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Vol. 12(11), pp. 136-141, 22 March, 2018
DOI: 10.5897/AJPP2018.4888
Article Number: 5A55B3A56360
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Antibacterial activity of crude extracts and pure compounds isolated from *Vernonia galamensis* leaves

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Received 11 January, 2018; Accepted 5 February, 2018

The aim of this study was to test the antibacterial property of the extract of the leaves and isolated compounds of *Vernonia galamensis* that is traditionally claimed to have diverse medicinal use. The disk diffusion method was used to test the successively extracted dried leaves of *V. galamensis* on *Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Shigella boydii*. Further fractionation of the acetone extract by a combination of column chromatography, gel filtration using Sephadex LH-20 and Prep-TLC afforded two compounds. The results showed that Vernonia Acetone Extract (VAE) of the leaves of *V. galamensis* showed weak to moderate antibacterial growth inhibition on the test bacteria. Two active compounds; C-I (vernolide) and C-II (vernonioside) were isolated that were not reported from *V. galamensis* before. C-I (0.6 mg/disc) showed antibacterial activity on all bacteria except *E. coli* with minimum inhibitory concentration (MIC) value of 2.5 mg/mL and C-II (0.48 mg/disk) showed growth inhibition only against *S. boydii* and *S. typhi* with MIC value of 1 mg/mL. In conclusion, *V. galamensis* leaves have been proved to possess antibacterial chemicals. The plant can possibly be exploited as a source of lead compounds for antibacterial drug development.

Key words: Antibacterial, *Vernonia galamensis*, *Staphylococcus aureus, Escherichia coli, Salmonella typhi*, *Shigella boydii*, vernolide, vernonioside.

INTRODUCTION

Plants synthesize secondary metabolites as defences against plant pathogens. The genus Vernonia, Family Asteraceae comprises about 1000 species of herbs and shrubs. Most members of the genus are well known for their bioactivities. V. galamensis is widely distributed in the tropics. The ethnobotanical importance has been

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reported before by Teklehaymanot and Giday (2010). In Ethiopia, it is distributed in the south and some parts of the north of the country (Baye and Becker 2005). *In vitro* studies done previously have shown that *V. galamensis* possess antidiabetic, sedative and analgesic properties (Johri et al., 1995; Adetutu et al., 2011). Many Vernonia species are reported to have antimicrobial properties. Worth mentioning are *V. amygdalina* (Erasto et al., 2006; Oboh and Masodje, 2009; Sharma and Sharma, 2010; Adetutu et al., 2011)*, V. auriculifera* (Hamill et al., 2003)*, V. cinerea* (Yoga-Latha et al., 2009), *V. colorata* (Kelmanson et al., 2000) and *V. leopoldii* (Mothana et al., 2009). *V. hymenolepsis* had shown antibacterial activity against Gram-negative multidrug-resistant bacteria (Noumedem et al., 2013). *V. galamensis* demonstrated antimicrobial activity against *E. coli, B. subtilis* and *S. aureus,* and the fungi *S. cerevisiae, Microsporum gypseum* and *Trichophyton mentagrophytes* (Sobrinho et al., 2015). Furthermore, the antibacterial activity and mode of action of *V. Adoensis* extracts against *S. aureus* and *P. aeruginosa* was reported (Mozirandi and Mukanganyana, 2017).

Therefore, the main aim of this study was to assess the antibacterial activity of the crude extract and isolated pure compounds from the leaves of *V. galamensis* on some pathogenic bacteria.

MATERIALS AND METHODS

Plant material

Fresh leaves of *V. galamensis* were collected from Bule-Hora area about 450 km south of Addis Ababa, Ethiopia, in November 2010. The plant was identified by a botanist and a specimen was kept in the National Herbarium of the Addis Ababa University with voucher number GT/005. Leaves were dried under shade to avoid any contamination and then ground with a blender to an appropriate size (about 0.5 mm) for extraction. The ground plant material was kept in closed container until used.

Extraction and isolation of pure compounds

The powdered leaves of *V. galamensis* (700 g) were macerated in *n*-hexane, acetone, ethanol and methanol successively in each solvent at a ratio of 1:7 (w/v) with some minor modifications as previously described by Pino-Rodriguez et al. (2003) for 24 h. The filtration was done using a double layer filter paper (Whatmann No.1) giving filtrate and residue in which the latter was subjected to the next maceration stage. Solvent was made to evaporate using Rotavapor R-210 with Vacuum Pump V-700 (Buchi) to collect crude extracts which were then weighed and kept at 4°C until further use. Preliminary antibacterial tests were conducted for each crude extract and the extract with the best activity was selected for further investigation. The extract Vernonia Acetone Extract (VAE) was then subjected to bioactivity-guided fractionation through flash column chromatography using the following solvent systems: pure *n*-hexane: chloroform, chloroform: methanol with increasing polarity. Different fractions (about 50mL each) were collected which were then combined based on their thin layer chromatography (TLC) profiles.

Further fractionation was done by gel-filtration using Sephadex LH-20 eluting with 1:1 chloroform/methanol. Non UV active compounds developed on TLC plate were visualized using vanillin sulfuric acid spray. Fractions with similar TLC profiles were combined and Prep-TLC was done for each combined fraction being eluted with ethyl acetate: chloroform (7:3). Bands made on Prep-TLC plate were scrapped off independently resulting in solid mixture of compound and silica. The obtained mixture was dissolved in chloroform and filtered out with filter paper (Whatmann No.1, 9cm) to separate the target compound from silica. Each compound was then poured in small container and kept in a hood being left open until the solvent completely evaporated. After completely dried, each pure compound was subjected to 1D (^{1}H and ^{13}C) and 2D (DEPT and HMBC) NMR for identification.

Antibacterial test

Four pathogenic standard bacterial strains namely, *S. aureus* (ATCC25223)*, E. coli* (ATCC23923)*, S. typhi* (ATCC13311) and *S. boydii* (ATCC9207), obtained from Ethiopian Health and Nutrition Research Institute (EHNRI) were used for the antibacterial tests. These bacterial strains were first grown on their own selective media prior to the test by incubating them at 37°C for 24 h. Each was incubated in nutrient broth and their turbidity was compared with McFarland (0.5 standard) before spreading them on the plates. Antibacterial tests were performed for the VAE crude extract and two major isolated compounds using the disk diffusion method (Tadeg et al., 2005). The four bacterial strains were streaked on different (independent) plates made of Muller-Hinton agar using sterilized swaps. VAE at 100, 200 mg and 300 mg and compound I (C-I) and compound II (C-II) dissolved in 1 mL of 3% Tween 80 at 20 and 16 mg respectively were used for the test.

Standard drugs were selected on the susceptibility of the bacteria to the drugs. Each drug at a concentration of 2.5 mg/mL in distilled water was prepared. Sterilized paper disks (6 mm each) were impregnated with 30 µL of each of 100, 200, 300, giving 3, 6 and 9 mg per disk of VAE; 0.6 mg/disk of C-I and 0.48 mg/disk of C-II. Similarly, four paper disks were impregnated with the standard drugs (75 µg/disk each): ampicillin, chloramphenicol, ciproflaxin and erythromycin. The same amount of 3% Tween 80 was loaded on different disks and all of them were left to dry. The paper disks were then kept on the allotted place of each plate that was partitioned externally into compartments. All plates were kept in incubator at 37°C for 24 h. After 24 h, zone of inhibitions were measured by ruler and recorded in mm. The antibacterial tests were done in triplicate for the crude extract and each compound.

Minimum inhibitory concentration

Minimum inhibition concentration (MIC) was conducted for C- I and C-II according to the methods in Taiwo et al. (1999) and Adebolu and Oladimeji (2005). C-I at concentrations of 20, 10, 5 mg/mL, 2.5, 1.25 and 0.65 mg/mL dissolved each in 3% Tween 80 and C-II at 16, 8, 4, 2, 1, 0.5 and 0.25 mg/mL dissolved each in 3 % Tween 80 were prepared. The same procedure used for the antibacterial tests was applied. The MIC of each pure compound was performed only on those bacterial strains that gave positive results.

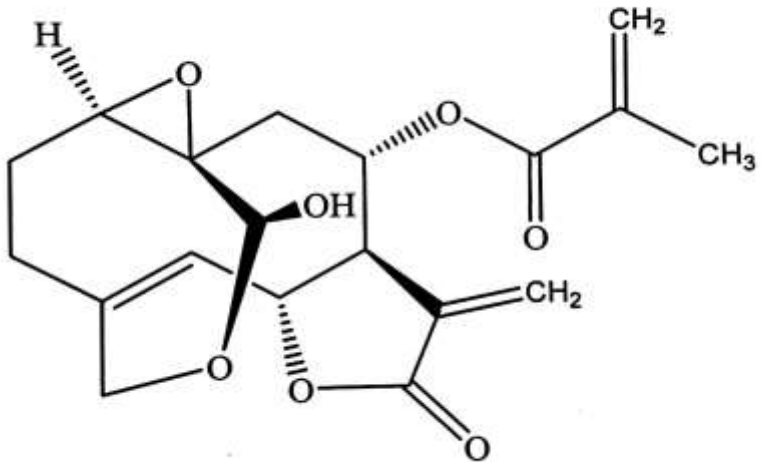

Figure 1. Structure of C-I, vernolide [2-Propenoic acid, 2-methyl-, (1aR,4Z,5aR,8aR,9S,10aR,11R)-1a,2,5a,7,8,8a,9,10-octahydro-11-hydroxy-8-methylene-7-oxo-3H-4,10a-(methanoxymethano)oxireno (5,6) cyclodeca (1,2-b)furan-9-yl ester]. PubChem CID: 6436299.

Statistical analysis

The mean (±SEM) value of zones of inhibition of each triplicate test was recorded and one way ANOVA (Turkey) was used to compare the results of the crude extract, the isolated pure compounds with both the negative and the positive controls; 95% confident interval was considered at significance level of *P*-value < 0.05.

RESULTS AND DISCUSSION

Crude extracts and isolated compounds

Subsequent extraction of the leaf powder of *V. galamensis* (700 g) resulted in four crude extracts namely Vernonia Hexane Extract (VHE, 13.5 g), Vernonia Acetone Extract (VAE, 25 g), Vernonia Ethanol Extract (VEE 15 g) and Vernonia Methanol Extract (VME, 17.4 g). Among these, VAE showed the best antibacterial activity during the preliminary test. Bioactivity guided fractionation of VAE resulted in identification of five compounds (compounds I – V).

Fractionation of VAE using hexane: ethylacetae (7:3) resulted in 11 fractions (Fractions A – K) among which fraction E and fraction J were active. Fractionation of E using Sephadex with chloroform: methanol (1:1) solvent system gave three fractions (E1 – E3). Fraction E_3 (40 mg) was a pure white crystal and resulted in compound I. Compound II (34 mg), III (7 mg) and V (5 mg) were obtained from fraction E_2 after Prep-TLC as band 2, 1 and 3 respectively.

C-I (Figure 1) gave a molecular ion at *m/z* = 362 corresponding to molecular formula $C_{19}H_{22}O_7$ and consistent with nine degrees of unsaturation (double bond equivalents) which accounted for three double bonds (six sp^2 hybridized carbons at δ_C 143.9, 135.8, 134.9, 128.7, 127.4 and 126.3) two carbonyls (at δ_C 169.5 and 167.4 ester carbonyls) and four rings. The ^{13}C NMR data showed a total of 19 carbon atoms and this together with ^{1}H NMR and 2D NMR data showed C-I to be a sesquiterpene lactone named as vernolide which was previously isolated from *V. amygdalina* by Erasto et al. (2006).

C-II (Figure 2) gave a molecular ion at *m/z* 546 corresponding to molecular formula $C_{31}H_{46}O_8$ and consistent with nine degrees of unsaturation. These nine degrees of unsaturation can be accounted for two double bonds (four sp^2- hybridized carbons at δ_C 143.5, 134.7, 121.4 and 118.4), one ester carbonyl (carbon at δ_C 170.5) and six rings. The ^{13}C NMR showed a total of 31 carbon atoms, which is consistent with a steroid nucleus. A comparison of our data with previously isolated steroids from other Vernonia species showed compound C-II to be an aglycone of a vernonioside previously isolated from *V. amygdalina* by Jisaka et al. (1993) from *V. cinerea* (Yao-Haur et al., 2003) and from *V. guineensis* by Donfack et al. (2012). Though the glycoside of C-II is well-known, this is the first report of the aglycone from *V. galamensis.*

Antibacterial activity

The level of inhibition obtained by VAE, C-I and C-II as compared to the standard drugs is shown in Table 1.

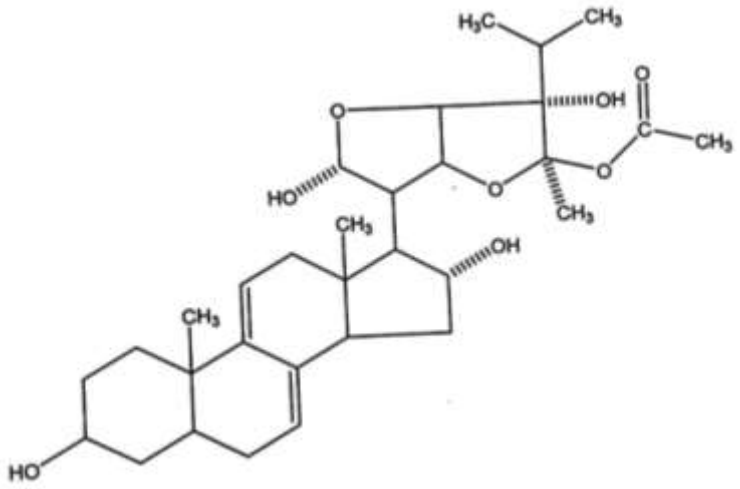

Figure 2. Structure of C-II, **vernonioside** [(2R,3S,5R)-hexahydro-6-((16R)-2,3,4,5,6,10,12,13,14,15,16,17-dodecahydro-3,16-dihydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl)-3,5-dihydroxy-3-isopropyl- 2-methylfuro[3,2-b]furan-2-yl acetate]; PubChem CID: 6324766.

Table 1. Level of inhibition of Vernonia Acetone Extract (VAE), C-I (vernolide), and C-II (vernonioside) on the test bacteria as compared to the controls.

Test material	Effect of inhibition Level					
	Stock Conc. (mg/mL)	Load/disk	Test organisms			
			S. typhi	*E. coli*	*S. aureus*	*S. boydii*
St. Drug	2.5	75 µg	++++	+++	+++	++++
	100	3 mg	-	-		-
VAE	200	6 mg	++	+	++	++
	300	9 mg	+++	++	++++	++++
C-I	20	0.6 mg	++	-	++	++
C-II	16	0.48 mg	+++	-	-	++++

St. Drug = Standard Drug: Chloramphenicol (for *S. typhi*), ampicillin (for *E. coli*), erythromycin (for *S. aureus*) and ciprofloxacin (for *S. boydii*); VAE = Vernonia leaf Acetone Extract; - = no effect; **+** = weak effect; **++** = moderate effect; **+++/++++** = strong effect.

VAE showed moderate (*S. typhi, E.coli)* to strong (*S. aureus, S. boydii*) dose dependent antibacterial activity at the dose of 6 and 9 mg/disc respectively. Table 2 shows C-I at the dose of 0.6 mg/disc showed mean growth inhibition of 18.7±0.6, 18±1.0 and 17.7±1.5 mm on *S. typhi, S. boydii* and *S. aureus* respectively. C-I is significantly different in inhibiting the growth of the test bacteria from the negative control (Tween 80) as well as the respective standard drugs in all tests (P = 0.00, Table 2). C-I showed antibacterial activity on all bacteria except *E. coli* with MIC value of 2.5 mg/mL (Table 3). Vernolide isolated from *V. amygdalina* was previously reported to have antibacterial activity with MIC of 0.5 mg/mL (Erasto et al., 2006). The difference in MIC values could be explained partly due to the species difference.

C-II at a dose of 0.48 mg/disc showed strong antibacterial activity only against *S. boydii* and *S. typhi* with mean inhibition of 28.7±1.5 and 25.7±5.9 mm respectively, while the standard drugs ciproflaxin (*S. boydii*) and chloramphenicol (*S. typhi*) inhibited the bacteria at mean inhibition of 30.0± 1.0 and 30.3 ±1.5 mm respectively (Table 2). The MIC value for C-II was 1

Table 2. Inhibitory activity of the Vernonia Acetone Extract (VAE), compound I (vernolide) and compound II (vernonioside) on the test bacteria.

Test material	Stock Conc. (load/disk)	Zone of Inhibition in mm (Mean + SEM)							
		Test organisms							
		S. typhi		*E. coli*		*S. aureus*		*S. boydii*	
		Mean± SEM	P-Value	Mean± SEM	P-Value	Mean± SEM	P-Value	Mean± SEM	P- Value
St. Drug	2.5 mg/mL (75 µg)	30.3± 1.5		25±1.0		25±1.0		30±1.0	
VAE	300 mg/mL (9 mg)	22±1.0	0.00	16±0.6	0.00	24±0.6	0.47	27.7±0.6	0.16
C-I	20 mg/mL (0.6mg)	18.7±0.6	0.00	0±0.0	0.00	17.7±1.5	0.00	18±1.0	0.00
C-II	16 mg/mL(0.48mg)	25.7±5.9	0.50	0±0.0	0.00	0±0.0	0.00	28.7±1.5	0.51

VAE = Vernonia leaf Acetone Extract; St. Drugs (Standard Drug): chloramphenicol (for *S. typhi*), ampicillin (for *E. coli*), erythromycin (for *S. aureus*), and ciprofloxacin (for *S. boydii*). P-value represents a comparison of the effect of the extract or pure compounds with the control drugs.

Table 3. Minimum inhibition concentration (MIC) of compound I (vernolide).

Test bacteria	Activity (mg/ml)						
	20	10	5	2.5	1.25	0.65	0.325
Salmonella typhi	++	+	+	+	-	-	-
Shigella boydii	++	+	+	+	-	-	-
Staphylococcus aureus	++	+	+	+	-	-	-
Escherichia coli	NT	NT	NT	NT	NT	NT	NT

NT= Not Tested; because this compound lacked activity against this bacterium.

Table 4. Minimum inhibition concentration (MIC) of compound II (vernonioside).

Test bacteria	Activity (mg/ml)						
	16	8	4	2	1	0.5	0.25
Salmonella typhi	++	+	+	+	+	-	-
Shigella boydii	++	+	+	+	+	-	-
Staphylococcus aureus	NT	NT	NT	NT	NT	NT	NT
Escherichia coli	NT	NT	NT	NT	NT	NT	NT

NT= Not Tested; because this compound lacked activity against this bacterium.

mg/mL (Table 4). Vernonioside, isolated from *V. guineensis* was reported to have antifungal activity with MIC value of 7.9 µg/mL (Donfack et al., 2012). Furthermore vernonioside B2 from *V. condensate* is reported to have analgesic and anti-inflammatory property (Valverde et al., 2001).

Previous work on *V. amygdalina* using disk diffusion method showed activity against some bacterial strains (Jisaka et al., 1993; Taiwo et al., 1999; Cos et al., 2002). Work on other species of Vernonia; namely *Vernonia hymenolepsis* (Noumedem et al., 2013), *V. galamensis* (Sobrinho et al., 2015) and *V. adoensis* (Mozirandi and Mukanganyana, 2017) have substantiated the antibacterial properties of the plant.

There was lack of activity against some tested bacteria by the pure compounds in the present work while the crude extract showed activity which might suggest the presence of synergism of compounds in the later. As reported by Erasto et al. (2006), vernolide is also inactive against some Gram negative bacteria, which might be supportive to the lack of activity by C-I and C-II against *E. coli* in the present study.

The results of the present study showed that *V. galamensis* contains phytochemicals that can control the

growth of some pathogenic bacteria especially those affecting the gastrointestinal tract normal function. Although vernolide was isolated from other *Vernonia* spp. it is the first report from *V. galamensis*. It is also the first report for vernonioside with aglycone from this plant. The effect of the crude extract and C- I against *S. aureus* can support the traditional use of this plant in wound healing treatments. The inhibitory activity of C- II against *S. typhi* and *S. boydii* might suggest that this plant can be a potential candidate for antibacterial drug development.

Conclusion

In conclusion, *V. galamensis* leaves have constituents that have inhibited the growth of pathogenic bacteria. C-II showed significant inhibition on the growth of *S. typhi* and *S.boydi*. Further study is recommended to fully exploit the medicinal importance of the plant.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations: VHE, Vernonia Hexane Extract; **VAE,** Vernonia Acetone Extract; **VEE,** Vernonia Ethanol Extract; **VME,** Vernonia Methanol Extract; **C-I,** compound I, vernolide; **C-II,** compound II, vernonioside.

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