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Antibacterial Triterpenoid from the Leaves Extract of Ehretia Cymosa

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Abstract

*Ehretia cymosa (Boraginaceae) is an indigenous plant in Ethiopia traditionally used against various diseases including toothache, tetanus, dysentery, gastric ulcers and skin diseases. In view of its traditional use, the leaves were successively extracted on maceration with n-hexane, EtOAc and MeOH which gave extract yield of 15 g (3%), 2.63 g (0.5%) and 8.13 g (1.6%), respectively. The extracts were subjected to qualitative phytochemical screening and the results revealed the presence of phytosterols, flavonoids, terpenoids, phenols, glycosides, and alkaloids. The n-haxane extract, after silica gel column chromatography, yielded four compounds identified with IR and NMR as compound **1**, α-amyrin (**2**), β-amyrin (**3**) and bauerenol (**4**). Similar attempt made to isolate compounds from the EtOAc extract has led to the isolation of same compounds. Compound 1 has not been reported from the genus Ehretia. The extracts and compound **1** were tested for their antibacterial activity using paper disc diffusion method against two bacterial pathogens such as Pseudomonas aeruginosa and Staphylococcus aureus. The methanol extract displayed remarkable activity against P. aeruginosa with inhibition zone of 30 mm at 25 mg/mL. The result is significant compared with gentamicin (22 mm at 25 mg/mL) used as positive control. The antibacterial activity presented herein has demonstrated that all the extracts and compound 1 were active against all tested bacterial species with the methanol extract showed appreciable activity comparable with the standard drug. Thus the present study corroborates the traditional use of the leaves of E. cymosa against bacteria.*

Keywords: *Boraginaceae, Ehretia cymosa, Antibacterial activity, NMR, α-amyrin, β-amyrin, bauerenol*

1. Introduction

Human beings have since time immemorial used plants for different purposes including as food, for flavor, cosmetic, dying clothes, and above all as medicine for treating a wide spectrum of diseases. Many medicinal plant extracts have been reported for their biological activities due to the presence of secondary metabolites which act as defense against pathogens such as bacteria, fungi and also insects and plant eating animals. Plants of the genus Ehretia comprises of about 33 species distributed in Africa and Asia, with a few

species in tropical America and the West Indies. Some species in the genus including *E. ovalifolia* and *E. thyrsiflora* were reported to have a variety of flavonoids including luteolin, apigenin, kaempferol, quercetin, quercetin - 3 – *O* – *α* - D - arabinoside, kaempferol - 3 – *O* –*α*-D-arabinoside, quercetin-3-*O*-*β*-D - glucopyranoside, hyperoside, kaempferol – 3 – *O* – *β* - D-galactopyranoside, kaempferol-3-*O*-*β*-D-glucopyranoside, kaempferol-3-*O*-arabinosylgalactoside, and quercetin-3-O-arabinosyl galactoside. Phenolic acids including caffeic and cinnamicacid were identified from *E. obtusifolia*, *E. thyrsifloraand E. ovalifolia* (Iqbal, 2005; Yoshikawa et al., 1995).The genus is rich in triterpenoids with bauerenol, bauerenol acetate, *α*-amyrin, betulin, lupeol and betulinicacid reported from *E.laevis, E. buxifolia and E. thrysiflora* (Thapliyal, and Yadav, 2003; Burkill, 1985).

Ehretia cymosa (Boraginaceae) is an indigenous plant widely distributed in different parts of Ethiopia where it is locally called *Game* in Amharic and *Hulaga*, *Ulaga*, *Garmi* in Afan Oromo. A leaf infusion and leaf sap is traditionally used to treat fever and as laxative agent, respectively. The leaves are also traditionally used against tooth ache, hyperthermia, gastric ulcers, tetanus and dysentery. The use of the root juice as a wound healing property was also reported (Maundu & Tengnäs, 2005). Despite the traditional use of this plant against various life threatening diseases, there is few scientific report in the literature that describes the chemistry and biological activities. Hence in this paper we present the results of the isolation of chemical constituents and antibacterial activities of the leaves extract of *E. cymosa*.

2. Material and Method

2.1. Plant material

The leaves of *Ehretia cymosa* (Figure 1) was collected in March, 2018 from Adama town, Kachema, East Shoa Zone, Oromia, Ethiopia. The plant material was authenticated by Shambal Alamu and voucher specimen (001) was deposited at the National Herbarium of Addis Ababa University.

2.2. Extraction and isolation

The air dried powdered leaves (500 g) of *E. cymosa* were extracted successively on maceration using each 2.5 L of *n*-hexane, EtOAc and methanol for 72 hours at room temperature. Each was filtered and concentrated under reduced pressure at 40^{o}C using rotary evaporator to give their corresponding extracts. The *n*-haxane extract (3 g) was adsorbed and subjected to column chromatography over silica gel with *n*-hexane: EtOAc as eluent to furnish 21 fractions, each 100 mL. Fraction **7**, eluted with *n*-hexane: EtOAc (7:3), was re-chromatographed over silica gel column chromatography which gave four fractions, each 40 mL. The second fraction was identified as compound **1.** Fraction **6** which was eluted with *n*-hexane: EtOAc (4:1) was found to be a mixture of compound **2**, **3** and **4**.

Figure 1. Leaves of *Ehretiacymosa* (picture taken by Gemechu Gutu from Kechama)

2.3 Phytochemical screening tests

The *n*-hexane, EtOAc and MeOH extracts were subjected to qualitative phytochemical screening for the presence secondary metabolites including alkaloids, saponins, flavonoids, phytosterols, phenols and glycosides were done following standard procedure (Harborne, 2005; Parekh & Chands, 2008).

2.3.1. Detection of alkaloids

Extracts from each solvent were dissolved separately in dilute hydrochloric acid and filtered. Filtrate was treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate confirms the presence of alkaloids.

2.3.2. Detection of saponins

Froth Test: The extracts were diluted with 20 mL distilled water and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam confirms the presence of saponins.

2.3.3. Detection of flavonoids

Alkaline Reagent Test: The extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, confirms the presence of flavonoids.

2.3.4. Detection of phytosterols

Salkowski's Test: The extracts was treated with chloroform and filtered. The filtrate was treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

2.3.5. Detection of phenols

Ferric Chloride Test: The extracts were treated with 3-4 drops of ferric

chloride solution. Formation of bluish black colour confirms the presence of phenols.

2.3.6. Detection of glycosides

The extract was hydrolyzed with dil. HCl, and then subjected to test for glycosides. Modified Borntrager's Test: The extracts was treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer confirms the presence of anthranol glycosides.

2.4 Structural elucidation of isolated compounds

The structures of the isolated compounds were elucidated based on data obtained from spectroscopic methods including IR and NMR. The spectral data were also compared with literature value reported for similar compounds.

2.5 Studying antibacterial activities

The extracts and isolated compounds from the leaves of *E. cymosa* were screened for their antibacterial activities *in vitro* (Baker, 2005). The antibacterial activities were determined using agar disc diffusion method against *S. aureus*and *P. aeruginosa*. Stock solution of the methanol, ethyl acetate, *n*-hexane extracts and compound 1 were prepared at concentration of 10 mg/mL using DMSO. MHA was stabilized at 45^{o}C and seeded with 20 microliters inoculated of a 24hr nutrient broth cultured of the test organism. It was rolled in the palm to ensure uniformly spread on the agar and the test organism. This was aseptically poured into a petridish and allowed to set. Then previously prepared filter paper discs were place onto the surface of the agar plate at equal distance from each other and 15 mm from edge of plate. The hole was filled with 1mL of the respective concentration of the test extract. The Petridish was pre- incubated for 30 min and incubated at 37^{o}C for 24 hr. Bacteria culture was incubated at 37^{o}C for 24 hr. Diameters of the clear zones of inhibition was measured in millimeters. DMSO and Gentamicin were used as negative and positive control respectively.

3. Result and Discussion

3.1. Phytochemical Screening

The *n*-hexane, ethyl acetate and methanol extracts of the leaves of *E. cymosa* were analyzed for the presence of secondary metabolites including alkaloids, phytosterols, flavonoids, phenolics, glycosides and saponin. Results of the phytochemical screening. Table 1 revealed that the *n*-hexane extract of the leaves of *E. cymosa* contains alkaloids, phytosterols and flavonoids while the EtOAc and methanol extract were found to have alkaloids, phytosterols, flavonoids, phenolics and saponin.

Table 1. Phytochemical screening of the leaves extracts of *E. cym*

Secondary metabolites	Methods/Reagents	Extracts of the leaves of *E. cymosa*		
		n-hexane ext	EtOAc ext	MeOH ext
Alkaloids	Dragendroff's	+	+	+
Glycosides	Modified borntragers	_	_	_
Saponins	Froth test	_	+	+
Phytosterols	Salkowski's	+	+	+
Flavonoids	Alkaline	+	+	+
Phenolics	Ferric chloride	_	+	+

Key: + Present, - Absent

The leaves of *E. cymosa* are rich in triterepenes and flavonoids (Table 1). Various reports showed that terepenes are used for the treatment of many diseases including cancer, malaria, inflammation, and a variety of infectious diseases including viral and bacterial (Lewis & Avioli, 1991). Numerous literature reports showed that flavonoids had remarkable antibacterial activity (Cushnie & Lamb, 2005). Hence the presence of these metabolites in the *n*-hexane, EtOAc and MeOH extracts of the leaves of *E.* cymosa likely accounts the traditional use of this against bacterial infection.

3.2. Characterization of isolated compounds

In the course of this work four compounds were isolated from the hexane extract of the leaves of *E. cymosa*. Herein is the characterization of these compounds.

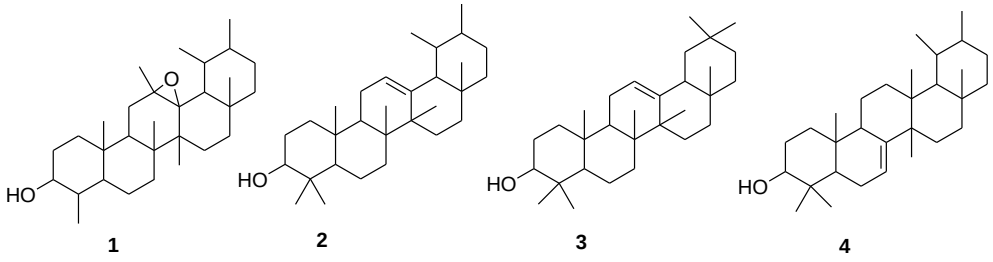

Figure 2. Structures of compounds isolated from the leaves extract of *E. cymosa*

Compound 1

Compound **1** was isolated as a white solid melting at 172°C. The TLC showed spot at Rf 0.43 with *n*-hexane:EtOAc (4:1) as a mobile phase which was visualized after dipping in vanillin /H_2SO_4. The IR spectrum of compound **1** showed absorption band due to hydroxyl stretching at 3375 cm^{-1}. The band due to C-H stretching of an alkane is evident at 2900 cm^{-1}. The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$, Appendix 1) showed proton signal at δ 3.24 (1H, *m*) accounted for the presence of proton on carbon bearing oxygen. The singlet signal appearing at δ 3.20 is likely due to the proton on oxygen. This supports the IR spectrum of compound **1** for the presence of aliphatic hydroxyl group. The ^{1}H-NMR spectrum also displayed five singlet proton signals on methyl groups at δ 0.72, 0.80, 1.12, 1.13 and 1.25 ascribed to the presence of five methyl groups on quaternary carbon. Also observed signals due to methyl groups are evident at δ 0.67, 0.85 and 0.91. The presence of seven methyl groups in the ^{1}H-NMR spectrum partly assures the presence of triterpenoid skeleton in the structure of compound **1**. The remaining proton signals were also observed in the aliphatic region.

The proton decoupled ^{13}C-NMR spectrum of compound **1** (Appendix 2) with the aid of DEPT-135 (Appendix 3) showed the presence of 30 carbon resonances of which six are due to quaternary, nine methylenes, seven methines and eight methyl groups. This indicates that the compound contain triterpenoid skeleton which supports the assertion of the ^{1}H-NMR spectrum. The quaternary carbons were observed at δ 77.2, 64.1, 39.8, 38.8, 38.3 and 35.1.The ^{13}C-NMR spectrum also showed methylene signals at δ 37.5, 36.7, 31.5, 30.9, 29.0, 27.3, 23.2, 22.9 and 16.0. The presence of an oxygenated methine carbon is apparent at δ 78.8 (C-3). The spectrum also displayed signals due to an oxygenated quaternary carbon at δ 77.2 and 64.1. The latter two signals are likely due to the presence of an epoxyidized carbon. The ^{13}C-NMR spectral data of compound **1** was compared with the NMR data presented in the literature for α-amyrin with the result depicted in Table 2.

Comparison of the ^{13}C-NMR spectral data of compound **1** (Table 2) with the data reported in the literature for α-amyrin was found in good agreement except for the fact that the signal due to the olefinic carbons (C-12 and C-13) of α-amyrin were too shielded in compound 1. This clearly indicates epoxidation of C-12 and C-13. The data generated well suited with a triterpenoids whose structure is depicted in Figure 2. Compound **1** has not been reported from the genus *Ehretia*.

Table 2. ^{13}C-NMR spectral data of compound 1 and literature reported for α-amyrin

Position	^{13}C-NMR spectral data of 1	Data reported for α-amyrin (Liliana, H.V. et al., 2016)
1	37.5	38.7
2	31.9	28.7
3	78.8	79.6
4	36.7	38.7
5	53.9	55.1
6	18.4	18.4
7	32.2	32.2
8	37.6	40.7
9	49.1	47.7
10	35.1	36.6
11	23.5	23.3
12	64.2	124.4
13	72.2	139.5
14	44.7	42
15	27.7	27.2
16	27.3	26.6
17	34.9	33.7
18	56.0	59.00
19	38.3	39.6
20	38.8	39.6
21	31.9	31.2
22	39.8	41.5
23	30.9	28.1
24	15.3	15.6
25	15.4	15.6
26	16.9	16.8
27	23.2	23.2
28	29.0	28.1
29	17.9	17.4
30	21.3	21.4

Fraction 6

Fraction **6** (500 mg) was obtained as a white solid melting at 180-181°C. The TLC profile showed spot at Rf 0. 57 with hexane:EtOAc (4:1) as a mobile phase which was visualized after dipping in vanillin/sulfuric acid. The IR spectrum of fraction **6** revealed the presence of hydroxyl stretching at δ 3380. The absorption band observed at δ 2932 is accounted to the presence of C-H stretching of a hydrocarbon. As observed from the ^{1}H and ^{13}C-NMR spectrum, fraction **6** is a mixture comprising of three triterpenoids. Purification using silica gel column chromatography was failed as the fraction showed same spot on TLC. Further attempt to purify this fraction using recrystallization was not successful. Therefore qualitative identification of these compounds in fraction **6** was done using NMR spectrum.

The proton decoupled ^{13}C-NMR spectrum of fraction **6** showed the presence of six well resolved carbon signals in the olefinic region. The signals observed at δ 124.3 and 139.6 are characteristics of α-amyrin (Liliana et al, 2016). On the other hand the olefinic signals due to β-amyrin were evident at δ 121.7 and 145.3 (Liliana et al, 2016). Also observed in the olefinic region is a signal at δ 116.4 which is characteristics of bauerenol (Sathish, et al, 2017).These indicate that Fraction **6** is likely a mixture comprising of α-amyrin (**2**), β-amyrin (**3**) and bauerenol (**4**). In agreement to this is the appearance of three signals in the oxygenated region at δ 79.2, 79.0 and 77.2 which are characteristics of C-3 of α-amyrin, β-amyrin and bauerenol, respectively (Liliana et al, 2016; Sathish et al, 2017). Furthermore the NMR spectrum clearly showed the presence of peaks characteristics of these compounds. Therefore the data generated agreed with the structures depicted in Figure 2 for compound **2**, **3** and **4**.

3.3 Antibacterial activity of extract of *Ehretia cymosa*

The n-hexane, ethyl acetate, methanol extract and compound **1** were assessed for their antibacterial using agar well diffusion method activities against *P. aeruginosa* and *S. aureus.* All the extracts exhibited activity against *P. aeruginosa* and *S. aureus* (Table 3). The zone of inhibition observed by the ethyl acetate extract against *P. aeruginosa* and *S. aureus* was found to be 16 mm and 13 mm at 25 mg/mL, respectively. The zone of inhibition observed by compound **1** against *S. aureus* was found to be 11 mm at 10 mg/mL. The zone of inhibition observed by the n-hexane extract against *S. aureus* was found to 13.5 mm at 25 mg/mL. On the other hand the methanol extract displayed remarkable zone of inhibition (30 mm at 25 mg/mL) against *P. aeruginosa*. This is significantly higher than the activity displayed by gentamicin (22 mm at 25 mg/mL) against same bacterial pathogens. The wide zone of inhibition of the methanol extract of *E.*

cymosa showed that it had great potential as a remedy for diseases caused by bacterial pathogens. Furthermore the antibacterial agent of the leaves of *E. cymosa* is likely residing in the polar fraction of the extract. The results displayed herein showed that the extracts showed better antibacterial activity against gram negative as compared to gram positive bacteria. The antibacterial activities displayed by the extracts of the leaves of this plant may be useful to prevent the occurrence of infections or treat opportunistic infections that may occur as a result of different infectious diseases in humans.

Table 3. Zone of bacterial growth inhibition (mm) for extracts and compound 1

Samples	Con. in mg/mL	Zones of inhibition	
		S. aureus	*P. aeruginosa*
n-hexane extract	25	13.5	7.0
EtOAc extract	25	13.0	16.0
MeOH extract	25	10	30.0
Compound **1**	10	11	9.0
Gentamicin		22	22

4. Conclusion

In conclusion, the presence alkaloids, phytosterols, flavonoids, phenolics and saponin in the extracts of the leaves of *E. cymosa* is significant as they may account for the traditional use of the species. Silica gel column chromatographic fractionation of the *n*-hexane and EtOAc extract has led to the isolation of four triterpenoids identified as *α*-amyrin (2), *β*-amyrin (3), bauerenol (4) and compound **1.** The latter compound was not yet reported from the genus. The work presented herein also demonstrated that the extracts and compound **1** had antibacterial activity with the methanol extract displayed pronounceable activity compared with the positive control.

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