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African Journal of Biotechnology

Full Length Research Paper

The use of microsatellites in the characterization of three plantain cultivars from the Democratic Republic of Congo

Mave Dhed'asi Gracia[1]*, Dhed'a Djailo Benoit[1], Kateregga John N.[2] and Nanyiti Sarah[3]

[1]Department of Biotechnology, University of Kisangani, P. O. Box 2012 Kisangani, Democratic Republic of Congo.
[2]College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, P. O. Box 7062 Kampala, Uganda.
[3]National Crop Resources Research Institute, Namulonge Uganda, P. O. Box 7084 Kampala, Uganda.

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Knowledge of the genetic variability of plantain cultivars in the Democratic Republic of the Congo (DRC) is limited. This study assessed genetic diversity and relationships in three plantain accessions differing in pseudostem color at seven microsatellite loci using SSR markers. DNA was extracted from banana leaves, and PCR was performed with unlabeled and fluorescent-labeled SSR primers and eventual capillary sequencing. Different population genetic analysis software was used to interpret the fragment analysis data. There was a high allelic power with a mean polymorphic information content of 0.83. A high average genetic diversity was indicated by Nei's Gene Diversity Index (h: 1.38) and the Shannon Information Index (I: 2.22). Pairwise genetic similarity between plantain genotypes averaged 0.82. A dendrogram grouped the 60 genotypes into three clusters. AMOVA showed high genetic variation within the population (98%) compared to the variation between populations, with no significant difference between the three cultivars regarding the color of the pseudostem. In contrast, these cultivars were closely related to each other. No correlation between the morphological and molecular characterization of cultivars was observed. We recommend a follow-up study with other plantain cultivars from other regions and large sample size for more representative data.

Key words: Characterization, microsatellites, genetic diversity, plantain cultivars.

INTRODUCTION

Bananas and plantains are of great economic, cultural, and nutritional importance worldwide, especially in developing countries, including the Democratic Republic of the Congo (DRC). They occupy the second position as a food crop, which also contributes greatly to the food security of the population of this mostly poor country, especially in the Oriental province (Onautshu, 2013). In 2019, banana production in the DRC was estimated at 64110596 tons, including 4856474 tons of plantains coming mainly from the lowland forest region (National Institute of Statistics (NIS), 2020). In addition, it is also an important source of household income, along with

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

cassava, rice, maize, and palm oil (Kasaka et al., 2021).

Most cultivated bananas are triploid and belong to the Eumusa section of the Musaceae family (Tenkouano et al., 2007). These cultivars are derived from intra- and interspecific crosses of two diploid ancestral species, *Musa acuminata* Colla (genome AA) and *Musa balbisiana* Colla (genome BB), originating from Malaysia and India, respectively (Lusty et al., 2006). Plantains that were characterized in this study belong to the AAB group (Perrier et al., 2011). The DRC has rich genetic diversity in bananas, especially plantains. The estimated plantain diversity in the Democratic Republic of the Congo is more than 100 cultivars. Most cultivars followed the traditional banana cultivar classification and identification system, which is mainly based on morphological and quantitative characteristics such as bunch type, crop size, bunch orientation, pseudostem color, and fruit peel color (Adheka, 2016).

However, it is challenging to classify accessions from the same variety using morphology since this method has some limitations when it comes to accurately identifying them. For example, there are only a limited number of traits available for characterization, and these traits can be strongly influenced by environmental factors for quantitative traits (Blazakis et al., 2017). Additionally, some heritable traits may show insignificant variations, and there can be difficulties in distinguishing physical traits due to the morphological similarity of the accessions (Rana, 2018). Color variation usually affects the pseudostem more than any other part of the plant. In the case of False Horn plantain accessions, for instance, the three cultivars used in this study share identical traits except for the color of their pseudostems. The pseudostem of Libanga Likale is green, that of Libanga Lifombo is red, and that of Libanga black is black (Adheka et al., 2018).

Some molecular characterization has been done, but not all plantain cultivars have been genetically characterized. Hence, the extent of molecular diversity among plantain varieties in the DRC is not very well understood. Various types of genetic markers are utilized to measure variation among genotypes. Microsatellites, also known as Simple Sequence Repeats (SSRs), are the most commonly used because they are dependable and can be used across different laboratories. These markers are codominant and can identify a large number of variable bands in a single sample rather than having high levels of variation at each genetic locus (Kitavi, 2015). SSRs are valuable because they are widespread, present throughout the genome, have multiple alleles, are highly reproducible, and can work with small quantities of material or degraded or ancient DNA (Senan et al., 2014). However, the mutation rates of SSRs are excessively high, leading to alterations in conserved regions between species and homoplasy (alleles that are identical in the state but not origin) (Barkley et al., 2009). There is currently a group of 22 SSR markers utilized for the molecular characterization of bananas that can distinguish subgroups and subspecies (Christelová et al., 2011; Hippolyte et al., 2012). This study was thus aimed at determining genetic diversity within and among the Congo basin plantain populations using microsatellite markers to determine if there is a correlation between morphological diversity and genetic diversity among the three plantain cultivars.

MATERIALS AND METHODS

Study location

Samples were obtained from two plantain collections in the Congo basin at the University of Kisangani, located in the North-Eastern region of the DRC. The first collection is located at the Faculty of Sciences, shown as the faculty collection on the map, and the second collection is the intendance collection (Figure 1).

Plant

Three False Horn plantain cultivars, which differ only in their pseudostem color (Figure 2), were used in this study. The samples (20 per cultivar) were collected by punching banana leaf discs directly into 2 mL microcentrifuge tubes (Changadeya et al., 2012). The leaf samples were put in the ice box containing ice packs and were authorized by the University of Kisangani and the Institute of Agriculture for transportation and analysis at NaCCRI, Uganda. The samples were kept in the deep freezer at -80°C for better preservation and to prevent denaturation of DNA.

DNA isolation

The extraction of DNA was done using Cetyltrimethylammonium Bromide (CTAB) as described by Changadeya et al. (2012) with a few modifications. Leaf discs of about 100 mg each were ground using a mortar and pestle and then transferred to the 2.0 mL microcentrifuge tube. 400 µL of extraction buffer (1.5% CTAB), 100 mM Tris-HCl, 20 mM EDTA, 1.4 mM NaCl, and 0.2% ß-mercaptoethanol were added to the sample and then incubated at 65°C for 30 min, shaking after 10 min. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added, and the homogenate was hand-mixed for 10 min at room temperature. The mixture was centrifuged at 12,000 rpm for 10 min. 300 µL of supernatant was transferred to a 2.0 mL microcentrifuge tube, and 210 µL of ice-cold isopropanol was added and incubated at -20°C for 1 h to precipitate DNA. The precipitated DNA was separated from the suspension by centrifugation at 13,000 rpm for 10 min; the supernatant was decanted, and the resultant pellet was washed in 400 µL of 70% cold ethanol to remove salt from the pellet and centrifuged for 10 min. The ethanol was decanted, and the DNA pellet was air dried for 10 to 15 min before dissolution in 100 µL nuclease-free water. The quantity and quality of the extracted DNA were determined using the Thermo Scientific Nanodrop 1000; DNA of good quality was defined as having an A260/A280 nm ratio between 1.8 and 2.0.

DNA amplification

To amplify the housekeeping actin gene in the extracted DNA, PCR was used as a check on the quality of the DNA extraction before using it in the upstream molecular analysis, including PCR

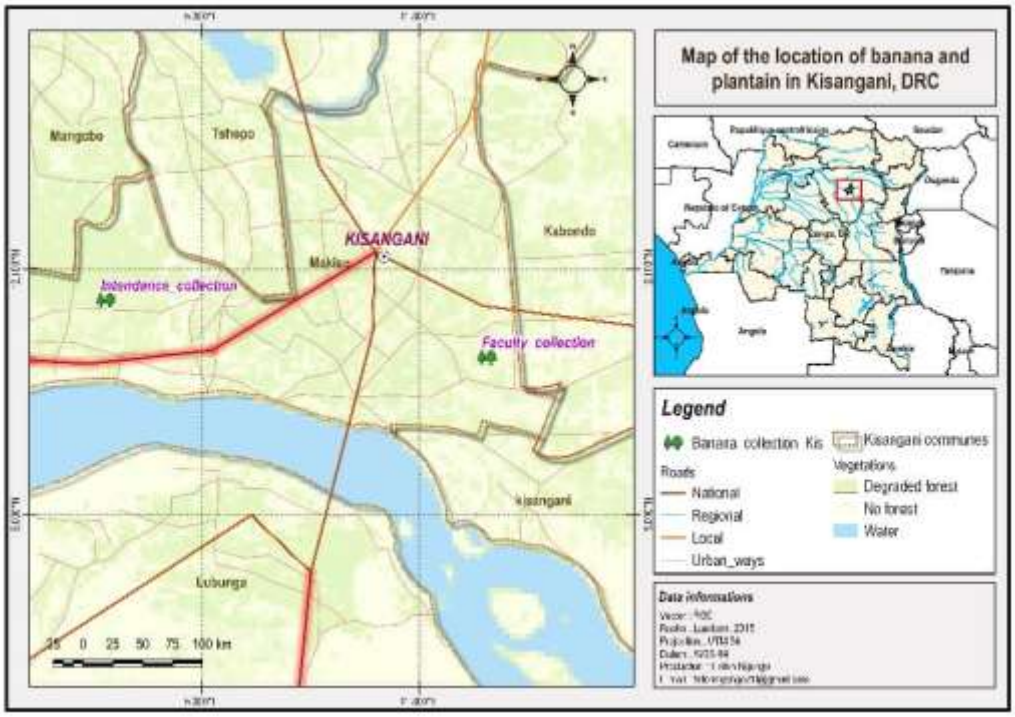

Figure 1. Map of the study area showing the banana collection sites including the Intendance collection and the Faculty of Sciences collection.
Sources: Fiston Ngongo Muselemu.

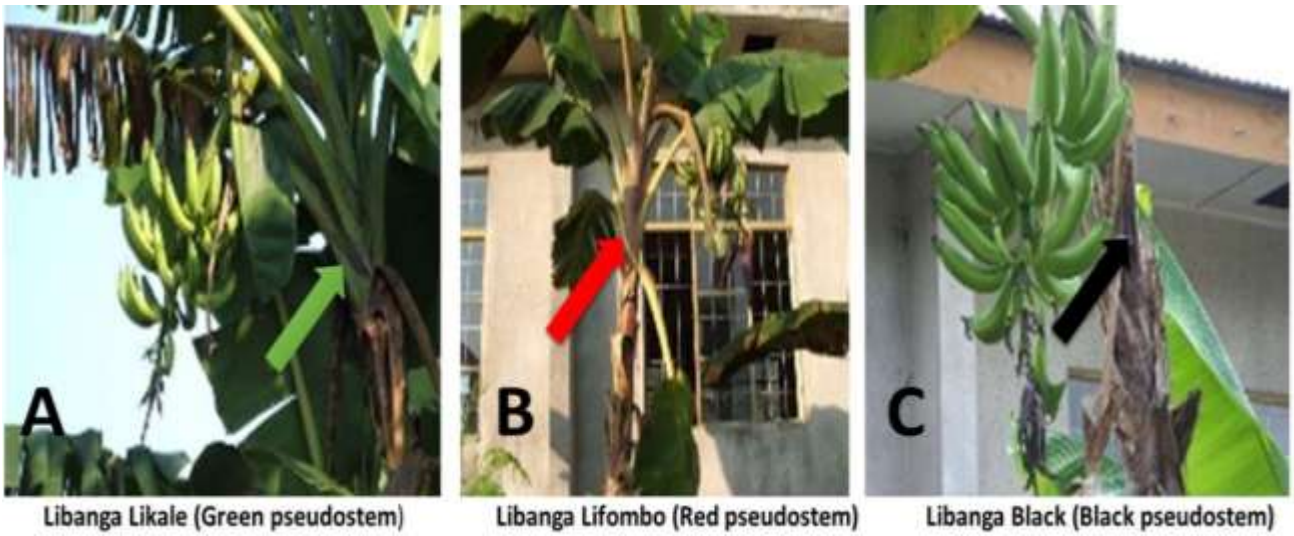

Figure 2. Plantain cultivars with different pseudostem colors used as biological material. (A) is the Libanga Likale with the green arrow pointing to the green pseudostem on the banana plant. (B) is the Libanga Lifombo with the red arrow pointing to the red pseudostem on the banana plant. (C) is the Libanga Black with the black arrow pointing to the black pseudostem on the banana plant. The three plantain cultivars are the most cultivated in the DRC.
Sources: Authors

amplification with SSR primers and capillary sequence analysis. Banana-specific actin gene primers forward, 5'ACCGAAGCCCCTCTTAACCC3' and reverse, 3'GTATGGCTGACACCATCACC5' reported in Tripathi et al. (2010) were used. The primers were manufactured by Macrogen (The Netherlands). The expected product size with the actin primers was

Table 1. Microsatellite primers used in the study.

Loci	Primes sequences	Expected product size	Ta (°C)
STMS1F/R	TGAGGCGGGGAATCGGTA GGCGGGAGACAGATGGAGTT	110-154	58
STMS7F/R	AAGAAGGCACGAGGGTAG CGAACCAAGTGAAATAGCG	234-252	54
STMS10F/R	ATGATCATGAGAGGAATATCT TCGCTCTAATCGGATTATCTC	116	53
AGMI93/94F/R	AACAACTAGGATGGTAATGTGTGGAA GATCTGAGGATGGTTCTGTTGGAGTG	124-158	57.5
Ma_1/16F/R	TTTGCCTGGTTGGGCTGA CCCCCCTTTCCTCTTTTGC	140-166	58.5
Ma_1/17F/R	AGGCGGGGAATCGGTAGA GGCGGGAGACAGATGGAGT	100-156	57.7
Ma_1/18F/R	TTTGCCTGGTTGGGCTGA CCCCCCTTTCCTCTTTTGC	138-182	57.5
Ma_1/24F/R	GAGCCCATTAAGCTGAACA CCGACAGTCAACATACAATACA	154-168	54
Ma_1/27F/R	TGAATCCCAAGTTTGGTCAAG CAAACACATGTCCCCATCTC	114-132	54
Ma_3/2F/R	GGAACAGGTGATCAAAGTGTGA TTGATCATGTGCCGCTACTG	204-238	56.2
Ma_3/90F/R	GCACGAAGAGGCATCAC GGCCAAATTTGATGGACT	128-162	53.2
Ma_3/103F/R	TCGCCTCTCTTTAGCTCTG TGTTGGAGGATCTGAGATTG	138-160	54

F-Forward; R- reverse; Ta- annealing temperature.
Sources: Authors

about 250 bp. A positive PCR with actin primers indicated good-quality DNA extraction and hence the DNA was subsequently used for molecular analysis using SSR primers. Polymerase Chain Reaction was carried out in the Thermocycler (BioRAD) in a 20 µL of final reaction volume that contained 10 µL One Taq Quick Load (New England Biolabs), 6 µL of Nuclease free water, 2 µL of genomic DNA and 1 µL of forward (10 µM) and reverse (10 µM) of banana actin primers. For each PCR, appropriate positive and negative controls were used. During the amplification process, denaturation at 94°C for 5 min was followed by 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and then 72°C for 1 min, followed by 7 min of extension at 72°C.

Twenty DNA samples chosen at random before labeling with fluorescent dyes were amplified using 12 different primer sets (Table 1) used by Changadeya et al. (2012) and manufactured by Macrogen (Netherlands). Each SSR marker was amplified separately using the Thermocycler (Bio-Rad) by conducting PCR reactions. The total reaction mixture volume of 20 µL comprised 10 µL of One Taq Quick Load, 1 µL of forward (10 µM) and reverse (10 µM) SSR primers, 6 µL of Nuclease free water, and 2 µL of genomic DNA. The PCR reaction was carried out with the following conditions: denaturation at 94°C for 30 s, followed by 35 amplification cycles consisting of denaturing at 94°C for 30 s, annealing at an optimal temperature (which varied based on the melting temperature (Tm) of the primers used) for a specific primer pair for 15 s, and elongation at 72°C for 30 s (the elongation time varied depending on the expected product size of the PCR product). The final extension was performed at 65°C for 20 min, followed by a 4°C soaking temperature. The markers such as Ma_3/90, STMS1, STMS10, and Ma_1/18, and Ma_3/2 were not

Table 1. Dyes and primers selected for capillary sequencing.

Panel	Marker	Annealing temp. (°C)	Allele size range (bp)	Dye color
1	Ma_3/103FP	54	138-160	FAM
	STMSTFP	54	234-252	FAM
	AGM193/94FP	57.5	124-158	HEX
2	Ma_1/24FP	54	154-168	FAM
	Ma_1/27FP	54	114-132	HEX
3	Ma_1/17FP	57.7	100-156	FAM
	Ma_1/16FP	58.5	140-166	HEX

Sources: Authors

labeled since they did not display the expected level of variation.

Seven of the twelve microsatellite primer pairs were chosen because of their reported level of variation in the Musa cultivar (Changadeya et al., 2012). For the remaining polymorphic markers, the forward primer was labeled at the 5' end with either FAM (blue color) or HEX (green color) as indicated in Table 2. To avoid allele overlap, primers with similar product sizes were dyed in various colors (Masi et al., 2003). The labeled primers were then used in PCR. The total reaction volume of 20 µL PCR mix contained 3 µL DNA, 1 µL each of the labeled forward primer (10 µM) and reverse primer (10 µM), 5 µL of Nuclease Free Water, and 10 µL One Taq Quick-Load (New England Biolabs). The amplification consisted of an initial denaturation at 94°C for 35 s, followed by 35 cycles of denaturation at 89°C for 30 s, annealing for 30 s with temperature variations according to the primers used, and extension at 72°C for 30 s. A final extension phase was carried out at 72°C for 5 min, and the PCR products labeled with dye and expected to have the same fragment size were combined in the same PCR mixture to create a single panel, which was then separated by capillary electrophoresis.

The samples were then placed on 96 microplate wells and sent to Macrogen Europe (Netherlands) for capillary sequencing of the amplicons. Genemarker v2.6.3 Software was used to analyze and identify alleles of the resulting data. The process of allele sizing and identification followed the instructions in the user manual, and the alleles were manually scored as fragment sizes in base pairs (Christelová et al., 2011).

Statistical analysis

Analysis of genetic variation

Polymorphism Information Content (PIC) was calculated as described by Botstein et al. (1980) using the formula:

$$PIC = 1 - \sum_{i=1}^{n} Pi^2$$

Pi represents the frequency of the ith allele out of the total number of alleles at the Simple Sequence Repeats (SSR) locus, while n indicates the total number of different alleles present at that locus. Each SSR allele was treated as a separate marker, and the presence or absence of each PCR amplification product was recorded as either "1" or "0," respectively, resulting in a binary data matrix because determining the number of copies of individual alleles in polyploid species is difficult (Lian et al., 2003; Mengoni et al., 2000). The genetic diversity parameters within populations and between banana cultivar collections were estimated using GenAlex Version 6.503 Software (Peakall and Smouse, 2012). The diversity was analyzed using these parameters: the number of alleles (Na), the number of effective alleles (Ne), gene diversity (h), Shannon's information index (I), and gene differentiation (Gst).

Analysis of cultivar similarity

The binary data matrix was generated and scored as either present (1) or absent (0) based on Nei's genetic distance to identify the genetic similarity among banana cultivars (Nei and Li, 1979). The Unweighted Pair Group Method Using Arithmetic Mean (UPGMA) cluster analysis was used to process similarity data using the R-3.6.1 Software Agnes package. A Principal Component Analysis (PCA) based on the SSR frequency was carried out to further evaluate the genetic linkages of plantain accessions as individual plants.

Structure analysis

The genetic structure of three cultivars was performed using the Bayesian model-based classification implemented in the STRUCTURE v2.3.4 software (Hilal et al., 2014). Using the mixing model, the value of K ranged from 1 to 10, with 100,000 MCMC, twenty iterations, and a break-in period of 200,000 (Workneh et al., 2022). The best probable K value was determined using the web-based structure harvester version 0.6.92 (Earl and Von Holdt, 2012).

Genetic differentiation and Analysis of Molecular Variance (AMOVA)

AMOVA was performed to compare differences within and between populations. The statistical significance of the AMOVA was assessed using a non-parametric permutation approach with 99 permutations. The degree of genetic differentiation among sub-populations was evaluated based on Gst values. Additionally, unbiased genetic distance (Nei, 1978) was used to calculate pairwise genetic distances.

RESULTS

Gene diversity and polymorphism

The genetic diversity of plantains was assessed using seven SSR markers, all of which showed variation, with a

Table 3. Number of alleles observed in the different populations.

Marker	Ma_1/103		STMS7		AGM93/94		Ma_1/24		Ma_1/27		Ma_1/16		Ma_1/17		Total
Population	TNA	ASR	TNA	ASR	TNA	ASR	TNA	ASR	TNA	ASR	TNA	ASR	TNA	ASR	
Libanga Likale	3	147-159	6	234-249	7	137-151	3	160-167	7	116-122	5	154-164	8	117-134	39
Libanga Noir	6	147-157	7	234-248	9	137-152	6	160-168	8	116-123	5	154-165	7	117-128	48
Libanga Lifombo	3	147-152	6	234-244	9	137-153	4	161-167	6	116-124	6	154-166	8	117-124	42
Total	12	-	19	-	25	-	13	-	21	-	16	-	23	-	129
PIC	-	0.67	-	0.97	-	0.85	-	0.85	-	0.95	-	0.7	-	0.72	-

TNA:Total number of alleles; ASR: allele size range; PIC: polymorphism information content.
Sources: Authors

total of 129 alleles detected across the loci, averaging 18.4 alleles per locus. The polymorphism information content (PIC) of the microsatellite primers ranged from 0.68 to 0.97, averaging 0.82 (Table 3.) The number of alleles observed ranged from 6.66 to 16.66, with an average of 11.61, while the number of valid alleles ranged from 6.31 to 14.64, with an average of 10.74. The average gene diversity (h) from Nei (1973) ranged from 0.28 to 2.72, with an average of 1.38 for the loci that produced polymorphic bands in 60 psyllium. This finding was further supported by Shannon's information index, with locus AGMI93/94 having the highest value of 4.11, while the lowest value of 0.57 was observed at locus Ma_1/103 (Table 4).

Similarity coefficient among the different plantain cultivars

Jaccard similarity coefficients based on seven SSR markers were used to assess the genetic similarities of all plantain genotypes under study. Among the sixty plantain genotypes, the similarity coefficient ranged from 0.35 to 0.94, with an average of 0.82. This finding suggests that the plantain accessions cultivated in the Congo basin have a high level of genetic diversity.

Genetic relationships among the plantain genotypes

A dendrogram was created based on the genetic similarities of the genotypes, and it showed that the three populations formed three distinct clusters (Figure 3). Observations in Cluster 1 are closer to those in Cluster 2 than those in Cluster 3. Sample 1 to sample 16 in population 1 is more similar to each other. PCA did not reveal any significant clustering among the 60 populations. The first component comprised 7.76% and the second principal component 10.15% of the total variation (Figure 4).

Genetic differentiation and AMOVA

AMOVA indicated that almost all of the genetic diversity was attributable to variation within individual populations (98%), while only a small proportion (2%) of the genetic variation was found among the three populations (green, black, and red) (Table 5). The statistical analysis (PhiPT= 0.019, P = 0.060) revealed no significant genetic differentiation between the plantain populations. The statistics on population structure and heterozygosity showed that the Gst values were estimated to be 43% of the total population differentiation. Using the model-based Bayesian algorithm, it was possible to identify six distinct clusters (k = 6). The Nei genetic distance pairwise population matrix revealed that the three accessions were closely related to each other, with values of genetic distance ranging from 0.014 to 0.016. The value of total heterozygosity (Ht) and gene diversity of individual relatives to their population (Hs) was high in all populations (1.43 and 1.40), respectively.

DISCUSSION

The results obtained in this work are similar to those of other work done in this direction. However, it should be noted that the discriminatory power of SSRs of the cultivars studied was not effective on Agarose compared to a study done by Dowiya et al. (2018) and on polyacrylamide gel

Table 4. The average genetic variability for plantain populations at 7 microsatellite loci.

Locus	Na	Ne	I	h
Ma_1/103	6.66	6.31	0.57	0.28
STMS7	12	12.1	2.23	1.36
AGMI93/94	16.66	14.64	4.11	2.72
Ma_1/24	7	8.02	1.15	0.69
Ma_1/27	14	12.03	3	1.9
Ma_1/16	10	9.73	1.85	1.15
Ma_1/17	15	12.39	2.65	1.6
Mean	11.61	10.74	2.22	1.38
SD	3.89	2.87	1.18	0.80

Sources: Authors

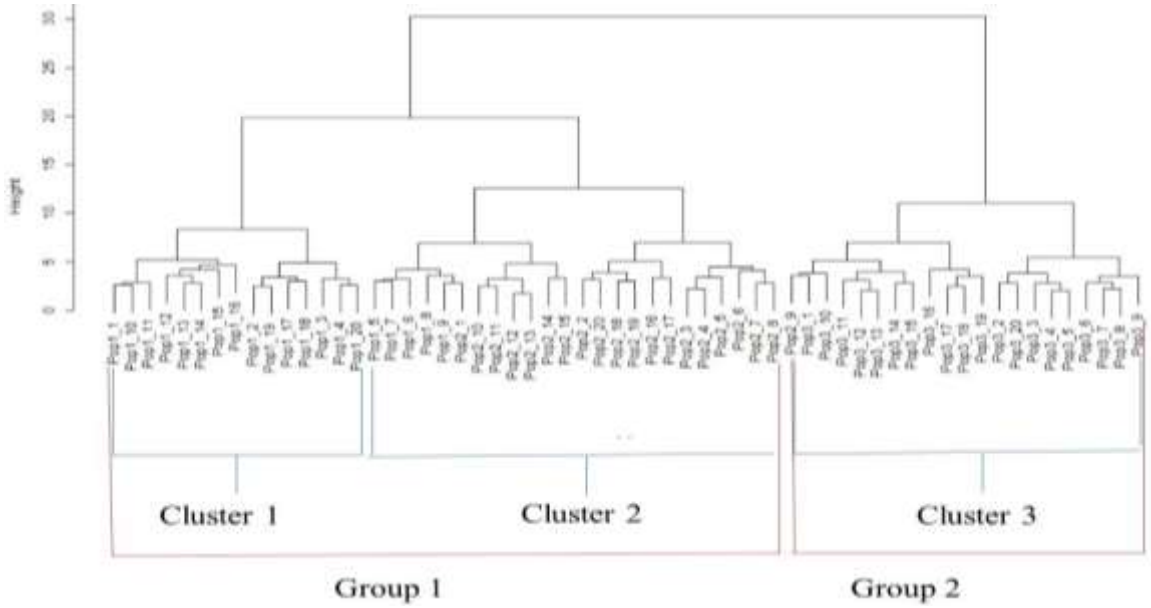

Figure 3. The dendrogram of genetic relationship among the three plantains cultivars generated by UPGMA. Cluster 1 represents a population of Libanga Likale with green pseudostem. Cluster 2: Population of Libanga Black with black pseudostem and Cluster 3: Population of Libanga Lifombo with red pseudostem. Samples 5, 6, 7, 8, and 9 of Population 1 crossed to Population 2, and sample 9 of Population 2 crossed to Population 3.
Sources: Authors

electrophoresis compared to a study done by Changadeya et al. (2012). This is due to the smaller difference in base pairs observed among the amplified fragments. Capillary electrophoresis is so far the best technique of fragment analysis with smaller base pair differences. Among the three accessions, Libanga Black provided a high number of alleles with high polymorphic information compared to Libanga Likale and Libanga Lifombo.

This study agrees with the findings of earlier research on Musa germplasm that employed SSrs, indicating a significant level of polymorphism. Christelová et al. (2011) analyzed 38 triploid accessions of plantains from the International Transit Center (ITC) in Leuven (Belgium) using 19 microsatellite primers and obtained 267 alleles in total with an average PIC of 0.85. Changadeya et al. (2012) tested 12 SSR primers to assess genetic diversity and relationship in 141 banana cultivars growing in Malawi and obtained a high allelic diversity (174 alleles) and the discriminatory power of the SSR primer used was high with a mean PIC of 0.74. Workneh et al. (2022) in Ethiopia characterized 96 banana genotypes and obtained PIC averaged 0.82. The dissimilarities in the number and nature of polymorphic markers utilized, the

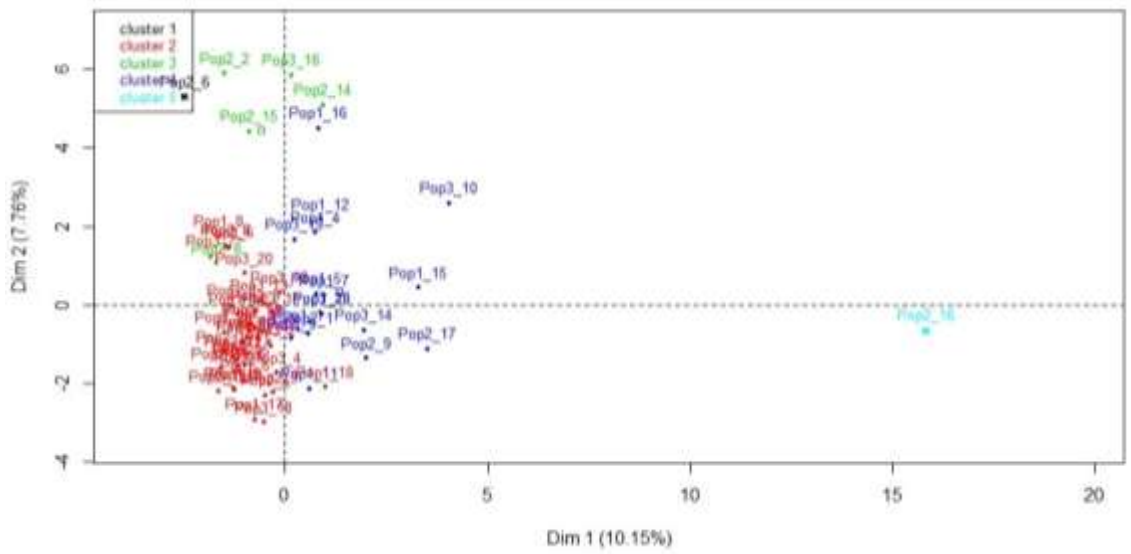

Figure 4. Principal component analysis (PCA) of populations of plantain cultivars.
Sources: Authors

Table 5. Genetic variance among and within the population of plantains.

Source	df	SS	MS	Est. Var.	%	Stat	Value	P-value
Among populations	2	14.417	7.208	0.102	2	PhiPT	0.019	0.060
Within populations	57	294.750	5.171	5.171	98			
Total	59	309.167	-	5.273	100			

Df= Degree of freedom, SS=sum of squares, MS=mean square.
Sources: Authors

sample sizes, the collection locations, and the geographical sources of the genotypes are plausible reasons for the substantial differences in the alleles found in this study versus those in previous studies on Musa germplasm (Gyang et al., 2017). The greater degree of polymorphism detected in this study relative to the research conducted by Changadeya et al. (2012) could be a consequence of the improved comprehensiveness of the plantain cultivar variation present in the Congo Basin and a higher resolution provided by capillary electrophoresis. The seven SSR markers in this research made it possible to differentiate between distinct genotypes. Studies have shown that SSR loci can cause large segregation between closely related individuals, even when only a few loci are used (Powell et al., 1996). According to Al-Badeiry et al. (2014), Polymorphic Information Content (PIC) is indicative of the strength of SSR loci and their ability to identify genetic distinctions between breeds. Hayden et al. (2010) classified PIC values into three categories: low information content (PIC < 0.25), moderate information content (0.25 ≤ PIC ≤ 0.5), and high information content (PIC > 0.5).

Shannon information index (I) and the Nei genetic diversity (h) values obtained in this study showed a significant degree of genetic diversity. This is consistent with findings from Quain et al. (2018), who also reported high genetic diversity in 40 Ghanaian plantain accessions (with a Nei genetic diversity of 0.61 and a Shannon information index of 0.41). By analyzing the genetic variation and structure of plantain (*Musa sapientum* L.) landraces from Côte d'Ivoire using Microsatellite markers, Kouamé et al. (2019) obtained a high genetic diversity (I = 0.726 0.023 and He = 0.506 0.008). The significant genetic diversity present in the collection is expected to enhance the effectiveness of selecting accessions for DRC plantain management and breeding initiatives.

The study found that the genetic variation, as determined by AMOVA, did not show statistical significance in differentiating strains based on the color of their pseudostems. Only 2% of the total genetic variation was attributed to differences between populations. Therefore, the primary source of genetic variation observed in the study was within the population itself. Nei's genetic identity analysis confirmed that the three populations had a close relationship. This finding is consistent with previous reports of a close relationship

among diploid banana cultivars in the *M. acuminata* group, including those by Changadeya et al. (2012), Kitavi (2015) for East African highland banana cultivars, and Quain et al. (2018) for forty plantain genotypes collected in Ghana. These results support the hypothesis proposed by several authors (De Langhe et al., 2005; Noyer et al., 2005) of a common origin of the plantain landraces.

Noyer et al. (2005), showed that all plantain cultivars originated from a single genotype, which underwent epigenetic mutations during successive vegetative propagations in the domestication process. The present study revealed high genetic variation within the plantain genotypes used but little differentiation among populations. This differentiation value was higher than the average for crop species (Gst = 0.34) (Oriero et al., 2006) and the Brazilian diploid and triploid banana accessions using SSR data (Rst=0.105) (Creste et al., 2004). The average differentiation value found by Changadeya et al. (2012) for *Musa* L cultivars was lower (0.14) than in this study. However, the differentiation value was lower than in studies of wild *Musa balbisiana* Colla using cpDNA PCR-RFLP (Gst = 0.77) (Ge et al., 2005). Domestication reduced genetic diversity in banana species due to human interventions and the artificial selection of preferred cultivars, leading to the disappearance of genes over time (Changadeya et al., 2012). Banana is a polyploid. The three selected plantain cultivars used in this study are triploid, but the molecular data was converted to binary and analyzed as haploid. This led to a loss of some information. Molecular analytical platforms for polyploidy species are still under development and currently unavailable. That was the limitation of the study.

Conclusion

There is significant genetic variability in plantain genotypes grown in the Congo basin, the variation of which remains to be confirmed in a larger number of samples considering different regions when collecting samples. The continuation of this study by analyzing other cultivars collected in distant regions and a follow-up study with tools such as RFLP, SNPs, and AFLP be used to verify the findings are recommended.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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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

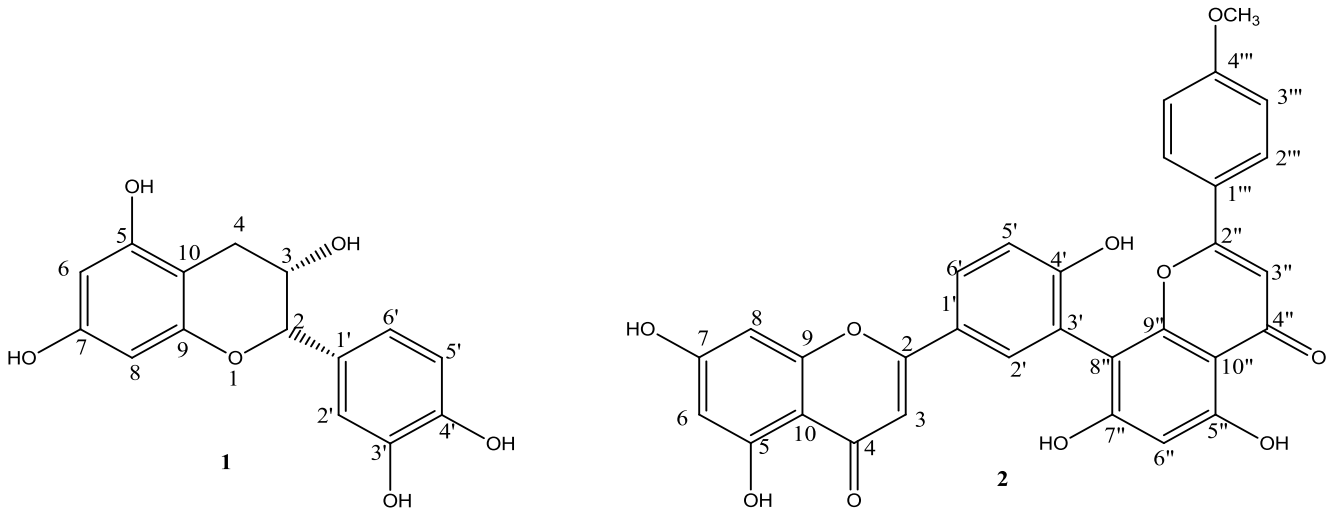

Figure 1. Structures of compounds 1 and 2 from *Juniperus procera*.
Source: Authors

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),

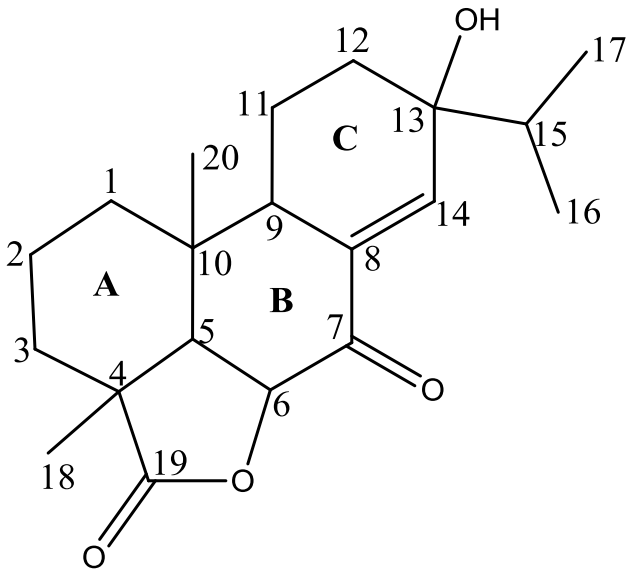

Figure 2. Structure of compound 3 isolated from *Juniperus procera*.
Source: Authors

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

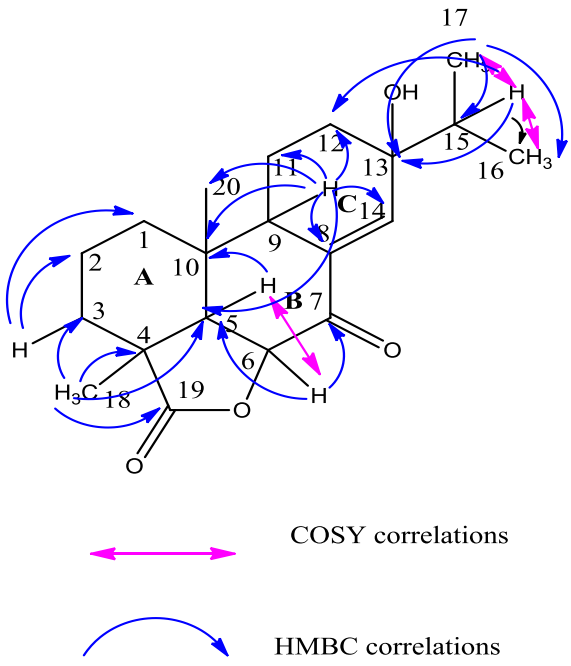

Figure 3. Structure of compound 3 showing selected HMBC and COSY correlations.
Source: Authors

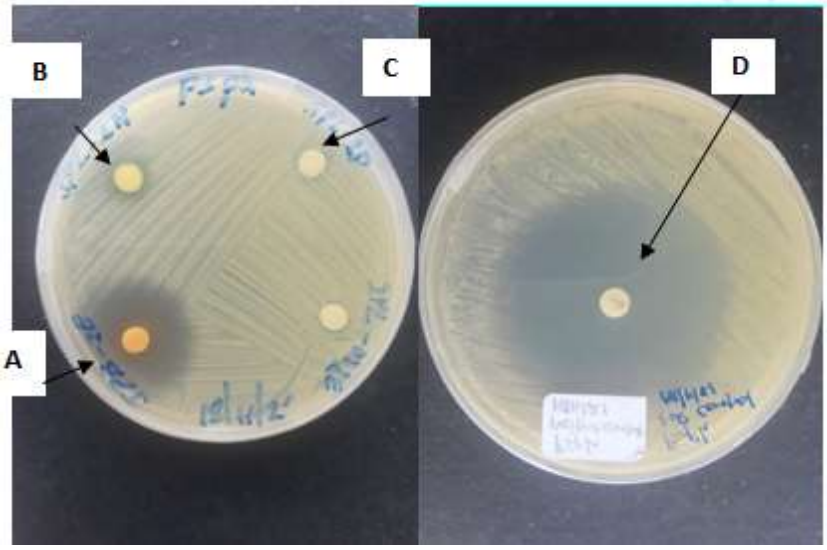

Figure 4. Inhibition of the isolated compounds against *Pseudomonas savastanoi* pv. *phaseolicola*: (A) compound 1, (B) compound 2, (C) compound 3, and (D) chloramphenicol.
Source: Authors

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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