

The hypoglycaemic activity of four plant extracts traditionally used in South Africa for diabetes are discussed in this chapter.

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**Hypoglycaemic activity of four plant extracts traditionally used in South Africa for diabetes**

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### 3.1 Abstract

*Aim:* To validate selected plant species for hypoglycaemic activity.

*Materials and methods:* Four plant species were investigated for hypoglycaemic activity by evaluating the inhibiting effects on carbohydrate-hydrolysing enzymes; alpha-glucosidase and alpha-amylase. Acetone plant extracts were screened against C2C12 myocytes, 3T3-L1 preadipocytes and Chang liver cells by measuring the glucose uptake. Cytotoxicity was conducted in preadipocytes and hepatocytes cell lines.

*Results:* Extract of *Euclea undulata* rootbark exhibited the highest activity, displaying a glucose uptake of 162.2% by Chang liver cells at 50 µg/ml. The fifty percent inhibitory concentration of the acetone extract of *E. undulata* was found to be 49.95 µg/ml and 2.8 µg/ml for alpha-glucosidase and alpha-amylase enzymes respectively. No cytotoxicity was recorded for *Euclea undulata*, while *Schkuhria pinnata* and *Elaeodendron transvaalense* exhibited cytotoxicity at 12.5 µg/ml. Alpha-glucosidase and alpha-amylase assays showed inhibitory activity on the enzymes for three plant extracts.

*Conclusion:* *E. undulata*, *S. pinnata* and *E. transvaalense* showed *in vitro* hypoglycaemic activity. *S. pinnata* and *E. transvaalense* indicated cytotoxicity on 3T3-L1 preadipocytes and Chang liver cells. *E. undulata*, *P. divaricata* and *E. transvaalense* inhibited alpha-glucosidase and alpha-amylase enzymes.

*Ethnopharmacological relevance:* The screening of plant extracts scientifically validated traditional use of *E. undulata* for treatment of diabetes. Cytotoxicity results revealed that the acetone extracts of *S. pinnata* and *E. transvaalense* are toxic and raised concern for chronic use.

Authors Keywords: Alpha-amylase; alpha-glucosidase; hypoglycaemic activity; *Euclea undulata*.

### 3.2. Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia and glucose intolerance, either due to insulin deficiency or to impaired effectiveness of insulin's action or to a combination of both. It is regarded as a non-curable but controllable disease. In Africa, diabetes mellitus is no longer a rare disease and recent investigations of non-communicable diseases indicated an increase in prevalence from 1% to 20% of the population. In South Africa the prevalence is between 4% and 6%. The global prevalence was estimated at 2.8% in 2000 (171 million people) and it is projected that 4.8% (366 million people) will be affected by 2030 if no action is taken (Wild *et al.*, 2004). The progressive nature of the disease necessitates constant reassessment of glycaemic control in people with diabetes and appropriate adjustment of therapeutic regimens. The burden of the disease is high not only because life-long treatment is necessary but also due to the prohibitive cost and unavailability of treatment in rural areas. The rate of limb amputations varies from 1.4% to 6.7% of diabetic patients, while foot complications and the annual mortality linked to diabetes worldwide is estimated at more than one million (World Health Organization, 2005). If glycaemic control is no longer obtained with a single agent, the addition of a second or third drug is usually more effective than switching to another single agent. Most patients with type II diabetes will ultimately require multiple anti-diabetic agents to maintain adequate glycaemic control (Gerich, 2001). The 2007 World Health Organization Regional office for Africa report stated that one of its strategy aims was to support research in community interventions, including traditional medicine. It is essential for the above reasons that the search for new anti-diabetic agents continues.

The most common problem encountered in the detection of pharmacological activity in plant extracts is that even extracts from single plants are mixtures of several compounds, and these can vary in concentration or composition depending on ecological changes (Farnsworth, 1993). Traditional remedies seldom comprise a single plant extract, and in many cases the therapeutic benefits are attributed to the consumption of plant mixtures in which whole plants or plant parts are prepared and consumed in combination (Etken, 1986). This complicates the pharmacological investigation of the preparation, because it has to be

determined which of the many constituents of a single plant is the active one. *In vitro* screening methods have a further inherent problem in that some compounds showing good activity in an *in vitro* assay may be metabolized *in vivo* into inactive metabolites. Conversely, some extracts show only *in vivo* activity due to the metabolism of inactive compounds into active compounds (Farnsworth, 1993). It is clear from the above that screening the activity of plant extracts may vary from batch to batch. However, the continued large scale use of plants in traditional medicines in developing countries, such as South Africa, necessitates the validation of the use of these plants for various diseases as being effective and safe.

The hypoglycaemic activity of four plant species traditionally used for the treatment of diabetes by South African traditional healers and herbalists was investigated. Species included the small herbaceous weed, *Schkuhria pinnata* (Lam.) Cabrera (Asteraceae). A decoction is prepared from the whole plant and used by traditional healers for the treatment of diabetes in the Ga-Rankuwa area, Gauteng, South Africa (Matibe, pers. comm.) (Deuschländer *et al.* 2009) *Pteronia divaricata* (P.J. Bergius) Less. (Asteraceae), is a twiggy, dense shrublet which grows up to 1 m tall. A tea is brewed from this plant and used in the treatment of diabetes by a traditional healer in the Clanwilliam district, Western Cape, (Maltz, pers. comm.) (Deuschländer *et al.* 2009). Both *Euclea undulata* var. *myrtina* Thunb. (Ebenaceae), a dense, erect, evergreen dioecious shrub or small tree and *Elaeodendron transvaalense* (Burt Davy) R.H. Archer (Celastraceae), a shrub or small, multi-branched tree from 5 to 10 m tall, are used in the Venda region, Limpopo Province, by local traditional healers and herbalists for the treatment of diabetes (Tshikalange, pers. comm.) (Deuschländer *et al.* 2009). A tea is brewed from the rootbark of *E. undulata* var. *myrtina* and from the stembark of *E. transvaalense* for this purpose.

### 3.3. Materials and Methods

#### 3.3.1 Plant material

Plant material was obtained from traditional healers as well as collected in the veld at various localities. *S. pinnata* (Deuschländer 112982) was obtained from the

Medical University of South Africa at Ga-Rankuwa, Gauteng, and additional material was collected from the University of Pretoria's experimental farm. *P. divaricata* was obtained from a traditional healer in the Clanwilliam district, Western Cape. *E. undulata* (Deutschländer 95254) was collected in the Lydenberg district, Mpumalanga whereas *E. transvaalense* was collected in the Venda region, Limpopo, (Tshikalange 092524). Plant material was authenticated by Ms M. Nel and Prof. A.E. van Wyk at the H.G.W.J. Schweickert Herbarium, University of Pretoria where voucher specimens are being kept.

Entire *S. pinnata* and *P. divaricata* plants were used for the preparations of the crude extracts. Acetone and ethanol extracts were prepared for *S. pinnata* whereas only acetone extracts were prepared for *P. divaricata* and the rootbark and stembark of *E. undulata* and *E. transvaalense* respectively. An ethanol extract was also prepared for *S. pinnata* to compare the hypoglycaemic activity of ethanol and acetone extracts. Results obtained from ethanol and acetone extracts were more or less similar but the toxicity for the ethanol extracts was higher, hence acetone extract was chosen for other investigations. Acetone / ethanol were originally chosen as solvents as their polarities are close to that of water.

### 3.3.2. Preparation of plant extracts

Initially the air-dried plant material was ground by using a mill and weighed (*S. pinnata* 70g; *E. undulata* 28g; *P. divaricata* 24g and *E. transvaalense* 94g). Ground plant material was extracted with cold acetone except in the case of *S. pinnata*, where a separate ethanol extract was also prepared. The volume of solvent varied from 300 ml – 500 ml depending on the quantity of ground plant material (24 g-94 g). This procedure was repeated three times to ensure that all possible compounds would be extracted. The filtered plant extracts of the three repetitions were combined and dried by making use of a Rotavapor (Büchi R-114) and liquid nitrogen. The percentage yield obtained for the various plant extracts were as follow: *Schkuhria pinnata* (1.92%); *E. undulata* (3.05%); *P. divaricata* (4.98%) and *E. transvaalense* (0.91%).

### 3.3.3 *In vitro* anti-diabetic and toxicity screening

The *in vitro* anti-diabetic and toxicity screening were done by using a method with slight modifications as described by Van De Venter *et al.* (2008). This method measures glucose utilization and can be used for long-term exposure of the cells to the sample.

Plant extracts prepared (reconstituted in undiluted DMSO, vortexed and left for 15 minutes, before further dilution with the respective growth medium) were tested on three cell lines namely: Murine C2C12 myoblasts, Chang liver cells and 3T3-L1 preadipocytes (Highveld Biological, South Africa). The final DMSO concentration never exceeded 0.25% and a vehicle control was always included in each experiment. At this concentration of DMSO, no effects were seen on cell viability. Hepatic, preadipocytes and myocytes were used because hepatic and, preadipocytes and muscle cells have different glucose transporters, and these react differently to insulin stimulation and have different roles in carbohydrate metabolism. This method has the potential to detect not only alterations in glucose uptake but any changes that might occur in the metabolic pathways where glucose plays a role.

### 3.3.4. *Routine maintenance of cell cultures*

All cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The Chang liver and C2C12 cell lines were fed fresh growth medium every two to three days, consisting of RPMI1640 (Highveld Biological, South Africa) medium supplemented with 10% fetal bovine serum. The 3T3-L1 cells were cultured in DMEM (1.5g/l NaHCO<sub>3</sub>) (Highveld Biological, South Africa) with 10% bovine serum.

#### 3.3.4.1 *Glucose uptake experimental procedures on C2C12 myocytes*

Cells in monolayer culture (at less than 70% confluence) were dislodged through brief exposure to 0.25% trypsin in PBSA, counted and suspended (25 000 cells/ml) in growth medium (RPMI1640 with 10% fetal bovine serum) and the 200 µl/well seeded into new 96-well plates. The seeded plates were incubated at 37°C for 3 to 4 days without changing the medium.

To measure glucose uptake, insulin (1  $\mu\text{M}$ ) was used as positive control whereas 50  $\mu\text{g/ml}$  plant extract (acetone for all four plants, as well as ethanol for *S. pinnata*) firstly in incubation medium and then the Glucinet reagent kit (Adcock Ingram) were used to execute the assays.

All medium were aspirated from the cells and then 50  $\mu\text{l}$  of the incubation medium/insulin/plant extract was added into the microtiter wells and incubated for one hour. Thereafter 20  $\mu\text{l}$  was transferred from each well to a clean well plate and 200  $\mu\text{l}$  Glucinet reagent was added per well. The plates were incubated at 37°C for 15 minutes and the absorbance was read at 492 nm using a microplate reader. Due to the short exposure of the C2C12 myocytes to the plant extracts no toxicity assay were done on this particular cell line.

#### *3.3.4.2 Glucose uptake experimental procedures on Chang liver cells and 3T3-L1 preadipocytes*

Chang liver cells and 3T3-L1 preadipocytes (at less than 90% confluence) were dislodged through brief exposure to 0.25% trypsin in PBSA, counted, suspended (30 000 cells/ml) in growth medium (RPMI1640 with 10% fetal bovine serum) and then 200  $\mu\text{l}$ /well seeded into new 96-well plates. The seeded plates were incubated at 37°C for five days without changing the medium. On day three the plant extracts or positive control was administered to the relevant vials according to the plate-well layout by adding 10  $\mu\text{l}$  extract or positive control to each well to a final concentration of 12.5  $\mu\text{g/ml}$  extract or 1  $\mu\text{M}$  metformin (for Chang liver cells). Incubation was continued until day five when the glucose uptake experiment was done. The positive control for 3T3-L1 preadipocytes was 1 $\mu\text{M}$  insulin but this was only added on day five during the glucose uptake experiment in order to avoid the development of insulin resistance. Fifty  $\mu\text{g/ml}$  crude plant extract in the incubation medium (RPMI1640 with 8 mM glucose, 0.1% BSA) and Glucinet reagent (Sigma) was used to execute the assays as was done for C2C12 cells, except the incubation period was 1.5 hours for 3T3-L1 and 3 hours for Chang liver cells. Toxicity assays were done by adding MTT (0.5 mg/ml in RPMI1640:10% fbs) to the wells of the last three rows of each plate. MTT is a yellow water soluble tetrazolium dye that is reduced by living

cells, but not dead cells, to a purple formazan product that is insoluble in aqueous solutions (Mosmann, 1983). Toxicity results were compared using Student's *t*-test (Two-sample assuming equal variances P (T<=t) two tail) with  $p < 0.05$  considered as significant.

#### 3.3.4.3. Dose response assay

The best hypoglycaemic effect was obtained from the acetone extract of *E. undulata*, a dose response assay was conducted on Chang liver cells for this extract. The concentration used ranged from 16  $\mu\text{g/ml}$  to 250  $\mu\text{g/ml}$ .

#### 3.4 Alpha-glucosidase inhibiting activity

Alpha-glucosidase is an enzyme produced by the villi lining the small intestine of mammals which is responsible for the hydrolysis of disaccharides to monosaccharide that can then be absorbed and consequently elevate blood glucose levels. Inhibition of intestinal  $\alpha$ -glucosidase has been used successfully to treat patients with both type I and II diabetes mellitus (Collins *et al.*, 1997; Hiroyuki, *et al.*, 2001).

The alpha-glucosidase inhibiting activity of the four acetone plant extracts was tested following the method described by Collins *et al.* (1997). This microplate assay offers convenience, speed and reproducibility. The alpha-glucosidase inhibitory activity was determined by measuring the release of p-nitrophenol from p-nitrophenyl- $\alpha$ -D-glucopyranose. The released p-nitrophenol yields a yellow colour when the stopping reagent, glycine (pH10), is added.

Two milligrams of each of the plant extracts were dissolved in 1 ml 100% DMSO to prepare a stock solution and 1mg acarbose was dissolved in 1 ml buffer to be used as the positive control (Subramanian *et al.*, 2008). The plant extract stock solutions were subsequently diluted with buffer (1,2-Morpholinnoethane sulfonic acid monohydrate-NaOH) (Mes-NaOH) (pH 6.5) (Fluka 69892) to obtain a final concentrations ranging from 0.02 to 200.0  $\mu\text{g/ml}$ . The substrate consisting of 1 g p-nitrophenyl- $\alpha$ -D-glucopyranose (Sigma-Aldrich, N1377-1G) was dissolved in 5 ml buffer and incubated at 37° C for 15 minutes. The enzyme ( $\alpha$ -glucosidase type 1 from Bakers Yeast) (Sigma 63412) was prepared and used at the highest concentration (0.58  $\mu\text{g}/\mu\text{l}$ ). The plant extract, positive

control, enzyme, buffer and substrate were placed in the 96-well microtiter plate to a volume of 200  $\mu$ l. After an incubation period of 15 minutes at 37° C in a Labcon incubator the reaction was stopped by adding 60  $\mu$ l glycine (pH10). The absorbance was read at 412 nm in a microtitre plate reader.

### 3.5 *Alpha-amylase inhibiting activity*

Alpha-amylase is a glycoside hydrolase enzyme that breaks down long-chain carbohydrates by acting on the  $\alpha$ -1,4-glycosidic bonds, yielding maltotriose and maltose molecules from amylose, or maltose and glucose from amylopectin. Two types of amylase are found in the human body, one in the saliva that breaks down starch into maltose and dextrin, better known as ptyalin and the other pancreatic  $\alpha$ -amylase that cleaves the  $\alpha$ -1,4-glycosidic linkages of amylose to yield dextrin, maltose or maltotriose.

The alpha-amylase inhibiting activity of the four plant extracts were tested based on the methods described by Park and Johnson (1949), Bernfeld (1955) and Slaughter *et al.* (2001). According to this method the reduction of ferricyanide ions in alkaline solutions followed by the formation of Prussian blue (ferric ferrocyanide) is measured quantitatively as the basis for the estimation of glucose levels. The alpha-amylase assay consists of two steps namely the enzyme and the Prussian blue assays.

#### 3.5.1. *Enzyme assay*

The enzyme assay was executed in 32, 20 ml Pyrex test tubes. Distilled water was added to the test tubes (blank  $2.5 \times 10^3$   $\mu$ l, negative control  $1.25 \times 10^3$   $\mu$ l, assay  $1 \times 10^3$   $\mu$ l and the colour test  $2.25 \times 10^2$   $\mu$ l). Plant extracts and the positive control (acarbose) were added to the test tubes for the assay as well as for the colour test (250  $\mu$ l) at four different concentrations namely  $1.25 \times 10^3$ ,  $1.0 \times 10^3$ ,  $7.5 \times 10^2$  and  $5.0 \times 10^2$   $\mu$ g/ml (Subramanian *et al.*, 2008). Alpha-amylase type VI-B from porcine pancreas (Sigma A; 3176-1MU) was prepared fresh by dissolving 20  $\mu$ g porcine pancreatic enzyme in 30 ml ice cold distilled water, sonicated for 15 minutes and placed on ice. The enzyme ( $1.25 \times 10^3$   $\mu$ l) was added to the test tubes containing the negative and positive controls as well as the assays and incubated at 25° C for 5 minutes in a warm water bath. Potato starch (Sigma EC232-686-4) (0.5%) was prepared by dissolving 60  $\mu$ g in  $120 \times 10^3$   $\mu$ l sodium phosphate buffer (pH 6.9).

Starch and buffer solution was heated for 20 minutes at a temperature of 60 – 70 °C. The prepared starch ( $2.5 \times 10^3 \mu\text{l}$ ) was added to all the test tubes and placed in a warm water bath at 37°C for 3 minutes to activate the enzyme reaction.

### 3.5.2 Prussian blue assay

Thirty two Eppendorf tubes (2ml) were prepared each containing 300  $\mu\text{l}$  sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) to which 300  $\mu\text{l}$  sample from each of the porcine enzyme test tubes were added and centrifuged. A second set of 32 Eppendorf tubes (2ml) was prepared containing 950  $\mu\text{l}$  of distilled water each to which 50  $\mu\text{l}$  from the first set of Eppendorf tubes were added.

A second set of 32, 20 ml Pyrex test tubes were used and 500  $\mu\text{l}$  of solution A (16 mM KCN, 0.19 M  $\text{Na}_2\text{CO}_3$ ) and 500  $\mu\text{l}$  of solution B (1.18 mM  $\text{K}_3\text{Fe}(\text{CN})_6$ ) were added to each of these test tubes. A 500  $\mu\text{l}$  of sample was taken from the second set of 32 Eppendorf tubes and added to the second set of test tubes containing solution A and solution B, these were vortexed, covered with foil and placed in boiling water for 15 minutes, removed and placed at room temperature for 15 minutes to cool down. Solution C ( $2.5 \times 10^3 \mu\text{l}$ ) (3.11 mM  $\text{NH}_4\text{Fe}(\text{SO}_4)_2$ ; 0.1 g sodium dodecyl sulphate in 1000 ml 0.05 N  $\text{H}_2\text{SO}_4$ ) was added and left for 2 hours and 30 minutes in the dark for colour development. The absorbance was read at 690 nm on a Beckman Coulter DU 720 General Purpose UV/Vis spectrophotometer.

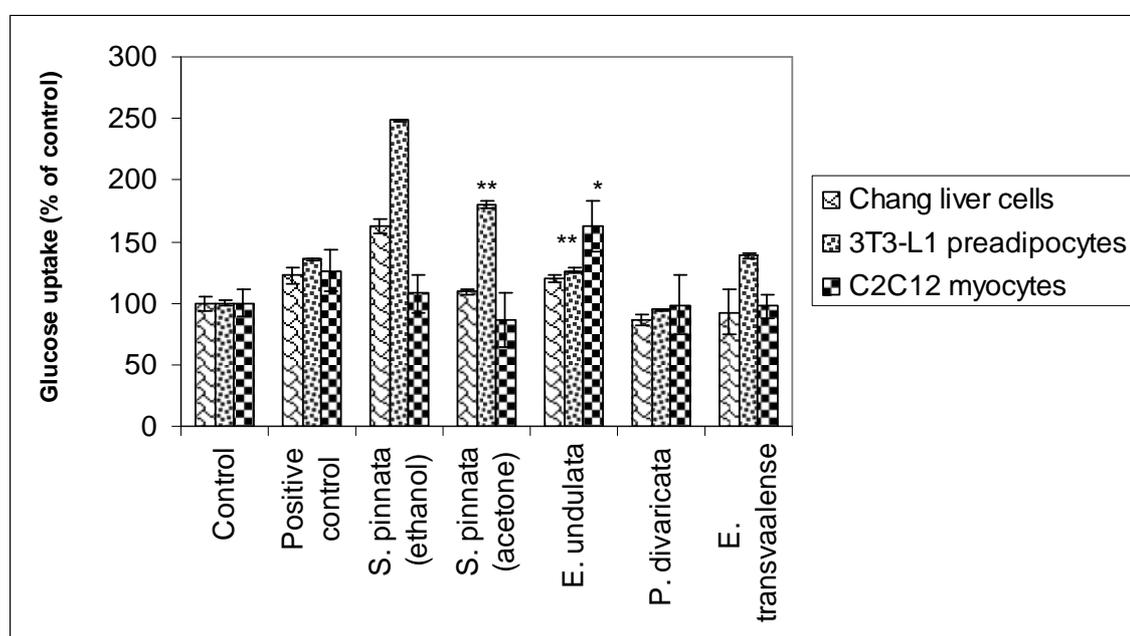
## 3.6. Results

### 3.6.1 In Vitro

The results obtained for *in vitro* assay on the C2C12 myocytes on the plant extracts (at 50  $\mu\text{g}/\text{ml}$ ), indicated that *E. undulata* showed some potential in lowering blood glucose levels (162.2%; 100% serves as base line). This was also observed for *S. pinnata* (ethanol) (107.5%; 100% serves as base line) (Figure. 3.1). Not much activity was observed when extracts of *S. pinnata* (acetone), *P. divaricata* or *E. transvaalense* were used (Figure. 3.1).

The toxicity of these plant extracts in C2C12 myocytes was not determined because of the short exposure time of the cells to the plant extracts (Van de Venter *et al.*, 2008).

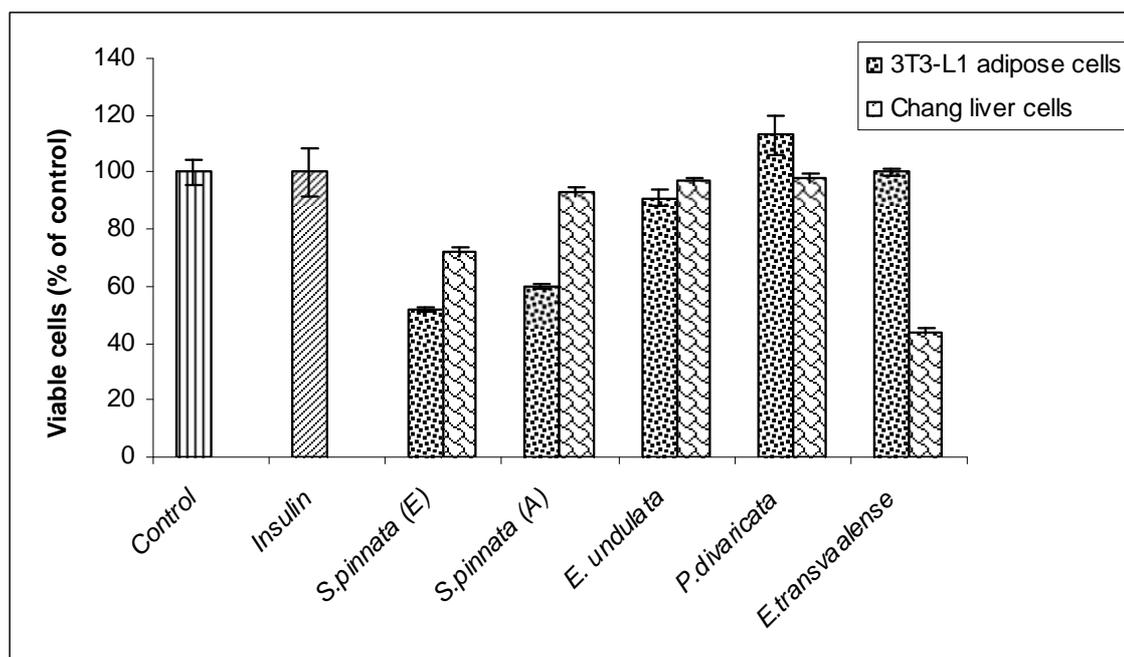
The *in vitro* assay in 3T3-L1 preadipocytes indicated that *S. pinnata* (ethanol) (248.2%) and *Schkuhria pinnata* (acetone) extracts (179.6%) had some potential to lower blood glucose levels at a concentration of 50 µg/ml as did that of *E. transvaalense* (138.6%), and to a lesser extent *E. undulata* (126.0%) whereas *P. divaricata* showed no glucose uptake (Figure.3.1).



**Figure 3.1.** Glucose uptake (as % of control  $\pm$  standard error of mean, N = 8 for C2C12 and 10 for Chang liver and 3T3-L1) of the various plant extracts tested for their hypoglycaemic activity. (\* P < 0.05; \*\* P < 0.005 compared to control using student's one and two-tailed t-test).

The toxicity assay revealed, however, that the results obtained with *S. pinnata* (ethanol) and (acetone) extracts were unreliable due to the toxicity of these extracts on 3T3-L1 preadipocytes (Figure.3.2). It should be noted that the toxic effect was induced by exposure of cells to an extract concentration of 12.5 µg/ml for 48 hours prior to measurement of glucose uptake in the presence of 50 µg/ml incubation medium for 1.5 hours. The *in vitro* assay in Chang liver cells indicated that *S. pinnata* (ethanol) extract (162.3%) and to a

lesser extent *E. undulata* extract (119.7%) had some potential at a concentration of 50 µg/ml. *P. divaricata* as well as *E. transvaalense* extracts showed no potential (Figure. 3.1). The toxicity assay revealed, however, that *E. transvaalense* and *S. pinnata* (ethanol) extracts were toxic to Chang liver cells (Figure.3.2).



**Figure 3.2. Toxicity (as % of control  $\pm$  standard error of mean, N = 6) of the various plant extracts tested for their hypoglycaemic activity.**

The *in vitro* assay results indicated that four of the five plant extracts tested namely *S. pinnata* (ethanol), *S. pinnata* (acetone), *E. undulata* and to a lesser extent *E. transvaalense* showed positive results in increasing glucose uptake by 3T3-L1 preadipocytes, C2C12 myocytes and by Chang liver cells (Figure. 3.1). *P. divaricata* showed no ability in increasing glucose uptake.

The results obtained were interpreted by making use of the scoring system developed by Van de Venter *et al.*, (2008) to determine which plant extract to analyze further. According to this scoring system the potential anti-diabetic activity as well as the toxicity of the plant extract is taken into consideration to assist in the selection of the most active and least toxic plant extract (Table 3.1).

According to the results obtained from the different *in vitro* assays done on the various plant extracts *E. undulata* (score +3) was chosen for further analysis because, it was not toxic and it showed some hypoglycaemic activity in all three cell lines tested.

**Table 3.1. Effect of plant extracts on glucose utilization and toxicity in Chang liver cells, 3T3-L1 preadipocytes and C2C12 myocytes using the scoring system of Van De Venter *et al.* ( 2008).**

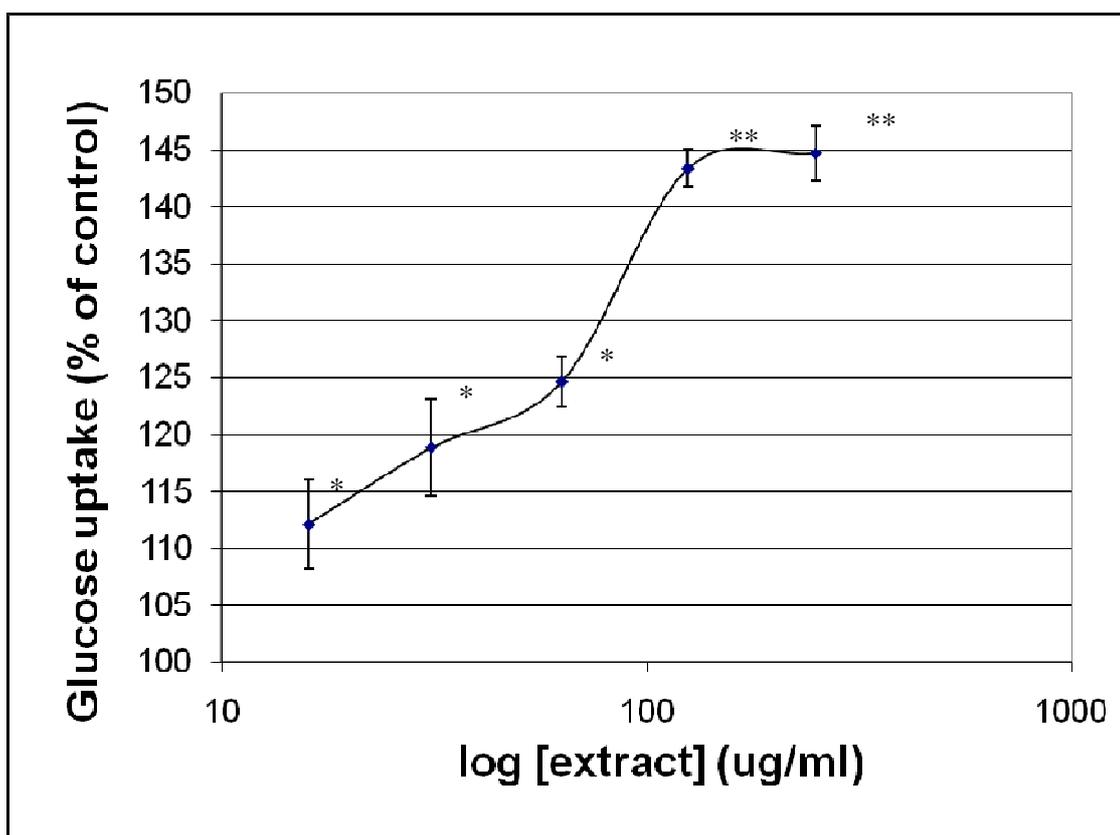
Species	Chang liver cells		3T3-L1		C2C12	Activity score (max +6)	Toxicity score (min -4)	Total score
	Active	Toxic	Active	Toxic	Active			
<i>S. pinnata (E)</i>	+2	-2	+2	-2	0	+4	-4	0
<i>S. pinnata (A)</i>	0	0	+2	-2	0	+4	-4	0
<i>E. undulata</i>	+1	0	+1	0	+1	+3	0	+3
<i>P. divaricata</i>	0	0	0	0	0	0	0	0
<i>E.transvaalense</i>	0	-2	+1	0	0	+1	-2	-1

Dose response assays were carried out *in vitro* on *E. undulata* extract to determine the hypoglycaemic effect on Chang liver cells at different concentrations. Figure 3.3 depicts the glucose uptake at different concentrations with the highest concentration being at 250 µg/ml and the lowest at 16 µg/ml. The highest glucose uptake was obtained at a concentration of 125 µg/ml (143.4%) and the lowest at 16 µg/ml (112.1%). The results obtained were statistically analyzed by making use of the Anova, One-way, Post Hoc, Tukey HSD test. According to the results, a value of  $p < 0.05$  was considered significant, obtained at concentrations 125 µg/ml (0.042), 25 µg/ml (0.030), 0.50 µg/ml (0.00014) and 1µg/ml (0.00014).

### 3.6.2 MTT toxicity assay

The MTT toxicity assay revealed that *S. pinnata* (ethanol) ( $p < 0.0001$ ) and (acetone) ( $p < 0.0001$ ) extracts were toxic to 3T3-L1 preadipocytes. The same was found for the *E. transvaalense* ( $p < 0.02$ ) extract. The *S. pinnata* ethanol ( $p < 0.0001$ ) and the *E. transvaalense*

( $p < 0.0001$ ) extracts were also toxic to Chang liver cells (Figure.3 2). The results were compared using the student's one and two tail t-test and P values obtained were  $< 0.0001$  for some of the results. This can be explained by the large differences between control and test values in these treatments (all greater than 40%) and small variations between replicates.



**Figure 3.3. Dose response of *E. undulata* plant extract *in vitro* in Chang liver cells (as % of control  $\pm$  standard error of mean, N = 5). Cells were exposed to 12.5  $\mu\text{g/ml}$  extract for 48 hours before glucose utilization was measured in the presence of the extract concentrations as indicated. \* $p < 0.05$ ; \*\* $p < 0.005$  compared to untreated control (using student's two-tailed t-test)**

### 3.6.3 Alpha-glucosidase and alpha-amylase assay

The alpha-glucosidase and alpha-amylase 50% inhibitory concentrations ( $\text{IC}_{50}$ ) of the plant extracts, as well as the positive control, acarbose, is depicted in Table 3.2. It is evident from the results that *P. divaricata* inhibited alpha-glucosidase the most, exhibiting an  $\text{IC}_{50}$  value

of  $31.22 \pm 0.35 \mu\text{g/ml}$ . It was evident from the results obtained with the alpha-amylase assays that acetone extracts of *E. undulata* and *E. transvaalense* inhibited alpha-amylase most with  $\text{IC}_{50}$  values of  $2.80 \pm 0.063$  and  $1.12 \pm 0.079 \mu\text{g/ml}$  respectively.  $\text{IC}_{50}$  value of  $4.75 \pm 3.18$  and  $22.92 \mu\text{g/ml}$  was obtained for the positive control, acarbose, alpha-glucosidase and alpha-amylase respectively. Acetone extracts of *S. pinnata* showed no inhibition.

**Table 3.2. Inhibitory effect ( $\text{IC}_{50}$ ) of four plant extracts and positive control, acarbose for alpha-glucosidase and alpha-amylase.**

Species	$\text{IC}_{50}^{\text{a}}$ ( $\mu\text{g/ml}$ ) alpha-Glucosidase	$\text{IC}_{50}^{\text{a}}$ ( $\mu\text{g/ml}$ ) alpha-Amylase
Acarbose	$4.75 \pm 3.18$	22.92
<i>S pinnata</i> (A)	NI <sup>b</sup>	NI <sup>b</sup>
<i>E undulata</i>	$49.95 \pm 0.007$	$2.80 \pm 0.063$
<i>P. divaricata</i>	$31.22 \pm 0.154$	$36.30 \pm 4.624$
<i>E.transvaalense</i>	$50.62 \pm 0.351$	$1.12 \pm 0.079$

$\text{IC}_{50}^{\text{a}}$  = Fifty percent inhibitory concentration

NI<sup>b</sup> = no inhibition

### 3.7 Discussion

The *in vitro* hypoglycaemic analysis revealed that the acetone extract of *S. pinnata*, *E. undulata* and *E. transvaalense* and ethanol extract of *S. pinnata* displayed hypoglycaemic activity in one or more of the various cell lines tested. While one acetone plant extract, *P. divaricata*, showed no hypoglycaemic activity in any of the cell lines tested. It was, however, determined that the high positive values obtained for *S. pinnata* could not be considered, as the toxicity assays revealed that the acetone and ethanol plant extracts are highly toxic to 3T3-L1 preadipocytes and the toxicity may be responsible for the high values obtained in the 3T3-L1 preadipocytes *in vitro* assays. Muthaura *et al.*,

(2007) noted a low cytotoxicity of methanol and water extracts of *S. pinnata* to Vero E6 cells but no toxicity was observed in *in vivo* mice tests. The acetone extract of *E. transvaalense* though displayed hypoglycaemic activity, but it was toxic to Chang liver cells. The only plant extract tested that displayed positive results in all three cell lines and no toxicity was *E. undulata*.

Some anti-diabetic drugs act through inhibition of digestion of complex carbohydrates in the gastrointestinal tract. To determine if some of the plant extracts could act at this level, they were tested to determine their inhibition of alpha-glucosidase and alpha-amylase. The results obtained for alpha-glucosidase and alpha-amylase indicated that the plant extracts of *E. undulata* ( $IC_{50}$   $49.95 \pm 0.007$ ;  $2.80 \pm 0.063$ ), *E. transvaalense* ( $IC_{50}$   $50.62 \pm 0.154$ ;  $1.12 \pm 0.079$ ) and *P. divaricata* ( $IC_{50}$   $31.22 \pm 0.351$ ;  $36.30 \pm 4.62$ ) displayed alpha-glucosidase and alpha-amylase inhibition. *S. pinnata* displayed no alpha-glucosidase or alpha-amylase inhibiting activity. Of interest is the fact that *P. divaricata* did not display any *in vitro* hypoglycaemic activity but did show some alpha-glucosidase and alpha-amylase inhibiting action, indicating a possible different mechanism through which it could function as an anti-diabetic treatment.

Tshikalange (2007) isolated various triterpenoides such as lup-20(30)-ene-3,29-diol, (3 $\alpha$ )-(9Cl), lup-20(29)-ene-30-hydroxy-(9Cl), taraxastanonol,  $\beta$ -sitosterol and a phenolic derivative and depside namely ataric acid and atranorin from *E. transvaalense*. These substances were however not tested for their anti-diabetic activity, but all except  $\beta$ -sitosterol demonstrated alpha-glucosidase and alpha-amylase inhibition (Nkobole, 2009). Drewes and Mashimye (1993) isolated the phenolic compound, elaeocyanidin, as well as the gallotannins and ouratea proanthocyanidin A from *E. transvaalense* and it is likely that these compounds are responsible for the hypoglycaemic activity (Gruendel *et al.*, 2007; Gorelik *et al.*, 2008). In literature the use of *S. pinnata* as an anti-diabetic agent is numerous but no literature could be found on the chemical analysis and testing for its hypoglycaemic activity. Zdero *et al.* (1990) isolated some neryl geraniol derivatives, clerodane and three diterpenes from *P. divaricata*. The triterpenes betulin and lupeol were isolated from *E. natalensis* by Khan and Rwekika, (1992) and Weigenand *et al.*, (2004). According to Sudhahar *et al.* (2006) lupeol and its derivative normalized the lipid profile in

Wistar rats that were fed a high cholesterol diet. Ali *et al.*, (2006) also found that lupeol inhibited alpha-amylase enzyme. The positive results obtained in the present study could be attributed to the above mentioned isolated compounds.

Based on previous phytochemical studies and the results from this study, we conclude that *E. undulata* should be further investigated to identify the compounds responsible for its promising *in vitro* anti-diabetic activity.

### 3.8 Acknowledgements

The author would like to thank the Diabetes Research Group in the Department of Biochemistry and Microbiology at the Nelson Mandela Metropolitan University for their support and Prof Gretel van Rooyen for her assistance in the technical editing of the article. This research was financially supported by the National Research Foundation.

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## CHAPTER 4

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Chapter 4 deals with the phytochemical study conducted on the root bark of *Euclea undulata* Thunb. var. *myrtina* and the isolation of various compounds as well as their hypoglycaemic activity.

**The isolation of a new triterpene and other compounds with hypoglycaemic activity, from *Euclea undulata* Thunb. var. *myrtina* (Ebenaceae) root bark**

**4.1 Abstract**

Phytochemical studies of a crude acetone extract of the root bark of *E. undulata* var. *myrtina*. afforded a new triterpene (**1**), in addition to three known compounds betulin (**2**), lupeol (**3**) and epicatechin (**4**). The chemical structures were determined by spectroscopic means.

The hypoglycaemic activity of the four compounds isolated 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 carbohydrate-hydrolysing enzymes were also established at concentrations ranging from 0.02 to 200.00 µg/ml. The *in vitro* results on C2C12 myocytes indicated that compound **4** has the potential to lower blood glucose levels, whereas compound **1** has the ability to inhibit alpha-glucosidase at a concentration of 200.0 µg/ml.

**4.2 Introduction**

*E. 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 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 (Deuschländer *et al.*, 2009).

**4.3. Materials and methods**

**4.3.1 Plant material**

Plant material was collected at De Wagensdrift, Gauteng Province in August 2005. Voucher specimens (Deuschländer nr 95254) have been deposited at the H.G.W.J. Schweickert Herbarium, University of Pretoria and authenticated by Ms M. Nel.

#### 4.3.2 Extraction of the plant material

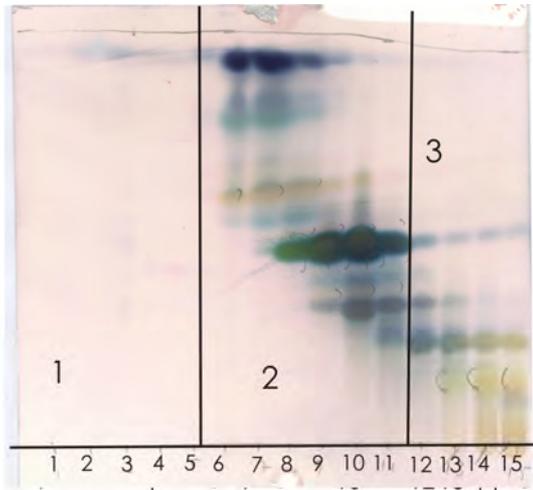
Plant material was air dried and the root bark stripped from the roots before it was ground. The ground root bark (215 g) was soaked in 0.5 l cold acetone for three days while on a shaker. After three days the extract was filtered and the residue extracted again with fresh cold acetone (3X). The plant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

#### 4.3.3 Fractionation of the crude extract

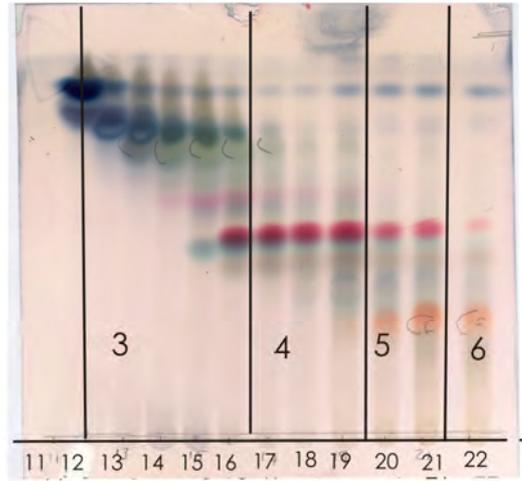
The crude acetone extract (35 g) was subjected to silica-gel column chromatography for the isolation of bioactive principles. The column was eluted with hexane: ethyl acetate mixtures of increasing polarity (0 – 100% ethyl acetate), washed with 100% methanol. Fractions containing the same compounds as determined by thin layer chromatography (TLC) were combined. Nine main fractions were obtained (Figure 4.1). All fractions except fractions 1 and 6 were not tested for glucose utilization using C2C12 myocytes as these fractions could not be dissolved in the solvent used for the bioassay. The bioactive fractions were further subjected to column chromatographic purification for the identification of bioactive principles. Fraction 2 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) lupeol **3**. Fractions 3 and 4 were combined and chromatographed over a sephadex column using ethanol and yielded a new triterpene **1** (14.28 mg; 0.04% yield) and betulin **2** (20.01 mg; 0.06% yield). Fraction 8 was chromatographed over a sephadex column eluted with ethanol and yielded (12.02 mg; 0.03% yield) epicatechin **4** (Figure 4.2).

#### 4.4 Determination of hypoglycaemic activity

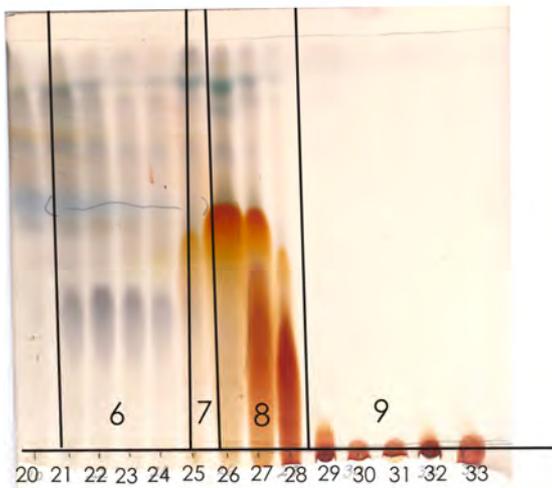
Seven of the nine main fractions obtained from the column chromatography were tested *in vitro* on C2C12 myocytes to measure glucose uptake at a concentration of 12,5 µg/ml and insulin (1 µM) in incubation medium was used as positive control. Glucinet reagent kit (Adcock Ingram) was used to execute the assays. According to the results obtained, fractions 2, 3 and 8 showed activity and subsequently submitted to chromatographic processes to isolate those compounds with probable hypoglycaemic activity.



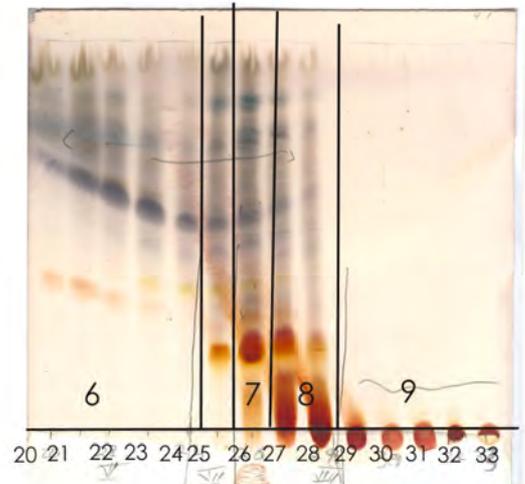
hexane: ethyl acetate (8:2)



hexane: ethyl acetate(1:1)



ethyl acetate : hexane (8:2)



methanol : chloroform (9:1)

**Figure 4.1.** Silica gel column fractionation of the acetone extract of *E. undulata*.

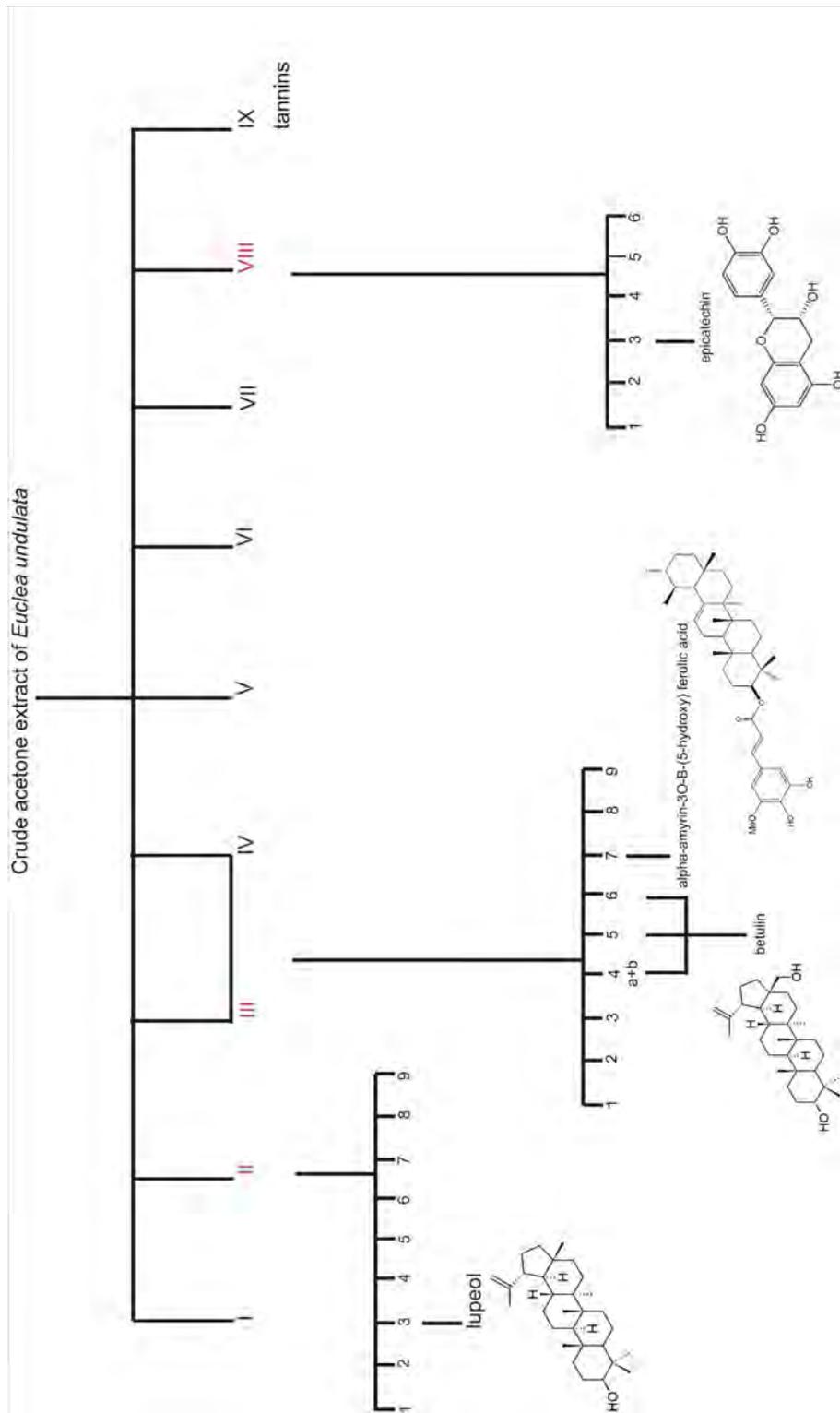


Figure 4.2. Dendrogram indicating the isolation of compounds from the crude acetone extract of *E. undulata*.

The hypoglycaemic activity of the isolated compounds **1** - **4** were 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 epicatechin (290.3) compared to that of betulin (442.7), lupeol (426.7) and  $\alpha$ -amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (619.5), it was decided to test epicatechin at 25 µg/ml and the others at 50 µg/ml to yield more comparable molar concentrations ranging from 86 µM for epicatechin to 117 µM for lupeol.

The alpha-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 alpha-glucosidase inhibitory activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl- $\alpha$ -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 1mg 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- $\alpha$ -D-glucopyranose (Sigma-Aldrich, N1377-1G) was dissolved in 5 ml buffer and incubated at 37° C for 15 minutes. The enzyme ( $\alpha$ -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. Due to the unavailability of sufficient amount of the purified compounds they could not be tested for alpha-amylase inhibitory activity.

unavailability of sufficient amount of the purified compounds they could not be tested for alpha-amylase inhibitory activity.

## 4.5. Results and discussion

### 4.5.1 *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. It was found that fractions II, III and VIII were active (Figures 4.1 and 4.,2).

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 4.3) 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**.

The *in vitro* assay on C2C12 myocytes of the different compounds revealed that **4** was active (266.3%) in lowering blood glucose levels at a concentration of 25 µg/ml and **2** was active to a lesser extent (121.4%) at a concentration of 50 µg/ml (100% used as base line) (Figure 4.4). In literature no evidence could be found of compounds 1- 4 being tested for hypoglycaemic activity on C2C12 myocytes.

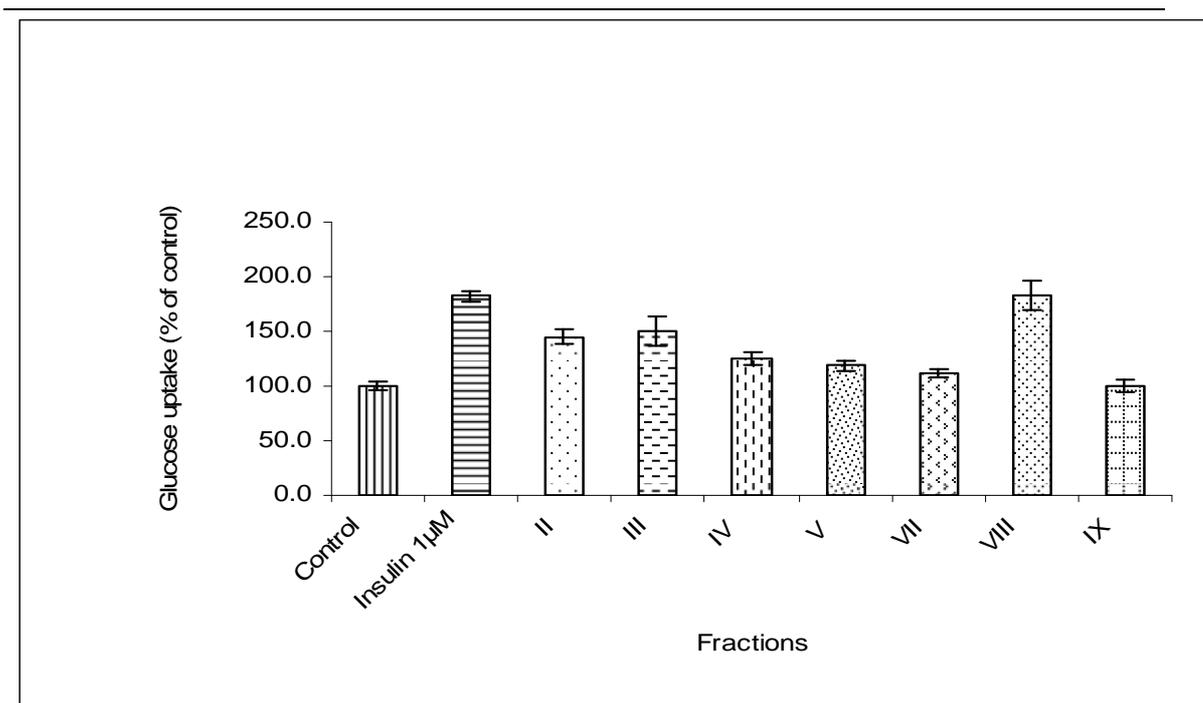
### 4.5.2 *Alpha-glucosidase* assay

According to the results obtained from the alpha–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, (Figure 4.5). IC<sub>50</sub> 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 4.1) In literature according to Parimaladevi *et al.* (2004) betulin isolated from *Cleome viscosa* (25, 50 mg/kg) exhibited

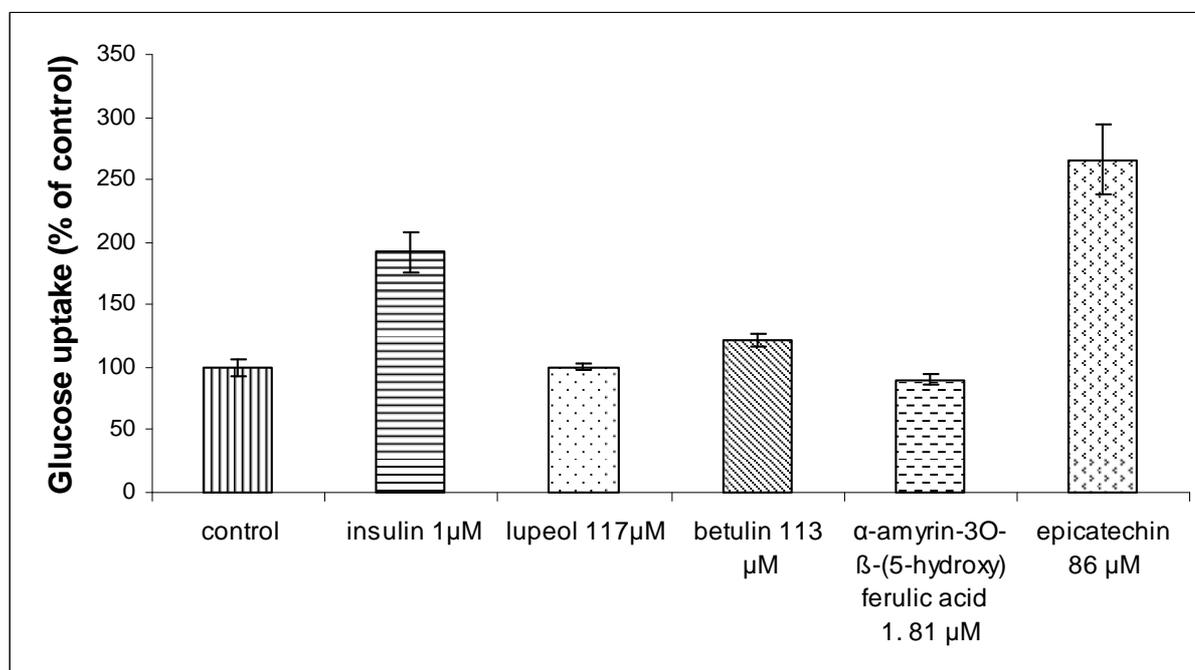
significant hypoglycaemic activity in both normal and streptozotocin induced diabetic rats compared with that of the standard drug glibenclamide (10 mg/kg). The results obtained by Rahman *et al.* (2008) indicated that betulin obtained from the methanolic extract of the seeds of *Cichorium intybus* was inactive in inhibiting alpha-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.

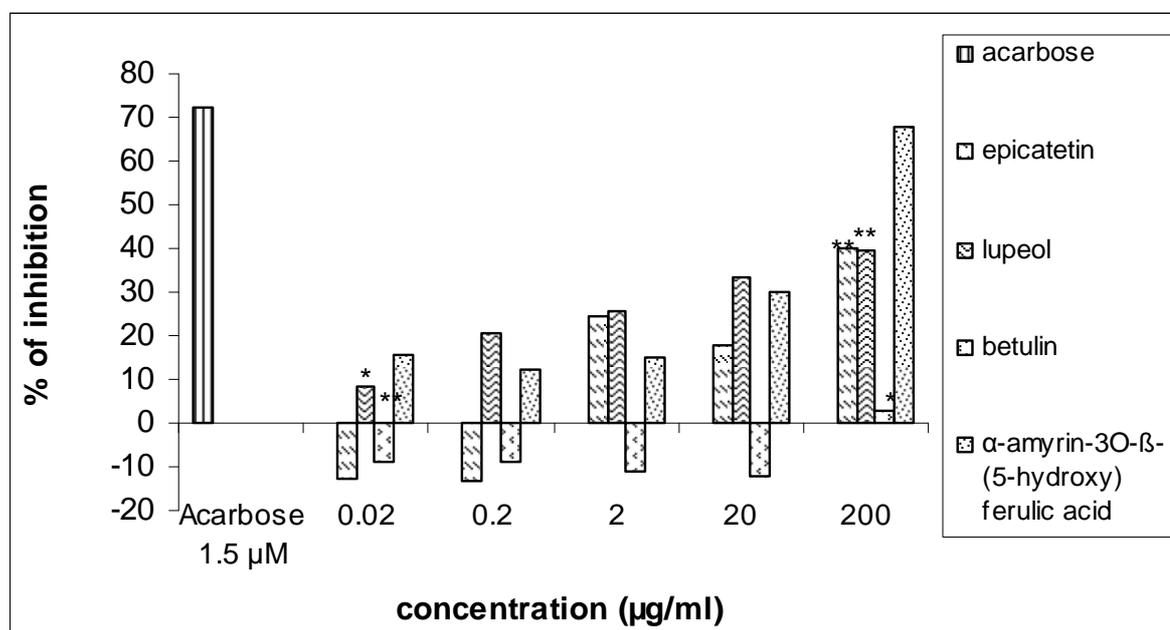
According to literature epicatechins displays antidiabetic activities and is one of the most active antioxidant constituents (Berregi *et al.*, 2003). Cho *et al.* (2006) found that catechins enhanced the expression and secretion of adiponectin, an adipocyte-specific secretory hormone that can increase 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<sup>++</sup>-ATPase in both normal and type 2 diabetic patients. 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.



**Figure 4.3.** Glucose uptake (as % of control  $\pm$  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



**Figure 4.4.** Glucose uptake (as % of control  $\pm$  standard error of mean N=8) of the compounds isolated from *E. undulata* tested for their hypoglycaemic activity using C2C12 myocytes.



**Figure 4.5. Inhibition of alpha-glucosidase by the different compounds isolated from the acetone extract of *E. undulata* (Statistic analysis were only done for the highest and lowest concentrations; \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; compared to control using student's two-tailed t-test)**

**Table 4.1 Inhibitory affect ( $IC_{50}$ ) of the four compounds and positive control acarbose for alpha-glucosidase.**

Compound	$IC_{50}$ ( $\mu\text{g/ml}$ ) alpha-glucosidase	$\mu\text{M}$
Acarbose	$4.75 \pm 3.18$	7.35
À-amyrin-3O-β-(5-hydroxy) ferulic acid (1)	$4.79 \pm 2.54$	7.76
Epicatechin (4)	$5.86 \pm 4.28$	20.18
Lupeol (3)	$6.27 \pm 4.75$	14.69
Betulin (2)	$32.04 \pm 2.79$	72.37

#### 4.6 Phytochemical examination

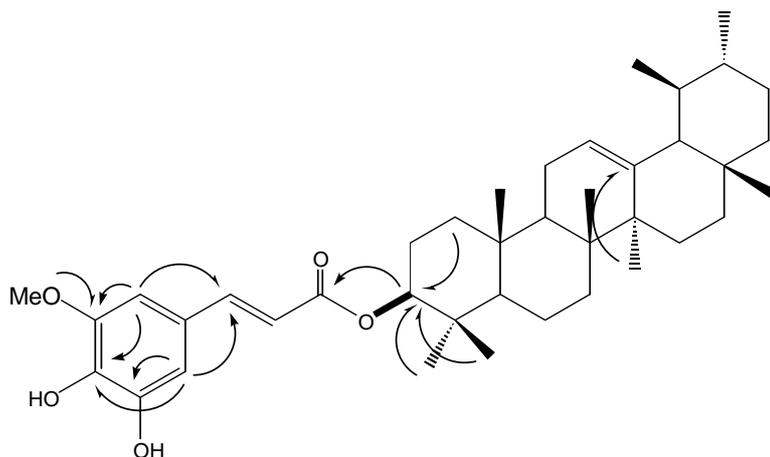
The phytochemical examination coupled with bioassay-guided fractionation of the acetone extract resulted in the isolation of a new  $\alpha$ -amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (**1**), the triterpenes: betulin (**2**) and lupeol (**3**), and the anthocyanin epicatechin (**4**) (Figures 4.1 and 4.2). Hydrolysis. A 5 mg portion of the compounds 1- 4 were added to 5ml of aqueous KOH and left under nitrogen overnight at room temperature. The reaction mixture was neutralized with 10% HCl. Compounds 1 - 4 were extracted with CHCl<sub>3</sub>, then purified by silica gel column chromatography using 30% EtOAc in hexane.

##### 4.6.1 Chemical constituents from the root bark of *E. undulata*

###### 4.6.1.1 $\alpha$ -Amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (1)

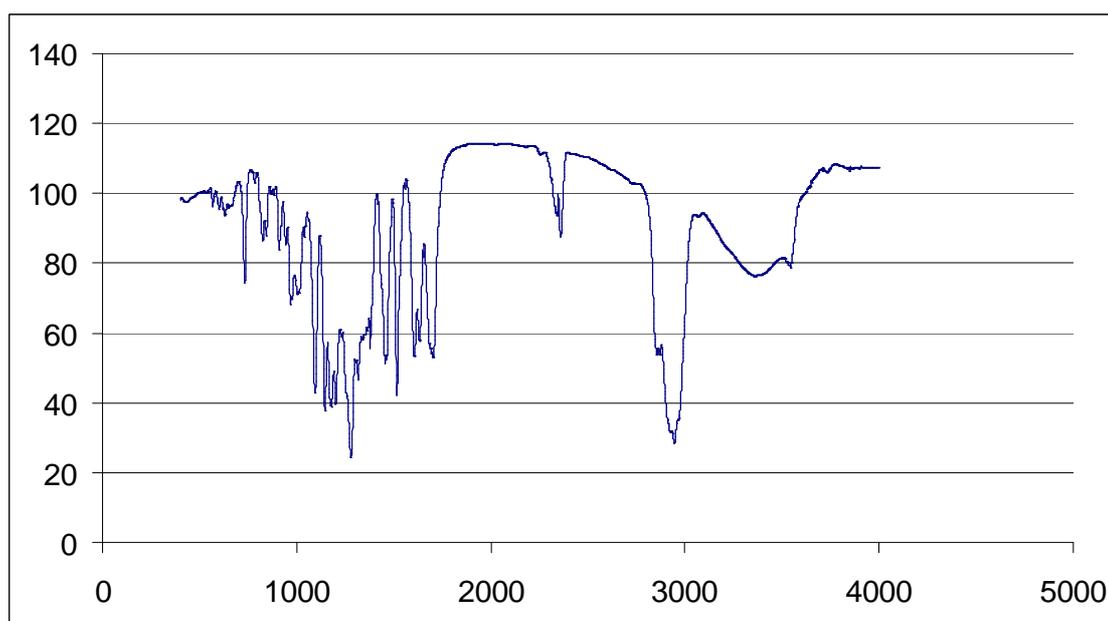
Compound (1), was purified with a Sephadex column eluted with dichloromethane and methanol, 99:1. HRESIMS showed a molecular peak at 619.47  $m/z$  ( $M^+ + H$ ) corresponding to the molecular formula C<sub>40</sub>H<sub>58</sub>O<sub>5</sub> (Figure 4.10). The IR spectra (Figure 4.7) revealed the presence of bands at 3362, 2945, 2870, 2359, 2341, 1700, 1643 and 1606 cm<sup>-1</sup> of  $\alpha$ ,  $\beta$ -unsaturated carbonyl, aromatic ring and cyclic alkane. The UV spectra gave two absorption bands at  $\lambda_{max}$  228, 320 nm of the conjugated aromatic system. The <sup>1</sup>H NMR data of 1 (Figures 4.8) showed signals for six singlet methyls ( $\delta_H$  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,  $\alpha$ ,  $\beta$  protons), 6.80, 6.62 (d,  $J= 0.5$  Hz, H-2', 6'), the aforementioned data indicated the presence of ursane type triterpene similar to  $\alpha$ -amyrin esterified in position C-3 with 5-hydroxy ferulic acid. The <sup>13</sup>C (Figure 4.9), DEPT-135 NMR and HSQC spectra of (1) (Figure 4.12) confirmed the proposed structure of  $\alpha$ -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 4.2). 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  $\alpha$ -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 (Figures 4.6 and 4.11) correlations which showed, amongst other correlations, a cross peak between MeO/C-3' and H-2'/C-3', C-4'.



**Figure 4.6** HMBC correlations of  $\alpha$ -amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (**1**).

The relative configurations of C-3 could not be determined from the recorded NOESY spectra of **1**; however, hydrolysis of **1** gave  $\alpha$ -amyrin with a 3 $\beta$ -OH configuration, which was identified on the basis of comparison of spectral data reported in literature.



**Figure 4.7** IR spectra of  $\alpha$ -amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (**1**).

**Table 4.2. NMR data of  $\alpha$ -amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (1),**

No.	C	H, J Hz	No.	C	H J Hz
1	38.37	1.13 m, 1.67 m	21	31.22	1.31 m, 1.39 m
2	23.68	1.6 m	22	41.51	1.29 m, 1.46 m
3	81.04	4.66, dd, $J= 10.3, 5.9$	23	28.1	-----
4	37.9	-----	24	16.84	0.92 s
5	55.27	0.87 m	25	15.71	0.99 s
6	18.23	1.41 m, 1.52 m	26	16.89	1.01 s
7	32.84	1.33 m, 1.55 m	27	23.22	1.06 s
8	40.01	-----	28	28.71	0.78 s
9	47.52	1.55 m	29	17.48	0.78 d, $J= 5.8$
10	36.83	-----	30	20.92	0.86 d, $J= 5.6$
11	23.34	1.91 m	1'	126.6	-----
12	124.3	5.10, t, $J= 1.5$	2'	109.3	6.82 d, $J=1.5$
13	139.59	-----	3'	147.1	-----
14	42.05	-----	4'	134.8	-----
15	26.58	0.96 m, 1.83 m	5'	144.0	-----
16	28.10	0.87 m, 1.83 m	6'	103.1	6.65 d, $J=1.5$
17	33.71	-----	$\alpha$	144.6	7.50 d, $J=15.9$
18	59.03	1.31 m	$\beta$	116.67	6.25 d, $J=15.9$
19	39.62	0.93 m	C=O	167.23	-----
20	39.78	1.31 m	OMe	56.20	3.86 s

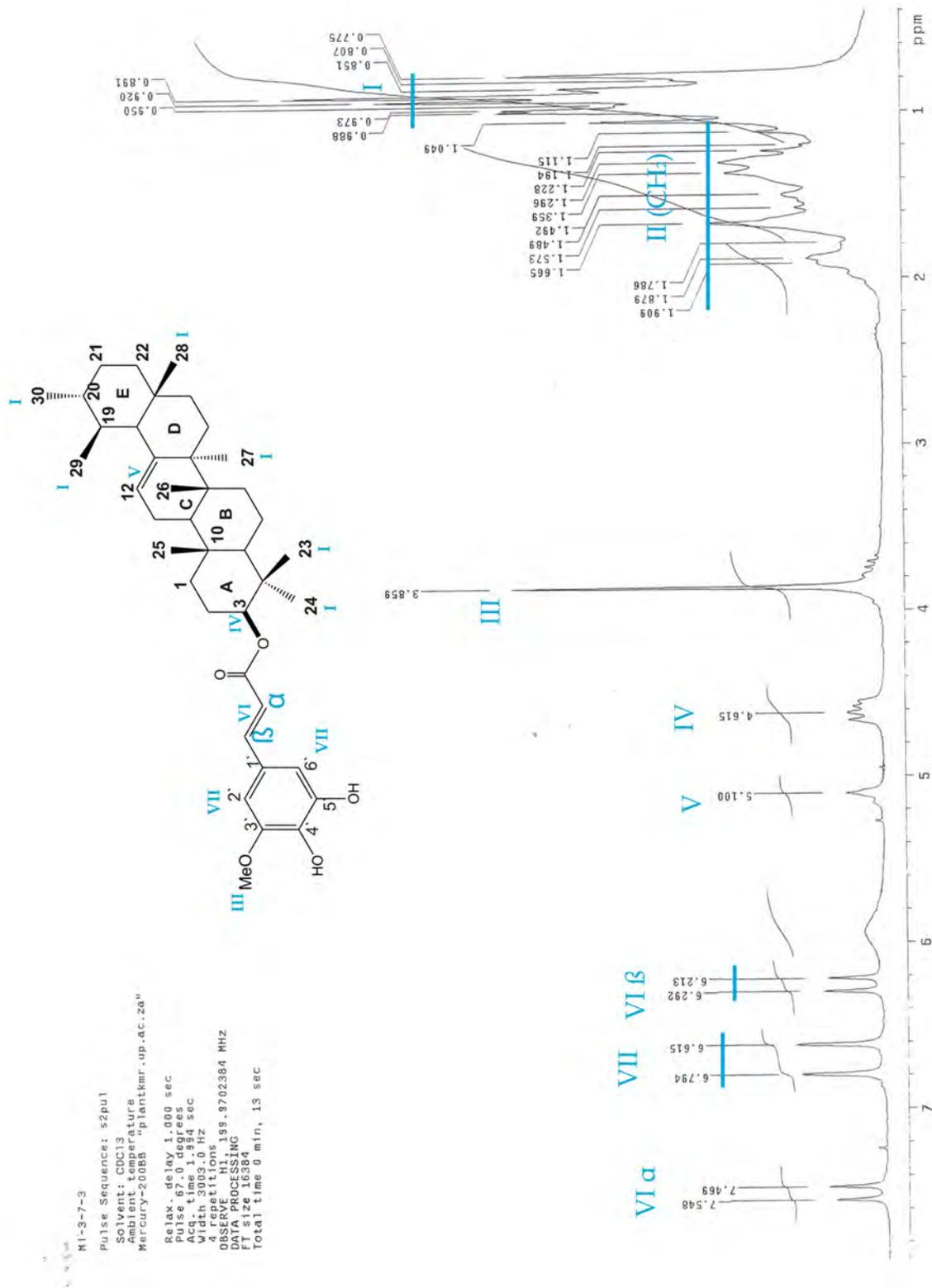


Figure 4.8 The  $^1\text{H}$  NMR spectrum of  $\alpha$ -amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (1)

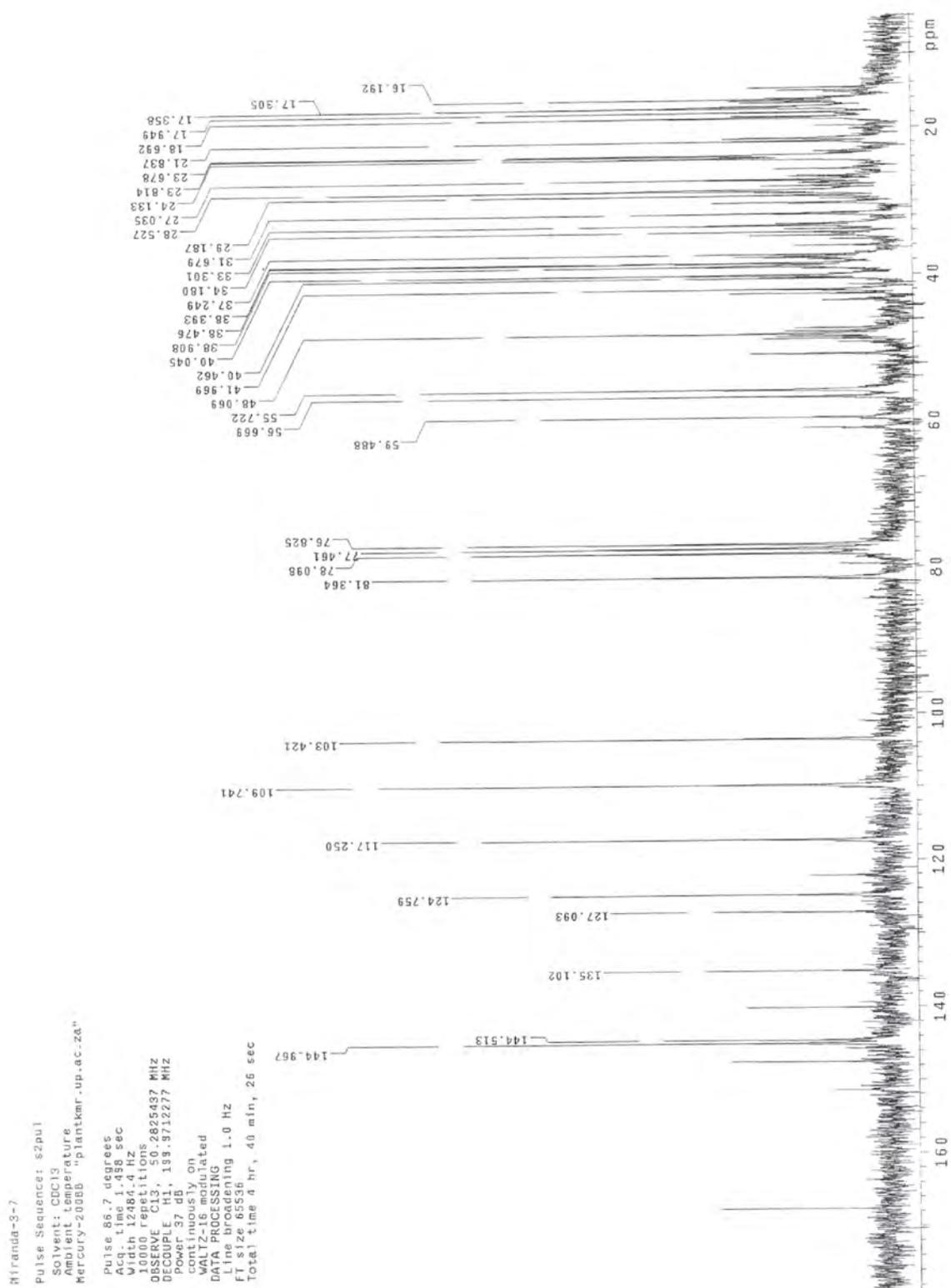


Figure 4.9 The  $^{13}\text{C}$  NMR spectrum of  $\alpha$ -amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (1)

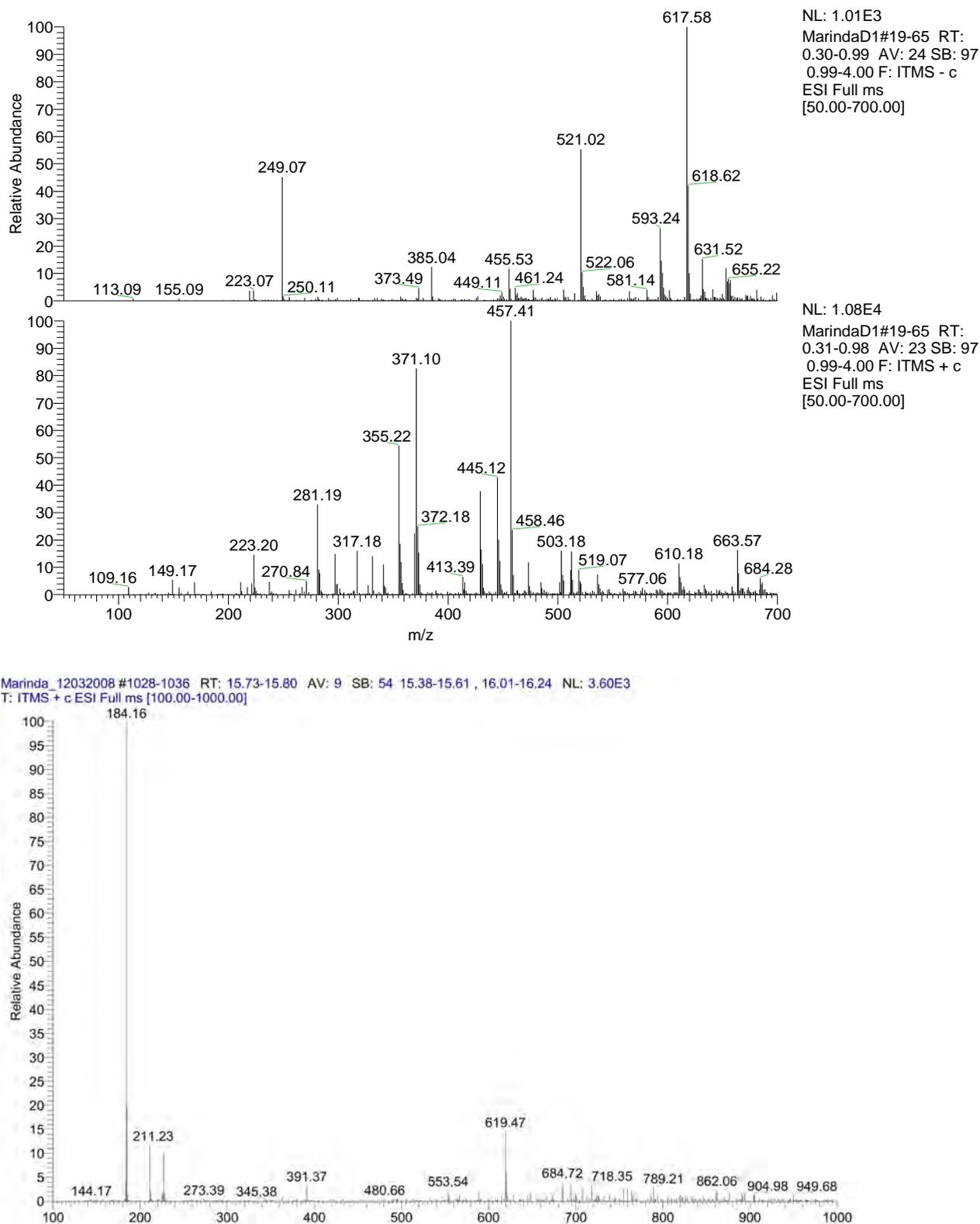


Figure 4.10. - + ESI spectrum of  $\alpha$ -amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (1)

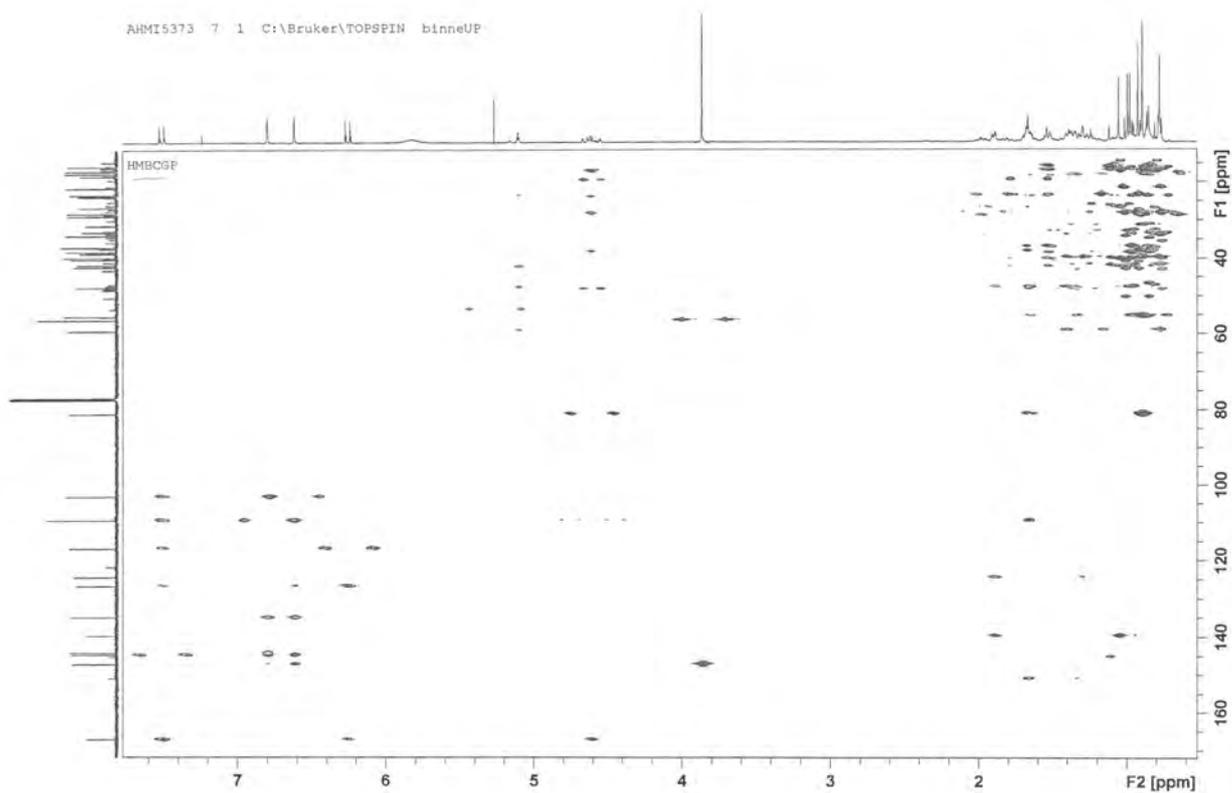


Figure 4.11. The HMBC spectrum of  $\alpha$ -amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (1)

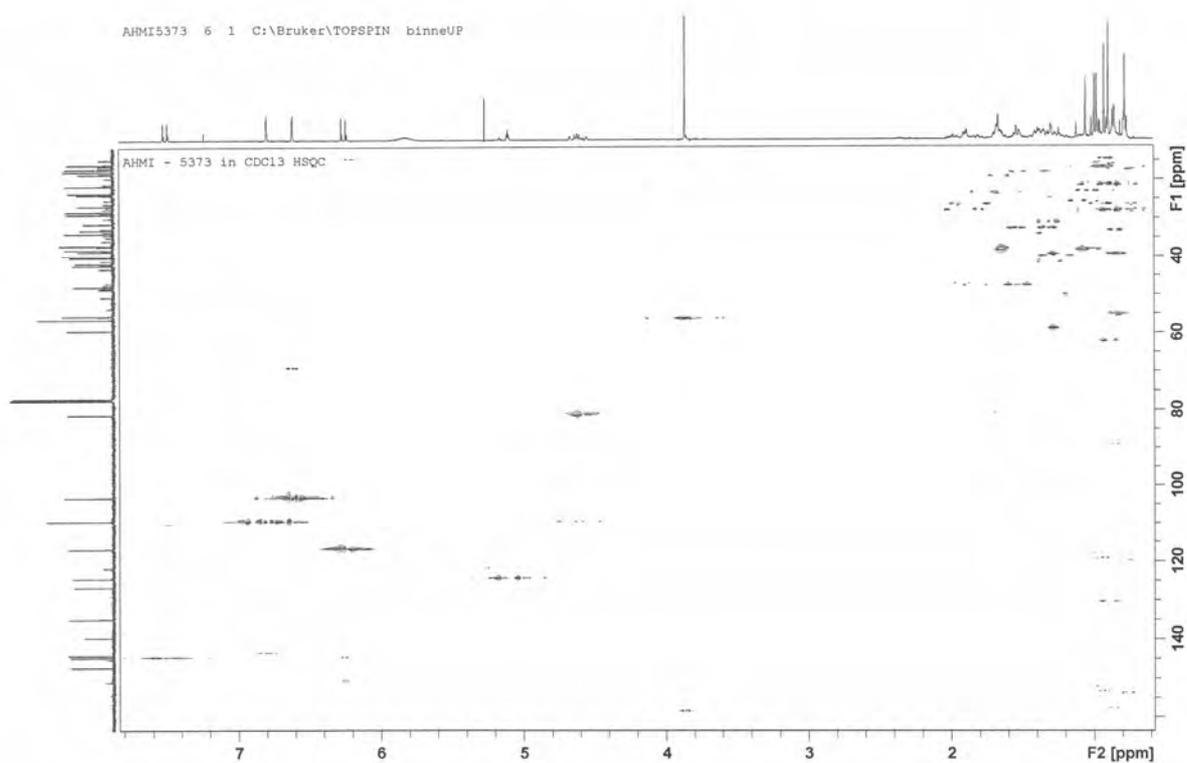


Figure 4.12. The HSQC spectrum of  $\alpha$ -amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (1)

#### 4.6.1.2 *Betulin* (**2**)

Fraction 3 yielded a white powdery substance. The  $^1\text{H}$  NMR data of **2** exhibited signals characteristic of the skeleton lupene triterpene, (Figure 4.13). Five signals of three proton singlets at  $\delta$  0.75, 0.81, 0.92, 0.96, 1.01 and 1.67 ppm were obtained as well as one proton signal at  $\delta$  2.4, ddd [H-19], one proton at  $\delta$  3.2, dd [H-3 $\alpha$ ] and two proton signals at  $\delta$  4.57, 4.66 ppm. The disappearance of one methyl group from the lupeol skeleton and appearance of two protons signal at 3.32, 3.77 ppm (d, each 1H, J=10.8 Hz) indicated the oxidation of this methyl group which is characteristic of the triterpene betulin which was isolated from the Ebenaceae family before by Mallavadhani *et al.* (1998) and Weigenand *et al.* (2004). Betulin was isolated from various plant species including *Cleome viscosas* (Parimaladevi *et al.*, 2004) and from the root bark of *Euclea natalensis* (Weigenand *et al.*, 2004), but this is the first report of the isolation of betulin from *E. undulata*.

#### 4.6.1.3 *Lupeol* (**3**)

Compound **3** was eluted from fraction 2 and identified as lupeol based on the  $^1\text{H}$  NMR data (Figure 4.14).

The  $^1\text{H}$ -NMR data exhibited seven signals of three protons at  $\delta$  0.75, 0.78, 0.82, 0.94, 0.96, 1.02 and 1.67 ppm. The proton signal at 2.39 ppm, ddd, was assigned to H-19, the signal at  $\delta$  3.2 ppm, dd, to 3 $\alpha$ -H, and two proton signals at  $\delta$  4.55 and 4.68 ppm of H-29A, 29B. One shows long range coupling with one methyl at  $\delta$  1.67. The last two protons are assigned to isopropenyl olefinic protons.

The  $^1\text{H}$ -NMR data indicated that the compound isolated is the commonly found triterpene, lupeol, which had previously been isolated from *E. natalensis* by Khan & Rwekika (1992) and Weigenand *et al.* (2004). Lupeol has been isolated from various plant species including *Spirostachys africana* (Mathabe *et al.*, 2008) and from the root bark of *E. natalensis* (Weigenand *et al.*, 2004), but this is the first report of the isolation of lupeol from *E. undulata*.

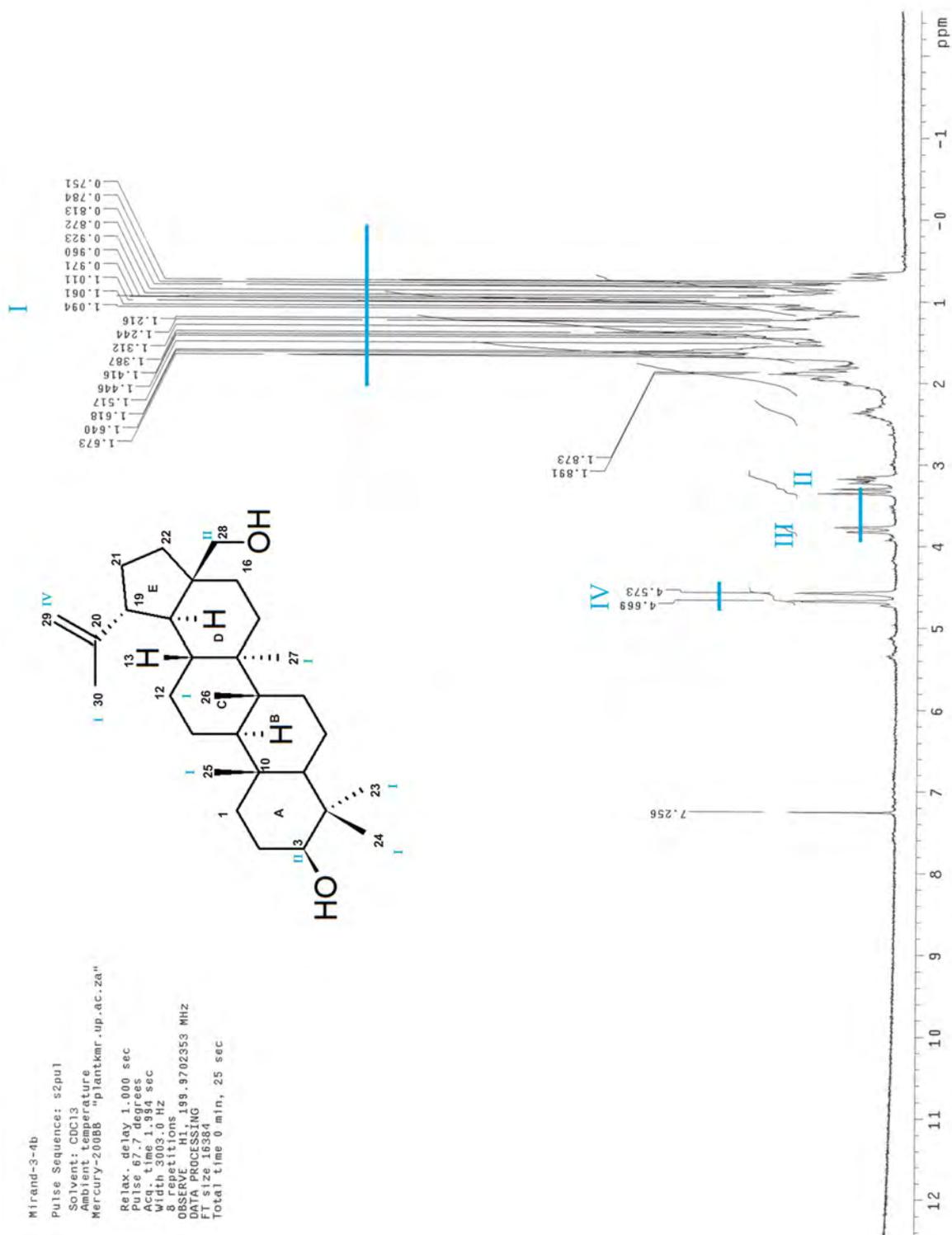


Figure 4.13. <sup>1</sup>H NMR spectrum of betulin (2)

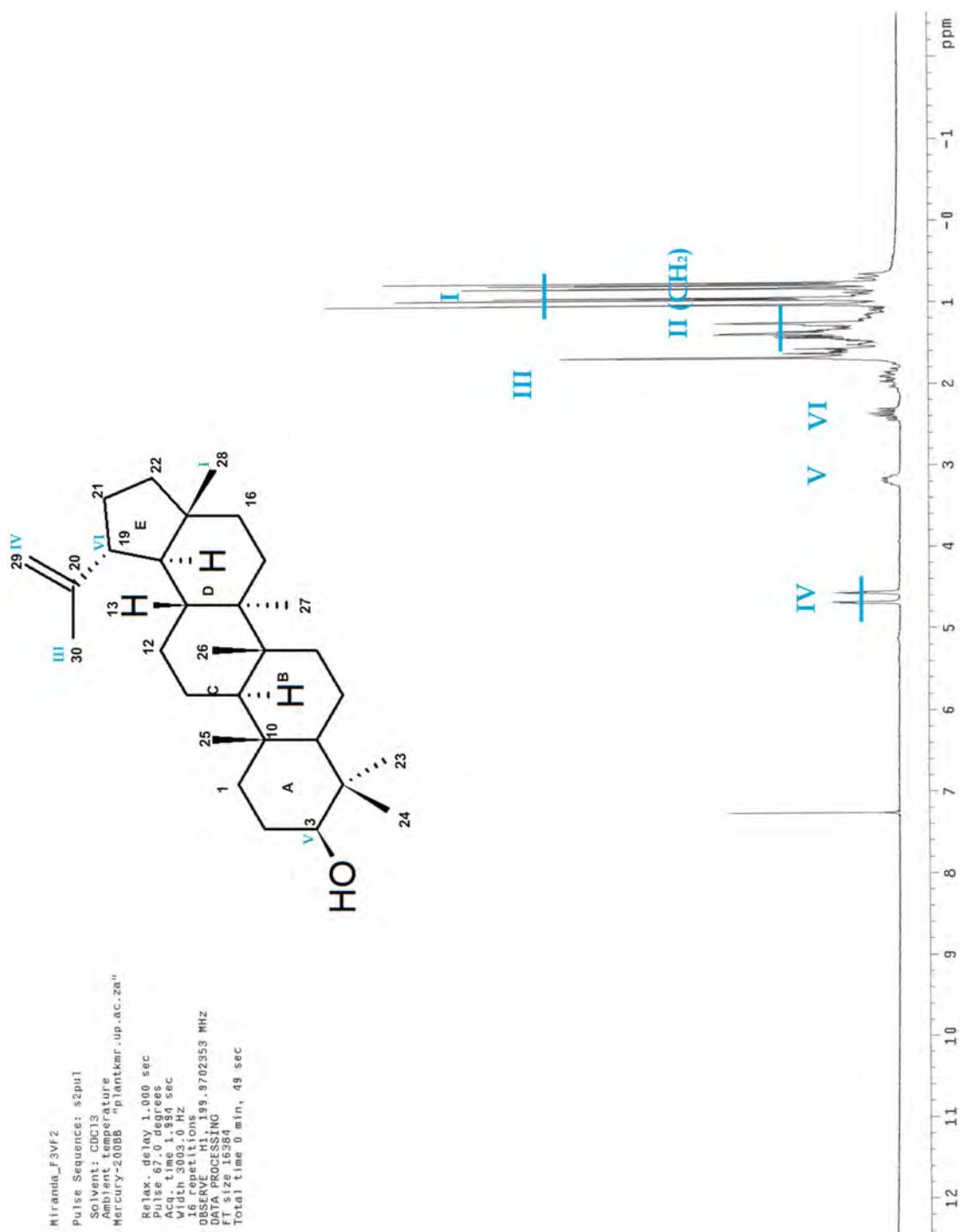


Figure 4.14. <sup>1</sup>H NMR spectrum of lupeol (3)

#### 4.6.1.4. Epicatechin (**4**)

A reddish brown powdery substance was eluted from fraction 8 and identified on the basis of  $^1\text{H-NMR}$  data (Figure 4.15). The  $^1\text{H-NMR}$  data of **4** exhibited signals identical with that of epicatechin. The  $^1\text{H NMR}$  data exhibited signals of 6 protons, at  $\delta$ 4.85 assigned to H-2, a proton signal at  $\delta$  4.18 ppm, to H-3, and two proton signals at  $\delta$  2.74 and 2.80 ppm were assigned to protons 4 $\alpha$  and 4 $\beta$  respectively, signal at  $\delta$ 6.01 was assigned to H-6 and the signal at 5.90 to H-8 (Okushio *et al.* 1998). Aromatic signals at  $\delta$ H 7.02, 6.75 and  $\delta$ H 6.80 corresponds with that of a B-ring. The basic structure was derived as a 3,3',4',5,7-pentahydroxyflavan and the broad proton singlet at  $\delta$ H 4.82 suggested a epicatechin (Figure 4.15) (Sun *et al.* 2006).

*In vitro* assays on C2C12 myocytes were done on the four compounds isolated from the root bark of *E. undulata* to determine their hypoglycaemic activity as well as their alpha-glucosidase inhibition potential.

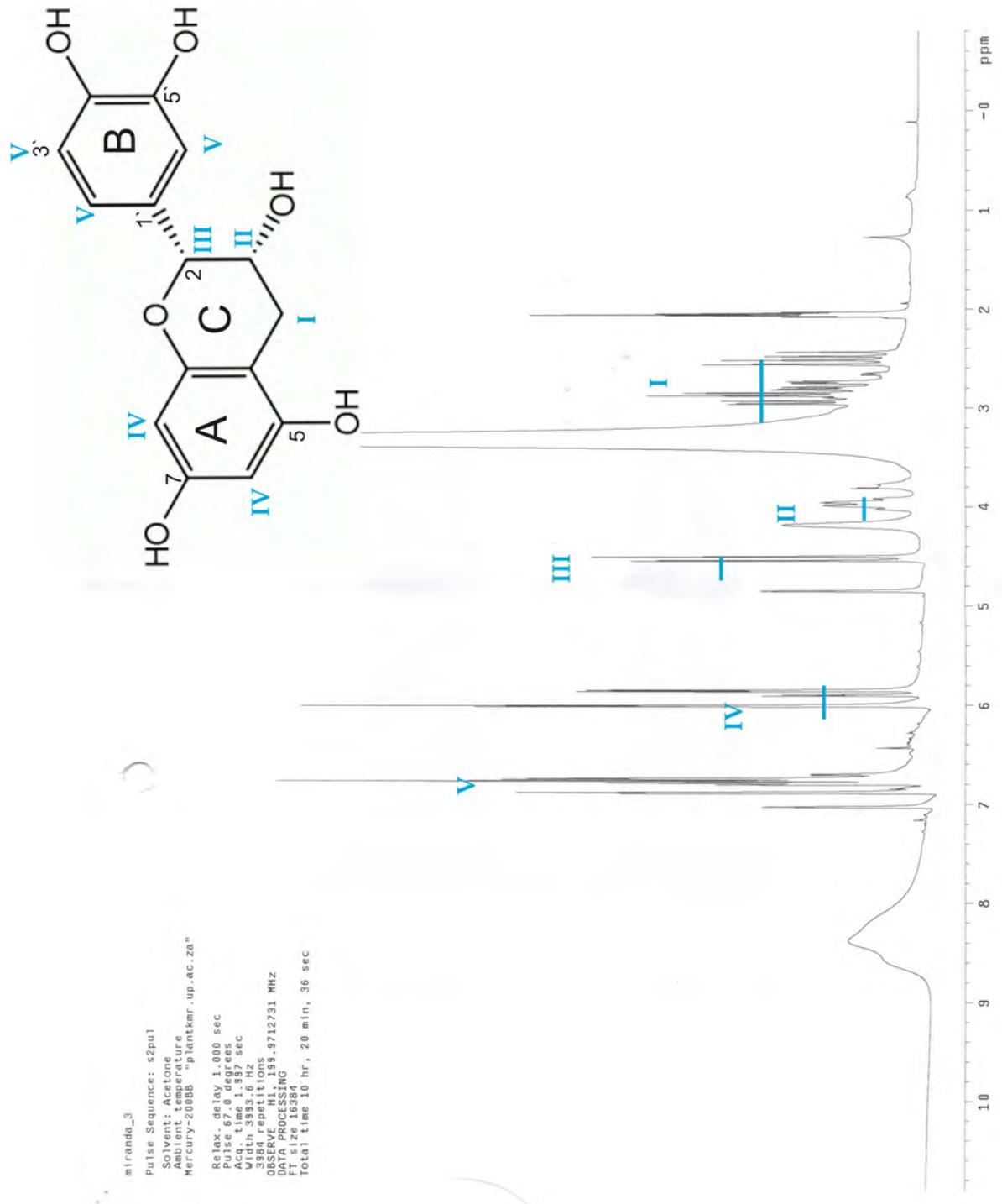


Figure 4.15. <sup>1</sup>H-NMR spectrum of epicatechin (4)

#### 4.7. Conclusions

Phytochemical studies conducted on *Euclea* species by Orzalesi *et al.*, (1970-71) and 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 *et al.* (1973; 1974). Chemical analysis indicated the presence of 3.26 % tannins in bark, saponins and reducing sugars in leaves 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).

The phytochemical examination coupled with bioassay-guided fractionation of the crude acetone extract of the root bark of *E. undulata* var. *myrtina* afforded a new triterpene and three other known compounds **1-4**. 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 anti-diabetic activity. The identified main fractions were sub-sequently subfractioned and four compounds isolated;  $\alpha$ -amyrin-3O- $\beta$ -(5-hydroxy) ferulic acid (**1**), betulin (**2**), lupeol (**3**) and epicatechin (**4**). 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-hydrolysing enzyme alpha-glucosidase. The present study reports for the first time the alpha-glucosidase inhibitory activity and glucose utilization by C2C12 myocytes of an acetone extract of *E. undulata* and its purified compounds.

The results indicated that epicatechin has the ability to lower blood glucose levels, whereas  $\alpha$ -myrin-3O- $\beta$ -(5-hydroxy) ferulic acid has the ability to inhibit alpha-glucosidase. These findings corroborate the ethnomedicinal use of *E. undulata* by traditional healers for the treatment of diabetes.

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