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Bioactive compounds from *Juniperus procera* (Cupressaceae) with activity against common bean bacterial pathogens

Teresia Wangui Nduati[1]*, Isabel Nyokabi Wagara[2], Waudo Walyambillah[3], Beatrice Were[4] and Josphat Clement Matasyoh[1]

[1]Department of Chemistry, Egerton University, P. O. Box 536-20115, Egerton, Kenya.
[2]Department of Biological Sciences, Egerton University, P. O Box 536-20115, Egerton, Kenya.
[3]Department of Chemistry, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya.
[4]Department of Biological Sciences, University of Eldoret, P. O. Box 1125-30100, Eldoret, Kenya.

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Common bean is an important source of protein in sub-Saharan Africa but its production is constrained by various factors especially bacterial diseases. The aim of this study was to evaluate compounds from *Juniperus procera* for bioactivity against common bean pathogens, *Pseudomonas savastanoi* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli.* Solvent extraction method was used to obtain crude extracts from the stem bark and leaf of *J. procera* that underwent fractionation and purification using various chromatographic techniques, leading to the isolation of three compounds, namely: epicatechin (1), podocarpusflavone A (2) and juniperolide (3). Structures of the compounds were elucidated based on NMR and HRESIMS analyses. The bioactivity of the compounds was determined by disc diffusion assay. The compound epicatechin showed the highest activity against *P. savastanoi* pv. *phaseolicola* (21.7±1.2 mm). On the other hand, podocarpusflavone A and juniperolide showed weaker activity of 8.0±1.7 and 8.0±2.0 mm, respectively against the same pathogen. The three compounds showed weak or no activity against *X. axonopodis* pv. *phaseoli* of 6.0±0.0, 7.0±0.0 and 6.0±0.0 mm, respectively. Therefore, epicatechin can be used for the development of biopesticides for the control of *P. savastanoi* pv. *phaseolicola.*

Key words: Antibacterial compounds, *Juniperus procera, Pseudomonas savastanoi* pv. *phaseolicola, Xanthomonas axonopodis* pv. *phaseoli*.

INTRODUCTION

Common bean (*Phaseolus vulgaris*) is an important grain legume in the world. In Kenya, farmers produce it both for sale and home consumption (Katungi et al., 2010). It is one of the main sources of plant proteins, dietary fiber

*Corresponding author. E-mail: teresiawnduati@gmail.com.

and micronutrients (Celmeli et al., 2018), therefore plays an important role to human health and the economy. *Phaseolus vulgaris* is also reported to possess pharmacological properties due to the presence of secondary metabolites, including flavonoids, phenols and tannins (Singh et al., 2020).

Production of *P. vulgaris* however, has been declining in Kenya partly due to pests and diseases (Duku et al., 2020). Halo blight and common bacterial blight caused by *Pseudomonas savastanoi* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli*, respectively, are among the major bacterial diseases that affect common bean (Singh et al., 2020). These diseases are mostly controlled using copper-based bactericides (Schwartz, 2011) which can lead to environmental pollution.

Juniperus procera (Cupressaceae family) is an evergreen tree that is found in the highland forests of East Africa. In Kenya, the plant can be found in lower slopes of Mt. Elgon, Mt. Kenya, Tugen Hills and Aberdares (Maundu and Tengnäs, 2005). It is a medicinal plant which is traditionally used as a remedy for gum bleeding by applying a spoonful of charcoal on the teeth and gums (Ngari et al., 2014). Also, a concoction prepared from boiled roots and bark is used for cancer treatment by the Kalenjin community in Kenya (Kigen et al., 2017). Studies have also reported that extracts from the plant possess phytochemical compounds like triterpenes, flavonoids, tannins, saponins, and alkaloids (Ali and Suleiman, 2018). Pharmacological studies of extracts have reported the presence of antimalarial, antioxidant, antileishmanial, nematicidal and antibacterial compounds (Mossa et al., 2004; Samoylenko et al., 2008; Alqasoumi and Abdel-Kader, 2012; Samaha et al., 2017). Antifungal activity of the leaf and fruit extracts against *Aspergillus fumigatus* and *Fusarium chlamydosporum* has also been reported (Bakri et al., 2020).

In this study, we report compounds from *J. procera* and their activity against *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli*, the causal agents of bean halo blight and common bacterial blight, respectively.

MATERIALS AND METHODS

Plant

The leaf and stem bark samples of *J. procera* were collected from Mt. Elgon National Park Forest (1.1493°N, 34.5930° E), Kenya. The samples were dried under shade to constant weight at room temperature and ground into a fine powder.

Extraction and isolation

The stem bark powder (700 g) was soaked in methanol (MeOH) for 24 h at room temperature and filtered using Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure in a rotary evaporator to obtain methanol crude extract. The crude extract was suspended in water and partitioned between hexane and ethyl acetate yielding aqueous, hexane and ethyl acetate extracts. Ethyl acetate extract was applied on column chromatography over silica gel and eluted with hexane/ethyl acetate and ethyl acetate/methanol mixtures of increasing polarities. Fraction 3 (100% ethyl acetate) was further separated by reverse phase preparative HPLC using Gemini C18 column (10 × 250 mm, 10 µm particle size, Phenomenex). The mobile phase used was double distilled water (with 0.1% formic acid) (A) and HPLC grade methanol (B). The gradient elution used 45 to 70% of solvent B for 18 min and then 100% solvent B for 7 min. The system returned to initial conditions of 45% solvent B within 0.5 min and equilibrated for 10 min. Ultraviolet (UV) monitoring was done at 230, 254, 275, 320 and 370 nm at a flow rate of 3 mL/min. Three fractions (JPB-1, JPB-2 and JPB-3) were obtained. Fraction JPB-2 was further purified using a VP 125/10 NUCLEODUR PolarTec column (10×125 mm, 5 µm, Macherey-Nagel) as the stationary phase with a flow rate of 3mL/min and isocratic conditions of 35% solvent B for 20 min to yield compound 1 (12 mg).

Similarly, ethyl acetate extract was obtained from the leaf as described earlier and applied on column chromatography over silica gel and eluted with ethyl acetate, hexane, and methanol mixtures in the ratio 6:3:1, respectively. Seven fractions were obtained and fraction 2 was further separated by reverse phase preparative HPLC. The gradient elution used 45 to 80% of solvent B for 20 min followed by 100% solvent B for 10 min. The system then returned to initial conditions of 45% solvent B within 0.5 min and equilibrated for 10 min. This yielded eight fractions (JPL 2A- 2H). Fraction JPL 2H was obtained as a pure compound 2 (3.8 mg). Fraction JPL 2D was further purified using 55% solvent B isocratic conditions for 20 min to afford compound 3 (3.5 mg).

Mass spectrometry

Mass spectrometry was carried out to determine the molecular masses of the three isolated compounds. MaXis electrospray ionization-time of flight (ESI-TOF) mass spectrometer was used to record high resolution electrospray ionization mass spectrometry (HR-ESIMS) data.

Nuclear magnetic resonance (NMR)

NMR experiments were performed on Bruker Avance III 700 MHz spectrometer equipped with 5 mm TCI cryoprobe (1H;700 MHz, 13C:175 MHz), which was used to measure one and two-dimensional NMR spectra.

Antibacterial assay

The bacterial pathogens, *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli* were isolated from infected bean leaves, purified and the pure cultures were preserved in sterilized 50% glycerol and stored in a freezer. The bacterial strains were revived 24 h prior to the bioassay using a sterile wire loop to scrape the frozen bacteria and streaking it on a plate containing nutrient agar. The inoculated plates were then incubated at 37°C for 24 h.

The antibacterial activity of compounds 1, 2 and 3 was screened against the bean pathogens. The dry compounds were first dissolved in dimethyl sulfoxide (DMSO) to make a solution of concentration 2 mg/mL. Sterile sensitivity discs (6 mm in diameter) impregnated with 100 µl of the solution were screened for bioactivity against the bacterial pathogens. This was done by placing them in Petri dishes containing Mueller Hinton agar and the

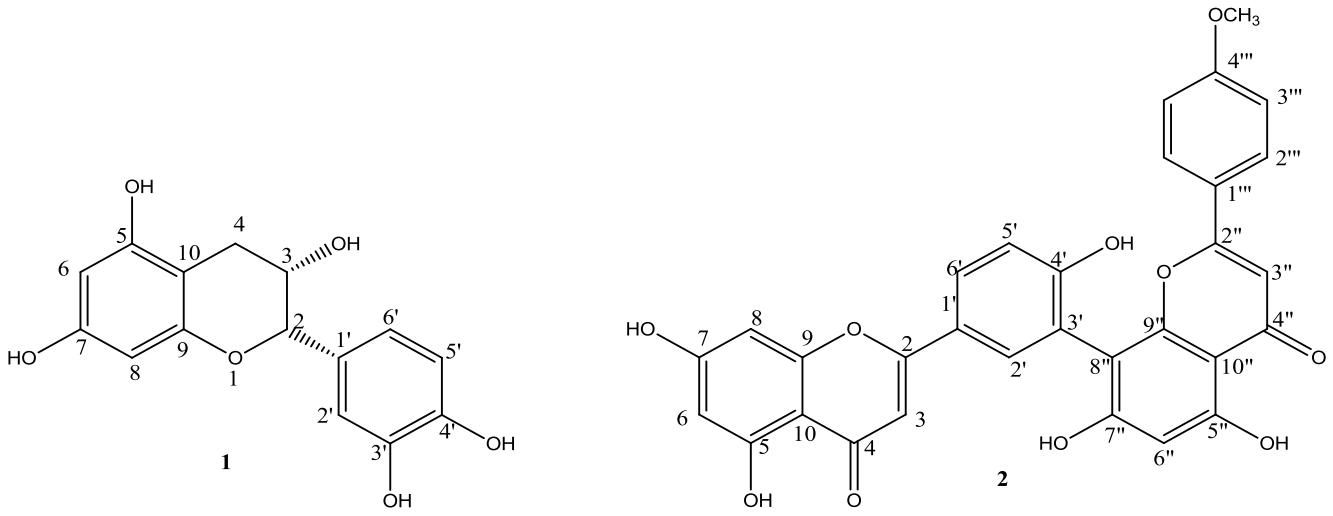

Figure 1. Structures of compounds 1 and 2 from *Juniperus procera*.
Source: Authors

respective bacterial strain followed by incubation for 24 h at 37°C. Sensitivity discs impregnated with DMSO and chloramphenicol were used as negative and positive control, respectively. The zones of inhibition were recorded in millimetres after 24 h. The experiments were done in triplicate.

Data analysis

The NMR spectra obtained were analysed using MestReNova NMR analysis software while Bruker Compass DataAnalysis 4.4 software was used to analyse data from mass spectrometry. The means of the inhibition zones from bioassays were calculated and one-way ANOVA used to determine the difference in mean inhibitory effect of the compounds. Tukey's Honestly Significant Difference (HSD) at 95% confidence level was used to separate the significant mean differences. This was done using Statistical Package for the Social Sciences (SPSS) software.

RESULTS AND DISCUSSION

Compound 1 (Figure 1(1)) was isolated as a brown amorphous solid. The data from mass spectrometry of this compound gave a molecular ion peak at *m/z* 313.0682 which corresponded to $[M+Na]^+$ ion. A peak observed at *m/z* 603.14703 corresponded to $[2M+Na]^+$ ion. This mass spectral data suggested molecular formula of the compound to be $C_{15}H_{14}O_6$. Structure elucidation of this compound was done by analysis of ^1H, ^{13}C, HSQC, NOESY, COSY and HMBC NMR spectra and also by comparison with data from previous literature (Yusuf et al., 2019). The ^1H and ^{13}C NMR spectral data (Table 1) suggested that compound 1 is a flavonoid. The ^1H NMR spectrum showed the presence of five aromatic protons at δ_H 5.94, 5.87, 6.86, 6.78 and 6.74. Signals at δ_H 2.52 and 2.87 corresponded to the two methylene protons. A total of fifteen signals were observed in ^{13}C NMR spectra. Five carbon signals at δ_C 144.8, 144.9, 155.5, 156.2 and 156.5 correspond to oxygenated aromatic carbon atoms. The signal at δ_C 27.1 corresponded to the methylene carbon on the aliphatic ring. The HSQC spectrum assisted in assigning protons that were directly attached to carbon atoms. The spectrum showed correlations between δ_H 4.58 with C-2, 3.99 with C-3, 2.52/2.87 with C-4, 5.94 with C-6, 5.87 with C-8, 6.86 with C-2´, 6.78 with C-5´ and 6.74 with C-6´. Furthermore, COSY correlations of H-2 (δ_H 4.58) with H-3 (δ_H 3.99), H-3 (δ_H 3.99) with H-4 (δ_H 2.57 and δ_H 2.87) identified the connectivity sequence in the aliphatic ring. NOESY correlation between H-2 (δ_H 4.58) and H-3 (δ_H 3.99) suggested cis configuration of the molecule at position 2 and 3. The connectivity of the three rings was further established by HMBC correlations. Correlations from H-2 (δ_H 4.58) to C-3 (δ_C 67.4), C-4 (δ_C 27.1), C-1´ (δ_C 130.8), C-2´ (δ_C 113.9), C-5´ (δ_C 114.7) and C-6´ (δ_C 118.6) and from H-4 (δ_H 2.52/2.87) to C-2 ($\delta_{C\ c}$ 81.5), C-3 (δ_C 67.4), C-10 (δ_C 99.4), C-5 (δ_C 155.5), C-6 (δ_C 94.9), C-9 (δ_C 156.2) and C-1´ (δ_C 130.8) were important in determining the connectivity of the rings in the molecule. This compound was identified as epicatechin, a flavonoid and has been previously isolated by Yusuf et al. (2019) from *Neocarya macrophylla.* It has also been previously isolated from Cupressaceae family (*Juniperus communis* and *Juniperus drupacea*) (Seca and Silva, 2006).

Compound 2 (Figure 1(2)) was isolated as a yellow powder. Its molecular formula, $C_{31}H_{20}O_{10}$ was determined from the molecular ion peak at *m/z* 551.0985 which corresponded to $[M-H]^-$ ion and 1103.2021 corresponding to $[2M-H]^-$ ion. The carbon skeleton was assigned using both 1D and 2D NMR spectra and also comparison

Table 1. ^{1}H NMR (700 MHz) and ^{13}C NMR (175 MHz) spectroscopic data of Compounds 1 and 2 from *Juniperus procera*.

No.	Compound 1		Compound 2	
	δ_C, Type	**δ_H, multiplet (J in Hz)**	**δ_C, Type**	**δ_H, multiplet (J in Hz)**
2	81.5, CH	4.58, d (7.5)	163.6, C	
3	67.4, CH	3.99, m (5.4, 7.5, 8.0)	102.6, CH	6.82, s
4	27.1, CH_2	2.52, dd (8.0, 16.1) 2.87, dd (5.4, 16.1)	181.5, C	
5	155.5, C		161.2, C	
6	94.9, CH	5.94, d (2.3)	98.5, CH	6.18, d (2.1)
7	156.5, C		163.8, C	
8	94.1, CH	5.87, d (2.3)	93.7, CH	6.43, d (2.1)
9	156.2, C		157.1, C	
10	99.4, C		103.5, C	
1′	130.8, C		120.6, C	
2′	113.9, CH	6.86, d (2.0)	131.2, CH	8.01, d (2.3)
3′	144.9, C		120.1, C	
4′	144.8, C		159.6, C	
5′	114.7, CH	6.78, d (8.1)	116.1, CH	7.14, d (9.3)
6′	118.6, CH	6.74, dd (2.0, 8.1)	127.5, CH	8.00, dd (2.3, 9.3)
2′′			162.9, C	
3′′			102.9, CH	6.88, s
4′′			181.9, C	
5′′			160.3, C	
6′′			98.6, CH	6.38, s
7′′			162.0, C	
8′′			104.1, C	
9′′			154.5, C	
10′′			103.5, C	
1′′′			122.8, C	
2′′′			127.7, CH	7.69, d (9.0)
3′′′			114.1, CH	6.91, d (9.0)
4′′′			161.9, C	
5′′′			114.1, CH	6.91, d (9.0)
6′′′			127.7, CH	7.69, d (9.0)
OMe-4′′′			55.2, CH_3	3.74, s

Compound 1 recorded in CD_3OD and compound 2 recorded in DMSO.
Source: Authors

from previous literature (Carbonezi et al., 2007). The spectral data obtained is as recorded in Table 1. ^{1}H NMR spectrum supported the presence of a methoxy group through a singlet peak at δ_H 3.74. The presence of aromatic protons was also evident through the signals at δ_H 6.18, 6.38, 6.43, 6.82, 6.88, 6.91, 7.14, 7.69, 8.00 and 8.01. The carbon atoms were assigned using the 2D NMR spectra. A total of 31 carbon atoms were displayed with two carbonyl carbons at δ_C 181.5 and δ_C 181.9. A signal at δ_C 55.2 was characteristic of a methoxy group. The HSQC spectrum showed correlation between δ_H 6.82 with C- 3, 6.18 with C-6, 6.43 with C-8, 8.01 with C-2′, 7.14 with C-5′, 8.00 with C- 6′, 6.88 with C- 3′′, 6.38 with C-6′′, 7.69 with C- 2′′′and C-6′′′, 6.91 with C- 3′′′ and C-5′′′ and 3.74 with CH_3O-4′′′. COSY correlation between H-5′ (δ_H 7.14) and H-6′ (δ_H 8.00), H-2′′′ (δ_H 7.69) and H-3′′′ (δ_H 6.91) assisted in establishing the connectivity of the methine groups in the molecule. The HMBC correlations from H-3 (δ_H 6.82) to C- 2 (δ_C 163.6), C-4 (δ_C 181.5), C-10 (δ_C 103.5) and C-1′ (δ_C 120.6) were important in connecting ring B and C. Further, the correlations from H- 3′′ (δ_H 6.88) to C-2′′ (δ_C 162.9),

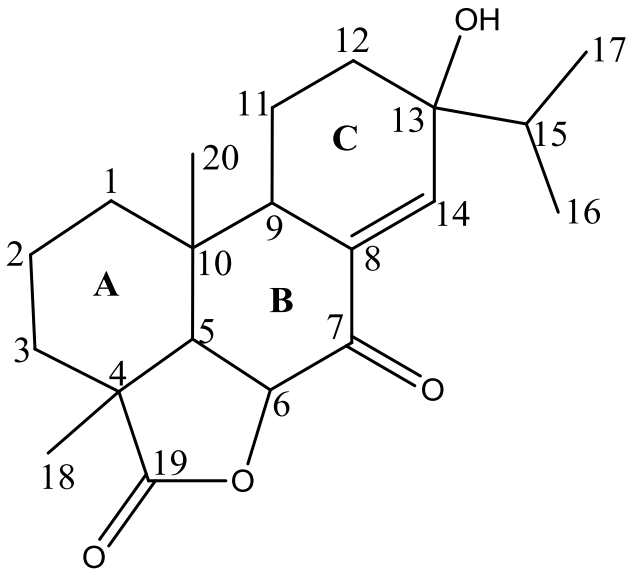

Figure 2. Structure of compound 3 isolated from *Juniperus procera*.
Source: Authors

C-4´´ (δ_C 181.9), C-10´´ (δ_C 103.5) and C-1´´´ (δ_C 122.8) established connectivity between ring B´´ and C´´. The HMBC correlation from H-2´ (δ_H 8.01) with C-2 (δ_C 163.6), C-4´ (δ_C 159.6), C-6´ (δ_C 127.5) and C-8´´ (δ_C 104.1) was important in determining the linkage between the two flavonoids. This is especially through the correlation with C-8´´ (δ_C 104.1) which showed linkage between C-3´ (δ_C 120.1) and C-8´´ (δ_C 104.1). Compound 2 was identified as podocarpusflavone A, a biflavonoid that has been previously isolated by Carbonezi et al. (2007) from *Ouratea multiflora* (Ochnaceae). It was also isolated from Cupressaceae family (*J. communis*) by Hiermann et al. (1996) as cited by Seca and Silva (2006).

Compound 3 (Figure 2) was isolated as a brown amorphous solid. Mass spectrometry data gave a molecular ion peak at *m/z* 331.1915 which corresponded to $[M-H]^-$ ion. This suggested the molecular formular of compound 3 to be $C_{20}H_{28}O_4$. The carbon skeleton was assigned based on 1D (Table 2) and 2D NMR spectra and comparison with data from previous literature (Jim-Min et al., 1993). ^{13}C NMR data was determined using 2D NMR data and twenty carbon atoms were observed with two carbonyl carbons at δ_C 194.1 and δ_C 181.6 and two olefinic carbons at δ_C 136.9 and δ_C 142.8. The methyl groups of the isopropyl group were evident from ^1H NMR spectrum due to the strong doublet signals at δ_H 0.99 (H-17) and 0.88 (H-16). Methyl signals (singlets) were also observed at δ_H 0.73 (H-20) and δ_H 1.33 (H-18). The signal at δ_H 6.86 (H-14) was characteristic of the β-unsaturated ketone while the signal at δ_H 4.98 (H-6) was characteristic of the γ-H of a lactone. From the HSQC spectrum, there was correlation between δ_H 1.37/1.65 with C-1, 1.66 with C-2, 1.52/2.12 with C-3, 2.34 with C-5, 4.98 with C-6, 2.22 with C-9, 1.47/1.73 with C-11, 1.63/1.76 with C-12, 6.86 with C- 14, 1.81 with C-15, 0.88 with C-16, 0.99 with C-17, 1.33 with C-18 and 0.73 with C-20. The ^1H-^1H COSY between H-15 (δ_H 1.81) and H-16 (δ_H 0.88), H-15 (δ_H 1.81) and H-17 (0.99) (Figure 3) aided in assigning the carbon atoms on the isopropyl group. COSY correlation between H-5 (δ_H 2.34) and H-6 (δ_H 4.98) was also observed. HMBC spectrum assisted in determining the connectivity of the rings. HMBC correlations of protons H-3 (δ_H 1.52,2.12) to C-4 (δ_C 41.9), C-5 (δ_C 51.8), C-18 (δ_C 24.2) and C-19 (δ_C 181.6) as well as H-5 (δ_H 2.34) to C-4 (δ_C 41.9), C-18 (δ_C 24.2) and C-19 (δ_C 181.6) and also H-18 (δ_H 1.33) with C-3 (δ_C 28.1), C-4 (δ_C 41.9), C-5 (δ_C 51.8), C-19 (δ_C 181.6), were important in establishing the connectivity of ring A to the lactone ring.

Furthermore, correlations from H-20 (δ_H 0.73) to C-5 (δ_C 51.8), C-9 (δ_C 48.9) and C-10 (δ_C 32.8), and from H-9 (δ_H 2.22) to C -5 (δ_C 51.8), C-8 (δ_C 136.9), C-10 (δ_C 32.8), C-11 (δ_C 18.5), C-12 (δ_C 29.5), C-14 (δ_C 142.8) and C-20 (δ_C 15.8) aided in determining the linkage of ring B to ring C. Correlations from H-14 (δ_H 6.86) to C-7 (δ_C 194.1), C-8 (δ_C 136.9), C-9 (δ_C 48.9), C-12 (δ_C 29.5) and C-15 (δ_C 37.5) and H-6 (δ_H 4.98) to C-5 (δ_C 51.8), C-7 (δ_C 194.1) and C-10 (δ_C 32.8) were important in determining the position of the alpha-beta unsaturated group as well as connectivity of ring B to C. The position of the isopropyl group was supported by HMBC correlations from H-15 (δ_H 1.81) to C-12 (δ_C 29.5), C-13 (δ_C 71.7), C-14 (δ_C 142.8), C-16 (δ_C 16.4) and C-17 (δ_C 15.1). Compound 3

Table 2. ^{1}H NMR (700 MHz) and ^{13}C NMR (175 MHz) spectroscopic data of Compound 3 from *Juniperus procera*.

No.	δ_C, Type	δ_H, Multiplet (J in Hz)
1	32.0, CH_2	1.37,1.65
2	17.3, CH_2	1.66
3	28.1, CH_2	1.52,2.12
4	41.9, C	
5	51.8, CH	2.34, d (6.3)
6	76.8, CH	4.98, d (6.3)
7	194.1, C	
8	136.9, C	
9	48.9, CH	2.22
10	32.8, C	
11	18.5, CH_2	1.47,1.73
12	29.5, CH_2	1.63,1.76
13	71.7, C	
14	142.8, CH	6.86, dd (1.7, 3.0)
15	37.5, CH	1.81
16	16.4, CH_3	0.88, d (6.9)
17	15.1, CH_3	0.99, d (6.9)
18	24.2, CH_3	1.33, s
19	181.6, C	
20	15.8, CH_3	0.73, s

Recorded in CD_3oD.
Source: Authors

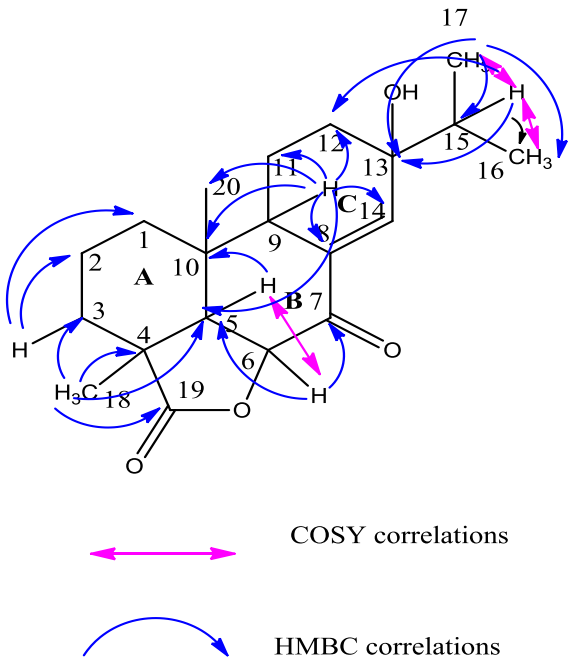

Figure 3. Structure of compound 3 showing selected HMBC and COSY correlations.
Source: Authors

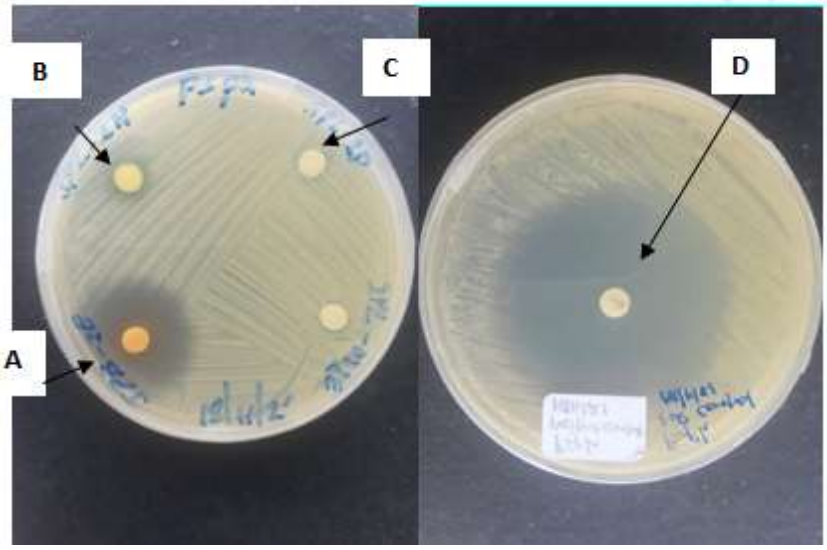

Figure 4. Inhibition of the isolated compounds against *Pseudomonas savastanoi* pv. *phaseolicola*: (A) compound 1, (B) compound 2, (C) compound 3, and (D) chloramphenicol.
Source: Authors

Table 3. Inhibition zones (mm) of compounds 1, 2 and 3 from *Juniperus procera* against test bacterial pathogens.

Compound/Treatment	Inhibition zones in mm (n=3)	
	P. savastanoi* pv. *phaseolicola	***X. axonopodis* pv. *phaseoli***
Compound **1**	21.7±1.2^{c}	6.0±0.0^{a}
Compound **2**	8.0±1.7^{b}	7.0±0.0^{a}
Compound **3**	8.0±2.0^{b}	6.0±0.0^{a}
Chloramphenicol	47.0±0.0^{d}	37.0±0.0^{b}
DMSO	6.0±0.0^{a}	6.0±0.0^{a}

Within a column, the inhibition zones of compounds sharing the same letter(s) are not significantly different while those with different letters are significantly different (α =0.05, Tukey's test).
Source: Authors

was identified as juniperolide, a diterpenoid that was first isolated by Jim-Min et al. (1993) from *J. chinensis* (Cupressaceae).

Antibacterial activity

Compounds 1, 2 and 3 were subjected to tests against two Gram-negative bean pathogens *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli*. Compound 1 was the most active against *P. savastanoi* pv. *phaseolicola* with an inhibition zone of 21.7 ± 1.2 mm (Figure 4) but was not active against *X. axonopodis* pv. *phaseoli* (Table 3). The activity of Compound 1 against *P. savastanoi* pv. *phaseolicola* can be attributed to the presence of the catechol moiety. This moiety has previously been reported to be an active site responsible for antioxidant bioactivity (Ruijters et al., 2013). Compounds 2 and 3 showed inhibition zones of 8.0 ± 1.7 and 8.0 ± 2.0 mm against this pathogen, respectively. Compounds 2 and 3 showed weak activity against both pathogens.

Conclusion

From the results, it is evident that *J. procera* is a potential source of antibacterial compounds for controlling bean pathogens. Compound 1 (epicatechin) has high anti-bacterial activity against *P. savastanoi* pv. *phaseolicola*

and can be used in development of biopesticides for the control of this pathogen.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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