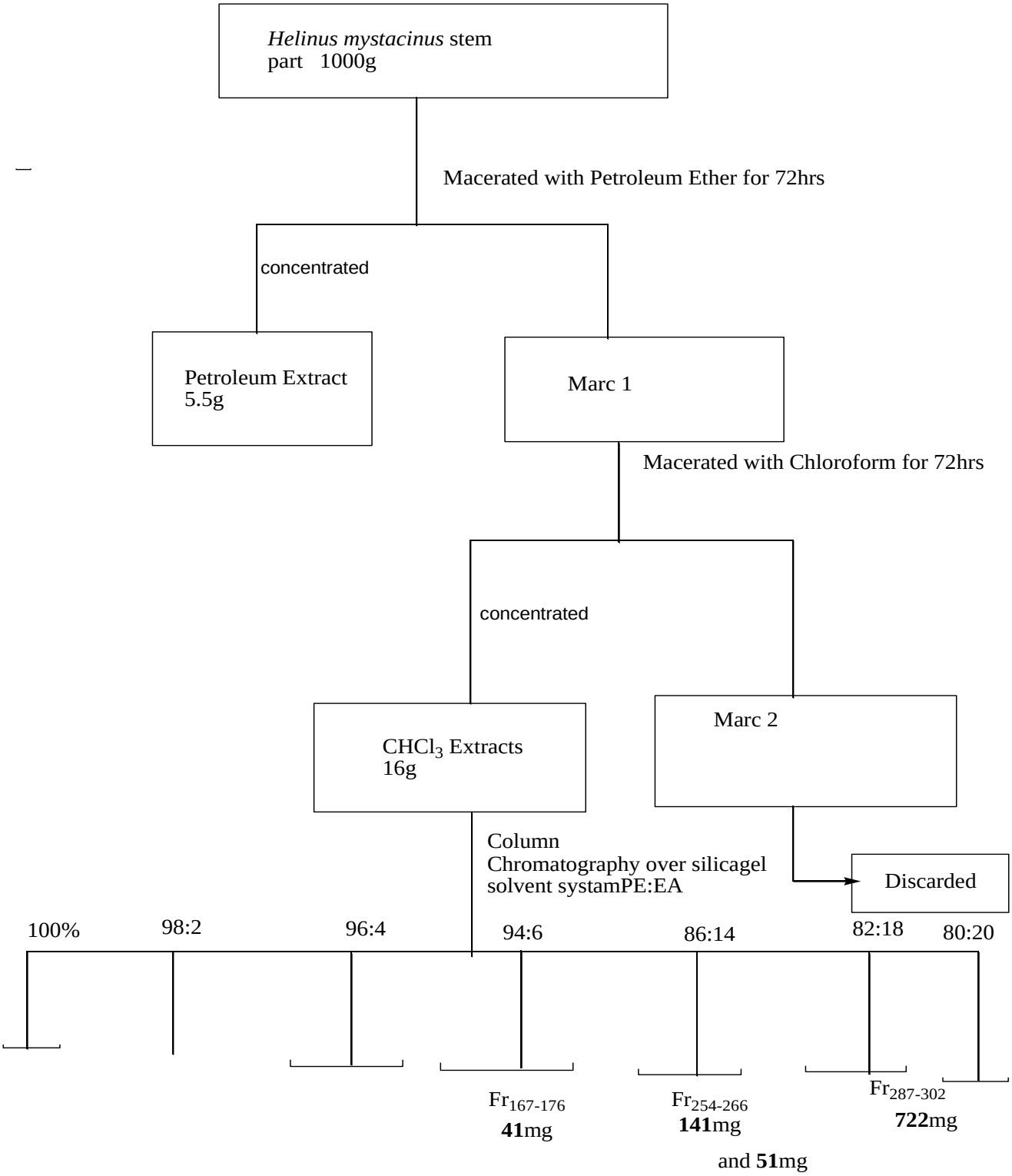
The air dried and powdered plant material (1000gm) was first soaked with 2.5litre petroleum ether for 72 hours and the extract was filtered and concentrated. The solvent free marc was then soaked with 2.5litre of chloroform for 72 hours and the extract was collected. This filtrate was evaporated under reduced pressure using the Rota vapor to afford 16g solid residue.

4.6 Isolation of compounds from stem of *Helinus mystacinus*

Eight (8g) crude extract was dissolved in chloroform and applied to a silica gel (100g) column which was packed with silicagel using petroleum ether (100%). The column was eluted using petroleum ether petroleum ether: ethyl acetate combination with increasing amounts of ethyl acetate. 427 fractions each of 20ml were collected. The following are the various fractions collected in each polarity; petroleum ether (100%): fractions 1-42, petroleum ether: ethyl acetate (98:2): fractions 43-81, petroleum ether: ethyl acetate (96:4): fractions 82-140, petroleum ether: ethyl acetate (94:6): fractions 141-182, petroleum ether: ethyl acetate (92:8): fractions 183-201,petroleum ether: ethyl acetate (90:10): fractions 202-224, petroleum ether: ethyl acetate (88:12): fractions 225-250, petroleum ether: ethyl acetate (86:14): fractions 251-276, petroleum ether: ethyl acetate (84:16): fractions 277-305, petroleum ether: ethyl acetate (82:18): fractions 306-330, petroleum ether: ethyl acetate (80:20): fractions 331-355,

petroleum ether: ethyl acetate (70:30): fractions 356-376, petroleum ether: ethyl acetate (60:40): fractions 377-402,and petroleum ether: ethyl acetate (50:50): fractions 403-427.

Fractions from 167-176 and 287-302 showed the single spots on TLC in UV light and iodine vapor. These fractions were pure and after the removal of the solvent using the Rota vapor afforded **41**mg of the compound **HMS-1** and **722**mg of compound **HMS-3** from fractions 167-176 and 287-302 respectively. Fraction 254-266 showed two spots on TLC using the solvent system chloroform: ethyl acetate (4:1) one spot in iodine vapor and one spot under UV light (254 nm).These fraction were subjected to further purification. It was seen that one of compound was soluble in petroleum ether and the other insoluble. The compounds were then separated based upon this solubility; the purity was checked again using TLC. **141**mg of **HMS-2** and 51mg of **HMS-4** in the pure form was obtained after removal of solvents. Scheme 1 shows general procedures of mass extraction and various compounds isolated from *Helinus mystacinus* stem.



Scheme 1. General procedures followed in the extraction and isolation of compounds from *Helinus mystacinus.*

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5.0 Result and Discussion

5.1 Yield of various extracts obtained from prelimnary extraction

The amounts of the crude extracts and their percent yields obtained from pilot extraction of plant material are given below (Table 1).

Table 1. Amounts of crude extracts obtained from stem of *Helinus mystacinus.*

Solvent	Stem of used=100gm	
	Weight of crude extract (g)	% yield
Petroleum Ether	0.33	0.33 %
Chloroform	1.72	1.725 %
Acetone	0.455	0.464 %
Methanol	3.699	3.92 %

5.2 Evaluation of antibacterial activities of crude extracts from stem of *Helinus mystacinus*

The antibacterial activity of the petroleum extract, chloroform extract, acetone extract and methanol extract of *Helinus mystacinus* was tested using agar disc diffusion method (section 4.4) against gram-negative pathogens (*S. thyphimurium*, *E. coli* and *P. aeruginosa)* and gram-positive pathogen (*S. aureus)* respectively. Two different concentration of each plant extracts were checked , that were 100mg/ml and 50mg/ml .All extracts showed moderate activity towards most of the bacterial strain. Chloroform extract showed relatively better activity at both 50mg/ml and 100mg/ml concentration. So based on this activity chloroform extract was selected for further isolation of compounds by column. The zone of inhibition obtained for the various extracts are summarized in (Table 2) below**.**

Table 2. Antibacterial inhibition zones (in millimeter) of crude extracts of stem of *Helinus mystacinus* .

Strain	concentration	Pet. Ether extract	Chloroform extract	Acetone extract	Methanol extract	Ciprofloxacin	DMSO
E. coli	100mg	-	14	12	12	28	-
	50mg	13	19	16	12	36	
	Average	13	16.5	14	12	32	
P. aeruginosa	100mg	-	11	9	11	31	-
	50mg	13	19	16	12	36	
	Average	13	15	12.5	11.5	33.5	
S. thyphimurium	100mg	16	11	16	12	31	-
	50mg	17	33	24	26	31	-
	Average	16.5	22	20	19	31	
S. aureus	100mg	9	13	15	11	35	-
	50mg	16	29	17	26	36	-
	Average	12.5	21	16	18.5	35.5	

5.3 Characterization of compounds

In this phytochemical investigation, four compounds (**HMS-1, HMS-2, HMS-3**and **HMS-4,)** were isolated from chloroform extract of the stem of *Helinus mystacinus*. The first two compounds (**HMS-1** to **HMS-2)** were fully characterized based on spectroscopic (NMR and IR) data and literature reports. Where as the rest two compounds (**HMS-3**&**HMS-4**) were characterized partially. The details of structural elucidations of the compounds are discussed in the following sub-sections.

5.3. 1 Structural elucidation of HMS-1

HMS-1 was isolated as white crystalline needle like substance obtained from the combined fractions of 167-176 that were eluted from column using 6% ethyl acetate in petroleum ether. Its Rf value was determined as 0.87 in chloroform-ethyl acetate.

The IR spectrum of **HMS-1** (Appendix.1) showed absorption band at 3431cm^{-1} that indicated characteristic absorption of -O-H stretching. Absorption at 2919 $cm^{-1}$and 2834 cm^{-1} was also seen which showed the aliphatic asymmetric and symmetric C-H stretching of CH_2. Other absorption frequencies include 1596 cm^{-1} as a result C=C stretching. The absorption frequency at 1054 cm^{-1} corresponds to cycloalkane. The out of plane C-H vibration of unsaturated part was also observed at 881cm^{-1}.

The proton NMR (Appendix.2) analysis exhibited a broad triplet at δ 5.37 and a multiplet at δ3.50 corresponding to H-6 olefinic proton and H-3α proton, respectively. Angular methyl protons at δ0.69(s), and δ 0.82(s) were seen which corresponds to C-18 and C-19, proton respectively. The observed ^{1}H-NMR spectral values are summarized in (Table 3) below.

Table 3. ^{1}H-NMR ($CDCl_3$, 400MHz) data of compound **HMS-1** along with reported data of β-sitosterol [27-37].

H	**^{1}H-NMR** data of **HMS-1** (ppm)	Reported **^{1}H-NMR** data of β-sitosterol[27-37]
H-6	5.37	5.34
H-3	3.54	3.52
Me-19	0.94	1.01
Me-21	0.93	0.92
Me-29	0.86	0.84
Me-26	0.84	0.83
Me-27	0.82	0.81
Me-18	0.69	0.68

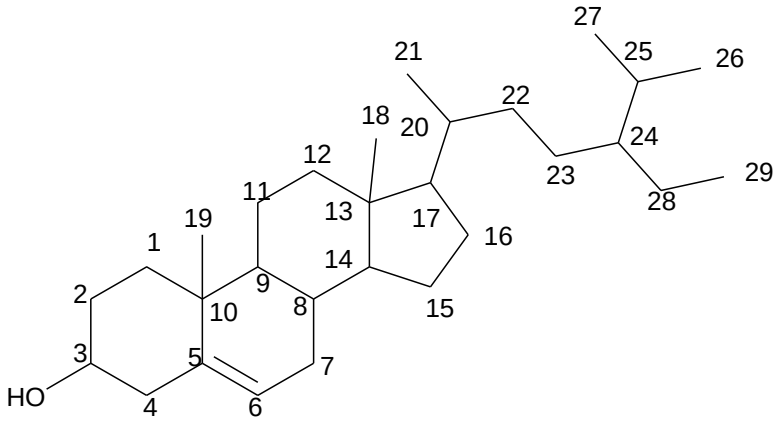
The ^{13}C-NMR spectrum of **HMS-1**(Appendix. 3) showed 29 signals which indicated 29 carbons including six methyls, nine methylenes, eleven methine and three quaternary carbons in **HMS-1.** The presence of one double bond was confirmed to the presence of two olefinic carbons signals at δ140.75 and δ121.74. A down field signal at δ71.82 was assigned to C-13 which was connected to –OH.

DEPT-135 spectrum also confirmed the presence of methyl carbon at δ11.95(C-29),11.99 (C-27), 19.41 (C-25), 18.78 (C-21) and 11.87 (C-18) , methine carbons at 71.82 (C-3), 121.74 (C-6), 31.89 (C-8), 50.12 (C-9), 56.76 (C-14), 56.04 (C-17), 36.15 (C-17) and δ23.06 (C-24) . The absence of peaks at δ140.75 (C-5), δ36.51 (C-10) and δ42.3 (C-13) in the DEPT-135 spectrum which were observed in the ^{13}C-NMR spectrum also confirmed presence of quaternary carbon atoms in **HMS-1**. The experimental results of IR, ^{13}C-NMR and DEPT-135 chemical shift value on spectral data (Table. 4) were consistent with that of reported β-sitosterol in literature [33-37]. To the best of our knowledge this is the first to report of isolation of β-sitosterol from *Helinus mystacinus.*

Table 4. ^{13}C-NMR and DEPT-135 ($CDCl_3$ 100MHz) spectral data for compound **HMS-1** and comparison with reported data of β-sitosterol [31-37].

Carbon	^{13}C-NMR of **HMS-1** (ppm)	DEPT-135 of **HMS-1** (ppm)	^{13}C-NMR of β-sitosterol [31-37] (ppm)	Nature of carbon
1	37.25	37.25	37.31	C H_2
2	31.65	31.65	31.82	C H_2
3	71.82	71.82	71.92	CH
4	42.32	42.32	42.43	CH_2
5	140.75	-	140.90	C
6	121.74	121.74	121.90	CH
7	31.92	31.92	32.12	CH_2
8	31.90	31.89	32.02	CH
9	50.12	50.12	50.83	C H
10	36.51	-	36.60	C
11	21.08	21.08	21.13	CH_2
12	39.77	39.77	40.32	CH_2
13	42.28	-	42.62	C
14	56.76	56.76	56.81	CH
15	24.31	24.31	24.31	CH_2
16	28.26	28.26	28.42	C H_2
17	56.04	56.04	56.22	CH
18	11.87	11.87	11.94	CH_3
19	19.03	19.03	19.45	CH_3
20	36.15	36.15	36.30	CH
21	18.78	18.78	19.10	CH_3
22	33.93	33.93	34.02	CH_2
23	28.26	28.26	29.33	CH_2
24	45.7	45.7	45.7	CH
25	29.2	29.2	29.2	CH
26	19.6	19.6	19.6	CH_3
27	18.9	18.9	18.9	CH_3
28	23.0	23.0	23.12	CH_2
29	11.8	11.8	11.95	C H_3

The proposed structure of **HMS-1** is represented in figure below.



β -Sitosterol

Figure 6. Proposed structure of **HMS-1**

5.3.2 Structural elucidation of HMS-2

HMS-2 (43 mg) was obtained as white needle-like solid from the fractions 254-266 eluted with 14% ethyl acetate in petroleum ether and the R_f value of the compound was determined as 0.8 in chloroform-ethyl acetate (90:10).

Analysis of IR ($CDCl_3$) spectrum of **HMS-2** indicated that it has acidic carbonyl group, which is confirmed by presence of strong and sharp bands ranging 1810-1680 cm^{-1}. Also the IR spectrum of the **HMS-2** (Appendix. 5) showed characteristic absorption band for a hydroxyl group at 3469 cm^{-1} and the medium intensity at 1237 cm^{-1} indicates C-O stretching. Sharp absorption at 707cm^{-1} is the indicative for =C-H bending.

The ^{1}H-NMR spectrum of **HMS-2** displayed signals for olefinic hydrogen's at δ 4.47, δ 4.33 (2H, s) for H-29 and hydrogen attached to carbon bearing -OH (H-3) at δ 3.08 (1H, s), respectively. The ^{1}H NMR spectrum also exhibited signals for methyl groups at δ 0.52, 0.60, 0.72, 0.75, 1.05 and δ1.15.

Table 5. ^{1}H-NMR ($CDCl_3$, 400 MHz) data of **HMS-2** along with reported data of Betulinic acid [15, 38-43].

^{1}H	^{1}H-NMR data of **HMS-2** (ppm)	^{1}H-NMR reported data of Betulinic acid
3-H	3.08(1H,m)	4.2(1H,m)
24-H	0.72s	0.72s
25-H	0.60s	0.64s
26-H	0.75	0.86s
27-H	1.05s	0.92s
29-H	4.47,4.33(1H,m)	4.68 ,4.56(1H,m)each
30-H	1.71s	1.80s

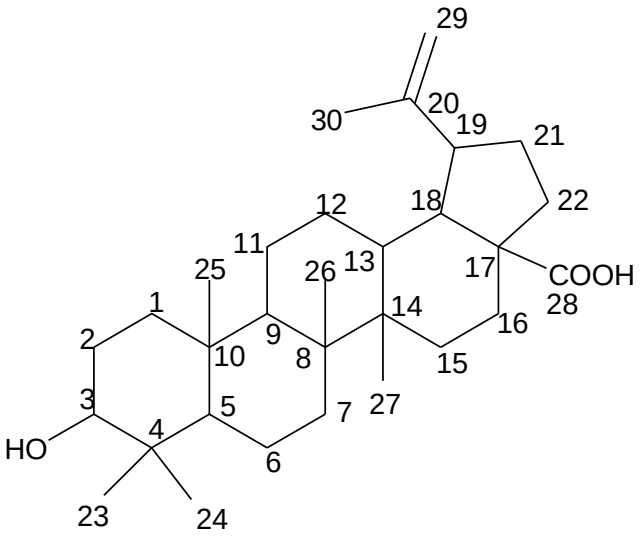
The ^{13}C NMR spectrum of **HMS-2** showed signals for thirty carbon atoms. The peak at δ 179.1 indicated the presence of acidic carbonyl group. The signals at δ150.7 and δ109.4 were attributed for the presence of olefinic carbons in the compound. These signals have been assigned to the isopropylene group at C-20 and C-29.

The DEPT-135 spectra of the compound showed 24 signals out of which eleven are negative signals indicating the presence of eleven methylene carbons in the compound. Based on the ^{13}C NMR and DEPT-135 spectral data, compound **HMS-2** was accounted to have 30 carbons comprising of six methyl, eleven methylene, six methine and seven quaternary carbons. Comparing the observed IR and NMR data with literature reports enabled us to propose the chemical structure of **HMS-2** to be identical with that of Betulinic acid [15, 38-43] (Table. 6) shows the ^{13}C-NMR and DEPT-135 data of **HMS-2** and Betulinic acid reported.

Table 6. ^{13}C NMR and DEPT-135 ($CDCl_3$ 100MHz): data of compound **HMS-2** and Betulinic acid [15, 42].

Carbon	^{13}C-NMR data of **HMS-2** (ppm)	DEPT-135dataof **HMS-2**	^{13}C-NMR of Betulinic acid[15,42]		Nature of carbon
1	38.39	38.39	38.6		CH_2
2	26.9	26.9	27		CH_2
3	78.71	78.71	78.7		CH
4	38.6	-	38.7		C
5	55.51	55.51	55.3		CH
6	18.35	18.35	18.2		CH_2
7	34.41	34.41	34.2		CH_2
8	40.73	-	40.5		C
9	50.65	50.65	50.4		CH
10	37.2	-	37		C
11	20.95	20.95	20		CH_2
12	25.61	25.61	25.4		CH_2
13	38.1	38.1	38.2		CH
14	42.49	-	42.3		C
15	30.62	30.62	30.5		CH_2
16	32.33	32.33	32.1		CH_2
17	56.3	-	56.1		C
18	49.27	49.27	49.1		CH
19	47.1	47.1	46.9		CH
20	150.7	-	150.6		C
21	30.60	30.60	30.54		CH_2
22	29.73	29.73	29.6		CH_2
23	27.82	27.82	27.5		CH_3
24	15.33	15.33	15.2		CH_3
25	15.84	15.84	15.8		CH_3
26	16.04	16.04	16		CH_3
27	14.60	14.60	14.5		CH_3
28	179.1	179.1	180.3		C=O
29	109.4	109.4	108.9		CH_2
30	19.15	19.15	19.2		CH_3

Moreover, the experimental melting point of **HMS-2** (283-285 ^{o}C) was found to be comparable to the reported melting point value of Betulinic acid (i.e., 283-285) [15, 38-43]. Therefore, based on the above information, the structure of **HMS-2** was deduced as Betulinic acid.



Betulinic acid (**18**)

Figure 7. The proposed structure of **HMS-2**

5.3.3 Structure elucidation of HMS-3 (partially characterized)

The **HMS-3** (722mg) was obtained as a white solid, soluble in methanol, from the fractions 287-302 that were eluted with 18% ethyl acetate in petroleum ether. The Rf value of the compound was 0.714 in chloroform- ethyl acetate (90:10%).

IR spectrum of **HMS-3 (**Appendix.9), the strong and sharp absorption bands at 1686 cm^{-1} indicated the presence of acidic carbonyl group in the compound. And the medium intensity at 1239 cm^{-1} indicated the C-O stretching. The ^{1}H-NMR spectrum of **HMS-3** (Appendix.10) shows the presence of acidic and olefinic protons at δ5.66,δ 5. 46, δ5.147 and δ5.10 respectively. The signals between δ1.02-0.5 were also indicate for the presence of methyl hydrogen's. ^{13}C-NMR spectrum (Appendix.11) signals at 183.00 and 182.28 shows presence of two carboxylic carbons and signals at 154.2, 144.51, 142.7 and 113.5 indicated for the presence of two olefinic carbons. The signals at 154.2 and 113.5 are characteristics values for the exomethylene carbons in lupane triterpenoids [42-43].Analysis of DEPT-135 indicated the

presence of 8 quaternary carbons and 9 methylene carbons. ^{13}C-NMR values were comparable with that of triterpenoids of lupane skeleton but have additional one carboxylic acid group and one double bonds in the ring. Review literature shows as it is similar with a compound not fully characterized from the root of this plant and from the species of the family (Rhamanceae), similar triterpenes has isolated namely zizyberanal (**21**) acid and zizyberanalic acid (**23**) from *Ziziphus jujuba* [15].

Table 7. ^{13}C- NMR and DEPT-135 ($CDCl_3$ 400MHz), data of compound **HMS-3.**

Carbon	^{13}C-NMR data of **HMS-3**(ppm)	DEPT-135 data of **HMS-3**(ppm)	Nature of Carbon
1	38.10	38.10	CH_2
2	31.87	31.87	CH_2
3	63.72	-	Quaternary
4	66.31	66.31	CH
5	21.30	21.30	CH_2
6	34.21	34.21	CH_2
7	43.43	-	Quaternary
8	54.38	54.38	CH_2
9	53.30	-	Quaternary
10	26.79	26.79	CH_2
11	29.99	29.99	CH_2
12	41.32	41.32	CH
13	45.12	-	Quaternary
14	142.70	142.70	CH
15	144.51	144.51	CH
16	59.87	-	Quaternary
17	51.02	51.02	CH
18	51.84	51.84	CH
19	154.2	-	Quaternary
20	33.46	33.46	CH_2
21	40.94	40.94	CH_2
22	33.1	33.1	CH_2
23	182.28	-	Quaternary
24	25.02	25.02	CH_3
25	23.82	23.82	CH_3
26	22.44	22.44	CH_3
27	183.00	-	Quaternary
28	113.58	113.58	CH_2
29	21.71	21.71	CH_3

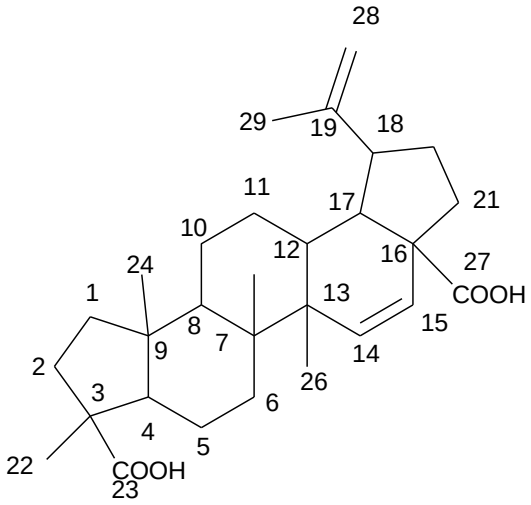


Figure 8. The proposed structure of **HMS-3**

5.3.4 Partial characterization of HMS-4

HMS-4 was isolated as green solid substance. It has an R_f value of 0.818 in a solvent system of chloroform-ethyl acetate mixture (90:10%).

IR spectrum analysis of **HMS-4** peak at 3425cm^{-1} indicated as the compound has –OH functional group, and peaks at 2918cm^{-1} and 2849cm^{-1} indicated C-H stretching of CH_2 and CH_3 respectively. Weak Peak at 1736.cm^{-1} indicated olefinic bond in **HMS-4**.

^{1}H-NMR peak at δ4.1(m) indicated proton attached to olefinic carbon and δ3.5 indicated proton attached to carbon attached to more electronegative element, and peak δ2.06 indicated proton attached oxygen.^{13}C-NMR indicated presence of fifteen carbon in **HMS-4** .DEPT-135 also confirmed presence of fifteen carbon without quaternary carbon.

5.3.5 Summary of spectral data of the isolated compounds

Compound HMS-1: the compound is a white crystalline compound, 136-138^{0}C; **^{1}H-NMR***:* 1.01 (2H, *m*, H-1), 1.37 (2H, *m*, H-2), 3.82 (1H, *m*, H-3), 2.62 (2H, *m*, H-4), 5.32 (1H, *t*,H-6), 1.93 (2H, *m*, H-7), 1.54 (1H, *m*, H-8), 0.94 (1H,*m*, H-9), 1.44 (2H, *m*, H-11), 1.69 (2H, *m*, H-12),1.10 (1H, *m*, H-14), 1.51 (2H, *m*, H-15), 4.61 (2H,*m*, H-16), 1.74 (1H, *m*, H-17), 0.67 (3H, *s*, H-18),0.98 (*s*, 3H, H-19), 1.90 (1H, *m*, H-20), 0.92 (3H, *d*,*J* = 2×9 Hz, H-21), 1.62 (2H, *m*, H-22), 1.65 (2H, *m*,H-23), 1.58 (1H, *m*, H-24), 1.56 (1H, *m*, H-25), 0.82(3H, *d*, *J* = 7

Hz, H-26), 0.80 (3H, *d*, *J* = 7 Hz, H-27), 1.52 (2H, *m*, H-28), 0.84 (3H, *t*, H-29)(Appendix-1) **^{13}C-NMR***:* 37.0 (C-1), 29.5 (C-2), 79.9 (C-3), 38.7 (C-4), 140.2 (C-5), 121.9 (C-6), 31.7 (C-7), 32.2 (C-8), 50.5 (C-9), 36.6 (C-10), 21.0 (C-11), 39.6 (C-12), 42.2 (C-13), 56.6 (C-14), 24.2 (C-15), 28.1 (C-16), 55.9 (C-17), 11.6 (C-18), 19.2 (C-19), 36.0 (C-20),18.6 (C-21), 34.0 (C-22), 26.0 (C-23), 45.7 (C-24), 29.2 (C-25), 19.6 (C-26), 18.9 (C-27), 23.0 (C-28), 11.8 (C-29).

Compound HMS-2: a White crystal partially soluble in chloroform and Rf value of 0.8 chloroform: EtOAC (9:1). **IR(KBr): Vmax**:3471.87, 239.52, 2929.87, 1685.79, 1643.35, 1454.33, 129.31, 1236.37, 707.88, 542.00; **^{1}H-NMR** (400MHz;$CDCl_3$/MeOD); δ 0.64, 0.76, 0.86, 0.92, 1.43, 1.64, 4.68 (1 H, J=2 Hz), δ 4.58 (1H, J=2Hz.1.5Hz), 1.64; **^{13}C-NMR** (100 MHz; DMSO-d6); δ 177.69 (C-28), 150.77 (C-20), 110.09 (C-29), 77.27 (C-3), 55.87 (C-5), 55.35 (C-19), 50.38 (C-9), 48.9 9 (C-18), 47.07 (C-17), 42.46 (C-14), 40.55 (C-8), 38.95 (C-4), 38.06 (C-1), 37.18 (C-22), 37.06 (C-10), 36.79 (C-13), 34.37 (C-7), 32.16 (C-16), 30.54 (C-21), 29.65 (C-15), 28.55 (C-2), 27.59 (C-23), 25.54 (C-13), 20.91 (C-11), 19.39 (C-30), 18.42 (C-6), 16.39 (C-26), 16.25 (C-25), 16.19 (C-24), 14.84 (C-27).

Compound HMS-3: the compound is a white crystalline compound, 299-302^{0}C; **^{1}H-NMR** (400MHz;$CDCl_3$/MeOD);δ5.66,δ5.64,δ 5.14 ,δ5.12,δ5.10 ;**^{13}C-NMR***:* 381.0, 31.87 , 63.72 , 66.31 , 21.3 , 34.21 , 43.43 , 54.38 , 53.3 , 26.79 , 29.99 , 41.32 , 45.12 , 142.7 , 144.51 , 59.87 , 51.02 , 51.84 , 154.2 , 33.46 ,40.94 , 33.1 , 182.28 , 25.02 , 23.82 , 22.44 , 183.0, 11.58, 21.71.

Compound HMS-4: the compound is a green crystalline compound,**^{1}H-NMR** ($CDCl_3$, 400 MHz)**:** δ4.16,414,4.13.4.11,3.5,2.06.1.43.1.3,1.27,1.12,0.94,0.89,0.8; **^{13}C-NMR***:* ($CDCl_3$, 400MHz)**:**60.44,59.52,38.14,33.93,31.94,31.24,29.71,29.38,24.68,22.7,21.06,21.0,18.38,18.26,14.13.

5.3.6 Antibacterial activity test of the isolated compounds

In the present study, the antimicrobial activity of the purified compounds were evaluated .Pure isolate (**HMS-1)** has antimicrobial activity against *E.coli*(12mm), *S. aureus(*10mm*)* ,*P.aeruginosa(*8mm*)* and *S.typhi* (9mm) .**HMS-2** did not show any activity against *S. aureus*, *p.aeruginosa* and, *S.typhi* but has less activity against *E.coli(*9mm*)*.**HMS-3** has less anti bacterial activity against *p.aeruginosa (*8mm*)*but has no activity against *S. aureus, E.coli and E.coli.* **HMS-4** has also less activity against the strains. Thus, the antimicrobial activity of the

pure isolates was comparable to the standard Ciprofloxacin as appositive control and DMSO as a negative control. Results are summarized in the Table-8 below.

Table 8.Growth inhibition zone (mm) of organisms tested for activity against isolate compounds in disc diffusion method.

Strains	Growth inhibition zone (in mm)						
	HM-1	**HM-2**	**HM-3**	**HM-4**	**DMSO**	Ciprofloxacin	
E. coli	12	9	-	9	-	32	
P. aeruginosa	8	-	-	10	-	34	
S. typhimurium	9	-	-	8	-	33	
S. aureus	10	-	8	12	-	31	

6.0 Conclusions and recommendations

Medicinal plants used in the folk medicine may be interesting and largely unexplored sources for development of potential new compounds. But it is necessary to isolate the active principles and test their bacterial activity for the beneficial to human beings. It was our attempt to identify the new compounds and evaluate anti bacterial activity of the isolates in this plant that revealed four compounds. The isolation and identification of first compound (**HMS-1**) from the stem of was new from this plant ever to be reported but the second compound (**HMS-2**) was previously isolated from the root of the plant where as the third (**HMS-3**)and fourth (**HMS-4**) partially characterized and not identified .The anti bacterial activity of crude extracts and each isolates were evaluated.**HMS-1**and **HMS-4** showed less activity against four standard strains, where as **HMS-2** and **HMS-3** almost has no activity. Further investigations are recommended on the plant and test their activity against bacterial strains to decide their potential as candidates in development of antibacterial drugs.

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