



Research Paper

Quantification of Diosgenin content in *Dioscorea bulbifera* linn germplasm from Uganda

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ABSTRACT

Diosgenin is mainly found in wild *Dioscorea* sp. and is a well-known precursor of steroidal and contraceptive drugs. However, due to large market demand and increase in ecological damage, available wild *Dioscorea* spp. specifically *Dioscorea bulbifera* is gradually declining. This study therefore quantified the amounts of Diosgenin in *D. bulbifera* accessions in Uganda for conservation of high yielding germplasm. Mature aerial bulbs of *D. bulbifera* were collected from various regions of Uganda, that is; the Eastern (Mt Elgon forest reserve), Northern (Abur-buru forest reserve), Western (Kasyoha Kitomi forest reserve and Bugoma central forest reserve), Southwestern (Bwindi Impenetrable forest) and Central (Mabira central forest reserve). Samples were separately washed, shredded into small pieces and lyophilised. The freeze-dried samples were powdered mechanically and kept in polythene bags for extraction. The powdered samples were macerated with 70% ethanol, concentrated *in vacuo* to dryness. The extracts were reconstituted with water and defatted with hexane in a separating flask before they were partitioned with n-butanol. The butanol layers were concentrated and analyzed using High Performance Liquid Chromatography (HPLC) and also qualitative phytochemistry was done on the crude extracts. Results obtained from HPLC showed the highest concentration of diosgenin in Mabira forest reserve population with 0.996^a mg/g, while Bwindi impenetrable forest population and Mt. Elgon populations had significantly lower concentration of 0.36^e and 0.373^e mg/g respectively at a retention time of 2.803 min. The method employed in current study confirmed a significant variation in the diosgenin concentration in Ugandan *D. bulbifera* populations except for germplasm from Bwindi and Elgon. The Diosgenin quantities were highest in Mabira central forest reserve with 0.996^a mg/g implying the germplasm from Mabira would be ideal for conservation.

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INTRODUCTION

Diosgenin is the main skeleton for steroidal drugs and is derived primarily from *Dioscorea* sp. (Xiang et al., 2010). Steroidal compounds have been reported for strong anti-allergic, anti-infection and other medicinal effects which play an important role in the treatment of sore throat, breast cancer, type II diabetes mellitus, sub-acute thyroiditis, etc. (Ghosh et al., 2011). Traditional medicine uses decoctions

from *D. bulbifera* leaves and tubers to control women fertility, relieving painful cycles and peri- and post-menopausal symptoms (Wu et al., 2005). Diosgenin has also been used in recent years as an oral contraceptive with major patient demand (Shen et al., 2018) and recently, alarming demands call for the expansion of introduction and cultivation of *Dioscorea* sp. worldwide (Figure 1). China and

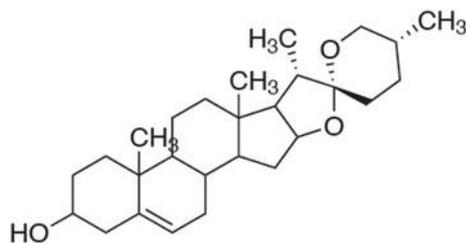


Figure1: Diosgenin (Aminkar et al., 2018)

Mexico have been the two main production countries, which account for 67% of diosgenin yield with the richest *Dioscorea* resource in the world (Yi et al., 2014). However, among other reasons, the yield and quality of diosgenin has declined due to the lack of high-quality germplasm (Shen et al., 2018) for this reason therefore, exploring approaches for conservation and cultivation of diosgenin-endowed *Dioscorea* sp. is indispensable. *D. bulbifera* Linn (Dioscoreaceae) is one of the unique medicinal plants among 600 species in the family which has found its importance in traditional medicine throughout the world (Ghosh, 2015). *D. bulbifera* has reported to contain highest composition of saponins, alkaloids and tannins as compared to other *Dioscorea* sp. in Nigeria (Ezeabara, 2018) the reason for our study to focus on it. Studies show that there still exist, some *D. bulbifera* species growing in Uganda (Eilu et al., 2007; Croxton et al., 2011; Ojelelet al., 2019) but what remains unknown is whether they contain high content of diosgenin or not across regions of Uganda. Studies about utilization and promoting the growing of *D. species* have already been reported in Uganda by (Byarugaba et al., 2007, Olanya et al., 2004) but none of them focus on variation of diosgenin content in different *D. bulbifera* populations of Uganda. Therefore, since no study has been done to quantify diosgenin in different populations of *D. bulbifera* for purposes of high-quality germplasm conservation, this highlights the cause of this study. This is why for the first time this study reports the concentrations of Diosgenin from different populations of *D. bulbifera* in Uganda.

MATERIALS AND METHODS

Collection of plant material and processing

Mature aerial bulbs were collected from forests of Uganda representing all regions including Bwindi impenetrable forest 00°57'S 29°47'E (South western), Mabira central forest reserve 0°37.026'S 30°39.376'E (Central), Kasyoha Kitomi forest reserve S00°15.602' E030°08.730' (western), Bugoma central forest reserve 1°14.148'N 31°3.085'E (western), Mt Elgon forest reserve N01°00.673 'E034°11.341' (Eastern), and Abur-buru forest reserve 33.24759 1.963797 (Northern). The samples were identified by the Taxonomist Dr Eunice Olet, Department of Biology,

Faculty of Science Mbarara University of Science and Technology. These were later authenticated by comparison with Herbarium specimens from the previously preserved samples in Makerere University Herbarium, Department of Botany, Kampala Uganda. The aerial tubers were washed and shredded into smaller pieces to increase surface area for drying and then later the cut pieces were freeze-dried using a lyophilizer (Lexicon® ULT, Freezer) for 24 h. The dried samples were pulverized mechanically to a fine powder.

Preparation of plant extracts

Extraction and fractionation of *Dioscorea bulbifera* Linn tuber

A modified extraction and fractionation procedures were carried out as described by Jayachandran et al. (2016). About 2 kg of the finely powdered tuber of each sample was separately extracted with 5 L of 70% ethanol by cold maceration process for 72 h with occasional agitation. The extracts were filtered and the process was repeated 3 times. The obtained extracts were bulked and concentrated *in vacuo* at 40°C to obtain the crude extract (CE). The total ethanolic CE was reconstituted with water, defatted using hexane and it was subjected to partitioning using n-butanol. The individual partitioned fractions were concentrated *in vacuo*, and stored in a desicator to free from moisture for further analysis.

Qualitative determination of phytochemicals

This was done according to Sheikh et al. (2013) and the methods description partly reproduces their wording.

Test for alkaloids (Mayer's test)

Wagner's test (iodine-potassium iodine reagent): To about 1 ml of the extract, a few drops of Wagner's reagent were added. Reddish brown precipitate indicates presence of alkaloids

Test for tannins

To 5 ml of the extract, a few drops of neutral 5% ferric

chloride solution were added. The production of dark green color indicates the presence of tannins.

Test for flavonoids (lead acetate test)

To 1 ml of the extract, a few drops of 10% lead acetate solution was added. Formation of yellow precipitate indicates presence of flavonoids.

Test for phenols

To 5 ml of the extract, 3 ml of 10% lead acetate solution was added and mixed gently. Formation of bulky white precipitate indicates the presence of phenols.

Test for saponins (froth test)

0.5mg of the extract was vigorously shaken with a few millimeters of distilled water in the test tube. The formation of frothing is a positive test for saponins.

Test for teroids

2 ml of extract with 2 ml of chloroform and 2 ml of concentrated sulphuric acid are added, the appearance of red color and yellowish green fluorescence indicates presence of steroids.

Test for anthraquinones

To 5 ml of the extract, a few millimeters of concentrated sulphuric acid was added and 1 ml of dilute ammonia was added to it. The appearance of rose pink confirms the presence of anthraquinones.

Test for terpenoids

3 ml of the extract was taken and 1 ml of chloroform and 1.5 ml of concentrated sulphuric acid are added along the sides of the tube. The reddish-brown color in the interface is considered positive for the presence of terpenoids.

Test for glycosides

To 2 ml of the extract, 3 ml of chloroform is added and shaken. The chloroform layer is separated and 10% ammonia solution was added. The pink color indicates the presence of glycosides.

Quantitative determination of total saponins

Fine bulbil powder amounting to 20 gm was put into a conical flask and 100 ml of 70% ethanol added to the

sample. The sample was macerated for 72 h. The mixture was then filtered and concentrated using a rotary evaporator and the residue re-extracted with another 40 ml n-hexane. The extract was then partitioned using 40 ml n-butanol and the samples are dried in the oven to a constant weight and values are expressed as mg/g of extract (Roghini and Vijayalakshmi, 2018) and the methods description partly reproduces their wording.

HPLC analysis of diosgenin

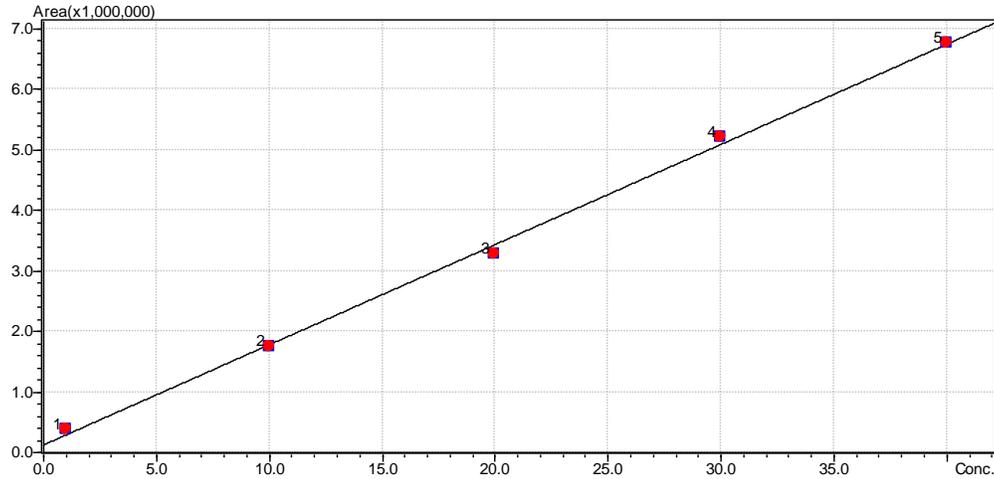
Diosgenin quantification was done as per Niño et al. (2007) with little modification and the methods description partly reproduces their wording. A stock solution of 1 mg/ml concentration was prepared by dissolving 10 mg of each standard extract in 10 ml of methanol. The dissolved standard samples were first filtered using EZ-pak® membrane filters of 0.22 µm and all the reagents used were of HPLC grade. Diosgenin quantification was performed on a HPLC instrument applying the external standard method, through a diosgenin calibration curve with five levels in a concentration range of 1, 10, 20, 30 and 40 µg/ml (Figure 2). The diosgenin concentrations in the different samples were calculated through a regression analysis from the peak area and the known concentrations of authentic diosgenin standard samples purchased from Toronto Research Chemicals (standard no D484700) and are the average of two consecutive readings for each tuber sample. HPLC was performed using Shimadzu Model HPLC system (Shimadzu, Tokyo, Japan) consisting of LC-20AD pump, SPD-20 Aphotodiode array detector, CBM-20A system controller and a rheodyne injection valve fitted with a 20 µl injection loop. Baseline resolution of diosgenin was obtained at 35°C using a Phenomenex Luna C18 column (250 × 4.6 mm × 5 µm). The experimental conditions were an isocratic binary system of acetonitrile/water (90:10v/v), a flow rate of 1 ml min⁻¹. The mobile phase was passed through 0.45 µ PVDF filter and degassed before use. Detection was carried out at wavelength of 194 nm (Niño et al., 2007).

Statistical analysis

R language software was used in data presentation. All experiments were repeated at least three times and the Standard Error of the Mean (SEM) determined. The means were compared using R language version 10.04 in which a one-way ANOVA was performed to determine the significance of diosgenin concentration from different forest locations (Figure 3).

RESULTS

Bearing in mind that the study was comparing diosgenin quantities from *D.bulbifera* accessions from different regions



Y = aX + b
 External Standard Mean RF: 210804.7, a = 165546.8
 Curve Fit Type: Linear RF SD: 90800.33, b = 126211.5
 Origin: Not Forced RF %RSD: 43.07321
 R² = 0.9982151 Weight: None R = 0.9991072.

Figure 2: High Performance Liquid Chromatographic calibration curve of standard diosgenin

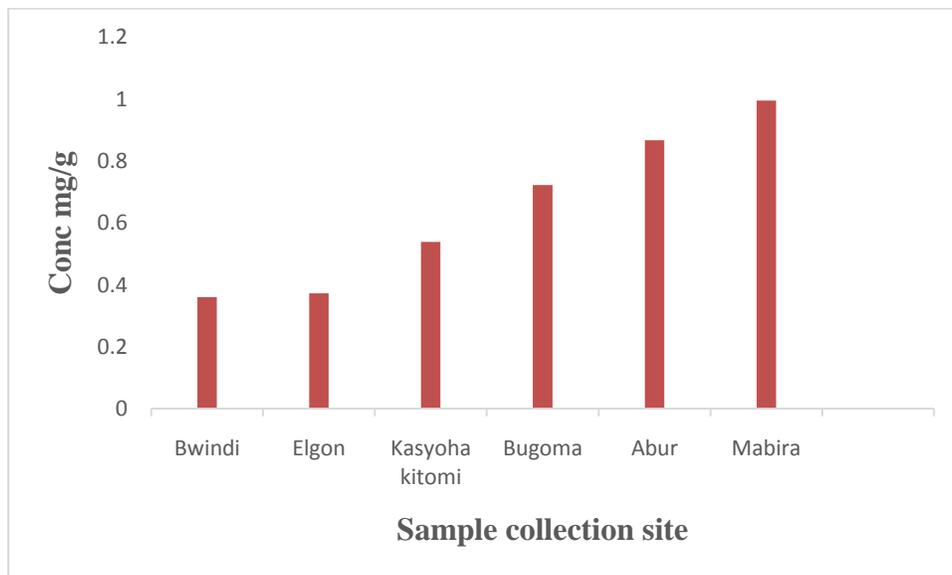


Figure 3: Diosgenin concentrations in *Dioscorea bulbifera* populations in different forests of Uganda

of Uganda, this map shows the forest locations where the samples were collected (Figure 4). The result of the preliminary phytochemical screening from tubers in Table 1 shows the presences of various phytochemicals prepared in Ethanol extract. Flavonoids, terpenoids, saponin, steroid, glycosides, anthraquinones, phenolic and tannins are present in the test. Analysis of variance was computed to ascertain the interactions within the source of variation. The results of one-way ANOVA showed a highly significant concentration of diosgenin for the location (Table 2). Further

analysis within source of variation indicated that the concentration of diosgenin from different forest locations was different with the highest diosgenin concentration coming from Mabira central forest reserve with and the least from Bwindi impenetrable forest as shown in Table 3. Similar results have been reported by Rosida et al. (2015) and Jayachandran et al. (2016). The diosgenin concentrations were calculated basing on the HPLC results obtained after the calibration curve (Figure 6) giving us peaks from different samples as in (figure 5).

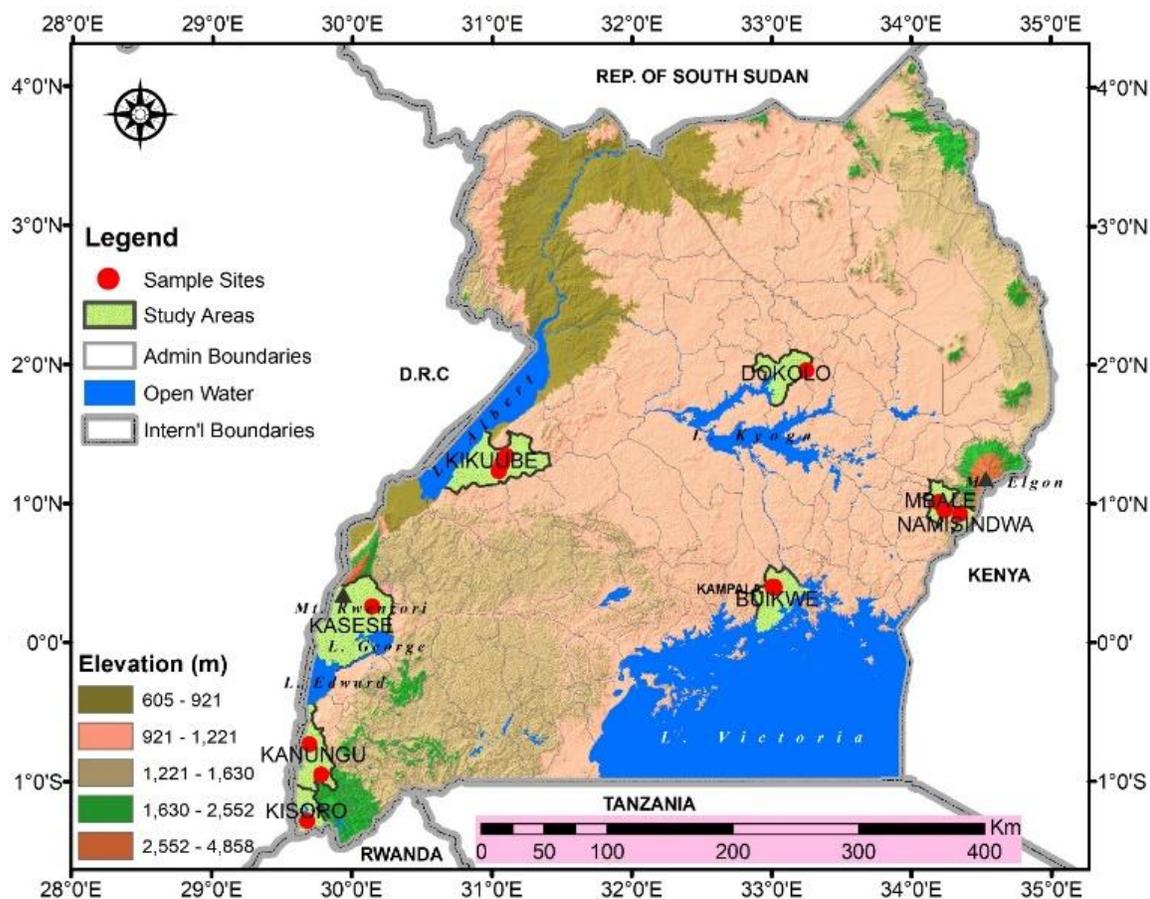


Figure 4: *Dioscorea bulbifera* germplasm sample collection sites in Uganda (drawn from the sample collection GPS)

Table 1: Phytochemical screening of the Ethanolic extract of six populations of *Dioscorea bulbifera*

Test	Populations					
	Bwindi	Mabira	Bugoma	Abur	Kasyoha Kitomi	Elgon
Anthraquinone	+	+	+	+	+	+
Quinones	-	-	-	-	-	-
Alkaloids	++	++	++	++	++	++
Glycosides	+	++	++	++	++	++
Phenol	+++	+++	+++	+++	+++	+++
Tannins	+++	+++	+++	+++	+++	+++
Flavonoids	++	++	++	++	++	++
saponins	++	++	++	++	++	++
Steroids	+	++	++	++	++	+
Terpenoids	+	++	++	++	++	++

+++strongly present, ++moderately present, +present, -absent

DISCUSSION

Maceration method of extraction with 70% ethanol at room temperature (Jayachandran et al., 2016) was used because it's a more efficient method of extracting saponins and its aglycones compared to the standard hydrolysis condition

with sulphuric acid (de Lourdes Contreras-Pacheco et al., 2013). Moreover, the medicinal properties or the therapeutic effect of many plants are attributed to the presence of saponins, alkaloids, flavonoids, terpenoids, tannins, phenolic acids and antioxidants (Roy and Geetha, 2013). Here the preliminary screening showed presence of

Table 2: Analysis of variance for germplasm locations

Diosgenin Concentration (mg/l)				
Source of Variance	DF	Sum of squares	Mean squares	P value
Location	5	0.85653	0.171305	0.0007635**
Residuals	6	0.04485	0.007475	
Diosgenin concentration (mg/g)				
Location	5	0.68613	0.137227	1.41e-08***
Residuals	6	0.00089	0.000148	

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

Table 3: Concentration of diosgenin in mg/l from different samples of *Dioscorea bulbifera*

Sample site	collection	No. of accessions collected	Weight of total saponin/g	Conc of diosgenin mg/l Mean \pmSD	Conc of diosgenin in mg/g
Elgon		07	0.0724 ^e \pm 0.014	5.152 \pm 1.756 ^a	0.373 ^e
Bugoma		08	0.1372 ^c \pm 0.007	5.277 ^a \pm 0.657	0.723 ^c
Bwindi		07	0.0666 ^e \pm 0.007	5.099 ^a \pm 1.498	0.360 ^e
Mabira		07	0.1855 ^a \pm 0.00	5.367 ^a \pm 2.227	0.996 ^a
Abur		08	0.1646 ^b \pm 0.00	5.265 ^a \pm 2.297	0.867 ^b
Kasyoha Kitomi		06	0.1186 ^d \pm 0.007	4.554 ^b \pm 0.590	0.539 ^d

Means within a column with different letters are significantly different (P = 0.05).

anthraquinones and glycosides in mild levels. Alkaloids, saponins, flavonoids, steroids and terpenoids are in moderate levels. The extract also showed high levels of phenolic compounds and Tannins while quinones were reported absent. The preliminary phytochemical analysis results obtained are reported in the Table.1. These results have a similarity with those reported by Preeti and Solanki (2016). It is not surprising therefore that *D. bulbifera* is used against various human and animal ailments due to the availability of these phytochemical compounds. The total saponin concentration of *D. bulbifera* from Uganda is so high with the highest concentration of 0.1855 \pm 0.00g and the least with 0.0666 \pm 0.007g when compared to *D. bulbifera* from Nigeria whose highest total saponin is 0.58 \pm 0.00 mg/100 g (Ezeabara and Anona, 2018). This further confirms the importance of this plant against ailments that are treated by saponins.

HPLC is a highly reliable technique for quantification of plant components. It is an attractive, simple, rapid and selective method for quantitative determination of steroidal saponins from *D. bulbifera* (Badole and Dighe, 2019). This is due to the fact that the experimental conditions used for diosgenin quantification by HPLC in the different *D. bulbifera* extracts gave a highly reproducible retention time (tR) equal to 2.803 \pm 0.02 min and all samples studied gave the same chromatographic pattern. This retention time is almost similar to the retention time reported in the literature for diosgenin even if it is from a different plant trillium govanianum reported as 2.9 min (Sharma et al., 2016). The

diosgenin peak in HPLC of all bulbil extracts displayed similar fragmentation patterns as diosgenin standard. The results of diosgenin quantification by HPLC are given in Table 2. The percentages of diosgenin obtained from the six populations of *D. bulbifera* on this work are in the range from 0.360 to 0.996 mg/g an equivalent of 0.004 to 0.12% respectively. Diosgenin concentration among populations was significantly different except for populations from Bwindi and Elgon (Table 3). Several studies have reported lower diosgenin content for example: *Dioscorea zingiberensis* cell cultures (0.1127 μ g/g of diosgenin) (Li et al., 2012), *Dioscorea alata* (0.0478mg/g) (Rosida et al., 2015), *Dioscorea bulbifera* (0.069%) (Jayachandran et al., 2016) and all others below 1% (Leunufna, 2020) even when different extraction methods were used. These findings correlate with the determination of the steroidal sapogenin contents in different *Tribulus terrestris* samples by HPLC, where significant differences were found depending on the origin and part of the plant used for extraction (Ganzera et al., 2001). The tubers with the highest diosgenin contents were collected in Mabira central reserve forest. This forest is located in Central Uganda with an altitude between 1070 and 1340m above the sea level with an average temperature 17-29°C and an annual precipitation ranging between 1250 and 1400 mm per year (Kizza et al., 2013) while the forest with the least content; Bwindi Impenetrable forest reserve has an altitude of 2000-2300 m above sea level with an average temperature Of 13.4-19.1°C, an annual precipitation of 1378 mm (Stanford and Nkurunungi, 2003). Therefore,

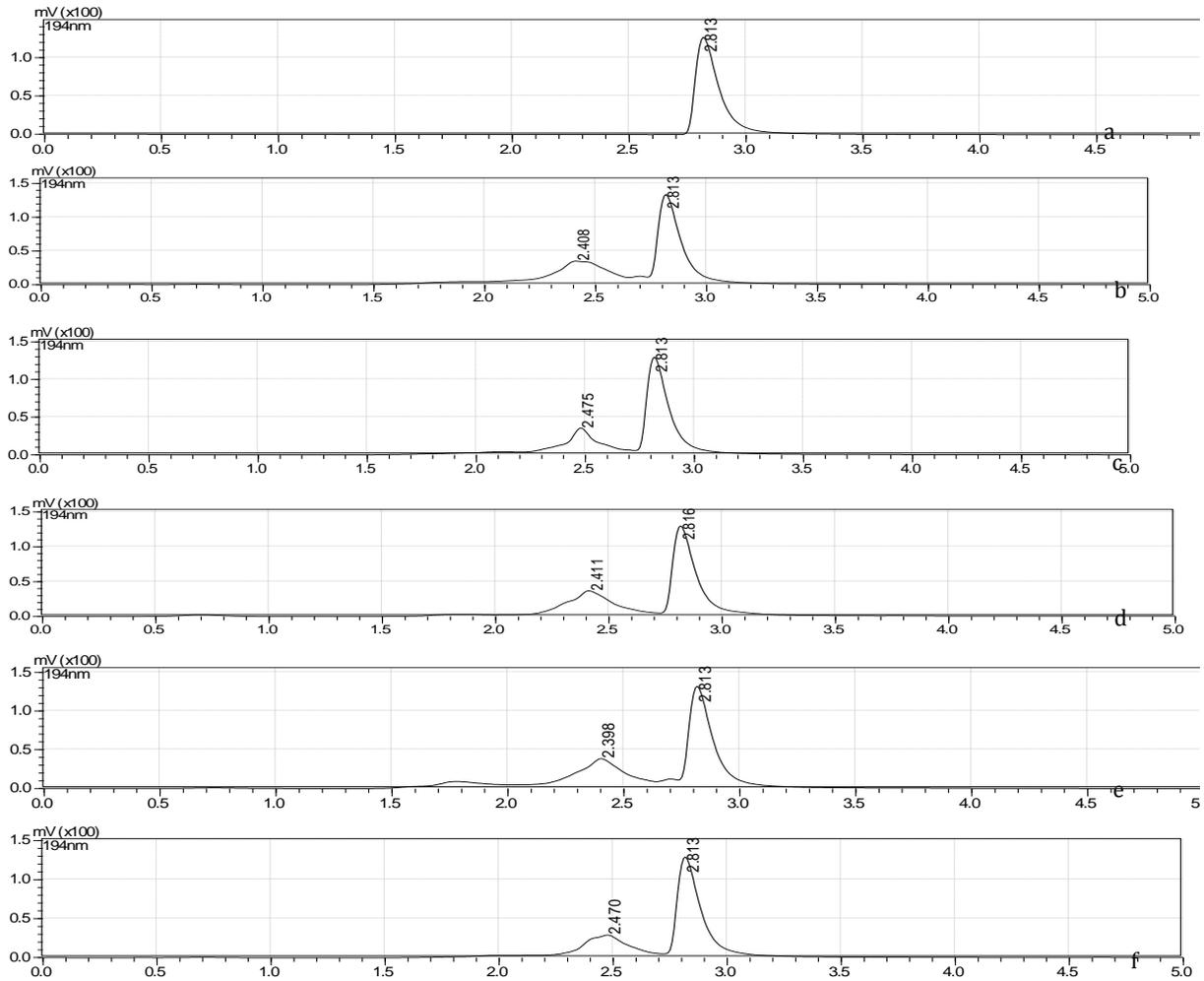


Figure 5: a-g' represent Chromatograms for diosgenin from Kasyoha Kitomi (a) Abur-buru (b) Mabira (c) Bwindi (d) Bugoma (e) Elgon

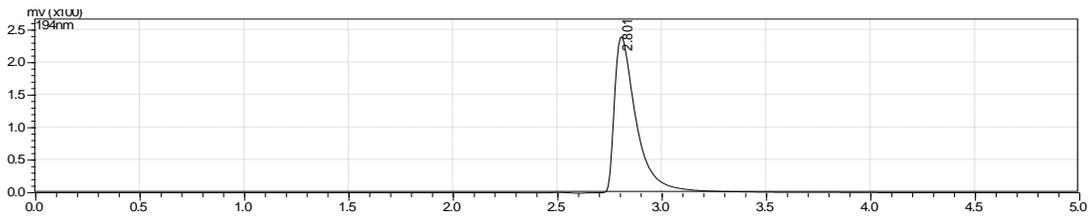


Figure 6: High Performance Liquid Chromatographic determination of diosgenin A typical HPLC chromatogram of standard diosgenin.

the physiological state, the climatic conditions as well as the geographic localization of plants may explain the heterogeneity found on the steroidal sapogenin contents (Bajad et al., 2019). Also the soil Nitrogen and Copper concentrations have a positive effect on the content of diosgenin in plants that contains it (Shams et al., 2014) and Mabira forest contains a higher concentration of nitrogen, 0.11-5.36 mg/kg and copper ions 1.88-7.38 mg/kg (Kizza et

al., 2013) compared to forests with low diosgenin content like Elgon that contains 0.12-0.27 mg/kg nitrogen ions and 4.0 mg/kg copper ions (Isaac, 2020). This difference could have brought about a difference in diosgenin amounts. More so, it was not easy to ascertain the age of different tubers and therefore the screening could have been done on tubers with different ages. This therefore implies that the tubers of *D. bulbifera* from Uganda could be a new source of diosgenin

supply if ways of improving on the content of diosgenin can be devised. This study would have been more beneficial if the genotype of the accessions was known but efforts are underway for this.

CONCLUSIONS

The Diosgenin quantities were highest in Mabira central forest reserve with 0.996^a mg/g implying the germplasm from Mabira would be ideal for conservation.

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