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Screening for antimicrobial compounds in *Gardenia volkensii* fruits (Rubiaceae)

Kinuthia E. Wanjiru, Mwangi E. M., Cheplogoi P. K.

Abstract— A modified iridoid (GV2) was isolated as a yellow powder in the yield of 0.3% of the dichloromethane crude extract of *Gardenia volkensii* fruits. It showed some antimicrobial activity on *Escherichia coli* with a Minimum Inhibitory concentration (MIC) of less than 20 µL of 10 mg/mL solution. The Inhibition Concentration (IC_{50}) for for the *Gardenia volkensii* stem was 1166.809 mg/mL and for Doxycycline® antibiotic was 0.335 mg/mL. The stem bark IC_{50} was found to be 3,483 times active as compared to Doxtcycline® antibiotic, an indication that the plant can be used to treat microbial diseases though a higher dose is needed.

Index Terms— *Gardenia, i*ridoid, modified, *volkensii*.

1 INTRODUCTION

Gardenia volkensii belongs to the Rubiaceae family that comprises of 10,700 species distributed in 637 genera [6]. *Gardenia volkensii,* known as "The wild Gardenia", *oltakurukuriet* (Maasai), *mukumuti* (Kikamba), *Ngenenet* (Kipsigis) and *Rayudhi* (Luo) is used by these tribes as herbal medicinal plant. It is a deciduous tree with a single grooved stem and a delicate fragrance fills the air in Spring when it blooms. Very good *bonsai* have been produced using this tree [1]. Interviews conducted among the Pokots people of Kenya showed that *Gardenia volkensii* fruits are used to treat malaria, headache, earache and as an emetic. Mutagenic and antimutagenic effects in *Salmonella* microsome and micronucleus tests of dichloromethane extracts of different parts of *Gardenia volkensii* have been investigated. The extract did not induce mutations neither did it modify the effect of the mutagen-4-nitroquinoline oxide, but it was genotoxic in the micronucleus test [7]. Previous phytochemical investigation of dichloromethane and methanol extracts of stem bark, twigs and seeds yielded iridoids, benzoids, cinnamates, aldehydes and flavonoids [4]. These compounds are antifungal, antibacterial, antioxidant, antipyretic, antiseptic, antifouling and phytotoxic [2], [3], [5], [8]. The aim of this project was to test for the antimicrobial activity of the crude extracts and isolated compounds in the *Gardenia volkensii*. The plant samples were collected from Baringo County in Kenya.

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- *Author name is currently pursuing Doctor of Philosophy degree program in Natural ProductsEgerton University, Kenya, P.O.Box 536-20107,Njoro. E-mail: author_esthermjumbe@yahool.com*

2 PROCEDURE

The air dried and ground plant materials of *Gardenia volkensii* parts were sequentially extracted using hexane, dichloromethane and methanol for 72 hours. The crude extracts for each solvent was screened for antimicrobial activity. Exactly 20 µL, 25 µL, 30 µL, 35 µL and 40 µL µL of 10,000 mg/L solution extract was spiked and the antimicrobial activities on Isolates of *Escherichia coli* (ATCC 11303), *Salmonella typhimurium* (C953), *Staphylococcus aureus* (Laboratory isolate), *Bacillus subtilis* (Laboratory isolate) and *Cadidas albican* (Laboratory isolate) studied. The crude dichloromethane extract showed more compounds and its fraction was purified by step gradient isolation (dichloromethane/methanol) followed by repeated column chromatography (ethyl acetate/hexane). Determination of **MIC** was carried out for all the crude extracts and isolated compounds in a serial dilution assay of 20 µL, 25 µL, 30 µL, 35 µL and 40 µL solution. The lowest concentration with the smallest inhibition zone was taken as the **MIC.** For the **IC_{50}** different concentrations of Doxycycline® antibiotic (10,000, 4,000, 1,000, 400, 100, 40, 10 and 4 mg/L in methanol) were prepared using serial dilutions method. The **IC_{50}** for Doxycycline® antibiotic was determined using probit analysis software (GraphPad Prism was used to plot inhibition zone against log of concentration of Doxycycline®). The **IC_{50}** for the crude extracts and the pure compounds were determined in a similar way. **IC_{50}** for the crude extracts and the pure compounds were then compared with those of Doxycycline® antibiotic.

Identification of pure compounds was achieved by ^{1}H, ^{13}C NMR and MS spectroscopy. NMR spectra were recorded at room temperature on a 500 MHz Bruker AVANCE NMR spectrometer at the School of Biomedical and Molecular Sciences, University of Surrey at Guildford UK. Chemical shifts **(δ)** are expressed in parts per million **(ppm)** relative to tetramethylsilane **(TMS)** as internal standard and coupling ***(J)*** are given in Hz

3 RESULTS

From the *Gardenia volkensii* dichloromethane crude extract, a **GV2** (34.80 mg) modified iridoid pure compound was isolated. It was a light yellow powder, UV inactive with an R_f of 0.14 (33% ethyl acetate in hexane) in the yield of 0.3%. It showed activity on *Escherichia coli* with an **MIC** of less than 20 µL of 10 mg/ mL solution as indicated in Table 2. There was no activity on *Cadidas albican* and *Salmonella typhimurium*

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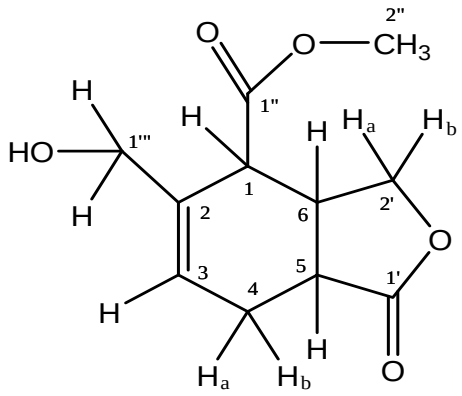
for all the crude extracts and the **GV2** compound. Methanol was used as a negative control while Doxycycline® antibiotic was used as a positive control for the pure compound. The $\mathbf{IC_{50}}$ (Table 2) for the stem bark, the most active crude extracts on *Bacillus subtilis* was determined using probit analysis (graphpad prism) and compared with the $\mathbf{IC_{50}}$ for Doxycycline® antibiotic which was 0.335 mg/mL. The $\mathbf{IC_{50}}$ for the stem bark was 1166.809 mg/mL 3,483 times less active as compared to the Doxycycline® antibiotic respectively. This is an indication that the plant can be used to cure microbial diseases though a higher dose is needed.

Structure elucidation of GV2 (modified iridoid)



GV2

The EI/FI-MS (Fig. 2) of **GV2** showed a molecular peak at m/z 226.08 corresponding to the molecular formula $C_{11}H_{14}O_5$. The degree of unsaturation for this compound was five. This was accounted for, by the cyclohexene, two carbonyl groups and a five membered cycloketone.

The ^{1}H NMR spectrum (Fig.3) of compound **GV2** showed one methoxy group (-OCH_3) resonance at δ 3.71 corresponding to the ^{13}C NMR resonance at δ 52.75. The proton resonance at δ 5.81 showed a characteristic of cyclohexene double bond corresponding to the ^{13}C NMR resonance at δ 129.44 in the HSQC-DEPT spectrum. Two coupled proton NMR resonance at δ 4.50 (H-2'a) and δ 4.47 (H-2'b) indicated the presence of non-equivalent protons, characteristic of butyrolactone corresponding to the ^{13}C NMR resonance at δ 67.87 in the HSQC-DEPT. Furthermore, two proton resonances at δ 2.73 (H-4a) and δ 2.28 (H-4b) indicated the presence of two non-equivalent protons, α to the double bond of the cyclohexene. The ^{13}C NMR resonance at δ 60.82 indicated the presence of an OH group next to a methylene with two proton singlets at a resonance of δ 4.29.

The eleven carbon resonances observed in the ^{13}C NMR spectrum were characterized by DEPT experiment which indicated that **GV2** was a monoterpenoid with a modified iridane skeleton. It consisted of one methoxy group (δ 52.75), three methylene (-CH_2) groups (δ 38.99, δ 67.87, δ 60.82, two of them oxygenated), four methine (-CH) groups (δ 49.49, δ 129.44, δ 50.72, δ 37.75, two oxygen bearing) and three quaternary (δ 140.26, δ 72.48, δ 171.60, two carbonyl) carbon signals. The chemical shift of one quaternary carbon (δ 140.26) and one methine (-CH) carbon (δ 129.44) indicated the presence of a cyclohexene while the chemical shift of the other two quaternary carbons (δ 172.48 and δ 171.60) indicated the presence of two carbonyl groups.

In the COSY spectrum, H-6 was correlated to H-1, H-4 and H-5. In the HMBC spectrum the correlation between C-2' and H-6, C-3 and H-5 indicated the presence of a cyclohexene. Also the correlation between C-3 and H-1''' indicated further the presence of a double bond of a cyclohexene. The NOESY spectrum confirmed the position of the methoxy group at C-1'' through correlation between the methoxy proton resonance and H-1. Also the position of the double bond at C-3 and C-2 was confirmed by the NOESY spectrum through correlation between the hydroxyl proton resonance and H-3. A summary of NMR data for **GV2** is shown in **Table 1**.

TABLE 1
NMR DATA FOR COMPOUND GV2

Position	δ ^{1}H ppm (J in Hz)	δ ^{13}C (ppm)	COSY	HMBC (H→C)	NOESY
1	3.74 d (13.5)	49.49 (CH)	6	3,4,6	1''',2''
2	-	14 0.26 (C)	-	-	-
3	5.81 m	129.44 (CH)	-	1,4,5,1'''	-
4	2.73 m, 2.28 m	38.99 (CH_2)	6	3,5,6'	6
5	3.72 m	50.72 (CH)	6	3,4,6	1'''
6	3.18 m	37.75 (CH)	1,4,5	1,4,5,2'	1,2''
1'	-	172.48 (C)	-	-	-
2'	4.50 t (3.5) 4.47 t (3.0)	67.87 (CH_2)	-	6	-
1''	-	171.60 (C)	-	-	-
2''	3.71(s)	52.75 (CH_3)		-	1'''
1'''	4.29(s)	60.82 (CH_2)	-	3	3

TABLE 2
MIC (mg/mL)

Crude extracts	*BSb*	*SA*	*EC*
Gardenia volkensii stem bark (CH_3OH extract)	0.20	0.30	0.30
Gardenia volkensii fruit seeds & fruit cover (CH_3OH extract)	0.35	>0.40	>0.40
Gardenia volkensii leaves (CH_3OH extract)	0.30	0.35	0.35
Gardenia volkensii fruit cover (CH_2Cl_2 extract)	0.25	0.35	0.35
Gardenia volkensii fruit seeds (CH_2Cl_2 extract)	0.25	>0.40	0.35
Gardenia volkensii fruits cover ($CH_3(CH_2)_4CH_3$ extract)	0.35	0.35	>0.40
Gardenia volkensii fruits seeds ($CH_3(CH_2)_4CH_3$ extract)	>0.40	>0.40	>0.40
GV2	>0.40	>0.40	<0.20

MIC=Minimum Inhibition Concentration, *BSb=Bacillus subtilis*, *SA= Staphylococcus aureus*, *EC= Escherichia coli*

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TABLE 3

Inhibition Zone Diameters (mm) of Doxycycline® antibiotic on *Bacillus subtilis*

Concentration.(µg/mL)	Inhibition zone (mm)
10,000	30
4,000	26
1,000	22
4000	21
100	17
40	12
10	10
4	10

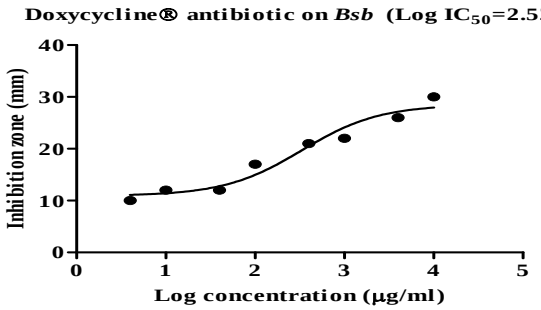

Fig.1. Doxycycline antibiotic on *Bacillus subtilis*

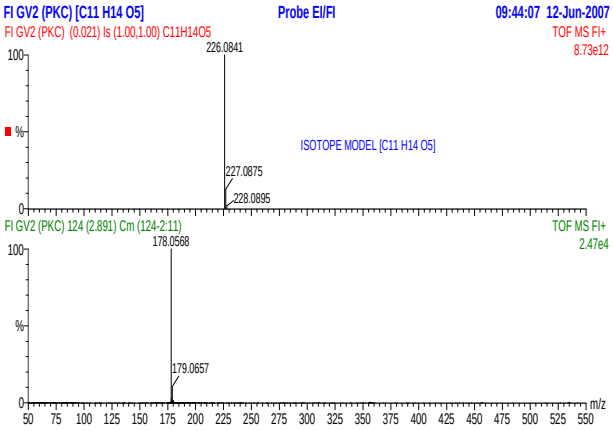

Fig. 2. MS of GV2

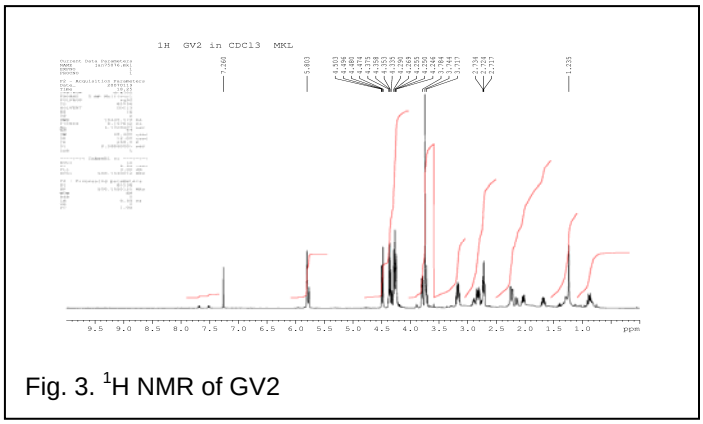

Fig. 3. ^{1}H NMR of GV2

4 Conclusion

In this research, all the crude extracts and the isolated pure **GV2** compound from the *Gardenia volkensii* showed some antimicrobial activity at a concentration of 40 µL in 10 mg/mL. **GV2** had an **MIC** of 20 µL-40 µL extract solutions, an indication that *Gardenia volkensii* contained compounds that are antimicrobial. All this validates the use of this plant by the Pokots of Kenya as anti-microbial agents.

The $\mathbf{IC_{50}}$ for the stem-bark of *Gardenia volkensii* crude extracts was too big as compared to the Doxycycline® antibiotics. Thus, the activity of the crude extracts was small and therefore a big dose of the concoction has to be administered for it to be effective. The proposed structures of was a novel monoterpenoid or modified iridoid.

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- *Kinuthia E. Wanjiru is an assistant lecturer at Laikipia University, Chemistry Department and is currenty pursuing PhD degree program in Chemistry at Egerton Univerity, Kenya, P.O. Box 536-20107, Njoro.E-mail:_ esthermjumbe@yahoo.com*
- *Dr. Mwangi E.M. is a Lecturer at Egerton University, Chemistry Department and one of the authors PhD supervisor, Kenya, P.O. Box 536-20107, Njoro. E-mail: _emmmwngi@yahoo.com*
- *Dr. Cheplogoi P.K. is a Senior Lecturer in Chemistry Department at Egerton University and is currently the Chairman of the Department, He is one of the authors PhD supervisor, Kenya, P.O. Box 536-20107, Njoro. E-mail: author_kiplagatpc@yahoo.co.uk*