3.1 Introduction

The importance of medicinal plants cannot be ignored. Several medicines for cancer, malaria, tuberculosis, and diabetes mellitus have been developed from plants. An important drug for the treatment of diabetes, 'metformin' (a derivative of plant-derived compound), has been used for diabetes for decades; however, the use of this drug is not without drawbacks. These include rigid and multiple dosing regimen. High costs, inaccessibility and untoward effects such as 'diarrheoa, abdominal plains and flatulence. These factors have contributed to the recent increase in the use of folkloric plant medicines. It is therefore, of vital importance to look for alternative drugs with less side effects. Acetone extracts of plants selected based on ethnobotanical use and phytochemical constituents were tested for their inhibitory activity on α-glucosidase and α-amylase enzymes. Plant extracts were also tested for their antioxidant activity by measuring free radical scavenging activity. In addition, extracts were tested for cytotoxicity on Vero cell lines.

Antioxidant supplements are very important in patients having diabetes mellitus. Low levels of plasma antioxidants is a risk factor associated with diabetes (McCune and Johns, 2002). Complications of diabetes include retinopathy; arterosclerosis, kidney failure, etc which are the leading cause of morbidity and mortality amongst diabetics. These complications arise as a result of low levels of plasma antioxidants which result in oxidative stress (Bayners, 1991). Plants produce biological active compounds. These compounds are known as secondary metabolites or secondary constituents. Amongst these constituents, there are those that act as antioxidants. Examples include tannins, carotenoids, and flavonoids. In addition plants also produce antioxidants such as ascorbic acid and tocophenols (Larson, 1988). It has therefore, been suggested that it will be an added advantage for plant-based medicines for diabetes which will also have antioxidant property.

3.2 Materials and Methods

3.2.1 Plant material

Eight plants used in present study were collected from different locations. *Terminalia sericea* stem bark, *Sclerocarrya birrea* stem bark and *Psidium guajava* leaves were collected from Venda (Limpopo province of South Africa). *Artemisia afra* leaves, *Aloe ferox* leaves, *Warbugia salutaris* leaves, *Euclea natalensis* root bark and *Spirostachys africana* stem bark were collected from the Botanical garden of the University of Pretoria (Table 3.1). The plants were botanically identified by the taxonomist, Prof Van Wyk, and submitted at the H.G.W.J. Schweickerdt Herbarium (PRU) of the University of Pretoria where voucher specimens were deposited.

3.2.2 Preparation of extracts

Plant extracts exist as mixtures of trillions of compounds. It is therefore, of critical importance to find a suitable extractant. For the current study, 50g of dried plant materials were ground to fine powder. Each plant material was soaked in (50 ml) 100% acetone overnight at room temperature. The solvent was then removed and replaced with equal volume of solvent. This procedure was repeated two times. Extracts were filtered and concentrated to dryness using BUCHI rotary evaporator under reduced pressure. Dried extracts were reconstituted in dimethylsulfoxide (DMSO) at 2mg/ml and stored in the dark at 4^{o}C.

3.2.3 Assay for bakers yeast α-Glucosidase inhibitory activity

The assay method was adopted from Collins *et al.,* (1997) and modified accordingly to suite microtiter reading. The enzyme, α-glucosidase (EC 3.2.1.20) and the substrate, p-nitrophenyl α-D-glucopyranoside were purchased from Sigma Chemical Co., (St Louis, MO, USA). The glycohydrolase assay was performed in 96-well microtiter plates.

Table 3.1: Medicinal plants investigated in this study

Species	**Family**	**Part used**	**Voucher specimen no**
Terminalia sericea Burch. Ex DC	Combretaceae	Stem bark	Van Rensburg PRU 38564
Psidium guajava L	Myrtaceae	Leaves	PRU 54544
Sclerocarya birrea (A.Richi.) Hochst. subsp. caffra	Anacardiaceae	Stem bark	PRU 4558/100
Warbugia salutaris Bertol.f.) Chiov.	Canellaceae	Leaves	PRU 094845
Artemisia afra Jacq. ex Willd.	Asteraceae	Leaves	PRU 112085
Aloe ferox Mill	Aloaceae	Leaves	PRU 110308
Euclea natalensis A.DC	Ebenaceae	Roots bark	PRU 095059
Spirostachys africana Sond	Euphorbiaceae	Leaves	PRU 8434

The enzyme was diluted in 50mM Mes-NaOH, pH 6.5. The extracts were allowed to interact with enzyme at room temperature for 5 minutes before the reaction was started by the addition of appropriate substrate. The total reaction volume was 0.2ml. The reaction was allowed to proceed at room temperature for 15 minutes before it was stopped by the addition of 60µl 2M glycine-NaOH, pH 10. The assay was performed in triplicates. The final concentration of plant extracts in the wells ranged from 0.02 to 200µg/ml. The concentration of positive drug control 'Acarbose' ranged from 0.01 to 100µgml. The highest % of DMSO (1%) did not have any effect on the inhibition of α-

glucosidase enzyme. The coloured product was detected in a Bio-Tek® model microplate reader at 412nm (reference 490nm). Results were analysed using the formula:

Percent Inhibition= Sample absorbance/Control absorbance x 100

3.2.4 Assay for porcine pancreatic α-Amylase inhibitory activity

3.2.4.1 Digestion of starch with porcine pancreatic α-amylase

Alpha amylase inhibition assay was performed using the chromatogenic method adopted from Sigma-Aldrich which was adapted from Bernfeld (1955). Porcine pancreatic α-amylase was dissolved in ice cold distilled water to give a concentration of 10 units/ml solution. Potato starch (0.5%, w/v) in 20mM phosphate buffer (pH 6.9) containing 6.7mM sodium chloride, was used as a substrate solution. The assay was carried out on glass tubes. Equal amounts (300µl) of solution from the tubes were transferred to eppendorfs containing 300µl of 3.18M sodium carbonate ($NaCO_{3)}$. Frozen samples were allowed to thaw and immediately put on ice and vortexed just before use for the Prussian blue assay.

3.2.4.2 Determing reducing sugars (Prussian Blue Assay)

The assay was adapted from Slaughter *et al* (2001) with slight modifications. Except for *Terminalia sericea*, the final concentration of plant extracts in the tubes ranged from 0.025 to 1.25mg/ml. During preliminary tests, *Terminalia sericea showed* activity at higher concentrations hence its concentrations ranged from 0.05 to 1.2mg/ml to determine IC_{50} values**.** The concentrations of positive drug control 'Acarbose' ranged from 0.016 to 1mg/ml. The highest % of DMSO did not have any effect on the inhibition of α-glucosidase enzyme. Briefly, equal portions (0.5ml) of sample (diluted with distilled water), solution A (1.6 mM KCN, 0.19 Na_2CO_3); solution B [1.18 mM $K_3Fe(CN)_6$]; were mixed in glass test tubes capped with aluminium foil. The tubes were placed in vigorously boiling water for exactly 15 minutes and then removed and allowed to cool at room

temperature for 15 minutes. Once cool, 2.5ml of solution C [3.11mM NH4Fe $(SO_4)_2$; 0.1 sodium dodecyl sulphate, 0.42% v/v H_2SO_4] was added to the tubes. The tubes were left to stand for 150 minutes for the development of colour. The absorbance was read on a Beckman Coulter Du 720 spectrophotometer at 690nm. Maltose standards covering the range of 0-10µM in the final assay were assayed by the same procedure. Reducing sugar concentrations are expressed as maltose equivalents. Readings were taken using blanks containing the amounts of inactivated enzyme and starch equivalent to that present in test assays.

3.2.5 DPPH assay

Antioxidant activities of acetone extracts and purified compounds were investigated using the 1,2 diphenyl-2-picrylhydrazil (DPPH) (Sigma-Aldrich, South Africa) antioxidant assay. Following the procedures as described by Toit *et al*., 2001) for each sample, a dilution series (8 dilutions) was prepared in a 96-well ELISA plate by adding distilled water (100µl) as a dilution medium. Final concentrations of the acetone plant extracts ranged from 3.125 to 200µg/ml and of compounds ranged from 0.78 to 100µg/ml. Each concentration was tested in triplicates. Ascobic acid (AA)/ vitamin C was used as a positive control and was tested at the concentrations ranging from 0.52 to 67µg/ml. Ninety microlitres (90µl) of ethanolic DPPH was added to each well. The plates were covered with aluminium foil and incubated at room temperature for 30 minutes before being read by a BIO-TEK Power-Wave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa).

The radical scavenging capacities of the samples were determined by using a BIO-TEK plate reader to measure the disappearance of DPPH at 550nm. The radical scavenging activity was measured in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (EC_{50}) (Toit *et al*., 2001). The EC_{50} was determined graphically by plotting the absorbance of DPPH as a function of the sample concentration in µg/ml for the standard and samples. The EC_{50} is the amount of antioxidant necessary to decrease the initial DPPH absorbance by 50%. The results are

expressed as the mg AA equivalents / g dry weight and are calculated as follows: EC_{50} AAmg/ml/ EC_{50} sample (g/ml) =xmg AA equivalents / g dry weight Zero mg/ml was taken as 100%

3.2.6 Toxicity screening (XTT viability assay)

The cytotoxicity of crude extract of *Terminalia sericea* was investigated by using XTT-based colorimetric assay Cell Proliferation Kit II (Boehringer-Mannheim) following the method as described by Roche (2004). As the extract of *Terminalia sericea* showed good hypoglycemic activity, this extract was further selected for its cytotoxicity evaluation. The final concentration of crude extract tested ranged from 3.125-400µg/ml. The final concentration of 'Doxorubicin', a positive control, in the wells ranged from 0.104-12.5µg/ml. The final concentration of the extract at which 50% (IC_{50}) of the Vero cells were alive until the 4^{th} day was considered to be the highest concentration which is non-toxic to the cells. These values were calculated using Graph Pad Prism 4 programme.

3.3 Statistical analysis

The final results are expressed as the mean (standard deviation, ± SE.S). The group means were compared using ANOVA test (MSTATC software, East Lansing, MI, USA) and the Duncan's Multiple range Test was applied to compare the means. Values were determined to be significant when p was less than 0.05 ($p<0.05$).

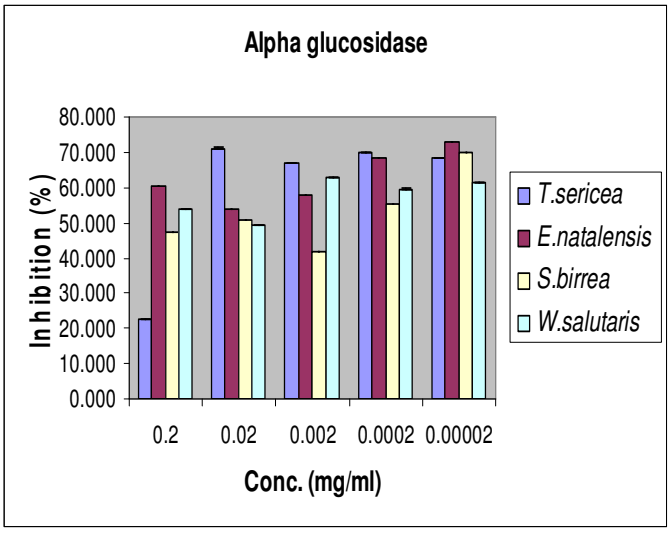
3. 3 Results and Discussion

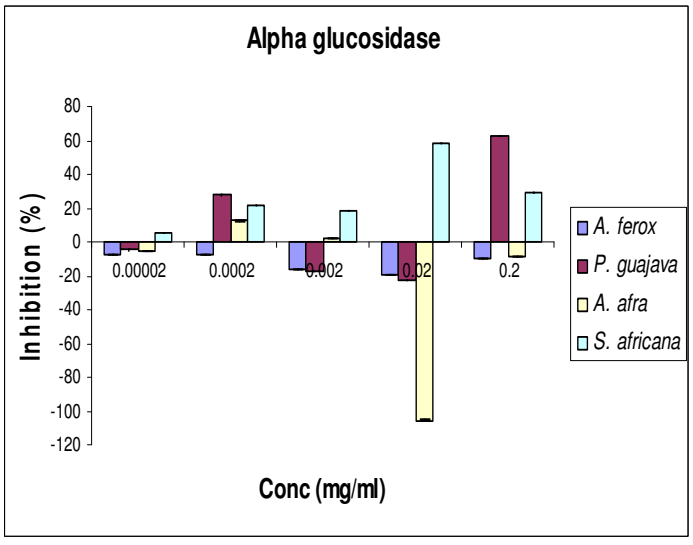
In Africa many plants are traditionally used for the management and control of diabetes mellitus, however few of such have received scientific scrutiny despite the fact that WHO has recommended that medicinal and scientific examination of such plants should be undertaken (WHO Expert Committee of Diabetes Mellitus, 1980). Alpha glucosidase and amylase are enzymes that are

involved in hydrolysis of starch hence they contribute to postprandial hyperglycaemia (Gowri *et al*., 2007). Inhibitors of these enzyme delay digestion of glucose hence prolonging overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the post-prandial plasma (Bhandari *et al*., 2008). Plant or plant products have been investigated with respect to the suppression of glucose production from carbohydrate in the gut.

3.3.1 α-Glucosidase and Amylase inhibitory activity

Eight plant extracts were tested for alpha glucosidase and amylase inhibition activity (Table 3.2, figures 3.3-3.5). The inhibition percentage of extracts ranged from 47.15 ± 0.02 to 97.57 ± 0.01 at 0.2mg/ml. The extracts of *A. ferox* and *S. africana* showed no inhibition against α-glucosidase at the highest concentration tested (0.2mg/ml) whereas *A. afra* showed weak inhibition (47.15%). *T. sericea* showed to be a potent inhibitor of α-glucosidase exhibiting 97.44 % inhibition of the enzyme ($p<0.05$). *W. salutaris, S birrea and E. natalensis* also showed good activity on α-glucosidase as they demonstrated 71.84; 97.44 and 92.60 % inhibition respectively ($p<0.05$). Other plant extracts such as *A. ferox* and *S. africana* did not exhibit any activity on α-glucosidase.(Table 3.2, figure 3.1 and 3.3)





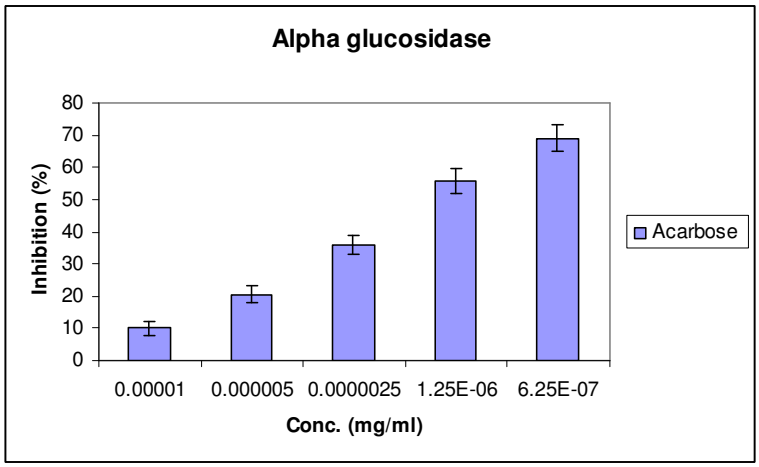


Figure 3.1: Inhibition of α-glucosidase using p-nitrophenyl α-D-glucopyranoside as a substrate, by the extracts and positive drug-control; Acarbose

T. sericea and *S. birrea* showed the best inhibitory activity on α-amylase enzyme, exhibiting 91.91 and 94.94 % inhibition respectively at 1.25mg/ml. *A. afra, E. natalensis, P. guajava* and *W. salutaris* also showed good inhibitory activity on α-amylase enzyme at 1.25mg/ml which was the highest concentration tested ($p<0.05$). (Table 3.2, figure 3.2 and 3.3). For determining the inhibitory activity against each enzyme, all extracts were tested at 1.25mg/ml for alpha amylase and 0.2mg/ml for alpha glucosidase and 50% inhibition or higher was taken as significant ($p<0.05$).

T. sericea stem bark demonstrated good activity on both enzymes. *T. sericea* has been used in the folk medicine for the treatment of many ailments including diabetes. Several compounds have been isolated from the plant (Fyhrquist *et al*., 2002). In a study done by Tshikalange *et al*., (2008), it was found that *T. sericea* inhibited α-glucosidase by 90.00 ± 0.01%. In a similar study, *Terminalia superba* and its constituents, gallic and methyl gallade showed significant α-glucosidase inhibitory activity (Wansi *et al*., 2007). On the other hand Gao *et al*, 2007 indicated that *T. chebula* also showed good inhibitory effect against rat intestinal maltase activity.

W. salutaris, S. birrea, *E. natalensis* and *P. guajava* also demonstrated good α-glucosidase and amylase inhibitory activities. *S. birrea* dichloromethane: methanol (1:1) extract has been found to decrease blood glucose levels while increasing plasma insulin levels in STZ rats (Dimo *et al*., 2007). A considerable improvement was seen in glucose tolerance during an oral glucose tolerance test in diabetic rats treated with the extract and this led to the speculation that this improvement could be associated with stimulation of insulin. In a study done by Venter and colleagues (2008), organic extract (methanol) of *S. birrea* caused a noted increase in glucose utilization in Chang liver cells. Quite a few studies have reported hypoglycemic activity of *P. guajava* extracts. A study done recently has revealed that the *P. guajava* methanol extract has shown significant inhibition of alpha-glucosidase activity in the small intestine of diabetic mice (Wang *et al*., 2007). In another study, aqueous leaf extracts of *P. guajava* were tested in STZ-induced (Ojewule, 2005) and alloxan-induced diabetic rats (Mukhtar *et al*., 2004) an ethanol extract was tested on alloxan-induced hyperglycemic rats (Mukhtar *et al*., 2006), a butanol-soluble fraction of the leaves was tested $Lepr^{db}/Lepr^{db}$ mice and has shown to significantly decrease the blood glucose levels in rats (Oh *et al*,. 2005). Other plant extracts such as *A. ferox* and *S. africana* showed weak inhibition on alpha glucosidase (inhibition less than 50%). In a study done by Jong-Anurakkun *et al*., 2008; Aloeresin A isolated from *A. ferox* have been reported to demonstrated dose-dependent α- glucosidase inhibitory activity with IC_{50} values of 11.94 and 2.16mM against intestinal sucrose and maltase respectively. In the present study the extract of *A. ferox* did not exhibit any α-glucosidase activity. *E. natalensis* also demonstrated good α-glucosidase and α-amylase inhibitory activities.

There are no reported data on the activity of *E. natalensis* on both enzymes; however, it is rich in pentacyclic triterpenoids. Triterpenoids have been reported to possess both potent α- glucosidase and α-amylase inhibitory activities (Luo *et al*., 2008). *W. salutaris* which also exhibited α-glucosidase and α-amylase inhibitory activities is rich in sesquiterpenoids. In a study done by Choudhary *et al*.,

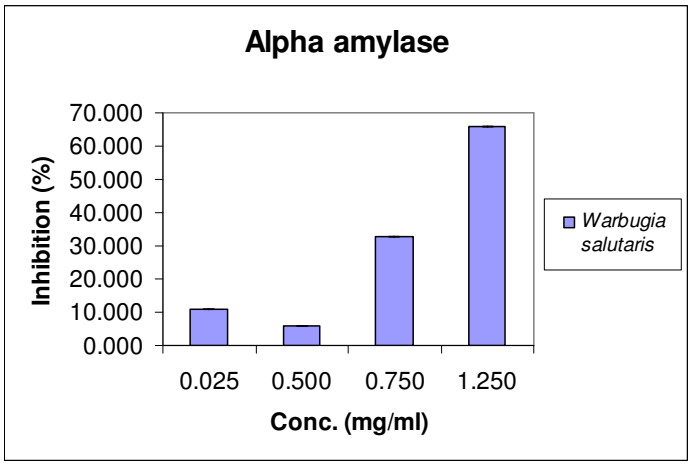
(2001), sesquitepenoids isolated from the air-dried roots of *Ferula mangolica* exhibited significant α-glucosidase inhibitory activity (IC50 value =79.87µM).

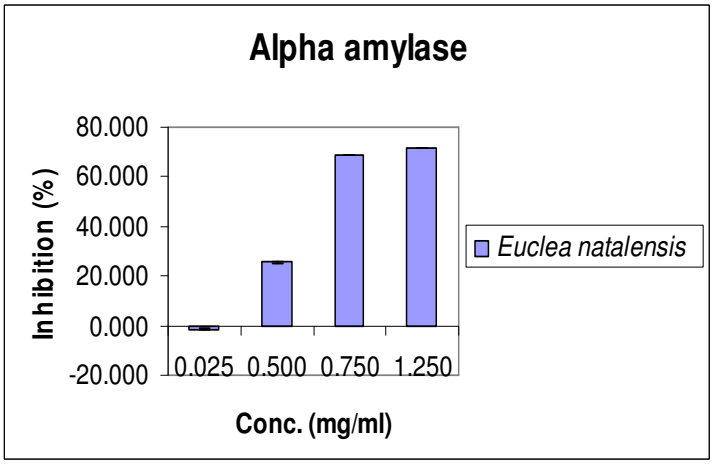
Table 3.2: Effect of plant extracts on the inhibition of α-glucosidase and α-amylase enzymes

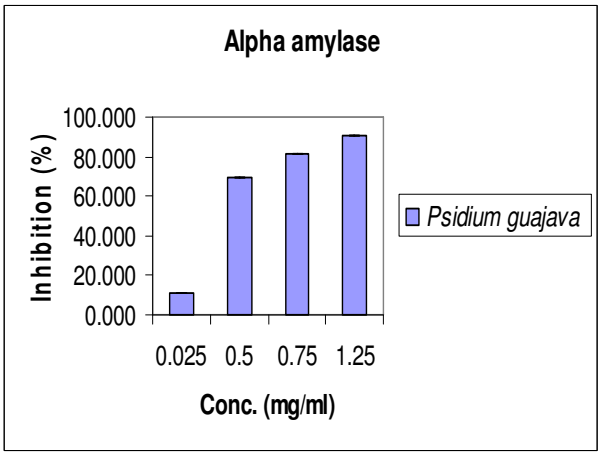
Plant	**α-glucosidase**	**α-amylase**
	Inhibition % (SD) at the concentration of 0.2mg/ml	**Inhibition % (SD) at the concentration of 1.25mg/ml**
Terminalia sericea	97.44 ± 0.03	91.91 ± 0.10
Sclerocarya birrea	97.44 ± 0.04	94.94 ± 0.01
Artemsia afra	47.15 ± 0.02	74.00 ± 0.01
Euclea natalensis	92.60 ± 0.04	74.54 ± 0.04
Psidium gaujava	62.74 ± 0.19	89.14 ± 0.01
Warbugia salutaris	71.84 ± 0.27	89.21 ± 0.06
Aloe ferox	**ni**[a]	**ni**[b]
Spirostachys africana	**ni**[a]	**ni**[b]
Acarbose	80.63 ± 0.03	73.40 ±0.03

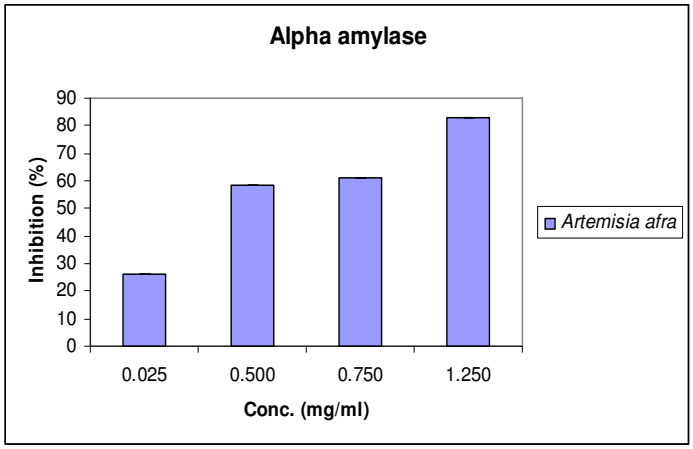
[a]: no inhibition of α-glucosidase enzyme at the highest concentration tested (0.2mg/ml)

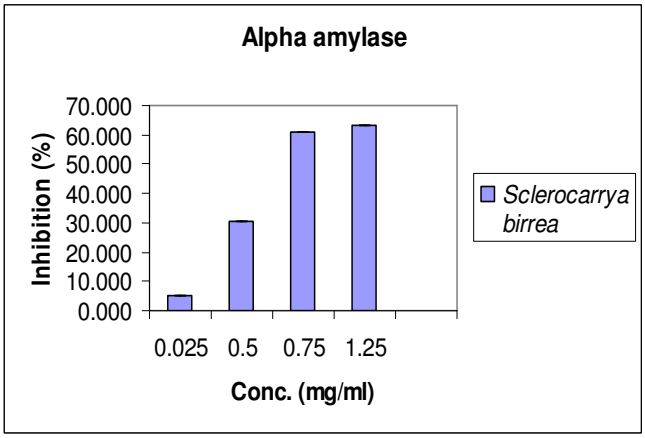
[b:] no inhibition of α-amylase enzyme at the highest concentration tested (1.25mg/ml)

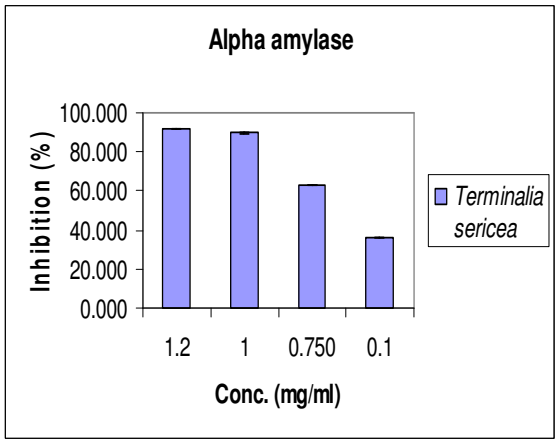

Alpha amylase
Inhibition (%)
70.000
60.000
50.000
40.000
30.000
20.000
10.000
0.000
0.025
0.500
0.750
1.250
Conc. (mg/ml)
Warbugia salutaris


Alpha amylase
Inhibition (%)
80.000
60.000
40.000
20.000
0.000
-20.000
0.025
0.500
0.750
1.250
Conc. (mg/ml)
Euclea natalensis


Alpha amylase
Inhibition (%)
100.000
80.000
60.000
40.000
20.000
0.000
0.025
0.5
0.75
1.25
Conc. (mg/ml)
Psidium guajava


Alpha amylase
Inhibition (%)
90
80
70
60
50
40
30
20
10
0
0.025
0.500
0.750
1.250
Conc. (mg/ml)
Artemisia afra


Alpha amylase
Inhibition (%)
70.000
60.000
50.000
40.000
30.000
20.000
10.000
0.000
0.025
0.5
0.75
1.25
Conc. (mg/ml)
Sclerocarrya birrea


Alpha amylase
Inhibition (%)
100.000
80.000
60.000
40.000
20.000
0.000
1.2
1
0.750
0.1
Conc. (mg/ml)
Terminalia sericea

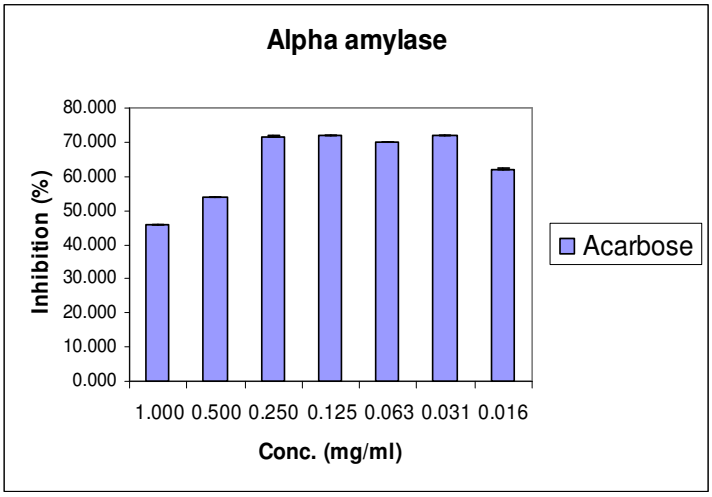


Figure 3.2: Inhibition of α-amylase using soluble potato starch as a substrate, by different concentrations of the extracts and acarbose (standard control).

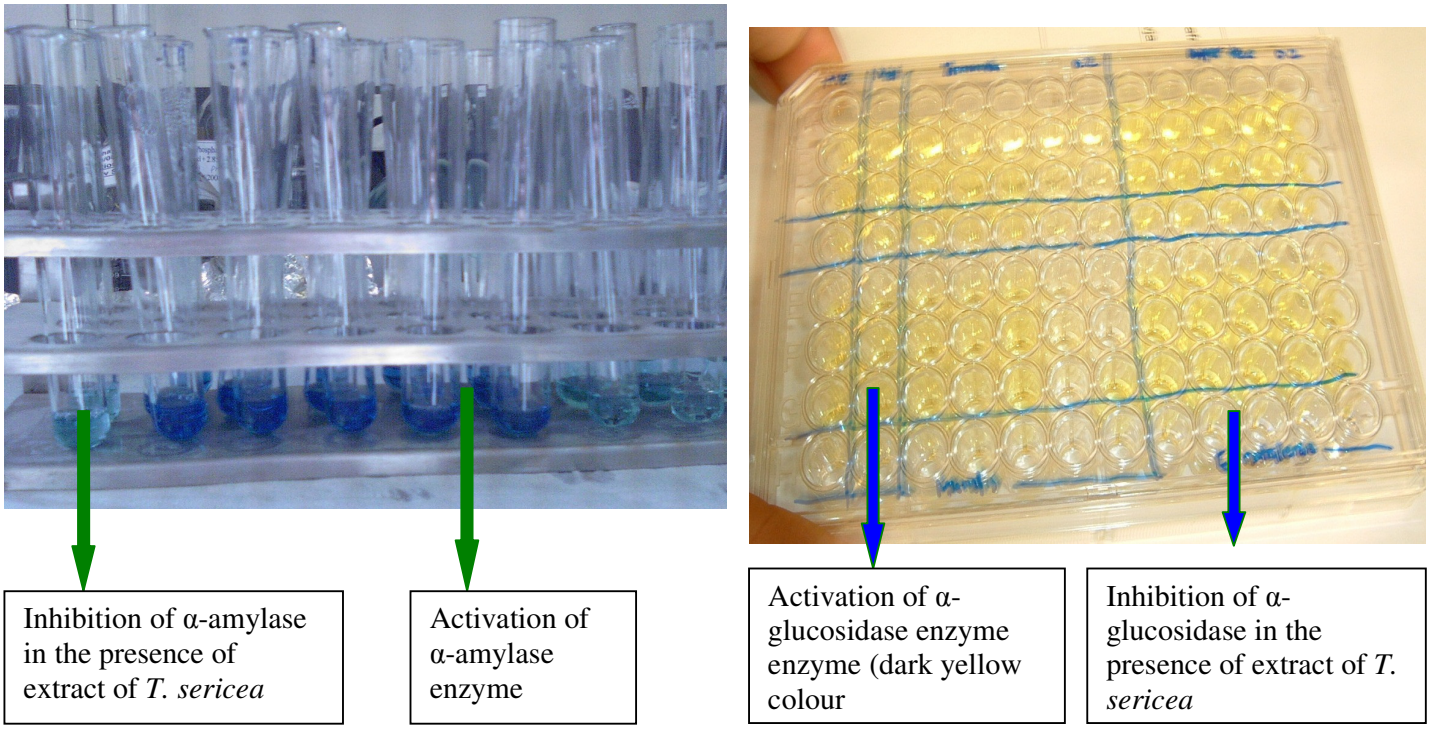


Figure 3.3: A) α- Amylase in glass tubes: activation of α-amylase enzyme results in the formation of dark blue colour, when this activity is inhibited; a light blue colour results

B) Inhibition of α-Glucosidase enzyme by the plant extracts. Activation of the enzyme results in formation of yellow colour on 96-well plates, inhibition is indicated by light-yellow colour as compared to the wells where enzyme is activated.

3.3.2 Antioxidant activity

Cellular damage/ oxidative injury arises from the formation of free radicals which appears to be the fundamental mechanism underlying a number of human neurodegenerative disorders such as cancers, diabetes and inflammation (Perez Guitierrez *et al*., 2008). Free radicals are natural by products of human metabolism which attack cells, proteins and enzymes present in cells (Masoko and Eloff, 2007). They are produced as a result of body's use of oxygen such as respiration and some cell-mediated immune response, however, environmental factors such as cigarette smoke; automobile exhaust, radiation and air pollution contribute to the formation of free radicals (Li and Trush, 1994). The body does produce antioxidants that scavenge/quench these free radicals; however, the antioxidant defence systems in the body can only protect the body when the quantity of free radicals is within the normal physiological level.

Plants contain a variety of free radical scavenging molecules. These may include phenolic compounds (flavonoids, phenolic acids, quinones, tannins); nitrogen containing compounds (alkaloids) Terpenes (carotenoids) (Zeng and Wang, 2001). The antioxidant activity of plant extracts was carried out using DPPH assay. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. As shown in table 3.3, figure 3.4 and 3.5 all six tested plant extracts showed good activity. *P. guajava* and *T. sericea* exhibited the highest activity with the IC_{50} values of 6.97± 0.03µg/ml and 5.56 ± 0.01 µg/ml respectively when compared to ascorbic acid (IC_{50}=2.52 ±0.01 µg/ml), a well-known potent antioxidant. These plant extracts demonstrated significant results ($p<0.05$). This was followed by *W. salutaris* (IC_{50}=5.08 ±0.01 µg/ml); *E.*

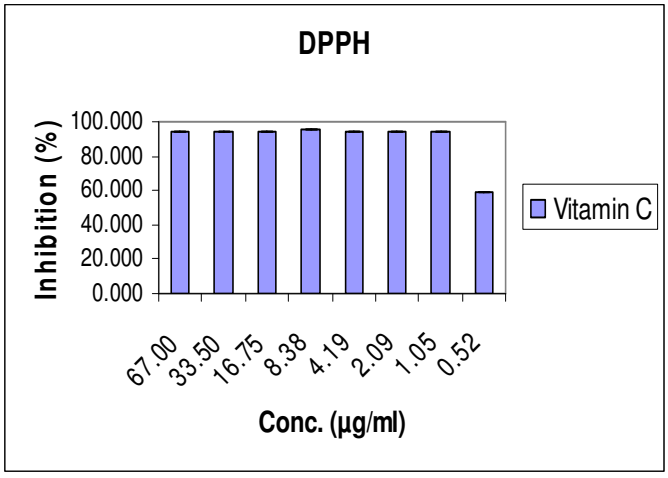
natalensis (IC_{50}= 8.46 ±0.01 µg/ml) and *S birrea* (IC_{50}= 9.41 ±0.01µg/ml). *A. ferox* showed IC_{50} value of 48.53 ± 0.01µg/ml (Table 3.3).

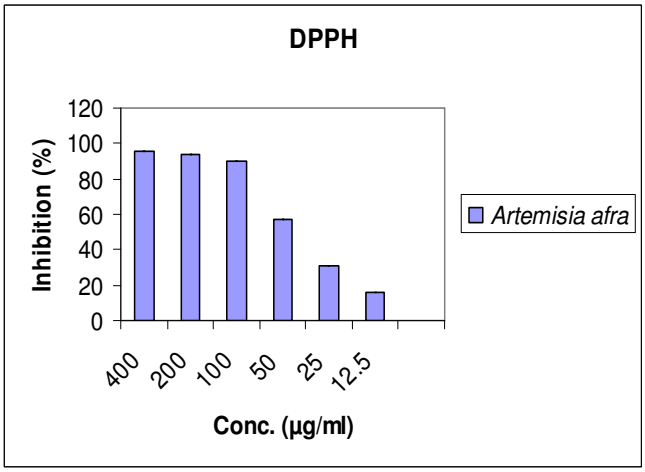
The antioxidant activity of these traditional medicinal plants may come in part from the antioxidant vitamins; phenolic compounds most particularly flavonoids or tannins. *P.guajava* contains high levels of phenolic compounds, which may attribute to its high antioxidant activity (Qian and Nihoimbere, 2004). It has been previously shown to posses antioxidant properties (Gutierrez *et al*, 2008); which are associated with its phenolic compounds such as protocathechin acid, ferulic acid, quercetin and guavin B (Thoipong *et al*., 2005). In qualitative analysis of antioxidant activity using DPPH on a thin layer chromatography (TLC) plate developed with acetone and methanol extracts of *T. sericea*, antioxidant compounds in extracts were clearly indicated as clear sports (Masoko and Eloff, 2007). Several flavonoids that demonstrated antioxidant properties have been isolated from *T. fagifolia*, a species belonging to *Terminalia* genus (Garcez *et al*., 2006).

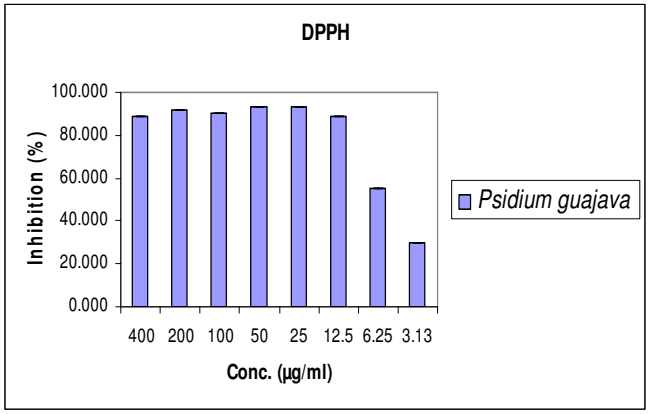
Table 3.3: A summary of fifty percent inhibitory concentration (IC_{50} values) of extracts on alpha (α) - glucosidase, α-amylase and DPPH

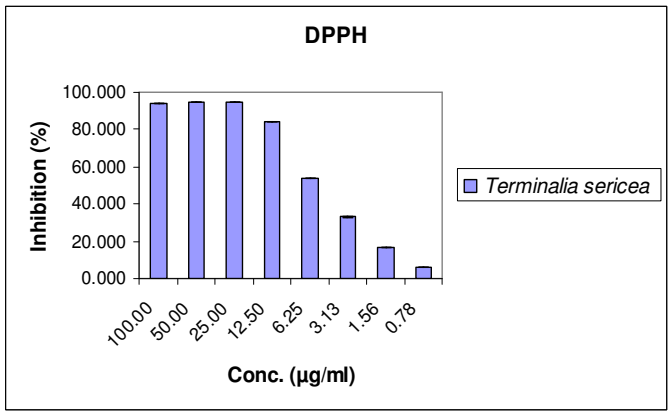
Plant	IC_{50} α-Glucosidase (µg/ml)	IC_{50} α-Amylase (µg/ml)	IC_{50} DDPH (µg/ml)
Terminalia sericea	83.41± 0.05	55 ± 0.04	5.56 ± 0.05
Sclerocarya birrea	100± 0.03	100 ± 0.01	9.41 ± 0.03
Artemsia afra	N/A	150 ± 0.01	48.53± 0.01
Euclea natalensis	95.55 ± 0.08	60.50± 0.05	8.46 ± 0.01
Psidium gaujava	96.67 ± 0.15	85.36 ± 0.06	6.97 ± 0.03
Warbugia salutaris	188± 0.01	71.23±0.09	7.50 ± 0.01
Aloe ferox	N/A	N/A	N/T
Spirostachys africana	N/A	N/A	N/T
Acarbose[a]	60 ± 0.09	42 ± 0.49	
Vitamin C[b]	-	-	2.52 ± 0.89

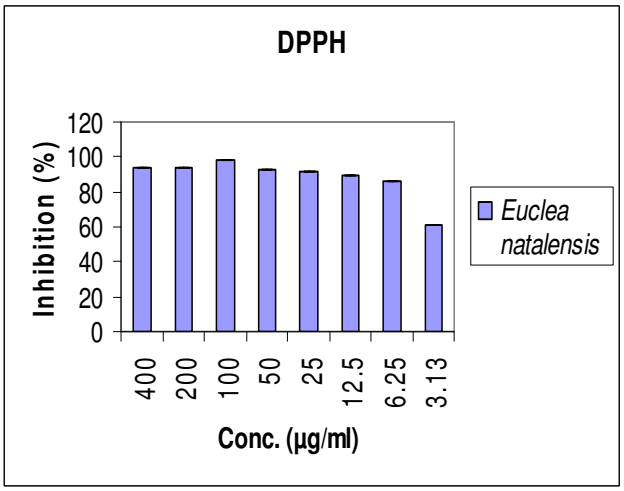
N/A: not active at the highest concentration tested
N/T: not tested (these plants did not show activity on α-glucosidase and α-amylase hence they were not tested for DPPH)
a: positive drug control for α-glucosidase and α-amylase inhibition assay
b: positive drug control for antioxidant bioassay

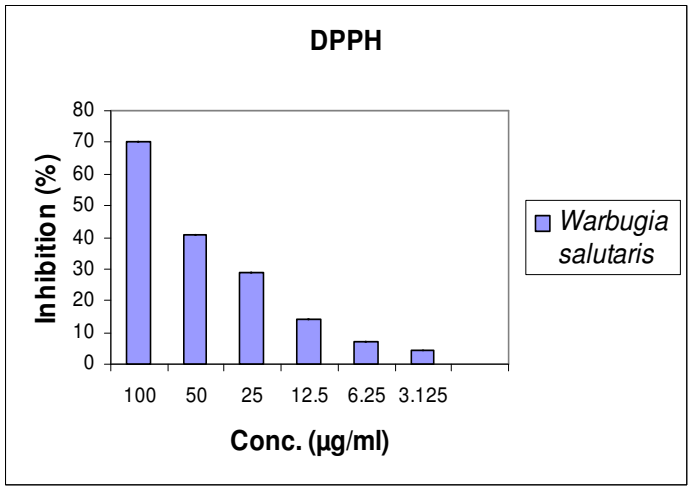












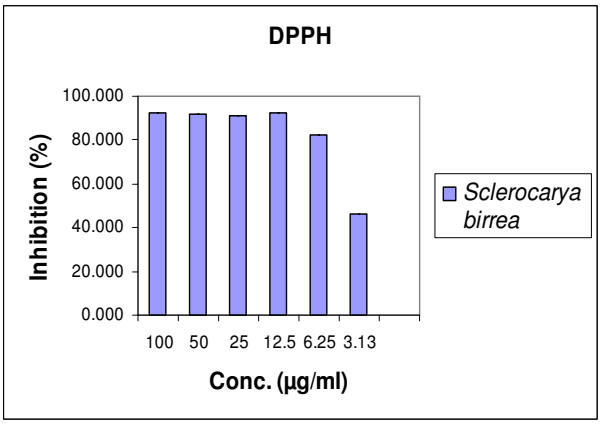


Figure 3.4: The percentage inhibition of DPPH by the acetone extracts of selected plants and Vitamin C (standard control)

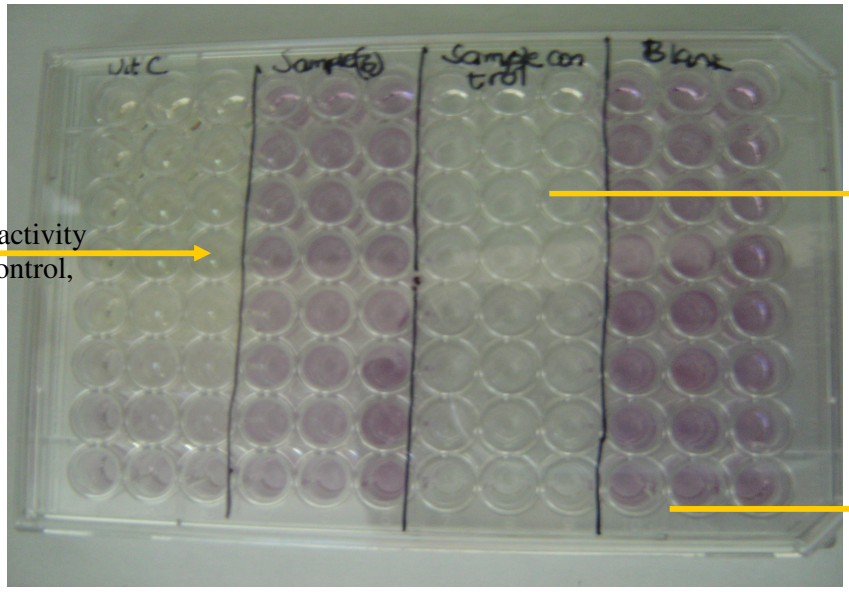


Figure 3.5: Antioxidant activity of *Terminalia sericea*.

The expression of the antioxidant activity of plant extracts in mg vitamin C equivalent has the benefits that the antioxidant is quantified and different plant extracts are compared. The IC_{50} values (the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50%) were calculated (IC_{50} VIT C mg/ml/IC_{50} sample (g/ml) = x mg vitamin C equivalents/g dry weight) and are listed in Table 3.4.

Table 3.4: EC_{50} values of acetone extracts of selected plants and number of cups equivalent in RSA (Radical scavenging capacity) to a single 200mg vitamin C capsule.

Extracts	**IC_{50} (µg/ml)**	**Mg vitamin C equivalents/g dry weight (IC_{50} value)**
Terminalia sericea	5.56	453.24
Sclerocarya birrea	9.41	260.09
Artemisia afra	48.53	51.93
Euclea natalensis	8.46	297.87
Psidium guajava	6.97	361.54
Warbugia salutaris	7.50	336.00
Vitamin C	2.52	336.00

Standard deviation values (present as error bars on graphs) were all ≤ a 3.1 % confidence interval

3.3.3 Cytoxicity of acetone extract of *T. sericea* on Vero cell lines

Cytotoxicity is a mechanism of cell-killing property that is carried by either a chemical compound, or a mediator cell called T. cell (Roche, 2004). Various methods for the determination of in vitro cytotoxicity exist; examples include brine shrimp, lactate dehydrogenase (LDH) and colorimetric assays. Lall and Meyer have reported that plant extracts and isolated compounds must undergo toxicity tests before drug discovery is taken into consideration. In this study, cytotoxicity of compounds isolated from *Terminalia sericea* has been investigated on Vero cell lines based on the tetrazolium reagent: sodium-2-3-bis-[2-metoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT). This method has been described as straightforward, reliable, efficient and inexpensive means of determining the toxicity of extracts and isolated compounds (Williams *et al*., 2003).

The IC_{50} (dose that inhibits 50% cell growth after incubation period) of the plant extract is reported in table 3.5, figure 3.6. *Terminalia sericea* acetone extract showed significant toxicity against VK cells exhibiting IC_{50} value of 20.94 ± 1.32µg/ml respectively. These findings correlate with those of Tshikalange *et al*., (2005) where it was found that methanol extract of *T.sericea* exhibited similar results (IC_{50} value was found to be 24.00µg/ml). In a study done by Fyhrquist *et al*., 2006; root extract of *T. sericea* was found to be more cytototoxic against T- 24 cancer cell lines (IC_{50}=25.35 µg/ml).

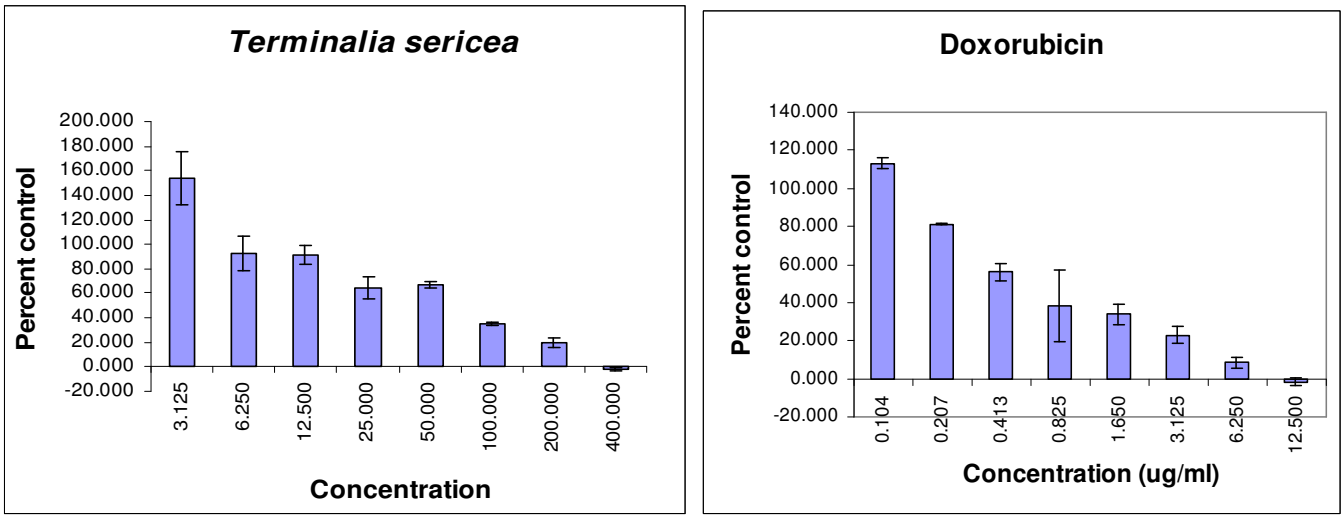


Figure 3.6: Effect of *T.sericea* crude extract and (Doxorubicin) on normal Vero cells

Table 3.5: IC_{50} values of crude extract of *T. sericea* after 4 days on Vero cells

Plant extract/ compound	Vero Cell lines IC_{50} (µg/ml) ±SD
Doxorubicin	0.2449 ± 0.120
Terminalia sericea	20.94 ± 1.32

3.4 Conclusion

This study investigated the antidiabetic activity of eight selected plants, focusing on inhibitory effects of alpha glucosidase and alpha amylase. *Psidium guajava* leaves, *Terminalia sericea* bark, *Sclerocarya birrea* bark and *Euclea natalensis* roots showed good α-glucosidase and α-amylase inhibitory activities. Several factors have been considered for choosing a plant for the isolation and identification of compounds. Factors included:

- The availability of the plant as isolation of pure compounds requires a large amount of material (2kg).

- It is feasible to collect the leaves and the bark of the plant as harvesting of the roots (as in the case of *P. guajava*) of the plant could destroy the plant.
- The plant that has been traditionally used for the treatment of diabetes and which has no scientific evidence of its hypoglycemic activity.

Due to the above mentioned factors, *T. sericea* was chosen for the isolation and identification of pure compounds. This study is the fist to report α-glucosidase inhibitory activities of all the plants except *T. sericea* which has been previously reported to possess α-glucosidase inhibitory activities. In addition, all the plants except *A. ferox* have never been reported for α-amylase activity.

3.5 References

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4. 1 Introduction

Recently medicinal plants have become the focus of intense study in terms of conservation and to whether their traditional uses are supported by pharmacological effects (Masoko *et al*., 2005) The acetone extract of the stem bark of *T. sericea* showed good hypoglycaemic activity and hence was selected for the identification of bioactive principle(s). *Terminalia* belongs to the family Combretaceae which consists of 20 genera and 300 species (Tan *et al*., 2002). The largest genera include *Combretum, Terminalia* and *Quisqualis*. About 32 *Terminalia* species occur in tropical Africa (North of the tropic of Capricorn) and about 7 occurring only in subtropical Africa.

4.1. 1 Medicinal uses and biological activity of *T. sericea*

There are many reports on the medicinal uses of *Terminalia sericea* for different ailments (Tshikalange *et al*., 2008; Moshi and Mbwambo, 2005; Fyhrquist *et al*., 2002). It has been selected as one of the fifty most important medicinal plants in Africa by the Association for African Medicinal Plant Standards (http://www.aamps.net). Dried fruits are traditionally used for the treatment of tuberculosis (TB). Roots decoctions are used for stomach troubles, wounds, diarrhoea, inflammation and sexual transmitted diseases (Eldeen *et al*., 2006). Aqueous and organic extracts made form leaves, roots and bark have been reported to have antimicrobial activity (Bruneton, 1995). In Tanzania, the bark of *T. sericea* is used for the treatment of diabetes, diarrhoea and gonorrhoea (Moshi and Mbwambo, 2005*)* Figure 4.1

It has been reported that biological activities of *Terminalia sericea* were mainly attributed to triterpenoids, saponins and tannins (Bombardelli *et al*., 1974). Several pentacyclic triterpenoids have been isolated from *Terminalia* species. The triterpenoids sericoside, arjunglucoside and an aglycon of sericoside have been found in the roots and stem bark of *Terminalia sericea* (Fyhrquist *et al*., 2002; Bombardelli *et al*., 1974)

A) **B)**

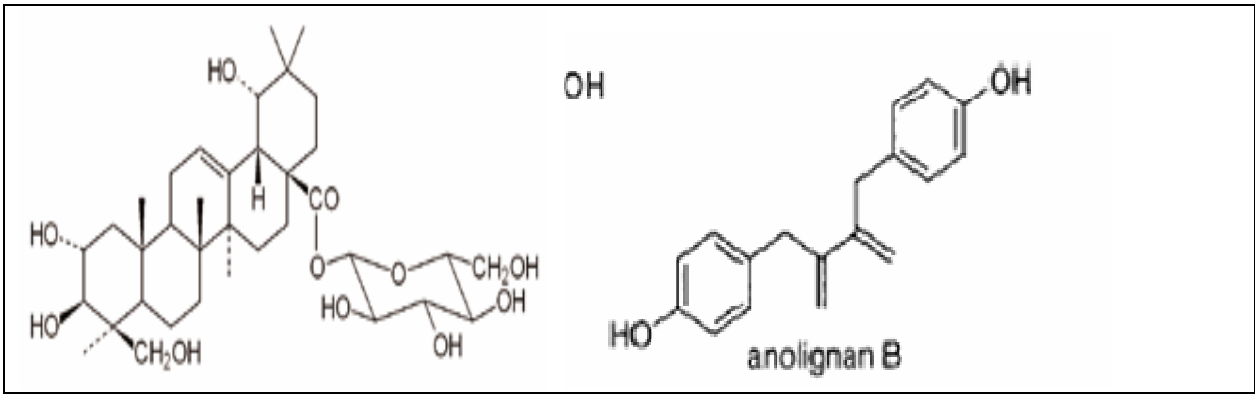
C)

Figure 4.1: Different plant parts belonging of *Terminalia sericea* (Combretaceae)
A=bark, B=fruit, C=leaves and twigs

(http://calphotos.berkeley.edu/cgi/img_query?statTerminalia+sericea&title_tag=Terminalia+sericea)

Anolignan B (a polyphenol) was isolated from the roots of *T. sericea* (Eldeen, 2006). It was reported that anolignan B with its constituent, anolignan A inhibited the enzyme HIV-reverse transcriptase. In a study done by Rimando *et al*., 1994, anolignan B was reported to have *in vitro* cytotoxic effects against fibrosarcoma cell line. Other medicinal activities that have been reported on anolignan B include: antibacterial activity, anti-inflammatory, inhibition of COX-1 and COX-2 (Eldeen *et al*., 2006).

Other compound that has been isolated from *T. sericea* includes resveratrol-3-O-β-D-rutinoside. It has been reported that *Terminalia* species possesses imino sugars [polyhydroxyalkaloids (PHA) or aza sugars]. These compounds are of significance because it has been reported earlier that the compounds inhibited glycohydrate enzymes thus are useful in carbohydrate-mediated disorders such as diabetes, HIV and various cancers (Durant *et al*., 2007) (Appendix A).



A) B)

Figure 4.2: Chemical structures of sericoside (A) and Anolignan B (B) isolated from *T. sericea*

4.2 Materials and methods

4.2.1 Extraction and isolation of pure compounds

Air-dried powdered stem bark of *Terminalia sericea* (1.8kg) was extracted with 100% acetone for 48 hours. The acetone extract was filtered and evaporated under reduced pressure. The total concentrated extract (80g) was subjected to silica gel column chromatography (CC, size 10cm x 20cm, figure 4.3) using hexane/ethyl acetate (EtOH) mixtures of increasing polarity (0-100%) followed by 100% methanol (MeOH), a total 30 sub-fractions (250ml) were collected (Figure 4.4) . The fractions were combined on the basis of thin layer chromatography (TLC) leading to 13 main fractions (Figure 4.4). TLC of 13 pooled fractions was developed with Methanol: Dichloromethane: 95:5. TLC plates were then examined under (254 and 366nm) after development and also dipped in vanillin (15g vanillin, 500ml ethanol and 10ml concentrated sulphuric acid) and heated to detect compounds which do not absorb UV. The 13 main fractions were tested on alpha glucosidase enzyme to evaluate their inhibitory activity. Results are shown in table 4.2. Fraction 1, 3 and 6 showed good inhibitory activity on α-glucosidase exhibiting IC_{50} values of 22.50, 40.85 and 50.01µg/ml respectively. Fraction 1 was subjected to silica gel CC and eluted using hexane/ ethyl acetate of increasing polarity which yielded compound **1** and 5 sub-fractions. Sub-fraction 1 of the main fraction 1 was rechromatographed on silica gel column which was eluted with ethyl acetate-hexane to give compounds **2** and **3**. Fraction 3 was subjected to sephadex CC and eluted using 100% ethanol which yielded compound **4**. Fraction 6 was further separated on silica gel CC using hexane/ethyl acetate mixtures of increasing polarity followed by 100% chloroform to give compound **5** and **6** (Figure 4.5). The structural elucidation of isolated compounds were identified by their physical (mp, $[\alpha]_D$) and spectroscopic (^{1}H and ^{13}C NMR) data. All the NMR data of all isolated compounds are illustrated in appendix A.

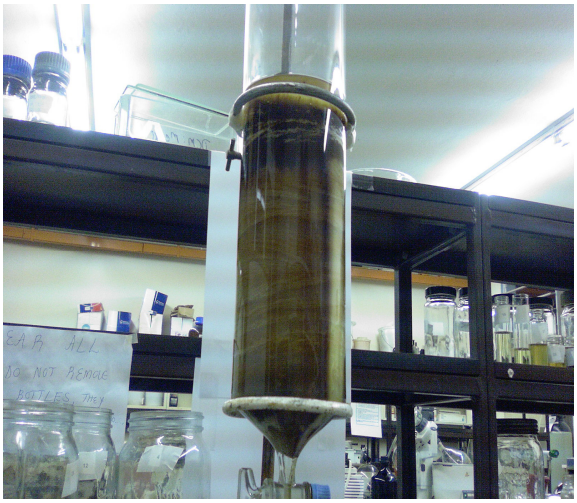
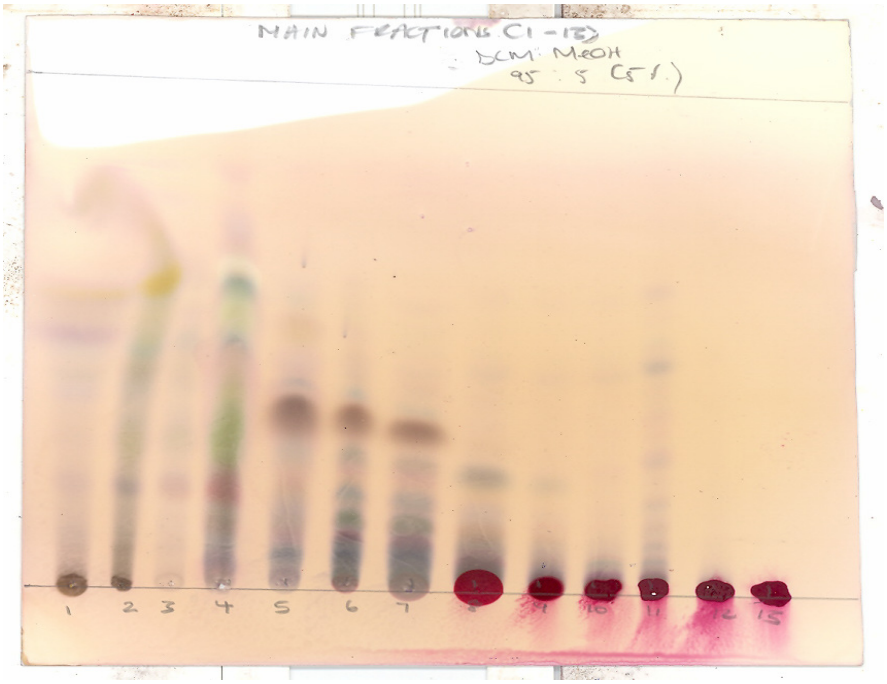
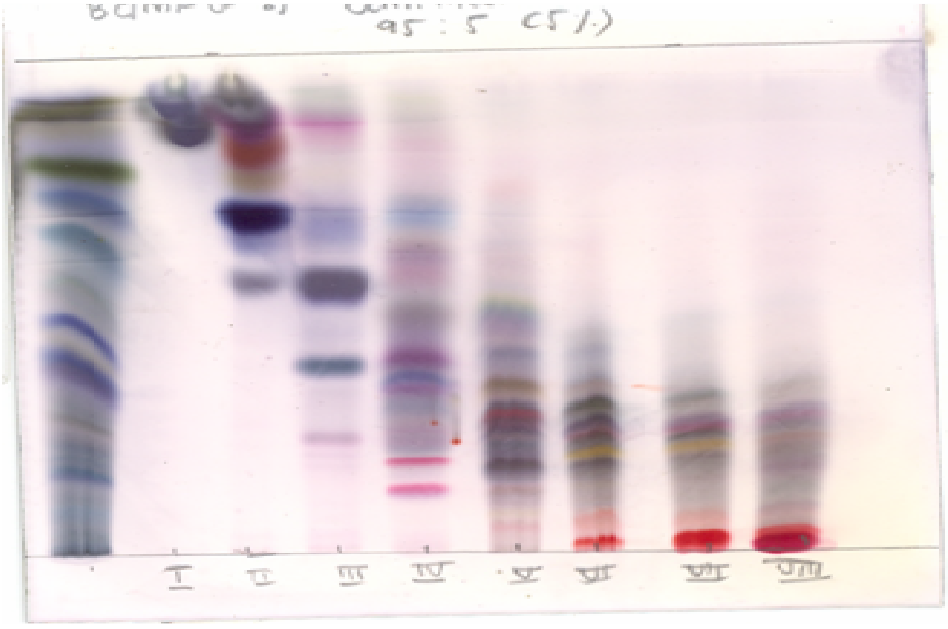


Figure 4.3: Silica gel column chromatographic purification of acetone extract of *T. sericea*



A)



B)

Figure 4.4: TLC plates of fractions obtained from chromatographic separation of acetone extract of *T. sericea*

Solvent system: A) and B) DCM: MeOH (95:5)

Detection: Vanillin in H_2SO_4

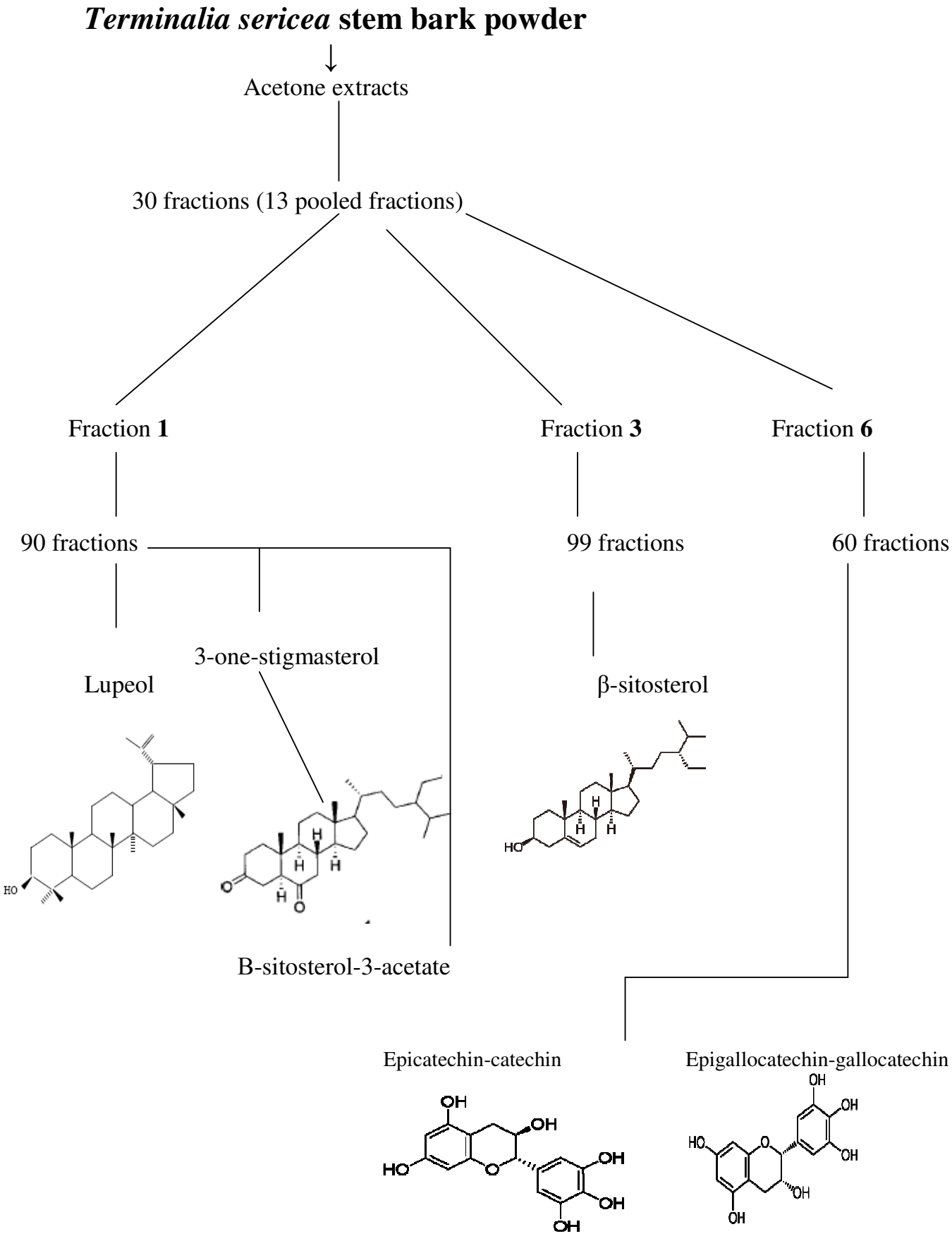


Figure 4.5: Isolation of compounds from the acetone extract of *Terminalia sericea*

4.2.2 Determining alpha glucosidase inhibition by the fractions

Alpha glucosidase activity of 13 main fractions of acetone extracts of *Terminalia sericea* was determined as described in chapter 3 (section 3.2.4). The fractions were tested at the final concentration of 0.1mg/ml.

4.2.3 Statistical analysis

The final results are expressed as the mean (standard deviation, ± SE.S). The group means were compared using ANOVA test (MSTATC software, East Lansing, MI, USA) and the Duncan's Multiple range Test was applied to compare the means. Values were determined to be significant when p was less than 0.05 ($p<0.05$).

4.3 Results and Discussion

4.3.1 Alpha glucosidase activity

Alpha glucosidase inhibition by the fractions was conducted to determine the most active fraction. Fractions 1, 3 and 6 showed the most significant alpha glucosidase inhibitory activity exhibiting 95.56 ± 0.25; 81.33 ± 1.25 and 89.50 ± 0.85% inhibition respectively ($p<0.05$) (*figure 4.3*)

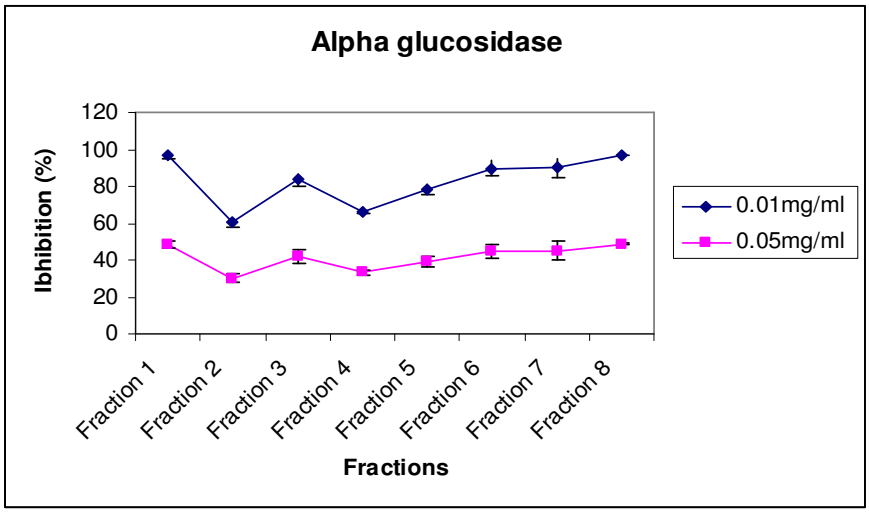


Figure 4.6: Dose dependent inhibition of alpha glucosidase enzyme by fractions using p-nitrophenyl α-D-glucopyranoside as a substrate.

Table 4.1: Fifty percent inhibitory concentration (IC_{50} values) of fractions on alpha (α) - glucosidase

Fraction(s)	*IC_{50} α-Glucosidase (µg/ml)*
Fraction 1	22.50
Fraction 3	40.85
Fraction 6	50.01

4.3.2 Characterization of compound (1+2)

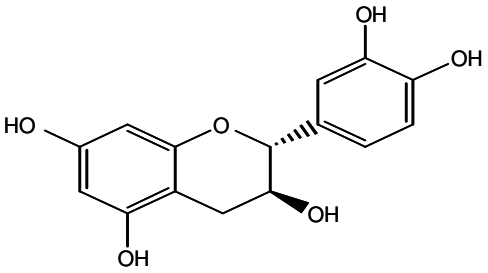
Mixture (1+2), Catechin and epicatechin

The compounds were isolated as inseparable mixture, the NMR data from the spectra obtained (^{1}H and ^{13}C) in CD_3OD are listed in Table 4.2 and show excellent agreement with the spectra obtained from authentic catechin and epicatechin, respectively, in the same solvent. The vicinal J_{HH} couplings for protons at positions 2–4 confirmed the relative stereochemistry at these centers (Khallouki *et al.*, 2007; Jesus *et al*., 2007). The mixture was identified as catechin-epicatechin (Figure 4.7).

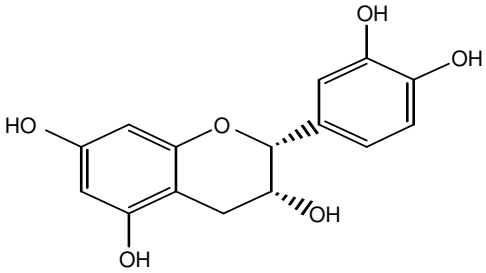
4.3.3 Characterization of compound (3+4)

Mixture (3+4) Gallocatechin and galloepicatechin

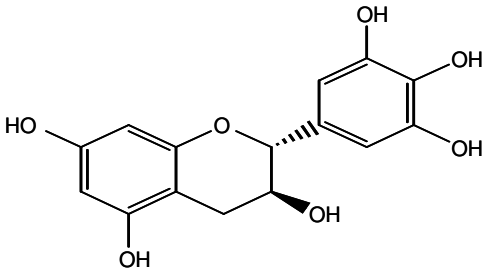
The mixture gave the same NMR profile of ^{1}H and ^{13}C (Table 4.2) of the previous mixture except for the ring B which showed a single peak (at 6.22 or 6.33) with the corresponding carbon signal at 115 (**3**) or 106 (**4**). The relative percentage of both mixtures was determined from their peak intensities either in ^{1}H or ^{13}C NMR signals. The mixture was identified as gallocatechin-galloepicatechin (Figure 4.7).



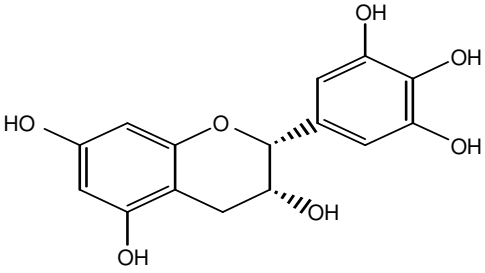
Catechin, **1**



Epicatechin, **2**



Gallocatechin, **3**



Galloepicatechin, **4**

Figure 4.7: Chemical structure of catechin, epicatechin, gallocatechin and galloepicatechin isolated from the bark of *T. sericea*

Table 4.2: ^{1}HNMR and ^{13}CNMR data of catechin-epicatechin and gallocatechin-epigallocatechin

	Mixture 1 + 2				Mixture 3+4			
	Catechin (~ 59.0%)		Epicatechin (~41.0%)		Gallocatechin (~32.0%)		Epigallocatechin (~68.0 %)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2	4.56, d, 7.5	82.6	4.81 m	79.3	4.40 d, 9.7	81.0	4.84 m	78.1
3ax	3.97, m	68.2		66.8	3.74, m	66.3		65.0
3eq		68.8	4.19 m	66.8		66.3	3.95, m	65.0
4ax	2.52, dd, 15.8, 8.0	29.0	2.91 dd,16.5, 5.5	28.7	2.33 m	27.4	2.33, m	28.1
4eq	2.79, dd, 12.8 5.4	29.0	2.74, m	28.7	2.68, m	27.4	2.68, m	28.1
5		157. 1		156. 8		155.3		155.8
6	5.88, d,2.2	96.0	5.91, d, 2.2	95.6	5.85, s	95.0	5.85,s	95.0
7		157. 6		157. 5		156.2		156.2
8	6.01, d, 2.2	95.4	6.01, d,2.2	96.0	5.66, br s	93.8	5.68, br. s.	94.1
9		157. 8		157. 4		156.5		156.4
10		100.. 6		99.5		99.0		98.6
1`		132.		132.		132.5		132.1

		1		2				
2`	6.89, d, 1.8	115.2	7.04, d, 1.8	115.2	6.22, s	106.0	6.35, s	106.0
3`		145.6		145.5		145.6		145.4
4`		145.2		129.3		129.7		129.7
5`	6.74 (d, *J* 8.0 Hz	115.6	6.7, d, 8.2	145.3		145.6		145.4
6`	6.70, dd, 8.0, 1.8	120.0	6.84, dd, 8.2, 1.8	115.4	6.22, s	106.0	6.35, s	106.0

4.3.4 Characterization of compound 5 (1)

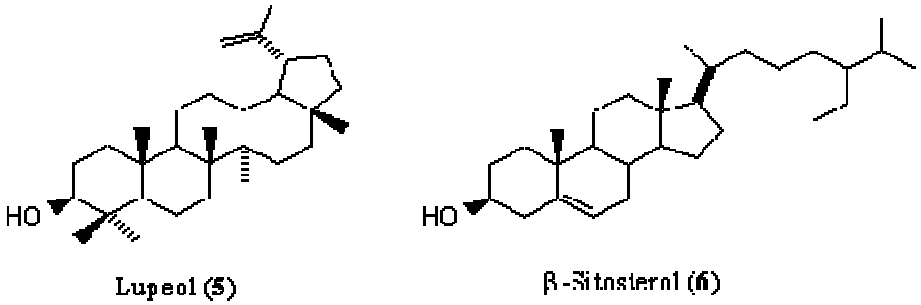
^{1}H-NMR (CDCl3), 400 MHz): δ4.69 and 4.56 (each 1H,m, H-29), 3.18 (1H, dd, H-3), 2.39 and 1.93 (each 1H, m,H-19, 21A), 1.71 (1H, t, H-15A), 1.69 (3H, s, H-30), 1.68 (2H, d, H-12A, 1A), 1.67 (1H, t, H-13), 1.61 (1H, d, H-2A), 1.54 (1H, q, H2B), 1.54, 1.49 and 1.42 (each 1H, d,H-64, 16A, 11A), 1.42 (1H, m, H-22A), 1.41 (2H, m, H-7), 1.39 (1H, q, H-6B), 1.38 (1H, t, H-16A), 1.37 (1H, t,H-18), 1.33 (1H, m, H-21B), 1.28 (1H, d, H-9), 1.29 (1H,q, H-11B), 1.20 (1H, m, H-22B), 1.07 (1H, q, H-12A),1.04 (3H, s, H-23), 1.01 (1H, d, H-15A), 0.98 (3H, s, H-23), 0.97 (3H, s, H-27), 0.91 (1H, t, H-18), 0.27,0.84, 0.79 (each 3H, s, H-25, 28, 24) and 0.69 (1H, d, H-5).

^{13}CNMR (CDCl3, 100 MHz): δ 150.8 (C-20), 109.3 (C-29), 78.9 (C-3), 55.2 (C-5), 50.3 (C-9), 48.2 (C-18), 47.9(C-19), 42.9 (C-17), 42.7 (C-14), 40.7 (C-8), 39.9 (C-22),38.8 (C-4), 38.6 (C-1), 38.0 (C-13), 37.1 (C-10), 35.5 (C-16), 34.2 (C-7), 29.8 (C-21), 27.9 (C-23), 27.4 (C-15),
27.3 (C-2), 25.0 (C-12), 20.9 (C-11), 19.2 (C-30), 18.2(C-6), 17.9 (C-28), 16.1 (C-25), 15.9 (C-26), 15.3 (C-24) and 14.5 (C-27).

The compound was isolated from the non-polar fractions through different silica gel columns. The compound obtained as a white powder. The identification of the compound was completed using NMR spectra (^{1}H and ^{13}C) and TLC comparison with authentic sample exist in our lab. The presence of seven methyl singlets and an olefinic function in the ^{1}H-NMR spectrum revealed that compound **1** may be pentacyclic tri-terpenoidal type in nature. The comparison of ^{1}H-NMR chemical shits with that of the reported data similar type of compounds and lupeol has led to the conclusion that compound **1** is lupeol (Figure 4.8). This is a very common chemical compound found in higher plants (Imam *et al*., 2007). This compound has been previously isolated from bark of *Heitiera utilis* and *Euphorbia laterifolia* (Blair *et al*., 1970; Lavie *et al*., 1968).

4.3.5 Characterization of compound 6 (4)

The isolated pure compound was characterized and identified by spectral analysis. Compound 4 was obtained as colourless crystals, m.p. 137. in ^{1}H NMR: it showed signals at 3.52 (1H, s, H-3), 5.35 (1H, s, 6-H), in addition to cluster of methyl signals in the aliphatic region, this compound was effectively identified as β-sitosterol (Figure 4.8) based on comparison of NMR data obtained with those in literature and comparison with the authentic exist in our lab. (Chen *et al*., 2008).



Lupeol (5) β-Sitosterol (6)

Figure 4.8: Chemical structure of lupeol and β-sitosterol isolated from the bark of *T. sericea*

4.3.6 Characterization of compound 7 (6)

IR: (KBr) cm_1: 3429, 3373 (AOH), 2959, 2866, 1463, 1367(C@C); MS (m/z): 414 (M+), 400, 387 329, 303, 213, 99, 85, 71, 57, 43, 29;

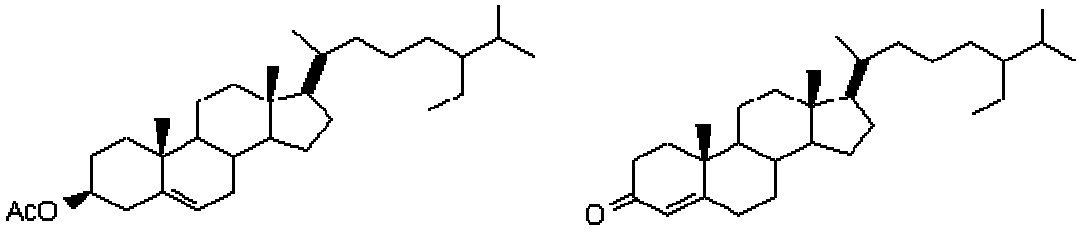
^{1}H NMR: (CDCl3, 500 MHz): 3.52 (1H, s, AOH), 5.35 (1H, s, 7-H);

13C NMR: (CDCl3, 125 MHz): 140.7 (C-5), 121.7 (C-7), 71.7(C-1), 56.7 (C-17), 56.0 (C-14), 50.1 (C-21), 45.8(C-10), 42.3 (C-13), 19.8 (C-29), 19.4 (C-28), 19.0 (C-25), 18.7 (C-26), 11.9 (C-27), 11.8 (C-4).

The compound was isolated as colorless powder and showed similar pattern of NMR like the previous compound except the downshift of proton H-3 at 4.50 and appearance of acetate signal at 2.04, which indicated the acetylation of the 3-OH, and the compound finally identified as β-sitosterol-3-*O*-acetate (Figure 4.9). The identification was confirmed by comparison of the isolated compound with the authentic acetate derivative of β-sitosterol that exists in the lab.

4.3.7 Characterization of compound 8 (7)

The isolated pure compound was characterized and identified by spectral analysis. It was isolated as white needles. The isolated compound showed a typical ^{1}HNMR spectra like the previous two compound except the downfield shift of the olefinic proton to 5.72 and the disappearance of H-3 signal. The compound was identified as stigma-4-ene-3-one from comparison of the NMR data with those published in literature (Figure 4.9) (Seca *et al.,* 2000).



β-Sitosterol-3-*O*-acetate (7) stigm-4-ene-3-one (8)

Figure 4.9: Chemical structure of β-sitosterol-3-acetate and stigma-4-ene-one isolated from the bark of *T. sericea*

4.4 Conclusion

Isolation of triterpenes

Triterpenes represent a diverse class of natural products. There have been reports of thousands of triterpenes structures as well as hundreds of new derivatives that are discovered each year. Pentacyclic triterpenes have 30-carbon skeleton that comprise five, six membered rings (ursanes and lanostanes) of four, six-membered rings and one, five-membered ring (lupanes and hopanes) (Chaturvedi *et al*., 2008).The production of pentacyclic triterpenes is based on the arrangement of squalene epoxide molecules. These molecules are predominantly present in fruits, vegetables and many medicinal plants. At least 4000 known triterpenes have been reported to most of which occur freely while others occur as glycosides (saponins) or in special combined forms (Jiri, 2003). Pentacyclic triterpenes have a wide continuum of biological activities that can be useful in medicine. Among this class of compounds, lupeol [lup-20 (29)-en-3b-ol] occurs across a multitude of taxonomically diverse genera.

The isolation of all the compounds (1-6) was achieved by means of various physical (solvent extraction, chromatography) and spectral techniques. Compound 3 was the first ever compound to be isolated from *T. sericea.*

In conclusion, lupeol and β-sitosterol have previously been isolated from *Terminalia sericea* and *T. glautens*. In addition, epicatechin and catechin have been isolated from *Terminalia catappa*, a same genus as *T. sericea***.** Stigma-4-ene-3-one has previously been isolated from *Hibiscus cannabinus*. A flavan-3-ol, gallocatechin, was first isolated from the leaves and twigs of *T. arjuna*. (Lin *et al*., 2000). This study is the first to report the isolation of β-sitosterol-3-acetate, stigma-4-ene-3-one,mixture of epicatechin-catechin and epigallocatechin-gallocatecin from *Terminalia sericea*.

4.5 References

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