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Phytochemical investigation and antimicrobial activity of leaves extract of *Vernonia auriculifera* Hiern

[Investigación fitoquímica y actividad antimicrobiana de extractos de hojas de *Vernonia auriculifera* Hiern]

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Abstract

Resumen

Context: The genus *Vernonia* is one of the largest groups in the family *Compositae* constituting more than 500 species distributed widely in tropical and sub-tropical regions of Africa, Asia, and America. Traditionally the genus is used for the treatment of schistosomiasis, amoebic dysentery, gastrointestinal problems, malaria, venereal diseases, wounds, hepatitis, and diabetes. *Vernonia auriculifera* Hiern is used for healing wounds as ointment around the injured areas.

Aims: To investigate the phytochemical constituents and evaluate antimicrobial activity of leaves extract of *Vernonia auriculifera* Hiern.

Methods: Phytochemical screening tests were conducted to identify the class of compounds present in the leaves extracts of *V. auriculifera*. Silica gel column chromatographic technique was applied to separate the constituents of the extracts. Various spectroscopic techniques (IR, ¹H NMR, ¹³C NMR, DEPT-135, COSY, gHSQC, and gHMBC) were applied to determine the structures of isolated compounds.

Results: Phytochemical screening of the methanol leaf extract revealed the presence of tannins, flavonoids, terpenoids, saponins and absence of anthraquinones, steroids, and alkaloids. Silica gel column chromatography of the methanol leaves extract yielded one compound. The hexane, chloroform, methanol and water extracts were tested against *Staphylococcus aureus*. The methanol and water extracts showed promising growth suppression at minimum inhibitory concentration of 200 µg/mL.

Conclusions: The polar extracts of the leaves of *Vernonia auriculifera* Hiern possess antimicrobial activity.

Contexto: El género *Vernonia* es uno de los mayores grupos en la familia *Compositae*, constituyendo más de 500 especies distribuidas ampliamente en las regiones tropicales y sub-tropicales de África, Asia y América. Tradicionalmente el género es usado para el tratamiento de esquistosomiasis, disentería amebica, problemas gastrointestinales, malaria, enfermedades venéreas, heridas, hepatitis y diabetes. *Vernonia auriculifera* Hiern es usada para cicatrizar heridas como ungüento alrededor de las áreas dañadas.

Objetivos: Investigar los constituyentes fitoquímicos y evaluar la actividad antimicrobiana de los extractos de hojas de *Vernonia auriculifera* Hiern.

Métodos: Pruebas de cribado fitoquímico fueron conducidas para identificar metabolitos de los extractos de hojas de *V. auriculifera*. Columnas cromatográficas de sílica gel fueron utilizadas para separar los constituyentes de los extractos. Varias técnicas espectroscópicas (IR, ¹H NMR, ¹³C NMR, DEPT-135, COSY, gHSQC, and gHMBC) fueron aplicadas para determinar las estructuras de los compuestos aislados.

Resultados: El cribado fitoquímico del extracto metanólico de las hojas reveló la presencia de taninos, flavonoides, terpenoides, saponinas y ausencia de atrainonas, esteroides y alcaloides. Mediante columna cromatográfica de sílica gel del extracto metanólico de las hojas fue aislado un compuesto. Los extractos en hexano, cloroformo, metanol y agua fueron probados contra *Staphylococcus aureus*. Los extractos metanólico y acuoso mostraron supresión del crecimiento a la concentración mínima inhibitoria de 200 µg/mL.

Conclusiones: Los extractos polares de las hojas de *Vernonia auriculifera* Hiern poseen actividad antimicrobiana.

Keywords: Antimicrobial activity; phytochemical screening; *Staphylococcus aureus*; terpenoids; *Vernonia auriculifera*.

Palabras Clave: Actividad antimicrobiana; cribado fitoquímico; terpenoides; *Staphylococcus aureus*; *Vernonia auriculifera*.

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INTRODUCTION

There has been a great deal of interest recently in the role of complementary and alternative medicines for the treatment of various acute and chronic diseases. Of the different classes of phytochemicals, much interest has focused on the anti-inflammatory and antioxidant properties of polyphenols found in various botanical agents (Aruoma et al., 2006). The genus *Vernonia* (*Compositae*) contains more than 500 species distributed in the tropical and subtropical areas of the world, especially Africa and South America (Bremer, 1994). *Vernonia amygdalina*, known as a bitter leaf, is a shrub or small tree of 2-5 m with a petiolate leaf of about 6 mm diameter and elliptic shape. The leaves are green with a characteristic odour and a bitter taste. It is a well known medicinal plant for diabetes, fever reduction, headache, venereal diseases, wounds, hepatitis and gastrointestinal problems (Igile et al., 1994; Akah et al., 1995; Akinpelu, 1999; Moundipa et al., 2000). Members of the genus *Vernonia* are good sources of sesquiterpene lactones such as vernolide, vernolepin, vernodaline and hydroxyvernolide known for their cytotoxic and antitumor activity (Kuo et al., 2003).

In our ongoing study to analyze the chemical constituents of medicinal plants of Ethiopian flora, we conducted a comprehensive phytochemical analysis of the methanol leaves extract and evaluate antibacterial activity of different solvent system extract of *Vernonia auriculifera*. *Vernonia auriculifera* Hiern is a shrub, small tree or woody herb that grows 1 - 7.5 m high; a stem branching from low down, it is relatively fast growing and prefers altitude of 2438.4 m or above. In Ethiopia, the leaf of the plant is used for healing wounds by rubbing (as ointment) around the injured areas after soaking the fresh leaf with water or tie the injured areas with fresh leaf after heating over the flame (Abiyu et al., 2014).

MATERIAL AND METHODS

Materials and instruments

Melting point was determined with Mettler Toledo Model FP62 machine. ^1H NMR, ^{13}C NMR,

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DEPT-135, COSY, HSQC and HMBC spectra were recorded on a Bruker Avance 400 MHz spectrometer (solvent: CDCl_3) with TMS as an internal standard. Infrared (IR) spectra were obtained on Perkins-Elmer Bx Infrared Spectrometer using KBr disc in the range $4000\text{--}400\text{ cm}^{-1}$. TLC analysis was carried out on 0.2 mm thickness TLC plates of Merck silica gel 60 F₂₅₄ coated on the aluminum plate. Compounds on TLC were detected using UV light (λ_{max} 254 and 366 nm). Silica gel column chromatography was carried using silica gel 60 (mesh).

Plant material

The leaves of *Vernonia auriculifera* Hiern were collected from Southern Ethiopia (Kembeta Zone), 300 km West of Addis Ababa. The plant was identified by botanists at the National Herbarium of Ethiopia, Department of Biology, Addis Ababa University, and a voucher of the specimen (BA-V008-10) was deposited at the National Herbarium, Department of Biology, Addis Ababa University.

Extraction and isolation of compounds

Extraction

Powder of air-dried leaf of *V. auriculifera* (360 g) was successively extracted with 1 L *n*-hexane (3.3% yield), chloroform (6.1% yield), methanol (11.1% yield) and water (8.4% yield) each for 24 x 3 h. The crude extracts were concentrated with the help of rotary evaporator (at 40°C) and under reduced pressure. The methanol extract was further suspended in water and partitioned with diethyl ether to give dark green crude extract (7 g, 1.94% yield). All the crude extracts were analyzed by TLC using 30% ethyl acetate in chloroform and 30% ethanol in ethyl acetate as eluent.

Isolation

The methanol extract (6 g) was subjected to silica gel column chromatography (80 g silica gel) and eluted with increasing gradient of ethyl acetate in chloroform (20 fractions, F₁-F₂₀), followed by increasing gradient of methanol in chloroform (20 fractions, F₂₁-F₄₀), followed by 1-5%

water in methanol (11 fractions, F_{41-51}). Fraction 28 ($\text{CH}_3\text{OH}:\text{H}_2\text{O}$, 9.5:0.5) was analyzed by TLC and showed a clear spot upon spraying with 0.5% sulphuric acid in vanillin. The solvent system used for the TLC examination of fraction 28 was 10% MeOH in ethyl acetate and observed under UV lamp. Fraction 28 yielded a yellowish compound (**1**) (30 mg), which was further analyzed by spectroscopic methods.

Phytochemical screenings tests

The methanol leaves extract was analyzed for the presence of secondary metabolites following standard protocols (Prashant et al., 2011; Pradeep et al., 2014).

Test for phlobatannins

Aqueous extract (3 mL) was added to 2 mL of 1% HCl and the extract was boiled. Deposition of a red precipitate was taken as evidence for the presence of phlobatannins (Prashant et al., 2011; Pradeep et al., 2014).

Test for flavonoids

A portion of crude powder was heated with 10 mL of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution. The instant disappearance of yellow coloration indicated the presence of flavonoids in the crude extract (Prashant et al., 2011; Pradeep et al., 2014).

Test for alkaloids

The crude powder (0.5 g) was defatted with 5% ethyl ether for 15 min. The defatted sample was extracted for 20 min with 5 mL of aqueous HCl on a boiling water bath. The resulting mixture was centrifuged for 10 min at 3000 rpm. One mL of the filtrate will be treated with few drops of Mayer's reagent and a second 1 mL with Dragendorff's reagent. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloid (Prashant et al., 2011; Pradeep et al., 2014).

Test for saponins

The crude powder (0.5 g) was shaken with water in a test tube, and it was warmed in a water

bath. The formation of stable foam was taken as an indication of the presence of saponins (Prashant et al., 2011; Pradeep et al., 2014).

Test for tannins

The crude powder (0.5 g) was stirred with 10 mL of distilled water. It was filtered and ferric chloride reagent was added to the filtrate, a blue-black precipitate was taken as evidence for the presence of tannins (Prashant et al., 2011; Pradeep et al., 2014).

Test for terpenoids

The crude powder (0.5 g) was dissolved in 5 mL of methanol. Two mL of the extract were treated with 1 mL of 2, 4-dinitrophenyl hydrazine dissolved in 100 mL of 2 M HCl. A yellow-orange coloration was observed as an indication of terpenoids (Prashant et al., 2011; Pradeep et al., 2014).

Test for steroids

The crude powder (0.5 g) was dissolved in 5 mL of methanol. One mL of the extract was treated with 0.5 mL of acetic acid anhydride and cooled in ice. This mixed with 0.5 mL of chloroform and 1 mL of concentrated sulphuric acid was then added carefully using a pipette at the separations level of the two liquids, a reddish-brown ring was formed as an indication of the presence of steroids (Prashant et al., 2011).

Test for glycosides

The crude powder (0.5 g) was dissolved in 5 mL of methanol. Ten mL of 50% HCl were added to 2 mL of methanolic extract in a test tube. The mixture was heated in a boiling water bath for 30 min. Five mL of Fehling's solution was added and the mixture was boiled for 5 min to observe a brick red precipitate as an indication for the presence of glycosides (Prashant et al., 2011).

Test for anthraquinones

The crude powder (0.5 g) was shaken with 10 mL of benzene and filtered. An aliquot of 0.5 mL of 10% ammonia solution was added to the filtrate, and the mixture will be shaken well and the presence of the violet color in the layer phase

indicated the presence of the anthraquinones (Prashant et al., 2011).

Antibacterial test of the leaves extracts of *Vernonia auriculifera*

Following the traditional use of *V. auriculifera* hiern to treat the wound in various parts of Ethiopia, we have analyzed the hexane, chloroform, methanol and water extracts against *Staphylococcus aureus*, according to standard protocols (Demarsh et al., 2001; NCCLS, 2012). Pus from the wound of a patient was collected aseptically in the sterile specimen container. The patient was injured in his legs from a car accident was admitted to Surgical Department of Hawassa University referral hospital. The ethical clearance was obtained from the Institutional Review Board of the Hawassa University College of Medicine and Health Sciences. Informed consent was taken from the patient.

The pus sample was inoculated on to appropriate culture media (Manitol Salt Agar). The inoculated media was incubated for 24 h at 37°C. The biochemical test was performed by using catalase and coagulase to identify the species, which was *Staphylococcus aureus*.

Muller Hinton agar plates were used to plate out agar for whether the plant extracts inhibited or suppressed the bacteria. A sterile wire loop was used to place the test bacteria into a test tube with peptone water over an open flame. The concentration of the inoculum was 0.5 McFarland's standards (ca. 10^8 CFU/mL). The minimum inhibitory concentration (MIC) values of *V. auriculifera* extracts were determined using two-fold broth micro-dilution to prepare extract concentrations of 0.8, 1.6, 3.2, 6.3, 12.5, 25, 50, 100, 200, and 400 mg/mL. The antibiotic cloxacillin was used as reference compound.

One mL of each extract concentration was added to test tubes containing 1 mL of sterile Muller-Hinton (MH) media. The tubes were then inoculated with a drop of microbial suspension and incubated at 37 °C for 24 h. During this time, 1 mL of Muller Hinton Broth (MHB) and 1 drop of the organism; 1 mL of MHB and two drop of the organism; 2 mL of MHB and organism; MHB and no organism; sterilized distilled water as well as

extracts with free of the organism were used as a control. The MIC value was determined after 24 h of incubation in comparison with the growth and sterility controls. MH plates were divided into three different sections and labeled with the various concentrations on the base of the plates; these were used to plate out the contents of each tube in the respective sections of the plates. The plates were incubated for 24 h at 37°C after which the growth of bacteria was recorded. Three replicates were done for each extract concentration and controls against the bacteria.

RESULTS AND DISCUSSION

Phytochemical screening

The leaves extracts of the plant of *Vernonia auriculifera* was subjected to phytochemical screening that reveals the presence of various pharmacological active components. Results of the phytochemical screening revealed the presence of flavonoids, terpenoids, tannins and saponins, and the absence of anthraquinones, steroids, and alkaloids (Table 1).

Antibacterial activity of *V. auriculifera*

The hexane, chloroform, methanol, and water extracts were examined against *Staphylococcus aureus*, and the result indicate that the water and methanolic extract showed growth suppression at a concentration above 200 mg/mL (Table 2). MIC of methanol and water extracts of *Vernonia auriculifera* against *Staphylococcus aureus* was found to be 200 mg/mL. MIC of cloxacillin was found to be 500 µg/mL.

Characterization of compound 1

Compound (1) was obtained as a yellowish solid (m.p. 212-214°C) with R_f value of 0.68 (20% MeOH in EtOAc). The IR spectrum revealed the broad absorption bands at 3300 cm^{-1} (due to a hydroxyl group). The absorption peak at 2957 and 2850 cm^{-1} indicates the sp^3 C-H stretching vibration, 1710 cm^{-1} (due to the carbonyl group). The strong absorption band around 2250 and 2139 cm^{-1} indicates the presence of carbon-carbon triple bond and

two ring deformation bands at 887.2 cm^{-1} and 774.4 cm^{-1} due to the presence of epoxy groups. The ^1H NMR (400 MHz, CDCl_3) spectrum revealed peaks at δ 2.00 (s, 3H), δ 4.25 (brs, 1H, H-3), δ 4.45 (brs, 1H, H-4), δ 4.45 (dd, 1H, H-6), and δ 4.75 (dd, 1H, H-7') and two broad singlet peaks at δ 3.75 and δ 1.30 attributed to two hydroxyl groups. The ^{13}C NMR spectrum revealed 10 carbon peaks of which five quaternary, one methylene, and four methine carbons are evident. The peak at 169.8 is attributed to the carbonyl carbon of lactone moiety (C-1). The upfield chemical shift value of the methyl group (δ 4.67) is attributed to direct connectivity with sp hybridized carbon. The peaks at δ 64–82 are for carbon atoms that are connected to electronegative atom oxygen. The DEPT-135 spectrum revealed five peaks at δ 76.8 is attributed to methylene (C-7) and peaks at δ 4.67 attributed to methyl whereas the remaining are for the methine carbons. The difference in the number of carbon from the ^{13}C NMR and DEPT-135 spectrum is five suggesting the presence of five quaternary carbons [δ 64.6, δ 69.3, δ 78.0, δ 80.0 and δ 169.8]. The COSY spectrum revealed a correlation between protons δ 4.45 (dd, H-7) and δ 4.05 (dd, H-6). HSQC spectrum revealed direct connectivity between C 7-H7, C 6-H6, C 4-H4 and C 3-H3 (Table 3). Moreover, the proton at δ 2.00 (H-10) belongs to methyl (δ 4.63, C-10).

HMBC spectrum revealed correlation between protons at δ 4.25 (H-3), δ 4.05 (brs, 1H), δ 4.75 (H-7) and with the carbonyl carbon δ 169.8 (C-1), correlations of the methyl (δ 2.00) with quaternary carbons at C-8,9 (δ 78.0, 69.5), correlation of the oxygenated methine proton at δ 4.45 with alkyne carbons (C-8,9) and quaternary carbon (δ 64.5, C-5) and correlation of proton δ 4.75 (H-7) with quaternary carbons at δ 64.5 is clearly evident. Thus, based on the above spectral data, the following compound (**1**) is suggested (Fig 1).

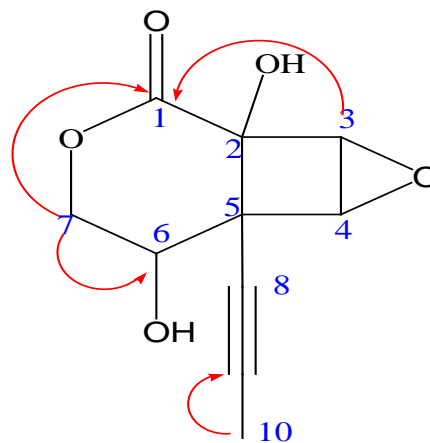


Figure 1. Important HMBC correlations of compound **1**

Table 1. Phytochemical screening of the methanol leaves extract of *V. auriculifera*.

Phytochemicals	Reagent used	Present(+)/Absent (-)
Phlobatannins	1% aqueous hydrochloric acid (HCl)	+
Flavonoids	1 mL of dilute ammonia solution	+
Alkaloids	Mayer's + Dragendroff's reagent	-
Tannins	Ferric chloride	+
Terpenoides	2,4-DNPH	+
Anthraquinones	Benzene + 10% NH_3 solution	-
Glycosides	Fehling's solution	-
Steroides	Acetic acid anhydride in ice + chloroform + concentrated sulphuric acid	-
Saponins	Warming in water bath	+

Table 2. The antibacterial activity at different concentrations of the crude extracts of *V. auriculifera*.

No.	Conc. (mg/mL)	With <i>Staphylococcus aureus</i>					Without organism as a control					
		HE + MHB	CFE + MHB	ME + MHB	WE + MHB	MHB	HE	CFE	ME	WE	MHB	Distilled water
1	0.8	+	+	+	+	+	NG	NG	NG	NG	NG	NG
2	1.6	+	+	+	+	+	NG	NG	NG	NG	NG	NG
3	3.2	+	+	+	+	+	NG	NG	NG	NG	NG	NG
4	6.3	+	+	+	+	+	NG	NG	NG	NG	NG	NG
5	12.5	+	+	+	+	+	NG	NG	NG	NG	NG	NG
6	25	+	+	+	+	+	NG	NG	NG	NG	NG	NG
7	50	+	+	+	+	+	NG	NG	NG	NG	NG	NG
8	100	+	+	+	+	+	NG	NG	NG	NG	NG	NG
9	200	+	+	-	-	+	NG	NG	NG	NG	NG	NG
10	400	+	+	-	-	+	NG	NG	NG	NG	NG	NG

Key: (-): Bacteria suppressed, (+): Bacteria not suppressed, NG: No growth, HE: Hexane extract, MHB: Muller Hinton Broth, ME: Methanol extract, CFE: Chloroform extract, WE: water extract.

For methanol and water extracts (MIC = 200 mg/mL). In this experiment was used cloxacillin as a reference antibiotic (MIC = 500 µg/mL).

Three replicates were done for each extract concentration and controls against the bacteria.

Table 3. Complete NMR data of compound **1** (400 MHz, CDCl₃) extracted from *V. auriculifera* leaves.

Position	δ _H , multiplicity	δ _C	COSY	HSQC	HMBC
1	-	169.8	-	-	-
2	-	80.5	-	-	-
3	4.25 (brs, 1H)	75.0	H ₃ ↔H ₄	H ₃ ↔ C ₃	H ₃ →C _{1,2,4,5}
4	4.05 (brs, 1H)	69.5	H ₃ ↔H ₄	H ₄ ↔ C ₄	H ₄ →C _{2,3,5,8}
5	-	64.5	-	-	-
6	4.45 (dd, 1H)	76.8	H ₆ ↔H ₇	H ₆ ↔ C ₆	H ₆ →C _{2,5,7,8}
7	4.75 (dd, 1H); 4.45 (dd, 1H)	74.8	H ₆ ↔H ₇	H ₇ ↔ C ₇	H ₇ →C _{1,5,6}
8	-	79.3	-	-	-
9	-	78.1	-	-	-
10	2.00 (s, 3H)	4.67	-	H ₁₀ ↔ C ₁₀	H ₁₀ →C _{8,9}

CONCLUSIONS

Vernonia auriculifera is one of the prominent plants traditionally used for healing wounds and gastrointestinal problems in different parts of Ethiopia. The results of the present study revealed that tannins, flavonoids, terpenoids, and saponins are present in the methanol leaves extract whereas anthraquinones, steroids, and alkaloids are absent. The antibacterial test against *Staphylococcus aureus* showed promising growth inhibition at concentration 200 mg/mL (MIC) for both methanol and water extracts. Since methanol and water extracts constitute polar constituents of the plant, the higher antibacterial activity against this strain may be attributed to the presence of polar polyphenols. Silical gel column chromatographic separation of the methanol extract afforded one compound, which is fully characterized under the present study. More, phytochemical work is recommended on the methanol and water extracts of the plant and an exhaustive *in vitro* and *in vivo* antibacterial examination of the crude extracts is recommended on various strains of micro-organisms.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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