Hypoglycemic evaluation of a new triterpene and other compounds isolated from *Euclea undulata* Thunb. var. *myrtina* (Ebenaceae) root bark

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Abstract

Aim of the study: Investigate the hypoglycaemic activity of the four isolated compounds from a crude acetone extract of the root bark of *Euclea undulata* var. *myrtina,* which is used by traditional healers in the Venda area, Limpopo Province in the treatment of diabetes.

Material and Methods: The hypoglycaemic activity of the four compounds isolated from *E. undulata* was determined by *in vitro* screening of glucose utilization by C2C12 myocytes at a concentration of 25 µg/ml or 50 µg/ml. The inhibition of α-glucosidase was also tested at concentrations ranging from 0.02 to 200.00 µg/ml.

Results: Assay-guided isolation of the crude acetone extract of the root bark of *E. undulata* var. *myrtina.* afforded a new triterpene, α-amyrin-3*O*-ß-(5-hydroxy) ferulic acid (**1**)**,** in addition to three known compounds; betulin (**2**), lupeol (**3**) and epicatechin (**4**). The *in vitro* results on C2C12 myocytes suggest that compound **4** may have some effect to lowers blood glucose levels, whereas compound **1** has the ability to inhibit α-glucosidase at a concentration of 200.0 µg/ml with an IC_{50} value of 4.79 that correlates with that of the positive control acarbose IC_{50} value 4.75.

Conclusion: The results suggest that **4** may have some ability to lower blood glucose levels, whereas **1** has the ability to inhibit α-glucosidase.

Ethnopharmacological relevance: These findings corroborate the ethnomedicinal use of *E. undulata* by traditional healers for the treatment of diabetes as two substances was isolated from the acetone plant extract that exhibit hypoglycaemic activity.

Authors Keywords: α-glucosidase; C2C12 myocytes, Ebenaceae, *Euclea undulata;* hypoglycaemic activity, new triterpene.

1. Introduction

Diabetes mellitus comprises a collection of heterogeneous diseases that differ in their etiological, clinical, and epidemiological characteristics, but have hyperglycaemia and glucose intolerance in concurrence, which are either due to insulin deficiency or to the impaired effectiveness of insulin's action or a combination of both (Roussel, 1998). Parameters to assess hyperglycaemia varies for the different types of diabetes, a fasting plasma glucose level higher than 126 mg/dl and a plasma glucose level above 200 mg/dl 2 hours after a 75 g oral glucose load is considered as a good indication of hyperglycaemia. Type 1 is characterized by an excess in weight loss, slender built, ketoacidosis and low insulin levels. Type 2 is associated with obese persons, with normal to elevated insulin levels and the presence or absence of ketoacidosis. Hypoglycaemia is the most common acute complication of Type 1 diabetes mellitus and may also occur in Type 2 diabetic patience treated with sulfonylurea. Symptoms of hypoglycaemia include confusion, dizziness, seizures, comas etc. It is a chronic disease with major long-term implications, not only for the health and well-being of affected individuals, but also for costs to the society as a whole (Szava-Kovats and Johnson, 1997). As a chronic metabolic disorder, diabetes mellitus can affect all the body's major organ systems leading to complications that are a source of significant morbidity and premature mortality, making it a costly disease (Szava-Kovats and Johnson, 1997). According to the World Health Organization it will affect an estimated 366 million people in 2030 (Motala *et al.*, 2008).

Until the 1980s, the few reported studies on diabetes in Africa indicated a low prevalence of diabetes that is between 0 and 1.0 % in sub-Saharan Africa. However, over the past few decades, type 2 diabetes has emerged as an important medical problem in this region (Motala *et al.*, 2008). Recent estimates by the International Diabetes Federation indicated that the largest increase in the prevalence of diabetes is expected to occur in developing regions of the world, including Africa (Motala *et al.*, 2008). The projected increase in diabetes for Africa is from 3.1% in 2007 to 3.5% in 2025 with the corresponding increase in numbers from 10.4 to 18.7 million

(International Diabetes Federation, 2006). Currently there are approximately 6.5 million diabetics in South Africa (Health 24, 2006).

Diabetes is a growing concern as African populations become westernized, urbanized and adopt a Western diet that often leads to overweight and obesity. It was the sixth leading natural cause of death in South Africa for the 2004 – 2005 period (South Africa Government Information, 2007).

Unfortunately, there is no cure yet for diabetes, but by controlling blood sugar levels through a healthy diet, exercise and medication, the long term complications of diabetes can be minimized. The progressive nature of the disease necessitates constant reassessment of glycaemic control in people with diabetes, and the appropriate adjustment of therapeutic regimes when glycaemic control is no longer maintained with a single agent. The addition of a second and third drug is usually more effective than switching to another single agent (Gerich, 2001).

The indigenous people of southern Africa have a long history of traditional plant usage for medicinal purposes and primary health care. A large portion of the population relies heavily on traditional healers and herbalist to meet primary health care needs.

Euclea undulata Thunb. var. *myrtina* (Ebenaceae), a dense, erect, dioecious shrub or small tree was selected for the identification of bio-active principles after preliminary *in vitro* screenings were done for hypoglycaemic activity on an acetone extract of the root bark. This selection was based on the facts that the crude acetone extract of *E. undulata* root bark gave positive results (hypoglycaemic activity) in the *in vitro* assays done on C2C12 myocytes, 3T3-L1 preadipocytes and in Chang liver cells without displaying any toxicity and scored a +3 total score, according to the scoring system developed by Van de Venter *et al*. (2008). The carbohydrate-hydrolysing enzymes alpha-amylase and alpha-glucosidase were also inhibited to some extent (Deutschländer *et al*., 2009).

2. Materials and methods

2.1 Plant material

Plant material was collected at De Wagensdrift, Gauteng Province in August 2005. Voucher specimens (Deutschländer no 95254) have been deposited at the H.G.W.J. Schweickert Herbarium, University of Pretoria and authenticated by Ms M. Nel.

2.2 Extraction of the plant material

Plant material was air dried and the root bark stripped from the roots before it was ground. The ground root bark (215 g) was soaked in 0.5 l acetone for three days while on a shaker. The extract was filtered and the residue extracted again with fresh acetone (3X). The plant extracts were combined and evaporated using a rotator evaporator to yield 87 g (40 %) total extract.

2.3 Determination of hypoglycaemic activity

The α-glucosidase inhibiting activity of the isolated compounds, 1- 4, was tested following the colorimetric micro-plate method as described by Collins *et al*. (1997). The α-glucosidase inhibitory activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-α-D-glucopyranose. The released *p*-nitrophenol yields a yellow colour when the stopping reagent glycine (pH 10), is added. The various compounds (1 mg) were dissolved in 1 ml 100% DMSO to prepare a stock solution and 1 mg acarbose was dissolved in 1 ml buffer (pH 6.5) (1,2-morpholinnoethane sulfonic acid monohydrate-NaOH) (Mes-NaOH) (Fluka 69892) to be used as a positive control (Subramanian *et al*., 2008). Final concentration of acarbose used was 1 µM. The stock solutions of the different compounds were subsequently diluted with Mes-NaOH buffer to obtain final concentrations ranging from 0.02 to 200.0 µg/ml. The substrate consisting of 1 g *p*-nitrophenyl-α-D-glucopyranose (Sigma-Aldrich, N1377-1G) was dissolved in 5 ml buffer and incubated at 37° C for 15 minutes. The enzyme (α-glucosidase type 1 from Bakers Yeast) (Sigma 63412) was prepared and used at the highest concentration (0.58 µg/ml). The different compounds, positive control, enzyme, buffer and substrate were respectively placed in a 96-well microtiter plate well to a volume of 200 µl. After an incubation period of 15 minutes at 37° C in a Labcon incubator the reaction was stopped by adding 60 µl glycine (pH 10). The absorbance was read at 412 nM using a

microtitre plate reader. The various methods were applied to the isolated compounds to establish if the different compounds exhibit different mechanisms to lower blood glucose levels.

Seven of the nine main fractions obtained from the column chromatography were tested *in vitro* on C2C12 myocytes to measure glucose uptake at a concentration of 12,5 µg/ml and insulin (1 µM) in incubation medium was used as positive control. Glucinet reagent kit (Adcock Ingram) was used to execute the assays. According to the results obtained, fractions II, III and VIII showed activity and were subsequently submitted to chromatographic processes to isolate those compounds with probable hypoglycaemic activity.

The hypoglycaemic activity of the isolated compounds **1-4** was also determined in C2C12 myocytes by applying the above mentioned assay. Concentrations used were 50 µg/ml for compounds **1** (81 µM), **2** (113 µM) and **3** (117 µM) and 25 µg/ml for compound **4** (86 µM), while insulin (1 µM) was used as positive control. Due to the lower molecular weight of **4** (290.3) compared to that of **3** (442.7), **2** (426.7) and **1** (619.5), it was decided to test **4** at 25 µg/ml and the others at 50 µg/ml to yield more comparable molar concentrations ranging from 86 µM for **4** to 117 µM for **3**.

2.4 Fractionation of the crude acetone extract of E. undulata.

The crude acetone extract (35 g) was subjected to silica-gel column chromatography (MN Kieselgel 60; 0.063-0.2mm / 70–230 mesh ASTM; Macherey – Nagel GmbH & Co. KG; Düren, Germany) for the isolation of bioactive principles. The column was eluted with hexane: ethyl acetate mixtures of increasing polarity (0 – 100% ethyl acetate) and washed with 100% methanol. Fractions containing the same compounds as determined by thin layer chromatography (TLC) were combined. From the nine fractions pool obtained. The bioactive fractions were further subjected to column chromatographic purification for the identification of bioactive principles. Fraction II was chromatographed over a silica column eluted with hexane: ethyl acetate mixtures of increasing polarity (0–100% ethyl acetate) and yielded (2500 mg; 7.14% yield) compound **3**. Fractions III and IV were combined and chromatographed over a sephadex column using ethanol and yielded compound **1** (14.28 mg; 0.40% yield) and **2** (20.01 mg; 0.57% yield). Fraction VIII was chromatographed over a

sephadex column eluted with ethanol and yielded (12.02 mg; 0.34% yield) compound **4**.

2. 5 General procedures:

Melting points were determined on a Kofler block and are uncorrected. Optical rotations: in $CHCl_3$ solution (Perkin-Elmer 241 MC polarimeter). IR: in KBr (Perkin-Elmer Spectrum One spectrophotometer). ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Bruker 500 MHz spectrometer operating at 500 MHz and 125 MHz, respectively. ^{1}H and ^{13}C NMR chemical shifts are reported with respect to the solvent ($CDCl_3$) signals (δ 7.24 for proton and δ 77.00 for carbon). All the ^{1}H and ^{13}C NMR assignments were in agreement with COSY, HSQC and HMBC spectra. Mass spectra: positive EI mode, 70 eV, CH_2Cl_2 (Hewlett-Packard 5973 spectrometer). HRESIMS: Agilent 6520 Accurate-Mass QTOF LC/MS apparatus.

3. Results and discussion

3.1 In vitro assay results

Seven of the nine main fractions were investigated for hypoglycaemic activity *in vitro* on C2C12 myocytes by using a method developed by Van de Venter *et al*. (2008). This method measures glucose utilization and can be used with long-term exposure of cells to the sample.

The results obtained from the *in vitro* assay on C2C12 myocytes indicated that fractions II (44.8%) (100% used as base line), III (50.6%) and VIII (82.8%) showed potential to lower blood glucose levels (Figure 1). Consequently fractions II, III and VIII were subjected to the isolation processes using different chromatographic techniques to isolate the pure, active compounds. The purification process of the above mentioned fractions resulted in the isolation of compounds **1-4**.

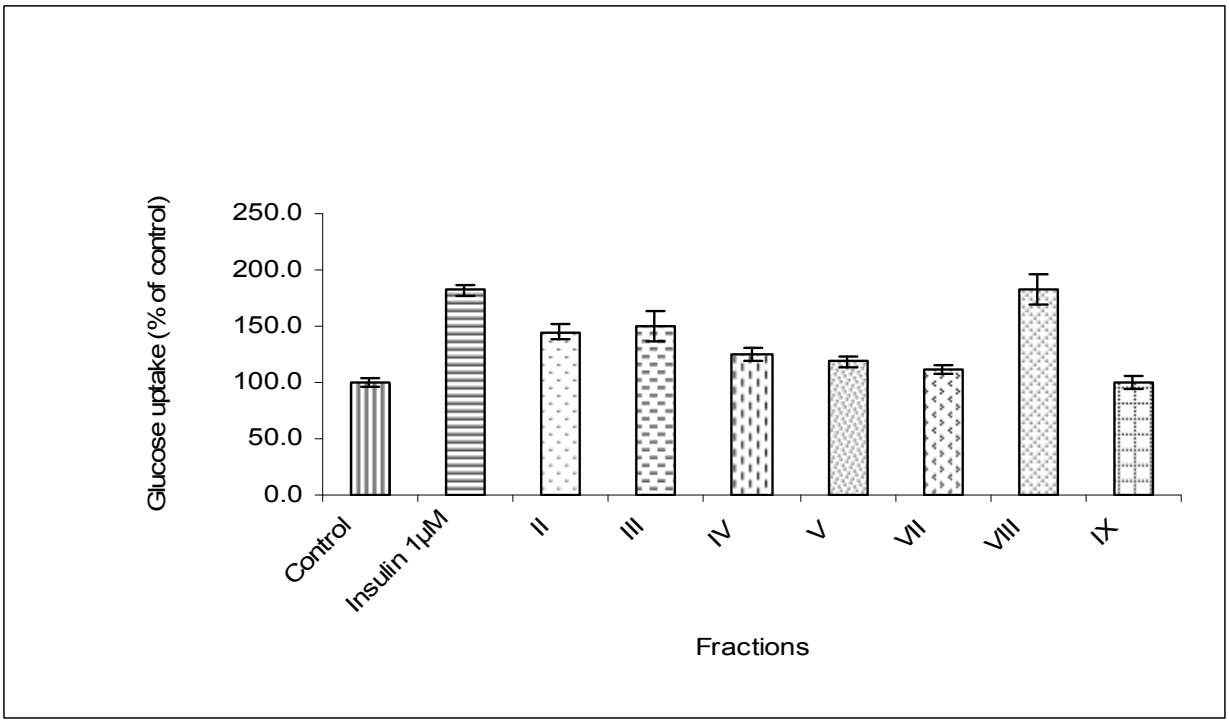


Figure 1. Glucose uptake (as % of control ± standard error of mean N= 8) of the different fractions of the crude plant extract of *E. undulata* (12.5 µg/ml) tested for their hypoglycaemic activity in C2C12 myocytes

The *in vitro* assay on C2C12 myocytes of the different compounds suggest that **4** may have some effect *in vitro* (266.3%) in lowering blood glucose levels at a concentration of 25 µg/ml and **2** has a lesser effect (121.4%) at a concentration of 50 µg/ml (100% used as base line) (Figure 2). In literature no evidence could be found of compounds **1**-**4** being tested for hypoglycaemic activity on C2C12 myocytes.

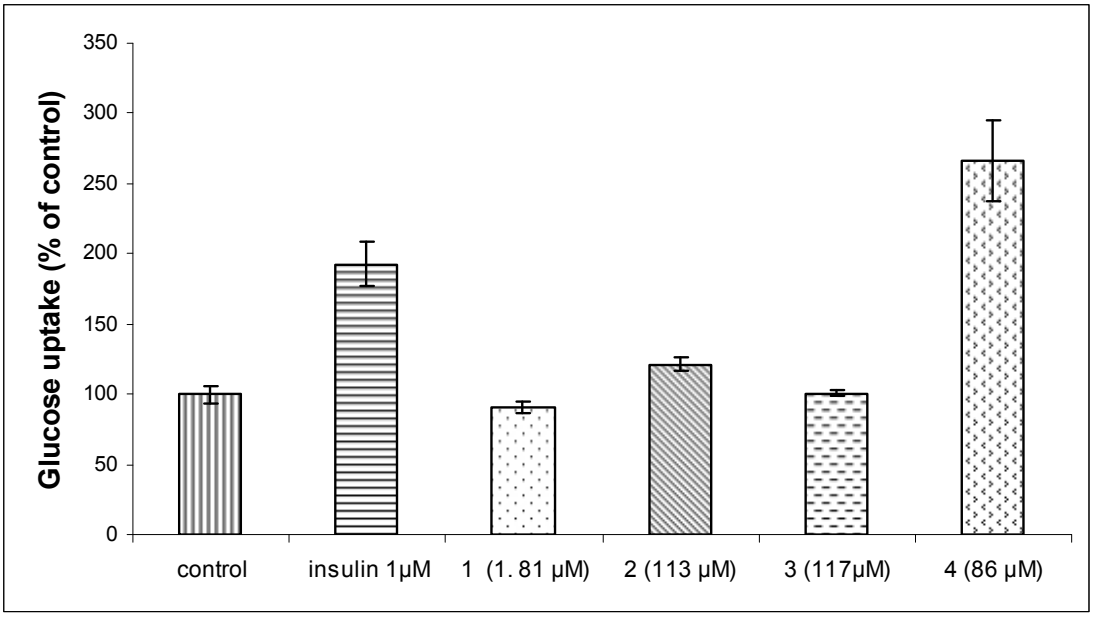


Figure 2. Glucose uptake (as % of control ± standard error of mean N=8) of the compounds isolated from *E. undulata* tested for their hypoglycaemic activity using C2C12 myocytes.

3.2 α-Glucosidase assay

According to the results obtained from the α–glucosidase assays on the different compounds isolated from the root bark of *E. undulata* an inhibition of 68%, 39% and 40% was found on exposure of compounds **1**, **3** and **4** respectively at a concentration of 200.0 µg/ml. IC_{50} values for compounds **1**, **3** and **4** were found to be 4.79 ± 2.54, 6.27 ± 4.75 and 5.86 ± 4.28 µg/ml respectively (Table 1).

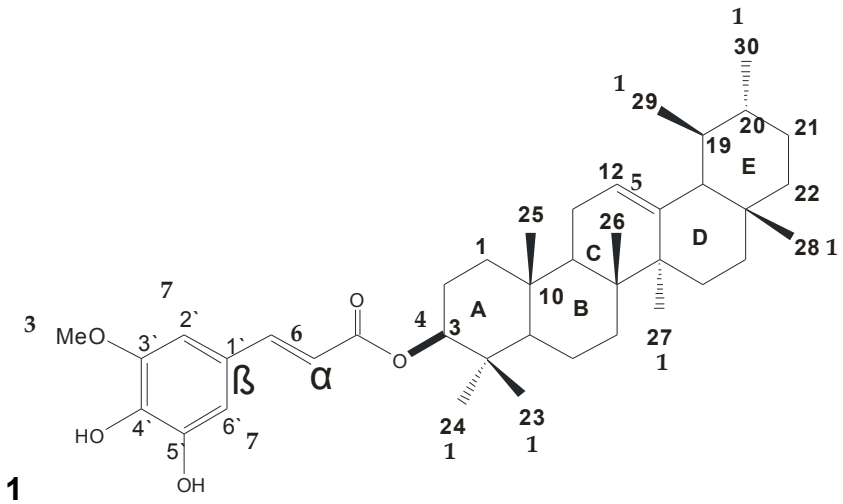
Table 1. Inhibitory effect (IC_{50}) of the four compounds and positive control acarbose for α-glucosidase.

Compound	IC_{50} (µg/ml) α-glucosidase	µM
Acarbose	4.75 ± 3.18	7.35
Crude extract	49.95 ± 0.01	
1	4.79 ± 2.54	7.76
2	32.04 ± 2.79	72.37
3	6.27 ± 4.75	14.69
4	5.86 ± 4.28	20.18

In literature according to Parimaladevi *et al*. (2004) betulin isolated from *Cleome viscose* (25, 50 mg/kg) exhibited significant hypoglycaemic activity in both normal and streptozotocin induced diabetic rats compared with that of the standard drug glibenclamide (10 mg/kg). The results obtained by Rahman *et al*. (2008) indicated that betulin obtained from the methanolic extract of the seeds of *Cichorium intybus* was inactive in inhibiting α-glucosidase.

Mbaze et al. (2007) and Rahman et al. (2008) found that lupeol isolated from *Fagara tessmannii* and *C. intybus* respectively did not inhibit alpha-glucosidase.

According to literature epicatechins displays hypoglycaemic activities and is one of the most active antioxidant constituents (Berregi *et al.,* 2003). Cho *et al.* (2006) found that catechins enhanced the expression and secretion of adiponectin, an adipocyte-specific secretory hormone that can increase insulin sensitivity and promote adipocyte differentiation. They also found that catechin treatment increased insulin-dependent glucose uptake in differentiated adipocytes and augmented the expression of adipogenic marker genes. In search of the molecular mechanism responsible for the inducible effect of (-)catechin on adiponectin expression they found that catechin suppressed the expression of Kruppel-like factor 7 protein. This protein inhibited the expression of adiponectin and other adipogenesis related genes that play an important role in the pathogenesis of type 2 diabetes. Zaid *et al*. (2002) found that treatment with epicatechin (1mM) resulted in a significant increase in the activity of erythrocyte Ca^{++}-ATPase in both normal and type 2 diabetic patience. According to Jalil *et al*. (2009) the intake for 4 weeks of a cocoa extract supplemented with polyphenols (2.17 mg epicatechin, 1.52 mg catechin, 0.25 mg dimer and 0.13 mg trimer g-1 cocoa extract) and methylxanthines (3.55 mg caffeine and 2.22 mg theobromine g-1 cocoa extract) significantly (P, 0.05) reduced the plasma total cholesterol, triglycerides and low-density lipoprotein cholesterol of obese-diabetic rats compared to non-supplemented animals. A study done by Kobayashi *et al*. (2000) using a rat everted sac showed that tea polyphenols consisting mainly of catechins, epicatechin gallate, epigallocatechin and epigallocatechin gallate inhibited sodium-dependent glucose transporters. This indicated that tea polyphenols interacts with sodium-dependent glucose transporters as antagonist-like molecules, possibly playing a role in controlling dietary glucose uptake in the intestinal tract.



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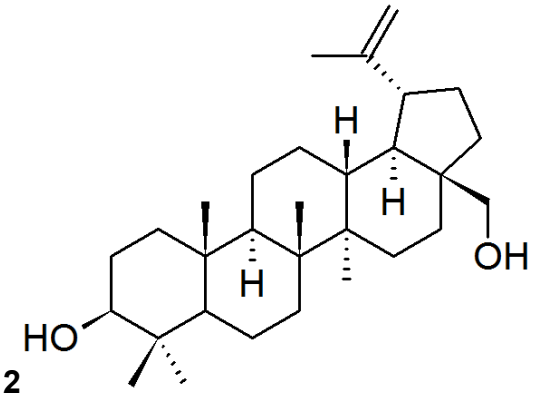

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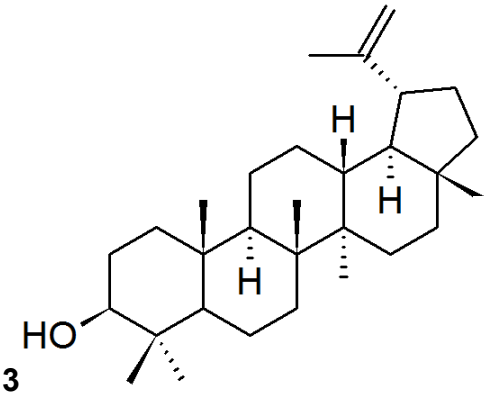

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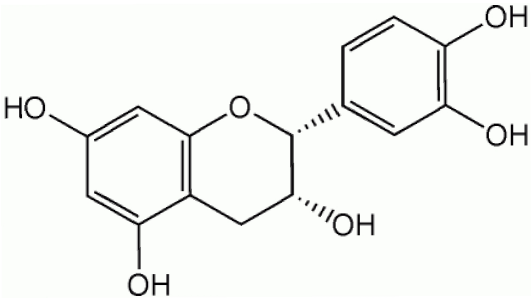

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Figure 3.Chemical structures for compounds 1 – 4.

4. Phytochemical examination

Bioassay-guided fractination of the acetone extract *in vitro* in C2C12 myocytes resulted in the isolation of a new α-amyrin-3*O*-ß-(5-hydroxy) ferulic acid,in addition to three known compounds: betulin and lupeol, and epicatechin (Figures 3).

Hydrolysis. A 5 mg portion of compound **1** was added to 5 ml of aqueous KOH and left under nitrogen overnight at room temperature. The reaction mixture was neutralized with 10% HCl. The mixture was extracted with $CHCl_3$, and then purified by silica gel column chromatography using 30% EtOAc in hexane.

*4.1 Compound **1***

Compound (1), was purified with a Sephadex column eluted with dichloromethane and methanol, 99:1. HRESIMS showed a molecular peak at 619.47 *m/z* (M^++H) corresponding to the molecular formula $C_{40}H_{58}O_5$ -$[\alpha]_D^{22}$ +24,3 (*c* 0.50, $CHCl_3$)

The IR spectra revealed the presence of bands at 3362, 2945, 2870, 2359, 2341, 1700, 1643 and 1606 cm^{-1} of α, ß-unsaturated carbonyl, aromatic ring and cyclic alkane. The UV spectra gave two absorption bands at λ_{max} 228, 320 nm of the conjugated aromatic system. The ^1H NMR data of **1** showed signals for six singlet methyls (δ_H 1.06, 1.01, 0.99, 0.92, and 0.78), and two methyl doublets (0.86, d, *J*=5.6 Hz; 0.78, d, *J*=5.8 Hz), methoxyl singlet (3.86), a methine proton bearing an ester at 4.66 (dd, *J*= 10.3, 5.9 Hz), olefinic proton at 5.10 (t, *J*= 1.5 Hz), in addition to 5-hydroxy ferulic acid signals at 7.50, 6.26 (d, *J*=16.1 Hz, α, ß protons), 6.79, 6.61 (d, *J*= 0.5 Hz, H-2', 6'), the aforementioned data indicated the presence of ursane type triterpene similar to α-amyrin esterified in position C-3 with 5-hydroxy ferulic acid. The ^{13}C DEPT-135, HSQC, HMBC and NOESY spectra of **1** confirmed the proposed structure of α-amyrin skeleton and showed eight methyl carbon signals, nine methylene, seven methane groups and six quaternary carbons, in addition to those signals attributed to 5-hydroxyferulic acid (Table 2). Thus, from the above data and comparing to the literature for similar structures (Garson *et al*., 2006; Nakagawa *et al*., 2004; Ohsaki *et al*., 2004) compound **1** was confirmed to be α-amyrin derivative esterified with 5-hydroxy ferulic acid at position C-3. The position of the methoxyl group at C-3' was deduced from the different chemical shifts of the aromatic proton H-2' and H-6' and confirmed by the HMBC correlations which showed, amongst other correlations, a cross peak between MeO/C-3' and H-2'/C-3', C-4'.

Table 2. ^{1}H and ^{13}C NMR spectral data of compound **1in $CDC1_3$.**

No.	**C**	**H,** *J* Hz	**No.**	**C**	**H** *J* Hz
1	38.4 t	1.13 m, 1.67 m	21	31.2 t	1.31 m, 1.39 m
2	23.7 t	1.60 m	22	41.5 t	1.29 m, 1.46 m
3	81.0 d	4.66, dd, *J*= 10.30, 5.90	23	28.1 s	------
4	37.9 s	------	24	16.8 q	0.92 s
5	55.3 d	0.87 m	25	15.71 q	0.99 s
6	18.2 t	1.41 m, 1.52 m	26	16.9 q	1.01 s
7	32.8 t	1.33 m, 1.55 m	27	23.2 q	1.06 s
8	40.0 s	----------	28	28.7 q	0.78 s
9	47.5 d	1.55 m	29	17.5 q	0.78 d, *J*= 5.80
10	36.8 s	----------	30	20.9 q	0.86 d, *J*= 5.60
11	23.3 t	1.91 m	1'	126.6 s	-------
12	124.3 d	5.10, t, *J*= 1.50	2'	109.3 d	6.79 d, *J*=1.50
13	139.6 s	--------	3'	147.1 s	----------
14	42.1 s	----------	4'	134.8 s	----------
15	26.6 t	0.96 m, 1.83 m	5'	144. 0 s	----------
16	28.1 t	0.87 m, 1.83 m	6'	103.1 d	6.61 d, *J*=1.50
17	33.7 s	----------	α	144.6 d	7.50 d, *J*=15.90
18	59.0 d	1.31 m	ß	116.7 d	6.25 d, *J*=15.90
19	39.6 d	0.93 m	C=O	167.2 s	----------
20	39.8 d	1.31 m	OMe	56.2 q	3.86 s

The relative configurations of C-3 could not be determined from the recorded NOESY spectra of **1**; however, hydrolysis of **1** gave α-amyrin with a 3ß-OH

configuration, which was identified on the basis of comparison of spectral data reported in literature.

5. Conclusions

Phytochemical studies conducted on *Euclea* species by Costa *et al*. (1978) demonstrated the presence of triterpenoids in the stems and leaves. Two naphthoquinones, diospyrin and 7 methyl-juglone, were isolated from the root, stem and fruit of *E. undulata* var. *myrtina* by Van der Vyver and Gerritsma (1973; 1974). Chemical analysis indicated the presence of 3.26 % tannins in bark, saponins and reducing sugars in leafs and stems, but no alkaloids, naphthoquinones or cardiac glycosides (South African National Biodiversity Institute, 2005). In this study it seemed as if the root bark of *E. undulata* var. *myrtina* was devoid of naphthoquinones. These contradicting findings may be attributed to the extraction procedures and different environmental factors such as geographical and seasonal variation. Unfortunately the localities and time of collection by Van der Vyver and Gerritsma, (1973; 1974) could not be established. Khan (1985) reported that the relative amounts of 7-methyljuglone and lupeol in *E. natalensis* are season dependent and interrelated and could indicate some biogenetic relationship between the two natural products.

It was reported in literature that aqueous leaf extract of *E. undulata* demonstrated antimicrobial activity *in vitro*, at a concentration of 40 mg/ml, against *Staphylococcus aureus*. This result, together with the presence of tannins in the leaves, supports its use as anti-diarrhoeal and for the relief of tonsillitis. No activity against *Pseudomonas aeruginosa*, *Candida albicans* or *Mycobacterium smegmatis* was shown in the preliminary tests (South African National Biodiversity Institute, 2005).

Narender *et al*. 2009 reported on the antihyperglycaemic activity of synthesized α-amyrin derivatives *in vivo* as well as Singh *et al.* 2009 on the antihyperglycaemic activity of α-amyrin acetate isolated from the aerial roots of *Ficus bengalensis* in normal and diabetic rats as well as in db/db mice.

The phytochemical examination coupled with bioassay-guided fracination of the crude acetone extract of the root bark of *E. undulata* var. *myrtina* afforded a new triterpene and three other known compounds **2**-**4**. The results obtained from the *in vitro* assays on the main fractions with C2C12 myocytes, 3T3-L1 preadipocytes and Chang liver cells indicated that three of the main fractions showed hypoglycaemic activity. The identified main fractions were sub-sequently subfractioned and four compounds isolated*;* α-amyrin-3*O*-ß-(5-hydroxy) ferulic acid, lupeol, betulin, and epicatechin. These compounds, isolated for the first time from *E. undulata* var. *myrtina,* were evaluated for, their hypoglycaemic activities by executing *in vitro* assays on C2C12 myocytes, as well as their ability to inhibit the carbohydrate-hydrolising enzyme α-glucosidase. The present study reports for the first time the α-glucosidase inhibitory activity and glucose utilization by C2C12 myocytes of an acetone extract of *E. undulata* and its purified compounds.

The results suggest that epicatechin has some effect *in vitro* to lower blood glucose levels, whereas α-amyrin-3*O*-ß-(5-hydroxy) ferulic acid has the ability to inhibit α-glucosidase. These findings corroborate the ethnomedicinal use of *E. undulata* by traditional healers for the treatment of diabetes.

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