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## RESEARCH ARTICLE

# CHEMICAL CONSTITUENTS AND ANTIBACTERIAL ACTIVITIES OF THE LEAVES OF *EHRETIA CYMOSA*

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## ABSTRACT

Phytochemical screening result of the methanol and ethyl acetate extracts of the leaves of *Ehretia cymosa* (Borginaceae) revealed the presence of alkaloids, saponins, flavonoids, terpenes and steroids. Phenolics were detected neither in the methanol nor ethyl acetate extract. The EtOAc extract after silica gel column chromatography has led to the isolation of two isomeric triterepnes identified as *α*-amyrin and *β*-amyrin. This is the first report of the isolation of *α*- and *β*-amyrin from this species. The antibacterial activities of the methanol extract were tested against four bacterial strains such as *S. aureus*, *E. coli*, *P. aeruginosa* and *P. mirabilis*. The extract exhibited pronounceable activity against *E. coli* and *S. aureus*. This is significant as this plant may be used as a remedy to treat infectious diseases caused by these two pathogens such as diarrhea. Therefore, the antibacterial activity displayed by the leaves extract of *E. cymosa* corroborates the traditional use of this plant against bacteria. The methanol and ethyl acetate extracts displayed low percent inhibition of DPPH likely accounted to the absence of phenolics.



## INTRODUCTION

Plants have been associated with human from time immemorial and are used as medicine, food, cosmetics, etc (T. Mahajan *et al.,* 2012). The use of medicinal plants as a source of medicine has been inherited and is an important component of the health care system. 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 (R.A. Dixon, 2001; J.C. Schultz, 2002). These compounds are also remarkable as they are used in the production of semi synthetic drugs (D.J. Newman *et al.,* 2003; L.A. Wessjohann, 2000). *E. cymosa* (Borginaceae) is a species in the genus *Ehretia* distributed in different parts of Africa, some in bush land and others in riverine forest. It is a popular hedge plant in Ethiopia which is traditionally used as febrifuge, laxative, pain-killer and cure for toothache (T.I, Borokini *et al.,* 2012; M. Megersa *et al.,* 2013). It is also used against diarrhea, skin wounds, stomach pain, paralysis, epilepsy, tonsillitis, typhoid, malaria, asthma, convulsions and to regulate menstrual cycle (T.I, Borokini *et al.,* 2012; J.A. Sarkodie *et al.,* 2015).

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Its use in the management of diabetes and gastric ulcers were also reported (T. Bekele, 2007). Some of the traditional uses of this plant are supported by scientific investigation with the ethanolic extract shown to have antihyperglycaemic and antimicrobial activities (Sarkodie *et al.,* 2015). Furthermore, the flowers are a source of nectar and pollen for honey bees. *E. cymosa* is planted as an ornamental tree in some neighboring countries such as Kenya and Uganda. In Ethiopia, the decoction of the leaves of *E. cymosa* is used to improve the quality and quantity of milk product of livestock. It is also locally used to prevent the milk product from bacteria attacking. Although various parts of *E. cymosa* are traditionally used against various diseases in Ethiopia, to the best of our knowledge there is no scientific report on the chemical and biological activities of this species. Herein we report the chemical constituents, antibacterial and antioxidant studies of the leaves extract of *E. cymosa.*

## MATERIALS AND METHODS

### *Plant material*

The leaves of *Ehretia cymosa* were collected in November 2015, from Gamo Gofa zone, Merab Abaya Woreda, South Nations' Nationalities Peoples Region, 455 kilometers south of Addis Ababa, Ethiopia. It was authenticated by Mr. Wege

Abebe and a voucher specimen (BB/EC/002/2015) was stored at the National Herbarium of Addis Ababa University, Addis Ababa, Ethiopia.

### *Instruments and Chemicals*

Melting point was determined using digital melting point apparatus. TLC was run on a 0.25 mm thick layer of silica gel GF254 (Merck) on aluminum plate. Spots were detected by using vanillin-$H_2SO_4$ reagent. Silica gel (230-400 mesh) column chromatography was employed for isolation of compounds. The extracts were concentrated using rotary evaporator. The UV-Vis spectral measurement was done using GENESY`S 2PC UV-Vis scanning spectrometer (200-800 nm). NMR spectra (in $CDCl_3$) were recorded using a Bruker Avance 400 MHz spectrometer. The IR spectra of compounds were recorded using Perkin-Elmer BX infrared Spectrometer (400-4000 $cm^{-1}$) as KBr pellets. Solvents (n-hexane, ethyl acetate, methanol, and chloroform) used in this research are analytical grade.

### *Extraction and Isolation*

The powdered air-dried leaves (250 g) of *E. cymosa* were extracted successively with n-hexane (1.25 L), ethyl acetate (1.25 L) and methanol (1.25L) at room temperature each for 48 hours. Each were filtered by Whatmann No, 1 filter paper and concentrated using a rotary evaporator at 40°C to afford 3.5 g hexane**,** 10.2 g EtOAc and 7.9 g MeOH extracts. The TLC profile of the EtOAc and MeOH extract were almost similar. Hence, the ethyl acetate extract (5 g) was adsorbed and subjected to column chromatography over silica gel (230-400 mesh).The column was eluted with n-hexane:EtOAc as eluent of increasing polarities to give 13 fractions (each 300 mL). F1 to F15 were collected with hexane, hexane:EtOAc (95:5), hexane:EtOAc (9:1), hexane:EtOAc (4:1), hexane: EtOAc (7:3), hexane: EtOAc (3:2), hexane: EtOAc (1:1), hexane: EtOAc (1:3), hexane: EtOAc (3:7), hexane: EtOAc (1:4), hexane: EtOAc (1:9), hexane: EtOAc (5:9.5) and EtOAc, respectively. F4 (84 mg), eluted using hexane:EtOAc (4:1), was recrystalized in n-hexane. The crystal was identified as compound 1. The filtrate was dried and found to be compound 2.

### *Phytochemical screening tests*

The presence or absence of classes of secondary metabolites such as alkaloids, tannin, terpenes steroids, saponins, flavonoids and phenols were carried out on the ethyl acetate and methanol extracts of the leaves of *E. cymosa* following standard procedures described in the previous literature reports (T.I. Borokini *et al.,* 2012; G.E. Trease *et al.,* 1989).

### *Antibacterial testing*

The anti-bacterial activity of methanol extract of the leaves of *E. cymosa* was done *in vitro* using four bacterial strains obtained from Oromia Public Health Research, Capacity Building and Quality Assurance Laboratory Center, Adama, Ethiopia. Three Gram-negative bacterial strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 35032, *Proteus mirabilis* ATCC 25923) and one gram–positive (*Staphylococcus aureus* ATCC 25923) were selected for the study. The antibacterial activities were determined using well diffusion method against different strains of bacteria (I.A. Holder *et al.,* 1994; H. Agwa *et al.,* 2000). Stock solution of the methanol extract was prepared at concentration of 10 mg/mL using 10% Tween 60. The stock solution was sterile filtered and subsequently diluted to 1.5 and 0.5 mg/mL with sterile water. MHA was stabilize at 45°C and seeded with 0.1 mL inocula of a 24 hour nutrient broth cultured of the test organism. It was rolled in the palm to ensure uniformly smeared or mixing of the agar and the test organism. This was aseptically poured into a Petridish and allowed to set. Well (6mm) was made in each petridish. The holes were filled with 1 mL of the respective concentration of the test extract. The petridishes were pre-incubated for 30 min and incubated at 37°C for 24 h. Bacteria cultures were incubated at 37°C for 24 h. Diameters of the clear zones of inhibition were measured in millimeters. Gentamicin and vancomycin were used as standard drug for bacteria strains. Chloroform was used as negative control. This experiment was done in triplicate for cultured bacteria strain.

### *Radical Scavenging Assay*

The antioxidant activities of the methanol extract was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical following the method described in (K. Ghasemi *et al.,* 2009). The methanol extract (12.5 mg/mL) and a stock solution of 0.04 % DPPH in methanol were prepared separately. 1 mL of the extract was mixed with 0.04 %DPPH (4 mL). After an incubation period of 30 min at 37°C in an oven, the absorbance was determined against a blank at 517 nm. The percent of DPPH discoloration of the samples was calculated according to the formula (S.Y. Qusti *et al.,* 2014):

$$(\%)\ \text{inhibition} = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \times 100$$

Where A control was the absorbance of the DPPH solution and A sample was the absorbance in the presence of plant extract.

## RESULTS AND DISCUSSION

The ground leaves of *E. cymosa* were successively extracted with n-hexane, EtOAc, and MeOH to afford 1.4% hexane, 4.6% EtOAc and 7.9% MeOH extracts. The TLC profile of the EtOAc and MeOH extracts were similar and found promising with the spots visualized after dipping in vanillin-$H_2SO_4$.

### *Phytochemical Screening*

The methanol and ethyl acetate extracts of the leaves of *E. cymosa* were screened to check for the presence/absence of secondary metabolites. Results showed that as the extracts are rich in secondary metabolites including saponins, tannins, alkaloids, flavonoids, terpenes and steroids (Table 1).

On the other hand tannins were detected in the methanol but not in the ethyl acetate extract. Phenolics were detected neither in methanol nor in the ethyl acetate extract of the leaves of *E. cymosa*. The presence of these secondary metabolites in the leaves of this plant is significant as they are reported as a remedy against a wide array of diseases. This likely accounts for the traditional uses of this plant against various ailments

**Table 1. Phytochemical analysis of EtOAc and MeOH extracts of the leaves of E. cymosa**

| S. No. | Phytochemical | Tests/methods | MeOH extract | EtOAc extract |
|---|---|---|---|---|
| 1 | Tannins | Potassium hydroxide test | + | - |
| 2 | Saponins | Froth test | + | + |
| 3 | Alkaloid | Wagner's test | + | + |
| 4 | Terpenoids | Salkowski Test | + | + |
| 5 | Flavonoids | Sodium Hydroxide Test | + | + |
| 6 | steroids | Salkowski Test | + | + |
| 7 | Phenols | 10% $FeCl_3$ | - | - |

+ indicates presence and – indicates absence

### *Structure Elucidation of Isolated Compounds*

Compound 1 was obtained as a white crystalline solid from the ethyl acetate extract of the leaves of *E. cymosa.* It was detected as pink spot when dipped in 1% vanillin-$H_2SO_4$ (Rf 0.73 in n-hexane/ethylacetate (4:1)). The UV-Vis spectrum of compound 1 showed no significant absorption maxima showing the absence of conjugated chromophore in the structure of the compound. The IR spectral analysis showed stretching frequency at $\delta$ 3383 $cm^{-1}$ due to the presence of hydroxyl group. The band at $\delta$ 2946 $cm^{-1}$ suggests the presence of C-H stretching of methyl group. Also observed was a band at $\delta$ 2855 and 1462 $cm^{-1}$ due to C-H stretching of methylene, and $CH_2$ bending, respectively. The presence of carbon-carbon double bond is evident at 1655 $cm^{-1}$. The $^1$H-NMR spectrum ($CDCl_3$) of compound 1 showed a triplet at $\delta$ 5.29 due to the presence of olefinic proton. The spectrum also showed signal at $\delta$ 3.22 (1H, *dd*) evident for the presence of proton on carbon bearing oxygen. The remaining proton signals integrating for 48 protons were observed in the region $\delta$ 2.3-0.80. The $^{13}$C-NMR ($CDCl_3$) with the aid of DEPT-135 spectrum of compound 1 showed carbon signals for 30 carbon atoms of which six are quaternary, nine methylenes, seven methine and eight methyl groups. The carbon signals at $\delta$ 124.4 and 139.6 are characteristics of $\alpha$-amyrin (H.V. Liliana *et al.,* 2016) (Figure 1). Furthermore the NMR spectral data generated for compound 1 is in good agreement with the spectral data reported in the literature for $\alpha$-amyin (H.V. Liliana *et al.,* 2016).

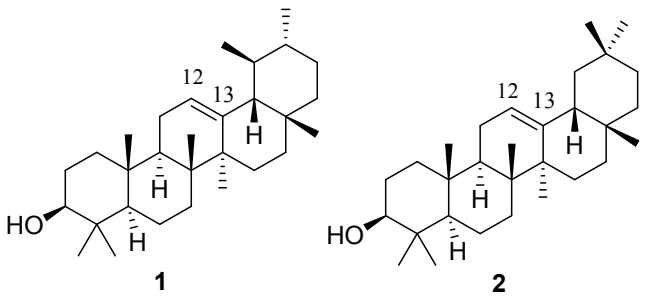


**Figure 1. Chemical structures of $\alpha$-amyrin (1) and $\beta$-amyrin (2) isolated from *E. cymosa***

On recrystalizing F4, the filtrate obtained was dried and found to be compound 2. This compound exhibited almost similar TLC, UV-Vis and IR spectra with compound 1. In contrast the $^{13}$C-NMR in combination with DEPT-135 of compound 2 ($CDCl_3$) showed the presence of 30 carbon signals of which seven are quaternary, five methine, ten methylene and eight methyl groups. The $^{13}$C-NMR spectrum showed characteristics signals at $\delta$ 121.7 and 145.3 accounting for olefinic carbons of $\beta$-amyrin (H.V. Liliana *et al.,* 2016). The presence of an oxygenated aliphatic carbon is also evident at $\delta$ 79.0. Therefore the spectral data generated for characterization of compound 2 fits very well with the NMR spectral data reported in the literature for $\beta$-amyrin (H.V. Liliana *et al.,* 2016). These two compounds were not yet reported from this species.

### *Antibacterial activity*

The methanol extract of the leaves of *E. cymosa* was assessed for its antibacterial activities against various strains of bacterial pathogens. The extract exhibited remarkable activity against *E. coli* and *S. aureus.* However, it displayed low activity against *P. aeruginosa* and *P. mirabilis* compared with standard drugs. The zone of inhibition observed by the extract against *E. coli* and *S. aureus* were found to be 12 and 9 mm at 1.5 mg/mL, respectively. This agreed very well with the study made on ethanolic extract which have shown to have antimicrobial activity (J.A. Sarkodie *et al.,* 2015). The activity displayed here was superior against *E. coli* than the other bacterial pathogens. Some *E. coli* strains are the most common causal agents of diarrhea in farm animals and humans. This makes the leaves of this plant remarkable as it may be useful to treat the occurrence of infectious diseases that may occur as a result of *S. aureus* and *E. coli*. The zone of inhibition observed against *E. coli* was also significant compared with gentamicin and vancomycin used as positive controls.

### *Radical Scavenging Activity*

The antioxidant activity of the methanol extract of the leaves of *E. cymosa* was assessed using DPPH by measuring its absorbance at 517 nm. The percent inhibition observed for the extract was found to be insignificant compared with ascorbic acid which was used as positive control. Many literature reports showed that the antioxidant activity of extract is correlated with the presence of phenolics compounds. Therefore, the absence of phenolics in the phytochemical screening results of this study supports the low radical scavenging activity of the extract of the leaves of *E. cymosa.*

In conclusion, silica gel column chromatographic fractionation of the ethyl acetate extract of the leaves of *E. cymosa* furnished two isomeric triterpenes identified as $\alpha$-amyrin and $\beta$-amyrin. These two compounds were not yet reported from this species. The methanol extract of the leaves were shown to be active against two bacterial strains namely *E. coli* and *S. aurus*. This finding may accounts for the traditional use of the leaves of *E. cymosa* to treat diseases caused by bacterial pathogens such as diarrhea, skin wounds and stomach pain. The methanol extract, however, displayed low percentage inhibition of DPPH showing its low radical scavenging activity. This is likely due to the absence of phenolics in the extract of the leaves of this species

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