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Isolation and Antioxidant Activity of Harmalol from *Grewia villosa* Willd. Stem and Root Bark

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***Grewia villosa* Willd. is a deciduous shrub found in arid parts of Kenya and used as both food and medicine. The study aimed to isolate compounds present in *Grewia villosa* stem and root bark extract and assess their antioxidant activity. *Grewia villosa* stem and root bark were collected from Loitokitok, Kajiado County, Kenya. They were dried in the shade, pulverized to fine powder, and extracted in methanol/dichloromethane 1:1 mixture for 72 h. The solvent was removed by rotary evaporation. The crude extract was subjected to column chromatography over silica gel to obtain one pure compound. Analysis by mass spectrometry and nuclear magnetic resonance spectroscopy identified the isolated pure compound as 1-methyl-4,9-dihydro-3H-β-carbolin-7-ol (harmalol). Antioxidant activity was performed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) standard protocol. Harmalol displayed significant antioxidant activity (115.22 µg/ml) comparable to that of the methanol extract. This compound was previously isolated from the root of *G. villosa* but was not fully characterized spectroscopically. This study is the first report of NMR characterization of harmalol.**

Keywords: *Grewia villosa*, isolation, harmalol, antioxidant

INTRODUCTION

The genus *Grewia* comprises about 150 species of small trees and shrubs distributed in tropical and subtropical regions of Africa and Asia. Different plant parts of the genus *Grewia*, have been used for decades in several parts of Africa as both food and medicine. Classes of compounds isolated from *Grewia* species include harman alkaloids, flavonoids, triterpenoids, coumarinolignans, neolignans, sterols and anthocyanidins among many others [1]. *Grewia villosa* Willd (Malvaceae), commonly known as round leaf grewia or mallow-leaved ross berry, is a deciduous shrub, that reaches a height of about 3m. It grows in tropical and sub-tropical areas of Africa and India, usually on riverbanks, stony ground or under the shade of larger trees. In Kenya, *G. villosa* is found in the arid south and northern parts of the country [2]. The Maasai use it as both food and medicine as its fruits are highly nutritious and its leaves have been used to treat stomach ache and coughs [3]. Compounds detected from *Grewia villosa* include α-amyrin, ursolic acid, uvaol, harman alkaloids, quinovaic acid, β-sitosterol, oleic and linoleic acids [2,4].

Harman alkaloids are indole alkaloids and have demonstrated biological activities including anxiolytic, hypotensive, anti-inflammatory, antidiabetic, anticancer, antinociceptive and hallucinogenic effects [5]. The anti-inflammatory effect is related to their antioxidant activity which is due to its conjugated ring system which allows for favourable electron delocalisation when neutralizing radicals [6]. From previous studies[7], the methanol extract of *Grewia villosa* had high total phenolic content (136.8 tannic acid equivalent in mg/g of extract) and high total

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flavonoid content (127.17 catechin equivalent in mg/g of extract). The extract also showed good antioxidant activity with a half maximal inhibitory concentration of 107.53 µg/ml comparable to ascorbic acid at 50.32 µg/ml [7]. *Grewia villosa* methanol extract also showed higher anti-inflammatory activity 1 h and 24 h after the carrageenan-induced rat paw oedema assay than diclofenac at the respective time points [8]. Traditional plants with nutritional and health benefits such as *Grewia spp*. can be used to develop medicines and functional foods that will benefit millions of people and act as a source of livelihood for the indigenous communities. This formed the basis for further research into the isolation of phytochemicals from *G. villosa*. The aim of the present study was to isolate and characterize compounds from *Grewia villosa* Willd. stem and root bark and assess their free radical scavenging activity.

MATERIALS AND METHODS

Plant material collection and identification

Grewia villosa stem and root bark were collected from Loitokitok in December 2014 and identified by a taxonomist. It was catalogued and a voucher specimen deposited at the University of Nairobi Herbarium (NAI) under voucher number JW2014/18.

Solvents and reagents

Analytical grade or general grade dichloromethane, *n*-hexane, ethyl acetate, acetone, toluene, dichloromethane and methanol were obtained from Kobian Chemicals, Nairobi, Kenya. The general grade solvents were double distilled before use. Iodine crystals were obtained from Alpha Chemika, Mumbai, India. Potassium bromide used for infrared spectroscopy and ascorbic acid standard and 2,2-diphenyl-1-picrylhydrazyl (DPPH) used in the antioxidant assay were obtained from Sigma Aldrich Co. (St Louis, MO, USA). Methanol-d4 and chloroform-d4 were sourced from Merck KGaA, Darmstadt, Germany.

Materials

Analytical thin layer chromatography (TLC) was performed on aluminium-backed normal-phase 0.2 mm thick silica gel 60 F_{254} pre-coated plates (Merck & Co. Inc., NJ, USA). Preparative thin layer chromatography (PTLC) was performed using normal phase silica gel 60 F_{254} (Merck & Co. Inc., NJ, USA) coated on glass plates (20 × 20). Silica gel for column chromatography (60-120 mesh, Alpha Chemika, Mumbai, India) was used for open column chromatography. Size-exclusion chromatography was carried out using Sephadex® LH-20 (Merck & Co. Inc., NJ, USA).

Equipment

Pulverisation of plant material was performed using a multifunctional grinder GRT-06B (Yongkang Tiange Electric Co., Zhejiang, China). Solvent evaporation was carried out on a Büchi Rotavapor R-200 rotary evaporator (Büchi Labortechnik, Flawil, Switzerland). Custom made glass columns (90 cm by 50 mm diameter or 60 cm by 25 mm diameter) were used for open column chromatography. Detection was performed using a handheld UV Lamp ENF-260C 365nm/254nm (Spectronics Corp. NY, USA). NMR spectra were run on a Bruker Avance III HD 800 spectrometer (Bruker BioSpin AG, Fallanden, Switzerland) equipped with a TXO cryogenic probe, using the residual solvent peak as the reference. Melting points were measured on a Büchi Melting Point M-565 apparatus (Büchi Labortechnik, Flawil, Switzerland). Fourier-transform infrared (FTIR) spectra were scanned on a Shimadzu 8400 FTIR-Spectrophotometer (Shimadzu Inc., Kyoto, Japan). Ultraviolet-visible (UV-Vis) spectra were recorded on a Shimadzu 1800 UV-Vis spectrophotometer (Shimadzu Inc., Kyoto, Japan). The electron ionization mass spectrometry (EI-MS) spectra were recorded on a Micromass GC-TOF mass spectrometer (Micromass, Wythenshawe, Waters Inc., Manchester, UK), using direct inlet, and 70 eV ionization voltage.

Plant material preparation and extraction

The plant material was dried at room temperature away from direct sunlight for a week and pulverised to a powder using a

commercial mill. Ground stem and root bark (1000 g) was extracted by maceration with 2.5 l of a 1:1 methanol/dichloromethane mixture. The extract was filtered using Whatman No. 1 filter paper and concentrated to dryness under reduced pressure at low temperature. This procedure was repeated three times for exhaustive extraction.

Fractionation of the crude plant extract

A 40 g aliquot of the crude extract was adsorbed onto silica gel by mixing the sample and 20 g of silica gel 60 in a minimal volume of dichloromethane. This mixture was dried under reduced pressure and ground to a fine powder using a mortar and pestle. Meanwhile, silica gel for column chromatography (400 g) was suspended in hexane, the slurry packed into a glass column (90 cm by 50 mm diameter) and left to equilibrate for a few hours. The adsorbed sample was loaded onto the packed column and fractionated using a gradient of hexane and ethyl acetate (100% hexane to 100% ethyl acetate) followed by an ethyl acetate and methanol gradient (100% ethyl acetate to 100% methanol). A total of 350 fractions (400 ml each) were collected, reduced under pressure to about 30 ml volume and transferred to marked glass vials. Aliquots of all the fractions were spotted on TLC plates and developed in appropriate mobile phases. Visualization of the TLC spots under UV light and by exposure to iodine gave seven major fractions (JK1, JK2, JK3, JK4, JK5, JK6 and JK7). The last major fraction (JK7) that eluted with 30-70% methanol was repeatedly passed through sephadex to separate the main compound from a brown pigment. Fraction JK7 was further purified by column chromatography over silica gel on a smaller column (60cm by 25mm diameter) eluted with ethyl acetate-methanol in an increasing gradient. A pure compound was obtained at 10% methanol in ethyl acetate.

Spectral characterization of the isolated compound

Structural elucidation of the pure compound obtained was carried out using nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy (MS) and infrared (IR) spectroscopy. Assignments were derived from the 1D and 2D proton and carbon-13 NMR (^{1}H-NMR and ^{13}C-NMR), correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT), heteronuclear single quantum coherence spectroscopy (HSQC), and heteronuclear multiple bond coherence (HMBC) spectra. Chemical shifts were recorded in δ (ppm).

Assay for antioxidant activity

The assay for antioxidant activity of JK7A was carried out using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as the oxidant. The choice was based on its reproducibility as previously described in the literature [9]. The test solutions of the pure compound were prepared in 95% v/v methanol to obtain concentrations in the range 250- 2000 µg/ml. Ascorbic acid was used as a standard and serial dilutions at 6.25-75 µg/ml were prepared using methanol. The oxidant was prepared by dissolving 3.94 mg of DPPH in 100 ml of 95% v/v methanol just before use and stored in the dark to minimize degradation. A 200 µl aliquot of the test/standard solution was placed in a vial and 2800 µl of DPPH added. The mixture was kept in the dark for 30 min and optical density measured spectrophotometrically against a blank at 517 nm. The blank constituted only 2800 µl of DPPH with 200 µl methanol added as a replacement for the sample/standard. The assay was done in triplicate.

The antioxidant activity of the pure compound was expressed as half-maximal inhibitory concentration (IC_{50}), which is the concentration (expressed in µg/ml) of sample required to cause a 50% reduction in DPPH radicals. The IC_{50} was calculated from the graph of inhibition percentage against concentration (Supplementary Data 1). Inhibition of free radical of DPPH in percentage terms (percentage inhibition-PI %) was calculated as shown in Equation 1.

$$\text{PI } \% = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \quad \text{Equation 1}$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the

sample) while A_{sample} is the absorbance of the pure compound (or the standard).

RESULTS AND DISCUSSION

Structure elucidation of harmalol (1-Methyl-4,9-dihydro-3H-β-carbolin-7-ol)

The structure was elucidated using data from 1D (^{1}H, ^{13}C and DEPT) and 2D-NMR (COSY, NOESY, HSQC and HMBC) experiments (Table 1, Supplementary Data 2-6). Compound JK7A (Figure 1) was obtained as a yellow solid with the chemical formula $C_{12}H_{12}N_2O$ consistent with the molecular ion peak m/z 200.09 on MS (Supplementary Data 7). Compound JK7A had a melting point of 100-105°C and $UV_{\lambda max}$ of 373 nm (Supplementary Data 8). The ^{1}H NMR spectra showed the presence of aromatic protons (7.69 and 6.93 ppm). There were two ortho coupled aromatic protons (δ_H 7.69 (d, J = 9Hz, H-5) and δ_H 6.93 (d, J = 9Hz, H-6)) and a third aromatic proton (δ_H 6.94 (s H-8)). The ^{1}H NMR spectra showed two sets of down shifted methylene protons at δ_H 4.03 (t, J = 8.6 Hz, H-3) and δ_H 3.37 (t, J = 8.4, H-4) and methyl protons at δ_H 2.80 (s) due to proximity to an electronegative amine group. ^{13}C NMR and HSQC spectra were used to assign the corresponding carbons as follows: δ_C 124 (C-5), δ_C 115.8 (C-6), δ_C 97 (C-8), δ_C 43.1 (C-3), δ_C 20.3 (C-4) and δ_C 18.4 (1-Me) respectively. The ^{13}C NMR spectrum further revealed the presence of five quaternary carbons: δ_C 165.9 (C-1), δ_C 145.6 (C-8a), δ_C 126.4 (C-9a), δ_C 127.8 (C-4a) and δ_C 120 (C-4b). There was an additional oxygenated quaternary carbon at δ_C 161.5 (C-7) attached to a hydroxyl group which gave an IR signal at 3490 cm-1 (Supplementary Data 9). The NMR assignments were further confirmed by HMBC where the methyl protons δ_H 2.80 (s) showed correlation with C-1 (^2J) and C-9a (^3J). The aromatic proton δ_H 6.94 (s, H-8) showed correlation with C-7 (^2J) and C-4a (^3J). The other aromatic proton δ_H 7.69 (d, J = 9Hz, H-5) showed correlation with C-4a (^3J) and the methylene proton δ_H 4.03 (t, J = 8.6 Hz, H-3) showed correlation with C-1. The spectral data confirmed the compound to be an indole alkaloid with an IUPAC name 1-Methyl-4,9-dihydro-3H-β-carbolin-7-ol and common name harmalol (Figure 1). This compound is a harman alkaloid previously isolated from *Peganum harmala* [10] and the plant root of *G. villosa* [11]. Although it has previously been isolated, characterization by HPLC-MS method had not been carried out.

Table 1: ^{1}H, ^{13}C NMR Data of Compound JK7A in CD_3OD at 500 MHz and 125 MHz (δ in ppm, *J* in Hz)

Position	^{13}C NMR (δ)	^{1}H NMR (δ, (multiplicity), *J*)	HMBC
1	165.9		
3	43.1	4.03, (t), 8.6	1, 4
4	20.3	3.37, (t), 8.4	4a, 4b, 3
4a	127.8		
4b	120		
5	124	7.69, (d), 9.5	8, 4b, 4a, 8a, 7
6	115.8	6.93, (d), 9.5	4b, 5*, 8a*
7	161.4		
8	97	6.94, (s)	6, 4b, 8a*
8a	145.6		
9a	126.4		
1-Me	18.4	2.80, (s)	1, 9a

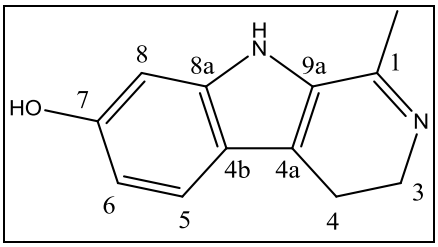

Figure 1: Chemical structure of compound JK7A

Harman alkaloids are indole alkaloids with diverse structures. The alkaloids derive their name from the plant species from which they were first isolated, *Peganum harmala* [10]. They have also been isolated in high levels from other plants such as *Passiflora edulis* and *Banisteriopsis caapi* [12,13]. Harman alkaloids are endogenously produced in animal tissues and have been detected in alcoholic drinks, meat, coffee and tobacco smoke [14]. These alkaloids have demonstrated various biological activities such as antianxiety, hypotensive, anti-inflammatory, antidiabetic, anticancer, antinociceptive and hallucinogenic effects [5]. Harmalol is a 3,4-dihydro-β-carboline with a C_7 alkyl substitution common in naturally occurring β-carbolines [14].

Antioxidant activity of isolated harmalol

The antioxidant activity of the isolated harmalol in this study was 115.22 µg/ml. The activity is comparable to that for the methanol extract (107.50 µg/ml) as previously described [7]. Harman alkaloids contain an intact β-carboline skeleton and are sometimes referred to as simple β-carbolines. These β-carbolines show different modifications that give rise to several compounds with slightly different activities. Examples of simple β-carbolines include norharman, harman, harmol, harmine, harmaline and harmalol [14]. β-carbolines are derived from tryptophan which is known to have antioxidative activity by scavenging reactive oxygen species and forming a stable indole radical [15]. Structural modifications of the β-carbolines give rise to different levels of antioxidant activity. For example, dehydrogenation of the pyridyl ring (e.g., harmalol to harmol, harmaline to harmine) resulted in a significant decrease in antioxidant activity while replacement of the hydroxyl group with a methoxy also decreased the antioxidant effect (e.g., harmalol to harmaline, harmol to harmine). Thus harmalol was found to have the highest antioxidant activity among the β-carbolines tested [16]. Harmalol is able to cross the blood brain barrier and may have a protective effect on neuronal cells and delay the onset of neurodegenerative diseases [17].

CONCLUSION

Harmalol (1-methyl-4,9-dihydro-3H-β-carbolin-7-ol), a harman alkaloid, was isolated from *Grewia villosa* Willd. stem and root bark extract. Its chemical structure was conclusively elucidated using 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. The alkaloid exhibited remarkable antioxidant activity that may be related to its anti-inflammatory activity and lend credence to the traditional use of *G. villosa* in treating aches and respiratory conditions.

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SUPPORTING INFORMATION

Supporting spectroscopic and activity data is available free of charge at https://uonjournals.uonbi.ac.ke/ojs/index.php/ecajps/libraryFiles/downloadPublic/29.

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