An evaluation of the anti-diabetic properties *Asparagus africanus* Lam. root extracts

by

ONYEKA DAMIANFRANKLIN OKOLIE

Dissertation submitted in partial fulfilment of the requirements for the degree

MAGISTER TECHNOLOGIAE: BIOMEDICAL TECHNOLOGY

Department of Health Science

Faculty of Health and Environmental Sciences

at

Central University of Technology, Free State

Supervisor: Professor S.S. Mashele (PhD)

Co-Supervisor: Dr I.T. Madamombe-Manduna (DSc)

2014

DECLARATION OF INDEPENDENT WORK

I, Onyeka Damianfranklin Okolie, with identity number A02170054 and student number 212087193, do hereby declare that this research project submitted to the Central University of Technology, Free State for the degree of MAGISTER TECHNOLOGIAE: BIOMEDICAL TECHNOLOGY, is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

....

SIGNATURE OF STUDENT **DATE**

Dedication

This work is dedicated to almighty God in heaven and all the people who contributed in one way or another; may God continue to bless them all abundantly, Amen.

Acknowledgements

Thanks to God almighty, who made this study possible and also:

- Prof. S.S. Mashele and Dr I.T Madamombe-Manduna for their steady supervision and support, as well as their wholehearted assistance in the preparation of this dissertation. All staff and postgraduate students in the Department of Health Sciences, Central University of Technology University, for their continual support and inspiration.
- The Research and Innovation Fund of the Central University of Technology, Free state for financial support.
- My Mom and Dad (Chief and Mrs. Akudo Chukwuebuka Basil Okolie) , Dayo, Simba, Jerry, Mrs. Polori, Refilwe, Rose, family and friends for their unconditional love and support.

Summary

Medicinal plants could be a good alternative in the prevention and treatment of diabetes and other diseases. Type II diabetes is becoming prevalent in society and it has become necessary to start looking at medicinal plants as potential antidiabetic treatments. The objective of this project was to evaluate the efficiency of *Asparagus africanus* root extracts for their potential application as sources of antidiabetic entities.

Glucose uptake by Chang liver and C2C12 muscle cells treated with methanol and water extracts of *A. africanus* was investigated. Furthermore, the antioxidant (free radical scavenging) activity of *A.africanus* was carried out using 2, 2- diphenylpicrylhydrazyl assays. Cytotoxicity was analysed using the SRB assay. The phytochemical content of the extracts was determined through standard qualitative screening methods and liquid chromatography mass spectrometry.

The extracts did not cause an increase in glucose uptake by the C2C12 muscle cells, but the methanol extract caused a marginal increase in glucose uptake by the Chang liver cells, suggesting that the antidiabetic activity exerted may actually take place in the liver. The antidiabetic test using the Chang liver cells and C2C12 muscle cells line showed some active glucose uptake compared to the control and reference drug. The plant also demonstrated moderate antioxidant properties with the concentration of a drug that gives half-maximal response (EC values) of EC_{50}≤14.723 mg/ml, while the ascorbic acid standard of EC_{50} was 0.1157 mg/ml. The total phenolic concentration of the *A.africanus* at 2.5 mg/ml was 0.04% and had a Gallic acid equivalence of 0.01. Phytochemical screening indicated several bioactive constituents, such as alkaloids, carbohydrate, cardiac glycosides, protein, flavonoids, tannins, steroids, terpenoids and saponins contained in *A.africanus* roots. These might modulate the activities of ligand-dependent transcription factors, namely peroxisome proliferator-activated receptors (PPARs), PPARs are dietary lipid sensors that control energy homeostasis, daily consumption of these constituents might be useful for the management of obesity-induced metabolic disorders, such as type 2 diabetes, hyperlipidaemia, insulin resistance and cardiovascular diseases.

Conclusively, the presence of aforementioned phytochemical constituents in *A. africanus* suggests that the plant might be a good inhibitor to reduce the risk of any cardiovascular diseases such as coronary heart diseases, heart failure and stroke, these diseases has similar symptoms as diabetes and hypertension.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

JNC 6	Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure
AGE	Advanced glycation end
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
DEX	Dexamethazone
DM	Diabetes mellitus
DMEM	Dulbecco's modification of Eagle's medium
EMEM	Eagle's Minimum Essential Medium
ECACC	European Collection of Cell Cultures
DMSO	Dimethyl sulphoxide
g	Gram
GAE	Gallic acid equivalent
GLUT	Glucose transporter
EC_{50}	The concentration of a drug that gives half-maximal response.
IDDM	Insulin-dependent diabetes mellitus or juvenile diabetes
IRS	Insulin receptor substrate
mg/ml	Milligram per millilitre
l	Litre
m	Milli

<	Greater than
&	And
>	Less than
M	Molar
n	Nano
NMR	Nuclear magnetic resonance
NIDDM	Non-insulin-dependent diabetes mellitus or adult onset diabetes
P	Probability
PI3-kinase	Phosphatidylinositol 3-kinase
PP	Postprandial
PPAR-γ	Peroxisome proliferator-activated receptor γ
r^2	Correlation coefficient
RPMI 1640	Roswell Park Memorial Institute culture medium
SD	Standard deviation
SEM	Standard error of mean
SRB	Sulphorhodamine B
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
TZD	Thiazolidinediones
WHO	World Health Organisation
α	Alpha

µ	Micro

CHAPTER 1: INTRODUCTION

1.1. Introduction

Traditional medical systems such as the Indian Ayurveda have a long history of plant use (Shrivasta and Leelavathi, 2010). Approximately 70–85% of the world's population rely on plant-based medicines for health care (Cordell, 1995). Moreover, traditional medicine is the only health resource available to about 60% of the world population. According to estimates by the World Health Organization (WHO), (2007), between 60 and 90% of Africa's population rely on medicinal plants to meet their health care needs totally or partially. This is also true of South Africa, where traditional medicine is an integral part of South African cultural life and up to 60% of the population consult one of an estimated 200 000 traditional healers, especially in rural areas where traditional healers are more numerous and accessible than Western doctors (Brandt *et al*., 1995). For example in KwaZulu-Natal, approximately 80% of the population seek medical advice from traditional healers, in preference to, or in addition to, Western medicine (Brandt *et al*., 1995).

Formal and informal (oral-tradition) systems of medicine exist together in South Africa, the first dating back only 300 years with the influx of European settlers and the latter possibly to Paleolithic times (Van Wyk *et al*., 1997). However, there is still a lack of detailed documentation on the use of medicinal plants in South Africa. This is becoming an urgent issue, based on the fragility of oral tradition and the rapid pace of urbanisation and acculturation in this country. For example, the informal (oral) traditional medical systems of the Khoi-San, the Nguni and the Sotho-speaking peoples of South Africa have not yet been systemised (Van Wyk *et al*., 1997).

Traditional medicine has been a fertile source of novel lead molecules, which are then subjected to investigation using the techniques of modern drug discovery. Several success stories of modern drugs originate from medicinal plant remedies, such as aspirin and digoxin (Alexander *et al*., 1992; Leslie Taylor, 2000; Gilani and Atta-ur-Rahman, 2005; Parekh and Chanda, 2007). Modern isolation and screening technologies have enhanced the search for new lead molecules and increased interest in folk medicinal plant

extracts in drug companies (Balunus and Kinghorn, 2005). Simultaneously, enthusiasm for medicinal herbs and natural products has increased among the public. However, there are some safety concerns about traditional remedies, although they have been used for hundreds of years. This fact has generated more questions for research in South Africa and globally regarding the use of medicinal plants.

The current treatments for diabetes have severe side effects such as kidney failure, stomach discomfort, insomnia, a decreased appetite and diarrhoea, hence safer and less toxic treatment alternatives need to be discovered (Andrade-Cetto and Wiedenfeld, 2004). Natural sources such as plants are proving to be a better option because they seem to be less toxic to humans (Kadarian *et al.*, 2002; Andrade-Cetto and Wiedenfeld, 2004; Liu *et al*., 2007).

Medicinal plants have been widely used in various ways to treat diabetes and other ailments across the globe. Plants such as *Asparagus africanus* Lam. have also been used in India as a herbal tea, which controls the blood sugar and glucose levels (Desta, 1993). *Asparagus* species have a long history of use as folk medicines in the form of juice, tea or soup for liver, kidney, heart and bladder problems (Negi *et al.*, 2010). This study focuses on the antidiabetic properties of crude extracts from *A.africanus* roots.

1.2. Aim

The general aim of this study was to evaluate *A.africanus* Lam. root extracts for antidiabetic properties.

1.3. Objectives of study

1. To assess the *in vitro* glucose-lowering effects of *A.africanus* extracts using C2C12 muscle and Chang cells.
2. To evaluate the antioxidant activity of *A.africanus.*
3. To screen extracts of *A. africanus* for cytotoxicity.

4. To determine the phytochemical content of *A. africanus.*

1.4. Presentation

The evaluation of the antidiabetic properties of *A.africanus* Lam. root extracts is presented in four sections. Chapter two of this dissertation reviews the literature on *A.africanus* and its importance. The importance of ethno-pharmacological research and the economic importance of plants, as well as its in the treatment of diabetes, are also presented. The third chapter of this dissertation presents the antidiabetic activity of *A.africanus*, while the fourth, fifth and sixth chapters present analyses of the antioxidant activity, cytotoxicity and phytochemical constituents of *A. africanus* respectively. The final chapter gives a general discussion and conclusion of the work.

REFERENCES

1. Alexander, D. M., Bhana, N., Bhika, K. H., & Rogers, C. B. (1992). Antimicrobial testing of selected plant extracts from Combretum species. *South African Journal of science*, 88(6), pp.342-344..
2. Andrade-Cetto, A. and Wiedenfeld, H. (2004). Hypoglycemic effect of Acosmium panamense bark on streptozotocin diabetic rats. *Journal of Ethnopharmacology*, 90(2-3), pp.217-220.
3. Balunas, M. and Kinghorn, A. (2005). Drug discovery from medicinal plants. *Life Sciences,* 78(5), pp.431-441.
4. Brandt H.D., Osuch E., Mathibe L., & Tsipa P. (1995). Plants associated with accidentally poisoned patients presenting at Ga-Rankuwa hospital. *South African Journal of Science*, 91, pp.57-59.
5. Cordell, G. (1995). Changing strategies in natural products chemistry. *Phytochemistry*, 40(6), pp.1585-1612.
6. Desta, B. (1993). Ethiopian traditional herbal drugs. Part II: Antimicrobial activity of 63 medicinal plants. *Journal of Ethnopharmacology*, 39(2), pp.129-139.
7. Gilani, A. and Atta-ur-Rahman, (2005). Trends in ethnopharmacology. *Journal of Ethnopharmacology*, 100(1-2), pp.43-49.

8. Kadarian, C., Broussalis, A., Miño, J., Lopez, P., Gorzalczany, S., Ferraro, G. and Acevedo, C. (2002). Hepatoprotective activity of Achyrocline satureioides(Lam) D. C. *Pharmacological Research*, 45(1), pp.57-61.
9. Leslie Taylor, N. (2000). *Plant-Based Drugs and Medicines by Leslie Taylor*. [online] Rain-tree.com. Available at: http://www.rain-tree.com/plantdrugs.htm#.VCPeq_mSxh4. [Accessed 23 Apr. 2013].
10. Liu, I., Tzeng, T., Liou, S. and Lan, T. (2007). Improvement of Insulin Sensitivity in Obese Zucker Rats by Myricetin Extracted from Abelmoschus moschatus. *Planta Med*, 73(10), pp.1054-1060.
11. Negi, J. S., Singh, P., Joshi, G. P., Rawat, M. S., & Bisht, V. K. (2010). Chemical constituents of Asparagus. *Pharmacognosy reviews*, 4(8), pp.215.
12. Parekh, J. and Chanda, S. (2007). In vitro antibacterial activity of the crude methanol extract of Woodfordia fruticosa Kurz. flower (Lythraceae). *Brazilian Journal of Microbiology*, 38(2), pp.204-207.
13. Shrivastava, S., and Leelavathi, S. (2010). Preliminary Phytochemical Evaluation of Leaf Extracts of Catunaregum Spinosa Thunb. *International Journal of Pharmaceutical Sciences Review & Research*, 3(2), pp.114-118.
14. Van Wyk, B-E., Van Oudtshoorn, B., and Gericke, N. (1997). *Medicinal plants of South Africa*. South Africa, Pretoria: Briza Publications.
15. WHO (2007). *The medicinal plant uses and test of toxicity in some various plants*. Pp 139-203. UK, Newcastle: Academic Press. Biomed.

CHAPTER 2: LITERATURE REVIEW

2.1. Medicinal use of *Asparagus*

Asparagus has a long history of use as a traditional medicine for liver, kidney, heart and bladder ailments, in the form of juice, tea or soup (Negi *et al.*, 2010). There is some scientific support for these uses, as *Asparagus* shoots contain the non-essential amino acid *asparagine*, which is diuretic in large amounts (Negi *et al.*, 2010). In its wild form in ancient Greece and Rome, the *Asparagus* family (*Asparagaceae*) was used as a diuretic (increasing urine flow) to flush out the kidneys and prevent the formation of kidney stones (Grieve, 1992). In Asian medicine, *Asparagus* root is given for cough, diarrhoea and nervous problems. It is reported that the *Asparagus* family's roots and leaves are used in Ayurvedic (Indian medicinal drink). In India, where it is also known as Shatavari, asparagus has been a treatment for infertility and lack of libido in women for thousands of years, and is regarded as one of the most important herbal tonics for women (Grieve, 1992). The virtues of *Asparagus* are well known as a diuretic and laxative; and for those of sedentary habits who suffer from symptoms of gravel, it has been found very beneficial, as well as in cases of dropsy (Grieve, 1992).

2.2. *Asparagus africanus* Lam.

In earlier classification systems, *Asparagus* was in the *Liliaceae* family. However, the APG III system (2009) allowed circumscription of the family to *Asparagaceae sensu lato* to include families such as *Agaveceae*, *Ruscaceae*, *Themidaceae* etc. *A.africanus* is widespread in tropical Africa, in open forests, occurring in clumps in most sandy savannah (Hans, 1996). In Nigeria, this plant is known as "Shekan bera" in Hausa and "aluki" in Yoruba (Dalziel, 1999). The common names for *A. africanus* in South Africa include "haakdoring" (Afrikaans) and "leunyeli" (Sotho) while in Australia the plant is called by the botanical name (Raimondo *et al.*, 2009). A description of *A. africanus* is shown in Figure 2.1.

Asparagus africanus Lam (Asparagaceae) is a fast-growing climbing plant. Without a host, the plant can grow as a scrambling low (1.5 m) shrub. (Dalziel, 1999)

Leaves: (Cladodes) in umbel-like turfs of 7-20 cladodes, rigidly subulate, sharply angular, 1-2cm long (Hans Diester, 1996).

Flowers: In the axils of the leaf turfs, solitary or in turfs, greenish-white. Stalk 5-8Mm long, six small petals, and one stamen at the base of each petal (Hans Diester, 1996).

Fruit: A globosely reddish berry, 8Mm thick (Hans Diester, 1996).

Stems: Up to 12m long, very fine and thorny.

Roots: Woody rhizomes that appear swollen and thick (Hans Diester, 1996Dalziel, 1999).

Figure 2. 1. Botanical description of *Asparagus africanus*

Adapted and modified from Baerts and Lehmann (1991).

A.africanus is used for the treatment of headache, backache and stomach pain and as an aid in childbirth (Msonthi and Magombo, 1983). The plant is also used for heamaturia, heamorrhoids (Desta, 1993), malaria, lishmaniansis, bilharziasis, syphilis and gonorrhoea, and diabetes (Oketch-Rabah *et al*., 1997). The root extract is applied externally for the relief of pain, rheumatism and chronic gout (Watt and Breyer-Brandwijk, 1996). It is also used as a diuretic, for sore throat and otitis (Oliver, 1990). Three steroidal saponins (two new monodesmosidic spirostanosides and a new bisdesmosidic furostanol glycoside) have been isolated from the roots of *A. africanus* (Debella *et al*., 1999). Most species of *Asparagus* are used as vegetables, for medicines and as ornamental plants.

2.3. Medicinal plants and treatment of diabetes mellitus

Medicinal plants have been part of the great healing traditions around the world, especially in Africa. According to WHO, traditional medicine is defined as “health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, and manual techniques applied singularly or in combination to treat, diagnose and prevent illness or maintain well-being” (WHO, 2002). In traditional medicine the use of plants has become more widely acceptable because they are available in various species. Furthermore, in Africa, traditional medicine is used by up to 80% of the population to meet primary health care needs and is crucial in the fight against diseases (Wilson, 2006). Overall, this highlights the need to focus on medicinal plants for their reliability and affordability at local level.

Diabetes is an endemic disease that has affected the world population at large. More than 1123 species of plants have been used ethnopharmacologically or experimentally globally, and in South Africa, to treat symptoms of diabetes (Grover *et al*., 2002; Wilson, 2006). Some medicinal plants with antidiabetic properties are shown in Table 2.1. They mostly function by decreasing blood glucose levels.

Table 2. 1. Medicinal plants with antidiabetic properties

Name of plant	Mechanism	Reference

Abelmoschus moschatus Medik (Malvaceae)	Improves insulin sensitivity through increased post–receptor. Also used for the development of antidiabetic compounds.	(Liu *et al*., 2007)
Acacia arabica (Lam) Wild. Mimosaceae	It induces hypoglycaemia in control rats but not in alloxanised animals.	(Wadood *et al*.,1998)
Achyranthes aspera L (Amaranthaceae)	It decreases blood glucose levels in normal and alloxan diabetic rabbits.	(Akhtar and Iqbal, 1991)
Achyrocline satureioides (Less) DC (Asteraceae)	It significantly increases the depleted level of liver glutathione and bile flow in rats.	(Kadarian *et al*., 2002)
Acosmium panamense Schott. (Leguminosae)	It is used to lower the plasma glucose level in diabetic rats within 3 hours in streptozotocin.	(Andrade-Cetto and Wiedenfeld, 2004)
Aegle marmelose (L) Corr. (Rutaceae)	It decreases the blood urea and serum cholesterol. *A. marmelose* leaf extract is used for hypoglycaemic activity in insulin treatment.	(Panda and Kar, 2007)
Artemisia herba-alba Asso. (Med). (Asteraceae)	The aqueous and methanolic extracts are used to reduce the blood glucose level.	(Khazraji *et al*., 1993)
Aloe barbadensis Mill. (Liliaceae)	Commonly known as dragon herb, the extract increases the binding of glucagon-like peptide to its receptor in vitro.	(Ribnicky *et al*., 2006)
Astragalus membranaceus Bunge (Fisch): (Leguminosae)	It decreases blood glucose and increases plasma insulin levels.	(Yu *et al*., 2006)

2.4 Ethno-pharmacological research and economic importance of plants

The study of medicinal plants used in traditional medicine requires information on the chemical composition of extracts, pharmacological activities of isolated compounds in

various plants, as well as indigenous knowledge of traditional healers. Obtaining ethno-botanical information remains a realistic aspect in any such study (Soejarto, 2005). The process of isolating and identifying lead compounds from a complex mixture requires a number of specific resources, including comprehensive knowledge, specialised equipment and skill. The urgency of the discovery of new agents is a result of impenetrable factors that come into play, including the outbreak of new killer diseases, known antimicrobial treatment resistance, the inefficiency of synthetic drugs and the high cost of bringing to market a single drug. A modification towards natural product research, which is further driven by remarkable advances in plant extract technology, biotechnology and analytical chemistry, is therefore certain (Paraskeva, 2007).

There is a great need and ethical obligation to document investigative findings on plants used for health purposes accurately. This will in addition aid in the efficient preservation and conservation of traditional knowledge, thus preventing the further disappearance of indigenous systems of medicine, which may potentially benefit society in general. According to the Southern African Trade Directory of Indigenous Natural Products, more than 1 000 species of plants are used traditionally in southern Africa (Izimpande, 2005). The economic exploitation of South Africa's rich natural plant resources is limited. At present only the indigenous flower industry has relatively successfully established small and medium scale entrepreneurs (Mander, 1997). The indigenous medicinal plant industry is large, but fully based on harvesting from the wild. This is not sustainable and will have to be supplemented with cultivation. The commercial utilisation of food types is limited, with the exception of the buchu industry, which has already been established as a cultivated industry with an export market. Only aloe and devil's claw (*Harpagophytum procumbens* DC.) are exported for medicinal use (Deliwe, 1998).

The utilisation of South African indigenous flora can only be successfully explored if the existing indigenous knowledge of the inhabitants is made available to science. By forming associations between natural healers and scientists, medicinal plants can be investigated. From these associations, industries can be formed to commercialise the products. Commercial utilisation will promote the creation and development of rural small, micro and medium enterprises. This will enable communities to create wealth from indigenous

technologies and plants (Deliwe, 1998 unpubl. report) and will ensure that natural habitats are protected. The problem is to prohibit illicit exploitation of plant material, as well as other prejudicial actions. At present no official legislation exists, but a proposed law known as the "Protection of Indigenous Knowledge Act" is being prepared to advance the promotion and protection of indigenous knowledge. This act does not prohibit the exploitation of indigenous plants. On the contrary, it attempts to promote and develop the use of indigenous genetic material. The primary aim is to ensure that the lawful owner is recognised in the development. Africa and the world generally are blessed with a diversity of medicinal plants; some of these plants are yet to be studied for medicinal properties. *A.africanus* can also be a source of economic importance, if proven to be a good source of natural treatment of diabetes and other afflictions.

REFERENCES

1. “Izimpande” - eThekwini Medicinal Plants Sector Support Programme. 2005/20061:14. [Online] Available: http://www.durban.gov.za/eThekwini/Business/mm/izimpande/plant, Accessed 1 August 2013.
2. Akhtar, M. and Iqbal, J. (1991). Evaluation of the hypoglycaemic effect of Achyranthes aspera in normal and alloxan-diabetic rabbits. *Journal of Ethnopharmacology*, 31(1), pp.49-57.
3. Al-Khazraji, S., Al-Shamaony, L. and Twaij, H. (1993). Hypoglycaemic effect of Artemisia herba alba. I. Effect of different parts and influence of the solvent on hypoglycaemic activity. *Journal of Ethnopharmacology*, 40(3), pp.163-166.
4. Andrade-Cetto, A. and Wiedenfeld, H. (2004). Hypoglycemic effect of Acosmium panamense bark on streptozotocin diabetic rats. *Journal of Ethnopharmacology*, 90(2-3), pp.217-220.
5. APG III (2009). An update of the Angiosperm Phylogeny Group for the orders and families of flowering plants classification: APG III. *Botanical Journal of the Linnean Society*, 161(2), pp.105-121.

6. Baerts, M. and J. Lehmann. (1991). Medicinal plants veterinarians in the region of the Nile-Zaire watershed peaks in Burundi. Royal Museum for Central Africa, *Tervuren. Ann. Sc. Eco.*, 21: 133.
7. Dalziel, J.M. (1999). *The useful plants of west tropical Africa*. Pp 477. London: Crown Agents for Overseas Government and Administration.
8. Debella, A.J. Hasliger, E.J., Kunert, O.J., Michl, G. and Abebe, D. (1999). Steroidal saponins from *Asparagus africanus*. *Phytochemistry*, 51(8), pp.1069 – 1075.
9. Deliwe, M. (1998 unpubl. report), In Coetzee, C., Jefthas, E. & Reinten, E. (1999), *Indigenous plant genetic resources of South Africa*, Reprinted from J. Janick (ed.) Perspectives on new crops and new uses, Alexandria, VA: ASHS Press.
10. Desta, B. (1993). Ethiopian traditional herbal drugs. Part II: Antimicrobial activity of 63 medicinal plants. Journal of Ethnopharmacology, 39(2), pp.129-139.
11. Grieve, M. (1992). *A modern herbal*. Hafner Press, New York. p. 574.
12. Grover, J. K., Yadav, S., and Vats, V. (2002). Medicinal plants of India with anti-diabetic potential. *Journal of ethnopharmacology*, 81(1), pp.81-100.
13. Hans, D.N. (1996). *African ethanobotany: poisons and drugs: chemistry, pharmacology, toxicology*. Weinheim: Chapman & Hall. Pp.287-293.
14. Kadarian, C., Broussalis, A., Miño, J., Lopez, P., Gorzalczany, S., Ferraro, G. and Acevedo, C. (2002). Hepatoprotective activity of Achyrocline satureioides(Lam) D. C. *Pharmacological Research*, 45(1), pp.57-61.
15. Liu, I., Tzeng, T., Liou, S. and Lan, T. (2007). Improvement of Insulin Sensitivity in Obese Zucker Rats by Myricetin Extracted from Abelmoschus moschatus. *Planta Med*, 73(10), pp.1054-1060.
16. Mander, M. (1997). *Medicinal plant marketing and strategies for sustaining the plant supply in the Bushbuckridge area and Mpumalanga Province*. Nelspruit: Unpublished report, Department of Water Affairs and Forestry.
17. Msonthi, J. D., and Magombo, D. (1983). Medicinal herbs in Malawi and their uses. *Hamdard*, 26(2), pp.94-100.
18. Negi, J. S., Singh, P., Joshi, G. P., Rawat, M. S., & Bisht, V. K. (2010). Chemical constituents of Asparagus. Pharmacognosy reviews, 4(8), pp.215.

19. Oketch-Rabah, H., Dossaji, S., Christensen, S., Frydenvang, K., Lemmich, E., Cornett, C., Olsen, C., Chen, M., Kharazmi, A. and Theander, T. (1997). Antiprotozoal Compounds from Asparagus africanus. J. Nat. Prod., 60(10), pp.1017-1022.
20. Oliver, B. (1990). Medicinal plants in Nigeria. Nigerian College of Art, Science and Technology, Ibadan, Nigeria. pp 19.
21. Panda, S. and Kar, A. (2007). Antidiabetic and antioxidative effects of Annona squamosa leaves are possibly mediated through quercetin-3-O-glucoside. *BioFactors*, 31(3-4), pp.201-210.
22. Paraskeva, M.P., 2007. *A Phytochemical and Pharmacological Study of Ten Commiphora Species Indigenous to South Africa*. MSc Dissertation, University of the Witwatersrand, South Africa.
23. Raimondo, D., von Staden, L., Foden, W., Victor, J.E., Helme, N.A., Turner, R.C., Kamundi, D.A. & Manyama, P.A. (2009). *Red list of South African plants*. Strelitzia 25. Pretoria: South African National Biodiversity Institute.
24. Ribnicky, D., Poulev, A., Watford, M., Cefalu, W. and Raskin, I. (2006). Antihyperglycemic activity of Tarralin, an ethanolic extract of Artemisia dracunculus L. *Phytomedicine*, 13(8), pp.550-557.
25. Soejarto, D. (2005). Ethnographic component and organism documentation in an ethnopharmacology paper: A “minimun” standard. *Journal of Ethnopharmacology*, 100(1-2), pp.27-29.
26. Wadood, A., Wadood, N., & Shah, S. A. (1989). Effects of Acacia arabica and Caralluma edulis on blood glucose levels of normal and alloxan diabetic rabbits. *Journal of Pakistan Medical Association*, 39(8), pp.208-212.
27. Watt, J.M. and Breyer-Brandwijk, M.G. (1996). *The medicinal and poisonous plants of Southern and Eastern Africa*, 2nd ed. Edinburgh and London, U.K: E. and S. Livingstone.
28. WHO Traditional medicine strategy 2002–2005. (2002). [Online] Available: http://www.who.int/medicines/organization/trm/orgtrmmain.shtml. Accessed: 27July 2013.

29. Wilson, G.P. (2006). *Optimisation of an in vitro model for antidiabetic screening*. Master's thesis in the Faculty of Science at the Nelson Mandela Metropolitan University, South Africa.
30. Yu, J., Zhang, Y., Sun, S., Shen, J., Qiu, J., Yin, X., Yin, H. and Jiang, S. (2006). Inhibitory effects of astragaloside IV on diabetic peripheral neuropathy in rats. *Canadian Journal of Physiology and Pharmacology*, 84(6), pp.579-587.

CHAPTER 3: ANTIDIABETIC ACTIVITY OF *ASPARAGUS AFRICANUS*

3.1 Introduction

Diabetes is one of the major causes of premature deaths worldwide. Every ten seconds a person dies from diabetes-related causes, mainly from cardiovascular complications (Narkhede, 2011). For example, diabetes caused 3.5 million deaths globally in 2007 (Das, 2008). Estimation further shows that 25% of the world population is affected by this disease. According to Thylefors (1990), diabetes is a metabolic syndrome of aetiologies characterised by chronic hyperglycaemia with abnormalities in carbohydrate, fat, and protein metabolism due to defects in insulin secretions. Furthermore, this ailment is associated with long-term damage, such as the malfunctioning of eyes, kidneys, nerves, heart and blood vessels. It is the major endocrine disorder, responsible for renal failure, blindness or diabetic cataracts (Thylefors, 1990), poor metabolic control and increased risk of cardiovascular disease, including atherosclerosis and advanced glycation end (AGE) products (Yokozawa and Nakagawa, 2004).

There are two major types of diabetes. Type I diabetes mellitus (DM) results from the body's failure to produce insulin and requires the person to inject insulin. It is also alleged that Type II DM results from insulin resistance, a condition in which cells fail to use insulin properly, sometimes combined with an absolute insulin deficiency. Furthermore, Type II DM is a heterogeneous disorder due to a combination of inherited and acquired factors that adversely affect glucose metabolism (Ningsih, 2013). It is thought that these factors lead to diabetes, mainly by affecting β-cell function and tissue insulin sensitivity. If the amount of insulin produced is too little to allow for glucose to be used or stored, or if the insulin being produced does not work effectively, glucose accumulates in the blood. In the normal individual, the concentration of glucose in blood is maintained at about 90 mg/dL of plasma. However, fasting blood glucose in diabetics may be 300-400 mg/dL and may even reach 1000 mg/dl (Johnson, 1998).

The entry of glucose into cells is a crucial step in life-supporting processes, since glucose is the main monosaccharide in nature that provides carbon and energy for almost all cells.

Its passage into cells depends on different parameters, including expression of the appropriate glucose transporters in the target tissues and hormonal regulation of their function (Gorovits and Charron, 2003). Glucose is a hydrophilic compound; it cannot pass through the lipid bilayer by simple diffusion and therefore requires specific carrier proteins to mediate its specific transport into the cytosol. Cells take up glucose by facilitated diffusion, via glucose transporters (GLUTs) associated with the plasma membrane (Medina and Owen, 2002). The transporter is envisioned as a conformational oscillator, which shifts the binding pocket or glucose between opposite sides of the cell membrane (Lienhard *et al*., 1992).

The liver plays a central role in glucose homeostasis. During the post-absorptive and fasting period, >90% of glucose production is derived from the liver. When glucose enters the blood following feeding, the liver switches from net glucose production to net glucose uptake to lessen the rise in blood glucose. Such switching is regulated by raised blood glucose per se and plasma insulin. One third of an oral glucose load is taken up by the liver. Glucose uptake by the liver is dependent on the amount of glucose reaching it, the insulin level within the hepatic sinusoids and a signal generated by portal glucose delivery. Impaired suppression of hepatic glucose production and a defect in hepatic glucose uptake in response to the raise in plasma glucose and insulin are major pathogenesis in fasting and excessive postprandial hyperglycaemia, that are common features in obesity and diabetes (Rutter *et al*., 2003). Liver cell membranes have noninsulin-dependent glucose transporters and therefore do not require insulin for glucose entry. However insulin, acting through intracellular signalling systems, stimulates the liver cell enzymes that promote utilisation of glucose for the synthesis of glycogen, amino acids, proteins and fats, particularly fatty acids.

Oral diabetic drugs are used in the treatment of type II diabetes, which is a disorder involving resistance to secreted insulin. Type I diabetes involves lack of insulin and requires insulin for treatment. There are now basically four classes of hypoglycaemic drugs and different sites of action, as indicated in Table 3.1. These drugs have been approved for use only in patients with type II diabetes and are also used to treat patients who do not respond to diet, weight reduction and exercise. These four classes have not

been approved for the treatment of pregnant women with diabetes (Kavishankar *et al*., 2011).

Table 3. 1. Synthetic Drug and Side Effects.

Agent	Mechanism	Site of Action	Advantages	Side Effects
Metformin	Decreases insulin resistance	Liver	Weight loss, does not cause hypoglycaemia	Nausa, diarrhoea, hypoglycaemia occurs when combined with sulphonylurea or insulin
Repaglinida	Decreases blood sugar	Liver	Weight loss, does not cause hypoglycaemia	Diarrhoea, abdominal disorder
Thiazolidinediones	Reduce insulin resistance by activating PPAR-γ	GI tract	Low risk	Increased liver enzymes, weight gain, oedema, mild anaemia
Sulphonylureas	Stimulating insulin production by inhibiting the K-ATP channel	Pan-creatic beta cells	Effective and inexpensive	Hypoglycaemia and weight gain
α-glucosidase inhibitors	Reduce intestinal glucose absorption	Fat, muscle	Decreases postprandial plasma triglyceride levels	Diarrhoea, abdominal disorder, serum levels of transaminases increases at doses

The choice of an oral antidiabetic agent may be influenced by a large number of factors, but often comes down as much to personal preference or experience as to detailed knowledge of the different actions of each molecule (Kavishankar *et al*., 2011). However, all the insulin resistance drugs have one or two side effects. Medicinal plants such as *A.africanus* might be less toxic alternatives that can be used to control insulin resistance and other cardiovascular disease, because the plant contains terpenoids and saponins,

which are responsible for removal of toxic substances from the cell membrane (Alberts *et al*., 2002; Okolie *et al*., 2013).

In search of treatment of diabetes, a series of plants have proven to be effective and less toxic than conventional treatment. These include *Allium sativum, Aegle marmelos, Azadirachta indica, Curcuma longa, Emblica officinalis, Gymnema sylvestre, Mangifera indica, Momordica charantia, Syzygium cumunii, Trigonella foenum-geaecum, Terminelia bellerica, Terminelia chebula* and *Zingiber officinale* (Mall & Sahani 2013). According to Liu *et al*. (2007) and Kavishankar *et al*. (2011), Abelmoschus *moschatus* Medik (Malvaceae), native to India, contains myricelin, which improves insulin sensitivity through increased post-receptor insulin signalling mediated by improvement in IRS-1-associated P13-Kinase and GLUT4 activity in muscles of obese Zucker rats. Myricelin might be used as a model substance for the development of antidiabetic compounds. Many closely related species of *Asparagaceae* or other families, such as *Allium cepa, Allium sativum,* and *Aloe barbadensis* (Kavishankar *et al*., 2011), have been shown in Table 2.1 with their antidiabetic activities.

Some members of the *Asparagus* genus, such as *Asparagus curillus* and *Asparagus racemosus,* contain a diverse array of bioactive compounds that treat ailments such as dysuria, diabetes, inflammation and dysentery. However, the fruit of *A. curillus* causes abortion in pregnant women, while *A. racemosus* causes drowsiness in humans after consumption (Gaur, 1999). *A.africanus* has been used in India as a tea to cure headache and enhance lactation in women (Kirtikar and Basu, 1984), without any documented side effects. Moreover, *A.africanus* is widely distributed globally and may therefore provide a safer and more accessible alternative for the treatment of diabetes. This study investigates the glucose uptake of C2C12 muscle cells and Chang liver cells treated with crude root extracts of *A.africanus* as a measure of the plant's antidiabetic activity.

3.2. Materials and Methods

3.2.1. Preparation of plant material and extracts

A.africanus was purchased and authenticated at the National Botanical Gardens in Bloemfontein, South Africa. The collected plant roots were oven-dried at 40°C and stored

in a cool and dry place. A voucher specimen, CUT01/11/2012, was kept in the laboratory at the Central University of Technology.

The dried roots of *A. africanus* (10 g of the dried roots) were weighed, pulverised and soaked separately in 100 ml of methanol and distilled water and then mixed on an orbital shaker for 72 hours. The extracts were filtered using Whatman No. 1filter paper disc and the solvent (methanol) was removed completely under vacuum by a rotary evaporator (at 40˚C) and stored in a refrigerator until use. The water extract was dried to a powder using lyophilisation. This extraction method was applied for the rest of the study (Chapters 3-6).

3.2.2. Antidiabetic activity of *Asparagus africanus*

The plant extracts *(A. africanus)* were tested for potential antidiabetic activity by exposing C2C12 muscle cells and Chang liver cells to the extracts and determining the amount of glucose taken up. Each cell line represents a different *in vivo* organ that is known to be involved in glucose homeostasis, each with its own different metabolic pathways and mechanism of glucose uptake.

3.2.2.1. Glucose uptake by C2C12 muscle cells

The C2C12 skeletal muscle cell line was purchased from Highveld Biological (Johannesburg, South Africa) and a passage < 10 of the cells was used in this study. Prior to this, a study was carried out to optimise the growth and differentiation conditions of this cell line (Askew, 2003). It was determined that after being cultured in RPMI 1640 (including 10% FCS) for three days, the cells were differentiated and ready to be used for experimentation. After this period, the cells could not be left indefinitely, otherwise they died suddenly. For the glucose uptake experiment, cells were seeded at 4 000 cells/well into 96-well microtiter plates and cultured for three days. The C2C12 cells were differentiated for a further two days in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 2% horse serum (GIBCO Invitrogen, Paisley, UK). Following differentiation, cells were acutely exposed for three hours to the relevant extracts or fractions at a concentration of 50 µg/mL; metformin (1 µM), insulin (1 µM) or solvent

control (dimethyl sulphoxide [DMSO]/water 1%) was added to modified DMEM media supplemented with 8 mM glucose. After three hours' exposure to the media, the glucose concentrations remaining in the wells were determined using a commercial fluorimetric kit (Biovision). The glucose uptake was determined after three hours using a glucose oxidase fluorimetric assay. Glucose uptake was calculated by subtracting the glucose concentration remaining in the test wells from the glucose concentration of media measured in wells not containing cells. Three replicate samples were used in a single experiment.

3.2.2.2. Chang cell line glucose uptake

The Chang liver cell line is derived from non-malignant human tissue. These cells are epithelial in morphology and tend to pile up in cultures with high population density (www.biotech.ist.unige.it). Cells of the Chang liver cell line were seeded at 6 000 cells/well into 96-well microtiter plates and cultured for five days in growth medium. On the day of the experiment, all procedures were carried out at 37°C. 50 µl of incubation medium (8 mM glucose) was added to the appropriate wells for three hours. Thereafter cells were acutely exposed for three hours to the relevant extracts or fractions at a concentration of 50 µg/mL; metformin (1 µM), insulin (1 µM) or solvent control (DMSO/water) was added to modified Eagle's Minimum Essential Medium (EMEM) media supplemented with 8 mm glucose. The glucose uptake was determined after three hours using a glucose oxidase fluorimetric assay. The glucose uptake from the media was calculated as described for C2C12 muscle cells. The cell passage number was <10. The treatments tested for their effect on glucose uptake in Chang liver cells was observed by exposing the cells to the treatments only on the day of the experiment for the three-hour incubation time. The Chang cells were also calculated in the same manner as described for the C2C12 muscle cells.

3.3. Results and discussion

The effect of *A. africanus* methanol and water extracts on C2C12 muscle cells is shown in Figure 3.1. The percentage glucose uptake of the methanol extract was 100.3% and that of the water extract was 74.9%. This was significantly lower than that of insulin

(192.2%) and metformin (157%). DMSO had the same effect as methanol extract. The water extract reduced glucose uptake, which may be a sign of cytotoxicity.

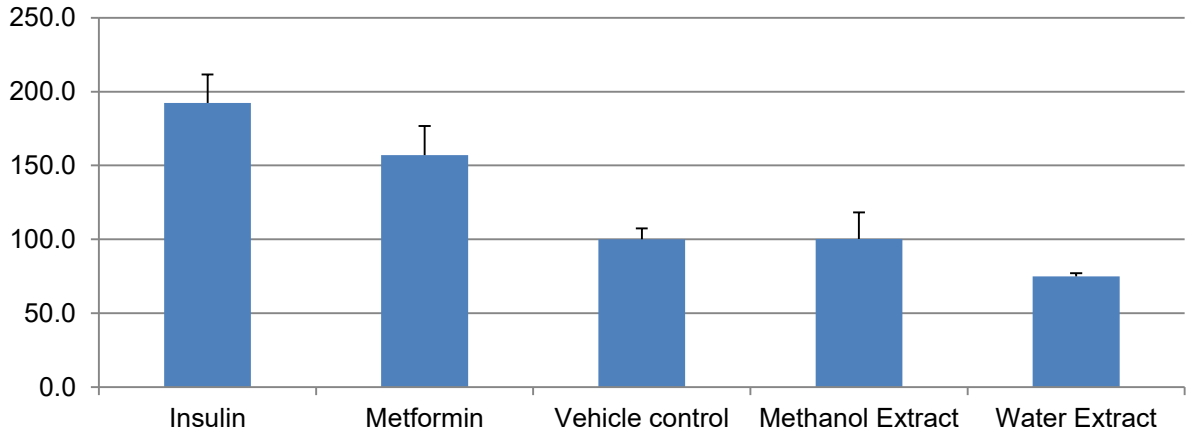


Figure 3. 1. Effects of methanol and water extracts of A.africanus on glucose uptake in C2C12 cells.

Values shown are the mean ± standard error of mean (SEM) (n=10 wells) from individual experiments.

There was a marginal increase in glucose uptake by the methanol (105.4%) and water (100.3%) extracts of *A. africanus.* Insulin and metformin yielded 114% and 117% glucose uptake respectively. DMSO (101%), which was used as a control, had the same glucose uptake as that of water extract of *A. africanus*, as shown in Figure 3.2.

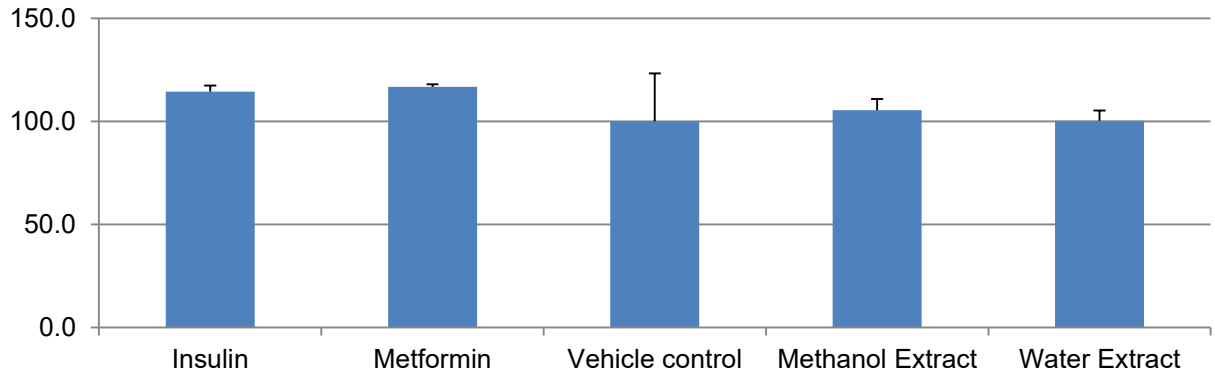


Figure 3. 2. Effects of methanol and water extracts of A.africanus on glucose uptake in Chang cells

Values shown are the mean ± SEM (n=10 wells) from individual experiments.

Insulin promotes the postprandial clearance of glucose from the blood primarily into skeletal muscle in both humans and rodents. Glucose transportation into skeletal muscle is regulated by translocation of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane (Aslesen *et al*., 2001). Several lines of independent experimentation support the notion that insulin-dependent regulation of GLUT4 movement is similar or identical in skeletal muscle and adipocytes. However, GLUT4 translocate in response to exercise and hypoxia in the former but not in the latter tissue. Insulin-dependent GLUT4 translocation is PI3-kinase dependent in both tissues (Tortorella & Pilch, 2002).

The results of this study however, show that the potential antidiabetic activity of *A.africanus* is not due to the promotion of glucose uptake by skeletal muscle. Antidiabetic activity may actually take place in the liver and may function in a similar way to metformin, which decreases blood glucose levels by reducing hepatic glucose output (Moriaka *et al*., 2005). For any experiment carried out, one needs a positive control in order to substantiate the result obtained (Aslesen *et al*., 2001). Taking into consideration that crude extracts of *A. africanus* were used in this research and not purified compounds to compare to metformin and insulin, the plant might be a good antidiabetic agent when purified, because the metformin and insulin are well purified and the plant extract might contain some bioactive compound that might enhance or retard any chemical process during this assay.

3.4. Conclusion

The *in vitro* assays of glucose uptake plant root crude extract suggest that *A.africanus* may promote glucose uptake in hepatic cells. More screening using alternative antidiabetic tests and the purification of bioactive compounds may conclusively show the antidiabetic potential of the plant. Moreover, previous studies on members of the *Asparagus* family, such as *A. racemosus,* also showed positive results on antidiabetic and antihyperlipidemic properties (Vadivelan *et al*., 2011; N.Lal Mahammed, G.Jyothi and Hasan, 2012). However, these studies were based on *in vivo* assay, without considering

the phytochemical constituent and cytotoxicity test. Considering the *in vitro* assays used in this study, it is evident that *A. africanus* has various phytochemical constituents and also poses a low hazard to the normal human cell line. In addition, further study on *A. africanus* using *in vivo* assays for antidiabetic properties, so as to validate this study for future reference, is suggested.

REFERENCES

1. Alberts, B., Johnson, A., and Lewis, J. (2002). *Molecular biology of the cell*. 4th ed. New York: Garland Science. ISBN 0-8153-3218-1.
2. Askew, S.L. (2003). *Evaluation of methods to culture C2C12 skeletal muscle cells and detects differentiation markers*. Honour's Thesis, Department of Biochemistry, University of Port Elizabeth.
3. Aslesen, R., Engebretsen, E. M., Franch, J., and Jensen, J. (2001). Glucose uptake and metabolic stress in rat muscles stimulated electrically with different protocols. *Journal of Applied Physiology*, 91(3), pp.1237-1244.
4. Das A.K.R. (2008). *A world without diabetes and its complications: A preventive program*. In: Jayaram B.M. (Ed.) *Type 2 diabetes and its complications: A preventive program*. pp. 1-2. Bangalore: Microlab Limited.
5. Gaur, R.D. (1999). *The flora of the district Garhwal North West Himalaya*. Garhwal: Srinagar. pp. 170.
6. Gorovits, N. and Charron, M. (2003). What we know about facilitative glucose transporters: Lessons from cultured cells, animal models, and human studies. Biochemistry and Molecular Biology Education, 31(3), pp.163-172.
7. Johnson, L.R. (1998). *Essential medical physiology*. 2nd Ed. Lippincott-Raven Publishers, New York, pp. 565-581.
8. Kavishankar, G.B, Lakshmidevi, N., Mahadeva Murthy, S., Prakash, H.S., and Niranjana S.R. (2011) Diabetes and medicinal plants - *A review, International Journal of Pharmaceutical Biomed Science*, 2 (3), pp.65-80.

9. Kirtikar, K.R., and Basu, B.D. (1984). *Indian medicinal plants*. In: Singh B., Singh and M.P. (Eds.) Dehradun India: New Connaught Place. pp. 2499.
10. Lienhard, G.E., Slot, J.W., James, D.E., and Muecklar, M.M. (1992). How cells absorb glucose. Scientific American, 206, pp.34-39.
11. Liu, I., Tzeng, T., Liou, S. and Lan, T. (2007). Improvement of Insulin Sensitivity in Obese Zucker Rats by Myricetin Extracted from Abelmoschus moschatus. Planta Med, 73(10), pp.1054-1060.
12. Mall, T.P. and Sahani, S. (2013). Diversity of ethno–medicinal plants for diabetes from Bahraich (U.P.) India, *International Journal of Interdisciplinary and Multidisciplinary Studies*, 1(1), pp.13-23.
13. Medina, R.A., and Owen, G.I. (2002). Glucose transporters: Expression, regulation and Cancer, Biol. Res. 35(1), pp. 9–26.
14. Moriaka, K., Nakatani, K., and Adachi, Y. (2005) Metformin-induced suppression of glucose-6-phosphatase expression is independent of insulin signalling in rat hepatoma cell. *Int j Mol Med* 15(3), pp.449-452.
15. N.Lal Mahammed, N., G.Jyothi, G. and Hasan, P. (2012). Antidiabetic and Antihyperlipidamic Activity of the Ethanolic leaf Extract of Glycosmis pentaphylla against Alloxan Induced Diabetes in Albino Rats. *IJSR*, 2(7), pp.429-432.
16. Narkhede, M. B., Ajimire, P. V., Wagh, A. E., Mohan, M., and Shivashanmugam, A. T. (2011). In vitro antidiabetic activity of Caesalpina digyna (R.) methanol root extract. *Asian Journal of Plant Science and Research*, 1(2), pp.101-106.
17. Ningsih, S. (2013). Improvement of quality of antidiabetic jamu through subcronic toxicity test in Sprague Dawley rat.
18. Okolie, O.D., Madamombe-Manduna, I.T. and Mashele, S**.** 2013. Anticancer screening of *Asparagus africanus* extracts. *Indigenous Plant Use Forum*. 1-4 July 2013, Nelspruit, South Africa.
19. Rutter, G.A., da Silva Xavier, G.D.S., and Leclerc, I. (2003). Roles of 5'AMP-activated protein kinase in mammalian glucose homeostasis. *Biochemical Journal*, 375, pp.116.
20. Thylefors, B. (1990). The WHO program for the prevention of blindness. *International Journal of Ophthal*, 14, pp.211.

21. Tortorella, L. L., and Pilch, P. F. (2002). C2C12 myocytes lack an insulin-responsive vesicular compartment despite dexamethasone-induced GLUT4 expression. American Journal of Physiology-Endocrinology and Metabolism, 283(3), pp.514-524.
22. Vadivelan, R., Dipanjan, M., Umasankar, P., Dhanabal, S.P., Satishkumar, M.N., Antony, S. & Elango, K. (2011). Hypoglycemic, antioxidant and hypolipidemic activity of *Asparagus racemosus* on streptozotocin-induced diabetic in rats. *Pelagia Research Library Advances in Applied Science Research,* 2(3), pp.179-185.
23. Yokozawa, T. & Nakagawa, T., (2004). Inhibitory effects of Luobuma tea and its components against glucose-mediated protein damage. *Food and Chemical Toxicology, 2004*. 42(6), pp.975-981.

CHAPTER 4: *ASPARAGUS AFRICANUS* ANTIOXIDANT ACTIVITIES

4.1. Introduction

Antioxidant compounds in food play an important role as a health-protecting factor. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds (Prakash *et al*., 2001). Evidence suggests that the presence of antioxidants in plants reduces the risk of chronic diseases such as coronary artery disease, diabetes, stroke, cancer, rheumatoid arthritis, certain neurodegenerative diseases, tissue damage and also ageing (Halliwell *et al*., 1992; Larkins, 1999). These plants contain large amounts of antioxidants in the form of polyphenols, which can play an important role in adsorbing and neutralising free radicals after human consumption to reduce any cardiovascular related diseases such and diabetes (Anderson *et al*., 2002). Free radicals can lead to a variety of biochemical and physiological injuries to living cells (Ames, 1998) and induce degenerative diseases (Halliwell *et al*., 1992). However, the main characteristic of an antioxidant is its ability to trap free radicals.

Antioxidants can prevent oxidation of substrates when present at low concentrations compared with an oxidisable substrate and prevent diseases such as diabetes and some infectious diseases, as mentioned before (Halliwell, 1995). Generally, the presence of antioxidant properties in agents (such as *A. africanus*) may retard the process of AGE products' formation by preventing further oxidation through rearrangement reaction and metal-catalysed glucose oxidation. In fact, inhibitors of AGEs that exhibit antioxidant activity may act as preventive agents against diabetic complications. The activities of antioxidants are in different phases of defence, which involve both enzymatic mechanisms that use specific enzymes such as superoxide dismutase, catalase and glutathione peroxidase, as well as non-enzymatic mechanisms that use nutrients and minerals (Aggarwal *et al*., 2005). These anti-oxidants can act at several different stages, by:

(i) The removal of or decrease in the local O_2 concentrations;

(ii) The removal of catalytic metal ions;

(iii) The removal of reactive oxygen species such as O^{2-} and H_2O_2;

(iv) Scavenging initiating radicals such as •OH, RO• and RO_2;

(v) Breaking the chain of an initiated sequence; and

(vi) Quenching or scavenging singlet oxygen (rearrangement of electrons that produces very rapid oxygen) (Gutteridge, 1994; Anderson *et al*., 2001).

Furthermore, antioxidant properties elicited by plant species have a wide range of applications in human health care, as they protect against free radicals. Knowledge of the potential antioxidant compounds present within a plant species does not necessarily indicate its antioxidant capacity, as the total antioxidant effect may be greater than the individual antioxidant activity of one compound, owing to synergism between different anti-oxidant compounds. Methods used to measure the antioxidant activity in plant material generally involve both the generation of radicals (their related compounds), and the addition of antioxidants, the latter resulting in the reduction of the radical and its consequent disappearance (Arnao et al., 1999).

Some species of the *Asparagus* genus are natural anti-oxidant sources, because most of the species' leaves are edible as vegetables; the Indians and Chinese also use them in place of coffee (Grieve, 1992). This chapter presents the evaluation of the antioxidant activity of *A. africanus* root extracts, using the 2-diphenyl-1-picrylhydrazyl (DPPH) method (Brand Williams et al., 1995).

4.2. Materials and Methods

The DPPH assay

The hydrogen-donating capacity of test samples is quantified in terms of their ability to scavenge the relatively stable, organic-free radical DPPH. The absorption of the deep violet DPPH solution is measured at 517 nm, after which absorption decreases due to decolourisation to a yellow-white colour, in the event of reduction. This decrease in absorption is stoichiometric according to the degree of reduction. The remaining DPPH is measured at a time interval of one hour after the addition of the DPPH, which corresponds

inversely to the radical scavenging activity of the sample extract or anti-oxidant (Brand Williams *et al*., 1995; Soares *et al*., 2009).

In this study the anti-oxidant activity of the crude methanol extract from the roots of *A. africanus* was determined at concentrations of 0.156, 0.3125, 0.625, 2.5 and 10 mg/ml respectively. Two millilitres of 0.004% DPPH solution was added to 0.02 mg/ml of the extract and shaken vigorously. After that the solution was incubated in the dark at room temperature for one hour. The absorbance (Abs sample) was then measured at 517 nm and converted into percentage of antioxidant activity (AA) using the following equation:

$$AA\ \% = 100 - \{[(Abs_{sample} - Abs_{blank})\ X\ 100]/Abs_{control}\}.$$

(Abs= absorbance)

2 ml of DPPH and a 0.1 ml solution of methanol were used as control and blank respectively. The experiment was done in triplicate. The radical scavenger activity was calculated as the amount of antioxidant necessary to decrease the initial DPPH absorbance by 50% (EC_{50}) (Brand Williams *et al*., 1995).

Results and discussion

The use of methanol extract was considered in this study because it is often recommended for the extraction of phenolic antioxidant components from plant material (Paschel *et al*., 2006). The methanol extract of *A. africanus* showed an increase in DPPH scavenging activity with an increase in extract concentration (Figure 4.1). The free radical scavenging activity of *A. africanus* reached 39.7% at a concentration of 10 mg/ml. The extract had an EC_{50} of 14.7238 mg/ml.

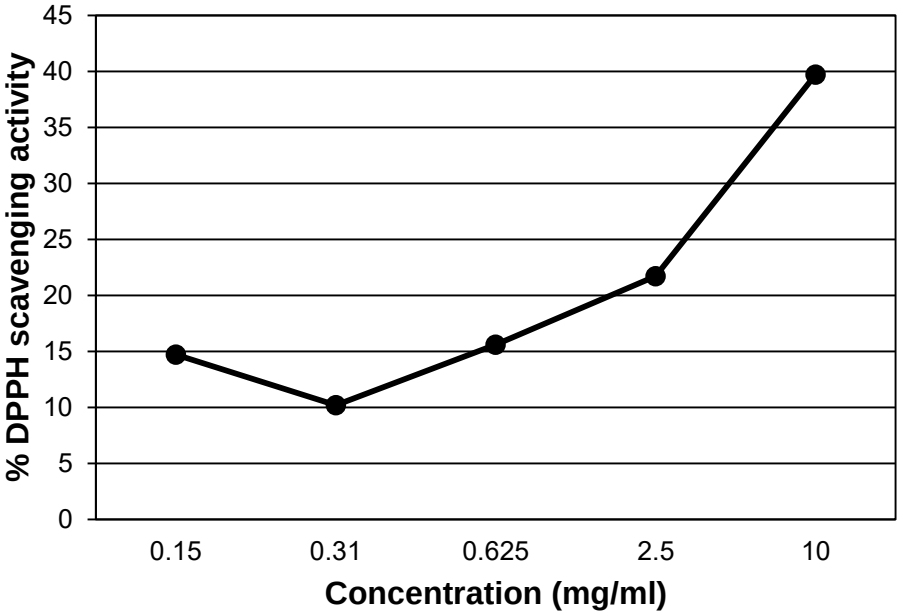


Figure 4. 1. DPPH activity at 517 nm of Asparagus africanus

Free radical scavenging activity demonstrated by ascorbic acid (the reference standard) was 102,5%. Ascorbic acid had an EC_{50} of 0.1157 mg/ml, which was significantly higher than that of *A. africanus*, as shown in Figure 4.2.

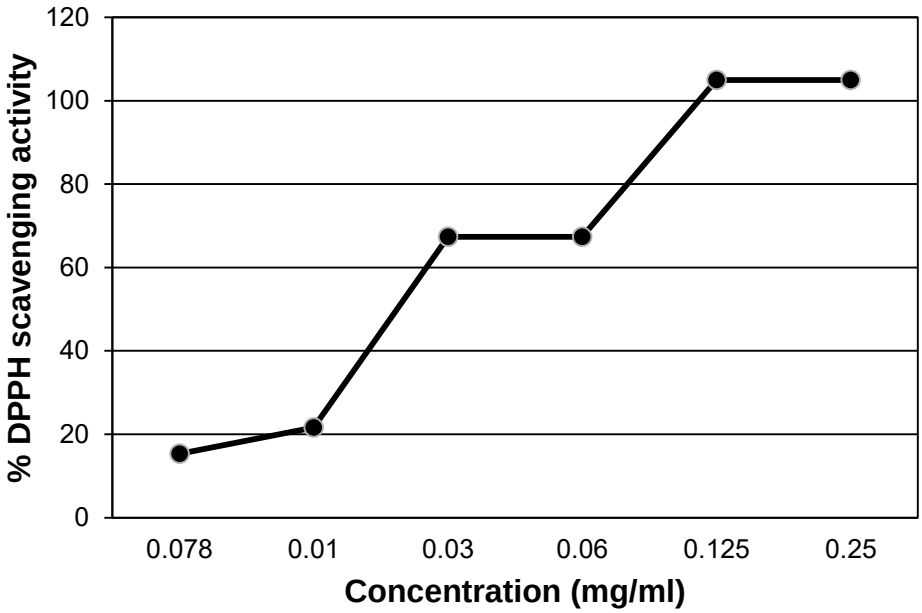


Figure 4. 2. DPPH scavenging activity of ascorbic acid

The concentration of antioxidant needed to decrease the initial DPPH concentration by 50% (EC_{50}) is a parameter widely used to measure the antioxidant activity (Sanchez-Moreno *et al*., 1998). A lower EC_{50} value corresponds with a higher antioxidant power.

4.3. Conclusion

Asparagus species also contain phytosterols, which have been clinically proven to lower blood cholesterol by removing dietary cholesterol from the body. Several anti-cancer compounds have been isolated from *asparagus* species, including ursolic acid, which induces apoptosis or cell death in *in vitro* cancer cell lines (Mashele, 2008). This is due to the antioxidant properties found in *Asparagus* species.

Synthetic anti-oxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, have been developed, but their uses are limited because of their toxicity. In the search for sources of novel antioxidants with low toxicity, medicinal plants have over the past few years been studied extensively for their radical scavenging activity (Molyneux, 2004). As plants produce a large number of anti-oxidants to control the oxidative stress caused by sunbeams and oxygen, it is clear that plants may represent a source of new compounds with antioxidant activity (Scartezzini and Speroni, 2000). The present findings suggest that *A. africanus* root extract may have potential but are not necessarily candidates for treating diabetes, since the plant root extracts were able to scavenge the DPPH at 45% in 10 mg/ml concentration of the plant extracts, which shows low antioxidant activity compared to the DPPH ascorbic activities at 0.25 mg/ml, which were more than 100% (Figures 4. 1 and 4.2 respectively).

Overall, this investigation shows that the antioxidant activity of extracts from *A. africanus* roots has low antioxidant activities. Moreover, more tests should be carried out in regards to antioxidant analysis for more facts and as a good pharmaceutical potential drug agent.

REFERENCE

1. Aggarwal, B.B., and Shishodia, S. (2006) Molecular targets of dietary agents for prevention and therapy of cancer. *Biochemistry Pharmacology*, 71, pp.1397-1421.
2. Ames, B. (1998). Micronutrients prevent cancer and delay aging. *Toxicology Letters*, 102-103, pp.5-18.
3. Anderson, M., Cbrita. L. and Fossen, T. (2002). Colour and stability of the six common antho cyanins-3-glucoside in aqueous solution. *Food Chemistry,* 68, pp.101-107.
4. Arnao, M., Cano, A. and Acosta, M. (1999). Methods to measure the antioxidant activity in plant material. A comparative discussion. *Free Radical Research*, 31(0), pp.89-96.
5. Brand-Williams, W., Cuvelier, M. and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. LWT - *Food Science and Technology*, 28(1), pp.25-30.
6. Grieve, M. (1992). *A modern herbal*. Hafner Press, New York. p. 574.
7. Gutteridge, J. (1994). Biological origin of free radicals, and mechanisms of antioxidant protection. *Chemico-Biological Interactions*, 91(2-3), pp.133-140.
8. Halliwell B. (1995). Antioxidant characterization: Methodology and mechanism. *Biochemistry Pharmacology*, 49, pp.1341-1348.
9. Halliwell B., Gutteridge J.M.C., and Cross C.E. (1992). Free radicals, antioxidants, and human disease: Where are we now? *The Journal of laboratory and clinical medicine*, 119(6), pp.598-620.
10. Larkins, N.J. (1999). Free radical biology and pathology. *Journal of Equine Veterinary Science,* 19, pp.84-89.
11. Mashele, S.S. (2008) Use of *in vitro* assays to assess the anti-mutagenic, mutagenic and cytotoxic effect of Asparagus laricinus plant associated with the prevention and treatment of prostate cancer. *Advances in Pharmacology and Toxicology,* 9(2), pp.51-55.
12. Molyneux, P. 2004. The use of the stable free radical diphenylpicryl-hydrazyl (DPPH) for estimating anti-oxidant activity. *Songklanakarin Journal of Science and Technology*, 26, pp.211-219.

13. Paschel, W., Sanchez-Rabaneda, F., Dn, W., Plescher, A., Gartzia, I., and Jimenez D. *et al*. (2006). An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Food Chemistry*, 97, pp.137-150.
14. Prakash, A., Rigelhof, F., and MIller, E. (2001). Antioxidant activity. *Medallion Laboratories Analytical Progress*, 19(2), pp.1-4.
15. Sanchez-Moreno C., Larrauri J.A., and Saura-Calixto F. (1998). A procedure to measure the antiradical efficiency of poly-phenols. *Journal of Agricultural and Food Chemistry*, 47, pp.425-431.
16. Scartezzini, P. and Speroni, E. (2000). Review on some Indian traditional medicine with antioxidant activity. *Journal of Ethnopharmacology,* 71(1-2), pp.23-43.
17. Soares, P., Trejaut, J., Loo, J., Hill, C., Mormina, M., Lee, C., Chen, Y., Hudjashov, G., Forster, P., Macaulay, V., Bulbeck, D., Oppenheimer, S., Lin, M. and Richards, M. (2008). Climate Change and Postglacial Human Dispersals in Southeast Asia. *Molecular Biology and Evolution*, 25(6), pp.1209-1218.

CHAPTER 5: CYTOTOXICITY OF *ASPARAGUS AFRICANUS* EXTRACTS

5.1. Introduction

Biological testing has played an important role in cytotoxicity studies of plant extracts. Over the years, the use of medicinal plants to treat ailments has not been widely accepted because some of these plants contain toxic isolated compounds, which are not good for human consumption (Biloba, 1999; Fugh-Berman, 2000). Cytotoxicity tests are important during the development of drugs using plant extracts. Taking *Callilepis laureola*, *Lobelia berlandieri* and *Sassafras* as a case study, these plants have been widely used in South Africa and India (Bye & Dutton, 1991; Popat *et al*., 2001; George, 2011), yet various researchers have disapproved of their usage because of the toxic compounds isolated from them; *Callilepis laureola* (atractyloside), *Lobelia berlandieri* (Piperidine alkaloid lobeline*)* and *Sassafras* (Safrole) and because over-dosage could be hepatotoxic (Williams, Ray and Kim, 1987; Steenkamp, 2008; Harrelson, 2012). However, with the help of cytotoxicity tests, which enable researchers to achieve good validation in research on medicinal plants, the use of medicinal plants can be approved. Cytotoxicity studies using the Sulphorhodamine B (SRB) assay (foetal lung fibroblast cells) and MTT assay (colorimetric assay) (Chang cell and C2C12 cell) were conducted to ascertain which concentrations of the compounds and plant crude extracts of *A. africanus* could be toxic to the above mentioned cell line.

5.2. Cytotoxicity assay for *Asparagus africanus*

Methods

Sulphorhodamine B assay

Determination of cell growth and cell viability was performed by *in situ* fixation of cells, followed by staining with a protein-binding dye, SRB. The SRB assay was performed at the Council for Scientific and Industrial Research (CSIR) in accordance with the protocol of the Drug Evaluation Branch, NCI, and the assay was adopted for this screen.

The WI-38 cell line - normal human foetal lung fibroblast from European Collection of Cell Cultures(ECACC) - was routinely maintained as a monolayer cell culture at 37ºC, 5% CO_2, 95% air and 100% relative humidity in EMEM containing 10% fetal bovine serum, 2 mM L-glutamine and 50 µg/ml gentamicin. As a screening experiment, the cells (21-50 passages) were inoculated in 96-well microtiter plates at plating densities of 10 000 cells/well and were incubated for 24 hours. After 24 hours the cells were treated with the experimental drugs, which had previously been dissolved in DMSO and diluted in medium to produce five concentrations. Cells without added drugs served as control. The blank contains complete medium without cells. Etoposide was used as a standard.

The plates were incubated for 48 hours after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed by SRB. Unbound dye was removed and protein-bound dye was extracted with 10 mM Tris base for optical density determination at the wavelength 540 nm using a multiwell spectrophotometer (Vichai and Kirtikara, 2006).

Data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (EC_{50}) was determined by non-linear regression.

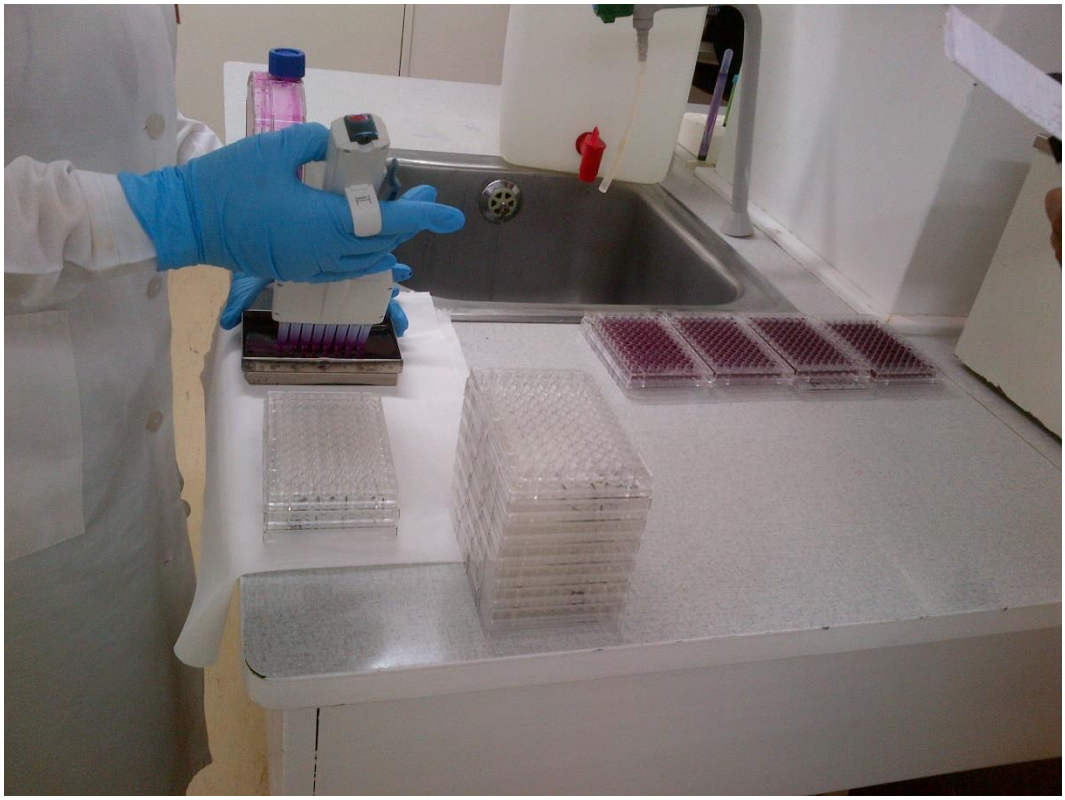


Figure 5. 1. Determination of cell growth and cell viability was performed by in situ fixation of cells, followed by staining with a protein-binding dye, sulphorhodamine B, at the CSIR laboratory.

MTT assay (Van de Venter *et al*., 2008; Deutschländer *et al*., 2009)

C2C12 cells were cultured in DMEM growth medium (Lonza, MD, USA) with 10% foetal bovine serum (Highveld Biological, Johannesburg, South Africa) at 37ºC in humidified air with 5% CO2. C2C12 cells were seeded at a density of 4 000 cells per well into 96-well culture plates and cultured for three days. The C2C12 cells were differentiated for a further two days in DMEM supplemented with 2% horse serum (GIBCO Invitrogen, Paisley, UK). Following differentiation, cells were acutely exposed for three hours to the relevant extracts or fractions at a concentration of 50 µg/mL; metformin (1 µM), insulin (1 µM) or solvent control (DMSO/water) was added to modified DMEM media supplemented with 8 mM glucose. After three hours' exposure to the media the glucose concentrations remaining in the wells were determined using a commercial fluorimetric kit (Biovision).

Chang cells were cultured in EMEM growth medium (Lonza, MD, USA) with 10% foetal bovine serum at 37ºC in humidified air with 5% CO2. Chang cells were seeded at 6 000 cells per well into 96-well culture plates and cultured for up to five days. Thereafter cells were acutely exposed for three hours to the relevant extracts or fractions at a concentration of 50 µg/mL; metformin (1 µM), insulin (1 µM) or solvent control (DMSO/water) was added to modify EMEM media supplemented with 8 mM glucose. Viability in representative wells was assessed using the MTT assay. The cell passage number was below 10. Three replicate samples were used in a single experiment.

Results and discussion

On SRB assay, according to the figures below (Figure 5.1; (a), (b) and (c)), the GraphPad prism was used to analyse the results below; these results were positive. The software used was able to calculate the EC_{50} and distribute the data. Overall, both extracts (methanol and water) of *A. africanus* posed a low hazard, because both plant extracts contained more than 100 µg/ml compared to the EC_{50} of the etoposide (5.1 µg/ml) used as standard during the experiment, according to the standard cytotoxicity criteria of EC_{50} .

Likewise, the MTT assay for *A. africanus* root extracts also shows moderate results, as is evident in the glucose uptake (Figure 3.1 and 3.2), which is suggested to be non-toxic. Similarly, the water extracts for both C2C12 and Chang cells shows a non-toxic result, as indicated in Figures 5.3 and 5.4 respectively. This MTT assay confirms the authenticity of the cytotoxicity of *A. africanus* root crude extracts.

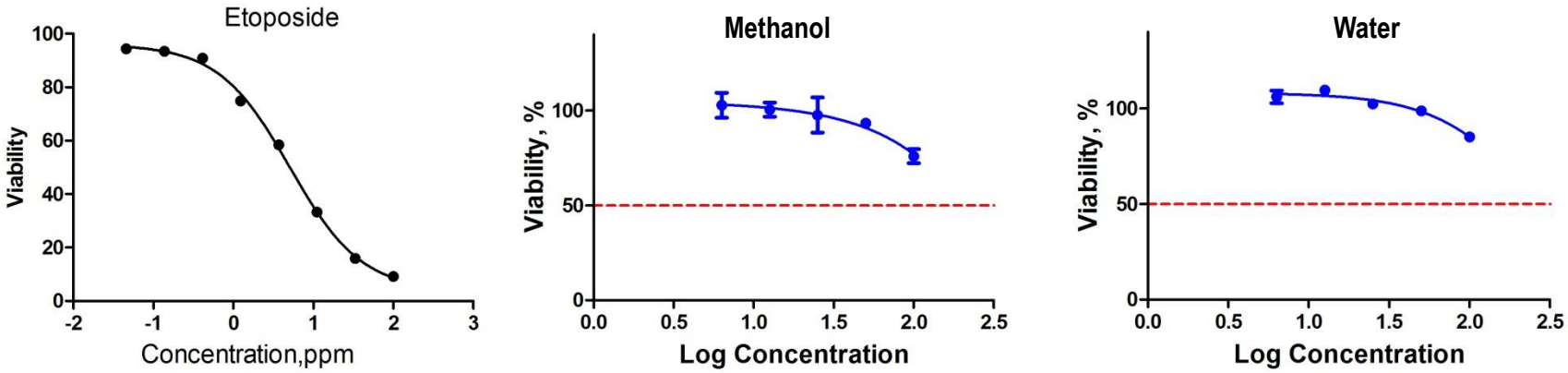


Figure 5. 2. Percentage viability of normal human foetal lung fibroblast cells after treatment with etoposide (a), methanol (b) and water (c) extracts of *A. africanus*

Standard cytotoxicity criteria key may be presented as follows, according to EC_{50}

The cytotoxicity criteria key was standardised according to the CSIR, where > 100 indicates a low hazard, < 100 ->30 a weak hazard, < 30 - >5 a moderate hazard and <5 a high hazard. *A. africanus* extracts showed a low hazard to the normal cell line used compared to the etoposide in figure 5.2 (a and b).

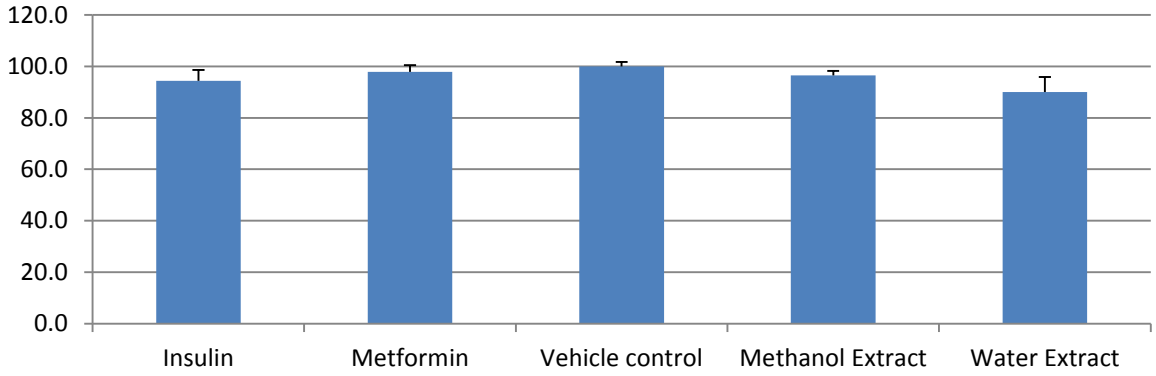


Figure 5. 3. Cytotoxicity for C2C12 cell line

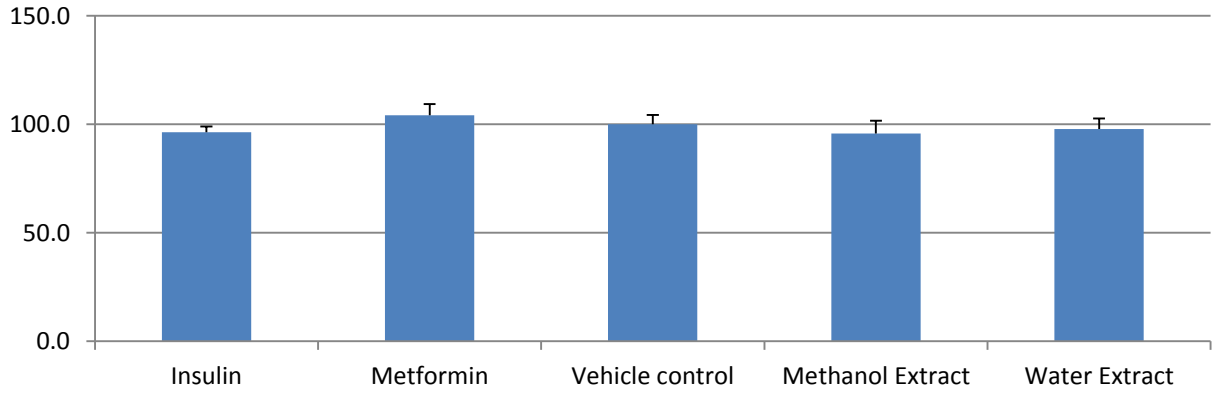


Figure 5. 4. Cytotoxicity for Chang Cell line

5.3. Conclusion

These results indicate that *A. africanus* might be good for human consumption and present a low hazard. The plant in question is regarded as edible in India (used as tea) and has been used as a medicinal herb to treat some ailments in ancient India, which

confirms its non-toxic properties (Negi *et al*., 2010). However; limited research has been conducted on this plant, despite its medicinal importance (Negi *et al*., 2010). Further studies should be conducted on the isolated compounds of *A. africanus*, in order to record more good and validated results for good consumption and pharmaceutical purposes.

REFERENCES

1. Biloba, G. (1999). Herbal remedies: adverse effects and drug interactions. *Am Family Physician*, 59(5), pp.1239-1244.
2. Bye, S. and Dutton, M. (1991). The inappropriate use of traditional medicines in South Africa. *Journal of Ethnopharmacology*, 34(2-3), pp.253-259.
3. Deutschländer, M., van de Venter, M., Roux, S., Louw, J. and Lall, N. (2009). Hypoglycaemic activity of four plant extracts traditionally used in South Africa for diabetes. Journal of Ethnopharmacology, 124(3), pp.619-624.
4. Fugh-Berman A. (2000). Herb-drug interactions. *The Lancet*, 355, pp.134–8.
5. George, P. (2011). Concerns regarding the safety and toxicity of medicinal plants - An overview. *Journal of Applied Pharmaceutical Science*, 1(6), pp.40-44.
6. Harrelson, T. (2012). *Healing Herbs - Toxic Or NOT?.* [online] Healingtools.tripod.com. Available at: http://healingtools.tripod.com/herbstoxic.html [Accessed 8 Jul. 2014].
7. Negi, J. S., Singh, P., Joshi, G. P., Rawat, M. S., & Bisht, V. K. (2010). Chemical constituents of Asparagus. Pharmacognosy reviews, 4(8), pp.215.
8. Popat, A., Shear, N., Malkiewicz, I., Stewart, M., Steenkamp, V., Thomson, S. and Neuman, M. (2001). The toxicity of Callilepis laureola, a South African traditional herbal medicine. *Clinical Biochemistry*, 34(3), pp.229-236.
9. Steenkamp, P. A. (2008). *Chemical analysis of medicinal and poisonous plants of forensic importance in South Africa*. PhD dissertation. University of Johannesburg, Johannesburg, South Africa.
10. van de Venter, M., Roux, S., Bungu, L., Louw, J., Crouch, N., Grace, O., Maharaj, V., Pillay, P., Sewnarian, P., Bhagwandin, N. and Folb, P. (2008). Antidiabetic

screening and scoring of 11 plants traditionally used in South Africa. *Journal of Ethnopharmacology*, 119(1), pp.81-86.

11. Vichai, V. and Kirtikara, K. (2006). Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature Protocols*, 1(3), pp.1112-1116.
12. Williams, H., Ray, A. and Kim, H. (1987). .DELTA.3-Piperideine alkaloids from the toxic plant *Lobelia berlandieri*. *Journal of Agricultural and Food Chemistry*, 35(1), pp.19-22.

CHAPTER 6: PHYTOCHEMICAL SCREENING OF *ASPARAGUS AFRICANUS*

6.1. Introduction

Plants have been in existence for a long time. Over 250 000 species of flowering plants are known and an estimated 155 000 species can be found in the tropics (Cordell *et al*., 1995). South Africa is recognised as one of the countries in the world with the richest biodiversity (http://www.gcis.gov.za/). Apart from fuel, fibre and food, plants have always been known to be a traditional source of medicines, since they contain secondary metabolites of high chemical diversity. Chemical diversity is one among several important factors that has given rise to continuing interest in research into natural products (Cordell *et al*., 1995). Most medicinal plants contain some organic compounds that cause a definite physiological action in the human body, as a result of the presence of bioactive substances such tannins, alkaloids, carbohydrate, terpenoids, steroids and flavonoids (Phillipson and Wright, 1996). These substances are also reported to be present in the roots of *Asparagus* species (Mashele, 2008).

In view of the reports on bioactive substances in natural plants, phytochemical screening of plants has become more important. Phytochemical analyses are conducted on medicinal plants to ascertain the presence of constituents that are known to exhibit medicinal factors, as well as physiological activities (Sofowora, 1993). Furthermore, recent studies have shown that phytochemicals present in leaves and roots have received a great deal of attention, mainly for their role in preventing diseases caused by oxidative stress, which releases reactive oxygen species, as stated in Section 4.1. Considering the importance of these substances, which fight against various illnesses and are good for human consumption, checking for their presence in any medicinal plant is a necessity. In addition, there is a need to validate the presence of these bioactive constituents through an organised infrastructure (phytochemical screening) if they are to be used as an effective therapeutic means in novel drug discovery (Timmermans, 2003). Therefore, in this study knowledge of the chemical constituents of *A. africanus* is desired and this will

be obtained through qualitative phytochemical screening and use of the Gallic acid equivalent (GAE).

6.2. Liquid chromatography–mass spectrometry of *Asparagus africanus*

Methods

Liquid chromatography mass spectrometry LCMS enables rapid initial screening of crude plant extracts. This technique provides a great deal of preliminary information about the content and nature of constituents of the extracts and is useful when large numbers of samples have to be processed because unnecessary isolation of compounds is avoided. LCMS allows the localisation and targeted isolation of new types of constituents such as high molecular weight secoiridoids and through LCMS it is possible to achieve the detection of specific target molecules (Hostettmann, 1998).

In order to identify the compounds, liquid chromatography mass spectrometry (LCMS) was carried out using the Waters Synapt G2 instrument with a ultra performance liquid chromatography (UPLC) at a flow rate of 0.4 ml/min. A 0.1% formic acid-to-acetonitrile gradient was used on a Waters UPLC BEH C18, 2.1x100 mm column. The electrospray was negative at a capillary voltage of 3 kV and the cone voltage was 15 v.

Mass spectrometry works by using magnetic and electric fields to exert force on charged particles in a vacuum. Therefore, the compounds must be charged or ionised and must be introduced in a gaseous phase. A beam of electrons accelerated from a filament, usually to energy of about 70 eV", passes through the sample and an electron collides with a neutral analyte molecule, which knocks out an electron, resulting in a positively charged ion. This involves ionisation energy and is termed electron-impact ionisation. The ionisation impact may produce either a molecular ion, which will have the same molecular weight and elementary composition as the starting material, or a fragment ion, which corresponds to a smaller piece of the analyte. Other ionisation techniques involve electrospray (ES) and fast atom bombardment (FAB) techniques.

In the FAB technique an analyte is dissolved in a liquid matrix such as glycerol, thioglycerol, *m*-nitrobenzyl alcohol or diethanolamine. A small amount of the solution is

placed on a target or emitter. The target is then bombarded with a fast atom beam, for example 6keV Xenon atoms that desorb molecular-like ions and fragments from the analyte. Cluster ions from that liquid matrix are also desorbed to produce a chemical background that varies with the matrix used.

By comparison, the electrospray ionisation technique works by spraying the sample solution across a high potential difference from the needle into the orifice in the interface. Heat and gas are used to desolate the ions existing in the sample solution. This ionisation technique can produce multiple charged ions, with the number of charges tending to increase as the molecular weight increases.

A high-resolution mass spectrum run using electrospray (ES either $[M+H]^+$ / $[M-H]^+$) ion within experimental error (14 in 10 000) is acceptable.

6.3. Determination of total phenolic contents

The total phenolic contents of *A. africanus* root crude extracts were determined using the Folin-Ciocalteu reagent according to the method of Lowman and Box, (1983), using Gallic acid as a standard. Three extract samples (methanol and water) were respectively measured and mixed in distilled water, 1 ml Folin-Ciocalteu reagent was added and the flask was thoroughly shaken. The mixture was allowed to react for few minutes and then 50 ml of 2% Na_2CO_3 solution was added. At the end of incubation at an interval of two hours at room temperature, the absorbance of each mixture was measured at 760 nm. The same procedure was applied to the standard solution of Gallic acid and a standard graph was obtained. The total phenol contents were expressed as mg/ml Gallic acid equivalents (GAE) per mg of the root crude extracts of *A. africanus.* All tests were carried out in triplicate, and GAE values were reported as X±SD of triplicates.

6.4 Phytochemical screening of *Asparagus africanus*

All the chemical tests were carried out on the methanolic and water extracts, using the standard procedure to identify the chemical constituents by colour changes (Harborne, 1973; Trease and Evans 1989; Sofowora 1993).

Test for saponins

Amounts of about 0.7 g of the extracts were dissolved in 3 ml of distilled water and shaken vigorously. The formation of emulsion was observed. The test was performed in triplicate.

Test for carbohydrates

Benedict's test was performed using 0.2 g of the extract dissolved in 2 ml of methanol; 1 000 ul of the sample was put into a test tube and the same proportion of Benedict's solution was added. The test was carried out in triplicate.

Test for protein

The test for protein was carried out measuring out 0.2 g of the extract dissolved in 2 ml of methanol; a few drops of 1% CuSO4 and 4% NaOH were used; these are also Biuret reagents. The test was performed in triplicate.

Test for flavonoids

Three methods were used to determine the presence of flavonoids in the plant sample (Sofowora, 1993; Harborne, 1987). 5 ml of dilute ammonia solution was added to both the methanol and distilled water extracts, followed by the addition of concentrated H2SO4. Yellow colouration observed in each extract indicated the presence of flavonoids. The test was done in triplicate.

Test for tannins

Amounts of about 0.7 g of the extracts were dissolved in 2 ml methanol and distilled water, then three drops of 0.1% ferric chloride solution were added. The formation of precipitates in the solution was observed; there was a blue- black or brownish green precipitate, which indicated the presence of tannins.

Test for steroids and terpenoids (Salkowski test)

About 2 g of each extract was dissolved in 2 ml of both methanol and distilled water. Both extracts were premixed with 1 ml of chloroform and afterwards concentrated sulphuric acid (H2SO4) was added to the sample. A layer was then formed. These experiments

resulted in a reddish brown colour precipitate at the border or interface of chloroform and H2SO4, which confirmed the presence of steroids and terpenoids.

Test for cardiac glycosides (Keller-Killani test)

About 0.7 g of each extract was dissolved in 2 ml of methanol and distilled water, and then the extracts where both treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlaid with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the deoxysugar characteristic of cardenolides. A violet ring appeared below the brown ring, while in the acetic acid layer, a greenish ring formed gradually throughout thin layer.

Test for alkaloids (Meyer's test)

Amounts of about 1 g of each extract were dissolved in 3 ml of methanol and distilled water; 1 ml of the Meyer's solution (potassium mercuric iodide solution) was added to 200 ul of the respective samples. The methanol extracts became creamish in colour, while the water extracts turned orange brownish in colour.

Test for alkaloids (Dragendorff's test)

About 1 g of each extract was dissolved in 3 ml of methanol and distilled water and 1 ml of the Dragendorff's solution (potassium Bismuth iodide solution) was added to 200 ul of the respective samples. Both the methanol and distilled water turned reddish brown in colour. This test was carried out in triplicate.

6.5. Results and discussion

A. africanus was also tested for the presence of phenolic content from the *Asparagaceae* family. Gallic acid has been identified as the key phenolic compound in the plant (Iwu, 2014). Gallic acid is a water-soluble polyhydroxyphenolic compound that can be found in plants such as grapes, strawberries, banana and many other fruits. The methanolic and water root crude extracts of *A. africanus* results showed similarities at 2.5 mg/ml concentrations with a GAE of 0.01 and 0.04% phenolic content found in the concentration. The *A. africanus* water crude root extract tested a little lower at the same concentration

of 2.5 mg/ml with a GAE of 0.02 and contained about 0.01% phenolic content in the same concentration. The standard curve equation is shown in Figure 6.1 and Table 6.1, y (absorbance) = 0.1869 x (µg Gallic acid) +0.0103, R^2 = 0.9994. The absorbance value was inserted in the above equation and the total amount of phenolic content was calculated. The Gallic acid, flavonoids and other plant phenolic content of *Asparagus laricinus* are reported by Mashele *et al*. (2008). Flavonoids are the most extensively studied phenolic and antioxidant compounds, which play more intensive roles in ethno-pharmacology.

Table 6. 1. Total Phenolic Compounds in Gallic Acid Equivalent

A.africanus root crude extracts	**Total Phenolic Compounds (GAE)**	**%Content**
Methanol	0.011401	0.0045604
Water	0.029904	0.0119616

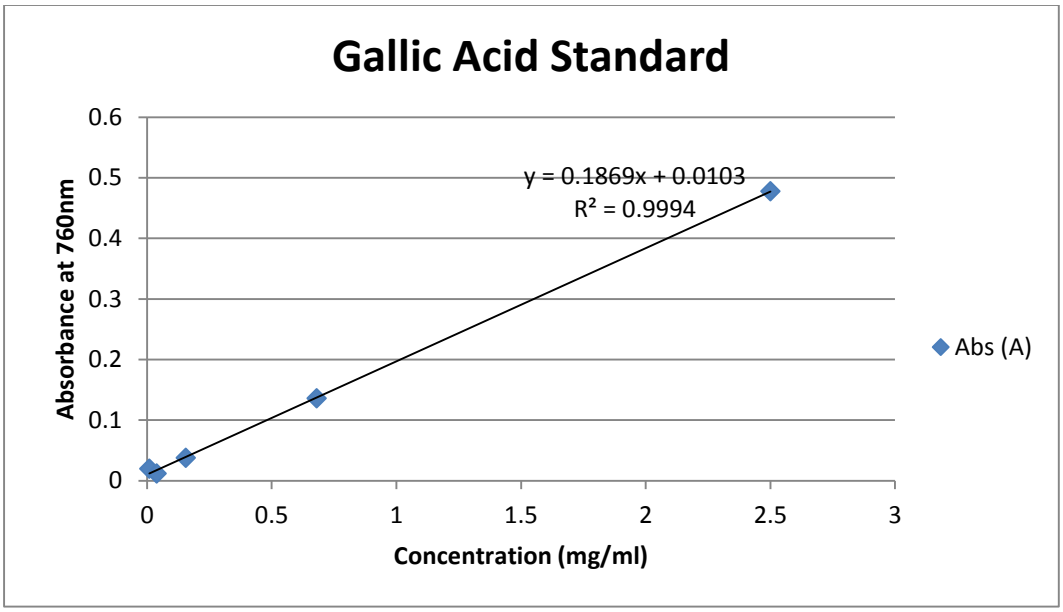
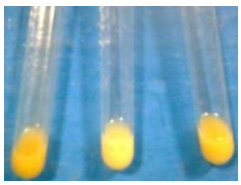


Figure 6. 1. Gallic acid standard (mg/ml).

Table 6. 2. Phytochemicals Screening (*A. africanus*)

Phytochemical	Methanol	Water	Image
Alkaloids (Meyer's test)	Cream colour(+)	Orange brownish(+)	
Alkaloids (Dragendorff's test)	Reddish brown(+)	Reddish brown(+)	
Carbohydrate (Benedit's test)	Reddish brown(+)	Brick reddish(+)	
Cardiac glycosides (Keller-Killani test)	Brown ring(+)	Purple (+)	
Protein (Biuret test)	Pale blue(+)	Pale blue(+)	

Flavonoids	Yellow precipitate(+)	Yellow precipitate(+)	
Tannins (ferric chloride test)	Brownish green(+)	Brownish green(+)	
Saponins (froth test)	Formation of emulsion(+)	Formation of emulsion(+)	
Steroids	Blue green(+)	Blue green(+)	
Terpenoids (Salkoski test)	Reddish brown(+)	Reddish brown(+)	

Positive (+) **Negative (-)**

Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, glycosides, tannins and steroids in crude root extracts of *A. africanus*. These results revealed that the plant has quite a number of chemical constituents, which may be responsible for the many pharmacological actions. Reports have shown that most active compound constituents in plants are alkaloids, phenolics, tannins and saponins

(Farnsworth, 1966). Alkaloids are very important in medicine and constitute most of the valuable drugs (Edeoga and Eriata, 2001). Phenolic are considered potentially toxic to the growth and development of pathogens, while tannins are fairly potent bioactive compounds used for therapeutic purposes (Singh and Sawhney, 1988; Khan *et al.*, 2011). All of these chemical constituents play a role in plants' resistance to disease. Further work should also be done to investigate the specific phytoconstituents responsible for these activities. Phytochemicals can serve as a valuable source of information and provide appropriate standards to establish the quality of this plant root material in future study or application.

The compounds found in the LCMS screening (major peaks) are listed in Table 6.3.

Table 6. 3. Liquid Chromatography Mass Spectrometry for Fractionation of *Asparagus africanus*

Peak	Chemical formula	Names	Mass
1.	C28H44O9	[(2R,2'R,5S,5'R,6'S,8a'S)-6'-Acetoxy-5-(2-acetoxyethyl)-2',5',8a'-trimethyldecahydro-2'H,3H-spiro[furan-2,1'-naphthalene]-5,5'-diyl]bis(methylene) diacetate	525.3064
2.	C60H50O4	(1R,5S,6S,7R,10S,11R,12R,16S,17S,18R,21S,22R)-25,28-Bis(9-anthrylmethoxy)-3,14-dimethoxynonacyclo[14.6.2.25,12.17,10.118,21.02,15.04,13.06,11.017,22]octacosa-2(15),3,8,13,19,23,26-heptae ne	835.3787

6.6. Conclusion

The results above indicate that all three solvents of *A. africanus* root crude extracts have a notable chemical constituent and phenolic content. The total phenolic contents in the extracts of *A. africanus* that were examined ranged from 0.01 to 0.004 mg GA/g .The highest concentration of phenols was measured in methanolic and water extracts of *A.*

africanus root crude extracts contain considerable phenolic content. *A. africanus* root crude extracts revealed the presence of alkaloids, flavonoids, tannins, terpenoids, cardiac glycosides, saponins and steroids by positive reaction with the respective test reagents.

The LCMS test was carried out; a compound was found and the compound in Table 6.3 was identified using the chemical compound website called www.chemspider.com. The nuclear magnetic resonance lipoprofile test should be carried out, so as to be specific about the compound present in the plant extracts.

These results indicate that *A. africanus* root crude extract is rich in phenolic, with low antioxidant and reducing activities. The findings of this study suggest that this plant root could be a potential source of natural antioxidants that could have great importance as therapeutic agents in preventing or slowing down the progress of ageing-associated oxidative stress related to degenerative diseases such as diabetes, cancer and cardiovascular diseases caused by lack of antioxidant capacity. Further studies of this plant should be carried out to determine the bioactive compounds and *in vivo* studies of its medicinal properties should be done in order to prepare natural pharmaceutical products of high value.

REFERENCE

1. Cordell, G. (1995). Changing strategies in natural products chemistry. *Phytochemistry*, 40(6), pp.1585-1612.
2. Edeoga, H.O. and Eriata, D.O. (2001). Alkaloid, tannin and saponin contents of some Nigeria medicinal plants. *Journal of Medical Aromatic Plant Science*, 23, pp.344-349.
3. Farnsworth, N. (1966). Biological and phytochemical screening of plants. *Journal of Pharmaceutical Sciences*, 55(3), pp.225-276.
4. Harborne, J.B. (1973). *Phytochemical methods: A guide to modern techniques of plant analysis.* Chapman and Hall, London.
5. Harborne, J.B. (1987). Flavonoids in the environment: structure-activity relationships. *Progress in clinical and biological research*, 280, pp.17-27.

6. Hostettmann, K. (1998). Strategy for the biological and chemical evaluation of plant extracts. *Pure and Applied Chemistry*, 70(11), pp.1-9.
7. Iwu, M. M. (2014). *Handbook of African medicinal plants*. Boca Raton, FL, CRC Press.
8. Khan, F.A, Hussain, I., Farooq, S., Ahmad, M., Arif, M and Ur Rehman, I. (2011). Phytochemical screening of some Pakistanian medicinal plants. *Middle-East Journal of Scientific Research*, 8 (3), pp.575-578
9. Lowman, M. and Box, J. (1983). Variation in leaf toughness and phenolic content among five species of Australian rain forest trees. *Austral Ecology*, 8(1), pp.17-25.
10. Mashele, S.S. (2008) Use of in vitro assays to assess the antimutagenic, mutagenic and cytotoxic effect of Asparagus laricinus plant associated with the prevention and treatment of prostate cancer. *Advances in Pharmacology and Toxicology,* 9(2), pp.51-55.
11. Phillipson, J.D. and Wright, C.W. (1996). Plants with antiprotozoal activity. In Trease and Evans, *Pharmacognosy*, 14th edn., WB Saunders Company, London, pp. 612.
12. Singh, R., and Sawhney, S. K. (Eds.). (1988). *Advances in frontier areas of Plant Biochemistry*. Prentice-Hall of India Private Limited.
13. Sofowora A. (1993). *Medicinal plants and traditional medicine in Africa*. Spectrum Books Ltd, Ibadan, Nigeria.
14. Timmermans, K. (2003). Intellectual property rights and traditional medicine: Policy dilemmas at the interface. *Social Science and Medicine,* 57(4), pp. 745-756.
15. Trease, G.E. and Evans, W.C. (1989). Pharmacognosy 13th Ed. London: Bailliere Tindall, pp: 176-182.

Chapter 7: General discussion and conclusions

The reliance of a greater percentage (70-85%) of the world population on plant-based medicine for health care and the increasing acceptance of this system in the modern age guided the entire process of this research work.

This study focussed on the evaluation of the antidiabetic properties of a medicinal plant, using *A. africanus* as a case study. In a bid to achieve the above,

- ✓ the *in vitro* glucose-lowering potential of *A. africanus* extracts was assessed, using C2C12 muscle and Chang cells;
- ✓ the antioxidant activity of *A. africanus* was determined;
- ✓ The screening for toxicity of the root crude extract of *A. africanus* was evaluated; and
- ✓ The phytochemical content of *A. africanus* was investigated.

A. africanus is a potential candidate for the treatment of diabetes and related ailments. It also has some antioxidant properties and is safe for human consumption. The therapeutic value of plants used in traditional medicine derives from the presence of some bioactive substances (Ayodele *et al*., 2003), which are found in parts of the plants. *A. africanus* root extracts contain alkaloids, tannins, flavonoids and saponins. Hence, *A. africanus* can be suggested as a rich and yet unexplored source that could potentially be useful as an antidiabetic drug in future replacement of synthetic drugs.

Few plant species from the family of *Asparagaceae* have been tested and found to be a good antidiabetic and good hypoglycaemic agents though recent studies of other families with plant species such as *Cuminum cyminum*, *Coriandrum sativum* and *Emblica offcinalis* have also proven them to be good antidiabetic agents. In *A. africanus*, the glucose uptake assay showed a positive result, where the cell lines used are liver and muscle cells. Moreover, the plant extract was also able to inhibit the glucose uptake of the cell lines, compared to the control and the treatment used in this study.

The highest DPPH scavenging activity of ascorbic acid at 0.25 concentrations was 105% and 15% at the lowest concentration (0.078 mg/ml). The Ec_{50} of the ascorbic acid was 0.1157 mg/ml, while the scavenging activity of *A. africanus* extract at the highest concentration of 10 mg/ml was at 40% DPPH scavenging activity at 0.15 mg/ml. The plant extracts was also able to scavenge at 15% DPPH scavenging activity.

The Ec_{50} of the plant extracts was 14.7238 mg/ml. The ascorbic scavenging activity was higher than the scavenging activity of the plant root crude extract of *A. africanus*. The phenolic content of the plant extracts at 2.5 mg/ml of the methanol and water extracts respectively was 0.0045604% and 0.0119616%. These results indicate that *A. africanus* has moderate phenolic content properties at a GAE of 0.01 and 0.04% phenolic content found in concentration. *Cuminum cyminum* (Apiaceae) has been proven to be an antidiabetic agent and has displayed antioxidant properties in the kidney and pancreas of diabetic rats (Kavishankar *et al*., 2011).

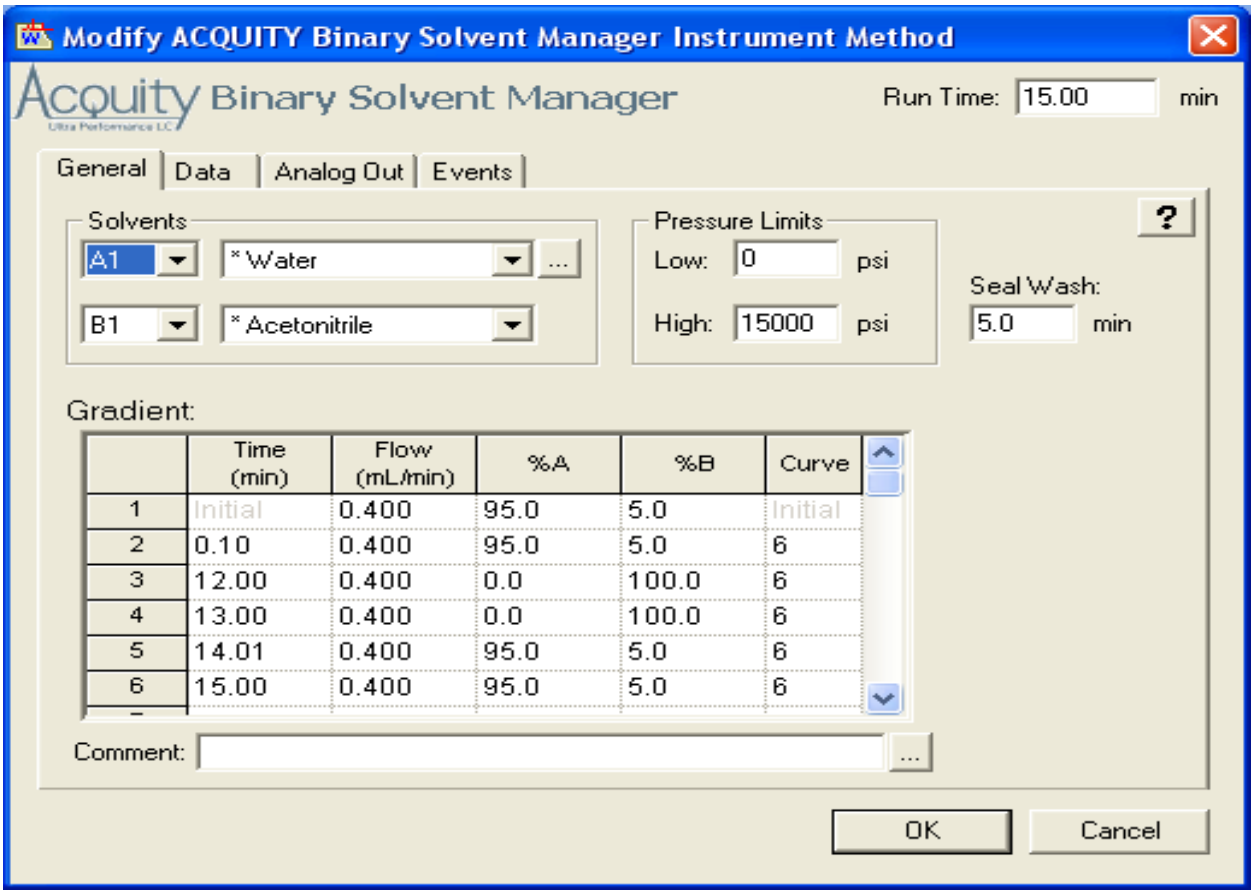
The cytotoxicity result indicated that the normal human cell lines used posed a low hazard compared to the etoposide. The plant extract showed a low hazard, compared to the etoposide result. The phytochemical screening indicated that *A. africanus* has all the constituents that proves it to have antidiabetic properties when compared to the plant species indicated above. The plant contains alkaloids, flavonoids, saponins, tannins, etc. All these compounds are present in *A.africanus*, as in other plants that were found to be antidiabetic agents and have good antioxidant properties. The (LCMS test shows some unknown compounds indicated above in Table. 6.3. Further studies should be done, using the NMR test to identify the compounds present in plant root crude extracts of *A. africanus*.

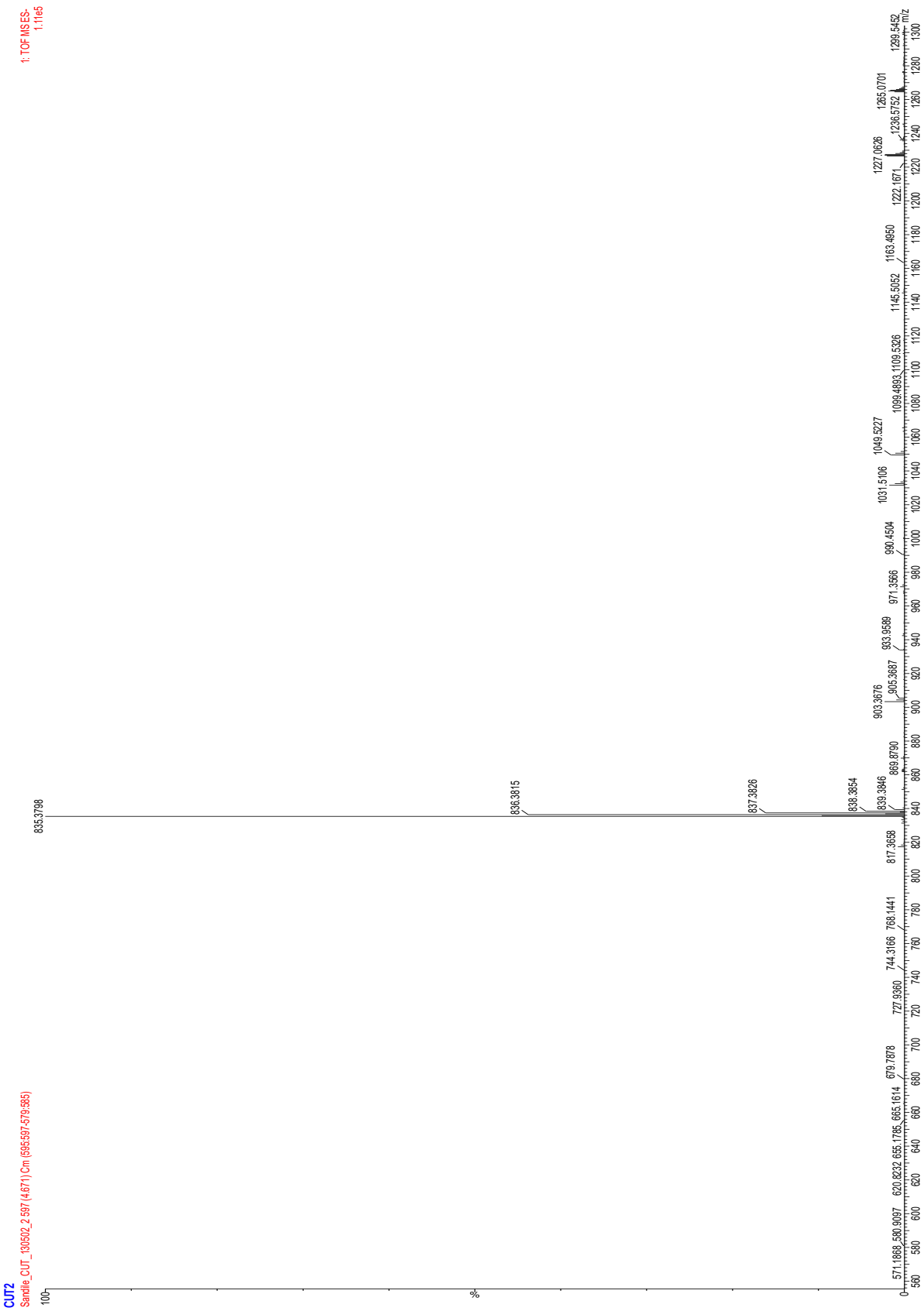
REFERENCE

1. Ayodele, A.E. Ibidun, D.K., and Ogunshe, A.O. (2003). The morphology and preliminary phytochemistry of *Albizia saman* (Jacq.) *F. Mull. Bioscience Research Communications*. 15(6): 463-46.

2. Kavishankar, G.B, Lakshmidevi, N., Mahadeva Murthy, S., Prakash, H.S., and Niranjana S.R. (2011) Diabetes and medicinal plants - *A review, International Journal of Pharmaceutical Biomed Science*, 2 (3), pp.65-80.

APPENDIX





Elemental Composition

File Edit View Process Help

Single Mass Analysis

Tolerance = 100.0 PPM / DBE: min = -0.5, max = 50.0

Element prediction: Off

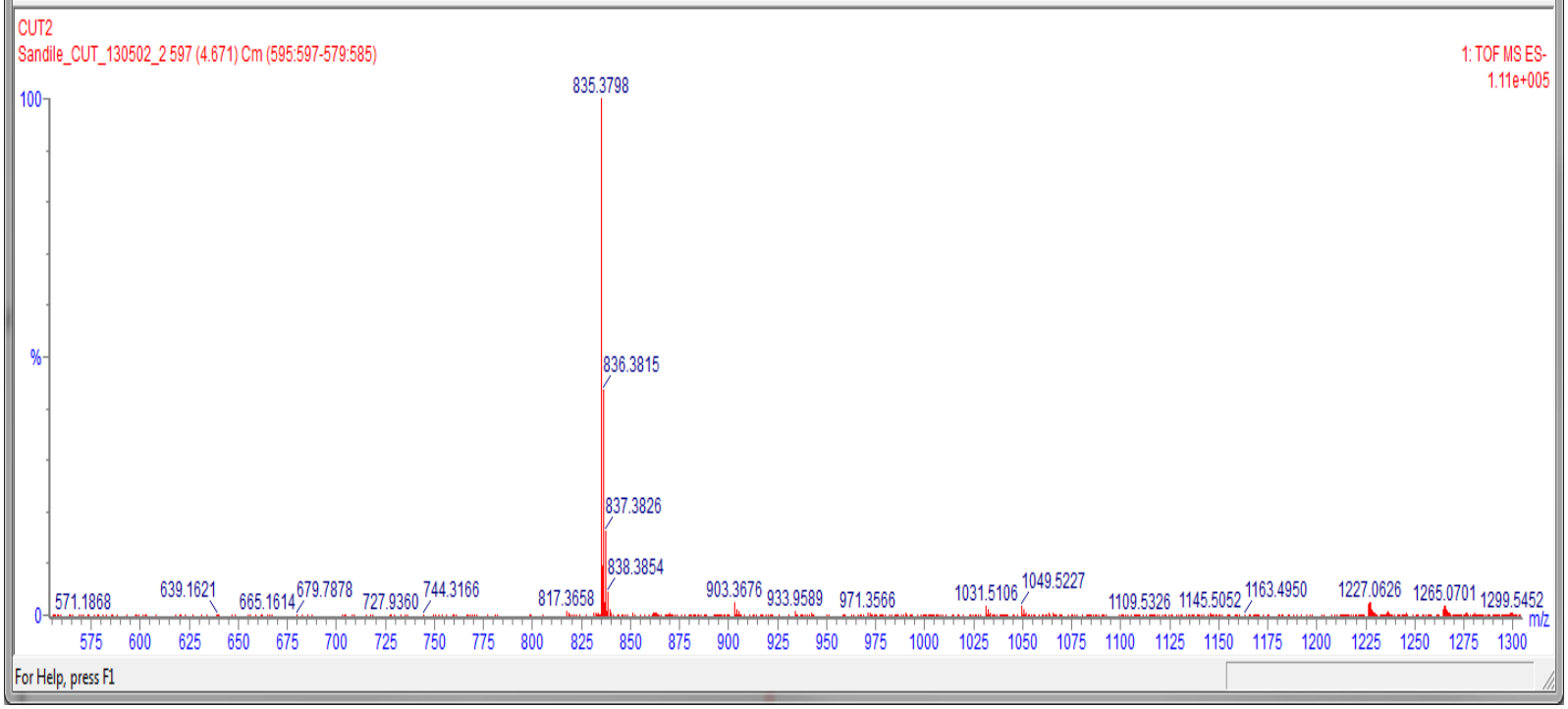
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

225 formula(e) evaluated with 47 results within limits (up to 50 closest results for each mass)

Elements Used:

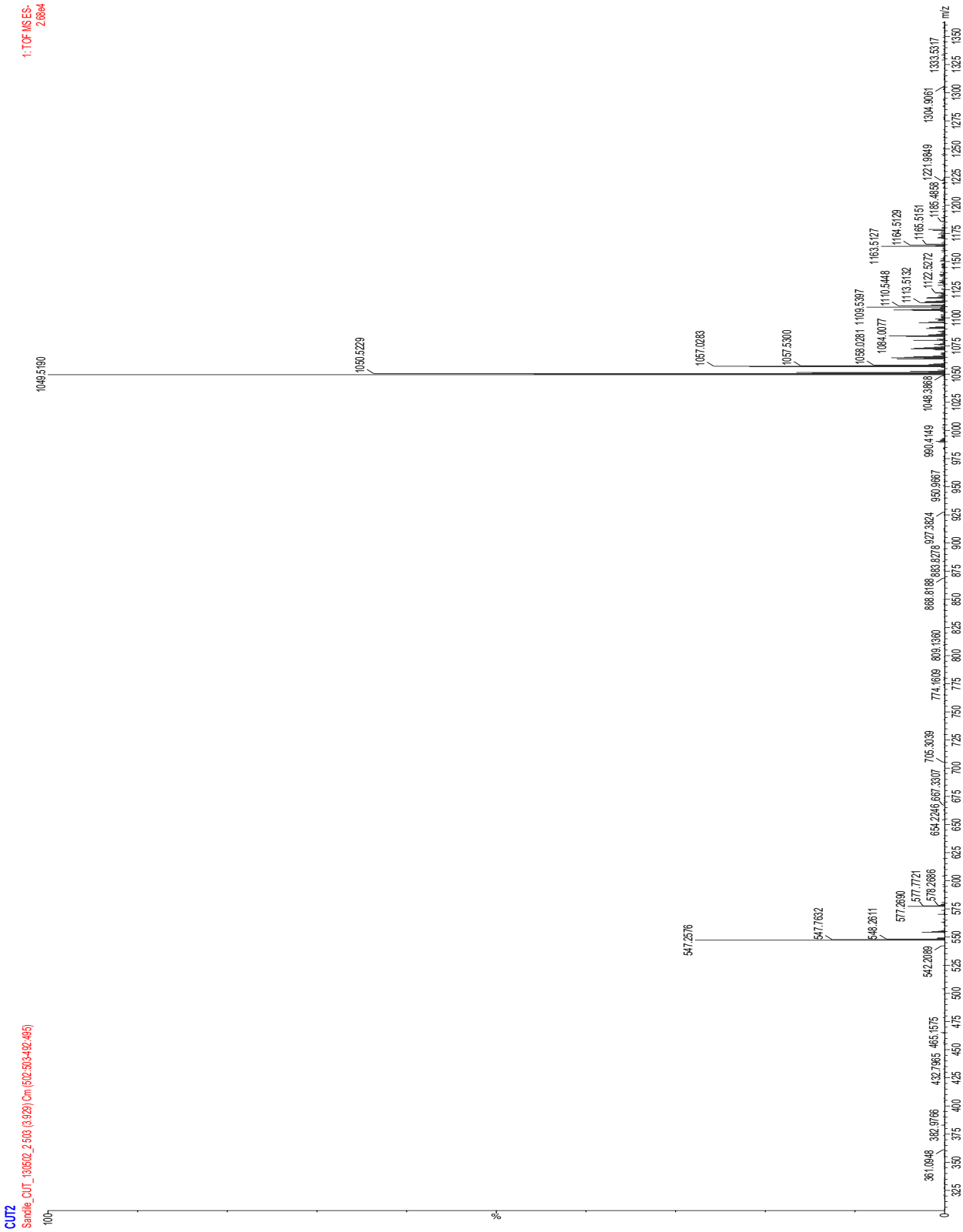
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
835.3798	835.3787	1.1	1.3	35.5	C60 H51 O4	75.9	7.824	0.04	60	51	4
	835.3811	-1.3	-1.6	4.5	C35 H63 O22	71.6	3.519	2.96	35	63	22
	835.3752	4.6	5.5	13.5	C42 H59 O17	70.1	1.978	13.83	42	59	17
	835.3846	-4.8	-5.7	26.5	C53 H55 O9	74.2	6.111	0.22	53	55	9
	835.3694	10.4	12.4	22.5	C49 H55 O12	73.0	4.865	0.77	49	55	12
	835.3905	-10.7	-12.8	17.5	C46 H59 O14	71.6	3.501	3.02	46	59	14
	835.3658	14.0	16.8	0.5	C31 H63 O25	73.1	4.965	0.70	31	63	25
	835.3940	-14.2	-17.0	39.5	C64 H51 O	76.6	8.538	0.02	64	51	1

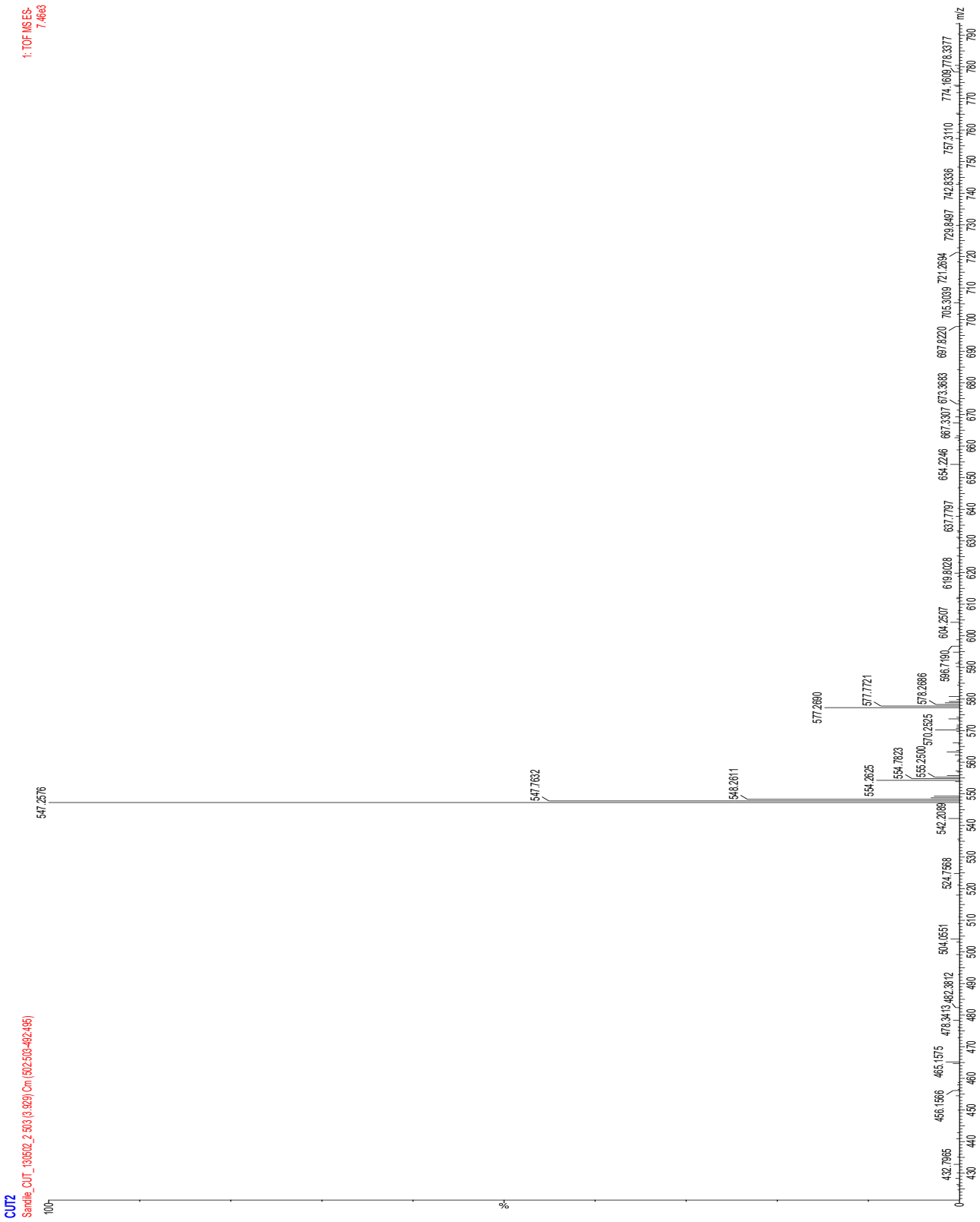


For Help, press F1

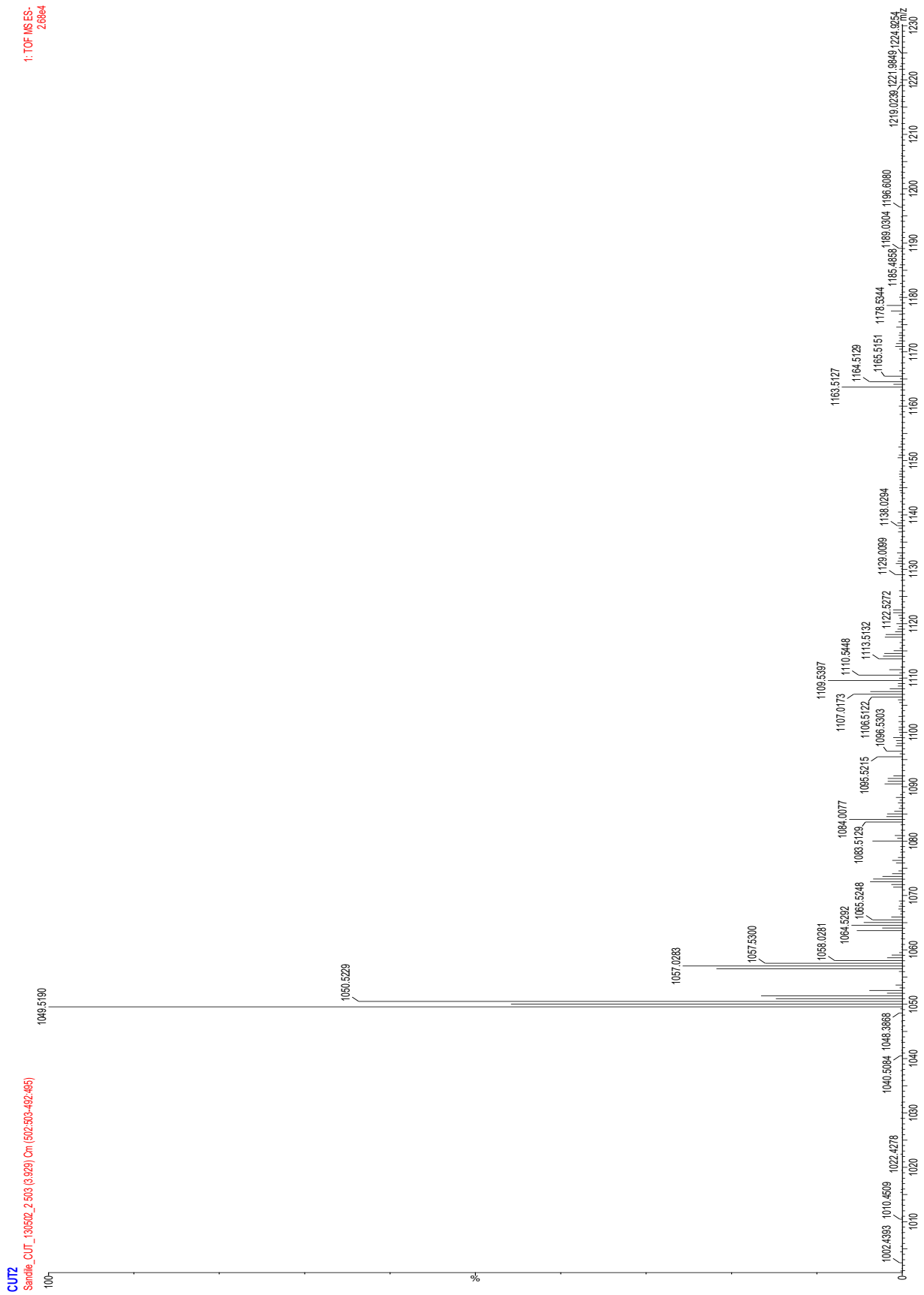
Not strong UV absorbance

>>>>>



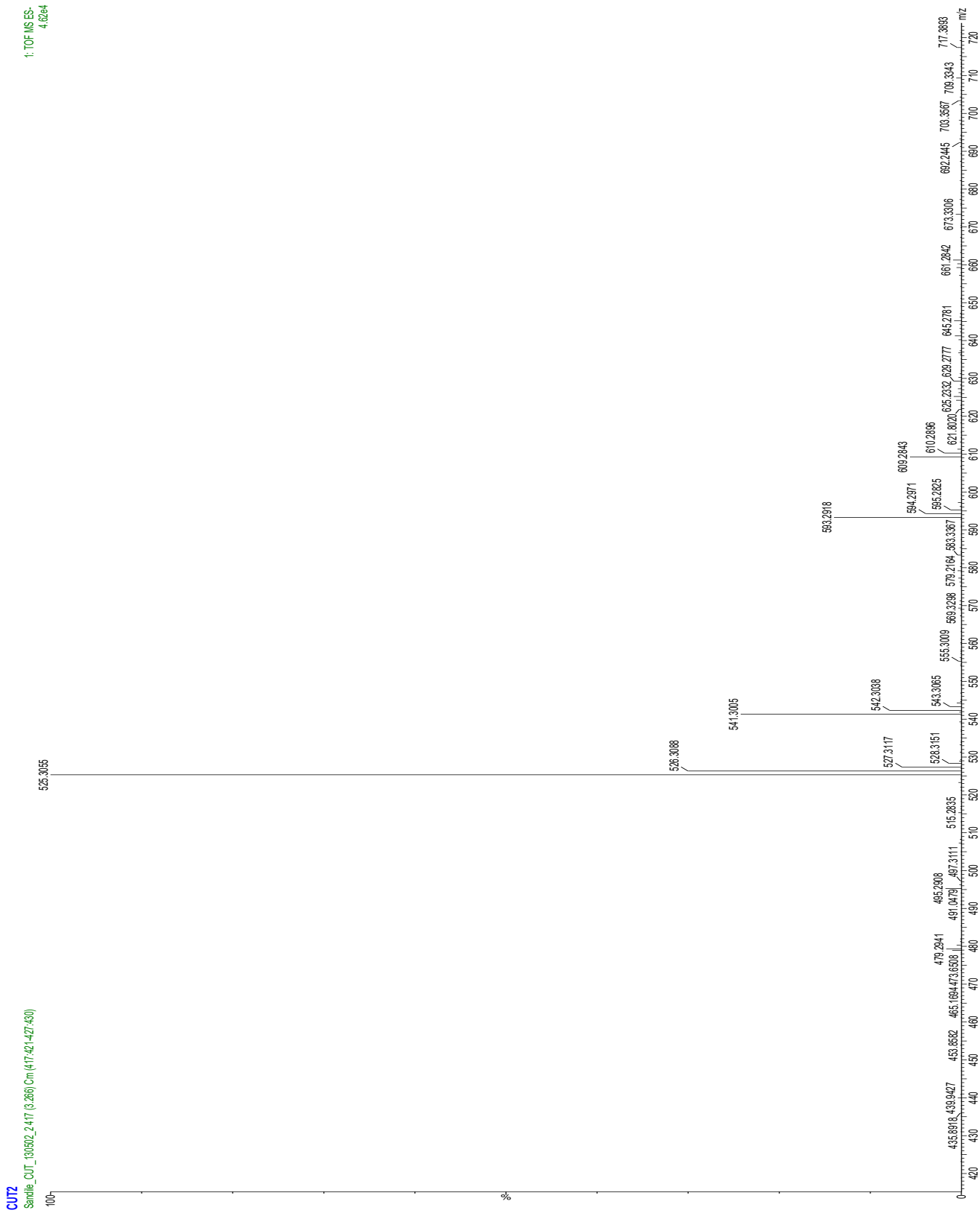


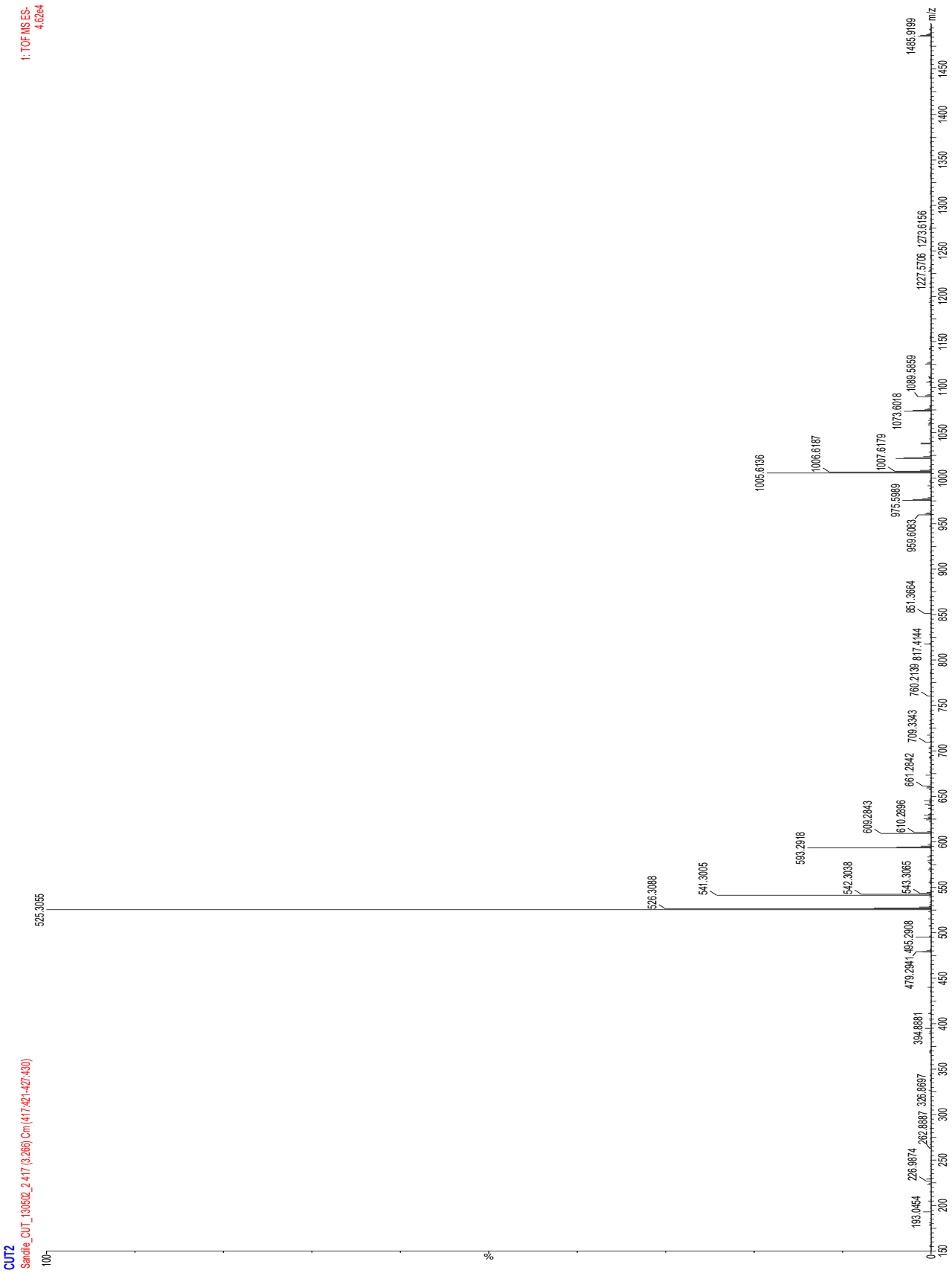
Charge state = 2

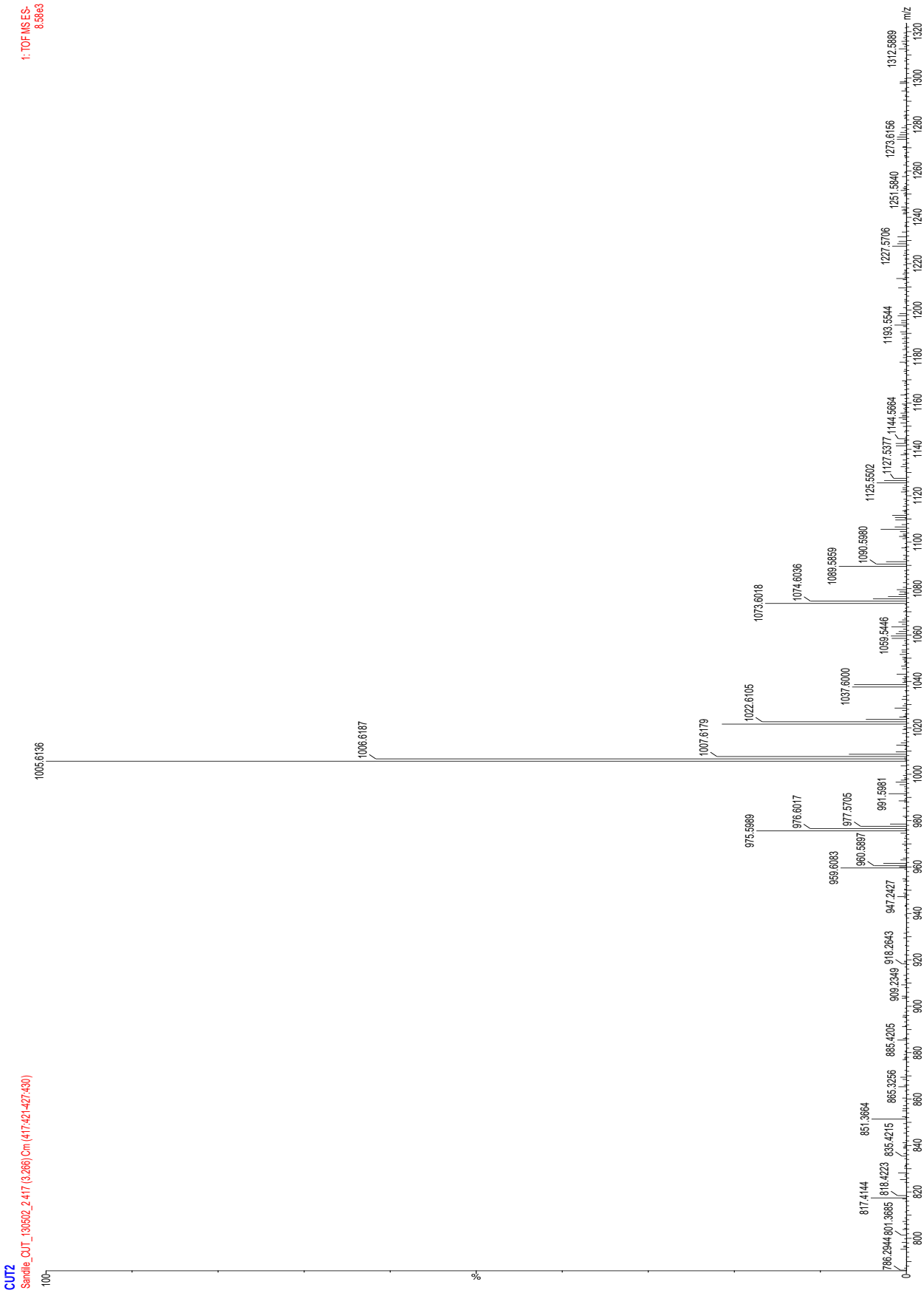


Not strong UV absorbance

>>>>>





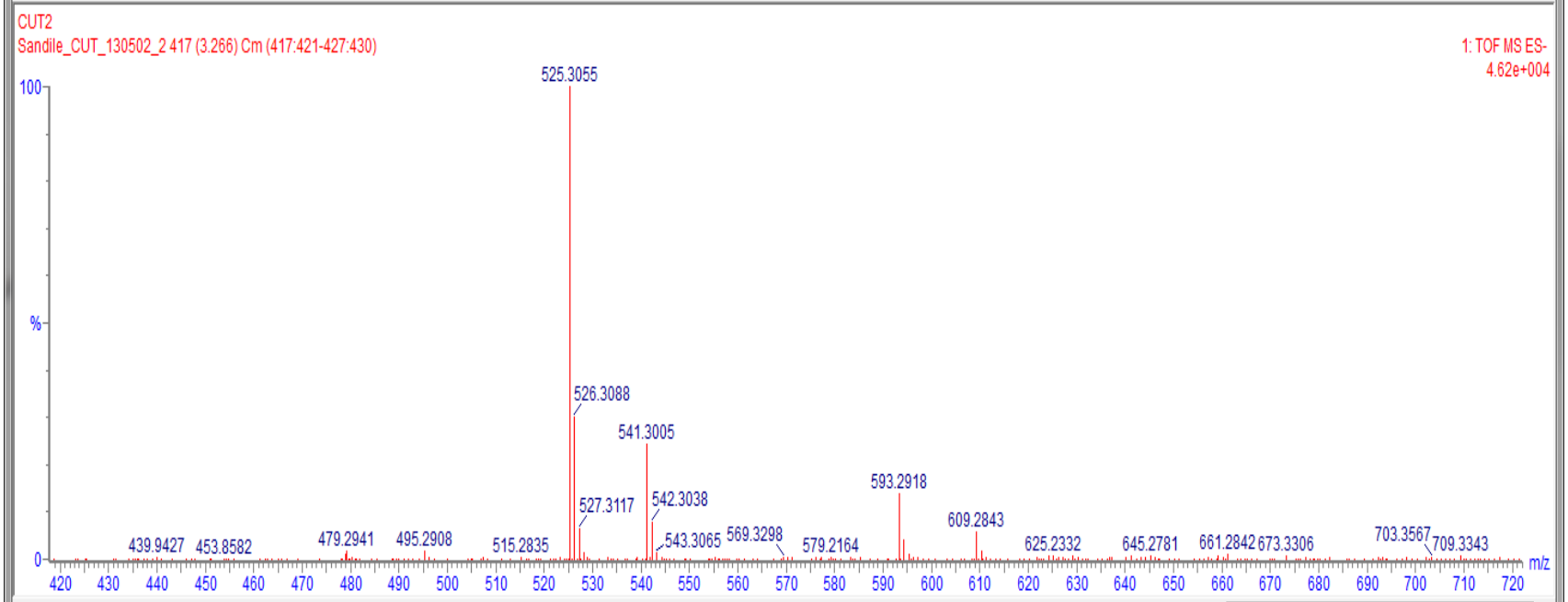


Elemental Composition

File Edit View Process Help

Tolerance = 100.0 PPM / DBE: min = -0.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
130 formula(e) evaluated with 16 results within limits (up to 50 closest results for each mass)
Elements Used:
C: 1-100 H: 1-100 O: 0-30

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
525.3055	525.3064	-0.9	-1.7	6.5	C28 H45 O9	27.7	1.561	20.99	28	45	9
	525.3005	5.0	9.5	15.5	C35 H41 O4	35.7	9.571	0.01	35	41	4
	525.3157	-10.2	-19.4	19.5	C39 H41 O	37.5	11.364	0.00	39	41	1
	525.2911	14.4	27.4	2.5	C24 H45 O12	32.5	6.437	0.16	24	45	12
	525.3216	-16.1	-30.6	10.5	C32 H45 O6	34.2	8.124	0.03	32	45	6
	525.2852	20.3	38.6	11.5	C31 H41 O7	33.6	7.528	0.05	31	41	7
	525.3275	-22.0	-41.9	1.5	C25 H49 O11	31.3	5.210	0.55	25	49	11
	525.2704	26.1	49.7	20.5	C38 H37 O2	38.2	12.058	0.00	38	37	2



For Help, press F1