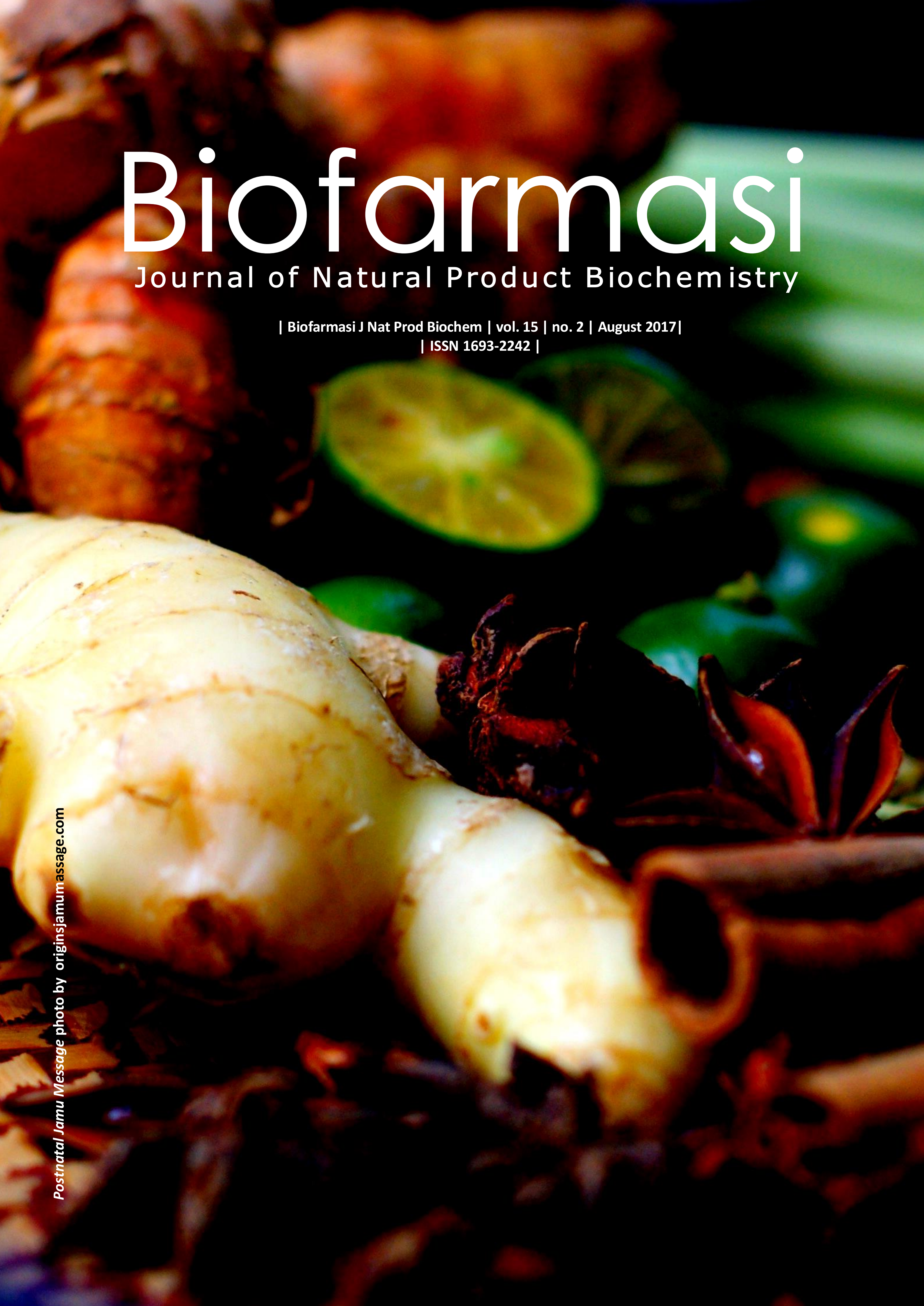
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Preservative effect of lupine extract (*Lupinus luteus*) on quality of raw cow’s milk during storage

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Abstract. *Mohammed AMM, Hamid OIA. 2017. Preservative effect of lupine extract (*Lupinus luteus*) on quality of raw cow’s milk during storage. Biofarmasi J Nat Prod Biochem 15: 45-52.* The experiment was conducted to assess the effect of various levels of lupine (*Lupinus luteus)* extract as milk preservative on the physicochemical and microbial load of raw cow’s milk in 2016. The milk samples were purchased from a milk dairy farm of the College of Animal Production Science and Technology, Sudan University of Science and Technology in the Kuku area. Five treatments were carried out in this study; in the first treatment, raw cow’s milk was left at room temperature without lupine extract, and, respectively, in 2nd, 3rd, 4th and 5th treatments, 0.5%, 1%, 1.5%, and 2% of lupine extract were added to fresh milk samples (four hundred mls of milk for each sample). The raw milk samples in all treatments were left for 0, 1, 2, 3, 4, and 5 hours at room temperature. The milk samples' physicochemical (protein, fat, titratable acidity, total solids not fat, pH and ash) and microbiological (total bacteria count) analyses were taken. The results showed that the lupine extract significantly ($p<0.05$) affected the milk samples' protein, fat, total solids, not fat, and pH contents. At the same time, no significant ($P\geq 0.05$) effect was found in raw milk samples' acidity and ash contents. The storage period significantly ($p<0.05$) affected the fat, pH, and acidity of raw milk samples, while no significant effect was reported in the protein, total solids, not fat, and ash contents. The microbial load of raw milk (Total bacterial count) was significantly ($p<0.05$) affected by the increased level of lupines. The storage period significantly ($p<0.05$) affected the microbial load of the fresh milk.

Keywords: cow’s milk, lupine extract, *Lupinus luteus,* quality, storage

INTRODUCTION

Preserving raw milk samples is common in numerous nations since the demonstrative research facilities are far from most dairy farming communities; transport of the samples to the research facility for analysis is inadequate (Dunham, 1985). These issues are aggravated by the requirement for facilities to keep the milk cool to minimize bacterial proliferation and sample spoilage before the examination. Nowadays, scientists have used various milk preservatives (hydrogen peroxide, sodium azide, bronopol, potassium dichromate, boric acid, milkofix, azidiol, and ortobor acid) to overcome these problems (Ng and Hayes 1982; Hanus et al. 1992a,b; Heeschen et al. 1994; Saha et al. 2003; FOSS Electric 2005). Applying an instrumental method in analyzing raw milk and using preservative agents is allowed (FOSS Electric 2005). The literature makes it possible to find various preservatives for each indicator (total bacteria count, fat and protein content, somatic cell count) (Seškēna and Jankevica 2007).

To optimize the instrumental method and exact estimation of milk content and quality markers, it is fundamental to discover an additive that could be utilized to appraise all of the markers from one test vial until the final one. In Sudan, it is common practice to utilize lupine seeds as an additive by wrapping them into a piece of clean cloth and inserting it into the fresh raw milk container while buying the milking process. The reason for the importance of this study is to know the possibility of using the extract of a lupine (*Lupinus luteus* L.) as an additive figure on the physicochemical and microbial degree of raw milk.

The objective of the study was: (i) To study the physicochemical properties of raw cow's milk with various levels of lupine extract; (ii) To determine the microbial load of raw cow’s milk with various levels of lupine extract, (iii) To determine the shelf life of raw cow’s milk with various level of lupine extract during storage.

MATERIALS AND METHODS

The study was conducted in 2016 at the College of Animal Production Sciences and Technology laboratories, Sudan University of Sciences and Technology, Khartoum, Sudan.

Materials

Source of milk

Six liters of fresh cow’s milk were purchased from the College of Animal Production Sciences and Technology, Sudan University of Sciences and Technology Dairy farm in the Kuku area.

Source of lupine:

Lupine seeds were bought at the Kuku market in the Khartoum State of Sudan.

Methods

Lupine extracts preparation

The lupine seeds were ground into a fine powder (flour) before being added to a hundred mLs of distilled water in various lupine powder weights; they were sterilized at 55 ºC for twenty-four hours (24) hrs. Then, 0.5%, 1%, 1.5%, and 2% of the total weight of the flour were soaked in distilled water and kept at the refrigerator temperature for 24 hrs; then, they were filtered by filter papers (size 42).

Treatments

In this study, five treatments were implemented. First was the control, in which a sample of raw fresh cow milk was left at room temperature without lupine extract addition. In contrast, in the second, third, fourth, and fifth treatments, respectively, 0.5%, 1%, 1.5%, and 2% of lupine extract were added to the raw cow's milk (four hundred mLs of milk for each sample) samples. The raw milk samples in all treatments were left for 0, 1, 2, 3, 4, and 5 hours at room temperature. The milk samples' physicochemical analysis was carried out to analyze the content of protein, fat, titratable acidity, total solids, non fat, pH and ash at each specified time. Each treatment was repeated three times.

Chemical analysis of milk

The chemical composition of milk and treatment samples (protein, fat, total solids, not fat, and PH) was determined by Lactoskan (made in BULGARIA, SUPPLY 12-14V DC50W) (a fresh milk analyzer). In contrast, the titratable acidity and ash were determined by AOAC (2009) methods.

Ash content

The ash content was determined in accordance with AOAC (2009). 10 mL of milk were weighed and poured into a suitable clean, dry container and evaporated to dry on the steam bath, and the container was placed in muffle fume at 55º C for 1.5 - 2 hrs, cooled in dissector, and weighed. The following formula calculated the ash content:

Ash% = w1/w0X100

Where:=
w1 : weight of ash
w0 : weight of sample.

Titratable acidity

Titratable acidity was decided in line with AOAC (2009). Ten mills of milk samples were put into a clean porcelain dish, and three to five drops of phenolphthalein marker were added; the sample was titrated against 0.1 NaOH until a faint color lasted for at least 30 seconds, then the titratable acidity of each sample was calculated with the following formula:

Titratable acidity = T/W

Where:
T : Titration figures
W : Weight of samples

Microbial analysis (total bacteria count)

The agar plate medium was used for the determination of the total bacteria count, according to Ramakant (2006).

The preparation of Nutrient Agar (the medium). The manufacturer's instructions were followed, namely, first, dissolving 28 grams of powder of agar plate medium in one liter of distilled water, second, boiling it and sterilizing it in an autoclave at 121 ºC for fifteen minutes.

Culturing. Serial dilutions were made for each sample, then from each dilution, fifty micro-milliliters (mmL) were transferred into sterile Petri dishes (duplicate), followed by the addition of melted, cooled (45-46ºC) plate count agar and mixed them thoroughly by rotating it, firstly, in one direction, and secondly, in the opposite direction. When the medium had solidified, the dishes were incubated in an inverted position at 37 ºC for 24 hours.

Counting. The quantity of colony-forming units (CFU) in each dilution was obtained by multiplying the quantity of colonies in the reciprocal to each dilution.

Statistical analysis

Statistical analysis was done using Statistical Package for Social Science (SPSS, version 16. 2007). General linear models were used to assess the impact of various levels of lupine concentrate, stockpiling periods, and the interaction between the various levels on the crude cow's chemical composition and microbial load. The least significant difference (LSD) was used to separate the treatment's mean. The level of significance (0.05) was used in this study.

RESULTS AND DISCUSSION

Results

Effect of various levels of the lupine extract on the chemical composition of fresh cow's milk

Results in Table 1 illustrate the effect of various levels of the lupine extract on the physicochemical characteristics of cow milk. The results indicated a significant difference ($p<0.05$) in the protein content of the treatments.

The data showed that the highest protein content (3.24±0.22%) was in control milk sample, while the lowest (3.02±0.17%) was recorded in the cow's milk treated with 0.5% lupine extract.

The fat content of the fresh milk sample was significantly ($p<0.05$) highest (4.04±0.76%), while the lowest value (3.35±0.21%) was found in the milk treated with 1% lupine extract (Table 1).

The results in Table 1) indicated that Total Solids Not Fat was significantly different ($p<0.05$) within the treatments. The control milk sample had a higher TSNF content (8.78±0.64%). However, a lower fat value (8.27±0.46%) was found in the milk treated with 0.5% lupine extract. pH content was significantly ($p<0.05$)

affected by various levels of lupine extract within all treatments. The lowest pH level (6.73±0.11) was scored by the milk with 0.5% lupine extract, while the highest one (7.01±0.36) was scored by the milk treated with zero lupine extract (Table 1).

The acidity of the cow milk samples was not significantly ($p<0.05$) affected by the treatments. Highest acidity percent (0.22±0.01) was for the control, treatment with 1.5% lupine extract, and 2% lupine extract, while the lowest value (0.21±0.02%) was for treatment with 0.5% and 1% lupine extract (Table 1).

The ash data in Table 1) showed that ash content was not significantly ($p<0.05$) affected by the concentration of lupine extract. The highest ash content (0.67±0.08%) was found in treatment with 0.5% and 1.5% lupine extract; however, the lowest value (0.63±0.19%) was recorded on control milk samples.

Effect of storage period on physicochemical characteristics of fresh cow's milk

Data in Table 2 showed the effect of storage time on the physicochemical characteristics of the fresh cow's milk. The result indicated that the storage time had a significant ($p<0.05$) effect on the acidity content of the fresh cow's milk (Table 2). The highest acidity content (0.23±0.01%) was at four hours of storage, and the lowest value (0.21±0.01%) was under control. The data indicated that the storage time had significantly ($p<0.05$) affected the fat content. The highest fat content (3.84±1.07%) was found at zero hour storage. The lowest one (3.23±0.26%) was reported at five hours of storage (Table 2).

The study demonstrated that (Table 2) the storage time had significantly ($p<0.05$) affected the T.S.N.F content. The highest T.S.N.F content (8.59±0.33) was recorded at two hours of storage, while the lowest (8.26±0.26) was found at five hours. Statistical analysis revealed that storage time had no significant ($p<0.05$) effect on ash content, total solids, not fat, and the fresh cow milk protein content samples (Table 2).

Effect of various levels of lupine extract and storage time on the physicochemical characteristics of fresh cow's milk

The protein content of fresh milk was not significantly ($p<0.05$) affected by the level of lupine extract and storage time. The lowest protein content (2.85±0.30%) was observed at zero hour storage in the milk added with 0.5% lupine extract, while the highest one (3.56±0.25%) was reported at zero hours storage in the control milk sample (Table 3).

Results in Table 4 showed that fat content was significantly ($p<0.05$) affected by the level of lupine extract and storage time. The lowest fat content (2.99±0.09%) was recorded at five hours of storage in the milk samples with 0.5% lupine extract. And the highest one (5.80±0.04%) was found at zero hours of storage in the milk sample with 0.5% lupine extract.

The total solids not fat content of the milk samples was not significantly ($p<0.05$) affected by the level of lupine extract and storage time. The control milk's highest T.N.F content (9.73±0.69%) was at zero hours of storage. While the lowest one (7.86±0.74%) was in milk with 0.5% lupine extract (Table 5).

Data in Table 6 shows the milk samples' pH content, which was significantly ($p<0.05$) affected by the various level of lupine extract and storage time. The lowest pH (6.57±0.02) was reported at five hours of storage in the milk with zero lupine extract, while the highest (7.53±0.60) was in the milk with 0.5% lupine extract and stored for one hour.

Table 1. Effect of various levels of the lupine extract on the physicochemical composition of fresh cow's milk

Treatment	Chemical composition					
	Protein%	**Fat%**	**T.S.N.F**	**pH**	**Acidity**	**Ash%**
Control	3.24±0.22a	4.04±0.76a	8.78±0.64a	6.73±0.11c	0.22±0.01	0.63±0.19
0.5%	3.02±0.17c	3.64±1.08b	8.27±0.46b	7.01±0.36a	0.21±0.02	0.67±0.08
1%	3.02±0.10bc	3.35±0.21c	8.29±0.26b	6.83±0.12b	0.21±0.01	0.66±0.11
1.5%	3.13±0.15b	3.41±0.26bc	8.53±0.40ab	6.82±0.09bc	0.22±0.01	0.67±0.08
2%	3.08±0.10bc	3.45±0.29bc	8.51±0.31ab	6.79±0.07bc	0.22±0.02	0.66±0.09
Sig	**	**	*	**	N.S	N.S

Note: Mean values bearing different superscripts within columns are significantly different ($p<0.05$). L.S = level of significance. * NS = not significance

Table 2. Effect of storage period on the physicochemical characteristic of fresh cow milk

Storage time	Chemical composition					
	Protein%	**Fat%**	**T.S.N.F**	**pH**	**Acidity**	**Ash%**
Zero hour	3.11±0.29ac	3.84±1.07a	8.54±0.77	6.78±0.05bc	0.21±0.01b	0.69±0.10a
1 hr	3.06±0.13ac	3.47±0.34bc	8.38±0.35	6.96±0.36a	0.21±0.00b	0.67±0.09ab
2 hrs	3.15±0.12ab	3.69±0.75ab	8.59±0.33	6.88±0.07ab	0.210.01b	0.60±0.18ab
3 hrs	3.13±0.19a	3.64±0.66ab	8.50±0.54	6.98±0.17a	0.21±0.01b	0.58±0.06b
4 hrs	3.00±0.12c	3.61±0.46ab	8.26±0.35	6.76±0.08ac	0.23±0.01a	0.70±0.09a
5 hrs	3.14±0.08a	3.23±0.26c	8.58±0.23	6.67±0.06c	0.23±0.02a	0.69±0.07a
Sig	NS	**	NS	***	***	NS

Note: Mean values bearing different superscripts within columns are significantly different ($p<0.05$). L.S = levels of significance. * NS = not significance

Table 3. Effect of various levels of lupine extracts and storage time on the protein of fresh milk

Storage time	Lupine concentration				
	Control	0.5%	1%	1.5%	2%
Zero hour	3.56±0.25	2.85±0.30	2.97±0.01	3.02±0.04	3.17±0.11
1 hr	3.26±0.07	2.99±0.04	2.99±0.05	3.11±0.11	2.96±0.03
2 hrs	3.10±0.29	3.25±0.07	3.14±0.08	3.17±0.04	3.12±0.01
3 hrs	3.27±0.04	2.90±0.07	3.17±0.04	3.32±0.25	3.01±0.04
4 hrs	3.11±0.27	3.02±0.09	2.89±0.00	2.99±0.04	3.01±0.01
5 hrs	3.15±0.12	3.13±0.00	3.03±0.01	3.19±0.05	3.20±0.09
Sig	*				

Note: Mean values bearing different superscripts within rows are significantly different <0.05). * L.S = levels of significance. * NS = not significance.

Table 4. Interaction between the various levels of lupine extract and storage time on fat content (%) of fresh cow's milk

Storage time	Lupine concentration				
	Control	0.5%	1%	1.5%	2%
Zero hour	3.20±0.69	5.80±0.04	3.13±0.04	3.49±0.11	3.59±0.13
1 hr	3.56±0.33	3.03±0.05	3.28±0.07	3.59±0.16	3.87±0.24
2 hrs	5.04±0.54	3.21±0.32	3.47±0.07	3.26±0.06	3.51±0.06
3 hrs	4.54±0.76	3.23±0.93	3.23±0.11	3.79±0.20	3.44±0.01
4 hrs	4.27±0.40	3.63±0.49	3.71±0.01	3.20±0.02	3.23±0.11
5 hrs	3.66±0.04	2.99±0.09	3.30±0.13	3.13±0.01	3.06±0.09
Sig			***		

Note: Mean values bearing different superscripts within rows and columns are significantly different ($p<0.05$). L.S = levels of significance. * NS = not significance.

Table 5: interaction between various levels of lupine extract and storage time on total solid not fat content (%) of fresh cow's milk.

Storage time	Lupine concentration				
	Control	0.5%	1%	1.5%	2%
Zero hour	9.73±0.69	7.86±0.74	8.10±0.01	8.35±0.25	8.67±0.28
1 hr	8.93±0.18	8.20±0.08	8.17±0.19	8.52±0.32	8.10±0.09
2 hrs	8.49±0.79	8.90±0.21	8.57±0.21	8.50±0.14	8.50±0.00
3 hrs	8.39±0.47	7.93±0.21	8.65±0.09	8.94±0.91	8.60±0.62
4 hrs	8.52±0.73	8.18±0.38	8.01±0.13	8.18±0.10	8.45±0.19
5 hrs	8.61±0.32	8.55±0.00	8.28±0.01	8.72±0.13	8.74±0.25
Sig			NS		

Note: Mean values bearing different superscripts within rows are significantly different $p<0.05$). L.S = levels of significance. * NS = not significance.

Table 6. Effect of various levels of lupine extract and storage time on pH of fresh cow's milk

Storage time	Lupine concentration				
	Control	0.5%	1%	1.5%	2%
Zero hour	6.78±0.04	6.79±0.04	6.72±0.11	6.81±0.02	6.79±0.02
1 hr	6.79±0.05	7.53±0.60	6.83±0.0	6.85±0.01	6.79±0.01
2 hrs	6.81±0.01	6.97±0.06	6.93±0.01	6.87±0.04	6.83±0.01
3 hrs	6.81±0.04	7.27±0.13	7.00±0.01	6.94±0.05	6.91±0.2
4 hrs	6.63±0.09	6.83±0.04	6.83±0.02	6.78±0.01	6.73±0.03
5 hrs	6.57±0.02	6.68±0.04	6.71±.02	6.67±0.02	6.71±0.00

Note: L.S = levels of significances. * NS = not significance.

The acidity content of the milk samples was not significantly ($p<0.05$) affected by the various level of lupine extract and storage time. The lowest acidity (0.20±0.00%) was recorded in the milk added with 0.5% and 1% lupine extract and stored for zero hours, while the highest one (0.25±0.04%) was found in the milk added with 2% lupine extract (Table 7) and stored for five hours.

Results in Table 8 show the milk samples' ash content, which was not significantly ($p<0.05$) affected by the various level of lupine extract and storage time. The lowest ash content (0.41±0.42) was reported at two hours of storage in the milk added with zero lupine extract, while the highest one (0.8±0.00) was found at zero hours of storage and four-hour storage in the milk made of zero lupine extract and 1% lupine extract.

Effect of various levels of the lupine extract on Total Bacteria Count of fresh cow's milk

This study explained (Table 9) that significant ($p<0.05$) variations were found in the TBC of different treatments. The highest total bacteria count (6.59±0.17log CFU/gm) was obtained from the control milk samples, while the lowest (6.46±0.12log CFU/gm) was obtained from the milk sample with 1.5% of the lupine extract.

Effect of storage time on total bacteria count of raw cow's milk

This study resulted in full disclosure (Table 10) that the total bacteria count was significantly ($p<0.05$ log CFU/gm) affected by the storage time. The highest total bacteria count (6.58±0.16 log CFU/gm) was obtained from four hours of storage, while the lowest (6.44±0.23 log CFU/gm) was obtained from one-hour storage.

Effect of various levels of lupine extracts and storage time on microbiological characteristics of fresh cow's milk

This study resulted in full disclosure (Table 11) that the total bacteria count was not significantly ($p<0.05$ log CFU/gm) affected by the level of lupine extract and storage period. The lowest total bacteria count (6.34±0.24 log CFU/gm) was recorded at one-hour storage and at the addition of 1% lupine extract, while the highest one (6.71±0.07 log CFU/gm) was gained from four hours storage in the milk sample containing zero lupine extract (control).

Table 7. Effect of various levels of lupine extract and storage time on titratable acidity content (%) of fresh cow's milk

Storage time	Lupine concentration				
	Control	**0.5%**	**1%**	**1.5%**	**2%**
Zero hour	0.23±0.01	0.20±0.00	0.20±0.00	0.22±0.01	0.22±0.00
1 hr	0.22±0.00	0.21±0.01	0.21±0.00	0.22±0.01	0.21±0.01
2 hrs	0.22±0.01	0.21±0.01	0.21±0.00	0.22±0.02	0.21±0.00
3 hrs	0.22±0.00	0.21±0.01	0.21±0.01	0.21±0.01	0.22±0.01
4 hrs	0.24±0.02	0.22±0.01	0.23±0.01	0.24±0.01	0.22±0.01
5 hrs	0.22±0.03	0.24±0.01	0.23±0.04	0.23±0.01	0.25±0.04
Sig			NS		

Note: Mean values bearing different superscripts within rows are significantly different ($p<0.05$). L.S = levels of significance. NS = not significance

Table 8. Effect of various levels of lupine extract and storage time on Ash content (%) of fresh cow's milk

Storage time	Lupine concentration				
	Control	**0.5%**	**1%**	**1.5%**	**2%**
Zero hour	0.80±0.00	0.70±0.14	0.60±0.14	0.70±0.00	0.65±0.07
1 hr	0.70±0.07	0.70±0.00	0.70±0.00	0.65±0.07	0.65±0.21
2 hrs	0.41±0.42	0.65±0.07	0.65±0.07	0.65±0.07	0.65±0.07
3 hrs	0.55±0.07	0.65±0.07	0.55±0.07	0.55±0.07	0.60±0.00
4 hrs	0.65±0.07	0.60±0.00	0.80±0.00	0.75±0.07	0.70±0.14
5 hrs	0.60±0.00	0.70±0.14	0.70±0.07	0.70±0.00	0.70±0.00
Sig			NS		

Note:Mean values bearing different superscripts within rows are significantly different ($p<0.05$). L.S = levels of significance. * NS = not significance

Table 9. Effect of various levels of the lupine extract on Total Bacteria Count of fresh cow's milk

Parameter	Lupine concentrations					
	Control	**.5%**	**1%**	**1.5%**	**2%**	**sig**
T.B.C	6.59±0.17a	6.58±0.18aa	6.51±0.17ba	6.46±0.12b	6.48±0.14b	**

Note: Mean values bearing different superscripts within rows are significantly different ($p<0.05$). L.S = levels of significance. NS = not significance

Table 10. Effect of storage Time on Total Bacteria Count of raw cow's milk

Bacterial count	Storage time						
	Zero hr	**1hr**	**2hrs**	**3hrs**	**4hrs**	**5hrs**	**sig**
T.B.C	6.53±0.13ab	6.44±0.23b	6.52±0.15ab	6.52±0.13ab	6.58±0.16a	6.57±0.14a	*

Note: Mean values bearing different superscripts within rows are significantly different ($p<0.05$). * L.S = levels of significance. * NS = not significance.

Table 11. Effect of various levels of lupine extract and storage time on Total Bacteria Count of fresh cow's milk

Storage time	Lupine concentration				
	Control	0.5%	1%	1.5%	2%
Zero hour	6.52±0.12	6.63±0.06	6.55±0.10	6.43±0.09	6.49±0.20
1 hr	6.57±0.16	6.42±0.32	6.34±0.24	6.44±0.19	6.45±0.26
2 hrs	6.57±0.14	6.62±0.09	6.44±0.20	6.42±0.16	6.54±0.05
3 hrs	6.51±0.29	6.48±0.11	6.48±0.02	6.55±0.08	6.55±0.07
4 hrs	6.71±0.07	6.75±0.04	6.59±0.09	6.43±0.11	6.41±0.13
5 hrs	6.69±0.12	6.56±0.13	6.67±0.10	6.50±0.04	6.44±0.11
Sig			NS		

Note: Mean values bearing different superscripts within rows are significantly different ($p<0.05$). L.S = levels of significance. NS = not significance

Discussion

Effect of various levels of the lupine extract on the physicochemical characteristics of fresh cow's milk

The protein of the untreated milk samples had the highest value compared to protein content in other treatments (Table 1). This could be attributed to the high moisture content in the milk samples with various levels of lupine extract, which may decrease the total solids of milk. These results agree with those reported by Wolko et al. (2011). They stated that the lupine extracts deactivate substances such as lectins and protease inhibitors, reducing protein digestion and availability. However, no variations were observed in the protein contents of the milk samples with the lupine extract.

The fat content of the milk samples increased with the increasing level of lupine extract (Table 1). This could be due to the high amount of fat in lupines, which coincided with Kroger's (1971).

The total solids not fat of the milk samples decreased with the increasing level of lupine extract (Table 1). This could be due to the proteolytic activities of lupine extract. These results were not in accordance with Gupta (2010), who studied the compositional change in crossbred and local cow milk, which were affected by 0.3 and 0.5% formalin preservatives. No significant difference was recorded in lactose, total solids, fat and specific gravity on the addition of formalin in milk.

The pH of the milk samples showed high values as an increasing level of lupine extract. This could be due to the breakdown of protein as the activity of the lupine extract; these results were not in line with those studied by Giolitti (1949), who found that no changes in lactose, fat, total nitrogen, and pH after the addition of 0.04% by weight of H_2O_2 to milk.

The acidity and ash contents of the milk samples were not affected by lupine extract addition (Table 1). These results contrasted with those reported by Sandhu et al. (1984).

Effect of storage period on the physicochemical characteristics of fresh cow milk.

The storage period did not significantly affect this study's protein, total solids, not fat, and ash content (Table 2). These results were not in accordance with those reported by ISO (1999), who studied the effect of $C_6H_7KO_2$ (potassium sorbate) on protein content, and it gave the reverse result, i.e., protein content in tested samples that were stored at 4°C and 20°C increased by 0.20% and 0.39%, correspondingly.

The fat content (Table 2) increased at zero hours of storage, but it decreased after five hours, which was the lowest one; this was probably due to the lipolytic activities. And these results were in line with the study of Seskena and Janevica (2007).

The PH (Table 2) of the sample with lupine extract was higher at one, two, and three-hour storage than at zero hours of storage. These might be due to the antimicrobial included in the lupines extract, which the lactic acid bacteria inhibited. These results are in line with those of Baltess (1998).

The acidity of raw milk samples showed no increase till four and five hours of storage (Table 2). The increase in acidity might be due to the breakdown of lactose into lactic acid by the lactic acid bacteria. These results are similar to those reported by Minzner and Kroger (1974).

Interaction between the various levels of lupine extract and storage period on the physicochemical characteristics of fresh cow milk

Table 3 showed the results that as the level of lupine extract decreased and the storage period changed, the protein values decreased. These were probably due to the breakdown of protein by the microorganisms in the raw milk sample with the lupine extract. These results agreed with those of Yuan (2001).

Table 4 showed that fat content was significantly ($p<0.05$) affected by the levels of lupine extract and storage period. The lowest fat content (2.99±0.09%) was recorded at five hours of storage in the milk without lupine extract, and this might be due to the hydrolysis of fats; the results agreed with those of Kroger (1985).

Total solids not fat content (Table 5), of the milk samples, were not significantly ($p<0.05$) affected by the levels of lupine extract and storage time. The highest T.N.F content (9.73±0.69%) was for the control milk at zero hours. The decrease in T.S.N.F values was in accordance with the increase in the level of lupine extract and storage time, and these might be due to the moisture content in the extract. These results agreed with those *of* Boghra and Borkhatriya (2003).

The lowest pH (6.57±0.02) was reported at five hours of storage in the milk without lupine extract; these results

mean the PH values increased as the level of lupine extract increased, and they decreased as the storage time increased, and these might be due to the preservative effect of the lupine extract. These results are in accordance with Baltess (1998), who stated that the important factors influencing the efficiency of preservatives were the initial microbial count in the product, microbial species, temperature, and pH of the environment.

Table 7 showed the results that the acidity content of the milk samples was not significantly ($p<0.05$) affected by the various level of lupine extract and storage time. This might be due to the low activity of antimicrobial factors in lupine extract; these results were not in line with Dawood et al. (1974), who found that an addition of 0.1% formalin to milk increased the titratable acidity from 0.175 to 0.190%.

Table 8 showed the results that the ash content of the milk samples was not significantly ($p<0.05$) affected by the various level of lupine extract and storage time.

Effect of various levels of the lupine extract on total bacteria count of fresh cow's milk

Table 9 showed that lupine extract significantly ($p<0.05$) affected the total bacterial count. The effective concentration of lupine extract is 1.5% to 2%, and the lowest total bacterial count of milk samples with 1.5% and 2% lupine extract could be due to the inhibition effect of the lupine extract on the total bacterial count; these results confirmed those of the Sesken and Janevica (2007).

Effect of storage period on total bacteria count of fresh cow's milk

Table 10 explained that storage time significantly affected the total bacteria count ($p<0.05$). The highest total bacteria count (6.58 ± 0.16 log CFU/gm) was reported at four hours of storage, and this agreed with that reported by Erdemoglu et al. (2009).

Interaction between the various level of lupine extract and storage period on total bacteria count of fresh cow's milk

Table1 1 demonstrated that the total bacteria count was not significantly ($p<0.05$) affected by the level of lupine extract and storage time. These might be due to the weak antibacterial effect of the lupine extract. These results were not in line with those reported by Al-Kerwi et al. (2005), who studied the antibacterial effect of milk proteins. This process was mediated by a hydrogen peroxide (H2O2) reaction that was thought to be a major antibacterial substance. Known as milk peroxidase, Lactoperoxidase, in combination with H_2O_2 and iodide, produce a potent anti-bacterial system known as the Lactoperoxidase system.

Based on the result of the study, the following conclusions were drawn: (i) The quality of raw cow's milk was improved with the addition of the lupines extract. (ii) The lupines extract significantly ($p<0.05$) affected the protein, fat, total solids, not fat, and PH contents of raw milk samples. (iii) The storage period significantly ($p<0.05$) affected the fat, PH, and acidity but did not significantly affect protein, total solids, not fat, and ash contents of raw milk samples. (iv) The microbiological characteristics of raw milk (total bacteria count) were significantly ($p<0.05$) affected by the increasing levels of lupines. (v) The storage period significantly ($p<0.05$) affected the microbiological characteristics of the fresh milk.

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Extraction and characterization of gelatin from *Lates niloticus* and potential industrial applications

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Abstract. *Kiplagat CS, Onyari JM, Mulaa F, Wabomba J. 2017. Extraction and characterization of gelatin from Lates niloticus and potential industrial applications. Biofarmasi J Nat Prod Biochem 15: 53-64.* This research aims to extract and characterize gelatin from *Lates niloticus* (Nile perch) scales, then blend it with polyvinyl alcohol (PVA). Hydrolysis of the scales was done using a crude alkaline protease harvested from a bacterium, *Bacillus cereus strain wwcp 1,* obtained from Lake Bogoria. The lyophilized solution yielded 16.3% of gelatin powder calculated from the dry weight of the scales. The sample was characterized using infrared spectroscopy and showed peaks at 3442 cm^{-1}, 1653 cm^{-1}, and ~ 1590 cm^{-1} corresponding to Amide A, Amide I, and Amide II bands, respectively. The amino acid analysis shows that glycine was the most abundant amino acid (21.7%), followed by a proline (14.6%) and alanine (11.8%). Isoleucine, Histidine, and Tyrosine were the least abundant (1.8, 1.4, and 0.9%, respectively). Polyvinyl alcohol-gelatin blend films of various compositions ranging from 10% to 90% PVA were prepared using the solution casting method. Differential Scanning Calorimetry (DSC) and Thermo-gravimetric Analysis (TGA) tests showed the films had glass transition, melting, and thermal decomposition onset temperatures intermediate between those of the respective individual polymers (PVA and gelatin). The thermal stability of the films reduced with the increase in the amount of the less thermally stable constituent. Lastly, potential applications of the prepared blend films were investigated. Batch experiments to assess the potential of the polymer blend films as an adsorbent material were done using Methylene Blue dye. The films were found to adsorb up to 64% of the dye, and the percentage of dye removal varied with the initial concentration of the dye and contact time.

Keywords: Gelatin, industrial applications, *Lates niloticus*

INTRODUCTION

Gelatin is a mixture of protein and peptides mainly derived from collagen. Collagen can be found in the skin, bones, cartilage, etc. It is also the most copious structural protein in animals. In humans, collagen makes up a third of the total protein in the human body and makes up 75% of the total weight of human dry skin (Shoulders and Raines 2009). When collagen is treated with partial hydrolysis, the gelatin will be generated. This also explains why gelatin is a water-soluble polypeptide. This characteristic makes gelatin one of the most used materials in the food and pharmaceutical industries. Depending on its utilization, gelatin can be produced in four grades, i.e., edible, pharmaceutical, photographic, and industrial (Tavakolipour 2011). In the form of an industrial grade, food industries widely use gelatin to perform texturization, gelling, stabilization, and water binding. While in the pharmaceutical industry, gelatin is frequently utilized to produce capsules, tablets, suppositories, and blood plasma substitutes (Nik Aisyah et al., 2014).

Conventionally, gelatin is produced from mammalian sources. A study of the total production output of gelatin found that Bovine and Porcine skins contribute to 46% of the total amount, Bones and Hooves contribute to 23% and 29%, respectively, and only 1% is generated from marine sources (Karim and Bhat 2009; Wang et al. 2010). It is also found that during gelatin production, the destruction of the secondary structure of the parent collagen and some aspects of primary and tertiary structures are inevitable. The production process normally consists of two stages, i.e., the pretreatment step and the main extraction step. In the pretreatment step, the raw materials are prepared for the extraction in either one of the acid pretreatment methods or the alkali pretreatment method, which is to be selected based on the source of the raw materials and the intended purposes of production of the gelatin.

To produce gelatin from connective tissue that exhibits highly interconnected structures such as that of the cattle, alkaline pretreatment is preferred over acid treatment. The alkaline treatment process is applied for up to twenty weeks (Karayannakidis and Zotos 2014). Acid pretreatment is preferred for materials such as pig skin and is usually performed for shorter periods due to a lower degree of collagen cross-linkage. The next stage in the extraction process involves neutralizing the pretreated material to neutral pH before gelatin is extracted using hot water.

The fish processing industry currently generates solid wastes that can be as high as 75% of the total weight of the catch. The wastes include scales, guts, heads, skins, and bones and are usually used to produce low-value goods such as fishmeal or are dumped in landfills or water bodies, posing potential environmental harm. The dry mass of fish is largely composed of protein. Therefore, fish processing by-products are candidates for alternative sources of high-

value protein ingredients such as food-grade gelatin (Karayannakidis and Zotos 2014).

Fish collagen possesses several intrinsic bioactive properties which are beneficial for skin health. It can stimulate skin collagen production and has anti-inflammatory, anti-wrinkle, and UV damage repair activity. Collagen has also been widely utilized in the pharmaceutical industry to produce wound dressings, drug delivery vehicles, and vitreous implants. However, Fish collagen has low thermal stability compared to its mammalian counterpart, posing a major problem for biomedical applications. The variation in thermal stability between mammalian and fish collagen is attributed to their amino acid content and distribution of molecular weights (Johnston-Banks 1990).

Amino acids have a key role in stabilizing the collagen helix structure. The amino acid content in mammalian collagen is higher than in fish collagen. Many reports indicated that collagen from cold-water fish contains fewer amino acids than collagen from warm-water fish. Hence, gelatin derived from mammalian sources and warm water fish have a higher melting point than gelatin derived from cold-water fish (Singh et al. 2011). Therefore, it is essential to stabilize fish collagen either physically or microbiologically to produce stable scaffolds for biomedical applications.

There are many fish processing plants in the country where fish filleting (both marine and freshwater fish) is done, both for domestic use and for export to European Union and other markets. Fish scales, a byproduct of that processing, are largely not commercially utilized in Kenya. Furthermore, the government's establishment of an economic stimulus package involving the establishment of fish ponds in all Counties in Kenya has increased the production and consumption of fish, which is expected to produce more scales. This project is aimed at the utilization of fish scale waste generated during fish processing to extract gelatin that was subsequently characterized and the investigation of potential applications.

The scales utilized were from *Lates niloticus*, commonly known as the Nile perch. This large freshwater fish species found extensively in Lake Victoria, East Africa, can grow up to 200 kg and two meters in length. Thus, many scales and other byproducts can be obtained from its processing.

The objectives of this research were (i): to extract the gelatin from *Lates niloticus* scales using a crude alkaline protease from *Bacillus cereus strain wwcp 1*. (ii) to determine the amino acid composition of gelatin. (iii) to prepare polymer blends containing gelatin and characterize them using FTIR, DSC, and TGA. (iv) to investigate potential industrial applications.

MATERIALS AND METHODS

Materials

Fresh Nile Perch (*Lates niloticus*) skin with intact scales was purchased from the local market in Nairobi, contained in a sealed plastic bag, and transported to the laboratory. Skins more than 1 kg were selected and stored at -20°C upon arrival if not processed immediately. The scaling process starts with thawing frozen skin at ambient temperature for two hours, washing, and rinsing thoroughly with warm water to help remove any grease from the surface.

After drying up, the skin was scaled on the bench using a knife, and the residual meat or lipids were rinsed entirely under running tap water. The scales were air-dry at ambient conditions, divided into an airtight plastic bag attached with dry weight information.

Enzyme preparation

Preparation of the crude alkaline protease refers to the method as described elsewhere (Wanyonyi et al., 2014). Briefly, a culture medium containing 0.5% casein and 0.25% glucose was prepared at 200 ml each in 500 mL conical flasks to allow aeration. The medium was then autoclaved at 121ºC (15 lb) for 15 min and allowed to cool to room temperature before inoculation. Five percent of overnight-cultured *Bacillus cereus strain wwcp1* was inoculated into the fresh medium and incubated at a rotary shaker (140 rpm) for 72 hours at 45°C. Following incubation, the medium was then centrifuged at 5000 rpm for 15 minutes to collect the crude enzyme's supernatant.

Gelatin extraction

The optimum pH for gelatin extraction referred to the method established by Wanyonyi et al. (2014). First, the supernatant that contains the crude enzyme was adjusted to pH 11 by using a pH meter and sodium hydroxide in a glass beaker. The supernatant was then poured over 1 kg of dry scales, ensuring submerged all parts. Hydrolysis was performed in the oven at 50°C. Every twelve hours, the pH of the solution was checked and restored to the optimum value. To ensure homogeneity of the process, the sample was stirred regularly.

Once the scales were completely hydrolyzed, the sample was removed from the oven and cooled down at ambient temperature. To obtain gelatin powder, gelatin solution (the liquid part) was separated by decantation and lyophilized. The yield was determined by the percentage of the total weight of dry gelatin powder per the total weight of dry scales.

Infrared spectroscopy

The sample's infra-red spectroscopic analysis was performed with minor alterations, as outlined by Muyonga et al. (2004). A 200 mg KBr disc containing 2-6 mg of the sample disc was prepared and used to obtain the spectrum. The analysis was performed on a Thermo Electron Corporation Nicolet 380 FTIR spectrometer.

Determination of amino acid composition

Amino acid analysis of Nile perch scale gelatin was performed using a narrow bore (2.1 x 200 mm) (Hypersil AA-ODS), 5 µm reverse phase column purchased from Thermo Electron (part # 30105-202130). Samples were weighed and placed into a 13 x 100 mm Pyrex tube along with 1ml N HCl and 11 µmoles of Internal Standards

(Norvaline and Sarcosine). After adding the Internal Standards, the samples, controls, and blanks were exposed to liquid-phase 6N HCl for 22 hours at 100°C. Amino acids were separated on an Agilent 1260 with a column heater, automatic injection programming UV, and fluorescence detection.

Five μL of the hydrolysate was dried down and re-suspended in 250 μL of 0.4 M Borate buffer. One μL was injected. The G1367E autosampler was used to perform pre-column derivatization and multiple sample handling. The derivatized amino acids were then eluted from the reverse phase column.

Primary amino acids (tagged with OPA, Agilent #5061-3335) and secondary amino acids (tagged with FMOC, Agilent 5061-3337) were detected by the Variable Wavelength (UV) detector (G1365D) at 338/390 nm and 266/324 nm, respectively. The fluorometric detector (G1321B) monitored the primaries at excitation/emission 340/450 and the secondaries at 266/305. The assay was calibrated by a standard (Agilent 5061-3331) subjected to the same treatment as the samples and control, including hydrolysis. The assay was controlled by a known protein, Human Serum Albumin. An aliquot from the same batch of HSA was run with every assay.

Preparation of polymer blends and films

The polymer blends were prepared in 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, and 10/90 PVA/gelatin proportions. The appropriate weights of PVA purchased from Merck (MW 60,000 and a degree of hydrolysis of 98%) were weighed out into 100 ml conical flasks and added to 30 ml distilled water at 95°C. This solution was swirled in a 90°C water bath to dissolve the PVA completely. The resulting solutions were labeled A.

Corresponding weights of gelatin were weighed into flasks and added with 10 ml of distilled water. The mixture was transferred to a water bath at 50°C and vigorously stirred for 15 minutes to ensure complete dissolution. The resulting gelatin solutions were then labeled solution B. Solutions B were poured into the appropriate solutions A to obtain film-forming solutions (FFS) with the predetermined PVA/gelatin ratios. The film-forming solutions were homogenized with magnetic stirring at room temperature for 30 minutes to ensure complete mixing.

In an attempt to create films with the same thickness, the same amount of FFS was then cast separately onto glass Petri dishes and subsequently placed in a chamber with air renewal circulation and then left to dry at room temperature. When the films had completely dried, it was peeled off from the Petri dishes and sent for further analysis.

Differential scanning calorimetry

Differential scanning calorimetry data were recorded for pure gelatin, PVA/ gelatin blends, and pure PVA. The tests were performed on a DSC Q100 V9.9 Build 303 (Universal V4.5A TA Instruments), with 1 to 9 mg samples sealed in aluminum pans. Thermal data (I and II heating scans) were recorded at a temperature ranging from -50 to 250°C.

Thermogravimetric analysis

Thermogravimetric analysis of pure gelatin, pure PVA, and the PVA/gelatin blends was performed on a TGA Q500 V20.13 Build 39 (Universal V4.5A TA Instruments) machine. The temperature was set between 0 to 200°C with sample sizes ranging from 9 to 27 mg. The onset decomposition temperature and weight loss profiles were obtained.

Dye adsorption experiments

Methylene blue dye (MB, $C_{16}H_{18}N_3ClS$) with a molecular weight of 319.85 and λ_{max} at 664 nm purchased from RANBAXY Fine Chemicals in Nairobi, Kenya, was used without any refinement. Batch experiments were performed in 100 ml conical flasks at 25°C. Films prepared from a 60/40 PVA/gelatin (w/w) blend were chosen to examine the adsorption properties.

Calibration curve

Stock of methylene blue solution (4.0 X 10^{-5} M) were diluted to obtain concentration 2.5 x 10^{-5}, 2.0 x10^{-5}, 1.25 x 10^{-5}, 1.0 x 10^{-5} and 0.75 x 10^{-5} M. Thirty ml portions of each concentration was measured out into separate 100 ml conical flasks. Samples were then picked from each flask, and the absorbance was measured on a UV-Vis spectrophotometer (Shimadzu UV-mini 1240) at 664 nm. The calibration curve was created by plotting the absorbance against concentration.

Effect of adsorbent concentration

The variation of equilibrium uptake of methylene blue with adsorbent concentration was determined in 100 ml conical flasks on an orbital shaker at room temperature. The film's varying weights (0.25, 0.50, 0.75, and 1 g) were measured and placed in four separate flasks. Thirty ml of 0.000025 M methylene blue was then added to each flask. Samples were then collected from each flask at regular intervals, and residual MB concentration was determined using a UV-Vis spectrophotometer (Shimadzu UV-mini 1240) at a wavelength of 664 nm.

Effect of initial dye concentration

The variation of equilibrium uptake of methylene blue with various adsorbent concentrations was determined in 100 ml conical flasks on an orbital shaker at room temperature. Films weighing 0.25, 0.50, 0.75, and 1 g were measured, placed into four separated flasks, and supplemented with 30 ml of 0.000025 M methylene blue. Samples were then collected from each flask at regular intervals, and the residual MB concentration was determined using a UV-Vis spectrophotometer (Shimadzu UV mini 1240) at a wavelength of 664 nm.

RESULTS AND DISCUSSION

Gelatin yield

In this study, the freeze-drying procedure of gelatin solution yielded 16.3% of dry gelatin (Figure 1). This amount is comparable to the previously reported gelatin

yield obtained from the scales of sea brass (*Dicentrarchus labrax*) using acetic acid (DinçEr et al., 2015). Another group has successfully produced 48.1% gelatin from cultured carp scales by employing the alkaline protease method. However, the scales were grounded into a fine paste, thus increasing the surface area for enzymatic reaction and proportionally resulting in a higher yield (Jiang 2013).

The long extraction process that took approximately 13 days might contribute to the lower yield of dry gelatin from Nile Perch, possibly because a significant amount of collagenous materials could have been lost. Moreover, since gelatin derived from collagen and the collagen content differ from the raw materials used, the expected yield obtained between species may vary. This might explain the differences in yield in three species of fish that have been examined above.

The type and quantity of cross-links determine gelatin extractability from different tissues in collagens from various tissues (Muyonga et al. 2004). Therefore, it is expected that the yields of gelatin extracted from different tissues will vary. The yield of gelatin obtained in this study (16.3%) is higher than that isolated from the head of Mackerel (*Scomber scombrus*) by using different organic acids with an average yield of 3.5 % (Khiari et al. 2011). It is also different from that reported from skin extraction of farmed Amur sturgeon (19.6%), catfish (*Clarias batrachus*) at 27.79%, snakehead (*Channa striatus*) at 16.57%, red tilapia (*Oreochromis niloticus*) at 11.75%, pangasius catfish (*Pangasius sutchi*) at 10.78%, bigeye snapper (*Priacanthus macracanthus*) at 6.5% and brown stripe red snapper (*Lutjanus vitta*) at 9.4% (Jongjareonrak et al. 2006; Nikoo et al. 2011; See et al. 2010). The yield in this study also differed from that reported for skin extraction from farmed Amur sturgeon at 19.6%, catfish (*Clarias batrachus*) at 27.79%, snakehead (*Channa striatus*) at 16.57%, red tilapia (*Oreochromis niloticus*) 11.75%, pangasius catfish (*Pangasius sutchi*) 10.78%, bigeye snapper (*Priacanthus macracanthus*) 6.5% and brown stripe red snapper (*Lutjanus vitta*) at 9.4% (Jongjareonrak et al. 2006; See et al. 2010; Nikoo et al. 2011).

IR analysis

The IR spectra of the gelatin obtained from Nile Perch scales show about nine characteristics of IR absorption bands which can be observed from the IR spectrum (Figure 2). These are the amide A, B, and I, as seen through the VII bands. The amide I band is very sensitive and is the most frequently used to study secondary protein configuration. The amide I peak occurs at 1653 $cm^{-1,}$ which corresponds to a combination of a C=O stretching vibration of the amide group and N-H bending and usually occurs at 1600 cm^{-1} to 1700 cm^{-1} (Ahmad and Benjakul 2011; Nikoo et al. 2011). Amide II primarily results from in-plane NH bending and CN stretching vibration and reveals less protein conformation than amide I. Other bands are rarely used in protein conformational studies (Nikoo et al., 2011).

The amide I peak absorption of 1653 cm^{-1} falls within the expected range of 1650 to 1660 cm^{-1}. This value is slightly higher than the absorption of 1630 to 1640 cm^{-1} observed in other studies (Muyonga et al. 2004a; Nikoo et al. 2011). The higher frequency of the amide I band suggests a higher degree of disruption of intermolecular bonding when gelatin extraction is done at elevated temperatures and for more extended periods (Kittiphattanabawon et al., 2012; Nikoo et al., 2011), as was the case in this study. The amide A in this study is the wide peak occurring at 3442 cm^{-1}. This peak corresponds to NH stretching coupled with hydrogen bonding. Hydrogen bonding between OH groups of the amino acids in gelatin is responsible for the width (Muyonga et al. 2004b).

As shown in Figure 2, it is evident that the Amide II and Amide III bands arose at 1590 cm^{-1} and 1230 $cm^{-1,}$ respectively, consistent with observations of Ahmad and Benjakul (2011). The low intensity of Amide III might be caused by weak molecular interaction in gelatins extracted under high-temperature conditions (Muyonga et al. 2004).

Amino acid analysis

The amino acid composition of gelatin obtained from the Nile Perch is presented in Table 1.

Figure 1. Nile perch scales (*left*), scales during hydrolysis (*right*)

Table 1. Amino acid composition of Nile perch scale gelatin

Amino acid	Composition (weight %)	SD
Asparigine/Aspartic acid	6.6	0
Glutamine/Glutamic acid	10.8	0.0707
Serine	2.4	0.0707
Histidine	1.4	0.1414
Glycine	21.7	0.7778
Threonine	2.1	0.0707
Alanine	11.8	0.2828
Arginine	8.9	0
Tyrosine	0.9	0.2121
Valine	3	0
Methionine	3.4	0.1414
Phenylalanine	3.1	0
Isoleucine	1.8	0
Leucine	3.3	0.0707
Lysine	4.3	0.1414
Proline	14.6	0.7778
Total %	100	

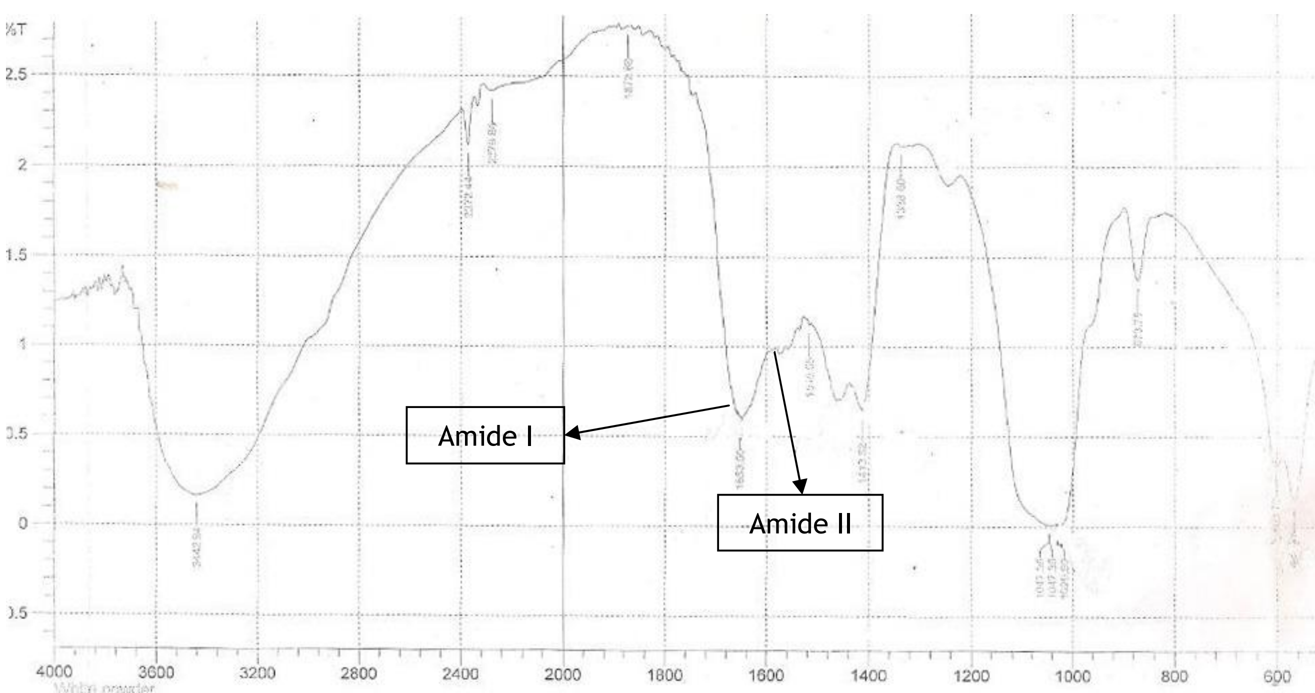

Figure 2. IR spectra of gelatin obtained from Nile perch scales

Among 16 amino acids examined in this study, glycine content was found to be the highest (21.7%), followed by a proline (14.6%). Gelatin consists of repeating Gly-X-Y motifs corresponding to glycine, proline, and hydroxyproline, respectively. Hence, these amino acids are the most abundant in any gelatin, irrespective of the source. For instance, chicken skin comprises 33.7% of glycine and 13.42% of proline, dominating the other types of amino acids present in the skin (Sarbon et al., 2013). Tavakolipour (2011) also reported that gelatin from silver carp fish waste (skins and fins) contains glycine and proline, making up one-third of all amino acid residues at 31.7% and 12.4%, respectively. The proline content of Nile Perch scale gelatin observed in this study is lower than that of bovine gelatin and porcine gelatin, yet, higher than that seen for Pollock (10.09%) and Salmon (10.79%) skin gelatins (Avena-Bustillos et al. 2006). Gelatin from animals that inhabit low-temperature habitats has lower amino acid content than those from animals inhabiting high-temperature habitats. Consequently, fish gelatins have lower amino acid concentrations than mammalian gelatin. Furthermore, warm-water fish tend to have a higher level of amino acids than cold-water fish. In this regard, it can be easily understood that Pollock and Salmon, classified as cold-water fishes, have a lower concentration of total amino acids. Proline and hydroxyproline make up the amino acid complex, which is particularly important in determining the gelatin's gel properties by stabilizing the gel network by hydrogen bonding. The higher the amino acid content is, the better the properties they could make (Gomez-Guillen et al., 2002; Nikoo et al., 2011).

Alanine was another major amino acid found to be the third-highest in its content (11.8%) after glycine and proline (Table 1). Alanine occupies the non-polar regions in which gly-pro-y sequences are predominant, with the third position coupled with either hydroxyproline or alanine. Methionine and Threonine were substantially low. The lowest amino acids in composition were tyrosine (0.9%), histidine (1.4%), and isoleucine (1.8%), while tryptophan and cysteine were completely absent. All of these are inherent properties of gelatin (Mahmoodani et al., 2014). The amino acid analysis results were further confirmed in the chromatograms in Figures 3_a and 5_b. The height of the peak corresponds to the amount of amino acid.

These findings are similar to the earlier study that reported the absence of tryptophan and cysteine from the list of amino acids analyzed from fish skin gelatin (Karim and Bhat 2009). Nevertheless, another group has also demonstrated that the content of glycine (19.3%) and proline (13,.4%) account for the most abundant type of amino acid in bigeye snapper skins, as was the case in this study (Jongjareonrak et al. 2006).

This is also true for the amino acid profile isolated from the lizardfish scale that yielded three major amino acids, glycine (18.3%), proline (16.5%), and alanine (12.4%). Similarly, threonine and histidine were the least abundant amino acids weighing 0.9% and 1.52%, respectively (Wangtueai and Noomhorm 2009). One plausible explanation for this similar amino acid profile is that both Nile Perch and lizardfish originated from warm water habitats.

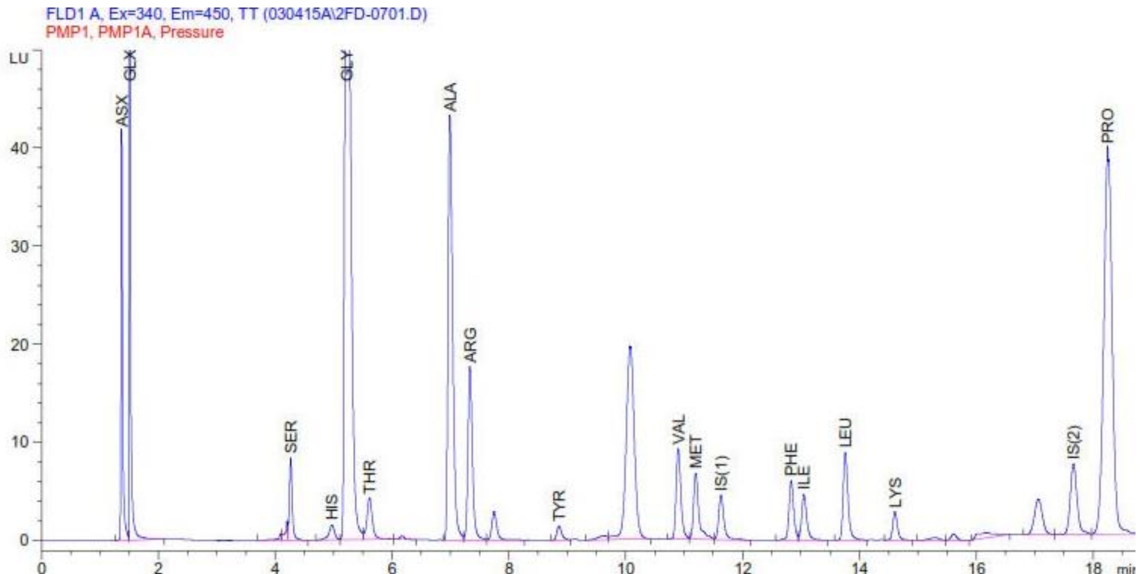

Figure 3.A. HPLC chromatogram of the amino acid analysis of Nile perch scale gelatin, the first repetition

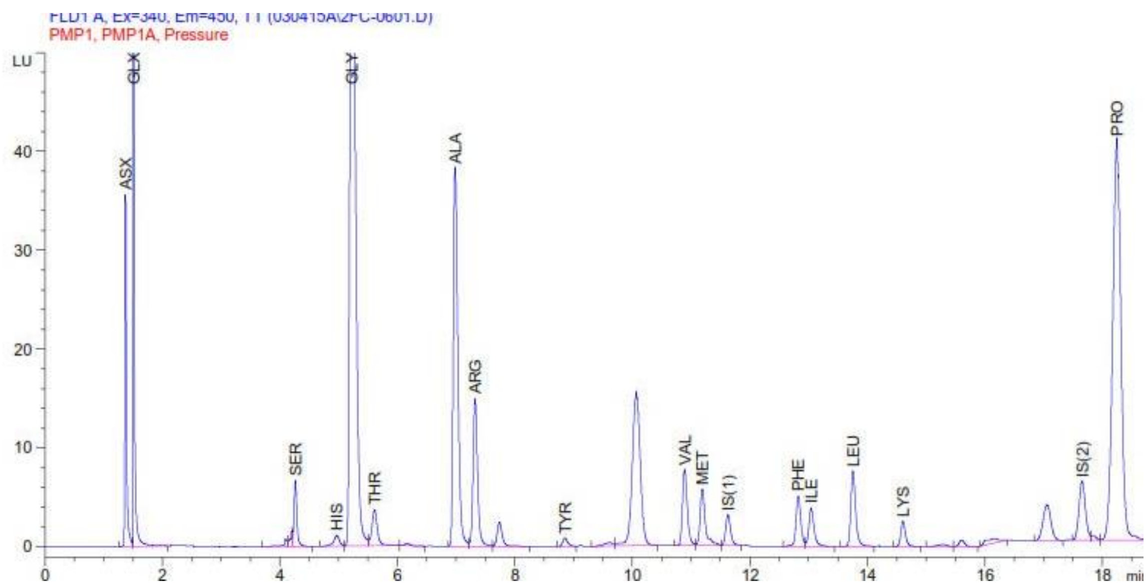

Figure 3.B. HPLC chromatogram of the amino acid analysis of Nile perch scale gelatin, the second repetition

Although amino acid composition might be tissue-dependent, to some extent, two different tissues within one species may display a similar profile of amino acids. For example, analysis of gelatin from sea bream bones and scales suggested that the chemical composition of type I collagen is conserved across tissues (Akagündüz et al. 2014). This finding could explain the similarity in the amino acid profiles of Nile Perch bone gelatin (Muyonga et al. 2004a) and that of Nile Perch scales reported in this study. The slight variations could be attributable to the different extraction methods (Amiza et al., 2015). Muyonga et al. (2004a) employed an acid pretreatment before extraction, while in the present study, an enzymatic extraction was used instead.

Blend films preparation

Figure 4 shows PVA/gelatin blend solutions drying for four days in a petri dish. Once completely dry, PVA/gelatin films of various compositions were peeled off the glass Petri dishes. Figure 5 below shows the 60/40 PVA/gelatin films being peeled off. All films were able to be peeled off except for PVA/gelatin ratios of 10/90. In general, films made from proteins are delicate. Therefore, plasticizers are often added to lower the protein-protein chain interactions stabilizing the film's network and increasing the mobility of protein molecules (Peesan et al. 2005; Vanin et al. 2005).

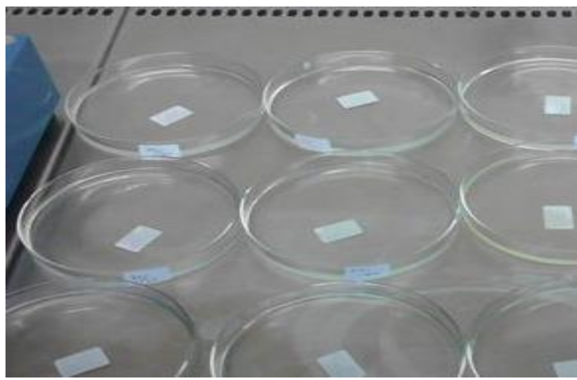

Figure 4. PVA/gelatin solutions of various compositions drying in Petri dishes

Figure 5. PVA/gelatin films of 60/40 PVA/gelatin composition

In this study, however, no plasticizer was used. Consequently, after completely drying the films, blend films with the highest gelatin content tend to be brittle and difficult to peel off. Gelatin exhibits a strong hydrophilic character and is highly soluble in water. Therefore, it has poor mechanical properties, such as tensile strength in damp environments (Yu et al. 2006). The low tensile strength of gelatin explains the ineffectiveness of moisturizing to allow peeling off of the 10/90 blends, unlike other blends containing lower gelatin content.

Thermal analysis of the polymer blends

DSC Analysis

DSC analysis of pure gelatin and pure PVA

The DSC thermogram obtained from pure gelatin analysis from Nile Perch scales is shown in Figure 6.

The thermogram shows no endothermic peak except for an inflection corresponding to the glass transition temperature (T_g) at approximately 157°C. The high glass transition temperature suggests a low degree of crystallinity. As a polymer gets more amorphous, its chains become more entangled, thus increasing the amount of energy required to disentangle them during glass transition, raising the temperature of the glass transition (Elsergany 2014). The T_g of gelatin in this study exhibits a higher value than that observed by Gao et al. (2014) and Silva et al. (2008), who reported 121.9°C and 57.5°C, respectively. Pawde et al. (2008) reported a much higher value of 220°C to 230°C. T_g is a kinetic event. Thus, the same substance may have different T_g‘s as reported in the literature, depending on the manufacturing conditions (Elsergany 2014). As shown in Figure 7, T_g and T_m values of 84.9°C and 190.8°C were obtained for pure PVA.

The endothermic peak indicates semicrystalline properties when the heat of fusion reaches 29.7 J/kg. Semicrystalline systems typically display a peak after the T_g transition (Silva et al. 2008), indicative of the melting of the crystalline domains of the polymer. They are absent in the case of amorphous polymers.

DSC analysis of PVA/gelatin blends

Table 2 summarizes the DSC data obtained for the various compositions of the prepared blends.

The glass transition temperature is used to determine the miscibility of two polymers in the amorphous phase, thereby serving as an essential tool in polymer blend technology. The presence of a single, composition-dependent glass transition temperature intermediate between individual constituents (Peesan et al. 2005) usually indicates the miscibility of polymer blends. Table 2 above represents the DSC data obtained for all the blend films studied.

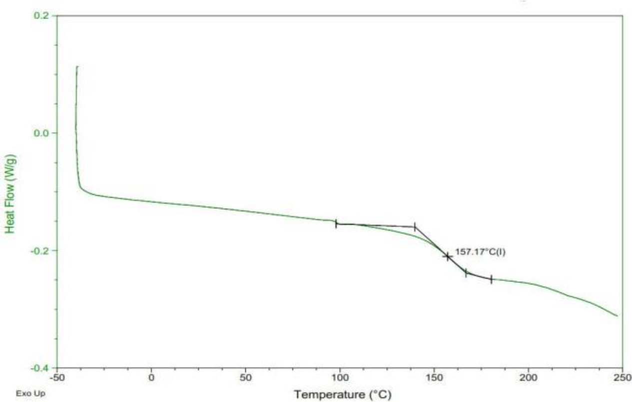

Figure 6. DSC thermogram of gelatin obtained from Nile Perch scales

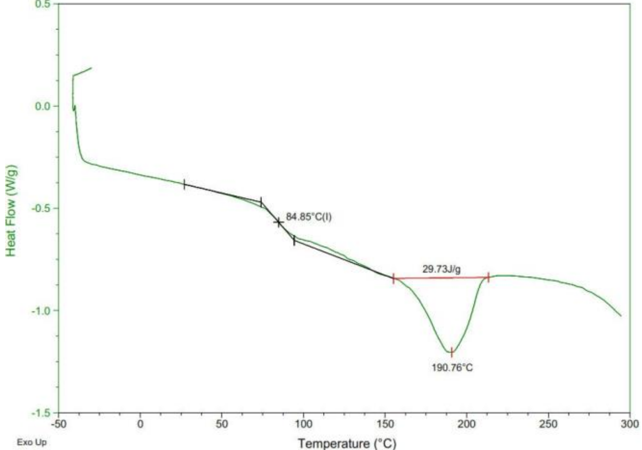

Figure 7. DSC thermogram of pure PVA

Table 2. DSC data (T_g and T_m) for PVA/Gelatin polymer blends

PVA/gelatin	T_g (°C)	T_m (°C)
20/80	97.14	-
30/70	96.78	-
40/60	97.47	187.6
50/50	96.81	188.77
60/40	96.95	197.88
70/30	96.79	207.54
80/20	96.75	207.0
90/10	87.36	173.55
Gelatin 100%	157.2	-
PVA 100%	84.85	190.76

For all films investigated, the T_g falls in between those observed for gelatin and PVA, and therefore miscibility between PVA and gelatin can be determined. A single T_g intermediate between the T_g's of the homopolymers implies a higher degree of miscibility in the amorphous phase (Onyari and Huang 2009). It can, therefore, be assumed that miscibility is highest in the 90/10 PVA/gelatin blend (Table 2).

This study highlights that the T_g of the PVA/Gelatin blends studied lies between 96°C to 98°C for all compositions except 90/10 PVA/gelatin blend film. The relatively constant value of T_g could be due to the restricted mobility of PVA molecules in the amorphous phase due to the presence of gelatin molecules, as observed by Peesan et al. (2005).

The T_g inflections in the DSC curves for all films in this study were followed by significant endothermic peaks, as seen in Figure 8, the DSC thermogram of the 40/60 PVA/gelatin blend.

Melting is a first-order transition ahead of glass transition, which is in the second order. A first-order transition occurs with heat capacity and enthalpy changes, while a second-order occurs with a change solely in the heat capacity (Chiellini et al. 2001; Zhang and Li 2009). This explains the presence and association of the peaks to the melting process (melting peak). The apex of the melting peak gives the melting temperature point (T_m) of a polymer, which is the temperature at which the polymer's crystallites lose the highly ordered arrangement in their structure.

Crystalline polymers are characterized by defined melting points, while amorphous polymers are featured with glass transition. The DSC curves of the PVA/gelatin films presented here showed both T_g and T_m values suggesting semi-crystalline properties, concurring with the findings of other studies (Mendieta-Taboada et al. 2008; Silva et al. 2008). The T_m of the films occurs between 174°C to 207°C for different compositions. In the films with 40, 50, and 90% PVA composition, there is a T_m depression (187.6, 188.77, and 173.56°C, respectively) from the observed T_m value for pure PVA (190.76°C). This, as observed by Pawde et al. (2008), is indicative of strong intermolecular interactions between PVA and gelatin molecules.

The area under the endothermic peak corresponds to the enthalpy value of fusion of the crystalline portion. It is qualitatively related to the number of crystallites/degree of crystallinity in a film sample. The larger the area, the larger the number of crystallites and the more crystalline a substance is.

The DSC curves of the films of various compositions are shown in Figures 9-13. As the gelatin content increases and the PVA reduces, the size of the area under the endothermic peak decreases, indicating a decline in the level of crystallinity associated with PVA. Almost no endothermic peak is observed in Figure 9. This graph represents the thermogram of the film with PVA and gelatin composition of 30% and 70%, respectively. The higher the gelatin content in a sample, the more properties resemble gelatins.

Gelatin is amorphous, while PVA is more crystalline. An increase in gelatin or a decrease in the PVA content in the sample films evidently reduces the number of crystallites in the blend samples, as also observed by Pawde et al. (2008).

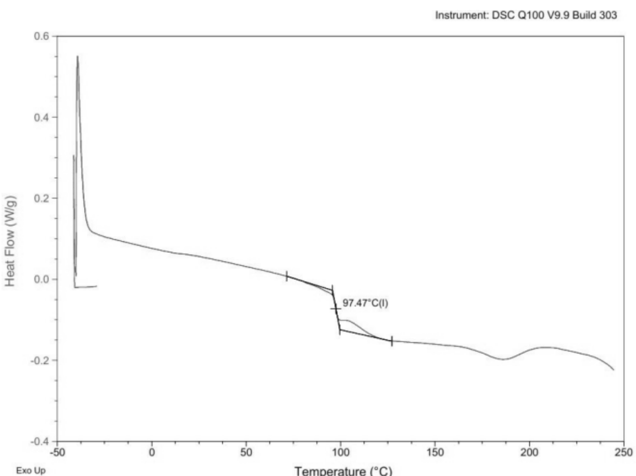

Figure 8. DSC thermogram of PVA/gelatin blend film in 40/60 composition.

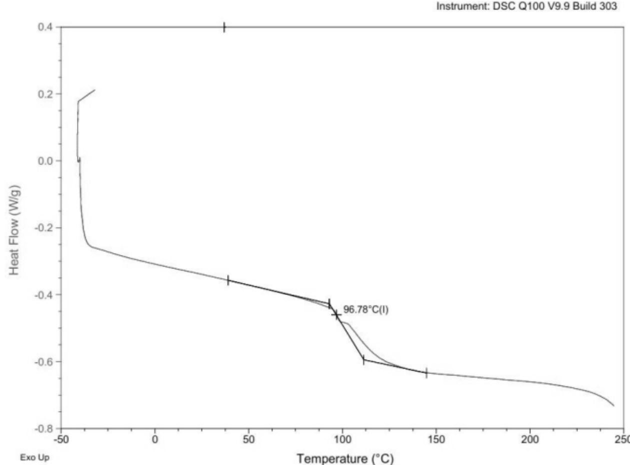

Figure 9. DSC thermogram of the 30/70 PVA/gelatin blend.

Figure 10 displays the DSC thermogram of the film made of the blend of PVA/ gelatin (50/50%). As shown above, the effect of PVA amount was prominent since increasing the amount of PVA will effectively increase the area under the endothermic peak. An increase in the quantity of PVA increases the number of crystallites and, therefore, the degree of crystallinity. The phenomenon is further illustrated in the thermogram of the film made from the blend with 70% PVA composition in Figure 11.

Thermogravimetric analysis

Thermogravimetric analysis (TGA) is the method of thermal analysis used to evaluate the thermal stability and decomposition behavior of polymer samples over time. A sample is exposed to high temperatures in a controlled environment, and its weight loss profile is plotted against the temperature in the TGA curves. The TGA thermograms of pure PVA and pure gelatin are displayed in Figures 14 and 15, respectively.

Figure 14 shows the DSC thermograms for the 30/70 PVA/gelatin blend composition film.

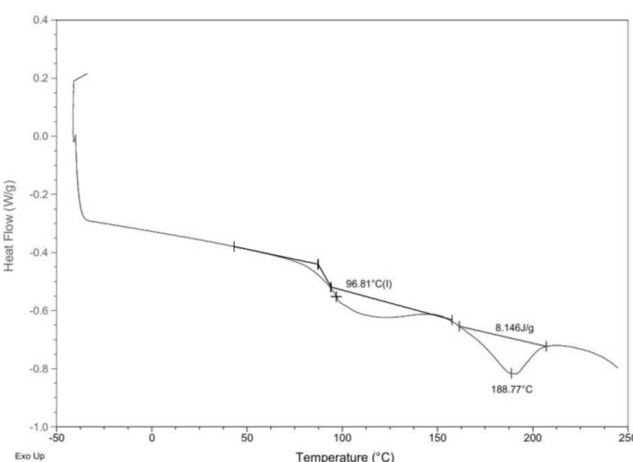

Figure 10. DSC thermogram of 50/50 PVA/gelatin blend film

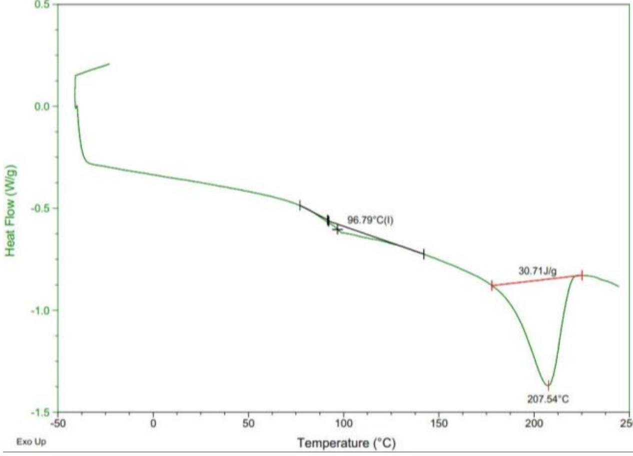

Figure 11. DSC thermogram of 70/30 PVA/gelatin blend film

Both curves show two zones of weight loss. The first regions are due to moisture loss and the evaporation of volatile components. The second region is due to pyrolytic reactions/thermal decomposition. The onset temperature for the decomposition of PVA is approximately 260°C. After the onset decomposition temperature, the slope of the curve is very steep, indicating a fast decomposition rate. Between 250°C and 400°C, PVA loses most of its weight, remaining with just 20% at around 400°C. The rate slows down and completely levels out at about 475°C. As shown in Figure 12, PVA loses 97.2% of its weight and a char yield of 2.82% between the temperature range of 100°C - 700°C.

Previously, Chiellini et al. (2001) reported a difference in temperature of approximately 40°C. In this study, referring to Figure 13, the degradation of onset temperature was approximately 210°C, around 50°C lower than PVA. Further analysis also revealed that the degradation rate of gelatin is slower than that of PVA. At 400°C, PVA had lost 80%, while gelatin had lost about 65% of its weight. Moreover, the slope of the curve of the gelatin is less steep, which explains that gelatin has a char yield of 19.9% and leads to conclude that gelatin thermally decomposes slower and partially as compared to PVA.

Two regions of weight loss can be attributed to the evaporation of moisture (in the first region) and pyrolytic reactions (in the second region). The onset temperature for the film occurred at 230°C, an intermediate between the onset temperatures of the individual pure components. Although the curve is not as steep as PVA, it is steeper than gelatin, suggesting an intermediate degradation rate. The film had lost 60% of its weight at the onset temperature of 400°C. All these observations display an alteration of individual polymer properties by introducing the other polymer component during blending. The properties of the 30/70 PVA/gelatin blend film are more similar to those of gelatin than PVA, probably due to the higher gelatin content.

The thermal properties of the films are getting similar to that of PVA as the percentage of the PVA in the film increases, i.e., the rate of degradation increases and the residue left at 700°C decreases. Figure 15 and 18 display TGA thermograms of the 50/50 and 70/30 PVA/Gelatin blends, which indicate that the thermal stability of the films significantly varies from that of the individual polymers depending on the ratio of each component in the blend. This reduction in thermal stability and an increase in the less stable component were also reported by Chiellini et al. (2001).

Dye adsorption

This study also aimed at determining the potential industrial applications of the films. The Methylene blue dye was used to examine the potential use of the films in the adsorption of dyes from an aqueous solution. Factors influencing the dye adsorption process, such as contact time, adsorbent amount, and initial concentration, were measured, and the result is given below.

Calibration curve

The calibration graph for the dilute solutions of methylene blue prepared from a stock solution of 4.0×10^{-5} is displayed in Figure 17.

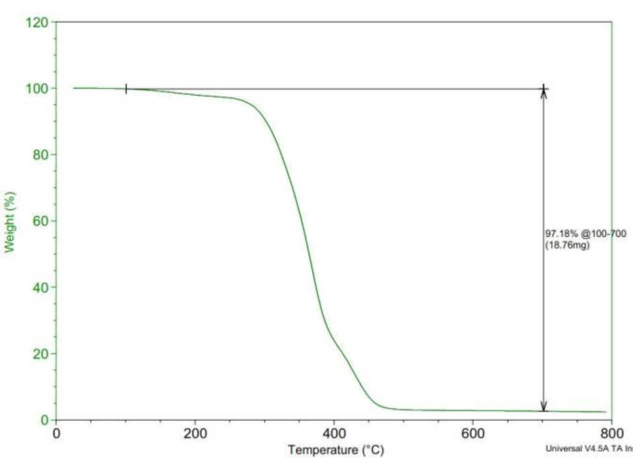

Figure 12. TGA thermogram of pure PVA

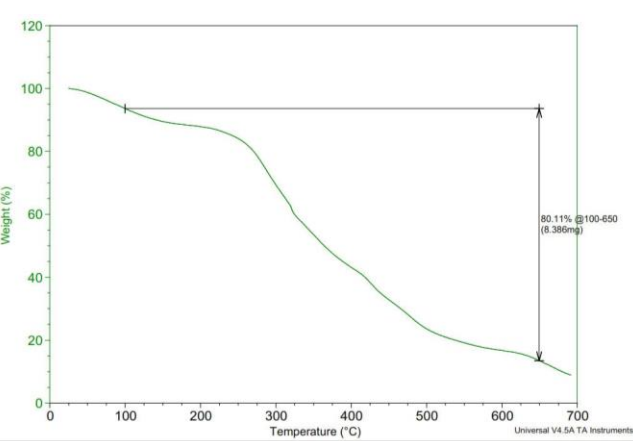

Figure 13. TGA thermogram of pure gelatin obtained from Nile Perch fish scales

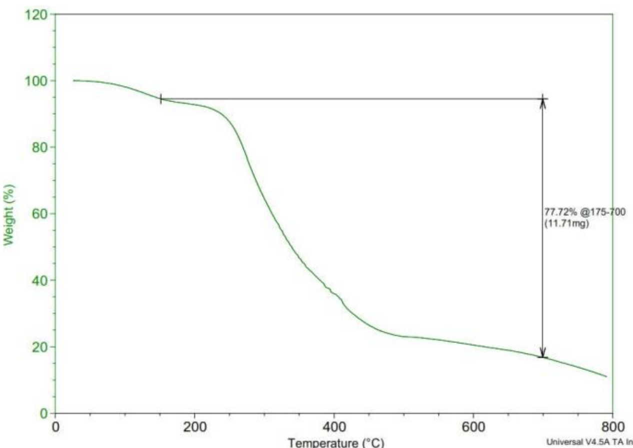

Figure 14. TGA thermogram of 30/70 PVA/gelatin blend film

Figure 15. TGA thermogram of the 50/50 PVA/gelatin blend film

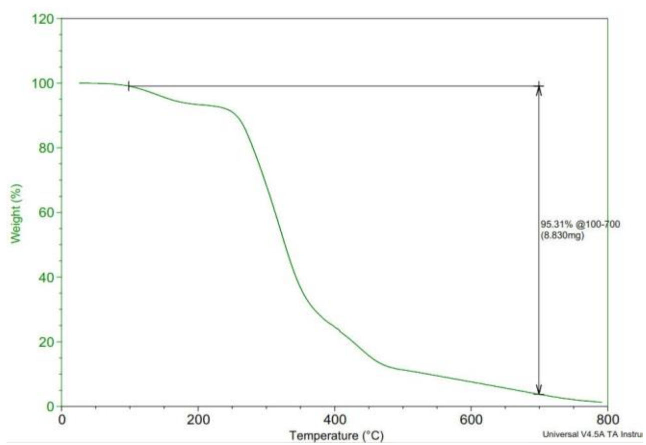

Figure 16. TGA thermogram of the 70/30 PVA/gelatin blend film

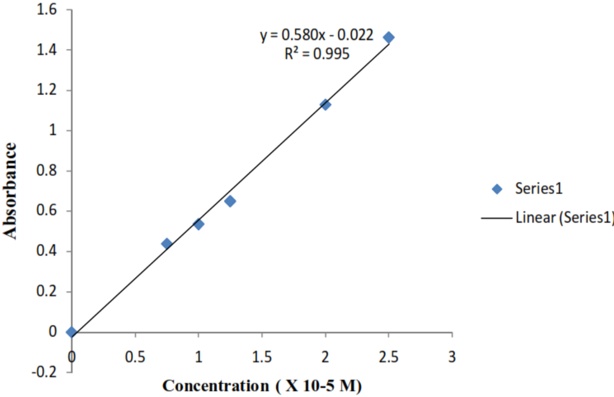

Figure 17. Calibration curve adsorption of methylene blue solutions onto 60/40 PVA/gelatin blend film

Effect of contact time on adsorption of methylene blue

The contact time between the adsorbent and dye molecules is essential for determining the efficiency, kinetics, and equilibrium adsorption time (Shee 2011). From Figure 18, it is evident that initially, the amount of dye adsorbed increased rapidly but decreased in approaching equilibrium. At equilibrium, the graph completely flattens, which indicates little or no adsorption. It can be seen that the adsorption reaches equilibrium after 110 minutes when the percent dye adsorbed is 64%.

The adsorption process can be divided into three regimes based on the duration of dye uptake. The first region shows speedy uptake, which gradually slows down in the second region. Finally, the dye uptake reached a plateau entering the third region. This is because an abundant amount of free surface area is available for adsorption in the first region. More molecules occupy and reduce the number of available sites, decreasing absorption rate as time elapses. Moreover, repulsive forces between adsorbate molecules on the adsorbent surface and those in the bulk solution keep increasing (Wanyonyi 2011).

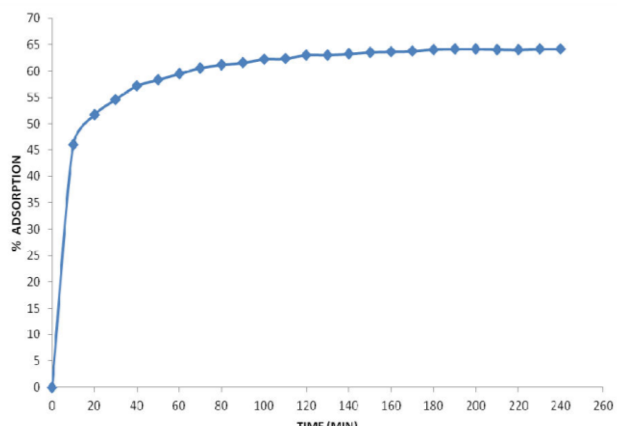

Figure 18. Effect of contact time on the adsorption of methylene blue dye solution, 2.0 X 10^{-5} M concentration onto 60/40 PVA/gelatin blend film

Effect of amount of adsorbent on adsorption

The variation of the percent adsorption of 0.000025M methylene blue dye solution with the amount of PVA-gelatin film (60/40 composition) is illustrated in Figure 19.

Increased weight of the PVA-gelatin film is positively correlated with increased the percent dye removal/ efficiency. At equilibrium, the percent dye removal is 43% for 0.25g of PVA-gelatin film and 50% for 1.0 g. When more adsorbent is used, binding/active sites become more available, thus increasing the adsorption efficiency. Wanyonyi (2011) and Shee (2011) have reported similar findings.

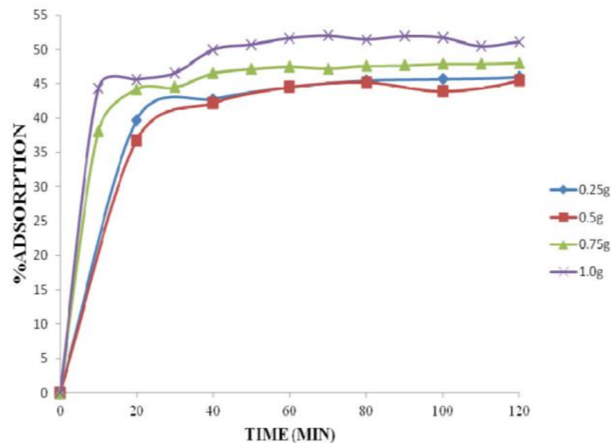

Figure 19. Effect of adsorbent weight on adsorption of 2.5 X 10^{-5}M by PVA-gelatin film

Effect of initial dye concentration

The adsorption kinetics is largely affected by the initial concentration of the adsorbed dye. This is due to the incorporation of driving force which is essential for overcoming mass transfer resistances between molecules in both the aqueous and the solid phases. The adsorption of methylene blue onto 0.25g PVA-gelatin film was studied at three different concentrations, and the results are displayed in Figure 20.

Figure 20 demonstrates that dye adsorption is directly proportional to the initial methylene blue concentration. For a dye concentration 4mg/L, the equilibrium uptake of a 0.25g PVA-gelatin film is 9.5mg/g, and when the dye concentration increases to 6 mg/L, the uptake value is 14 mg/g. The correlation between dye adsorption and initial dye concentration is explained as follows. Increasing the dye concentration will increase the amount of driving force available to overcome resistance to mass transfer of the dye onto the film (Wanyonyi 2011). It also means increasing the number of dye molecules in the solution, thereby increasing the interaction between the dye and the adsorbent, which effectively increases adsorption. Shee (2011) also reported similar findings in the adsorption of methylene blue dye onto mangrove bark, mangrove leaves, and coconut husks.

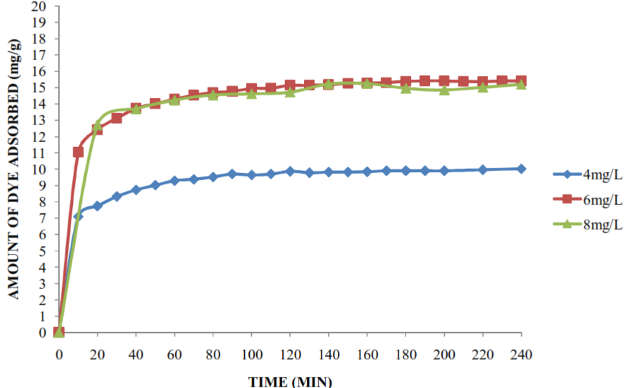

Figure 20. Effect of initial methylene blue dye concentration on equilibrium dye uptake using PVA/Gelatin blend film (60/40 composition)

Nevertheless, only a very little change in the percent of equilibrium is observed when the initial methylene blue concentration is elevated from 6 mg/L to 8 mg/L. Because the weight of the adsorbent remains the same throughout the time course, no change has occurred on the available binding sites. Therefore, at some point, all available binding sites are fully occupied by the dye molecules, so an increase in concentration will have no significant effect on adsorption.

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Determination of pyrrolizidine alkaloids levels in *Symphytum asperum*

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Abstract. *Onduso SO, Ng'ang'a MM, Wanjohi W, Hassanali A. 2017. Determination of pyrrolizidine alkaloids levels in* Symphytum asperum*. Biofarmasi J Nat Prod Biochem 15: 65-73.* The levels of the pyrrolizidine alkaloids (PAs) in the roots and leaves of comfrey (*Symphytum asperum* Lepech) from two agro-ecological zones in Kenya, Kiambu, and Kisii counties during wet (April 2012) and the dry (September 2012) seasons were determined in the present study. The samples were dried beneath the shade for four weeks, continued with ground and extraction of the PAs by applying different solvent systems. The result displayed no significant difference in the percentage of dry weights for the leaves and the roots samples collected from Kisii and Kiambu counties during the wet and dry seasons (p>0.05 at 95 % confidence limit). Gas Chromatography (GC) combined with a Mass spectrometer (MS) was used to analyze the samples and characterize and quantify major components. Among four PAs identified in the root samples (echimidine (**7**), 7-acetyllycopsamine (**17**), 3'-acetyllycopsamine (**18**), triangularine (**19**), and heliosupine (**24**)), a significant amount of 7-Acetyllycopsamine (**17**) was evident during the wet season as compared to the dry season (p=0.033, α=0.05, t-test). The other pyrrolizidine alkaloids measured between the two seasons did not vary significantly (p>0.05, α=0.05, t-test). All the compounds reported in the root samples are associated with toxicity, and their values exceed the tolerable levels recommended by relevant regulatory bodies in various countries. One of the most toxic PAs, echimidine (7), was found in all the root samples. However, the leaf samples showed PAs level below detectable limits using GC-MS. Further study is needed to examine whether PAs are produced from the leaves collected at different maturation levels and from a broader plant profile. Furthermore, it is necessary to know the levels of a particular PA and related analogs in a larger profile of the plants cultivated in other agroecological zones in Kenya. A study on the toxicity of heliosupine (**24**) is of future interest since it exists in significant amounts, yet such studies have not been done on this constituent. Also, preparative isolation of the PAs should be performed to provide reference standards in other quantification facilities that utilize Gas Chromatography because GC-MS is a rather expensive method.

Keywords: Alkaloids, *Symphytum asperum,* pyrrolizidine

INTRODUCTION

Pyrrolizidine alkaloids (PAs), compounds identified in plants, consist of two five-membered rings which share nitrogen atoms at position 4 (Crews et al. 2009). Typically, a hydroxy methylene group is attached at C-1 and a hydroxyl group at C-7 (Molyneux et al. 1991). This structure forms the necine base (**1**), with known major representative structures including retronecine (**2**), heliotridine (**3**), platynecine (**4**), and otonecine (**5**). Retronecine and heliotridine are both enantiomers at C-7 (Fu et al. 2001; FAO 2010). The necines can either be saturated or possess a double bond in the 1,2 positions (Roeder 1999).

Esterification of one or both of the hydroxyl groups in the necine base by a necic acid gives its structural diversity, comprising monoesters, non-macrocyclic diesters, and macrocyclic diesters (Roeder 1999; Boppre 2011). The most prominent necic acids that form esters with necines are given in Table 1 (Roedar 1999).

Over 660 PAs and their N-oxides have been identified, and more are being identified in over 6 000 plants belonging to some 13 families of the flowering plants (ANZFA 2001; Fu et al. 2001; Fu et al. 2004; COT 2008; Crews et al. 2009; Mei et al. 2010; Boppre 2011). This number represents about 3% of all the species of flowering plants, in which three principal families are involved in the synthesis of Pas that are considered toxic. These families are Boraginaceae (all *genera*-including *Symphytum)*, Compositae (tribes *Senecionae* and *Eupatoriae*), and Leguminoceae (genus *Crotalaria*) (Roeder 1999; Coulombe 2003; Fu et al. 2004; COT 2008; Pawar et al. 2010; CODEX 2011). A list of *Symphytum spp*. and the corresponding PAs produced are given in Appendix 1a (CODEX 2011). This study focused on the PAs obtained from comfrey (*Symphytum asperum*).

(1) (2) (3)

(4) (5)

Table 1. The most prominent necic acids

General structure	R1	R2	R3	R4	General names
Monocarboxylic acids occurring in PAs					
2-C Acids	Me-CO_2H				Acetic acid (Ac)
5-C Acids	**R_1**	**R_2**	**R_3**		
	H	Me	Me		Senecioic acid (Sen)
	Me	Me	H		Tiglic acid (Tig)
	Me	H	Me		Angelic acid (Ang)
	CH_2OH	H	Me		Sarracinic acid (Sar)
7-C Acids	**R_1**	**R_2**	**R_3**		
	H	H	OH		(-)-Viridifloric acid
	H	OH	H		(+)-Trachelanthic acid
	OH	OH	H		Echimidinic acid
	OH	OMe	H		Lasiocarpic acid
7-C Acids	**R_1**	**R_2**			
	H	OH			(-)-Trachelanthic acid
	OH	H			(+)-Viridifloric acid
Dicarboxylic acids used for the construction of 12-membered macrocyclic PAs					
10-C Acids	**R_1**	**R_2**	**R_3**	**R_4**	
	Me	H	H	H	Senecinic acid
	H	Me	H	H	Integerrinecic acid
	H	H	Me	H	Senecivernic acid
	Me	H	H	OH	Isatinecic acid
	H	Me	H	OH	Retronecic acid
10-C Acids	**R_1**	**R_2**	**R_3**		
	Me	H	H		Seneciphyllic acid
	H	Me	H		Spartioidinic acid
	Me	H	OH		Riddelliic acid
10-C Acids					Erucifolinecic acid
10-C Acids					Petasinecic acid
Dicarboxylic acids used in the construction of 11-membered macrocyclic PAs					
8-C Acids	**R_1**	**R_2**			
	OH	OH			Monocrotalic acid
	H	OH			Crotaleschenic acid
10-C Acids	**R_1**	**R_2**			
	H	H			Incanic acid
	H	OH			Trichodesmic acid
	OH	OH			Globiferic acid

Comfrey, a Latin word for "growing together," is a member of the plant family Boraginaceae (Raymond 1997; Squires 2010). Several plant species in the genus *Symphytum* belong to the group of comfrey, such as wild or common comfrey and *S. officianale* L., which is native to England and extends throughout most of Europe and Asia, and western Siberia. Others are prickly or rough comfrey (*S. asperum* L.), named for its bristly or hairy leaves, brought to Britain from Russia in about 1800. Quaker, Russian or blue comfrey (*S. uplandicum* N.), originated as a natural hybrid of S. *officianale* and *S. asperum*. This hybrid was called Russian or Caucasian comfrey in reference to its country of origin (Teynor et al. 1992). More known comfrey include: *S. bulbosum* Shimp, Bulbous Comfrey, *S. caucasicum* Bieb, Caucasian Comfrey, *S. ibericum* Steven, Creeping Comfrey, *S. orientale*, White Comfrey, *S. tauricum*, Crimean Comfrey, *S. tuberosum*, Tuberous Comfrey, (*S. asperum x officinale,* synonym: *S. peregrinum*) (Kartal et al. 2001; Mei et al. 2010), *S. sylvaticum, S. aintabicum* (Kurucu et al. 2001), *S. asperrimum*, *S. cordatum*, and *S. grandiflorum* (Squires 2010).

The major constituents found in the comfrey plant include allantoin, mucilage, amino acids, PAs (Teynor et al. 1992; Raymond 1997; Squires 2010), proteins, vitamins B_{12} (Teynor et al. 1992; Squires 2010), tannins, phytosterols, triterpenoids, polyphenols (Raymond 1997; Squires 2010). Other constituents include mucopolysaccharides (Neagu et al. 2010), coniferin, carotene, starch, mineral substances (Ca, K, P, Mg, Fe, Mn, Na, Zn) (Neagu et al. 2011), phenolic acids (rosmarinic, chlorogenic, caffeic and lithospermic), choline, asparagines, volatile oil, steroidal saponins (Squires 2010).

The objectives of this study were (i) To isolate and characterize the various types of PAs in leaves and roots of *S. asperum* collected from distinct parts of Kiambu and Kisii Counties using GC-MS. (ii) To determine the concentrations of PAs in the *S. asperum* obtained from different parts of Kiambu and Kisii counties during two seasons, wet and dry, throughout the year.

MATERIALS AND METHODS

Study area

The study was performed in the selected areas of Kiambu County (Githunguri, Kahawa, and Ruiru) and Kisii County (Kenyenya sub-County) of Kenya, where these plants are widely grown and used.

Sample collection and sample size

Samples were collected from the roots and leaves of comfrey grown at selected locations in Kiambu County and parts of Kisii during the wet (April 2012) and dry (September 2012) seasons of the year. Large leaves that were ready for consumption are preferable for this study. The samples were obtained from small-scale farmers who largely depended on the crop as a vegetable. A taxonomist from Kenyatta University verified the plant's identity. A sample specimen SO/SA/2012/1 was deposited in the university herbarium. The sample collection locations varied from close to water points (streams or near water taps) to behind kitchens at the farmer's homestead.

Sample preparation and extraction of PAs

Samples were air-dried at ambient temperature (Crews et al. 2009) and ground into fine powders for four weeks. From six selected locations, a total of twenty-four samples of roots and leaves were collected during the wet season and the dry season. Thirty grams of either ground roots or ground leaves were placed in a conical flask and added with 100 ml of chloroform/methanol (85:15) and 5 ml of ammonium hydroxide (25% solution). The mixture was extracted mechanically using a shaking water bath at room temperature for 90 minutes. The extraction process was followed by filtration and reaction with 30ml 2M HCl to allow separation of the two-phase solution, the organic layers, and the aqueous layers. A separating funnel separated both layers, and the aqueous layer was neutralized with sodium carbonate and further extracted with chloroform (25 ml x 3). The mixed chloroform extract was dried over anhydrous sodium sulfate and filtered. A portion of the filtrate was spotted on the TLC plate and eluted with a solvent mixture of chloroform: methanol. To visualize the presence of candidate PAs, the plate was then sprayed with Draggendorf reagent. The remaining filtrate was then concentrated in the rotary evaporator, and as many as ~1-2 ml were transferred into a glass pre-weighed vial. To remove the residual organic solvent, the filtrate was gently flushed with a stream of nitrogen gas. Finally, the weight of the blend of PA alkaloids was recorded, and then continued with the extract's analysis by using GC-MS. This procedure was repeated for all twenty-four samples (Wuilloud et al. 2004).

Gas Chromatography linked with Mass Spectrometer

The composition of the alkaloid extracts was determined using a gas chromatograph system (GC 8000 series) with a fused capillary column (15 m length; 0.25 mm i. d.; 0.25 μm film thickness; static phase methyl silicone SE-30) directly coupled to a quadrupole mass spectrometer (Hewlett Packard 5973). Electron impact ionization was performed at an energy of 70 eV. Helium was used as a carrier gas injector, and the detector was maintained at 200ºC and 250ºC, respectively. The analytical conditions were as follows: oven temperature was 2 min isothermal at 60ºC, then 60 to 240ºC at a rate of 15ºC/min, then held isothermal for 6 min. The instrument was scanned at a mass range from 60 to 400 amu.

The blank solution of Dichloromethane (DCM) was run in the GC-MS, and its TIC (Total Ion Chromatogram) and its mass spectrum were obtained. Methyl stearate (10 ppm) was prepared in DCM and run in the GC-MS equipment to obtain the TIC and the mass spectrum. The extract in the vials was resuspended in methyl stearate (10 ppm), then combined and shaken with 0.5ml of methyl stearate to entirely dissolve the extract. One microliter of the dissolved extract was injected into the GC-MS equipment. For each sample, two injections were done. GC-MS were set to the Tundis et al. (2006) protocol conditions. By

comparing mass spectral data on computer and NIST library and reviewing the literature mass-spectral fragmentation data, the candidate of the alkaloids can be identified. The total ion chromatograms and their corresponding mass spectra are given in Figures S1 to S10 (Appendix). The data obtained were analyzed using a t-test at a 95% confidence limit.

RESULTS AND DISCUSSION

The percentage weight of the pooled solvent extracts for the roots and the leaves during wet and dry seasons is displayed in Table 2. The t-test means the percentage of dry weight is given in Table 3.

The results showed no significant difference in the dry weight of leaves and root extracts between the wet and dry seasons ($p>0.05$ at 95 % confidence limit). However, there were significant differences in the dry weight extracts of the roots and leaves in both seasons ($p<0.05$ at 95% confidence level).

In both seasons, the weight of the root extracts was more than the weight of the leaf extracts. There were mixed results for the weights measured from the root extracts between the wet and dry seasons, whereby three locations (Kiamumbi (ZW), Ruiru (RR), and Githunguri (GB)) recorded higher weights during the wet season compared to the counterpart data of the dry season. Meanwhile, the other three sampling locations (Kahawa Sukari (KW), Kisii (KS), and Kiambu (KB)) recorded higher weights during the dry season.

Table 2. Percentage weight of dried extracts from dried leaves and roots, respectively, obtained during the wet and dry seasons obtained from Kisii and Kiambu, Kenya

Masses (%)	KW	ZW	RR	KS	KB	GR
Leaves wet season	0.069	0.047	0.020	0.015	0.009	0.012
Roots wet season	0.059	0.104	0.072	0.063	0.159	0.114
Leaves dry season	0.037	0.031	0.041	0.049	0.023	0.060
Roots dry season	0.164	0.096	0.062	0.159	0.203	0.045

Note: KB: Kiambu, KW: Kahawa Sukari, KS: Kisii, ZW: Kiamumbi, GR: Githunguri, RR: Ruiru

Table 3. The mean (±SE) percentage dry weight of the roots and leaves during the dry and wet seasons

Part	Wet (Mean±SE)	Dry (Mean±SE)	p-values
Roots	0.10±0.02	0.12±0.03	0.403
Leaves	0.03±0.01	0.04±0.01	0.328
p-value	0.005	0.024	

For the leaf extracts, a higher dry weight in the wet season was recorded in Kahawa Sukari (KW) and Kiamumbi (ZW), while the rest recorded higher weights during the dry season than in the wet season. Therefore, the variation in weights of the samples between the two seasons suggests it did not follow a particular pattern. The variation is perhaps attributed to the differences in the maturity of the leaves collected for analyses, the leaf size, and the lifespan (age) of the plant. Where possible, larger leaves were used in this study regarding their size. Both leaf and root samples were taken in April 2012 (wet season) and September 2012 (dry season).

These plants seemed not to be physiologically affected by a lack of water supply during the dry season. They grew near water points or behind the kitchen, where regular access to water was available. Similarly, the sampling regions did not experience adverse effects due to the dry spells. The rainfall intensity in these regions would be minimal, occasional during the dry season. Thus, the plants would not, therefore, be said to lack water. This would also explain the variability of the weights between the wet and dry seasons.

TLC of concentrated root and leaf extracts sprayed with Dragendorff reagent exhibited brown-orange spots, indicating the presence of alkaloids (Eyad 2007). The GC-MS presenting the total ion chromatograms and their corresponding mass spectra are provided in Figures S1 to S10.

The total ion chromatogram for the root samples obtained from Kiambu during the wet season is given in Figure 1 for illustration purposes.

Based on TIC data, a total of three PAs (Table 4), namely echimidine (**7**), 3-Acetyllycopsamine (**18**), and heliosupine (**24**), were all identified from the database from the NIST library. The compound 7-Acetyllycopsamine (**17**) was identified from the mass spectral fragmentation data since the NIST library could only give the spectra but no identity. The mass spectra of the PAs identified in this study are shown in Figures 2 to 5.

Three compounds detected had characteristic ions at *m/z* 55, 80, 93, 94, 120, 136 and 220 (echimidine and heliosupine), at *m/z* 55, 80, 93, 94, 120, 136, 138 and 220 (3-acetyllycopsamine), and at *m/z* 55, 80, 93, 94, 120, 138, 180 and 220 (7-acetyllycopsamine). They all showed intense ions at *m/z* 93, 120, 136, and 220. Furthermore, 7-acetyllycopsamine showed an intense ion at *m/z* 180. These characteristics are typical of unsaturated PAs (Witte et al. 1992; Wuilloud 2004; Tundis et al. 2006; Rosemann 2006; Crews et al. 2009). The proposed structures of these fragment ions are given in Figure 6.

Table 4. PAs were obtained from root samples from Kiambu, Kenya, during the wet season

Retention time	Pyrrolizidine alkaloid
13.46	3-Acetyllycopsamine (18)
13.68	7-Acetyllycopsamine (17)
15.56	Echimidine (7)
15.73	Heliosupine (24)

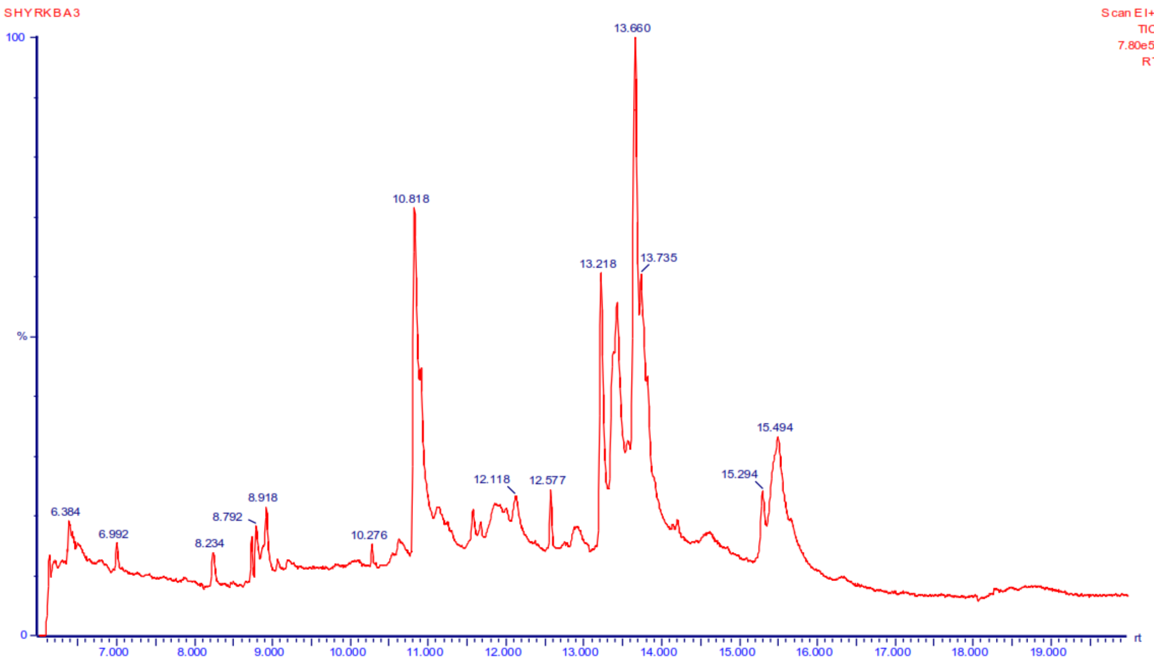

Figure 1. TIC of the sample collected from Kiambu, Kenya, during the wet season

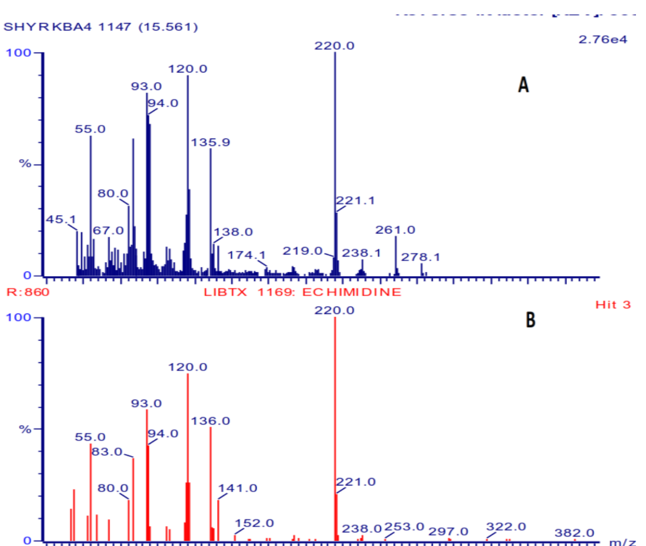

Figure 2. The mass spectra of echimidine (**7**) in a sample collected from Kiambu, Kenya, during the wet season (A: Sample spectrum, B: NIST Library spectrum)

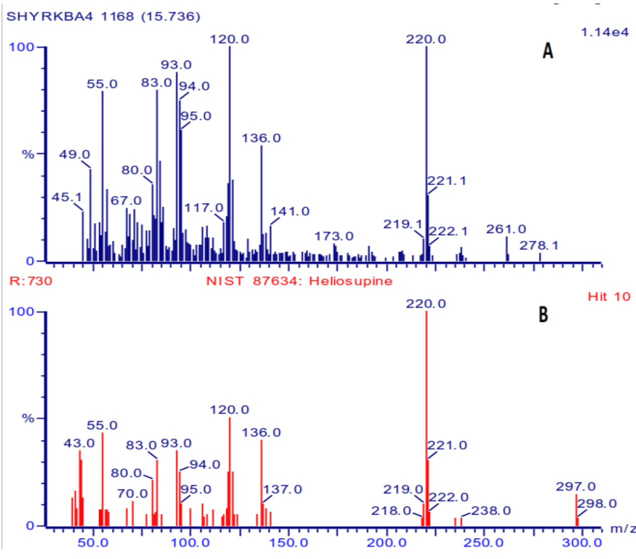

Figure 3. The mass spectra of heliosupine (**24**) in a sample collected from Kiambu, Kenya, during the wet season (A: sample spectrum, B: NIST library spectrum)

The fragmentation pattern of echimidine, whose characteristic ions are at m/z 55, 80, 93, 94, 120, 136, and 220. is shown below in Figure 7.

GC-MS analyses were done for all of the root and leaf samples. None of the leaf samples showed a positive test for the PAs. In contrast, the root samples gave a positive result for the PAs. The TICs and mass spectra of the whole root samples are presented in Figures S1 to S10. Identification of the pyrrolizidine alkaloids was determined regarding their mass spectra compared to the mass spectra published by the NIST library. In some cases (7-acetyllycopsamine in particular), identification was made from mass spectral fragmentation patterns by referring to the literature data because the NIST library did not provide identity information. The concentrations of each alkaloid were measured from their peak area ratios and compared to the peak area ratio of methyl stearate as the internal standard. The concentration of the internal standard has been determined. A summary of the PAs identified and their corresponding concentrations are presented in Table 5.

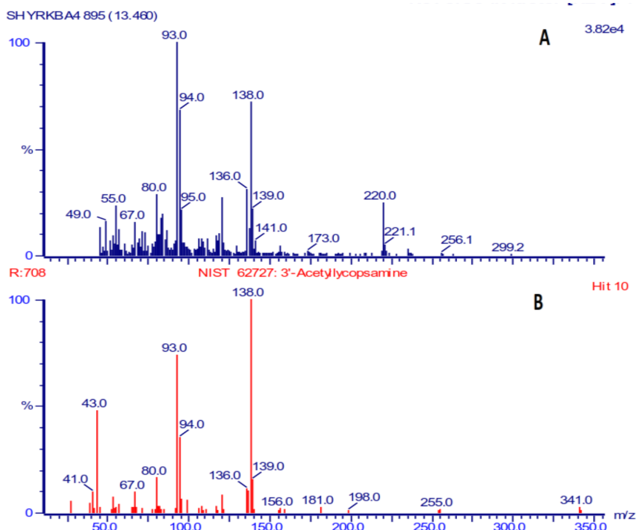

Figure 4. The mass spectra of 3'-acetyllycopsamine (**18**) in a sample collected from Kiambu, Kenya, during the wet season (A: sample spectrum, B: NIST library spectrum)

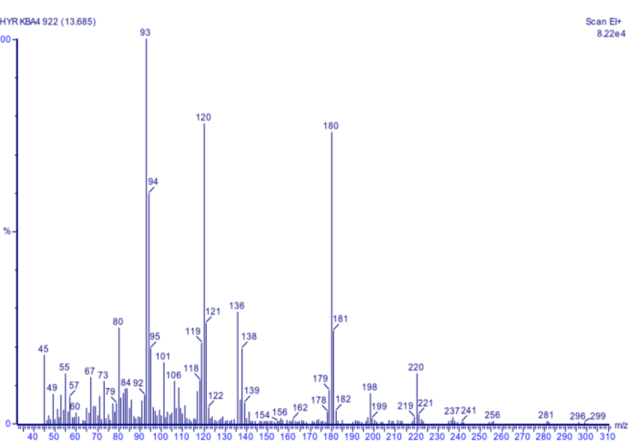

Figure 5. The mass spectra of 7-acetyllycopsamine (**17**) in the root sample collected from Kiambu, Kenya, during the wet season (Culvenor et al. 1980; Wuilloud et al. 2004)

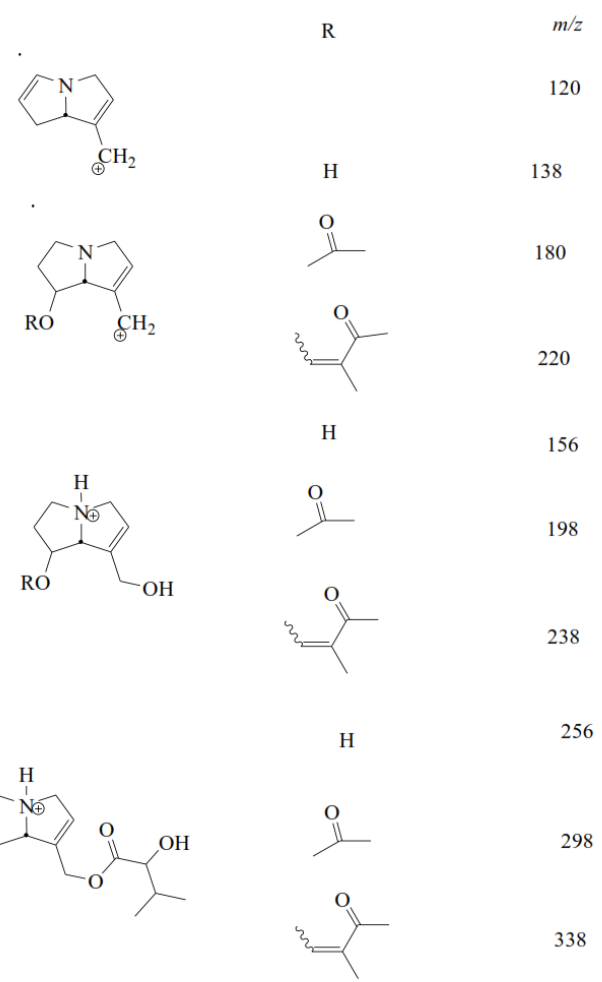

Figure 6. Proposed structures of fragment ions observed in the mass spectra of PAs (Witte et al. 1992; Wuilloud 2004; Tundis et al. 2006 and Rosemann 2006)

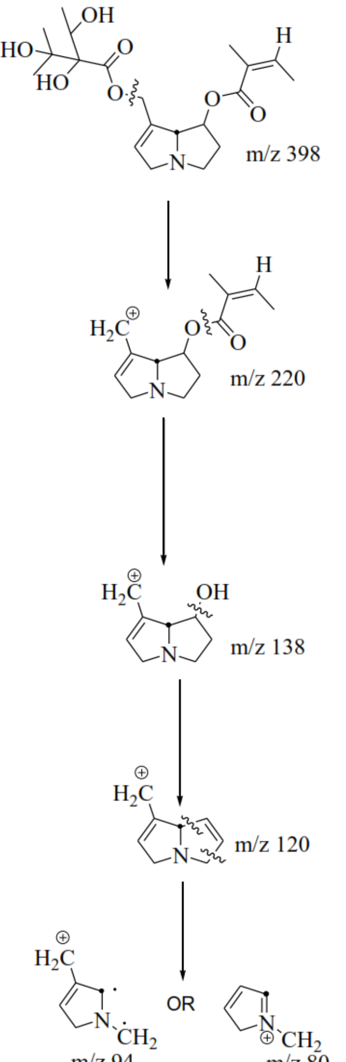

Figure 7. Proposed fragmentation pattern for echimidine (Rosemann 2006)

Echimidine (**7**) was found in the root samples in all sampling locations. At the same time, Heliosupine (**24**) was also detected from all locations except in Kiambu during the dry season and in Githunguri during the wet season. Other PAs identified were 7-acetyllycopsamine (**17**), 3-acetyllycopsamine (**18**), and triangularine (**19**). The concentration of the PAs (in ppm) ranged from 2ppm to 10ppm. The PAs were, however, below detectable levels in the leaf samples.

Some of the PAs reported in the *Symphytum spp* include echimidine (**7**), lycopsamine (**11**) and 7-acetyllycopsamine (**17**) (Awang et al. 1993; Culvenor et al. 1980), symphytine (**8**), 7-acetylintermedine (**10**), Symviridine (**12**), triangularine (**19**), uplandicine (**20**), symlandine (**21**), myoscorpine (**22**), echiumine (**23**) (Wuilloud et al. 2004; Culvenor 1983, Mei et al. 2010; WHO 1988), heliosupine (**24**) and asperumine (**25**) (Wuilloud et al. 2004). All these PAs are unsaturated and of retronecine type. Five of these PAs, echimidine (**7**), 7-acetyllycopsamine (**17**), (3-Acetyl-lycopsamine (**18**), triangularine (**19**), and heliosupine (**24**), were identified from the analyzed root samples.

Depending on the plant region, the PAs level varies, with the roots having the highest concentrations, followed by the small leaves. Previous studies indicate that mature leaves have the lowest concentrations (Culvenor et al., 1980; Mattocks, 1980; Awang et al., 1993; Betz et al., 1994; Fu et al., 2001; Oberlies et al., 2004; Mei et al., 2010; McCall and Fordyce 2010). Oberlies et al. (2004) note that the leaves of *S. x uplandicum* had a 16-fold higher concentration of total PAs in small leaves than in large leaves. On the other hand, a complete lack of PAs in the leaves of *S. officinale* has been reported (Betz et al. 1994), indicative of natural biological (epigenetic or chemotypic) variations associated with local environmental differences (Betz et al. 1994), as well as precise the age of the plant material (Mei et al. 2010). In the present study, undetectable amounts of PAs in the leaf samples could be attributed to similar factors. A more detailed investigation of the levels of the PAs in leaves of different sizes and different ages plants growing in different seasons under different conditions would help shed further light on this question.

Table 5. PA types and means levels (ppm) in the roots from Kiambu and Kisii counties of Kenya

Location	Season	Echimidine (mean±SD)	Heliosupine (mean±SD)	7-Acetyllycopsamine (mean±SD)	3-Acetyllycopsamine (mean±SD)	Triangularine (mean±SD)
KB	Wet	4.26±0.07	3.36±0.05	9.46±0.08	6.54±0.05	-
	Dry	4.23±0.06	-	5.51±0.05	-	6.95±0.11
KW	Wet	2.25±0.13	3.32±0.09	8.04±0.08	4.54±0.07	-
	Dry	2.54±0.08	2.45±0.08	2.14±0.07	-	2.53±0.12
KS	Wet	7.53±0.11	9.1±0.04	8.37±0.05	-	-
	Dry	4.27±0.04	2.53±0.07	-	3.44±0.08	-
ZW	Wet	3.32±0.09	3.20±0.06	-	8.52±0.11	-
	Dry	2.55±0.10	3.53±0.17	-	-	-
GR	Wet	2.28±0.12	-	3.54±0.08	-	2.59±0.10
	Dry	4,17±0.04	5.56±0.12	-	10.57±0.11	5.29±0.03
RR	Wet	2.41±0.04	2.44±0.11	3.50±0.08	-	4.64±0.15
	Dry	3.54±0.07	3.54±0.12	4.58±0.20	-	7.24±0.12

KB-Kiambu, KW-Kahawa Sukari, KS-Kisii, ZW-Kiamumbi, GR-Githunguri, RR-Ruiru,-: PAs not detected

Table 6. The pyrrolizidine alkaloids' mean (±SE) concentration (ppm) was obtained from the roots during the wet and dry seasons

Pyrrolizidine alkaloids	n	Dry (Mean±SE) ppm	Wet (Mean±SE) ppm	p-value
Echimidine	12	3.55±0.23	3.67±0.56	0.841
Heliosupine	10	3.52±0.38	4.27±0.80	0.408
7-Acetyllycopsamine	6	4.08±0.64	6.58±0.85	0.033
3-Acetyllycopsamine	4	7.00±2.06	6.53±0.73	0.840
Triangularine	8	5.50±0.71	3.61±0.60	0.070

A t-test was done on the PA identified between the wet and dry seasons, and the results are shown in Table 6.

The level of 7-Acetyllycopsamine was significantly higher during the wet season than during the dry season ($p=0.033$, $\alpha=0.05$, t-test). The other pyrrolizidine alkaloids did not vary considerably between the dry and wet seasons ($p>0.05$, $\alpha=0.05$, t-test).

Conclusions

GC-MS analyses of all the root samples detected a total of 5 PAs, in which Echimidine (**7**) was found in all of the root samples analyzed. Meanwhile, 7-Acetyllycopsamine (**17**), 3-acetyllycopsamine (**18**), and triangularine (**19**) were found only in some root samples. This variation might be attributed to differences in the age of the plant and epigenetic/chemotypic differences resulting from changes in soil and other environmental conditions in areas from where the plants were harvested (Betz et al. 1994; Mei et al. 2010). Heliosupine, which is the other PA identified in the roots of all extracts in the study, is a stereoisomer of Echimidine. Its toxicity is yet to be studied. No detectable levels of PAs were found in the leaf samples of *S. asperum* collected from any of the locations in both seasons. A previous study on *S. officinale* leaves described the total absence of PAs (Betz et al. 1994). However, other studies reported varying levels of PAs in the leaves, with the highest levels being in the small, young leaves, especially early in the season (Mattocks 1980). Oberlies et al. (2004) reported a 16-fold higher concentration of total PAs in small leaves than in large ones. In this study, larger mature leaves were mostly used since they were consumed.

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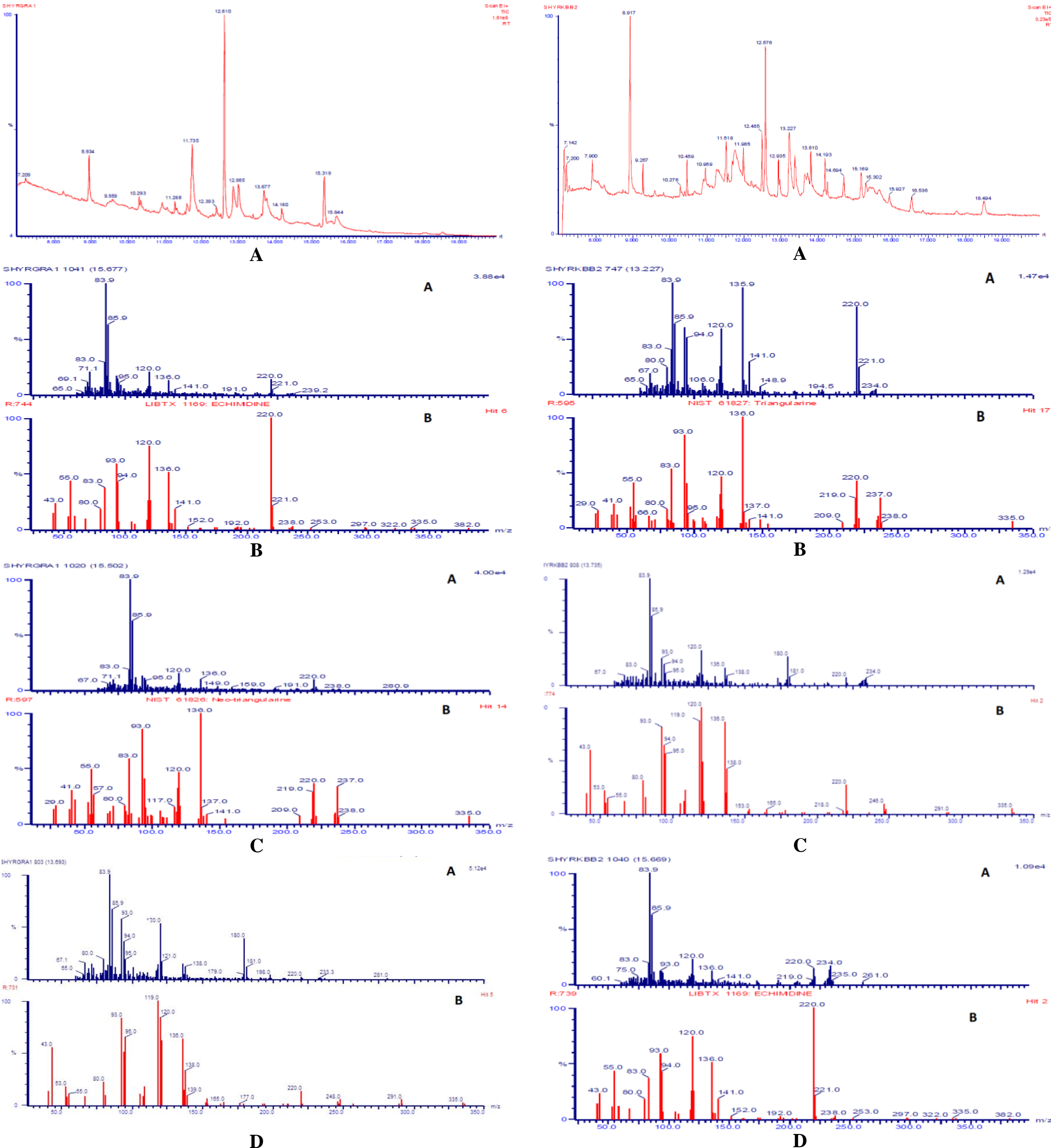

Figure S1.A. TIC of the sample collected from Githunguri, wet season. B. Mass spectra of echimidine in the sample collected from Githunguri, wet season. C. Mass spectra of triangularine in the sample collected from Githunguri, wet season. D. Mass spectra of 7-acetyllycopsamine in the sample collected from Githunguri, wet season. Note for Figure S1 to S10, Above/A: sample spectrum, Below/B: NIST library spectrum)

Figure S2.A. TIC of the sample collected from Kiambu, dry season. B. Mass spectra of triangularine in the sample collected from Kiambu, dry season. C. Mass spectra of 7-acetyllycopsamine in the sample collected from Kiambu, dry season. D. Mass spectra of echimidine in the sample collected from Kiambu, dry season

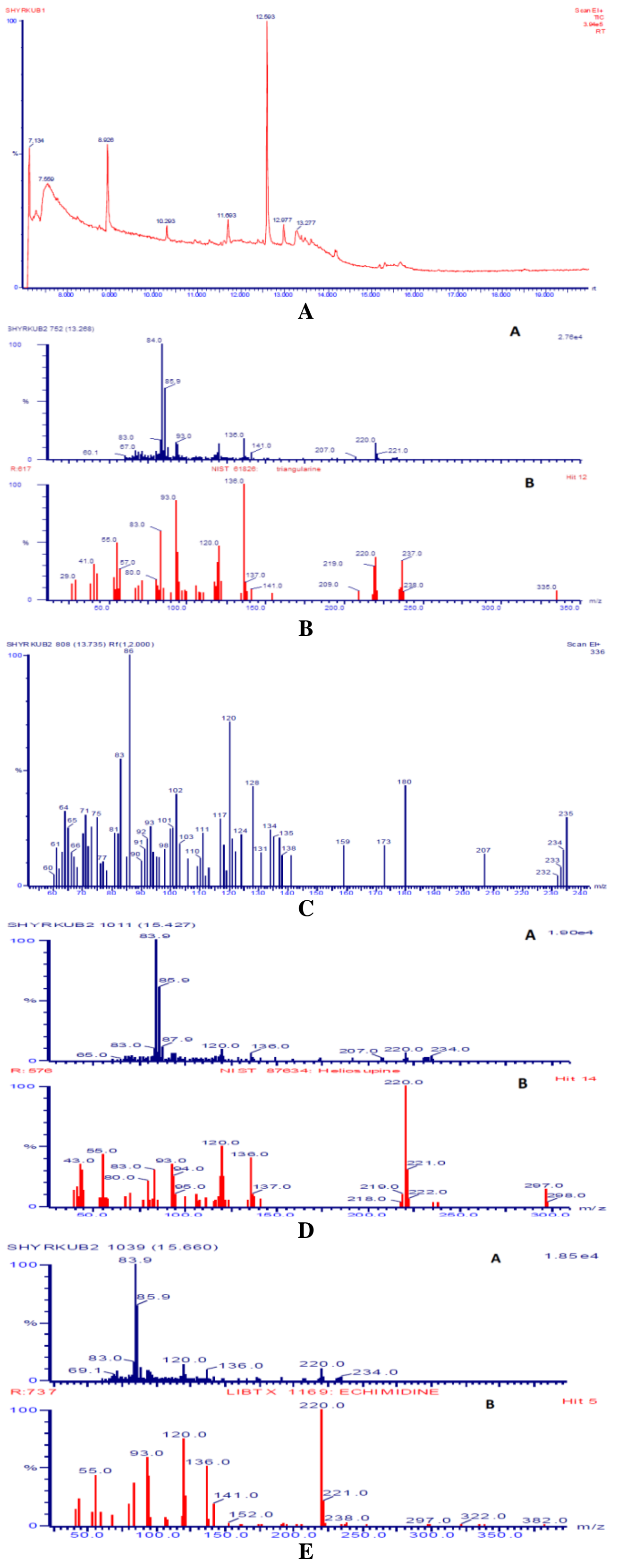

Figure S3 .A. TIC of the sample collected from Kahawa Sukari, dry season. B. Mass spectra of triangularine in the sample collected from Kahawa Sukari, dry season. C. Mass spectra of 7-acetyllycopsamine in the sample collected from Kahawa Sukari, dry season. D. Mass spectra of heliosupine in the sample collected from Kahawa Sukari, dry season. E. Mass spectra of echimidine in the sample collected from Kahawa Sukari, dry season

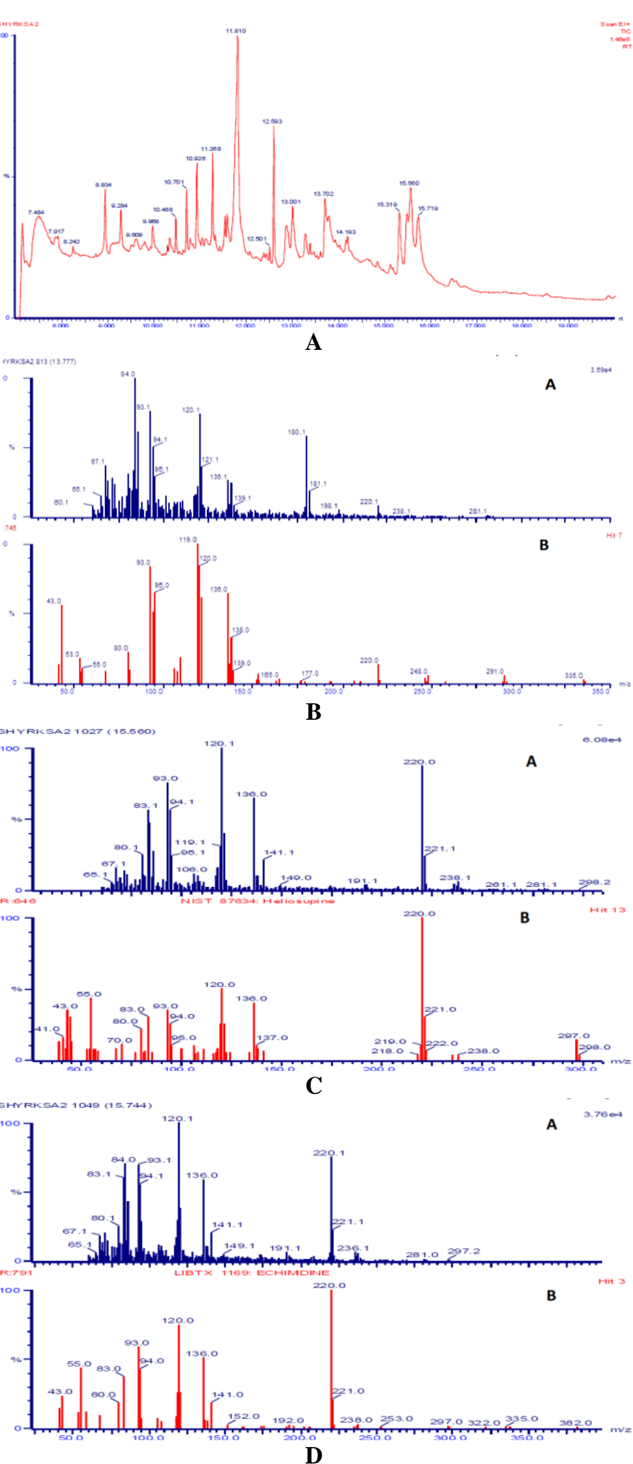

Figure S4 .A. TIC of the sample collected from Kisii, wet season B. Mass spectra of 7-acetyllycopsamine in the sample collected from Kisii, wet season. C. Mass spectra of heliosupine in the sample collected from Kisii, wet season. D. Mass spectra of echimidine in the sample collected from Kisii, wet season

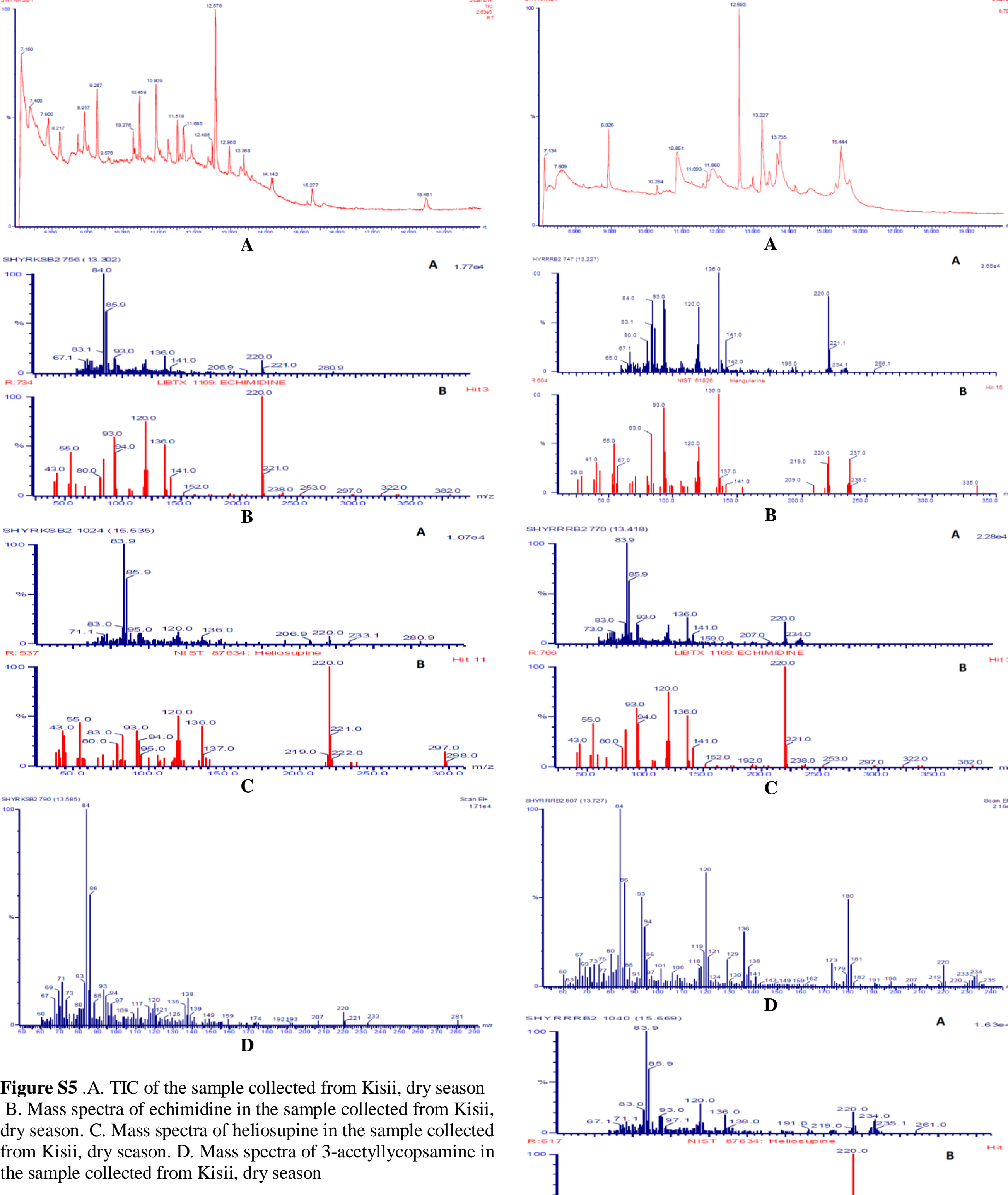

Figure S5 .A. TIC of the sample collected from Kisii, dry season B. Mass spectra of echimidine in the sample collected from Kisii, dry season. C. Mass spectra of heliosupine in the sample collected from Kisii, dry season. D. Mass spectra of 3-acetyllycopsamine in the sample collected from Kisii, dry season

Figure S6 .A. TIC of the sample collected from Ruiru, dry season. B. Mass spectra of triangularine in the sample collected from Ruiru, dry season. C. Mass spectra of echimidine in the sample collected from Ruiru, dry season. D. Mass spectra of 7-acetyllycopsamine in the sample collected from Ruiru, dry season. E. Mass spectra of heliosupine in the sample collected from Ruiru, dry season

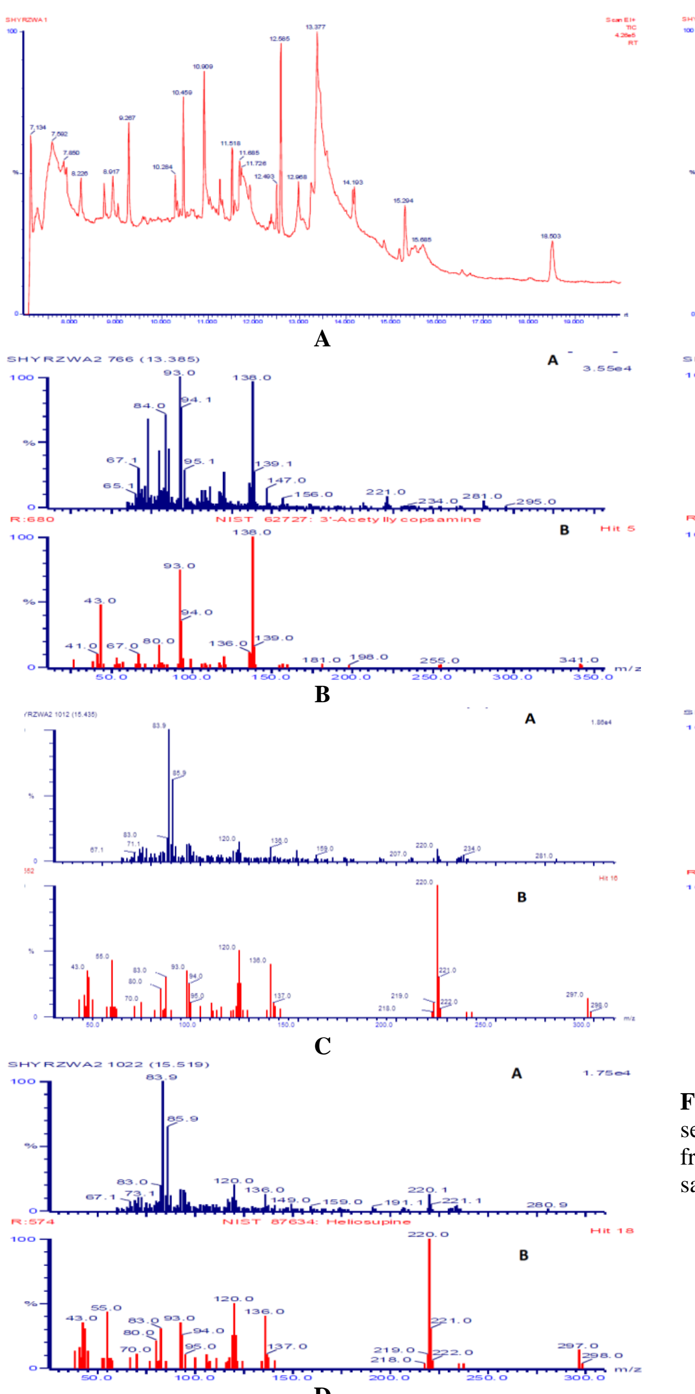

Figure S7 .A. TIC of the sample collected from Kiamumbi, wet season. B. Mass spectra of 3-acetyllycopsamine in the sample collected from Kiamumbi, wet season. C. Mass spectra of echimidine in the sample collected from Kiamumbi, wet season. D. Mass spectra of heliosupine in the sample collected from Kiamumbi, wet season

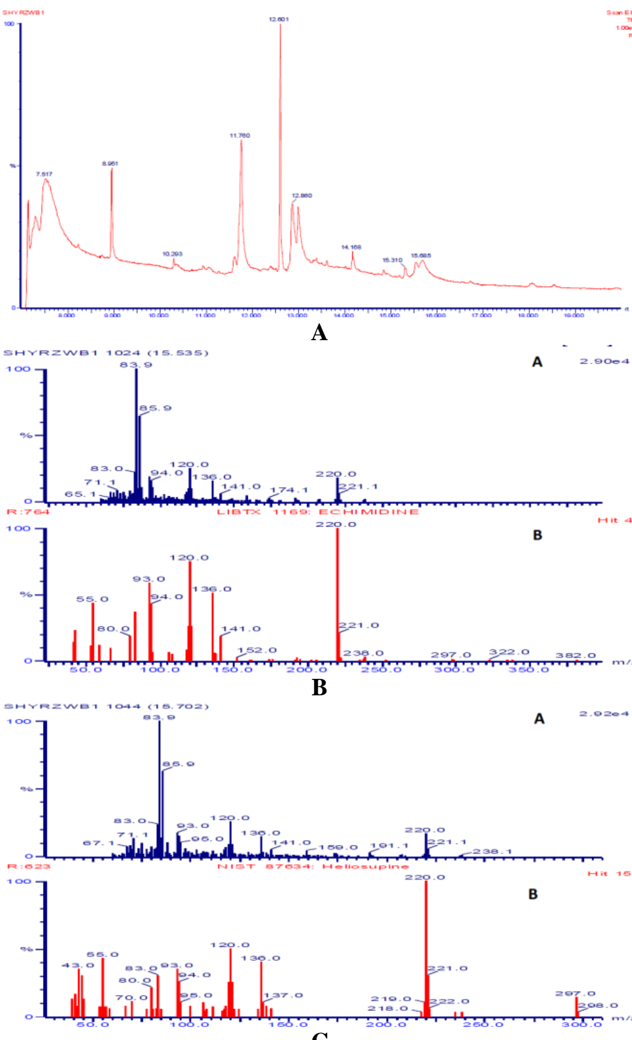

Figure S8.A. TIC of the sample collected from Kiamumbi, dry season. B. Mass spectra of echimidine in the sample collected from Kiamumbi, dry season. C. Mass spectra of heliosupine in the sample collected from Kiamumbi, dry season

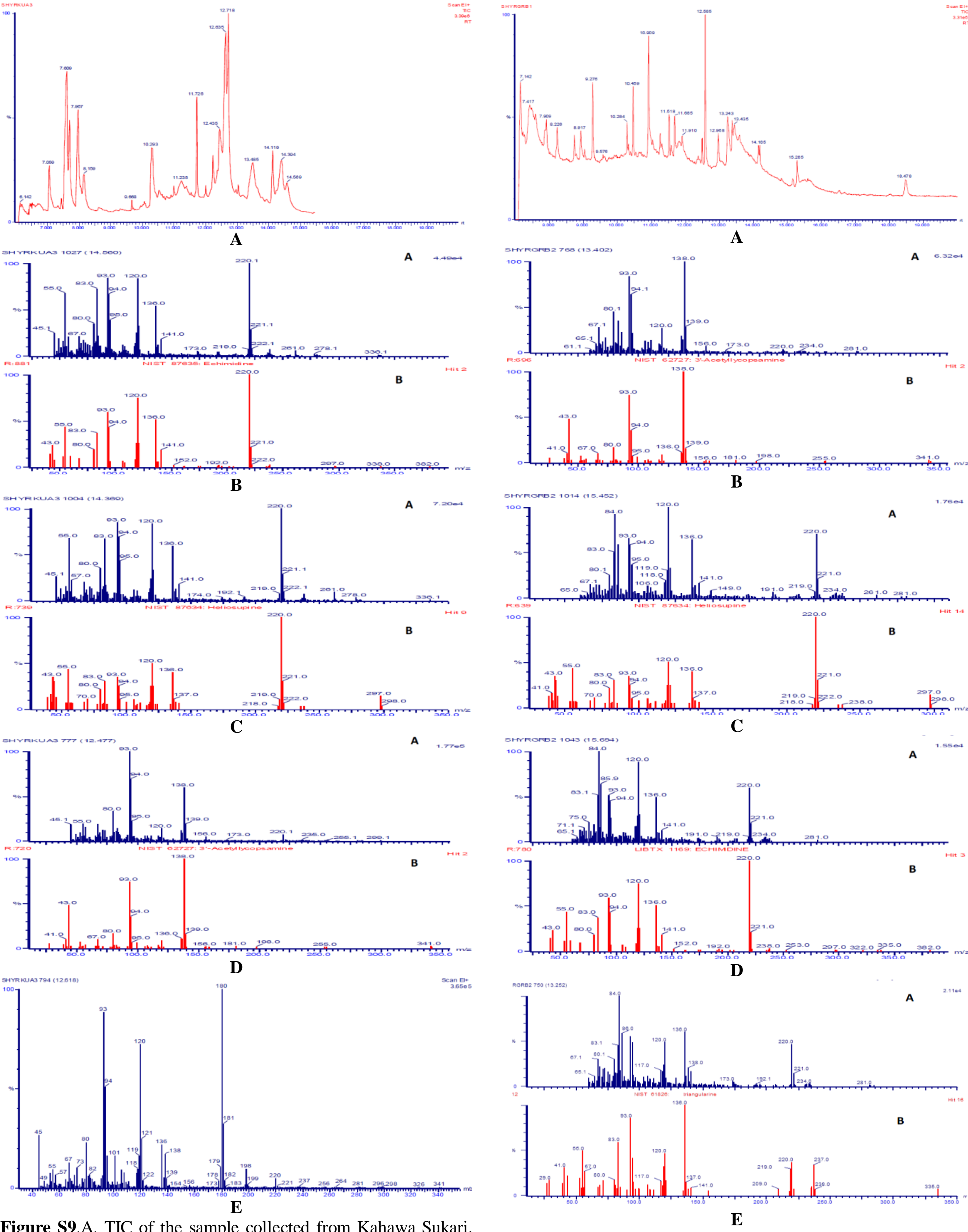

Figure S9.A. TIC of the sample collected from Kahawa Sukari, wet season. B. Mass spectra of echimidine in the sample collected from Kahawa Sukari, wet season. C. Mass spectra of heliosupine in the sample collected from Kahawa Sukari, wet season. D. Mass spectra of 3-acetyllycopsamine in the sample collected from Kahawa Sukari, wet season. E. Mass spectra of 7-acetyl-lycopsamine in the sample collected from Kahawa Sukari, wet season

Figure S10.A. TIC of the sample collected from Githunguri, dry season. (b) Mass spectra of 3-acetyllycopsamine in the sample collected from Githunguri, dry season. C. Mass spectra of heliosupine in the sample collected from Githunguri, dry season D. Mass spectra of echimidine in the sample collected from Githunguri, dry season. E. Mass spectra of triangularine in the sample collected from Githunguri, dry season

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Effects of water stress on phenolic content and antioxidant activity of African nightshades

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Abstract. *Okello OP, Gweyi JPO, Nawiri Mp, Musila W. 2017. Effects of water stress on phenolic contents and antioxidant activity of African nightshades. Biofarmasi J Nat Prod Biochem 15: 74-90.* This study aimed to map out the distribution of different African Nightshade species in Siaya and Kisii Counties of Kenya and determine the water stress effect on total antioxidant capacity and total phenolic content of two selected African nightshade, namely, giant nightshade (*Solanum scabrum*) and black nightshade (*Solanum villosum*). Before selecting the two varieties, the study involved field visits, mapping of nightshades present, and administering semi-structured questionnaires to farmers to determine the indigenous vegetables being grown, the nightshade species grown, and factors affecting their production. The experiments were conducted both in the field and in greenhouse conditions. Watering intervals were at 15 cbars, 50 cbars, and 85 cbars. Data on the number of secondary buds, leaf area, shoot height, and shoot and root dry weights were gathered. The total antioxidant capacity and the total phenolic content were recorded using the DPPH radical scavenging method and the Folin-Ciolcalteu method, respectively. The data collected were subjected to ANOVA. In both counties where production was 100% under small scale, Solanum scabrum was the main variety grown in Siaya County (36%), while in Kisii, the main variety was Solanum villosum (32%). There were significant differences ($P \leq 0.05$) among treatments in leaf area, plant height, shoot biomass, number of secondary buds, leaf and root total phenolic content, and leaf and root antioxidant activity. At all stress levels, Solanum scabrum exhibited the tallest plant with a maximum height of 45.17cm at 15cbars. At the same time, Solanum villosum had the shortest plants at all stress levels, with the shortest one being recorded at 16.65 cm at 85 cbars. S. scabrum also had the highest root dry weight (7.78g), shoot dry weight (50.78g), and highest leaf area (304.45cm2). However, Solanum villosum had the highest number of secondary buds at all stress levels, with the highest being 24 at 15 cbars. Concerning phytochemicals, *Solanum villosum* had a higher concentration of both the total phenolics and antioxidant activity in the shoots (46.41g GAE/Kg DM total phenolic content and 52.68% total antioxidant activity). Meanwhile, *Solanum scabrum* had a higher concentration in the roots (25.06gGAE/Kg DM total phenolic content and 27.18% total antioxidant activity). Water stress causes a decline in all growth parameters but increases phytochemical accumulation in nightshade accessions grown. Therefore, it is suggested that for better yields, irrigation should be performed at every 15 cbars; however, for adequate phytochemical accumulation, the irrigation should be carried out at 50 cbars. Further research must explore and quantify other phytochemical components affected by different watering regimes.

Keywords. African nightshades, antioxidant activity, phenolic contents, water stress

INTRODUCTION

African nightshades (*Solanum* spp.) are among the African indigenous leafy vegetables reported to be an important source of antioxidants such as lycopene, phenolics, and vitamin C in the human diet. Antioxidants have been linked to reduced risk of prostate and various other forms of cancer and heart disease and boosting the immune system of HIV/AIDS patients (Maundu et al. 1999). Growing demand for dietary antioxidants has triggered the search for newer, economic, nutritional, and multifunctional sources that possess free radical scavenging potential (Imungi 2002). With cancer and HIV/AIDS becoming a threat to economic development, a shift has been seen in food consumption trends both in urban centers and rural areas of Kenya, with a majority opting for traditional vegetables. About 56% of the population of Kenya lives below the poverty line, and about 50.6% of the population lack access to adequate food; moreover, even the little they get is of poor nutritional value and quality (GoK 2004). The high poverty levels manifest in malnutrition and poor health, mainly for most Kenyan rural populations. The poverty situation has been worsened by the high prevalence of HIV/AIDs, where 2.5 million Kenyans are already infected with an annual infection rate of 200,000 (GoK 1999). Nevertheless, Kenya is endowed with high agricultural biodiversity like African nightshades (ANS), which could significantly manage HIV/AIDS infected and affected persons (Irungu 2007).

African nightshades have high micronutrient content, medicinal properties (Maundu et al. 1999), and several agronomic advantages and contribute to food and nutrition security and income generation (Schippers 2002). African nightshades are indigenous or traditional vegetables whose

leaves, young shoots, and flowers are consumed. Communities in Western Kenya have used these vegetables for a long time (Imungi 2002). Though they have been underutilized or neglected for a long time, there has been a renewed interest in African nightshades by the policymakers and the international community to realize that these vegetables have a prominent benefit that has yet to be exploited (Olembo et al. 1995).

Phytochemical studies have revealed that the plant contains glycoalkaloids (solanine, solamargine, solanigrine, and solasodine), steroidal glycosides (β-solamargine, solasonine, and α, β- solansodamine), steroidal saponins (diosgenin), steroidal genin (gitogenin), tannin and polyphenolic compounds (Oh et al. 2010). In response to drought, plants typically accumulate a wide range of antioxidants, including enzymatic antioxidants (peroxidases, superoxide dismutase, and catalase) and non-enzymatic antioxidants to quench the reactive oxidative species (ROS) induced by water stress (Mittler 2005). In many plant species, water stress also induces some phytochemicals, including a-tocopherol, b-carotene, and flavonoids, in many plant species (Velikova et al., 2000).

A plant is affected by several stress factors (biotic and abiotic), and any particular factor may reinforce or compensate for the effects of others. The current climate change depicted by local changes and annual variations in the precipitation expose many plants to water stress (Gorai et al. 2010). To prevent the accumulation of activated oxygen species (AOS), plants have evolved highly efficient antioxidative defenses composed of both enzymatic and non-enzymatic antioxidants (Foyer et al. 1994). These products are good health indices whose quantities need to be documented. Therefore, this research sought to assess ANS responses to water stress effects on plant distribution based on the phytochemicals produced. Plant phytochemicals, for example, phenolics, are responsible for plant adaptation to variable environments.

The objective of this study was (i) To document and map out the distribution of African Nightshade species in Siaya and Kisii counties of Kenya. (ii) To evaluate biomass yields of African Nightshades species under different watering regimes. (iii) To determine the concentration of phytochemicals in cultivated species concerning soil water status and determine the trade-off between yield and phytochemicals in plant shoots.

MATERIALS AND METHODS

The study area

Two approaches to research were performed. Firstly, a field survey for preliminary data gathering was carried out in Siaya and Kisii Counties of Kenya in February (long rainy season) and August (short rainy season) of 2014. The survey covered determining nightshade species grown by farmers in the two counties. Moreover, the phytochemical concentration of the samples collected during the mapping period was also confirmed, and the soil moisture content at the time of mapping. In Siaya County, which has an area of approximately 2,530.5 Km^2, the survey covered the Agoro Oyombe, Ramba, Dominion farms, Yala, and Gem Ulamba areas. The county lies between latitude 0°26’ to 0°18’N and longitude 33°58’E and 34°33’W, receiving annual rainfall between 1,170 mm and 1,450 mm, with an average temperature of 21.75 ^{0}C and a range of from 15 ^{0}C to 30 ^{0}C. On the other hand, Kisii County, located in Western Kenya with approximately 1,317.4 Km^2, and the coverage includes Kisii town, Minyinkwa, and Keumbu. Kisii receives frequent convectional rains and enjoys a great highland climate because it is located between 0°41’0” S, 34°46’0 ”E. Thus, it enjoys two rainfall seasons; short rains (September-November) and long rains (February-June), and receives rainfall amount of over 1,500mm per annum with temperatures ranging from 16 ^{0}C to 27 ^{0}C. The second approach was field experiments in Kenyatta University Research and demonstration farm in Nairobi County. Kenyatta University is located between1°10’52 ”S, 36°55’38 ”E and 1.1°S, 36.9°E, receives rainfall of about 750 mm with temperatures between 25°C to 33°C.

Field survey

This preliminary data collection involves qualitative and quantitative data. The qualitative data collection is performed through direct discussions and interviews with public and private sector key stakeholders in the production and marketing of African nightshades, such as traders, farmers, and agricultural extension officers. The quantitative data collection is performed by analyzing semi-structured questionnaire results from randomly-selected 20 traders from various key markets and 60 farmers from different farms who are directly engaged in African nightshade production and marketing.

The questionnaire for traders involves some inquiries about the source of nightshade, the effect of rainfall, market availability, etc.; in contrast, inquiries for farmers involve some inquiries about the scale of production, dependence on rainfall and or irrigation, the effect of drought to taste, and the rationale of nightshade production. To ensure the validity of the questionnaire, several preliminary tests and adjustments were carefully conducted, and direct observation and informal interviews complement the questionnaire. Thus, plant samples from farmers were also collected and analyzed on the concentration of phytochemicals.

The greenhouse and field experiment

Materials

Open field at Kenyatta University’s main campus and a demonstration farm was selected as the place for the experiment in addition to the greenhouse. The experiment materials, such as seeds and fertilizers, were obtained from Kiambu agro vets. Loamy soils (4.5 kg) and DAP (Diammonium phosphate) fertilizer at a 5g/5 liters plastic pot rate were prepared for the greenhouse experiment.

Tensiometer installation

The installation included a portable Irrometer Model P tensiometer for the spot check of the soil moisture conditions. The measurement was made from 24cm root depth at a 3-hour interval. Treatments included irrigation at

15 cbars, 50 cbars, and 85 cbars. If the soil moisture conditions changed, irrigation began with 5 cbars referring to the soil moisture capacity.

The greenhouse experiment and design

The experiment started with preliminary preparation by transplanting six healthy seeds into an individual pot; watering them daily until they were ready for the experiment. One week elapsed, and four plantlets remained in each pot. The experiment began with treatment with a different range of water patterns, i.e., low (15 cbars), moderate (50 cbars), and high (85 cbars). The treatments were replicated four times, and the pots were randomly rearranged every week to remove errors that greenhouse conditions might generate. Unexpected weeds in the pots were removed by hand. At the end of the third week, various parameters data collection was performed thoroughly.

The field experiments

This was carried out at the Kenyatta University Research and demonstration farm in two distinct seasons. The first season (long rains) was between February and May in 2014, and the second one (short rains) was carried out between August and October the same year. The experiment was replicated four times in a Randomized Complete Block Design (RCBD) on split plots. The main plot was the two nightshade accessions (*S.villosum* and *S.scabrum*), and the subplots underwent watering patterns at 15, 50, and 85 cbars, respectively.

Data collection

Parameters collected are all related to growth in different treatments during six weeks, i.e., length of the root, length of the shoot length, weight of the fresh and dry plant, weight of the fresh and dry roots, and area of the leaves.

Number of secondary shoots

The number of secondary shoots emanating from the stem was counted and recorded.

Shoot height

Shoot height was measured using a meter rule from the stem base up to the shoot apex. This was done before uprooting the plant to counteract errors due to loss of turgidity.

Leaf area

Leaf area was determined using the formula LA=0.73 (L_L X W_L), where L_L is the leaf length, and W_L is the maximum width measured for each leaf on each plant. Four leaves were randomly measured, and leaf area was determined from the averages.

Root and Shoot fresh weights

The whole plant was uprooted, rinsed, and separated into shoot and root, and the weights were measured with an electronic weighing balance. Obtained samples were air-dried for two days before being taken to the laboratory for phytochemical analysis.

Table 1. The reagent used in the study

Chemical reagent	Percentage purity	Company of origin
Gallic acid	99.9	Sigma Aldrich, Germany
Methanol	99.9	Scharlab S.L, Spain
Folin and Ciocalteu phenol reagent	99.7	BDH Limited, Poole-England
Sodium Carbonate	99.5	Central drug house (P) Limited, New Delhi-India
DPPH(Diphenyl picryl hydrazyl)	99.9	Sigma Aldrich, Germany

Reagents used

Reagents used during the phytochemical analysis are shown in Table 1.

Preparation of plant materials for phytochemical analysis

The plant shoots and roots resulting from the experimental pots were dried in an oven for 48 hours and measured for their dry weight. Electric grinders were then used to separate the samples; five grams of the sample were taken and added with 50 mL of methanol and kept for 60 hours while covered with aluminum foil. The results were then filtered using Whatman filter paper No. 1, in which the filtrate was then kept in disposable sampling tubes in a freezer for further analysis.

Analysis of total phenolic content

Folin-Ciocalteu method (Rispail et al. 2005) was used in determining the total phenol content of the extracts by using Gallic acid as a standard; 0.5g Gallic acid was dissolved in 10 mL of methanol and diluted using distilled water to 1000 mL equivalent to 500 ppm; and subsequently to 250 ppm, 125 ppm, 62.5 ppm, 31.15 ppm, and 15.625 ppm. Each 1 mL of the different Gallic acid solutions was pipetted into separate test tubes, and 4 mL of distilled water was added, continued by 0.2 mL Folin reagent, and mixed well. After 10 minutes, 0.4 mL of freshly prepared sodium carbonate (prepared by adding 40 g of Na_2CO_3 in 200 mL of distilled water) was added, and the solution was incubated for 1 hour at 25ºC. 1 mL of plant extract was pipetted in a separate test tube to determine phenolic content in the plant extracts. The absorbance of each solution was determined at 765 nm against the blank.

Calculation of total phenolic content (TPC):

$$TPC = \frac{\text{Gallic acid equivalent (mg/L) x total volume of methanol extract x sample weight (kg/g)}}{\text{Dilution factor (L/mL)}}$$

Antioxidant determination

Akter *et al.* (2010) analyzed the free-radical-scavenging ability of the extracts against DPPH (2, 2-diphenyl-1picrylhydrazyl) free radicals. They resulted in the reactivity of the test compounds with a stable free radical providing a strong absorption band at 517nm in the visible region. Next step was preparing concentration of extracts with 0.05, 0.1, 0.5, 1.0, and 2.0 mg/mL methanol in cuvette placed inside spectrophotometer (Analar grade). For the

antioxidant standard, Vitamin C was used. One mL of the extract was placed in a test tube, added with 3 mL of methanol and 0.5 mL of 1 mM DPPH in methanol, mixed, and kept for 5 minutes. A blank solution was prepared to contain the same amount of methanol and DPPH. The absorbance of the resulting solution was measured at 517 nm with a UV-vis spectrophotometer (model Cecil CE: 2041; 2000 series, Shimadzu Corp., Kyoto, Japan). The experiments were replicated three times, and the radical scavenging activity was measured as follows:

$$\% \text{ total antioxidant activity} = \frac{\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Sample}}}{\text{Absorbance}_{\text{Control}}} \times 100$$

Data analysis

Analysis of variance (ANOVA) was carried out using SAS statistical computer package version 9.00 TS Level 00M0 XP-PRO platform. In contrast, SPSS software version 21 was used to analyze data gathered from the survey. Means were separated using the least significance difference (LSD) test at the 95% probability level.

RESULTS AND DISCUSSION

Indigenous vegetables and nightshade accessions grown and traded in Siaya and Kisii counties

The indigenous vegetables produced in the two counties included; *Vigna unguiculata*, *Crotalaria brevidens*, *Gynandropsis gynandra*, *Brassica carinata*, *Solanum, Corchorus olitorius*, *Amaranthus gracecizans*, and *Cucurbita* sp. The nightshade species grown included; *S. nigrum*, *S. villosum*, *S. americanum*, *S. florulentum*, *S. scabrum*, *S.grossidentatum*, *S. Tarderemotum*, and S. nigrum (Table 2). The major indigenous vegetable grown in the two counties was nightshade at 32% and 29% for Siaya and Kisii counties, respectively.

Cowpea was the second most dominant vegetable in Siaya (20%) and Kisii (28%) counties, followed by Amaranthus in Siaya (18%) and Kisii (13%). Similar results were obtained by Maundu et al. (1999). There was variation in their diversity across the two counties for other African indigenous vegetables, as shown in Table 2. The market share of cowpeas has reached 20%, followed by nightshade at 19% and Amaranthus at 17%, among the most traded African indigenous vegetables. The statistic differed in Kisii County; however, nightshade took the most shares of the indigenous vegetables traded at 43%, followed by cowpea at 18% and Amaranthus at 12%. The variation in production and trade levels is attributed to consumer preference, for instance, the bitter taste of each vegetable. Thus, the trade of pumpkin, jute mallow, slender leaf, spider plant, and African kale varied in the two counties. However, some consumers are cooking several African vegetables mixed to add flavor to the taste and reduce its bitterness.

As many as eight nightshade species were grown in Siaya County, the majority of which were found to be *S. scabrum* (36.15%). Meanwhile, only seven nightshade species had been grown in Kisii County, dominated by *S. villosum* at 32.31%. Dominion farm bordering Yala swamp had the wild relatives of black nightshades growing by themselves within the bushes. Some farmers, especially in Kisii County, produced the AIVs for their kitchen gardens' consumption. The dominance of *S. scabrum* in Siaya County is attributed to the farmers and the consumer preference. For example, because S. villosum is more bitter and has smaller leaves than S. scarbum, this has led to a general decline in *S villosum* production and consumption in Siaya County. On the other hand, *S. villosum* dominates Kisii County in the form of readily-available seeds. This is because the seeds are produced from the landraces, and there is less knowledge to process seeds in agro vets form.

A field survey conducted in this study demonstrated that Siaya County was richer in diversity in African nightshade accessions than Kisii County (Figures 1 and 2). Other groups have reported similar results (Schippers 2002; Abukutsa-Onyango 2003).

During the field survey, it was observed that Siaya County was richer in diversity in African nightshade accessions as compared to Kisii County (Figures 1 and 2). The results agreed with the findings of Schippers (2002) and Abukutsa-Onyango (2003).

Table 2. Indigenous vegetables and nightshade species in Siaya and Kisii counties, Kenya

Indigenous vegetables grown	Siaya (%)	Kisii (%)	Indigenous vegetables traded in market	Siaya (%)	Kisii (%)	Nightshade accessions grown	Siaya (%)	Kisii (%)
Vigna unguiculata	20	28	*Vigna unguiculata*	20	18	*S. nigrum*	9	15
Crotalaria brevidens	5	10	*Crotalaria brevidens*	3	4	*S. villosum*	21	32
Gynandropsis gynandra	2	6	*Gynandropsis gynandra*	12	7	*S. americanum*	5	4
Brassica sp.	5	3	*Brassica* sp.	7	3	*S. florulentum*	12	4
Solanum sp.	32	29	*Solanum* sp.	19	43	*S. scabrum*	36	25
Corchorus olitorius	8	9	*Corchorus olitorius*	12	8	*S.grossidentatum*	4	4
Amaranthus gracecizans	18	13	*Amaranthus gracecizans*	17	12	*S. tarderemotum*	4	0
Cucurbita sp.	10	2	*Cucurbita* sp.	10	5	*S. nigrum*	9	15

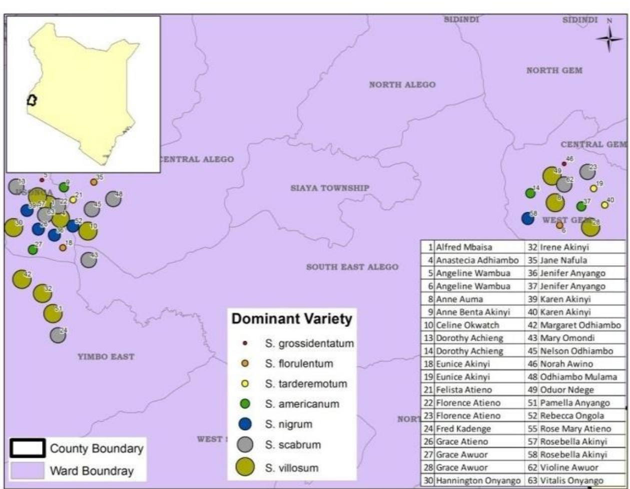

Figure 1. Nightshade accessions in Siaya County, Kenya

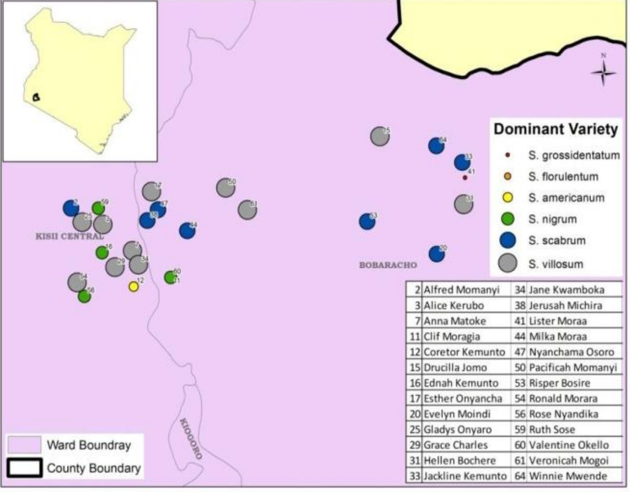

Figure 2. Nightshade accessions in Kisii County, Kenya

Some of the vegetable species were not preferred by certain farmers. They described them as tasting very bitter, except for the Giant nightshade (*Solanum scabrum*), which dominates most farms in Siaya County. The bitterness is probably associated with a higher degree of phytochemical accumulation. Hence, they were the most preferred by HIV/AIDS patients within Siaya County (Irungu et al. 2007). Giant nightshade was introduced and recommended by the World bank in 2012. Although common, its leaf's prolificacy tends to reduce faster after the first harvest than other grown accessions.

In Kisii County, wild relatives of black nightshade were found along the hedges of live fences, where they were considered to be used for herbal medicine rather than a vegetable source. The *Solanum villosum* is most preferred by consumers, thus dominating most farms in the Kisii region. Other accessions found in the two counties were mapped. *Solanum scabrum* and *Solanum villosum* were sold in all Siaya markets, in contrast to the *Solanum villosum* variety sold only in the Kisii market. Seventy-two point seven percent of traders in Siaya County sourced the ANS from their farms, while 27.3% had them supplied from other areas such as Kadenge, Anduro, Awello, and Umalla. Forty percent of traders in Kisii County were producers, while 60% obtained the vegetables from Transmara, Kilgoris, Magena, and Kiamabundo. The production in Kisii county does not meet consumers' demand due to the fragmentation of farms to more income-generating crops like sugarcane. This leaves little land for ANS production. Hence, the traders import ANS from the neighboring counties to satisfy consumer demand.

Gender and production system in Siaya and Kisii counties

Table 3 showed that men and women were both involved in the production of ANS. In Siaya County, 55% of males and 45% of females were involved in producing African nightshades. On the other hand, 15% of males and 8of 5% of females engaged in farming African nightshades in Kisii County.

In the two counties, the farmers produce ANS in an area below one acre on average. The irrigation system was more developed in 33.3% of Siaya County farmers, opposite to the situation of Kisii County farmers where no irrigation systems were in place. In the latter county, the entire farmers highly depend on the onset rainfall for ANS production, whereas in Siaya County, more than half the number of farmers (57.7%) did not rely on it.

Regarding gender composition in ANS production in Siaya County, the findings differ from Abukutsa-Onyango *et al*. (2005). They reported that 25% of males and 75% of females were African Indigenous Vegetable (AIV) farmers. The shift in gender composition might be due to the high returns from AIV sales, high poverty, and unemployment levels in Siaya County (GoK 1999), resulting in the majority of the males turning to farm, especially the production of ANS. The demand increases are due to WHO's efforts to create awareness of the health benefit of ANS to HIV/AIDS infected patients in Siaya County, which has an HIV/AIDS prevalence rate of 17.8% (GoK 1999). All the farmers interviewed agreed that ANS fetched higher returns than exotic vegetables such as kale and cabbages, with prices escalating to $100 per bag in certain markets such as Awelo.

Table 3. Gender and production systems in Siaya and Kisii counties, Kenya

Gender	Siaya (%)	Kisii (%)	Production scale	Siaya (%)	Kisii (%)	Irrigation system	Siaya (%)	Kisii (%)	Rainfall Dependence	Siaya (%)	Kisii (%)
Male	55	15	Below 1 acre	100	100	present	33.3	0	Dependant	43.3	100
Female	45	85	Above 1 acre	0	0	absent	66.7	100	Not dependant	57.7	0

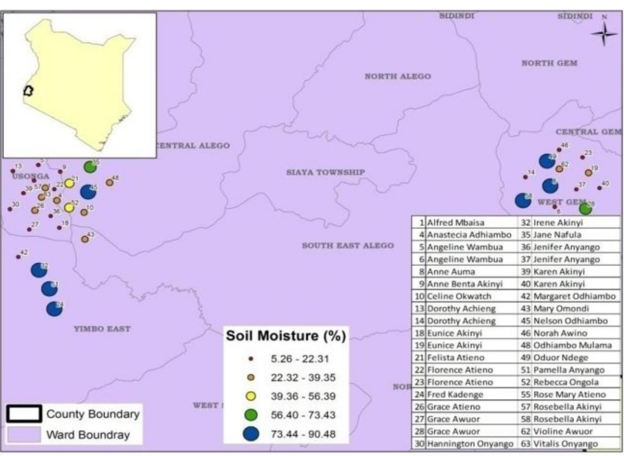

Figure 3. Soil moisture levels during the survey in Siaya County, Kenya

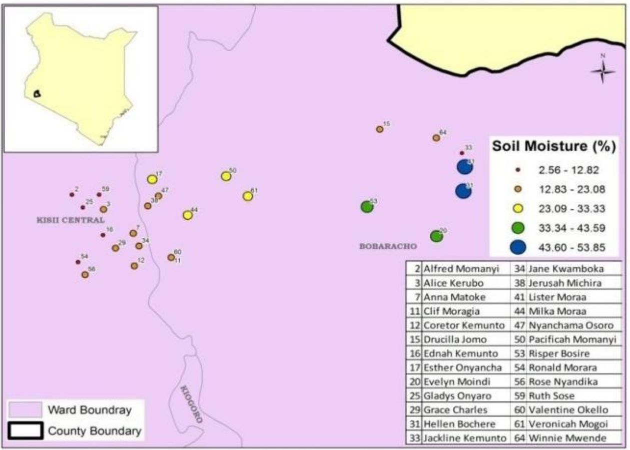

Figure 4. Soil moisture levels during the survey in Kisii County, Kenya

Production of African nightshade in Siaya County takes place along rivers or on swampy ground. The rivers provide water for irrigation as practiced in some regions, including Agoro Oyombe, Gem Kanyibuop, and Gem Kathomo. The vast Yala swamp region, especially farms surrounding the Dominion region, produce African nightshade on ever moist soil, which does not require irrigation. On the other hand, Kisii County lies within a high-altitude region and receives well-distributed rainfall throughout the year, promoting ANS production for the entire year.

Soil moisture content directly influences the phytochemical concentration among plant parts (Akinci and Lösel 2012); however, as admitted by farmers in the growing regions, the roles of specific genes on phytochemical accumulation cannot be ruled out. Areas with the highest soil moisture content were either lying within Yala Swamp (Figure 3) Yimbo East or under daily irrigation in some parts of West Gem in Siaya county. Kisii County had no irrigation practices; hence the soil moisture content may be directly influenced by differences in the altitudinal ranges and the prevailing climatic conditions. However, Kisii County recorded a lesser variation in soil moisture content than Siaya County (Figure 4).

Age, education levels, and optimal African nightshade production

There was variability regarding age, level of education, the reason behind the production of ANS, and the constraints involved in ANS production among interviewed farmers, as shown in Table 4. The majority of interviewed farmers were between the ages of 20 to 30 years, with a percentage composition of 40% and 35% in both Siaya and Kisii counties, respectively. The second dominant group was between the ages of 30 and 40 years. Their percentage composition was 24% and 33% in Siaya and Kisii counties, respectively. Among interviewed farmers, 65% in Siaya County and 51% in Kisii County had basic primary education. Twenty percent and 10% of Siaya County interviewees had secondary and college education, respectively. On the other hand, 29% and 12% of the interviewed farmers in Kisii County had secondary and college education, respectively. However, 5% in Siaya County and 8% in Kisii County admitted having had no education. This agrees with Irungu (2007) and Mwaura et al. (2013), that ANS farmers are more educated than the other categories of traders, implying that the production of ANS is a field for those endowed with human capital.

Farmers in Siaya County began producing ANS commercially due to high consumer demand and high returns, both at 35%. However, only 30% of interviewed farmers in Siaya County produced ANS due to their health benefits. In Kisii, however, the main reason behind ANS production was the high returns constituting 55% of the interviewed farmers. Only 20% of interviewed farmers in Kisii County knew about the health benefits of the African nightshades. Sixty percent and 80% of interviewed farmers in Siaya and Kisii counties
respectively admitted that the major constraint to ANS production was poor quality seeds. This is in agreement with Abukutsa-Onyango et al. (2005). Most farmers complained that seeds obtained from the agro vets were low-yielding due to the high fruiting rate after the second or third harvest.

Table 4. Age, education level, and optimal African nightshade production

Age	Siaya (%)	Kisii (%)	Education level	Siaya (%)	Kisii (%)	Reason for production	Siaya (%)	Kisii (%)	Production challenges	Siaya (%)	Kisii (%)
20-30	40	35	Primary	65	51	Health benefit	30	20	Poor quality seeds	60	80
30-40	24	33	Secondary	20	29	Consumer demand	35	25	Technical production	15	8
40-50	18	21	College	10	12	High returns	35	55	Marketing system	5	2
50-60	12	11	None	5	8				Inadequate research	20	10
60-Above	6	-	-			-			-	-	-

Fifteen percent in Siaya County and 8% in Kisii County had technical production problems. Twenty percent of interviewed farmers in Siaya County and 10% of interviewed farmers in Kisii County felt that inadequate research was being done to promote the production of ANS, though only 5% of interviewed farmers in Siaya County and 2% of interviewed farmers complained of poor market systems. Kenya has an unemployment rate of above 40% (GoK 1999). Hence more youths are opting for self-employment, and one of the ways is through indulging in Agriculture. This explains the high percentages of youth involved in ANS production. The high returns due to increasing ANS demand among consumers might be a reason behind the increase in the number of youthful farmers in the two counties. Finally, the low farmers' low acquisition of tertiary education does not render them competitive enough in the ever-shrinking job market; therefore, they opt for farming (Schippers 2002; Otieno et al. 2009).

The green, leafy African nightshades contain polyphenols which have beneficial physiological effects on humans as antioxidants. They are also known to be anticarcinogenic and anti-arteriosclerotic (Imungi 2002). A study in Nairobi showed that consumption of African nightshades is associated with treating various diseases, including therapy for patients with HIV/AIDS, diabetes, high blood pressure, and other common ailments (Kimiywe et al. 2006). The majority of farmers in Siaya County are aware of this due to WHO campaigns on ANS consumption. The awareness has created an increase in demand which has alternatively led to high ANS prices in the markets in Siaya County. However, in Kisii, there has been a tradition in the consumption of ANS; hence the demand has always been there, leading to ever-increasing returns from ANS production.

The lack of quality seeds has been a major hindrance to sustainable production and utilization of indigenous vegetables (Abukutsa-Onyango 2003), as shown in Table 4. Some of the vegetables perpetuate themselves untended. They were harvested whenever they occurred, and this system of seed procurement heavily depended on the soil-borne seed pool and the ability of these species to reproduce. Seed production has virtually remained in the hands of farmers for a long time, although seed sale in markets was common. For a long time, these vegetables were harvested from the wild, but as the pressure on land increased, they were domesticated and the need for quality seed set in. Usually, ANS is grown as a subsistence crop, and most farmers save their seed from season to season and sell the surplus to other growers. The quality of such seeds is poor regarding purity, viability, and seed dormancy issues. There is a need for the production and supply of quality seeds to increase yields and quantities produced to meet the unsatisfied market demands of priority indigenous vegetables, especially in urban centers (Irungu et al. 2007).

African nightshades have often been grown as intercrops with other vegetables or staples; however, there has been hardly any technical information on optimal production and appropriate cropping systems. There has been a lack of agronomic and preparation packages, and access to technical information has been very limited. Therefore, extension workers have limited knowledge to advise African nightshade growers. This necessitates research on optimal production packages for African nightshades and recipe development.

The marketing of African nightshade vegetables has been poorly organized, leading to significant product losses in transit or markets. The major constraint of marketing was the abundance of ANS vegetables during the rainy season leading to low prices and scarcity during the dry season. This calls for identifying and creating markets for ANS vegetables and possibly linking farmers to appropriate markets. The results were in concurrence with those of Abukutsa-Onyango (2003).

African nightshades have a short harvesting period but are more prolific in seed production. This is discouraging to producers; however, less or no research has been conducted to develop high-yielding varieties regarding leaves and low seed producers.

Phytochemical concentration

Phytochemical analysis of plant samples collected from different farms revealed that different nightshade accessions have different phytochemical concentrations (Figures 5 to 8).

Solanum villosum had the highest phytochemical concentration in both Kisii and Siaya counties at 61% and 55% total antioxidant activity and total phenolic content of 38.8gGAE/Kg DM and 31g GAE/Kg DM, respectively. The lowest total antioxidant activity in Kisii county was recorded in *S. grossidentatum* at 24%, with a total phenolic content of 3.57gGAE/Kg DM. Meanwhile, the lowest total antioxidant activity in Siaya county was recorded in *S. tarderemotum* at 16%, with a total phenolic content of 7.46gGAE/Kg DM. However, there was great variability in the phytochemical concentration among night accession samples collected from the field.

This shows that the production of various phytochemicals (total phenolic content and total antioxidant activity) may be genetically regulated. Hence, cells activate some responses such as an increase in the expression of genes for antioxidant functions and production of stress proteins, up-regulation of anti-oxidants systems, including antioxidant enzymes, and accumulation of compatible solutes differently in different plants.

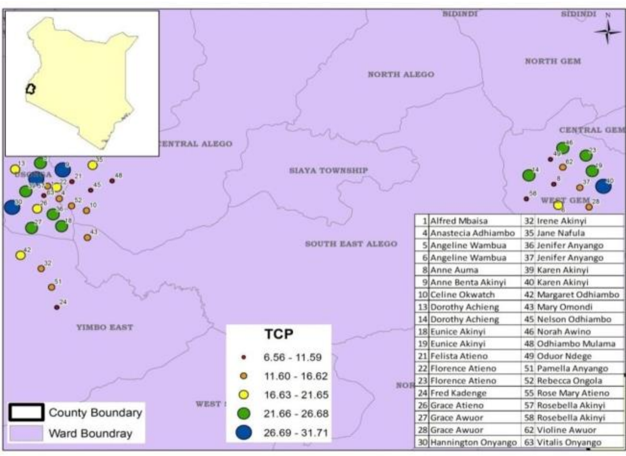

Figure 5. Total phenolic content of different nightshade species as distributed in Siaya County, Kenya

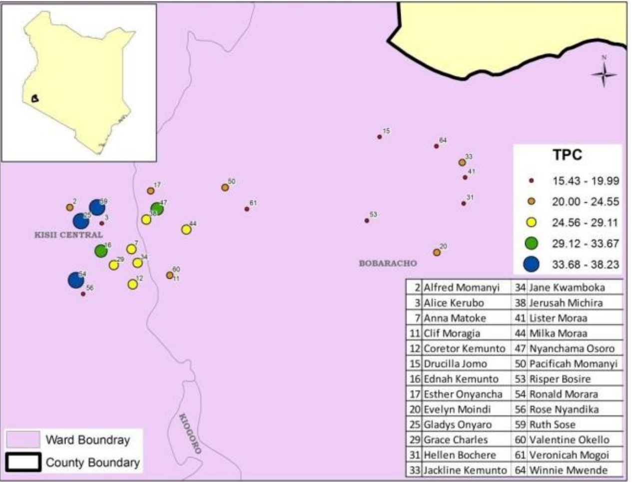

Figure 6. Total phenolic content of different nightshade species as distributed in Kisii County, Kenya

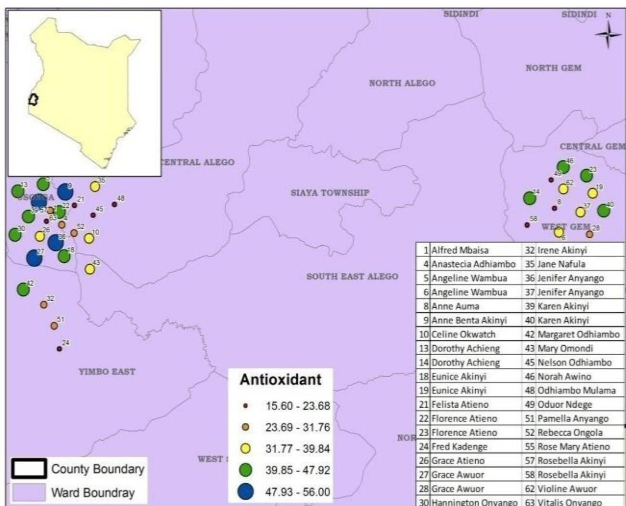

Figure 7. Total antioxidant activity of different nightshade species as distributed in Siaya County, Kenya

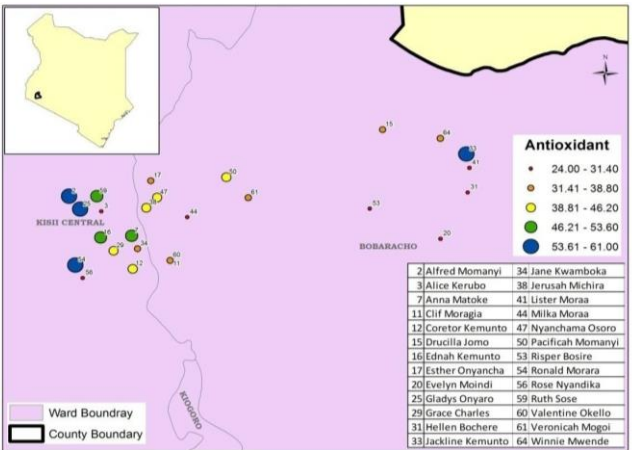

Figure 8. Total antioxidant activity of different nightshade species as distributed in Kisii County, Kenya

Among antioxidant enzymes are catalases (CAT), superoxide dismutase (SOD), peroxidases (POD), ascorbate peroxidases (APX), glutathione reductase (GR), and monodehydroascorbate reductase (MDAR) are prominent (Sairam et al. 2008). Besides, there are anti-oxidant molecules such as ascorbic acid (AA), glutathione, tocopherols, flavanones, carotenoids, and anthocyanins. Some other compounds like osmolytes (proline), proteins (peroxiredoxin), and amphiphilic molecules (tocopherol) also have ROS scavenging functions and may act as an antioxidant (Mustapha et al. 2014). Non-enzymatic plant antioxidants are either AA-like scavengers or pigments. Some of these compounds are multifunctional; for example, AA can react with H_2O_2, O_2, OH- and lipid hydroperoxidases and acts as the enzyme co-factor and a donor-acceptor of electrons (Apel and Hirt 2004).

The degree of activities of antioxidant systems under drought stress is extremely variable as the defining factors may include variation in plant species, the cultivars of the same species, development and the metabolic state of the plant, and the duration and intensity of the stress.

Effect of water stress on growth parameters of nightshades

Number of secondary buds

Water stress significantly affected the number of secondary buds at $P \leq 0.05$ in the greenhouse during long and short rains (Figure 9). The numbers of secondary buds were significantly affected by the different irrigation intervals.

Solanum villosum had the highest number of secondary buds when irrigation was done at a tensiometer reading of 15 cbars. However, *Solanum scabrum* developed the least number of secondary buds in the greenhouse and both seasons at a tensiometer reading of 85 cbars. The highest mean number of secondary buds of 24 was attained by *Solanum villosum* under irrigation at a water tension of 15 cba. In comparison, the lowest mean number of secondary buds of 5 was attained by Solanum scabrum under irrigation at a water tension of 85 cbars. *Solanum villosum* had the highest number of secondary buds at all stress levels measured. There was a significant interaction between water stress and the number of secondary buds (Table 5).

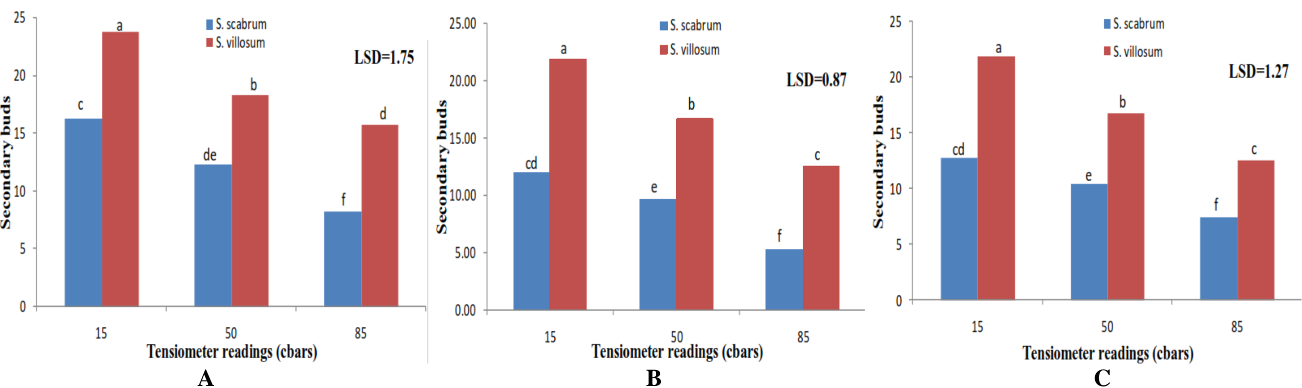

Figure 9. Effects of water stress on the number of secondary buds in (A) greenhouse, B. long rains, and (C) short rains. Bars with different letters were significantly different (P≤ 0.05).

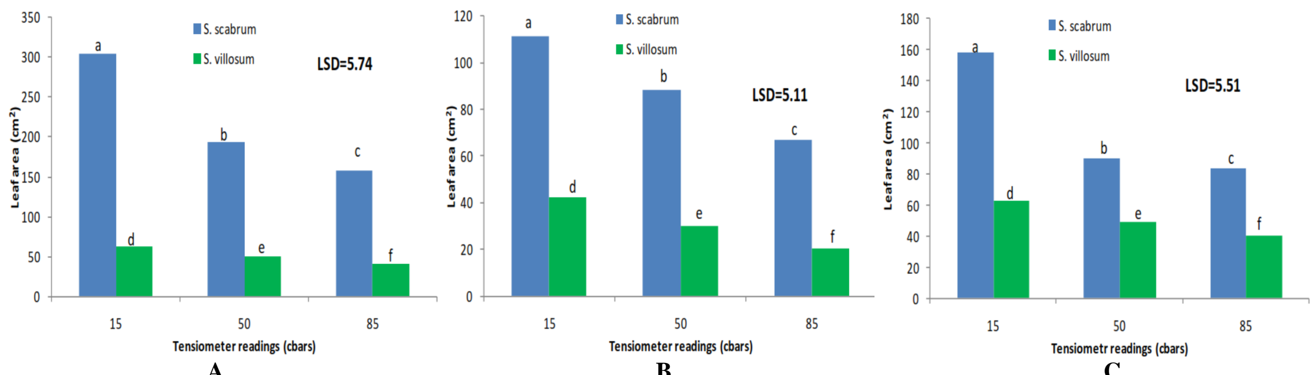

Figure 10. Effects of water stress on leaf area in (A) greenhouse, (B) long and short rains. Bars with different letters were significantly different (P≤ 0.05).

From the obtained data, moisture stress at the vegetative stage significantly reduced branch production in this study. This is in line with Mustapha et al. (2014), who found that water stress reduced branching in selected soya bean genotypes, and Summerfield et al. (1976), who reported that water-stressed cowpea plants were reduced in branching. Reduction in the number of secondary buds can be a phenomenon by the plants to decrease the surface area over which transpiration takes place.

Leaf area

There was a significant effect at P ≤ 0.05 between watering regimes and leaf area in the greenhouse, season one (long rains) and season two (short rains) (Figure 10).

Solanum scabrum had a higher total leaf area of 304.45cm^2 than *Solanum villosum* of 62.45cm^2 under irrigation at water tension of 15 cbars. The lowest leaf area of 20.62cm^2 was attained in *Solanum villosum* under irrigation at a water tension of 85 cbars. In moderate water deficit, leaf area among varieties had a declining order of *Solanum scabrum* › *Solanum villosum.* S. scabrum leaves had the highest leaf area of all the water stress levels. As soil water tension increased, the leaf area reduced. The reduction in leaf area was more pronounced in *S. scabrum* when subjected to water stress than in *S. villosum.* There was a significant interaction between irrigation interval and the variety of leaf areas (Table 6).

Leaf area development is directly related to the yield of African nightshades since the edible part is the leaf. Water stress reduces leaf growth by reducing cell division and expansion rates due to turgor loss (Akinci and Lösel 2012). It is well known that as soil water availability is limited, plant growth is usually decreased. This was previously considered due to turgor loss in the expanded cells. However, more recent studies show that stem and leaf growth may be inhibited at low water potential despite complete turgor maintenance in the growing regions due to osmotic adjustment (Tanguilig et al. 1987). This suggests that growth inhibition may be metabolically regulated, possibly serving an adaptive role by restricting the development of transpiring leaf areas in water-stressed plants. The impact of water stress on leaf growth can be explained as a method of adaptation to water shortage conditions to limit the rate of transpiration and maintain the water supply in the soil around plant roots to increase the chance of survival of plants. The mechanism by which plant leaf area is reduced under water stress is by reducing cell elongation, which leads to decreased cell size and, therefore, leaf area reduction (Markhart 1985).

Table 5. Interactions between irrigation interval and variety on the number of secondary buds

Variety	Tensiometer readings (cbars)	Greenhouse Secondary buds	Long rainy season Secondary buds	Short rainy season Secondary buds
Solanum scabrum	15	16.25c	12d	13d
	50	12.25e	9.6e	10.47e
	85	8.25f	5.25f	7.75f
Solanum villosum	15	23.75a	21.9a	22.47a
	50	18.25b	16.75b	17.25b
	85	15.75cd	12.55c	13.75c
LSD		**1.75**	**0.87**	**1.74**
TXV		*	*	*

*Means followed by the same letter in a column are not significantly different at P≤ 0.05

Table 6. Interactions between irrigation interval and variety on leaf area

Variety	Tensiometer readings (cbars)	Greenhouse	Long rainy season	Short rainy season
		Leaf area	Leaf area	Leaf area
Solanum scabrum	15	304.45a	111.27a	158.64a
	50	193.61b	88.45b	90.37b
	85	158.45c	66.92c	83.41c
Solanum villosum	15	62.45d	42.50d	56.88d
	50	49.55e	30.16e	47.26e
	85	40.81f	20.62f	35.51f
LSD		5.74	5.11	5.51
TXV		*	*	*

Note: *Means followed by the same letter in a column are not significantly different at P≤ 0.05

Emam et al. (2010) reported a reduction in the leaf area of dry beans when the plants were exposed to drought stress during the vegetative growth stage. Furthermore, Nielsen and Nelson (1998) observed significant leaf area reductions in black beans (*P. vulgaris* L) under drought stress in the vegetative phase. Markhart (1985) also found significant decreases in the leaf area under drought conditions 23 days after planting for two bean species (*P. vulgaris and P. acutifolius*). Muthomi and Musyimi 2009, found leaf area reduction on *Solanum scabrum* when they subjected the seedlings to water stress. Indeed, loss of leaf area, which could have resulted from the reduced size of younger leaves and inhibition of the expansion of developing foliage, is also considered an adaptation mechanism to moisture deficit (Emam et al. 2010).

Shoot height

Water stress significantly affected shoot height at P≤ 0.05 in the greenhouse during long and short rains, as in Figure 11. *Solanum scabrum* produced the tallest plants at all water stress levels.

This was related to the growth habit of this species. However, water stress depressed the plant height of both accessions, and the shortest plants were produced at higher water stress levels. The two varieties differed in height concerning plant height. Solanum scabrum was the tallest (40.17cm) under irrigation at 15 cbars, while *Solanum villosum* was the shortest (20.24cm) under irrigation at 85 cbars. The growth reduction in shoot length in irrigation interval at 85 cbars treatment compared to 15 cbars could be associated with a decrease in the cytokinin transport from roots to shoots or the increase in the amount of phytohormone abscisic acid (ABA). The hormone imbalance leads to changes in the cell wall extensibility and a decline in the concentration of photosynthetic enzymes, which results in reduced growth. (Kannan and Kulandaivelu 2011). In Table 7, the interaction between water stress and varieties of shoot height was statistically significant (P≤0.05).

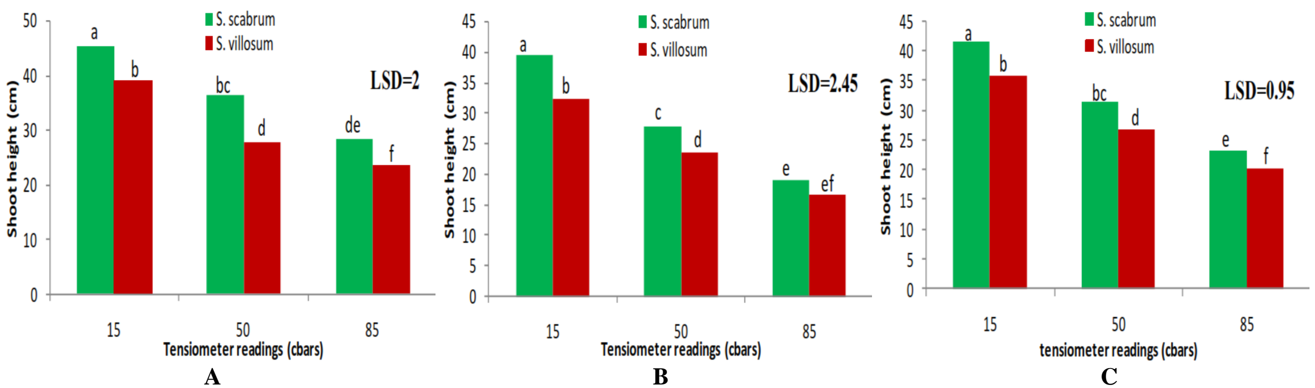

Figure 11. Effects of water stress on shoot height in (A) greenhouse, (B) long rains, and (C) short rains. Bars with different letters were significantly different (P≤ 0.05)

Table 7. Interaction between irrigation interval and species on shoot height

Variety	Tensiometer readings (cbars)	Greenhouse	Long rainy season	Short rainy season
		Shoot height	Shoot height	Shoot height
Solanum scabrum	15	45.17a	39.49a	41.42a
	50	36.24bc	27.85c	31.24bc
	85	28.29e	19.09e	23.29e
Solanum villosum	15	39.03b	32.22b	35.64b
	50	27.79d	23.58d	26.79d
	85	23.45f	16.65ef	20.24f
LSD		2	2.45	0.95
TXV		*	*	*

Note: *Means followed by the same letter in a column are not significantly different at P≤ 0.05

The effect of water stress on plant height was highly significant. Plant height was decreased at the vegetative stage. This is in concurrence with Desclaux et al. (2000), who found that water stress reduced the number of nodes and the main stem height of soybeans. The result also agrees with Hiler et al. (1972), who found that plant height in cowpea was substantially reduced by water stress. Similarly, a reduction in plant height in response to soil water deficit has been reported for wheat (Day et al. 1978; Abayomi 1992), barley (Day et al. 1978), grain sorghum (Blum et al. 1989, and cowpea (Aderolu 2000). This result is in agreement with the results of Emam (2010), Kannan and Kulandaivelu (2011), Nielsen and Nelson (1998), and Shenkut and Brick (2003), who reported on depression of plant height as a result of the severe stress treatment. Influence of environmental factors such as water stress. Drought stress affects both elongation and expansion growth (Jaleel et al. 2008). Due to the low turgor pressure, water deficiency significantly suppresses cell growth and expansion. Osmotic regulation can enable the maintenance of cell turgor for survival or assist plant growth under severe drought conditions (Shao et al. 2009). The reduction in plant height was associated with a decline in cell enlargement and more leaf senescence (Bhatt and Rao 2005). Stress inhibits the efficiency of the translocation and assimilation of photosynthetic products (Xiong and Zhu 2002) and might have caused a reduction in shoot growth.

Fresh and dry shoot weights

There was a significant (at P≤ 0.05) effect between water stress and the fresh and dry shoot weights (Figure 12). In well-irrigated soils (at 15 cbars), the *S. scabrum* variety had the highest fresh and dry weights of 152.27 and 50.78, respectively.

The difference in the weights was significant (P≤0.05) among *S. scabrum* and *S. villosum* varieties. In moderate water deficit, the two accessions' fresh and dry shoot weights had a declining order of *S. scabrum* › *S. villosum*. There was a great reduction in weight between fresh and dry shoot weights of *S. scabrum* than *S. villosum*. This indicates that *S. scabrum* harbors more water in the tissues and is more susceptible to water stress, as seen in the rapid reduction in fresh weight at different irrigation intervals.

Moisture stress reduced the shoot's dry weight (Table 8). Plants under irrigation at 15 cbars had large leaf areas and hence absorbed sufficient sunlight and so likely produced large quantities of dry matter through photosynthesis resulting in high fresh and dry weights compared to those under irrigation at 85 cbars. The reduction in the number of secondary buds, leaf area, and shoot height also significantly reduced shoot fresh and dry weights.

These results agree with the findings of Bradford and Hsiao (1982) and Chartzoulakis et al. (1993). Chartzoulakis et al. (1993) reported similar water stress effects on the dry matter in Kiwi fruit. Other researchers have published similar impacts of water stress on shoot biomass reduction for a range of different agricultural and horticultural crops, including sorghum (Chaudhuri and Kanemasu 1982), tomato (Rumit et al. 2010; peach (Tan and Buttery 1997), and strawberry (Kirnak et al. 2003).

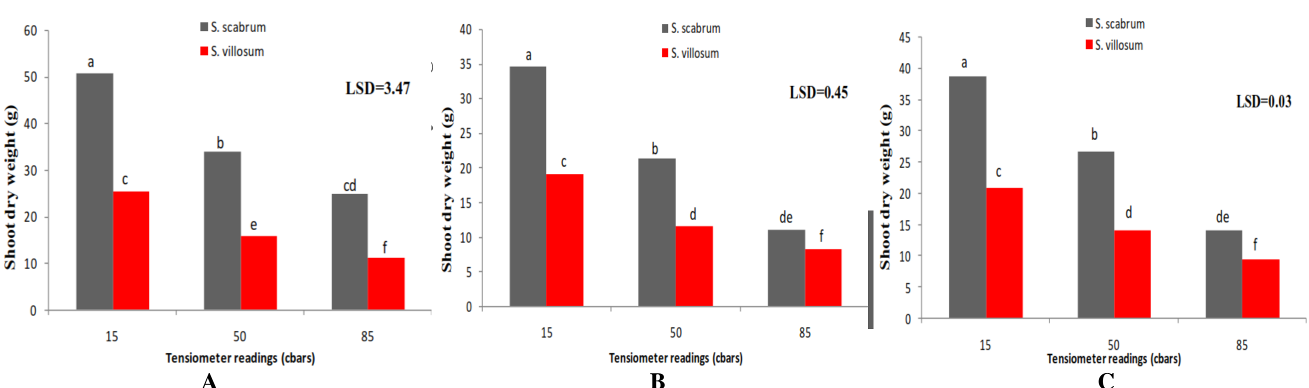

Figure 12. Effects of water stress on shoot dry weight in (A) greenhouse, (B) long rains, and (C) short rains. Bars with different letters were significantly different (P≤ 0.05)

Table 8. Interactions between irrigation interval and species on shoot fresh and dry weights

Variety	Tensiometer readings (cbars)	Greenhouse		Long rains		Short rains	
		Shoot fresh weight	Shoot dry weight	Shoot fresh weight	Shoot dry weight	Shoot fresh weight	Shoot dry weight
Solanum scabrum	15	152.27a	50.78a	84.21a	34.53a	87.44a	38.54a
	50	96.72b	33.87b	54.91c	21.39b	60.28bc	26.55b
	85	66.35c	24.73cd	37.86ef	11.02de	45.41c	14.01d
Solanum villosum	15	73.69cd	25.23c	58.48d	18.98c	61.26b	20.7c
	50	47.68e	15.82e	38.25e	11.45d	44.01d	13.99de
	85	28.63f	11.07f	26.73f	8.16f	30.27e	9.3f
LSD		7.34	3.47	2.42	0.45	0.98	0.03
TXV		*	*	*	*	*	*

Note: *Means followed by the same letter(s) in a column are not significantly different at P≤0.05

The reduction in fresh and dry weights of plants subjected to water stress can be attributed to declining plant shoot height, decreased plant leaf areas, and reduced number of secondary buds.

Fresh and dry root weights

Water stress significantly affected the two accessions' fresh and dry root weights. The effect on the dry and fresh weights was statistically significant at P≤0.05.

S. scabrum had the highest fresh and dry root weights (18.51 and 7.78, respectively) at irrigation intervals of 15 cbars. The lowest fresh and dry root weights (1.92 and 0.89, respectively) were recorded in *S. villosum* at an irrigation interval of 85 cbars. However, the reduction due to water stress was minimal compared to the same effect on shoot weight in terms of root weight. Table 9 shows the interaction between the different irrigation intervals and fresh and dry root weight, which was significant (P≤ 0.05).

Root growth was less inhibited than shoot growth under water stress. This agrees with Kirnak et al. (2003), who reported that some roots continue to elongate at low water potentials that completely inhibit shoot growth. In plants growing in drying soil, root system development is usually less inhibited than shoot growth. Likewise, roots tend to grow thinner under low water potential, and this change in morphology is believed to be adaptive such that roots can further concentrate their use of resources (Sharp 1996).

Effects of water stress on the phytochemical accumulation in nightshades

Total shoot and phenolic root content

There was a significant effect of water stress effect (P≤ 0.05) on the total leaf phenolic content (Figure 14).

The different irrigation intervals significantly affected the shoot and root total phenolic content (P≤0.05). Data showed that the highest concentration of total phenolic compounds was in the shoots of *S. villosum* at 46.41gGAE/Kg DM and roots of *S. scabrum* at 25.06gGAE/Kg DM. The phenolic contents of both the roots and leaves increased with increasing water stress levels; hence the highest results were obtained from plants irrigated at 85 cbars. Despite variation in the phenolic contents in different seasons, the trend remained the same (Figure 15).

During water stress, plants activate the synthesis of phenolic compounds (anthocyanin, proline, and flavonoids), carotenoids, and ascorbic acid (Mandal et al. 2009). Thus, phenolic compounds provide essential physiological and ecological functions, mainly involved in protection against different types of stress (Ayaz et al. 2000). Besides numerous enzymes (superoxide dismutase, peroxidase,

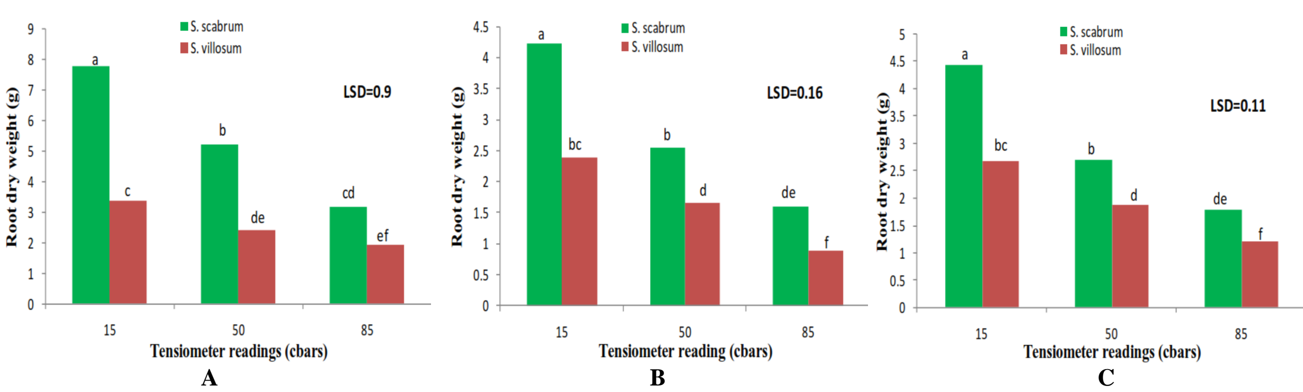

Figure 13. Effects of water stress on root dry weight in (A) greenhouse, (B) long rains, and (C) short rains. Bars with different letters were significantly different at P≤ 0.05)

Table 9. Interactions between irrigation interval and variety on root fresh and dry weights

Variety	Tensiometer readings (cbars)	Greenhouse Root fresh weight	Root dry weight	Long rains Root fresh weight	Root dry weight	Short rains Root fresh weight	Root dry weight
Solanum scabrum	15	18.51a	7.78a	7.78a	4.23a	12.41a	4.43a
	50	8.49b	5.23b	5.23b	2.55b	7.50bc	2.69b
	85	5.17bd	3.17cd	3.17c	1.59de	5.03e	1.78de
Solanum villosum	15	8.25bc	3.36c	3.36cd	2.39bc	8.26b	2.67bc
	50	4.47ce	2.41de	2.41e	1.66d	6.23d	1.86d
	85	3.49cf	1.92ef	2.23f	0.89f	2.80f	1.19f
LSD		3.32	0.9	0.76	0.16	0.81	0.11
TXV		*	*	*	*	*	*

Note: *Means followed by the same letter in a column are not significantly different at P≤ 0.05

Table 10. Interactions between irrigation interval and variety of shoot and root total phenolic content

Variety	Tensiometer readings (cbars)	Greenhouse		Long rainy season		Short rainy season	
		STPC	RTPC	STPC	RTPC	STPC	RTPC
Solanum scabrum	15	23.55f	9.49e	11.26f	8.56e	13.78f	6.92e
	50	29.86d	16.45c	19.71d	13.96c	24.65d	13.87b
	85	38.91b	21.81a	24.53b	20.17a	30.66b	16.67a
Solanum villosum	15	27.86de	7.59f	14.24e	7.83f	22.15e	5.57f
	50	36.75c	15.28cd	21.37c	10.65d	29.87c	10.40d
	85	46.41a	18.39b	32.81a	16.62b	36.26fa	13.29bc
LSD		2	1.17	1.21	0.51	0.15	1.23
T X V		*	*	*	*	*	*

Note: *Means followed by the same letter(s) in a column are not significantly different at P≤ 0.05. (STPC- shoot total phenolic content, RTPC- root total phenolic content).

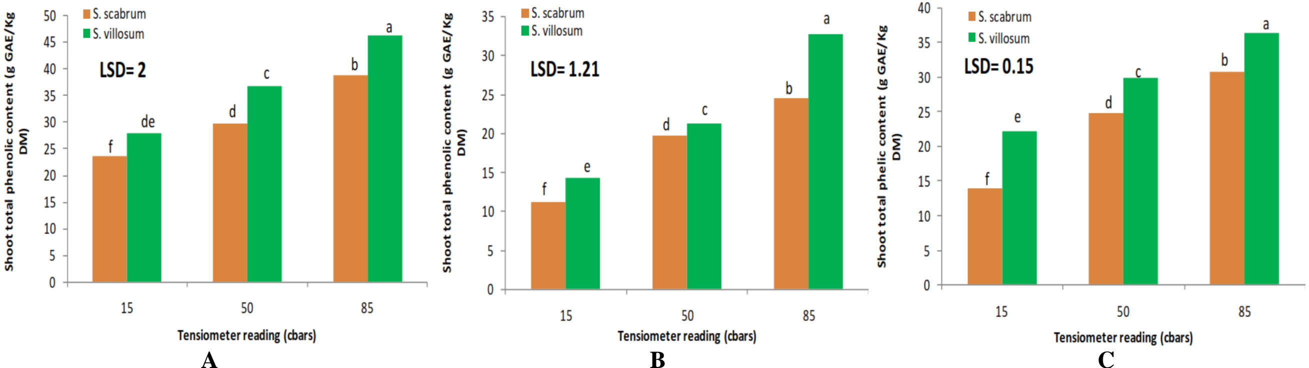

Figure 14. Effects of water stress on shoot total phenolic content in (A) greenhouse, (B) long rains, (B) and (C) short rains. Bars with different letters were significantly different (P≤ 0.05)

etc.), phenolic compounds are potent antioxidants that help plants to survive stress conditions (Mittler 2002). Antioxidant compounds such as phenolic compounds can prevent the oxidative burst of plant cells and thus protect plants from damage to proteins and lipids, DNA, and RNA molecules (Apel and Hirt 2004). In the study, the nightshade species were grown and revealed higher contents of total phenols under water stress conditions. The promotion of the synthesis of phenolic compounds due to drought has been documented in numerous studies (Ayaz et al. 2000; Alexieva et al. 2001; Sánchez-Rodríguezand Rubio- Wilhelmi 2010).

However, *Solanum scabrum* and *Solanum villosum* responded differently to the water stress conditions. *Solanum scabrum* had higher phenolic content in the roots, while *Solanum villosum* had higher phenolic content in the shoots. This can be explained as the differential partitioning of phenolics in different plant species under stress. *Solanum scabrum* portioned more phenolics in the roots to act as osmolytes to maintain root cell turgor and increase the osmotic potential that helps keep the gradient for water uptake. The higher phenolic content in the shoots of *Solanum villosum* would likely help in protecting enzymes, membrane structure, and integrity, scavenging free radicals, and stabilizing cellular macromolecule structures.

Shoot and root total antioxidant activity

There was a significant influence on watering regimes and the total leaf and root antioxidant activity in the greenhouse during long and short rains (Figure 16, Table 10).

Solanum villosum had the highest total antioxidant activity in the leaves at 52.68%, while *S. scabrum* had the highest concentration in the roots at 27.18% (Figure 17).

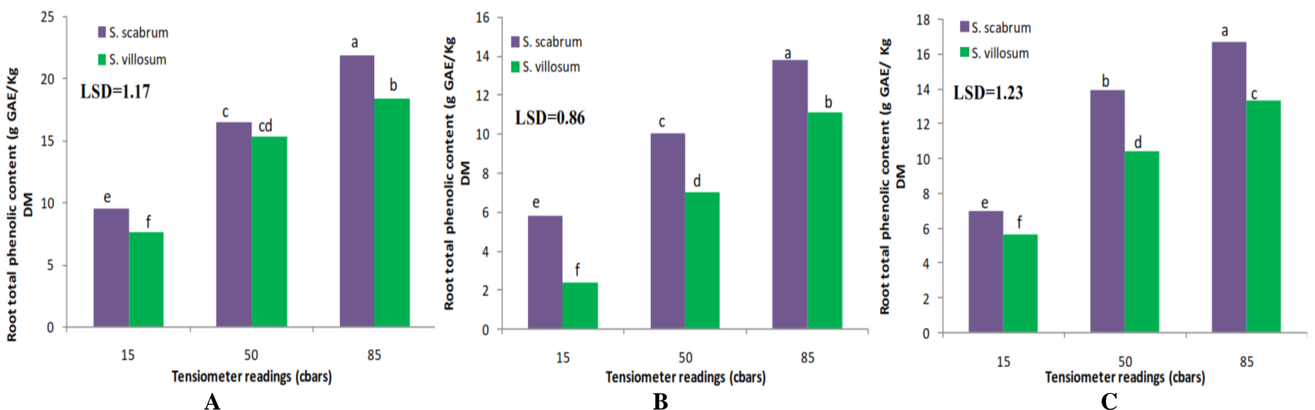

Figure 15. Effects of water stress on root total phenolic content in (A) greenhouse, (B) long rains, and (C) short rains. Bars with different letters were significantly different (P≤ 0.05)

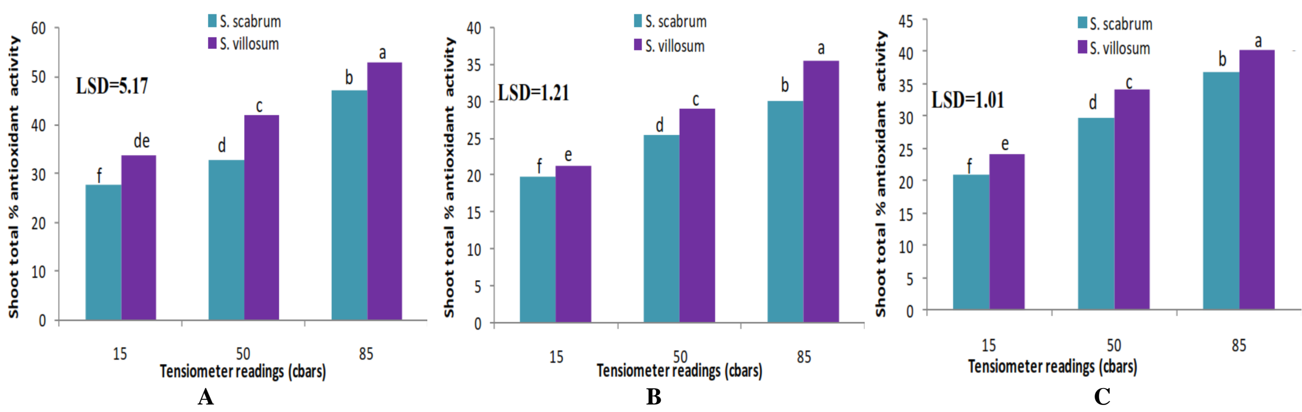

Figure 16. Effects of water stress on the shoot total antioxidant activity in (A) greenhouse, (B) long rains, and (C) short rains. Bars with different letters were significantly different (P≤ 0.05)

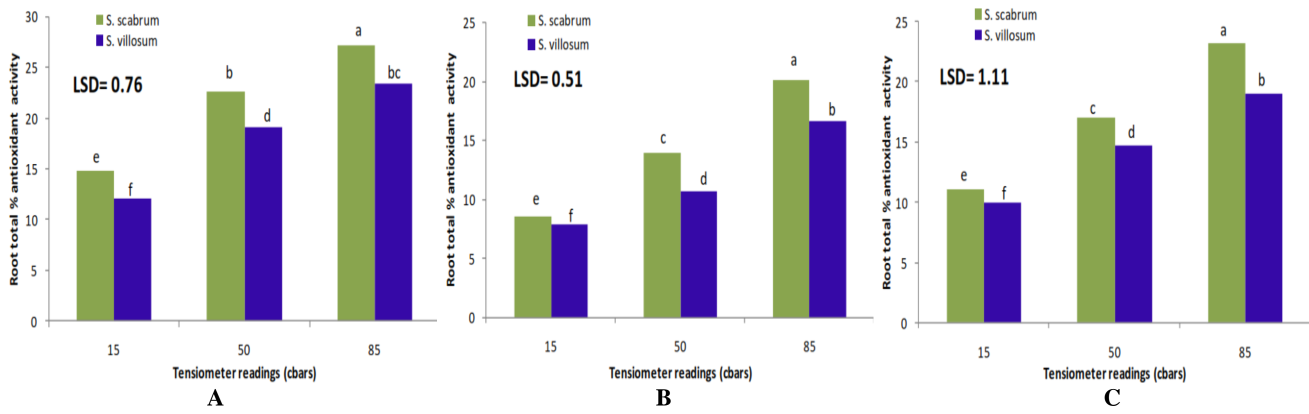

Figure 17. Effect of water stress on the root total antioxidant activity in (A) greenhouse, (B) long rains, and (C) short rains. Bars with different letters were significantly different (P≤ 0.05)

Table 4.10. Interactions between irrigation interval and variety on the total shoot and root antioxidant activity

Variety	Tensiometer readings (cbars)	Greenhouse		Long rainy season		Short rainy season	
		STAA	RTAA	STAA	RTAA	STAA	RTAA
Solanum scabrum	15	27.7f	14.84f	19.83f	8.56e	21.1f	11.05e
	50	32.66de	22.59bc	25.41d	13.96c	29.89d	17.01c
	85	47.04b	27.18a	30.14b	20.17a	36.87b	23.22a
Solanum villosum	15	33.64d	12.06e	21.34e	7.83f	24.19e	9.95f
	50	41.87bc	19.11d	28.95c	10.65d	34.22c	14.74d
	85	52.68a	23.35b	35.6a	16.62b	40.35a	19.04b
LSD		5.17	0.76	1.21	0.51	1.01	1.11
T X V		*	*	*	*	*	*

Note: *Means followed by the same letter(s) in a column are not significantly different at P≤0.05. (STAA- shoot total antioxidant activity, RTAA- root total antioxidant activity)

Changes in the antioxidant capacity of water-stressed plants paralleled those in the total phenolic compounds. The changes in the antioxidant capacity of nightshade plants were roughly reflective of the changes in the total phenolic content. At 50 cbars, the total antioxidant activity in the roots among the two species had an increasing order of *Solanum scabrum* greater than *Solanum villosum*. In the leaves, the order was *Solanum villosum* › *Solanum scabrum.* The lowest levels of total antioxidant activity were recorded in the plants irrigated at 15 cbars. This shows that an increase in water stress led to increased phenolic content in the species grown. The differential in the concentration of total antioxidant activity depended on nightshade species grown, which determines their differential in partitioning as in total phenolic content.

Drought affects not only water relations but also induces stomatal closure and decreases the photosynthetic rate and growth. Closure of stomata decreases CO_2 concentration in leaf mesophyll tissue and results in NADPH accumulation. Under such conditions, where NADP is a limiting factor, oxygen acts as an alternate acceptor of electrons from the thylakoid electron transport chain, resulting in the formation of a superoxide radical (O_2^-) (Cadenas 1989). Superoxide radical and its reduction product H_2O_2 are potentially toxic compounds and can also combine by the Haber-Weiss reaction to form the highly toxic hydroxyl radical (OH^-) (Sairam et al. 1998).

Under optimal conditions, leaves are rich in antioxidant enzymes and metabolites and can cope with reactive oxygen species (ROS), thus minimizing oxidative damage. Many reports deal with the deleterious effects of ROS, whose production is stimulated under water stress conditions (Malenčić et al. 2000, Blokhina et al. 2003, Foyer and Noctor 2005). ROS causes lipid peroxidation, membrane injuries, protein degradation, and enzyme inactivation (Sairam et al. 2008), thus inducing oxidative stress. Tolerant genotypes, therefore, should not only be able to retain sufficient water under drought but should also have a highly active system to protect against oxidative injury, and *Solanum villosum* exhibits more of this than *Solanum scabrum*. Plants possess several tissue antioxidant enzymes for protection against ROS, like superoxide dismutase (SOD), ascorbate peroxidase (APOX), guajkol peroxidase (GPOX), reductase (GR), and catalase (CAT). These enzymes either quench toxic compounds or regenerate antioxidants with the help of reducing power provided by photosynthesis (Zacchini et al. 2003).

During drought conditions, high activities of antioxidant enzymes are associated with lower levels of lipid peroxidation, being related to drought tolerance (Bowler et al. 1992). An increased metabolic capacity of these enzymes may be part of a general antioxidative system in plants involving the regulation of protein synthesis or gene expression (Foyer et al. 1994, Scandalios et al. 1997). Low-molecular-weight antioxidants are presented by carotenoids, tocopherols, glutathione, and ascorbic acid. Apart from their prominent role as enzyme substrates, they can react chemically with almost all forms of ROS. Among substances able to protect plant cells from oxidative attack, a specific function of polyamines in preventing photooxidative damage is reported (Tadolini 1988; Løvaas 1997). Genotypes of the same species respond differentially to environmental stresses and oxidative injury due to genetic differences in their antioxidant systems, such as in *Solanum villosum* and *Solanum scabrum.* That provides a valuable tool for insight into the physiological mechanism operative in stress-tolerant genotypes (Sairam et al. 1998).

According to Foyer et al. (1997), much of the injuries caused by exposure to biotic and abiotic stresses are associated with oxidative damage at a cellular level, the chloroplasts being an essential site of H_2O_2 generation. Zlatev et al. (2005) established that, at the end of the drought period, an increased H_2O_2 and OH^- production was observed in young bean plants, therefore revealing a state of oxidative stress in cells. H_2O_2 is a strong oxidant produced mainly due to the scavenging of superoxide radicals. Its higher concentration is injurious to cells, resulting in localized oxidative damage, lipid peroxidation, disruption of metabolic function, and losses of cellular integrity at sites where it accumulates (Menconi et al. 1995; Velikova et al. 2000).

It is well known that H_2O_2, similar to glutathione, has multi-functional interactive roles in the early stages of the plant stress response. H_2O_2 can diffuse relatively long distances, causing changes in the redox status of surrounding cells and tissues that, at relatively low concentrations, it may trigger an antioxidative response

(Foyer et al. 1997). Rather than just the scavenging capacity, a fine-tuning of H_2O_2 levels is essential for efficient control. The rationale of this assumption is that H_2O_2, while deleterious to some cellular components, is critical to plants in various biosynthetic reactions and, as suggested by some authors, possibly also in signal transduction pathways, which could contribute to plant defense (Schreck and Baeuerle 1991). The drought-induced production of H_2O_2 in the mesophyll cells may be associated with changes in the cell wall structure (Scandalios et al. 1997). Furthermore, H_2O_2 is necessary for the peroxidase-mediated oxidative polymerization of cinnamyl alcohols to form lignin. Several enzymatic systems have been proposed as responsible for hydrogen peroxide production on the surface of plant cells (Lütje et al. 2000).

Therefore, it may be suggested that the increased level of H_2O_2 observed by many authors in the drought-treated plants is due to oxidative damages but eventually may also have a signal function. H_2O_2, OH^-, and other ROS can be expected to be responsible.

For the lipid peroxidation (Zacchini et al. 2003). Sgherri and Navari-Izzo (1995) reported that increased activity of scavenging enzymes could be due either to an adaptive change in catalytic properties or the transcription of the corresponding silent genes. This could be related to enhanced levels of free radicals or other ROS in plant cells and correlate with temporal coordination of the production of H_2O_2 via SOD and destruction of this peroxide by APOX and CAT. Such coordinated responses are believed to promote plant tolerance to oxidative stress (Foyer et al. 1994). It is also possible that increased SOD activity could alter the expression of other metabolic processes associated with water stress. Gupta et al. (1993) have demonstrated that enhanced activity of Cu, Zn SOD in transgenic plants was associated with increased activity of APOX. Some other authors also reported an increase in SOD activity in plants under oxidative stress (Gupta et al. 1993; Zlatev et al. 2005).

The relative tolerance of plant genotypes, as reflected by its lower lipid peroxidation and higher membrane stability, is related to the levels of its antioxidant enzyme activity. APOX, Cu, Zn-SOD, and CAT are involved in overcoming oxidative stress. The increased activities of antioxidant enzymes act as a damage control system and, thus, protect from oxidative stress, resulting in lower LPO and higher membrane stability in tolerant genotypes. The literature analyzed in this review complexity of tolerance of plants to water deficit and supports the statements of many authors that the flexibility of cell metabolism and its acclimation to changes in environmental conditions is a first critical step in avoiding stress (Yordanov et al. 2000). The wider the range of adaptation capacity of plants, the better they are protected against various stresses. Plant development program changes are always associated with the physiological and biochemical program and activity changes.

Despite the intensive investigation of the problem of water deficit tolerance, many of its aspects remain to be explored. Water deficit induces expression of particular genes, which is associated in most cases with adaptive responses of stressed plants. The functions of many of them are still not established. Similar results were obtained by Oh (2010) on lettuce seedlings subjected to different water stress levels.

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Short Communication: Evaluation of antimicrobial activities of *Alchemilla vulgaris* and *Portulaca oleracea* ethanolic extracts and correlation with their phytochemical profiles

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Abstract. *Edrah SM. 2017. Short Communication: Evaluation of antimicrobial activities of* Alchemilla vulgaris *and* Portulaca oleracea *ethanolic extracts and correlation with their phytochemical profiles. Biofarmasi J Nat Prod Biochem 15: 91-94.* The ethanol extracts of leaves of *Alchemilla vulgaris* and *Portulaca oleracea* were studied for antimicrobial activity at 10 mg/mL concentrations by using the disc diffusion method on two gram-positive bacteria: *Staphylococcus aureus* and *Staphylococcus epidermidis;* three gram-negative bacteria: *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* and one fungus: *Candida albicans* were used in the study. After incubation for 24 hrs, the zone of inhibition was compared with standard antibiotics Gentamycin (10 µg/disc) used as a positive control. The dose-dependent study concluded that the ethanol extract of A. vulgaris was more potential than the leaf extract of *P. oleracea.* Almost all of the chemical ingredients present in both ethanol extracts, such as tannins, flavonoids, and phenols, may be responsible for the antimicrobial activity.

Keywords: *Alchemilla vulgaris,* antimicrobial activity, disc diffusion method, *Portulaca oleracea*

INTRODUCTION

Plants are great sources of new harmless, biodegradable, and renewable medications. The use of plants is as healing representatives in addition to existing medicine. Medicinal plants play an influential role in public health, especially in developing countries. It is considered that the great utilization of plants in healing activity seems not to be managed to intoxicate. The price of drugs in use today is too high for the bulk of people in the community in third world countries. Therefore, the search for cheap sources of antimicrobial substances in nature becomes inevitable.

Alchemilla vulgaris L. has different names in various ethnic groups; in Libya, it is known as "*rejel alasad*," while *Portulaca oleracea* L. is known as "*bleabsha*." The aerial parts of the *A. vulgaris* plant, a member of the family Rosaceae, are applied to heal inflammation, particularly to the intestinal and female reproductive tracts, including maintaining to stop minor bleeding and treat wounds. *A. vulgaris* is an herbaceous herb, and it is in Libyan folk; this medicine is applied to urinary diseases. Moreover, it is also used to treat ovarian infections in women as well as for the treatment of diarrhea and internal bleeding, to treat vaginal diseases, uterine and abdominal relaxations after childbirth, and repeated abortions. This plant is a favorite for a gynecologist and is also prescribed to treat obesity and diabetes. *P. oleracea,* a member of the family Portulacaceae, is a warm climate annual green herb; it was reported as a global remedy due to its various therapeutic uses (Iwu 1993; Lim and Quah 2007). It is broadly used to treat diarrhea in both humans and animals in China and has been established with anti-inflammatory effects by recent studies (Lee 2012; Abd El-Azime et al. 2014).

Intended for several years, the limitation of bacterial infections by Inhibiting microbial growth was a major advance concerning antimicrobial treatment. Such approved antimicrobial medications were regularly utilized for medical infectious illnesses of various ages. Nevertheless, in current ages, the random application of certain antimicrobials leads to increased bacteria resistance and influence. Consequently, natural antimicrobial agents are needed more by traditional people.

MATERIALS AND METHODS

Preparation of the crude ethanol extracts

Two traditional medicinal plants, viz., *A. vulgaris* and *P. oleracea* (leaves), were screened. The good leaves of the two plants were collected, washed with distilled water, dried, and powdered finely using a blender. 20 g of ground, air-dried material was shaken with 500 mL of ethyl alcohol (EtOH 96°) (w/v) separated at room temperature with stirring for 96 hours (150 cycles/ minute). The ethanol was evaporated to dryness after extraction using a rotary vacuum evaporator. The extract was weighed and dissolved in ethanol (2 mL) at a 200 mg/mL concentration and stored at 4°C for further experiments.

Phytochemical analysis

The presence of the main class metabolites was determined along with the standard methods (Harborne 1973; Matos 1988 et al.; Trease and Evans 1989; Sofowora 1993; Memelink et al. 2001; Raaman 2006). Freshly prepared extracts were subjected to standard phytochemical analyses to find the presence of the phytoconstituents.

Test for alkaloids. 10 mL of the crude extract was added to 2-3 mL of HCl (10%). This acidic medium was heated in a water bath. It was added by a volume of NH_4OH (10%) to obtain a medium with pH= 9, which was extracted with ethylic ether and then concentrated with a rotary evaporator. The residue will be added with 0.5 mL of HCl (2%) and divided into two equal parts. The first was treated with a few drops of Mayer's reagent and the second with Wagner's reagent. Observation: turbidity or precipitation.

Test for tannins. A few milligrams of crude extract were dissolved in 3 -5 mL of distilled water, and 1% ferric chloride solution drops were added. A change in color to dark green, blue, black, or the formation of a precipitate indicated a positive reaction showing the presence of tannins.

Test of saponins. 10 mL of the aqueous solution was added to a little water and then stirred strongly. Persistent foam indicated the presence of saponins.

Test for steroids. 3 mL of extract was dissolved in 3 mL of chloroform and 3 mL of concentrated H_2SO_4. Formation of Bluish red to cherry color in chloroform layer showed the presence of steroids.

Test for phlobatannins. 5 mL of plant extract was treated with 5 mL of 1% HCl and heated. Red color precipitate indicated the presence of Phlobatannins in the sample.

Test for terpenoids. 3 mL of plant extract, 5-6 mL of chloroform, and 8-9 mL of Conc. H_2SO_4 was mixed. A reddish-brown precipitate at the interface confirmed the presence of terpenoids.

Test for flavonoids. A few milligrams of extract were dissolved in 8- 10 mL of methanol. The mixture was filtered, and 3-5 drops of concentrated HCl and both 1 cm pieces of magnesium tape were added. A pink tint in the solution indicated a positive reaction.

Test for phenols. 10 mL of plant extract, when treated with a few drops of the $FeCl_3$ solution, gave blue-green color, and it confirmed the presence of phenols.

Test for proteins. About 50mg of the extract was dissolved in 10 mL of distilled water and filtered through Whatman no. 1 filter paper, and the filtrate was subjected to test for proteins.

Biuret test: 5 mL of filtrate was treated with 3 drops of 2% copper sulfate solution. To this, 3-4 mL of ethanol was added, followed by the addition of potassium hydroxide pellets. The pink color in the ethanol layer indicated the presence of proteins.

Test for glycosides. 5 mL of plant extract, 4-5 mL $Fecl_3$ (5%), and 4-5 mL of acetic acid were added, then a few drops of H_2SO_4 were added to the mixture. Greenish blue color indicates the presence of glycosides.

Antimicrobial assay. Two gram-positive bacteria: *Staphylococcus aureus* and *Staphylococcus epidermidis;* three gram-negative bacteria: *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa; and* one fungus*: Candida albicans* were used in the study.

Preparation of inoculum

Stock cultures were maintained at 4°C on slopes of nutrient agar. Active cultures for experiments were performed by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton broth (MHB) for bacteria and Sabouraud dextrose broth (SDB) for fungi that were incubated without agitation for 24 h at 37°C and 25°C respectively. To 5 mL of MHB and SDB, 0.2 mL of culture was inoculated and incubated until it reached the turbidity equal to that of the standard 0.5 McFarland solution at 600nm, equivalent to 106–108 CFU/mL (McFarland 1907).

Disc diffusion method

In vitro antimicrobial activity was screened using Mueller Hinton Agar (MHA). The MHA plates were prepared by pouring 15 mL of molten media into sterile Petri plates. The plates were allowed to solidify for 5-6 mins, 0.1 % inoculum suspension was swabbed uniformly, and the inoculum was allowed to dry for 4-5 mins. The same procedure was conducted on the fungi using Sabouraud dextrose agar. The extract concentration of 10 mg/mL, 50 µl/disc, was loaded on 6 mm sterile individual discs. The loaded disc was placed on the surface of the medium and was allowed to diffuse for 3-4 min, and the plates were set aside for incubation at 37°C for 24 h. The negative control was prepared using a respective solvent (10 µl of Ethanol). Gentamycin (10 µg/disc) was used as a positive control. At the end of incubation, inhibition zones formed around the disc were measured in millimeters (Baur et al. 1966). Each antimicrobial assay was performed in triplicate.

RESULTS AND DISCUSSION

Phytochemical analysis

Phytochemical constituents such as alkaloids, flavonoids, tannins, phenols, saponins, and several other aromatic compounds are secondary metabolites of plants that function as a defense mechanism against many microorganisms, insects, and other herbivores. The phytochemical constituents of the selected plants were studied as reviewed in Table 1. Analysis of plant extracts revealed the presence of these components in both selected plants, which could be responsible for the observed antimicrobial property.

It is fundamental to study medicinal plants to improve the proper use of medicinal plants and affirm their potential as sources for new medicines. The therapeutic properties of medicinal plants are essentially due to the presence of numerous chemical substances of various components that result in secondary metabolite products (Lozoya et al. 1989; Meckes-Lozoya et al. 1990; Karthikeyan et al. 2009). As shown in Table 1, each chemical constituent, alkaloids,

tannins, saponins, steroids, Phlobatanins, terpenoids, flavonoids, phenols, and glycosides, present in leaf extracts in both *A. vulgaris* and *P. oleracea.* Still, phlobatanins are not present in *P. oleracea,* and proteins are not present in both. Phlobatannins have the diuretic property (Awoyinka et al. 2007). Saponins remain a remarkable class of glycosides that own soapy properties and are active agents against fungi (Sadipo et al. 1991; Chung et al. 1998). The presence of phenol compounds makes the resistance to diseases in humans and plants. Tannins are similarly recognized as antimicrobial agents; additionally, it has the potential to prevent the development of microorganisms by precipitating microbial protein (Sadipo et al. 1991), as well as by inhibiting the growth of several Microorganisms such as bacteria and fungi. It also has physiological properties such as anti-parasitic, anti-secretolytic, and anti-phlogistic effects (Asquith and Butler 1986). Consequently, because of the good results of applications against human pathogens, these plants may preferably be used as medications.

Antimicrobial activities

The antimicrobial activity of ethanol extracts of leaves of *A. vulgaris* and *P. oleracea* against human pathogenic bacteria, *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and fungi: *Candida albicans*, is measured by quantifying the zone of inhibition in disc diffusion method (Table 2). The organisms and zone of inhibition to the corresponding extracts are shown in Table 2. The Zones of inhibition range from 7 – 13 mm for leaves of both extracts against bacteria and are 11 mm and 15 mm against fungi, respectively. The ethanol leave extract of *P. oleracea* has higher inhibition zones, i.e., 13, 7, 8, 9, and 10mm, against *E. coli, K. pneumonia, P. aeruginosa, S. aureus, S. epidermidis*, respectively, and lower inhibition zone, i.e., 11 mm, against *C. albicans*. At the same time, the ethanol leaf extract of *A. vulgaris* has low inhibition zones, i.e., 10, 9, 11, 7, and 12 mm against each of *E. coli, K. pneumonia, P. aeruginosa, S. aureus, S. epidermidis*, respectively, and highest inhibition zone, of 15 mm against fungi *C. albicans*.

Table 1. Phytochemical screening of *Alchemilla vulgaris* and *Portulaca oleracea* leaves extracts

Phytochemicals	*Alchemilla vulgaris*	*Portulaca oleracea*
Alkaloids	+	+
Tannin	+	+
Saponin	+	+
Steroids	+	+
Phlobatannins	+	-
Terpenoids	+	+
Flavonoid	+	+
Phenolics	+	+
Proteins	-	-
Glycoside	+	+

Note: + = present, - = not present

Table 2. Antimicrobial activities of *Alchemilla vulgaris, Portulaca oleracea,* and the positive control tested against microorganisms by disk diffusion method

Bacterial types and fungi	Plants names and antibiotic Mean diameter of growth inhibition zone (mm)		
	Alchemilla vulgaris	*Portulaca oleracea*	Positive control Gentamicin
Escherichia coli	10	13	18
Klebsiella pneumonia	9	7	22
Pseudomonas aeruginosa	11	8	19
Staphylococcus aureus	7	9	17
Streptococcus epidermidis	12	10	21
Candida albicans	15	11	19

Naturally, medicinal plants contain numerous phytochemical ingredients, which are significantly required to limit the growth of microorganisms. *A. vulgaris and P. oleracea* are utilized by Libyans as medications for treating many diseases. In conclusion, this research concludes that these plants' leaf extracts have satisfying activity against each of *S. aureus, S. epidermidis, E. coli, K. pneumoniae, P. aeruginosa,* and *C. albicans*.

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