5.1 Introduction

A bioassay guided fractionation led to the isolation of 4 pure compounds namely β-sitosterol, β-sitosterol-3-acetate, lupeol and 3-one-stigmasterol and two sets of mixtures of isomers (epicatechin-catechin and epigallocatechin-gallocatechin. Lupeol and β-sitosterol have been isolated from the roots of *Terminalia sericea* before. 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*. β-Sitosterol-3-acetate, stigma-4-ene-3-one, Epicatechin-catechin and epigallocatechin-gallocatecin from *Terminalia sericea* are reported for the first time. It was decided to evaluate the α-glucosidase, α-amylase, antioxidant and cytotoxicity activities of the isolated compounds.

5.2 Materials and Methods

The materials and methods for all the assays are described in chapter 3.

5.3 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 ($p<0.05$).

5.4 Results and Discussion

There are more than 200 pure compounds from plant sources that have been reported to show blood glucose lowering activity (Marles and Farnworth, 1994). The wide variety of chemical compounds contributes to the different mechanisms of lowering blood glucose levels they are responsible for (Ali *et al*., 2006). In addition, it has long been recognized that many naturally occurring substances have inhibitory effect of α-glucosidase and amylase in plant materials such as fruits, leaves, seeds etc (Shim *et al*., 2003). Studying those promising bioactive constituents open doors to new diabetic drugs discovery.

5.3.1 α-Glucosidase and Amylase inhibitory activity

The results of alpha glucosidase and alpha amylase inhibitory activities of compounds isolated from *Terminalia sericea* are shown in table 5.1, figure 5.1. This study reports that, from the six isolated compounds, β –Sitosterol and lupeol showed best inhibitory activity on α-glucosidase exhibiting 50% inhibitory concentration (IC_{50}) value of 54.49.± 0.01 µM and 66.48 ± 0.02 µM respectively ($p<0.05$). This was followed by epigallocatechin-gallocatechin (IC_{50}=119.34 ± 0.01 µM); β-sitosterol-3-acetate (IC_{50}=129.36 ± 0.01 µM); stigma-4-ene-3-one (IC_{50}=184.87 ± 0.01 µM) and epicatechin-catechin (IC_{50}=255.80 ± 0.02 µM).

During the evaluation of purified compound's inhibitory activity on α-amylase, compounds of interest were lupeol and β-sitosterol which exhibited IC_{50} values of 140.72 µM and 216.02 µM respectively as compared to the positive drug-control acarbose (IC_{50}=65.25 µM). Epicatechin-catechin and epigallocatechin-gallocatechin also demonstrated α-amylase inhibitory properties and the IC_{50} values were found to be lower than 100µg/ml. In a study done by Mai *et al*., (2007) it was

found that catechins posses α-glucosidase inhibitory activities (93% inhibition) at the final concentration of 0.8mg, suggesting that these compounds might be possible new sources of α-glucosidase inhibition.

Lupeol (Lup-20(29)-en-3-ol) is a naturally occurring triterpene that is abundant in various fruits, has been isolated from many medicinal plants including *Hieracium pilosella, Tamaindus indica, Crataeva nurvala, Arbutus unedo* (Gawronska-Grzywacz and Krzaczek, 2007; Imam *et al*., 2007). In a study done by Ali *et al*., 2006 it was found that lupeol inhibited alpha amyalse enzyme by 60% and these findings are similar of the present study where lupeol inhibited α-amylase enzyme by 70% at the highest concentration tested. In a recent study lupeol, β-sitosterol and stigmasterol obtained from a methanolic extract of seeds of *Cinchorium intybus* demonstrated good α-amylase inhibitory activities (IC_{50} values=250µM, 300µM and 500µM) respectively (Rahman *et al*., 2008).

Table 5.1: Fifty percent Inhibitory concentration (IC_{50}) values of compounds on alpha (α)-glucosidase and α-amylase enzymes

Compounds	***IC 50 á –Glucosidas (µM))***	***IC 50 á –Amylase (µM)***
Acarbose (positive drug Control)	93.22	60.25
β-sitosterol	54.49	215.95
β-sitosterol-acetate	129.36	**N/A**
stigma-4-ene-3-one	184.87	**N/A**
Epigallocatechin & Gallocatechin	119.34	328.06
Epicatechin & Catechin	255.796	304.89
Lupeol	66.48	140.72

N/A: NOT ACTIVE at the highest concentration tested

In a recent study by Shabana *et al.,* (2009)it was found that the millet seed coat inhibited both alpha glucosidase and pancreatic amylase in a dose dependent manner. Mass spectra of the finger millet extract showed the presence of naringenin, kaempferol, luteolin glycoside (+)-catechin/ (-)-epicatechin etc (Shabana *et al*., 2009). It has been reported that plant phenolic compounds modulate the enzymatic breakdown of carbohydrates by inhibiting amylases and glucosidases (McDougall *et al*., 2005). Furthermore, flavonoids, like antioxidants may prevent the destruction of pancreatic β-cells function due to oxidative stress thus reducing the incidence of type-2 diabetes (Song *et al*., 2005). Sabu *et al*., (2002) observed the antidiabetic and free radical scavenging activities of tea polyphenols such as gallocatechin (GC), epigallocatechin (EGC), epicatechin (EC), epicatechin gallate (EGCG) which correlates to the findings of the present study of isolated compounds from *T. sericea.*

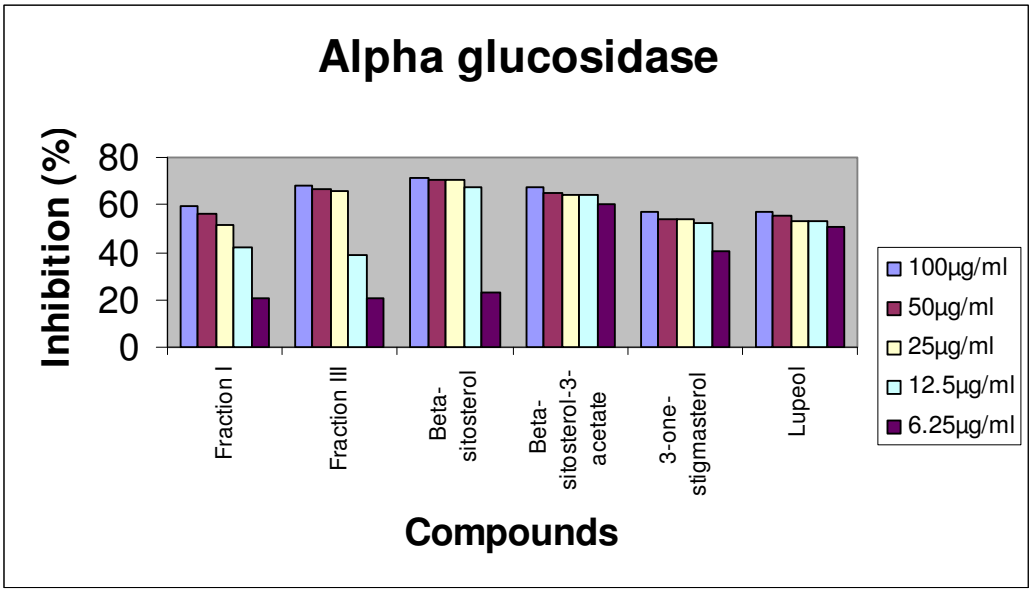


Figure 5.1: Inhibitory activity of compounds isolated *T. sericea* on α-glucosidase

5.3.2 Antioxidant activity

DPPH is a stable radical that has the maximum absorption of 517nm which can readily undergo scavenging in the presence of an antioxidant (Lu and Yeap, 2001). The advantages of using DPPH assay for the determination presence of antioxidant includes: This bioassay can accommodate multiple samples period, it is sensitive enough to detect active ingredients at low concentrations, as a result DPPH has been used to evaluate the antiradical activity of various samples (Pioa *et al*., 2004; Yu *et al*., 2002). Table 5.2 depicts the DPPH scavenging activity of the compounds isolated from *Terminalia sericea*. As established, epigallocatechin-gallocatechin, epicatechin-catechin and lupeol showed high radical scavenging activity as they inhibited DPPH by 98.19; 96.98 and 70.90 % at 100µg/ml respectively ($p<0.05$). The two isolated isomers namely epigallocatechin-gallocatechin, epicatechin-catechin are polyphenolic plant antioxidants. They belong to the family of flavan-3-ols (Li *et al*., 2007). Epigallocatechin–gallocatechin and epicatechin–catechin inhibited DPPH by more than 95% similar to our findings where it was found that similar compounds caused more than 95% inhibition on DPPH at 100µg/ml suggesting they had scavenged the whole amount of DPPH (Han *et al*., 2008). On the other hand, the activity of scavenging DPPH was very low in case of β-sitosterol (21.5% inhibition). β-Sitosterol-3 and its derivative, β-sitosterol-acetate-3-acetate did not show any activity, (table 5.2).

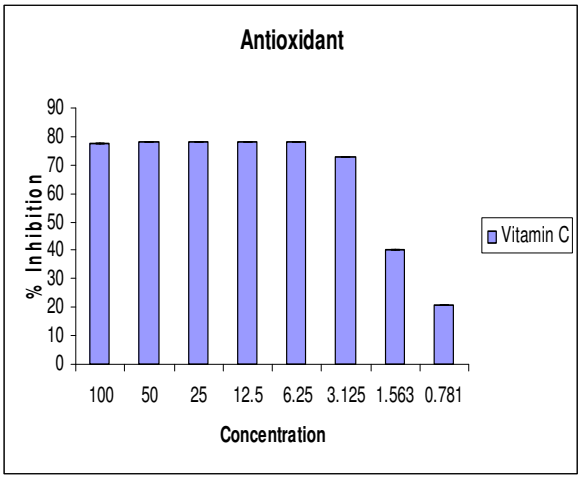
Catechin and epicatechin are epimers with (-)-epicatechin and (+) and they are common isomers that are abundant in nature. On the other hand, epigallocatechin and gallocatechin contain an additional phenolic hydroxyl group when compared to the former (Li *et al*., 2007). Flavonoids have been reported as being potential therapeutic agents for type 1 diabetes (Yazdanparast *et al*.,

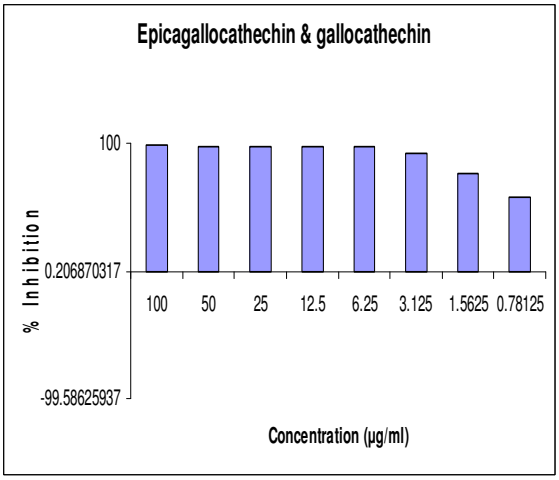
2007). Therefore, currently there is intensive focus on polyphenolic phytochemicals such as flavonoids (Coskun *et al*., 2005). Narvaez-Mastache *et al*., (2008) previously reported that catechin and epicatechin that were isolated from *Eysenhardtia subcoriacea* demonstrated strong radical scavenging properties against diphenylpicrylhydrazil (DPPH). Our results are in agreement with the findings of Yu *et al*., (2007), where it was found that epicatechin, isolated from *Garcinia mangostona* exhibited significant antioxidant activity when DPPH was used. Epicatechin-a flavan-3-ol has previously been isolated from *Hibiscus escullentus*. This plant demonstrated good *in vitro* antioxidant potential and the major antioxidant molecule was identified to be epigallocatechin (Shui and Peng, 2004). It has been reported that several derivatives of stigmasterol such as stigmasterol, stigmastadienol and stigmastadiene which were isolated from *Hibiscus tiliaceus* have demonstrated *in vitro* antioxidant effects using *Saccharomyces cerevista* defective in antioxidant defense and exposed to oxidative stress induced by hydrogen peroxide and tert-butylhdroperoxide (Rosa *et al*., 2006; Wang *et al*.,2000). Contrary to these finding, stigma-4-ene-3-one (a derivative of stigmasterol) which was isolated from *T. sericea* in our findings did not demonstrate antioxidant activity. This difference could be due to the different assays used and the difference in the chemical structure of these compounds. β- Sitosterol on the other hand did not show any DPPH scavenging effects and our study correlates with the study done by Han and colleagues (2008), where they did not find antioxidant effect of β-sitosterol.

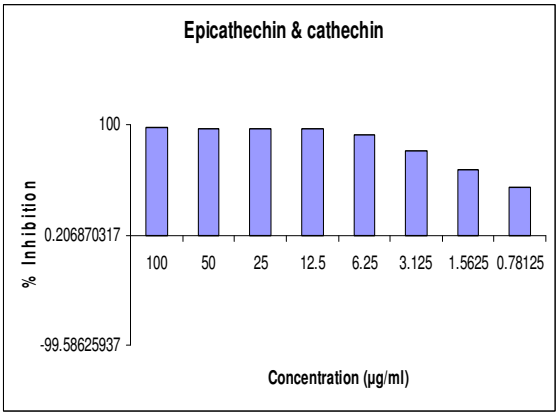
Table 5.2: Inhibition of DPPH (percent) by the compounds at the concentration of 100µg/ml

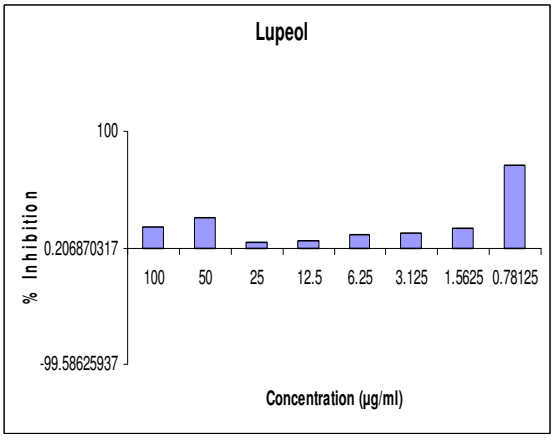
Compounds	DPPH (%) activity)
Vitamin C	2.5
β-sitosterol	21.504
β-sitosterol-3-acetate	N/A
Stigma-4-ene-3-one	N/A
Epigallocatechin - Gallocatechin	98.19
Epicatechin -Catechin	96.98
Lupeol	70.9

N/A=not active at the highest concentration tested









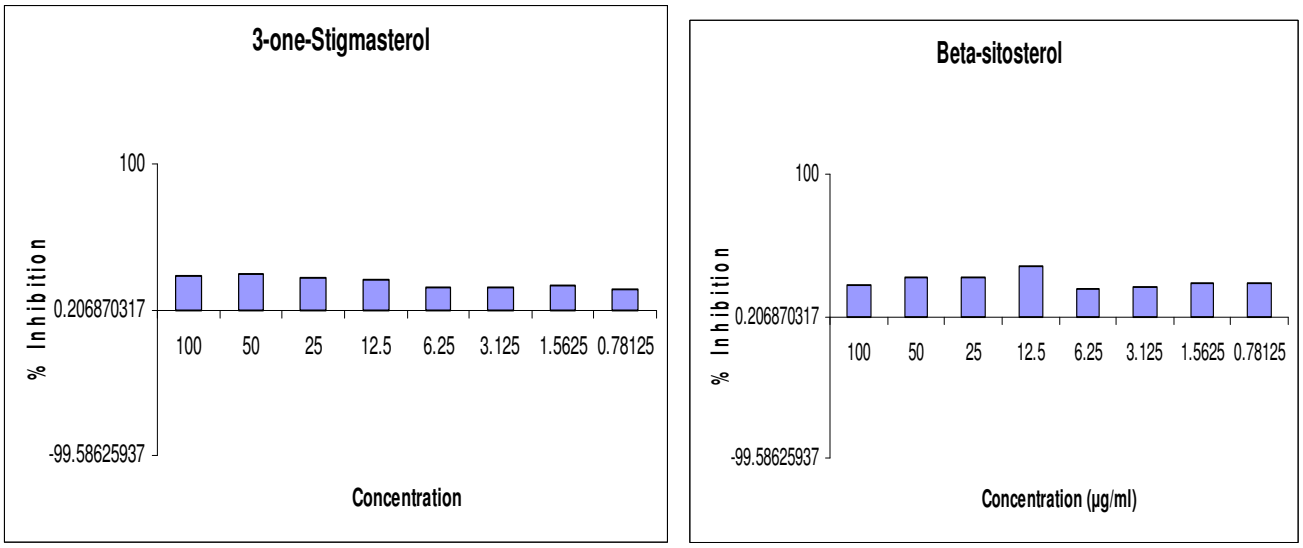


Figure 5.2: Antioxidant activity of isolated compounds from *T. sericea*

5.3.3 Cytotoxicity of isolated compunds on Vero cell lines

Compounds isolated from *Terminalia sericea* were evaluated for their *in vitro* activity against the growth of Vero cell lines. All the compounds except β-sitosterol did not inhibit the growth of these cells lines at the highest concentration tested (200µg/ml). β-Sitosterol showed moderate toxicity exhibiting IC_{50} values of 192.72 ± 2.8 µM. β-Sitosterol-3-acetate, epicatechin-catechin, lupeol and epigallocatechin-gallocatechin were found to be non-toxic to Vero cells as 100% cell viability was observed when Vero cells were exposed to these samples (table 5.4). β-Sitosterol did not demonstrate cytotoxicity on Vero cells, however, Moon *et al*., (2007) suggested that the same compound induced apoptosis in MCA-102 fibroblasts.

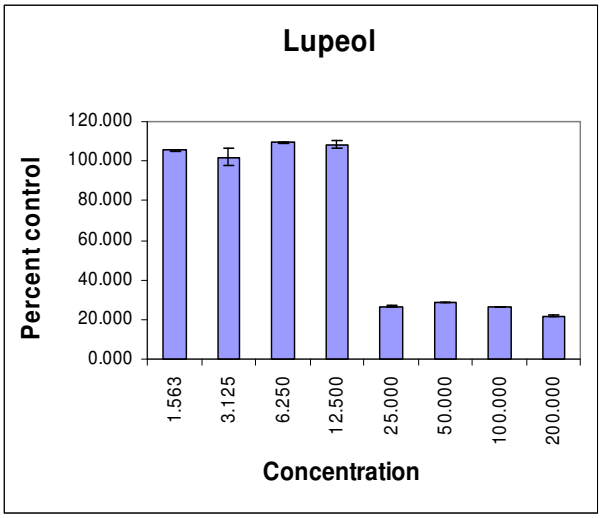
Table 5.4: IC_{50} values of isolated compounds from *T. sericea* after 4 days on Vero cells

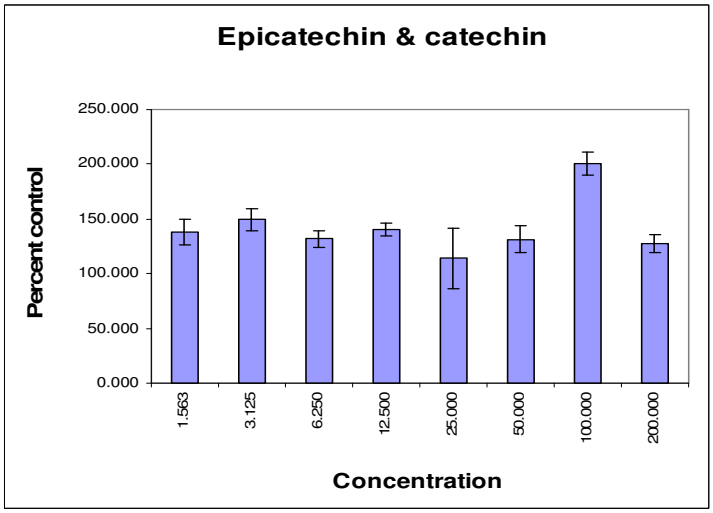
Plant extract/ compound	**Vero Cell lines IC_{50} (µg/ml) ± SD**	**Vero Cell lines IC_{50} (µM) ± SD**
Doxorubicin	0.2449 ± 0.120	0.41 ± 0.12
Lupeol	>300.9 ± 2.43	705.14 ± 0.12
β-sitosterol-3-acetate	>200.00 ± 0.659	482.25 ± 0.659
Epigallocatechin – gallocatechin	>200.00 ± 0.265	653.02 ± 0.27
Epicatechin - catechin	>200.00 ± 4.93	689.00 ± 4.93
β-sitosterol	82.0 ± 2.8	197.72 ± 2.80

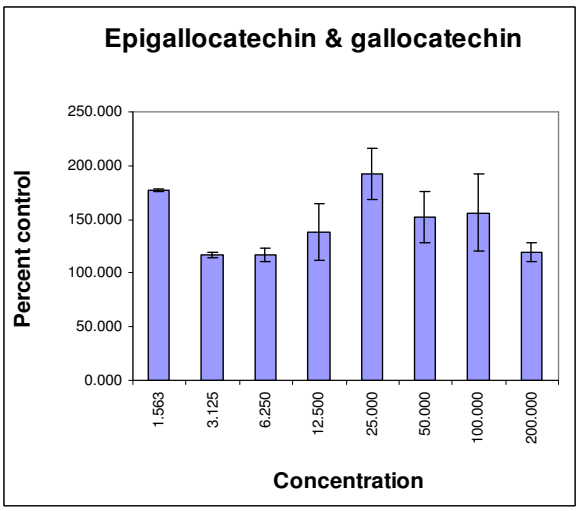
SD: Standard deviation

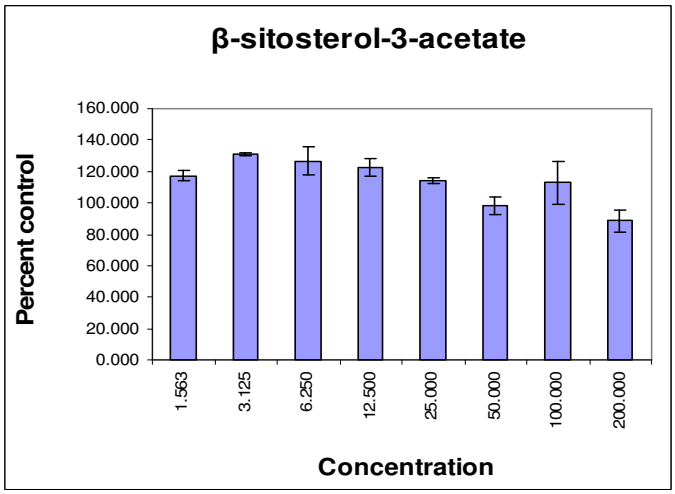
Lupeol isolated from *Spirostachys africana* had shown no toxicity on Vero cell lines with the IC_{50} value of 300.09µg/ml (Mathabe *et al*., 2008). You *et al*., (2003) have reported that lupeol did not inhibit the growth of tumor cell lines such as SK-MEL-2 and B16-F10 melanoma. On the other hand lupeol exhibited weak cytotoxicity (IC_{50}= >100µg/ml) when tested against melanoma B16 cells and human cancer cell lines (Chaturvedula *et al*., 2002; Liu *et al*., 2004).

Catechin derivatives: epicatechin-catechin and epigallocatechin-gallocatechin did not demonstrate any toxicity on Vero cell lines in the present study. This confirms the findings by Pragon *et al*., (2008) where *Erythroxylum cuneatum* extract was tested on Vero cells, demonstrated no toxicity (IC_{50} value of 366µg/ml). The active compound isolated from the plant was (+)-catechin (Pragon *et al*., 2008). This might explain non-toxicity properties observed from all catechin-derived compounds isolated.









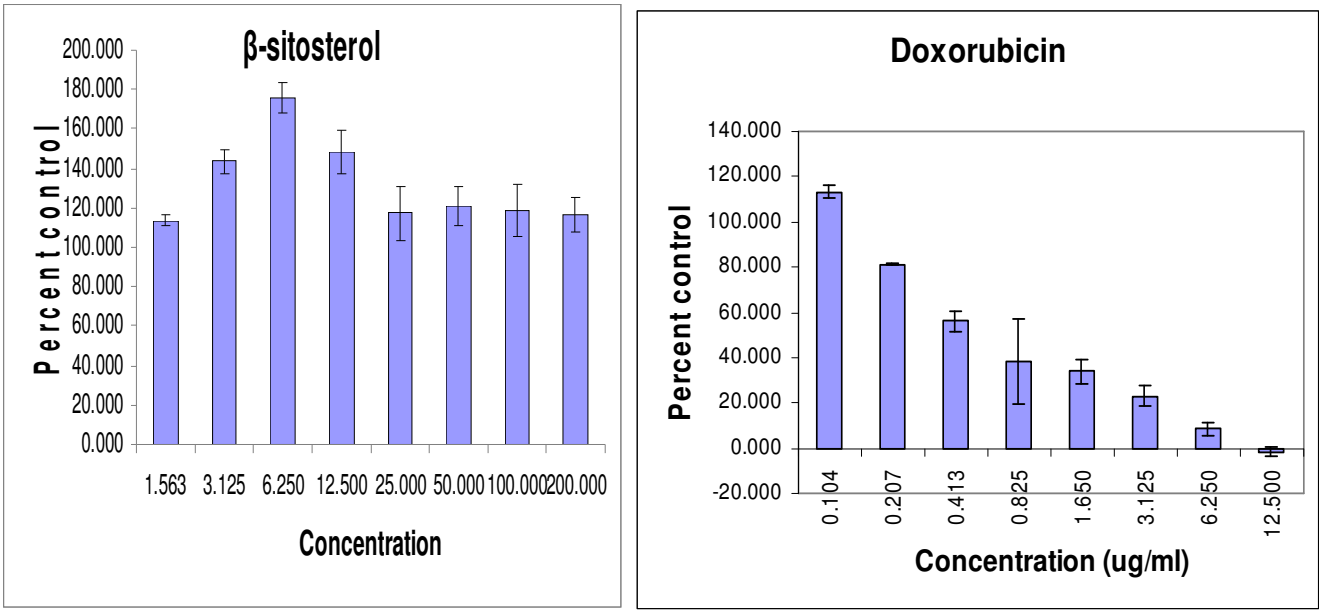


Figure 5.3: Effect of isolated compounds on the viability of Vero cells

5.4 Conclusion

Compounds belonging to triterpenes and flavonoids that were isolated from the stem bark of *Terminalia sericea* were tested on alpha glucosidase, amylase and DPPH assays for their antidiabetic and antioxidant properties. Compounds were also tested for cytotoxicity on Vero cell lines. This study is the first to report α-glucosidase, α-amylase and antioxidant properties of epicatechin-catechin, epigallocatechin-gallocatechin, β-sitosterol-3-acetate and stigma-4-ene-3-one isolated from *T. sericea*. In addition, epicatechin-catechin, epigallocatechin-gallocatechin, β-sitosterol-3-acetate and stigma-4-ene-3-one are isolated from *T. sericea* for the first time.

T. sericea is moderately toxic to Vero cells. This could be due to the solvent used. Ideally water extracts (which are less toxic) are used traditionally however due to their low activity other organic solvents are recommended for *in vitro* studies. Compounds have demonstrated good antioxidant

and hypoglycemic activities. As these compounds can be synthesized in the labs in large quantities, this will be an added advantage and will open doors for drug discovery.

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