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Research Article

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Isolation and characterization of natural products from *Helinus mystachnus* (Rhamnaceae)

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ABSTRACT

*In the present work phytochemical analysis of root extract of Helinus mystachinus was carried out. Root of the plant material was successively extracted with petroleum ether, chloroform, and methanol. Phytochemical screening tests conducted on petroleum ether, chloroform and methanol crude extracts indicated that choloroform extract constitutes saponins, terpenoids and glycosides. Methanol extracts contains alkaloids, saponins, tannins, terpenoids and glucosides. On the other hand, Petroleum extract does not contain any of these compounds tested. Based on the phytochemical screening tests the chloroform extract was subjected to column chromatography and yielded two compounds namely, betulinic acid (**1**) and, benzoic acid (**2**). The structures of the compounds have been elucidated with spectroscopic methods (IR and NMR).*

Key words: *Helinus mystachinus*, Betulinic acid, Rhamnaceae, Benzoic acid, Solvent extraction.

INTRODUCTION

Since ancient times, people have been exploring the nature particularly plants in search of new drugs. This has resulted in the use of large number of medicinal plants with curative properties to treat various diseases [1]. Nearly 80% of the world's population relies on traditional medicines for primary health care, most of which involve the use of plant extracts [2]. Phytochemical constituents are the basic source for the establishment of several pharmaceutical industries. The constituents present in the plant play a significant role in the identification of crude drugs. Phytochemical screening is very important in identifying new sources of therapeutically and industrially important compounds like alkaloids, flavonoids, phenolic compounds, saponins, steroids, tannins, terpenoids etc., [3].Phytochemical constituents are the basic source for the establishment of several pharmaceutical industries. Phytochemical screening is very important in identifying new sources of therapeutically and industrially important compounds like alkaloids, flavonoids, phenolic, compounds, saponins, steroids, tannins, terpenoids etc., [3].

Helinus mystachinus is a woody climber under Rhamnaceae family. The plant is distributed throughout Ethiopia, Uganda, Somalia and other East African countries [4]**.** *Helinus mystachinus* is one of an ethno medicinal plant used by the Shinasha people (Ethiopia) and its root is used as antimalra and anti-abdominal pain [5]. To the best of our knowledge, there was no phytochemical investigation on any part of this plant. Hence, the present study has been aimed at studying the phytochemical patterns of the plant by extracting and isolating compounds from this medicinally important plant.

EXPERIMENTAL SECTION

General: Melting points are recorded. TLC was performed on pre coated plates (Silica gel 60 F254, Merck) using *petroleum ether*/EtOAc (8:2) as solvent system. CC was performed on silica gel. IR spectra were measured on a Perkin Elmer 1600 using KBr. ^{1}H- and ^{13}C-NMR were recorded on a JEOL JNM-EX400 instrument at 400 MHz and 100 MHz, respectively, using $CDCl_3$ and DMSO as solvent and internal reference.

Plant material: The fresh plant material, root part of *Helinus mystachinus* Vernacular name Homachesa (Afan Oromo) was collected from Shinasha area, Metekle Zone in November 2010. The botanical identification of the plant was done by Dr. M. Remash, Jimma University and a Voucher specimen (number 001) was deposited in the herbarium of biology department, Jimma University.

Extraction and isolation: The powdered plant material (1 kg) was extracted successively twice for 24hrs with petroleum ether to yield (11g, 1.1%), chloroform and methanol by maceration techniques, respectively. It was then concentrated under a reduced pressure using a Rota vapor. The marc obtained from the extract was then soaked in petroleum ether again for 24 hours and the extract was concentrated. The marc from the petroleum extract was then soaked with chloroform for 48 hours (24 hours x 2) each to yield (23g, 2.87 %). Finally the marc from chloroform extract was extracted with methanol (24 hours x 2) to yield (26g, 2.97%). The chloroform extract and methanol extracts were filtered and concentrated under a reduced pressure using a Rota vapor and dried in desiccators. The chloroform extract (13g) was chromatographed on silica gel (290 g) using increasing amounts of petroleum ether EtOAc as eluents and fractions of 50 mL each were collected. The fraction (2.1 g) eluted with petroleum ether/EtOAc (98:2) was identified as benzoic acid. The fraction (7mg) eluted with petroleum ether/EtOAc (85:15) was identified as betulinic acid (**1**).

Preliminary phytochemical screening

Phytochemical screening is important in giving information about the class of chemical compound existing in the plant material and that would help the investigator to decide which extract to be further isolated. The phytochemical screen of the plant root extracts were carried out using the standard procedures [6- 12].

Alkaloidal Screening

0.2gm of the alcoholic extract was heated on a boiling water bath with 2N HCl (5ml).After cooling, the mixture was filtered and the filtrate was divided in to two equal portions. One portion was treated with few drops of Mayer's reagent and the other with equal amounts of Dragendeoff's .Turbidity of the resulting precipitate in the both reagents was taken as evidence for the presence of alkaloids [6, 8].

Tannins Screening

0.2gm of each extracts were mixed with10ml of distilled water and heated on water bath. The mixtures were filtered and to each filtrate 5 %(w /v) solution of ferric chloride was added and the formation of dark green solution was taken as an indication for the presence of tannins [6].

Saponins Screening

About 0.2 g of each extracts was shaken in test tubes with 5ml of distilled and heated on water bath to boil. Formation of strong and stable foam (1.7cm height) was taken as indication for the presence of Saponins [6, 7].

Terpenoids Screening

0.2 g of each petroleum ether, chloroform and methanol extracts were mixed separately with 2ml of chloroform ($CHCl_3$) and concentrated H_2S0_4 (3 ml) and then added carefully, to form a layer. A formation of reddish brown coloration of the solution at inert face was taken as the presence of terpenes [7, 8].

Flavonoids Screening

From each crude extracts 0.2 g was taken and dissolved in diluted NaOH and 1M of HCl was (5ml each)added. A yellow solution that turns to colourless was taken as the indication for the presence of flavonoids [7].

Anthraquinones Screening

0.5 g of the extracts was boiled with 10% of HCl for few minutes in a water bath and filtered. The filtrate was allowed to cool and equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% NH_3 was added to the

mixture and heated. The formation of rose-pink colour was taken as indication for the presence of authraquinones [6, 8].

Glycosides Screening

1.2g of the extract was hydrolyzed by 10 ml of 1% HCl solution and neutralized with10% of NaOH solution. A few drops of Fehling's solution A and B were added. The formation of red precipitate indicates the presence of glycosides [6, 8].

Spectral Data

Compound 1

A White crystal partially soluble in chloroform and R_f value of 0.39 in petroleum ether: EtOAC (8:2).

IR(KBr): V_{max}:3471.87, 239.52, 2929.87, 1685.79, 1643.35, 1454.33, 129.31, 1236.37, 707.88, 542.00; ^{1}H-NMR (400MHz;DMSO-d_6); 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-d_6); 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 2

Compound 2 was isolated as crystalline with melting point 122-124^{o}C and R_f value of 0.87 in petroleum ether: EtOAC (8:2) ^{1}H-NMR (400 MHz; $CDCl_3$): δ 7.48 (2H,t, J= 8Hz), 7.62 (2H,t J = 3.2 Hz), 8.15 (1H,d,J=1.4 Hz), 12.50 (1H,brs); ^{13}C-NMR (100 MHz; $CDCl_3$): δ128.41 (C-3), 128.41 (C-5), 129.49 (C-1), 130.31 (C-2), 130.30 (C-6), 133.76 (C-4), 172.46 (C-7).

RESULTS AND DISCUSSION

The plant root was extracted with petroleum ether, chloroform and methanol and phytochemical screening analysis was then conducted. The phytochemical screening tests indicated the presence of alkaloids, saponins, tannins, terpenoids and glycosides and absence of anthraqiunones and flavonoids in the methanol extract (Table 1). The chloroform extract contains saponins, terpenoids and glycosides. On the other hand, the petroleum extract does not contain any of these compounds tested.

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Table1 Phytochemical screening of the root extracts of *Helinus mystachinus*

Chemical component	Petroleum ether extract	Chloroform extract	Methanol extract
Alkaloids	-	-	+
Saponins	-	+	+
Tannins	-	-	+
Terpenoids	-	+	+
Glycosides	-	+	+
Anthraqiunones	-	-	-
Flavonoids	-	-	-

Note : *'+' indicates presence '-' indicates absence*

Isolation of compounds

The extraction of the root material (1kg) of *Helinus mystachinus* was carried out using petroleum ether, chloroform, and methanol and extraction with chloroform yielded a dark gray crude extract (23g, 2.30 %). The chloroform extract was then fractionated with column chromatography by using increasing polarity of Petroleum ether and EtOAC solvent system. The column chromatography furnished two compounds with R_f values of 0.87 and 0.39 in Petroleum ether: EtOAc (8:2).

Characterization of compound (1)

Compound **1** was obtained as a white crystalline solid, partially soluble in chloroform. The TLC of the compound showed no fluorescence when viewed in UV light but showed a single spot when put in iodine vapor.

In the IR spectrum of the compound showed characteristic absorption band for a hydroxyl group at 3471 cm^{-1}. An absorption band at 1685cm^{-1} was also observed indicating the presence of (C=O) group and the medium intensity at 1236 cm^{-1} indicates C-O stretching. Sharp absorption at 707cm^{-1} is the inductive for =C-H bending.

The ^{1}H-NMR spectrum of compound **1** exhibited signals for methyl groups at δ 0.64, 0.76, 0.86, and 0.92, 1.32 and 1.64. The ^{1}H NMR spectrum also displayed signals for olefinic hydrogens at δ 4.68, δ 4.58 (2H, s) for H-29 and hydrogen attached to carbon bearing OH (H-3) at δ 4.20 (1H, s), respectively.

The ^{13}C NMR spectrum of **1** showed signals for thirty carbon atoms. The peak at δ 177.69 indicated the presence of a carboxylic group. The signals at δ 150.77 and δ 110.09 were attributed for the presence of terminal 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 23 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 **1** was accounted to have 30 carbons comprising of six methyl, eleven methylene, six methine and seven quaternary carbons.

The ^{1}H NMR and ^{13}C NMR data (Table 2 and 3) indicated the compound is a pentacyclic triterpenoid of lupane type and comparison of its physical and spectral data (Table 2) with published values confirmed the identity of **1** as betulinic acid **[**13-17].

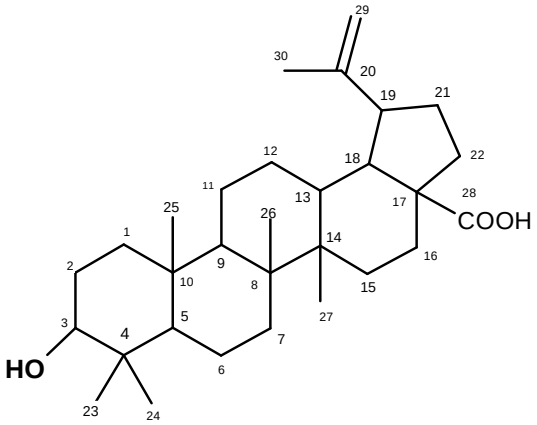

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Table 2: Comparison of the observed ^{1}H-NMR(400MHz) spectra of compound 1 in DMSO-d_6 with the reported values of betulinic acid (500MHz) in C_5D_5N.

	Observed data (400MHz,in DMSO-d_6) δ	Reported data (400MHz,in C_5D_5N) δ
3-H	4.20 (1H,m)	3.55(1H,m)
24-H	0.76 s	1.00 s
25-H	0 .64 s	0.81 s
26-H	0.86 s	1.05 s
27-H	0.92 s	1.06 s
29-H	4.68 (1H,m) 4.56(1H,m)	4.95 ,4.78 (1H,m) each
30-H	1.64 s	1.80 s

Characterization of (2)

Compound (2) was isolated as white crystal with melting point of 122-124^{o}C.

The ^{1}H-NMR spectrum of **2** showed two triplets at δ 7.48 and δ 7.61 integrating for two protons and one proton each, respectively. A doublet was also observed at δ 8.15 integrating for two protons. A broad downfield signal was also seen at δ 12.5 indicating the presence of carboxylic acid proton (Table 3). The ^{13}C-NMR spectrum of the compound exhibited signals at δ128.40, 129.49, 130.30, 133.67 and 172.46. The DEPT-135 spectrum of the **2** showed three signals at δ 128.40, 130.30, and 133.67 indicating the presence of three aromatic methine carbons (Table 4).

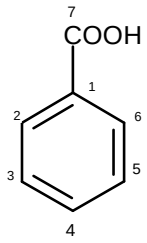
Table 3: Comparison of ^{13}C-NMR (100MHz) chemical shifts of compound 1 in DMSO-d_6 with reported values of betulinic acid in C_5D_5N

Carbon	δ(in ppm) for compound 1	DEPT 135 δ	δ(in ppm) reported value for Betulinic acid	Nature of the carbon
1	36.8	CH_2	39.0	CH_2
2	27.6	CH_2	27.6	CH_2
3	77.3	CH	78.2	CH
4	39.0	-	39.1	C
5	55.4	CH	55.5	CH
6	18.4	CH_2	18.4	CH_2
7	34.4	CH_2	34.5	CH_2
8	40.6	-	40.8	C
9	50.4	CH	50.7	CH
10	37.2	-	37.3	C
11	20.9	CH_2	21.0	CH_2
12	25.5	CH_2	25.7	CH_2
13	38.1	CH	38.1	CH
14	42.5	-	42.5	C
15	29.7	CH_2	30.2	CH_2
16	32.2	CH_2	32.9	CH_2
17	56.0	-	57.2	C
18	47.1	CH	48.1	CH
19	49.0	CH	49.2	CH
20	150.7	-	150.1	C
21	30.5	CH_2	30.6	CH_2
22	37.7	CH_2	37.0	CH_2
23	28.6	CH_3	27.9	CH_3
24	16.2	CH_3	15.5	CH_3
25	16.3	CH_3	16.4	CH_3
26	16.4	CH_3	16.7	CH_3
27	14.9	CH_3	15.0	CH_3
28	177.7	C=O	180.3	C=O
29	110.1	CH_2	108.9	CH_2
30	19.4	CH_3	19.6	CH_3

Table 4: ^{1}H NMR chemical shifts of compound 2 in $CDCl_3$ (δ 400MHz)

Proton	δ	Multiplicity
H-2, H-6	8.15	d, 1H, J=2.4 Hz
H-3, H-5	7.49	t, 2H
H-4	7.62	t, 2H, J=2 Hz, 1.2 Hz
H-7	12.50	br.s

The peak at δ 7.46 and δ 7.61 coupled with two protons each were accounted as two magnetically equivalent protons of two aromatic CH, as the DEPT-135 spectrum of the **2** showed the absence of methylene carbons. The ^{13}C-NMR spectrum showed only 5 signals, but a total of seven carbons were accounted for **2** as the two signals at δ 128.49 and δ 130.30 were of double intensity both in the ^{13}C-NMR and DEPT spectra, and hence accounted for two carbons each. So from the ^{13}C-NMR and DEPT-135 spectrum of **2** was concluded to have 5 aromatic CH, two quaternary carbons of which one is indicated for carboxyl carbon. The presence of five CH protons was also seen in ^{1}H-NMR of **2**. Based upon the above spectral data of **2** was identified as benzoic acid. The melting point and the spectral data of **2** also matched with the standard sample of of benzoic acid. TLC of **2** was also compared with an authentic sample of benzoic acid which showed that compound **2** have the same R_f value with authentic sample. Therefore based above information **2** was identified as benzoic acid.



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CONCLUSION

In order to promote Ethiopian herbal drugs, there is an urgent need to evaluate the therapeutic potentials of the drugs as per WHO guidelines [18]. Bioactive extracts should be standardized on the basis of phytochemical compounds [19].

Helinus mystachinus is the plant belonging to the family *Rhamnaceae*. The roots of this plant is traditionally clamed for its traditional medicinal value (anti-malarial and abdominal pain) in Ethiopia by Shinasha people [5]. The anti-malarial and anti-abdominal pain a activities of the plant may be attributed to its high alkaloids, tannins and terpenoids constituents. Only three species of *Helinus* are distributed all over the world. There was no report regarding the phytochemical studies of this plant.

Petroleum ether, chloroform, and methanol extracts of the roots were prepared. The chloroform crude extract was subjected to column chromatography using increasing polarities of petroleum ether and ethyl acetate mixture which results isolation of benzoic acid and betulinic acid. Benzoic acid was isolated in a large proportion from the chloroform extract.

These compounds are isolated for the first time from this plant and the literature review revealed that betulinic acid has much biological activities including anti-retroviral, anti-malarial, anti-inflammatory activities, as well as potential anti-cancer agent [20, 21]. As this is the first attempt of any phytochemical investigation from *Helinus mystachinus* further isolation and purification of other fractions of this plant is recommended which could yield some novel and bioactive compounds.

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