ANTI-DIABETIC AND ANTI-OXIDANT EVALUATION OF *ANTHOCLEISTA DJALONENSIS* A. CHEV AND *ANTHOCLEISTA VOGELII* PLANCH

by

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ABSTRACT

Diabetes a major degenerative disease of global concern accounts for about 3.2 million deaths annually. Alpha-amylase inhibitors from plants are effective in managing postprandial hyperglycaemia which is significant in Type 2 diabetes. Search for natural anti-oxidants has increased recently because free radicals production has been linked to a number of diseases including diabetes. *Anthocleista djalonensis* and *Anthocleista vogelii* are used traditionally in Nigeria and parts of Africa to treat diabetes. This study was aimed at evaluating the α-amylase inhibition, anti-oxidant and anti-diabetic effects of extracts and compounds of both plants to verify their traditional use.

The leaves, stem bark and roots of both plants were collected along Ijebu-Ode – Benin road and authenticated at the Herbarium of the Forestry Research Institute of Nigeria, Ibadan. The plant samples were macerated in 80% aqueous methanol for 72 h. Each crude extract, suspended in water: methanol (4:1) was partitioned into ethyl acetate. The crude extracts and ethyl acetate fractions of the leaves and stem bark of both plants were subjected to *in vitro* α-amylase inhibition assay with acarbose as positive control. The anti-oxidant activity was evaluated using 2, 2'-diphenyl-1-picrylhydrazyl with α-tocopherol as control, while anti-diabetic properties of the crude extracts were studied *in vivo* using 45 albino wistar rats (150-200 g) of both sexes. The rats were made diabetic with 80 mg/kg of alloxan and treated with the extracts (1 g/kg) for seven days; glibenclamide 2.5 mg/kg was used as reference. Blood glucose levels (BGL) were monitored daily. Bioassay-guided fractionation and chromatographic methods were used to isolate active compounds from the ethyl acetate fractions of both plants. Structures of the isolated compounds were elucidated using spectroscopic techniques: infra-red, mass spectrometry, nuclear magnetic resonance (one-dimensional and two-dimensional). Data were analysed statistically using ANOVA at $p < 0.001$.

Anthocleista djalonensis leaf and stem bark crude extracts gave highest α-amylase inhibition of 42.8% and 41% with their ethyl acetate fractions also producing the highest α-amylase inhibition of 50.0% and 36.6% at 1.0 mg/mL while acarbose gave 54.9%. The crude extract and ethyl acetate fraction of *A. vogelii* leaf gave 80.7% and 87.4% inhibitions at 1.0 mg/mL in the anti-oxidant assay while α-tocopherol gave 89.5%. Peak reduction in BGL was observed for *A. djalonensis* stem bark and leaf crude extracts at 72.6% and 45.7% on day-6 of treatment while the stem bark and leaf extracts of *A. vogelii* gave 68.9% and 60.4%, respectively on day-7. The root extracts of both plants also caused peak reduction in BGL at 48.5% on day-7 while glibenclamide had 57.4%. Bioassay-guided fractionation furnished djalonenol, a monoterpene diol with a significant α-amylase inhibition of 53.7% from fraction 11 of the stem bark of *A. djalonensis* and decussatin, a xanthone with significant inhibition of 78.0% from fraction 5 of the leaves and stem bark of *A .vogelii*.

The presence of α-amylase inhibitors, djalonenol and decussatin from both plants makes them important in the treatment of type 2 diabetes and could be responsible for their anti-diabetic effect. *Anthocleista vogelii* could be a source of anti-oxidant compounds.

Keywords: *Anthocleista* species, anti-diabetic activity, anti-oxidant effect, djalonenol, decussatin.

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DEDICATION

This work is dedicated to my Lord and saviour Jesus Christ for His faithfulness unto me.

CERTIFICATION

I certify that this work was carried out by Mrs. O.O. Olubomehin in the Department of Pharmacognosy, University of Ibadan under my supervision.

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CHAPTER 1

INTRODUCTION

Plants have been a source of medicines throughout human history. A great percentage of modern drugs have been isolated or developed from plant sources. The full treasure of natural resources, however, has by no means been fully investigated and utilized (Zakair, 2009). The biodiversity of developing countries is a source of natural products and vast resources of utmost importance. The priceless heritage which includes medicinal plants have been used by indigenous communities for centuries as drug substances for relief from illnesses, as healthcare products, fragrances, sweeteners and as materials for pest control (Adesina, 2005). Currently, plants may be the almost exclusive source of drugs for the majority of the global population. Substances derived from higher plants constitute about a quarter of all prescription medicines (Principe, 1989). Plants are therefore of great importance as many of them throughout the world are screened for one or more activities (Sofowora, 1993). Herbal extracts have been used directly or indirectly for the preparation of many modern medicines (Tamil *et al*., 2010). Natural products play a dominant role in pharmaceutical industry and systemic investigation of natural resources for the discovery of new drug molecules. This has been a primary objective for bio-prospection programs (Verpoorte *et al*., 2005). Traditional medicine exploiting the natural resources has been the main stay of managing several infections and diseases in indigenous communities (Sharma and Prasad, 2012).

Herbal preparations used for the treatment of diseases and its knowledge have been used in healthcare delivery in parts of Africa, China, India and globally. In Africa, there is need to complement orthodox medicine with traditional medicine to achieve an effective healthcare delivery system (Elujoba *et al*., 2005).

1.1 Botanical description

1.1.1   Plant Family: Loganiaceae

Anthocleista Afzel. ex R. Br. is known in English as the cabbage tree, fever tree, forest big-leaf or Muderer's mat (Mabberley, 1997). It belongs to the Loganiaceae family which contains about 30 genera and 600 species (Backlund *et al*., 2000). Several genera yield valuable drugs and poisonous substances, and some are grown, as ornamentals with showy clusters of flowers. The family is made up of mostly trees and shrubs with coloured juice (Watson and Dallwitz, 1992). Members of the family bear leaf-like appendages at the base of the leaf stalks and have terminal flower clusters. The ring of petals on each flower has four or five overlapping lobes. The fruit vary from capsules to fleshy drupes, and it is a 1-many seeded berry (Bruce, 1955).

The leaves are opposite; herbaceous' or leathery, or membranous; petiolate to sessile, connate or not connate simple. Lamina is entire; linear, lanceolate, oblong, or ovate, one-veined, or pinnately veined. The young stems are cylindrical with cork cambium present; initially deep-seated or superficial. The plants are usually hermaphrodite, or without dioecious. Gynoecium of female flowers is pistillodial. Plants are homostylous. The flowers aggregate in inflorescences usually, or solitary; in cymes, or in panicles (when not solitary). The inflorescences are usually terminal or axillary with the terminal inflorescence uniting cymosely. Flowers are bracteates, bracteolate; fragrant (sometimes), or odourless; regular, 4-merous or 5- merous; cyclic; tetracyclic (Watson and Dallwitz, 1992).

1.1.2 The Genus: *Anthocleista*

The genus, *Anthocleista* Afzel. consists of approximately 50 species native mainly to tropical Africa, Madagascar and Mascarene Islands. Representative species in the genus include *Anthocleista amplexicaulis* Baker., *A. djalonensis* A. Chev, *A. exelliana* Th. Monod, *A. grandiflora, A. keniensis* Summerh, *A. liebrechstiana* De Wild &T. Durand, *A. madagascariensis* Baker, *A. nobilis* G. Don, *A. nigrescens* Afzel.ex Gilg, *A. parviflora* Baker, *A. schweinfurthii* Gilg, *A. kamerunesis* Gilg, *A. oubagwensis* Aubrev & Pellegr, *A. vogelii* Planch, *A. auriculata* De Wild, *A. lanceolata* Gilg, *A. zenkeri* Gilg, *A. rhizophoroides* Baker, *A. procera* Lepr. ex Bureau, *A.laxiflora* Baker, *A. scandens* Hook. F. (Gentian Research Network, 2008; The plant list, 2010).

Among the 50 species, six are known to be of economic importance in various parts of Nigeria (Keay, 1989). Nine of the species are recorded in West Tropical African region out of which four: *A. nobilis, A. vogelii, A. djalonensis and A. procera* are relatively abundant and widespread (Burkill, 1995).

They are normally small trees or scrambling shrubs with soft white wood. Their leaves are glabrous, leathery and large and are often over one-foot long in mature trees and up to 1.52 m long in saplings. The base of the leaf stalk is dilated and sometimes more or less winged (Burkill, 1995).

1.1.3 The Species: *Anthocleista djalonensis*

Anthocleista djalonensis A. Chev (Loganiaceae) is a medium-sized tree of West tropical Africa, 9-14 m high, with blunt spines on the unbranched, pale grey trunk and wide spreading crown (Fig. 1.1). It is a small candelabrum-shaped tree found in the

semi-savannah tropical regions of West Africa, characterized by its inconspicuously spiny branches, secondary venation and creamy or white flowers (Dalziel, 1995; Iwu, 1993).

This species has sparingly spiny branches, a rather spreading inflores-cence with slender peduncles and comparatively long pedicels (0.5- 1.5 cm.). The calyx is small (7-8 mm.); the corolla club-shaped in bud and rounded at the apex, the tube is funnel-shaped and about 21 times the length of the lobes, which are generally about 10 in number ; the infructescence is dropping. The main feature of the species is the leaf-shape, which is very variable. The upper pair of leaves subtending the inflorescence is generally cuneate at the base and though normally petiolate may be subsessile. The leaves remote from the inflorescences are usually conspicuously rounded at the base and fairly long petiolate. The twigs may or may not have sparse spines while the leaves are variable, 15-40 cm long by 8 -25 cm broad (Bruce, 1955). The inflorescence is terminal with white fleshy flowers, of rather dry sites in savannah thickets from lowlands to 500 m altitude in Guinea Bissau and into East Cameroon. The plant produces tubular white flowers in Ghana from April to May, and in Nigeria from March to May. Green smooth fruits occur in Nigeria in October and November (de Ruitjer, 2007). The wood is white, soft, sappy and perishable appearing to have no usage. The stems are sometimes hollowed out in Northern Nigeria for use as quivers. *Anthocleista djalonensis* is commonly known in Hausa as *kwarii* (meaning quiver), in Ibo: *Okpokolo* and in Yoruba: *Sapo* (Burkill, 1995).

1.1.4 *Anthocleista vogelii*

Anthocleista vogelii Planch is a tree (of the Loganiaceae family) which commonly grows around river edges and banks or in marshy areas of the tropical humid forest of

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West Africa (Irvine, 1961). It can be found from Sierra Leone east to Kenya and south to Zambia and Angola (de Ruijter, 2007) with great concentration in Cameroon and Gabon (Leewenberg, 1972). It is known as *Apa oro* (meaning - poison antidote) by the Yorubas of South western Nigeria (Burkill, 1995). In Nigeria *A. vogelii* flowers from October to February and from March to May; it fruits from November to March. This plant (Fig.1.2) is 6 to 20 meters high, usually with buttrous roots, with branches having spikes, which also have sessile leaves and short petals (Bruce, 1955). Small to medium-sized tree up to 20 m tall; bole up to 55 cm in diameter, sometimes with stilt roots; twigs with 2(–4) divergent spines confluent at base. Leaves opposite, simple and entire, almost sessile; blade oblong-ovate to oblanceolate, 15–45 cm × 6–24 cm, in young plants up to 150 cm × 45 cm, base cuneate, auricled, apex rounded, margin recurved, papery or leathery (Bruce, 1955). Inflorescence erect terminal dichasia cymes 30–50 cm long, many-flowered with yellowish green or orange peduncle and branches thickened at the nodes. Flowers bisexual, regular; sepals 4, free, orbicular or broader than long, outer ones 4–12 mm long, inner ones about twice as long; corolla with cylindrical tube, 12–18 mm long, lobes 13–16, oblong-lanceolate, 12–19 mm long, spreading, creamy to pale yellow; stamens as many as corolla lobes and alternating with them, exserted, filaments partly or entirely fused, anthers whitish green; ovary superior, ovoid-cylindrical to ovoid-conical, 5–7 mm × 3–6 mm, 4-celled, stigma obovoid-cylindrical, apically 2-lobed. Fruit an ellipsoid berry 2.5–4.5 cm × 2–3.5 cm, rounded at apex, thick-walled, green or yellowish, many-seeded. Seeds obliquely ovoid-globose, 2–2.5 mm × 1.5–2 mm, dark brown (de Ruitjer, 2007).

Fig.1.1. *Anthocleista djalonensis* (picture taken by Olubomehin, O. O. along Ijesha-Ijebu road).

Fig. 1.2. *Anthoclestia vogelii* (picture taken picture taken by Olubomehin, O. O. at Olabisi

Onabanjo University, Ago-Iwoye).

1.2   Diabetes mellitus

Diabetes mellitus, commonly known as diabetes is one of the world's oldest known diseases. It is a heterogeneous group of disorders of carbohydrate, fat and protein metabolism characterized by chronic hyperglycaemia which is associated with increased risk of cardiovascular diseases (Jenkins *et al*., 2002). It is a multifactorial disease involving lipoprotein abnormalities (Scoppola *et al*., 2001), raised basal metabolic rate (Nawata *et al*., 2004; Okwu *et al*., 2006), defect in reactive oxygen species scavenging enzymes and altered intermediary metabolism of major food substances (Unwin *et al*., 2001). As a chronic metabolic disorder, diabetes mellitus can affect all the body's major organ systems leading to complications that are a source of significant morbidity and premature mortality, making it a costly disease (Szava-Kovats and Johnson, 1997).

Diabetes being a major degenerative disease is found in all parts of the world and it is becoming the third most lethal disease of mankind and rapidly increasing (Ogbonnia *et al*., 2008).

There are four types of diabetes mellitus: type 1, type 2, "Other specific types" and gestational diabetes. Each of the types of diabetes mellitus identified extends across a clinical continuum of hyperglycaemia and insulin requirements.

Type 1 diabetes mellitus (formerly called type I, IDDM or juvenile diabetes) is characterized by beta-cell destruction caused by an autoimmune process usually leading to absolute insulin deficiency (American Diabetes Association, 1997). It is a catabolic disorder in which circulating insulin is virtually absent, plasma glucagon is elevated and the pancreatic β-cells fail to respond to all insulinogenic stimuli (Nolte and Karam, 2001).

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The onset is usually acute, developing over a period of a few days to weeks. It is characterized by an excess in weight loss, slender build, ketoacidosis and low insulin levels (Szava-Kovats and Johnson, 1997). Hypoglycaemia is the most common acute complication of Type 1 diabetes mellitus.

Type 2 diabetes mellitus (formerly called type II, NIDDM or adult onset) is characterized by insulin resistance in peripheral tissue and an insulin secretory defect of the beta-cells (Harris, 1995). The pancreas compensates by secreting more insulin, but eventually the beta cells will fail to sustain this, at which stage the patient requires insulin treatment (Cerasi, 2000). It is the most common form of diabetes mellitus and is highly associated with a family history of diabetes, older age, obesity and lack of exercise, stress and a sedentary lifestyle. Due to the fact that it is triggered off by obesity, diet, stress and a sedentary lifestyle, diabetes has now been recognized as one of the lifestyle related diseases (Stohs, 1995; Valavanidis *et al*., 1995).

Other specific types: This group is made of types of diabetes mellitus of various known etiologies. This type of diabetes was formerly called MODY or maturity onset diabetes in youth. It includes persons with genetic defects of beta-cell function or with defects of insulin action: persons with diseases of the exocrine pancreas, such as pancreatitis or cystic fibrosis; persons with dysfunction associated with other endocrinopathies (eg. acromegaly, and persons with pancreatic dysfunction caused by drugs, chemicals or infections (Harris, 1995).

Gestational Diabetes: gestational diabetes mellitus is an operational classification (rather than a pathophysiologic condition) identifying women who develop diabetes mellitus during gestation (Harris, 1995).

1.3   Pathology of diabetes mellitus

Several pathogenic processes are involved in the development of diabetes. These range from, autoimmune destruction of the β-cells of the pancreas, with consequent insulin deficiency, to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrates, fat and protein metabolism in diabetes is deficient action of insulin on target tissues. This results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action (American Diabetes Association, 2008).

Diabetes is a result of the body's failure to adequately control blood sugar levels. The normal blood sugar levels are in the range of 75-115 mg/dL of blood. After a meal, the body tightly regulates increases in blood sugar to a level not exceeding 180 mg/dL in people without diabetes (Muhammed and Lakshmi, 2005).

Postprandial hyperglycaemia plays an important role in the development of type 2 diabetes mellitus and complications associated with the disease such as micro- and macro vascular diseases (Baron, 1998) and has been proposed as an independent risk factor for cardiovascular diseases (Cerriello, 1998). Therefore, control of postprandial hyperglycaemia is suggested to be important in the treatment of diabetes and prevention of cardiovascular complications (Li, *et al*., 2005; Mitra, 2008).

1.4   Diagnosis of diabetes mellitus

Insulin is the master regulator of glucose and lipid metabolism (Saltiel, 2003). Its deficiency and resistance play a key role in the pathogenesis of several human diseases like diabetes, obesity, hypertension and cardiovascular diseases (Nandi *et al*., 2004).

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The following tests are used for the diagnosis of diabetes.

i. A fasting plasma glucose test; this measures the blood glucose after an overnight fast for at least 8 hours. This test is used to detect diabetes or pre-diabetes.

ii. An oral glucose tolerance test (OGTT); this measures the blood glucose after one has gone for at least 8 hours without eating and 2 hours after one drinks a beverage containing 75 g of glucose. This test can be used to diagnose diabetes or pre-diabetes.

iii. A random plasma glucose test; here the blood glucose is checked without any regard to the time you ate your last meal. This test along with an assessment, of symptoms is used to diagnose diabetes but not pre-diabetes.

Confirmation of diabetes is done by repeating the fasting plasma glucose test or the oral glucose tolerance tests at least twice.

The oral glucose tolerant test (OGTT) previously recommended by the National Diabetes Data Group has been replaced with the recommendation that the diagnosis of diabetes mellitus be based on two fasting plasma glucose levels of 126 mg/dL (7.0 mmol/L) or higher (Mayfield, 1998).

Other diagnosis options include two, two-hour postprandial plasma glucose (2 h PPG) readings of 200 mg/dL (11.1 mmol/L) or higher after a glucose load of 75 g (essentially, the Criterion recommended by WHO) or two casual glucose readings of 200 mg/dL (11.1 mmol/L) or higher.

Measurement of the fasting plasma glucose (FPG) level is the preferred diagnostic test, but any combination of two abnormal test results can be used.

Fasting plasma glucose (FPG) was selected as the primary diagnostic test because it predicts adverse outcomes (e.g. retinopathy) as well as the 2 h PPG test but is much more reproducible than the OGTT or the 2 h PPG test and the procedure is relatively easier to do in clinical setting (Mayfield, 1998).

Table 1.1. shows the results of the range of glucose level using the diagnostic tests and their interpretation (American Diabetes Association, 1997).

Persons with fasting plasma glucose levels ranging from 110 to 126 mg/dL (6.1 to 7.0 mmol/L) are said to have impaired fasting glucose, while those with a 2 h PPG level between 140 mg/dL (7.75 mmol/L) and 200 mg/dL (11.1 mmol/L) are said to have impaired glucose tolerance.

Both impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) are associated with an increased risk of developing type 2 diabetes mellitus (Mayfield, 1998).

1.5 Management of diabetes mellitus

Currently, there is no cure for diabetes, but by controlling blood sugar levels through a healthy diet, exercise and medication, the long term complications of diabetes can be averted (Gerich, 2001).

Table 1.1. Impaired Glucose Homeostasis and Diabetes Mellitus Results

Glucose levels	Normal		Impaired Glucose homeostasis		Diabetes mellitus	
Venous Plasma	FPG	2h PPG	IFG	I G T	FPG	2h PPG
mmol /L	6.1	7.75	6.1-7.0	7.75-11.1	7.0	11.1*
mg/dL	<110	<140	110-126	140-200	126	≥ 200*.

Adapted from American Diabetes Association (1997).

*Values could be taken alone or in addition to the presence of symptoms like polyuria, polydipsia or unexplained weight loss.

FPG = Fasting plasma glucose

IFG = Impaired fasting glucose

IGT = Impaired glucose tolerance

2h PPG = Two hour postprandial glucose.

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1.5.1 Diet control and exercise

Lifestyle management including diet control and adequate exercise is essential to the successful treatment of type 2 diabetes. Experts on diet and health, and the American Diabetes Association (ADA) state that there is no single dietary regimen for diabetes. A diet made of high-glycaemic-index forms of carbohydrate produces higher blood glucose concentrations and greater demand for insulin and would increase the risk of type 2 diabetes, (Willett *et al*., 2002). In large prospective epidemiologic studies, both the glycaemic index and the glycaemic load (the glycaemic index multiplied by the amount of carbohydrate) of the overall diet have been associated with a greater risk of type 2 diabetes in both men and women. Conversely, a higher intake of cereal fibre has been consistently associated with lower diabetes risk. In diabetic patients, evidence from medium-term studies suggests that replacing high-glycaemic-index carbohydrates with a low-glycaemic-index form will improve glycaemic control and among persons treated with insulin, will reduce hypoglycaemic episodes, (Willett *et al*., 2002). These dietary changes can be accomplished by replacing products made with white flour and potatoes with whole-grain, minimally refined cereal products (Willett *et al*., 2002). This low-risk dietary pattern has also been associated with reduced incidence of coronary heart disease (Rimm *et al*., 1996; Willett, 1998; Wolk *et al*., 1999; Liu, 1999), a lower occurrence of diverticular disease (Aldoori, *et al*., 1994) and constipation (Dukas, 2000); as such it has become an appropriate component of recommendations for an overall healthy diet. In a related study, Ajala *et al*. (2013) concluded that low-carbohydrate, low-GI (glycaemic index), Mediterranean, and high-protein diets are effective in improving various markers of

cardiovascular risk in people with diabetes and should be considered in the overall strategy of diabetes management.

Dietary recommendations for diabetes may be developed based on the patient's requirement and treatment goals. Successful nutritional management of diabetes entails:

(i) Regular monitoring of metabolic parameters (including blood glucose, glycated haemoglobin, lipids, and blood pressure),

(ii) Maintaining healthy body weight,

(iii) Lifestyle management.

It is important that diabetics space meals adequately over the day to avoid glucose over-load and low blood sugar as seen in studies by Jenkins *et al*. (1995) which showed that lipid metabolism could be improved by dividing meals into small snacks, thus simulating the slow release of carbohydrates.

1.5.2 Therapeutic measures for the management of diabetes mellitus

The aim of therapy in diabetes is to maintain blood glucose at normal levels. Drug therapy includes glucose lowering agents as well as medications to treat or prevent the secondary complications of the disease (Muhammed and Lakshmi, 2005).

The progressive nature of the disease necessitates constant reassessment of glycaemic control in people with diabetes, and the appropriate adjustment of therapeutic regimes when   glycaemic control is no longer maintained with a single agent. The addition of a second and sometimes, a third drug is usually more effective than switching to another single agent (Gerich, 2001).

Postprandial hyperglycaemia plays an important role in the development of type 2 diabetes and has been proposed as an independent risk factor for cardiovascular disease (Li *et al*., 2005). Control of postprandial hyperglycaemia is imperative in the treatment of diabetes in an attempt to prevent cardiovascular complications. Inhibiting glucose uptake in the intestines may help diabetic patients to control the blood glucose levels in the postprandial state. Substances that inhibit amylase and glycosidase have been studied, and some of them have been developed as drugs to treat diabetes mellitus (Hara and Honda, 1990; Kobayashi *et al*., 2000).

Many diverse therapeutic strategies for the treatment of Type 2 diabetes are in use. The available therapies for diabetes include stimulation of endogenous insulin secretion, enhancement of the action of insulin at the target tissues, oral hypoglycemic agents, such as biguanides and sulfonylureas and the inhibition of degradation of dietary starch by glycosidases such as α-amylase and α-glucosidase (Rang *et al*., 2003).

In order to manage carbohydrate related metabolic disturbances at various levels, several synthetic medicines have been developed which are listed below:

1.5.2.1 Alpha-glycosidase inhibitors

This group of drugs which includes acarbose, miglitol and voglibose manage postprandial hyperglycaemia at digestive level. They block the actions of alpha-glucosidase enzymes in the small intestine, which is rate limiting in the conversion of oligosaccharides and disaccharides to monosaccharides, necessary for gastrointestinal absorption. Postprandial glucose peaks may be attenuated by delayed glucose absorption. The main benefits attributable to alpha-glucosidase inhibitors are

reductions in both postprandial glycaemic levels and in the total range of postprandial glucose levels (Lebovitz, 1997).

However, it is well documented that alpha-glucosidase inhibitors have undesirable side effects such as flatulence, diarrhoea and abdominal cramping. In addition, some of them may increase the incidence of renal tumours, serious hepatic injury and acute hepatitis (Carrascosa *et al*., 1997; Kihara *et al*., 1997; Diaz-Gutierrez *et al*., 1998; Charpentier and Riveline, 2000).

1.5.2.2 Sulphonylureas

Glypizide, glibenclamide, and chlorpropamide are included in this group. These drugs are responsible for insulintrophic action at β-cells of pancreas resulting in enhanced endogenous insulin secretion. The glucose production in the liver is simultaneously suppressed, and may lead to hypoglycaemia in some individuals who are on reduced calorie intake or are prone to general debility, renal impairment or alcohol consumption (Muhammed and Lakshmi, 2005; Tiwari, 2005).

1.5.2.3 Biguanides

The only one in use is Metformin. It enhances glucose uptake through multiple pathways at tissue/cellular levels. They prevent the synthesis of glucose in the liver thereby reducing blood sugar levels. There may also be mild reduction in intestinal glucose absorption. However, there may be high levels of lactic acid produced, resulting in lactic acidosis and gastrointestinal disturbances (Laurence *et al*., 1997).

1.5.2.4 Insulin Sensitizers

Drugs in this group include Glitazones which deal with the problems of insulin resistance. The principal mechanism of action appears to be the activation of an enzyme which facilitates the penetration of glucose through cell membranes including membranes of sub cellular organelles (Clark *et al*., 1992). Common side effects associated with insulin therapy include hypoglycaemia, local or systematic allergic reactions, lipoathropy and visual disturbances (Clark *et al*., 1992). The chemical structures of thes drugs are shown in Table 1.2.
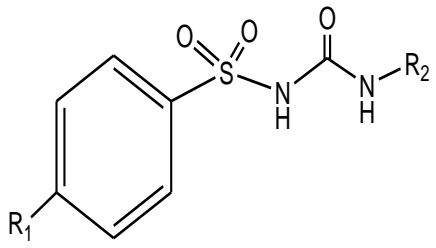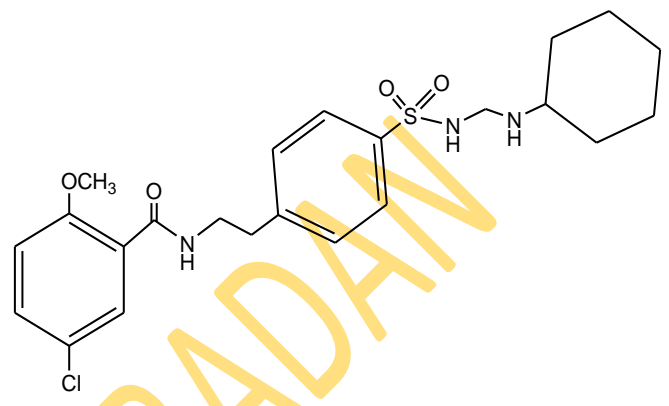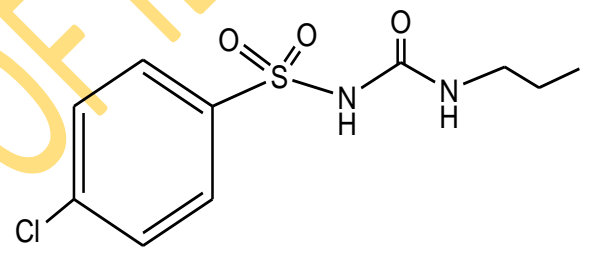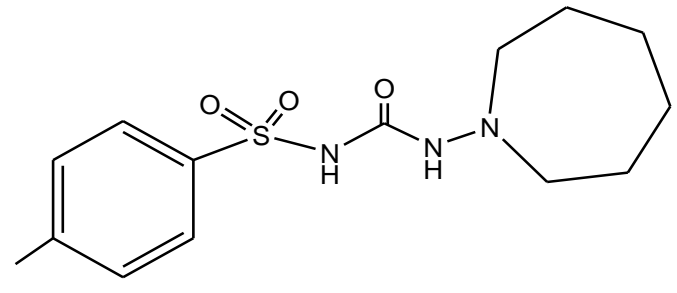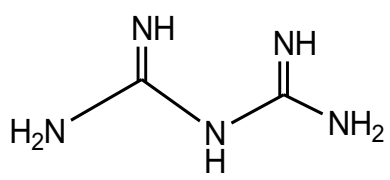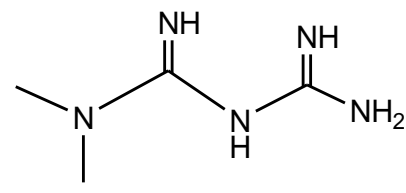
1.6   Need for alternative method of treating diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (ADA, 1997). It represents a growing health problem in Africa with rapidly increasing prevalence particularly in sub-Saharan regions (Gill *et al*., 2009).

In 1997, diabetes prevalence was introduced as a "basic health indicator" for member states by the WHO; which estimated in 1995, that the number of people with diabetes would escalate to 300 million by 2025. Currently 16 million people suffer from the disease globally. A publication by the WHO links 3.2 million deaths worldwide to diabetes each year (Unwin and Marlin, 2004). The global prevalence was estimated at 2.8% in 2000 (171 million people) and it is projected that 4.8% (366 million people) will be affected by 2030 if no action is taken (Wild *et al*., 2004).

Table 1.2 Chemical Structures of Some Oral Hypoglycaemic Drugs

Class of drug	Basic chemical structure	Examples
Sulphonylurea		Glibenclamide
		Chlorpropamide
		Tolazamide
Biguanides		Metformin

Thiazolidined
-ione (Insulin
sensitizers)
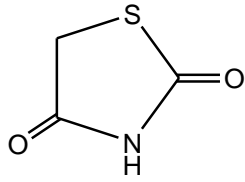
Pioglitazone
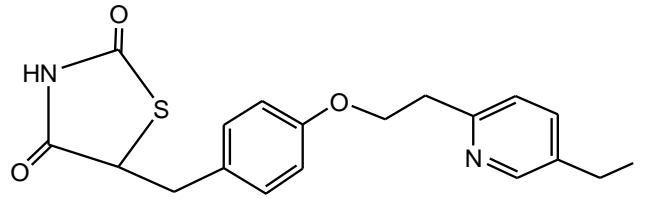
Rosiglitazone
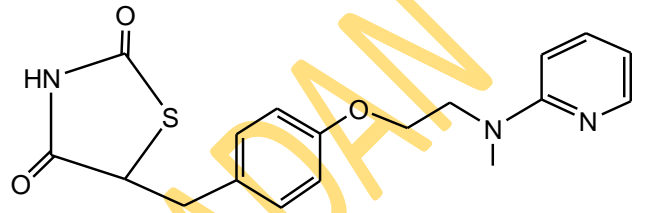
Alpha-
glucosidase
inhibitors

Miglitol
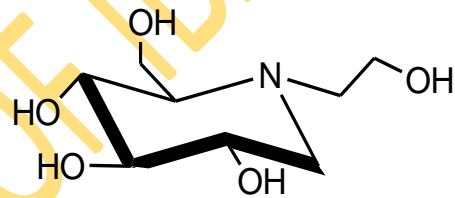
Acarbose
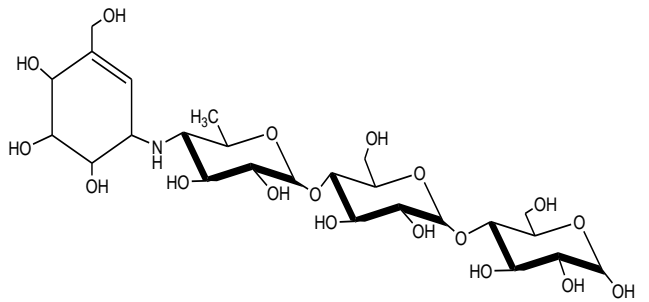
The burden of the disease is high, not only because lifelong treatment is necessary but also due to the prohibitive cost and unavailability of treatment especially in the rural areas. The rate of limb amputation varies from 1.4% to 6.7% of diabetic patients, while foot complications and the annual mortality linked to diabetes worldwide are estimated at more than one million (Carmona *et al.*, 2005).

The need for an alternative method of treating diabetes has led to the investigation of different plants for their anti-diabetic activity. Such plants include *Pterocarpus marsupium* (Ahmad *et al.,* 1991) *Gymnema sylvestre* (Yoshikawa *et al.,* 1997)*, Malpighia emarginata* DC (*Hanamura et al.,* 2005)*, Bauhinia monandra* (Aderogba *et al.,* 2006) *Lagaersroemia speciosa* (Klein *et al.,* 2007) and *Olea europea* (Khan *et al.,* 2007) to mention a few. Diabetes leads to high blood sugar level in the body which over protracted period of time causes "glycation" of key body proteins inducing secondary symptoms or complications. Many workers have outlined various complications that could result from diabetes or untreated diabetes.

i. Retinopathy – Diseased small blood vessels in the back of the eye causing the leakage of protein and blood in the retina. Spontaneous bleeding from new and brittle blood vessels can lead to retinal scorning and retinal detachment thus, impairing vision or leading to blindness.

ii. Ketoacidosis – this is due to insufficient insulin resulting from elevated blood sugar level.

iii. Neuropathy – nerve degeneration which is caused by disease of small blood vessels making blood flow to the nerves limited.

iv. Nephropathy – renal changes which may lead to kidney malfunctions.

v.   Atherosclerosis – hardening of the arteries of the larger blood vessels.

vi.   Angina – coronary heart disease leading to heart attack and stroke.

(Kissebah and Hennes, 1995; American Diabetes Association, 1997; Sreenan, 2001).

The treatment goal for patients with type 2 diabetes mellitus is generally agreed to be to maintain near-normal levels of glycaemic control, both in the fasting and postprandial states (Ratner, 2001). Although different types of oral hypoglycemic agents are available along with insulin for the treatment of diabetes mellitus, none offers complete glycemic control permanently (Jiang, 2003).

Utilizing modern tools and techniques, medicinal plants described in Ayurvedic texts for diabetes and related disorders have been observed to possess disease modifying therapeutic potentials (Tiwari, 2005). Therefore, they may play an important role in alleviating postprandial hyperglycaemia. The alpha-glucosidase inhibitors are observed to not only mitigate postprandial hyperglycaemic excursions but they also reduce triglyceride levels, postprandial insulin levels and reduce cardiovascular mortality due to diabetes (Johnston *et al*., 1994; Lebovitz, 1998; Mooradian and Thurman, 1999). In diabetic complications, these medicinal plants have been observed to attenuate renal hypertrophy, urine volume, albuminuria and ameliorate diabetic neuropathy and gastropathy (Grover *et al*., 2001, 2002).

The role of oxidative stress in complicating the disorders of diabetes mellitus which culminates in different disorders has been studied and diabetic complications can be prevented or retarded by administration of appropriate antioxidants, in addition to traditional therapeutic principles (Miyake *et al*., 1998; Parker *et al*., 2000). Therefore,

it has become important to have therapeutics addressing oxidative stress in concurrence with modifying or eliminating the cause of hyperglycaemia in order to check the outbreak of various diseases of diabetic complications (Tiwari, 2005). An imbalance between oxidative stress and anti-oxidative defense mechanisms in diabetics can result in cell and tissue damage and accelerate diabetic complications while the administration of appropriate antioxidants could prevent or retard diabetic complications to some extent (Parker *et al*., 2000).

1.7 Aims and Objectives

The World Health Organisation (WHO) Expert Committee on Diabetes has listed as one of its recommendations that traditional methods of treatment of diabetes be further investigated (WHO Expert Committee, 1980). Plants with known history of anti-diabetic potential could provide a useful source of new oral hypoglycaemic adjuncts to existing therapies (Bailey and Nattrass, 1988). The incidences of diabetes is on the increase and in 1997, diabetes prevalence was introduced as a "basic health indicator" for member states by the WHO.

Thus, the specific objectives of the study are to

(i)   Investigate *Anthocleista djalonensis* and *Anthocleista vogelii* for their alpha-amylase inhibitory activities

(ii)   Evaluate their anti-oxidant properties

(iii)   Investigate their anti-diabetic activities using *in vivo* rat models

(iv)   Isolate and characterise the compound(s) responsible for the activities

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CHAPTER 2

LITERATURE REVIEW

2.1   Ethnomedical and folklore uses of *Anthocleista* species

In Nigeria, particularly among the Ibo tribe, the seed, stem bark and root of *A. djalonensis* are widely used as antipyretic, laxative and remedy for various stomach disorders (Okoli and Iroegbu, 2004). Also, the Ibibios of Southern Nigeria use the leaves and stem bark as malaria remedy (Iwu, 2000). An ethnobotanical field research conducted in Imo and Anambra states of Nigeria revealed that Igbo healers mix the roots of *A. djalonensis* with potash, boil in water, and administer it orally for the treatment of fungal skin infections, filarial worm infections, Loa Loa infections and to enhance fertility in women (Bierer *et al*., 1995). Another preparation used by these Igbo healers is to chop up the soft outer portion of the roots, soak them in water, and then take the tea orally to treat Candida oral thrush (Bierer *et al*., 1995).

Aqueous extracts of the leaves mixed with lemon juice is used by the Abros of Ghana to cure epilepsy (Irvine 1961; Watt and Breyer-Brandwijk, 1962), while in Casamane, Senegal, the leaves of *A. djalonensis* are used as a diuretic (Keharo and Adam,1974). The root decoction of *A. djalonensis* and related species , *A. vogelii and A. kerstingii* have been used in the treatment of diabetes mellitus with a school teacher herbalist claiming a high percentage of cure (75%) in his patients treated particularly with *A. djalonensis* and *A. vogelii* (Ampofo,1977). *Anthocleista djalonensis*, is used in traditional medicine for the treatment of various diseases, such as haemorrhoids, syphilis, female infertility, diabetes, malaria, hernia, hypertension, and is known for its antipyretic, stomachic, analgesic and purgative actions (Chah *et al*., 2006; Gbolade, 2009). Traditional healers in Sibi, Mali report that *A. djalonensis* is used frequently in the treatment of malaria and abdominal pain (Togola, *et al*., 2005). The

decoction of the leaves is drunk in Sierra Leone as a treatment against jaundice. In Ivory Coast, the root is used as a diuretic and a vigorous purgative and also as a poison- antidote against leprosy, as an emmenagogue and in the treatment of Oedemas and elephantiasis in the scrotum (Burkill, 1995) reported that the plant is used as febrifuge, abortifacient and pain killer while the root decoction is used to treat dogs and such treated animals are claimed to pass out worms thereafter (Akubue *et al*., 1983). It is used for treatment against gonococci infection, intestinal pains, chest pains, and constipations and externally for furuncles and carbuncles (Le Grand, 1989) and for rheumatism (Akah and Nwambie, 1994). In Southern Africa, bark decoctions of *A. grandiflora* are used traditionally to treat malaria (Palmer and Pitman, 1972). Regionally, preparations of the bark have also found use as an anthelmintic (specifically for roundworm), anti-diarrhoeal and to treat diabetes, high blood pressure and veneral diseases (Watt and Breyer-Brandwijk, 1962; Mabogo, 1990). Further North in the continent epilepsy is remedied with the aid of the bark decoctions (Neuwinger, 2000).

In Cameroon, the stem bark of *A.vogelii* is reported in treating abdominal pains (Adjanohoun *et al*., 1986). In Ghana the wood-ash is used as a mordant to fix colours, the wood is used to make crates, while the potash of the wood is used in making soap, while a root decoction of *A. vogelii* and *Combretum mucronatum* Schumach & Thonn with pepper and ashes is taken to treat chest pain (Abbiw, 1990).

In Nigeria stems are hollowed out to make quivers. In Zambia trunks are cut for dugout canoes. In Congo the leaves are placed between tobacco leaves during drying to make the tobacco stronger, and a decoction of the leaves is known to prevent malaria and alleviate symptoms of malaria such as fever. It is also used to treat

jaundice and as haemostatic. The bark is noted for its anti-pyretic, tonic and purgative properties, fresh twig bark with manioc is eaten raw to treat aspermia. A stem bark decoction is taken to treat hernia and a root decoction is taken to treat stomach-ache in women, ovarian problems, venereal diseases, hernia, bronchitis and fever, and also as purgative and to induce labour. Sap of young leaves, root powder or bark pulp is used to treat sores, abscesses, as a haemostatic and for cicatrization. The sap is applied topically to treat otitis or ophthalmia. A plaster of pulp of terminal buds is used to draw out thorns or splinters and is applied to snakebites, (Adjanohoun *et al*., 1988; Burkill, 1995). In Libreville, Gabon, a decoction of the stem bark is used in the management of cardiovascular diseases (Madingou *et al*., 2012).

2.2 Previous phytochemical studies of *Anthocleista* species

The epicuticular waxes of the leaves of three *Anthocleista* species: *A. djalonensis, A vogelii* and *A. nobilis* were found to contain n-alkanes ranging from 24-37 carbon atoms. A total of fourteen alkanes were identified namely: tetracosane, pentacosane, hexacosane, heptacosane, octacosane, nonacosane, friacontane, heutriacontane, pentatriacontane, hexatriacontane and heptatriacontane in varying degrees amongst the species (Sonibare, *et al*., 2007). In a related study of the foliar trichome morphology of four *Anthocleista* species, the result of the microscopic observations showed differences in the type, morphology and frequency and abundance of these trichomes on foliar surfaces of the species studied. Though there could be similarity in terms of types of trichomes and morphology in both adaxial and abaxial surfaces of the species, *A. liebrechtsiana* showed a lesser abundance of trichomes on both surfaces while *A. nobilis* has a lesser abundance of trichomes on the abaxial surface.

Anthocleista djalonensis has in addition to the other types, conical, falcate and stellate trichomes (Edwin-Wosu *et al.*, 2012).

The inhibitive action of leaf extracts of *Anthocleista vogelii* on aluminum corrosion in water-ethanol mixture was studied using gravimetric techniques. The results showed that the leaf extract of *A. vogelii* functioned as a good inhibitor in a concentration dependent manner. The study revealed that the inhibition efficiency of the extract was found to vary with percentage of water present and the time of immersion at room temperature, increasing as the time of immersion increases and decreasing as the percentage water present increases. Weight loss was found to also decrease with an increase in time spent in the water-ethanol extract of *Anthocleista vogelii*. Physical adsorption mechanism was proposed for the adsorption of the inhibitor, (Adeyemi and Olubomehin, 2010a). In a related study, the inhibitive effect of water extract of the bark of *Anthocleista djalonensis* (WEAD) on acid corrosion of aluminum was investigated using the gravimetric method. Inhibition efficiency ranging from 39.76-96.50% was found to depend on the concentration of *Anthocleista djalonensis* extract and immersion time. It increased with concentration and decreased with immersion time. Corrosion penetration rate reached 0.0022 mm/y and decreased to 0.0006 mm/y at 50/50 vv, (Adeyemi and Olubomehin, 2010b).

A new steroid, schweinfurthiin and two known compounds, bauerenone and bauerenol which were found to be highly promising α-glucosidase inhibitors have been isolated from the dichloromethane/methanol extract of the roots of *Anthocleista schweinfurthii* (Mbouangouere, *et al.*, 2007). A new monoterpene diol (djalonenol), iridoid glucoside, dibenzo-α-pyrone- djalonensone and some steroids have been isolated from *A. djalonensis* (Onocha *et al.*, 1995). A phthalide djalonensin has been reported from the stem bark of *A. djalonensis* (which was the first report of its

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isolation from natural source). Llichexanthone and an uncharacterized triterpene have also been isolated from the hexane extract of the stem bark (Okorie, 1976).

Previous investigation of *A. grandiflora* yielded two iridoid glucosides: grandifloroside and methyl grandifloroside together with coumarin, scopoletin (Chapelle, 1976). Four novel triterpenoids: bauerenol, bauerenone, 6- ketobauerenone and grandiflorol have been isolated from the stem bark of *A. grandiflora* in addition to scopoletin and (+)-de-O-methyl asiodiplodin. The root bark yielded in addition to the above compounds lupeone and the iridoid – sweroside (Mulholland *et al*., 2005). In a study carried out to investigate the secoiridoid pattern of *A. nobilis,* a new secoiridoid anthocleistenol and a sweroside were isolated from the methanol extract of the root bark (Madubunyi *et al*., 1994).

The isolation and structural elucidation of a novel plant metabolite, 1,22-bis[[[2-(trimethylammonium)ethoxy]phosphinyl]oxy]docosane named Irlbacholine from *A. djalonensis* has been reported with its antifungal activity against pathogenic fungi *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus niger* (Bierer *et al*., 1995).

A new rearranged nor-secoiridoid, anthocleistenolide, along with the known 1-hydroxy – 3, 7-dimethoxyxanthone, 1-hydroxy- 3,7,8 – trimethoxyxanthone, 7-α-hydroxy sitosterol and sitosterol 3-0-β-D-glucopyranoside have been isolated from the stem bark of *Anthocleista vogelii* (Tene *et al.*, 2008).

Some of the compounds isolated from some *Anthocleista* species are shown in Table 2.1

Table 2.1.   Some Isolated Compounds From *Anthocleista* species

Plant name	Compound	References
Anthocleista procera	Heteroside swertiamarine	Koch, 1964.
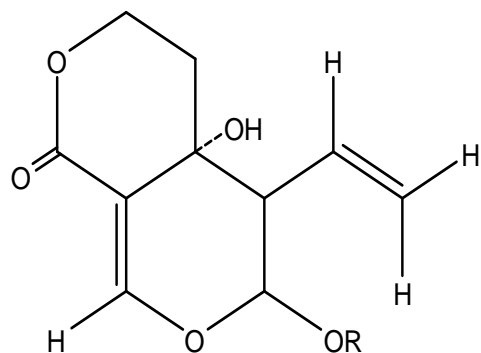
R= β-glucosyl.

Anthocleista zambesica	Sweroside	Chapelle, 1976.
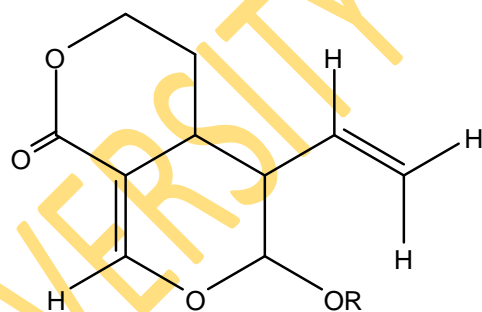
R= β-glucosyl.

Anthocleista djalonensis	Djalonensic acid	Okorie, 1976. .

COOH

H₃CO

H₃C

OCH₃

O

O
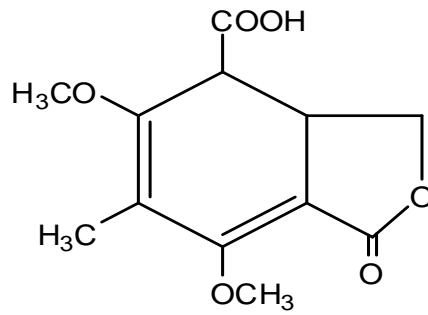
Anthocleista djalonensis	Djalonensin	Okorie, 1976.

COOCH₃

H₃CO

H₃C

OCH₃

O

O
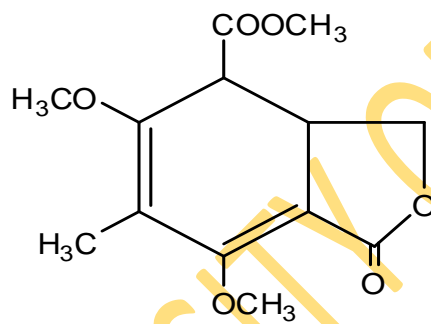
Anthocleista vogelii	1-hydroxy-2, 6, 8-trimethoxy-9*H*-xanthen-9-one	Okorie, 1976.

OH   O   OCH₃

H₃CO

OCH₃

O
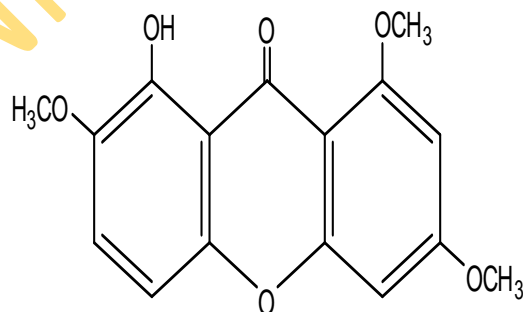
Anthocleista vogelii	Fagaramide	Okorie, 1976.
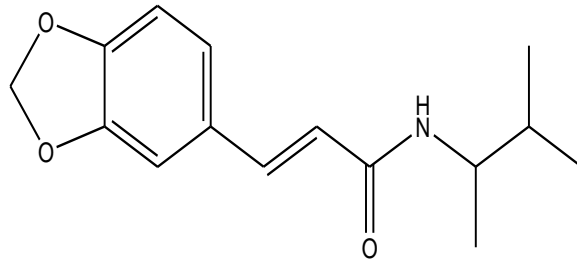
Anthocleista djalonensis	Iridoid glucoside- sweroside	Onocha *et al*., 1995.

OH
OH
OH
OH
HO
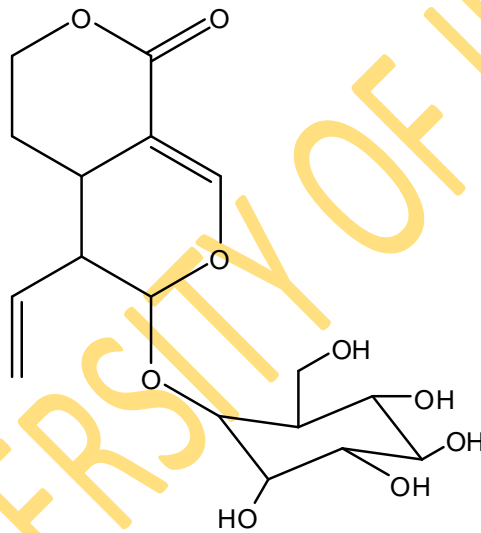
Anthocleista djalonensis	Ursolic Acid	Onocha *et al*., 1995.

COOH

RO
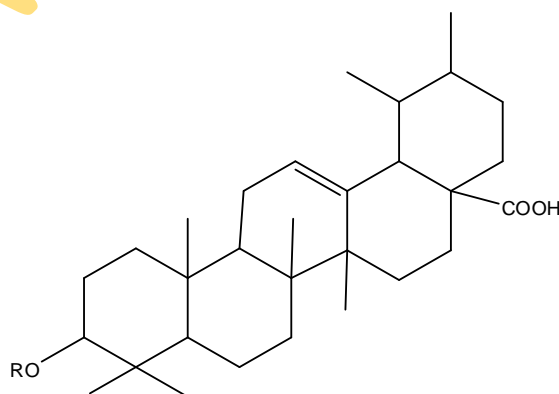
Anthocleista djalonensis	Djalonenol	Onocha *et al*., 1995.
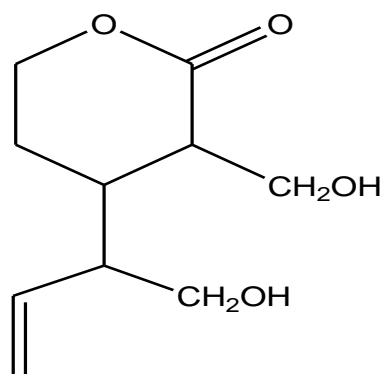
Anthocleista djalonensis	Djalonenol diacetate	Onocha *et al*., 1995.
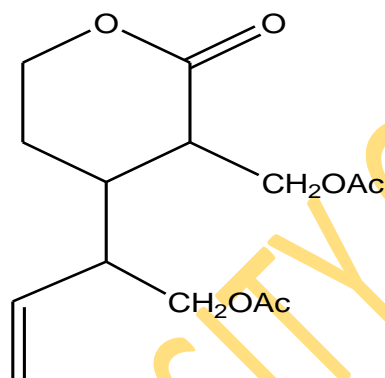
Anthocleista djalonensis	Sweroside tetraacetate	Onocha *et al*., 1995.

32
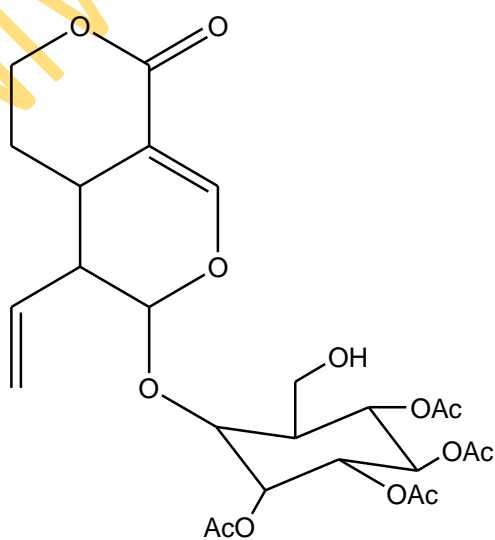
Anthocleista djalonensis	Dihydrosweroside	Onocha *et al.,* 1995.
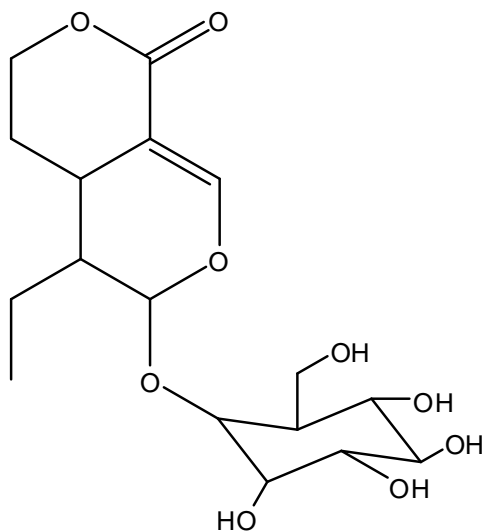
Anthocleista djalonensis	Djalonensone	Mulholland *et al.*, 2005.
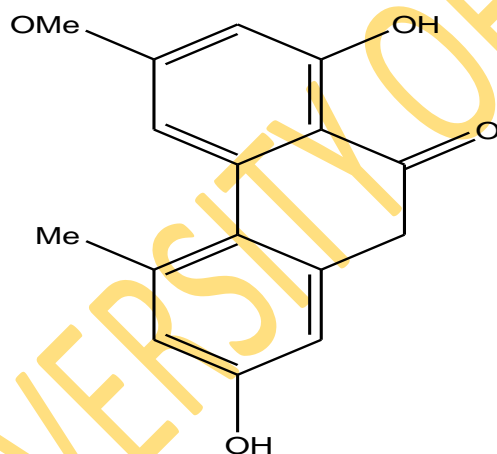
Anthocleista grandiflora	Grandiflorol	Mulholland *et al.*, 2005.
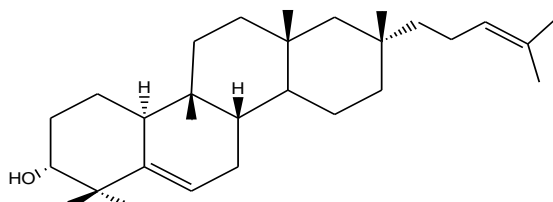
| *Anthocleista grandiflora* | Scopoletin | Mulholland *et al.*, 2005. |

| *Anthocleista grandiflora* | (+)-de-O-methyl-asiodiplodin | Mulholland *et al.*, 2005. |

| *Anthocleista grandiflora* | Iridoid sweroside | Mulholland *et al.*, 2005. |

| *Anthocleista grandiflora* | 1= baurenol | Mulholland *et al*., 2005. |
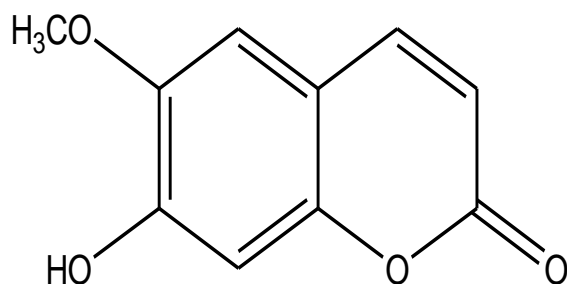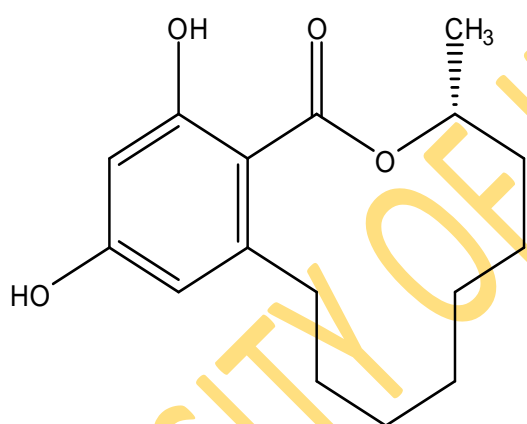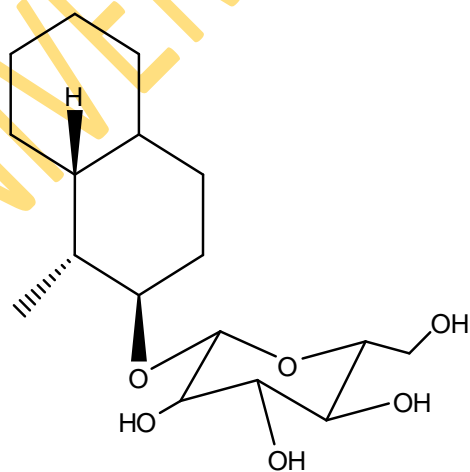
2= baurenone

1. R=B-OH
2. R=O
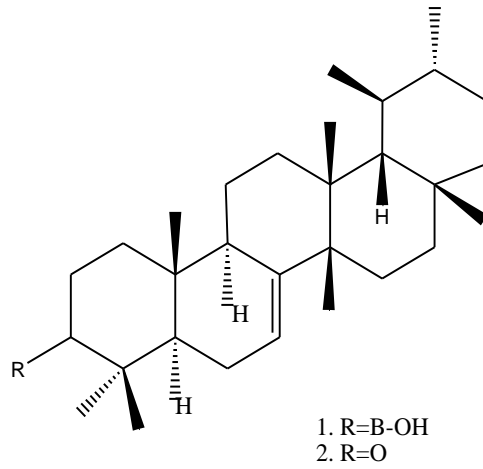
| *Anthocleista grandiflora* | Ketobauerenone | Mulholland *et al*., 2005. |

| *Anthocleista grandiflora* | Lupeone | Mulholland *et al*., 2005. |

| *Anthocleista vogelii* | Anthoclestenolide | Tene *et al*., 2008. |

| *Anthocleista vogelii* | (*S*)-3-(2-oxo-2,3-dihydro furan-3-yl) pentanedial | Tene *et al*., 2008. |

| *Anthocleista vogelii* | 1-hydroxy-3,7-dimethoxyxanthone | Tene *et al*., 2008. |

| *Anthocleista vogelii* | Sitosterol 3-O-β-D-glucopyranoside | Tene *et al*., 2008. |
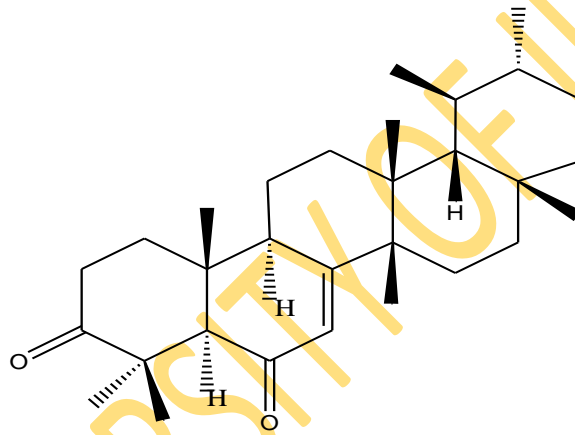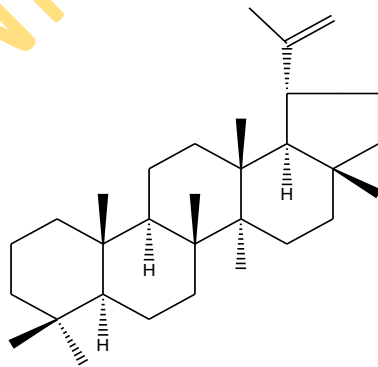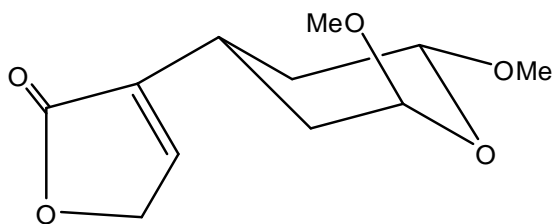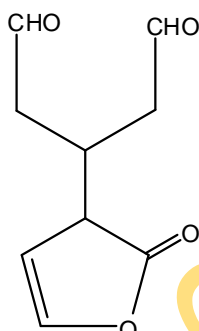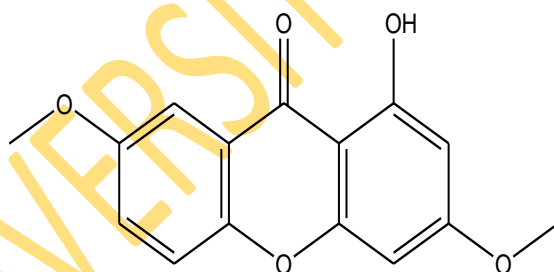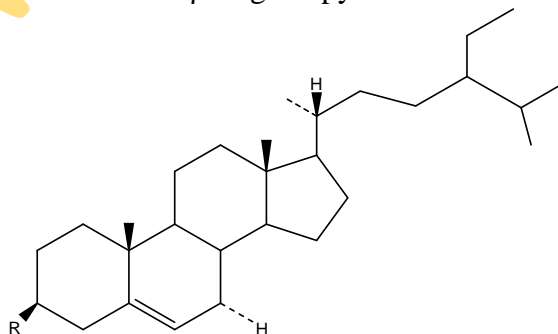
Anthocleista vogelii   7α-hydroxysitosterol   Tene *et al*., 2008.
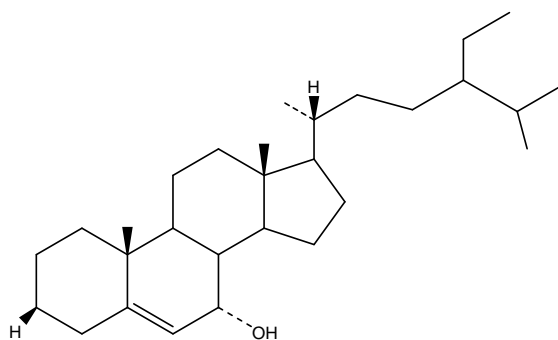
Anthocleista vogelii   1-hydroxy-3,7,8-trimethoxyxanthone   Tene *et al*., 2008; Alaribe *et al*., 2012.
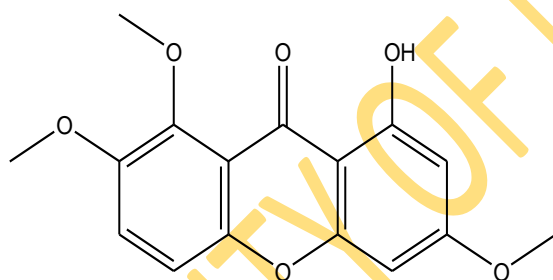
1,8-dihydroxy-3,7-dimethoxyxanthone   Mbouangouere *et al*., 2007.
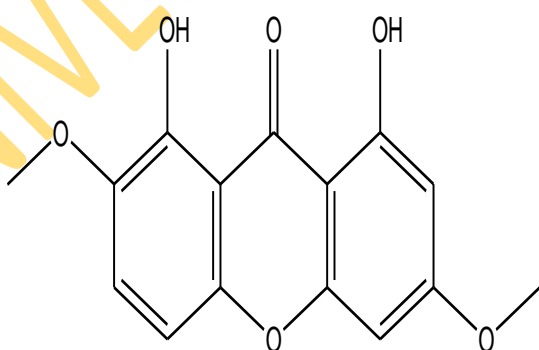
2.3   Previous biological work on *Anthocleista* species

Aqueous extracts of *A. djalonensis* was found to produce a rise in blood pressure of cats and an increase in tone and amplitude of movement of rabbit duodenum preparations (Ampofo, 1977). The cold water and ethanolic extracts of *A. djalonensis* showed a remarkable broad spectrum activity against both Gram positive and Gram negative bacteria (Okoli and Iroegbu, 2004). The bioactivity of isosaline extracts of the leaves of *A. djalonensis* caused a 77.7% reduction in fasting blood glucose of extracts treated diabetic rats when compared with untreated ones and resulted in a significant reduction ($p<0.05$) in blood cholesterol level (Olagunju *et al*., 1998). The anti-diabetic activities of ethanol root extract/fractions of *A. djalonensis* at 37-111 mg/kg were evaluated in alloxan-induced diabetic rats for 14 days. A significant reduction in fasting blood glucose level ($p<0.001$) of the diabetic rats was observed both in acute study and prolonged treatment-2 weeks (Okokon *et al*., 2012). The methanol extracts of *A. djalonensis* and *A. vogelii* leaves were investigated for their anti-diabetic activities in acute and chronic studies. A 13.0% and 15.7% significant reduction ($p<0.05$) in blood glucose levels were observed respectively for both plants at 180 min in the acute study, while in the chronic study, *A. djalonensis* exerted a 72.3% maximal reduction on day 3 and *A. vogelii* had a 60.7% maximal reduction on day 7 (Osiyemi *et al*., 2012).

The cytotoxicity activity of the constituents of *A. djalonensis* and their derivatives was carried out and the results of the test showed that cytotoxicity appreciated by evaluation of ED_{50} (effective dose median) up to the third day of treatment (Onocha *et al*., 2003).

According to Bassey *et al*., (2009) *A. djalonensis* leaf extract (1000 – 3000 mg/kg/day) exhibited a significant anti-plasmodia activity both in the 4-day early infection test and in the established infection with a considerable mean survival time, which was incomparable to that of the standard drug, chloroquine (5 mg/kg/day). The stem bark extract (220 – 660 mg/kg/day) also demonstrated a promising blood schizontocidal activity in early and established infections. Also, *A. djalonensis* leaf and stem bark extracts have moderate to negligible toxicity, as shown in their LD_{50} values of 5.0 g/kg and 2.23 g/kg for the leaf and stem bark extract, respectively, justifying the use of the plant in ethno-medicine to treat malaria. In a related study to evaluate the antimalarial activities of ethanolic root extract/fractions of *Anthocleista djalonensis* in *Plasmodium berghei* infected mice, Akpan *et al*., (2012) found the extracts and fractions to dose-dependently reduce parasitemia induced by chloroquine sensitive *Plasmodium berghei* infection in prophylactic, suppressive and curative models in mice. These results apart from being statistically significant ($p<0.001$), also improved the mean survival time from 13 to 28 days relative to control ($p<0.001$). The activities of the extracts/fractions were comparable to that of the standard drug used (chloroquine and pyrimethanmine). On pyrexia induced by dinitrophenol, amphetamine and yeast, the extract inhibited significantly ($p<0.05$-0.001) and in a dose-dependent fashion, temperature rise caused by these pathogens proving that *A.djalonensis* root extract has antiplasmodial and antipyretic activities.

In another study, Esimone *et al*. (2009) screened the aqueous and methanol leaf and root extracts of *Anthocleista djalonensis*, *Diospyros mespiliformis*, and their combinations for possible anti-mycobacterial activities using *Mycobacterium smegmatis* as a surrogate screen since these plants are reputed among folk practices as potent remedy in the management of tuberculosis and leprosy cases. In their results

only the methanol extracts of *A. djalonensis* and *D. mespiliformis* showed anti-mycobacterial activity, while the aqueous extracts exhibited no inhibitory activity on *M. smegmatis*, with the 8:2 ratio of *D. mespiliformis* and *A. djalonensis* exhibiting the greatest degree of anti-mycobacterial synergy against *M. smegmatis*. Thus, supporting the claims of efficacy reported in the folk use of these plants in mycobacterial infection.

The *in vitro* anthelmintic activity of *A. djalonensis* was studied using the L_3, larvae of *Heligmosomoides polygyrus*. The ethanolic extract (25, 50, 100, 200 mg/mL) exhibited a concentration-dependent lethal action on *H. polygyrus* larvae. After 24 h incubation, the percentage mortality of *A. djalonensis* extract was 41% at the highest concentration of 200 mg/mL while levamisole the positive control at 10 mg/mL had 91% (Nweze and Ngongeh, 2007). Also, the *in vitro* myometrial inhibition of the partitioned aqueous fraction of *Anthocleista djalonensis* leaves, by Enitome and Okunrobo (2010) investigated the effect on the uterus of the aqueous fraction of the partitioned methanol crude extract of the leaves of *A. djalonensis* and the possible mechanism of its activity. It was observed that the extract inhibited the concentration-response curves induced by oxytocin and $CaCl_2$ on rat uterus *in vitro* and significantly reduced the EC_{50} in a concentration-dependent manner (p <0.05). An evaluation of the extracts of *A. djalonensis* for activity against bacterial isolates from cases of non-gonococcal and urethritis revealed that the cold water and ethanol extract of the roots showed a remarkable broad spectrum activity against *Staphylococcus aureus* and *Escherichia coli* (Okoli and Iroegbu, 2004). Thus, the antibacterial activity exhibited by the extracts against these organisms justifies the use of the plant in the treatment of sexually transmitted diseases and the folklore use of the aqueous decoctions of the

plant in the treatment of dysentery and other gastrointestinal disease (Okoli and Iroegbu, 2004).

Aqueous, ethanol and chloroform extracts of some plants including *A. vogelii* used in Igala folkloric medicine of North-Central Nigeria for the treatment of typhoid fever were investigated for their antibacterial properties against *Salmonella typhi*. The polar extracts of *A. vogelii* were very effective against the test organism starting from 25 mg/mL concentration with a value similar to that of a standard antibiotic - amoxicillin. The polar extracts also had MIC of 25 mg/mL. These results suggest that there is a pharmacological rationale for the use of *A. vogelii* in Igala folkloric medicine for the treatment of typhoid fever (Musa *et al*., 2010).

The hypoglycaemic effect of roots of *Anthocleista vogelii* was studied in mice, rats and rabbits. The extract (100, 400 and 800 mg/kg) induced significant hypoglycaemic activity in a dose-related fashion at 2 h after oral administration in mice and rats with ED_{50} of 250 mg/kg and 350 mg/kg respectively. The extract (800 mg/kg) similarly induced statistically significant lowering of blood glucose levels at 8h in normoglycemic rabbits (Abuh *et al*., 1990).

A novel xanthone, 1-hydroxy-3,7,8-trimethoxyxanthone (decussatin) isolated from the methanol extract of the stem bark of *Anthocleista vogelii* produced a dose dependent effect on the tone and force of the spontaneous contraction of the rat ilea and stomach smooth muscle fragments at concentrations ranging from 2.50×10^{-2} - $1.60 \ \mu g/mL$. A concentration of $8.00 \times 10^{-1} \ \mu g/mL$ of *A. vogelii* produced maximal contractile effect in a cumulative as well as in a single concentration (Ateufak *et al*., 2007).

Anthocleista vogelii leaf extract was investigated for its anti-plasmodial activities against residual infection in chloroquine sensitive *Plasmodium berghei* infected mice. Phytochemical analysis and oral acute toxicity in mice were evaluated. Iron chelating ability of the extract and isolated compound were also determined. The extract was found to be safe at up to 2000 mg/kg dose producing a dose dependent reduction in parasite density compared to the control group when given intraperitoneally. Decussatin, stigmasterol (stigmasta-5,22-dien-3-beta-ol), swertiaperennin (1,8-dihydroxy-3,7-dimethoxy-xanthone), and hexadecanoic acid were isolated with decussatin demonstrating very weak reduction in parasite density at 10 mg/kg. The extract and decussatin demonstrated good iron chelating ability at the tested concentration (1 mg/mL), which may be involved in its antiplasmodial activities (Alaribe *et al*., 2012).

Also, anthocleistenolide, 1-hydroxy-3,7-dimethoxyxanthone, 1-hydroxy-3,7,8-trimethoxyxanthone, 7α-hydroxysitosterol, and sitosterol 3-*O*-β-*D*-glucopyranoside isolated from the stem bark of *A. vogelii* have been tested for activities against *Staphylococcus aureus* and *Enterococcus faecalis* with low activities recorded. Some of the compounds were also active against *Candida parapsilosis* (Tene *et al*., 2008).

The impact of the ethanolic root bark extract of *A. vogelii* on weight reduction in high carbohydrate diet (HCD) induced obesity in male Wistar rats was investigated. The extract was found to significantly decrease ($p<0.05$) food intake, body weight, total fat mass, adiposity index and low density lipoprotein cholesterol, but showed no significant difference ($p<0.05$) in body mass index, total cholesterol, triglycerides, high density lipoprotein cholesterol, and very low density lipoprotein cholesterol when compared with the HCD obese control. The results indicated that the ethanolic

root bark extract of *Anthocleista vogelii* has potential to reduce weight in animals, (Anyanwu *et al*., 2013).

2.4 Plants and compounds with anti-diabetic activity

Many plants from various families have been studied for their anti-diabetic activity with many different compounds isolated. These are shown in Table 2.2.

2.5 Methods of studying anti-diabetic activity and detecting blood sugar level.
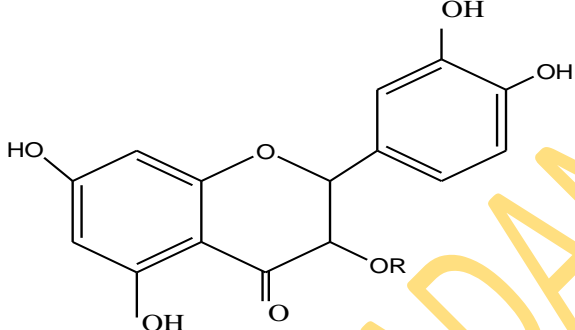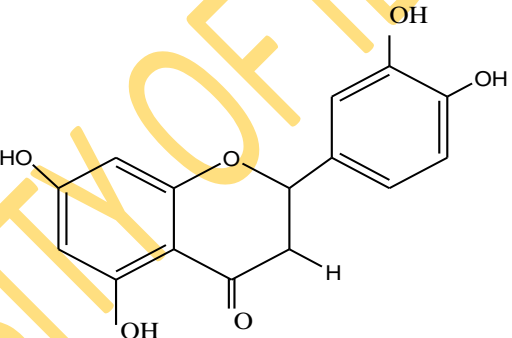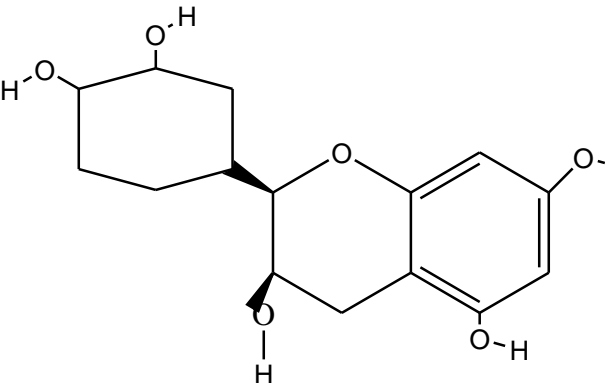
Two major types of diabetes are common, one associated with insulin deficiency called Type 1 or insulin dependent diabetes mellitus (IDDM) and the other associated with insulin resistance called Type 2 or non-insulin dependent diabetes mellitus (NIDDM). Type 1 is associated with a specific and complete loss of pancreatic β-cells while type 2 is associated with obesity, hyper-insulinemia and insulin resistance (Gupta, 2004).

2.5.1 Models for IDDM

(i)   Chemically induced Diabetes; chemically induced Type-1 diabetes is the most commonly used animal model of diabetes. Chemical agents which produce diabetes can be classified into three categories, and include agents that:

- Specifically damage β –cell,

- Cause temporary inhibition of insulin production and/or secretion, and

- Diminish the metabolic efficacy of insulin in target tissues.

In general, Chemicals in the first category are of interest as they produce lesions resembling IDDM. Alloxan and steptozotocin are the most usual and widely used chemicals to induce experimental diabetes in animals (Szkudelski, 2001).

Table 2.2. Some Plants and Isolated Compounds with Anti-diabetic Activity

lant name	Family	Compound	References
Bauhinia Monandra	Cesalpinaceae	Quercetin-3-O-rutinoside	Aderogba *et al.*, 2006.
		 R = rutinosyl	
Bauhinia Monandra	Cesalpinaceae	Quercetin	Aderogba *et al.*, 2006.
Pterocarpus marsupium	Leguminosae	(-) Epicatechin	Ahmad *et al.*, 1991.

| *Parkia biglobosa* | Leguminosae | Cis, cis-9,12-Octadecadienoic acid | Fred-Jaiyesimi, 2008. |

| *Spondias mombin* | Anacardiaceae | β-sitosterol | Fred-Jaiyesimi, 2008. |

| *Gymnema sylvestre* | Ascelpiadaceae | Gymnemic acid | Yoshikawa *et al*., 1997. |

| *Gymnema sylvestre* | Ascelpiadaceae | Gymnemoside (a) | Yoshikawa *et al*., 1997. |

| *Gymnema sylvestre* | Ascelpiadaceae | Gymnemoside (b) | Yoshikawa *et al*., 1997. |

| *Pterocarpus marsupium* | Leguminosae | Pterostilbene | Manickam *et al*., 1997. |
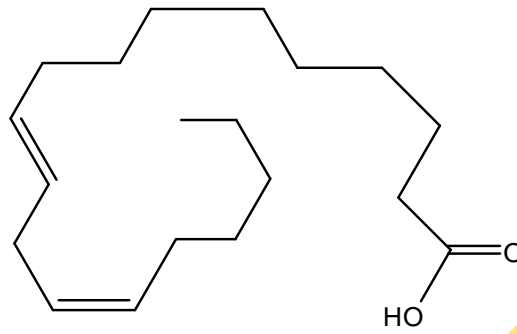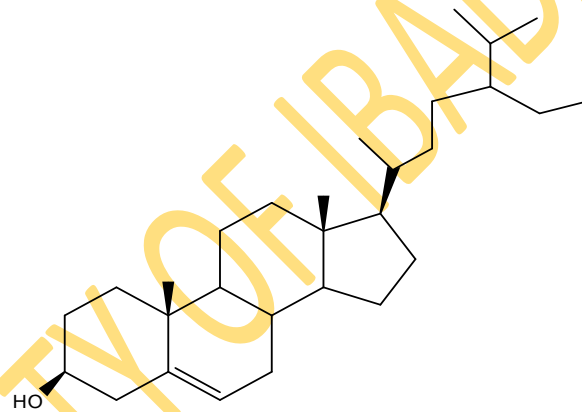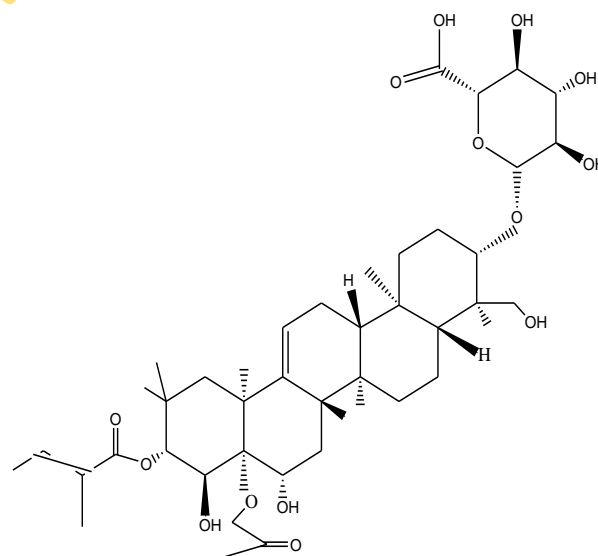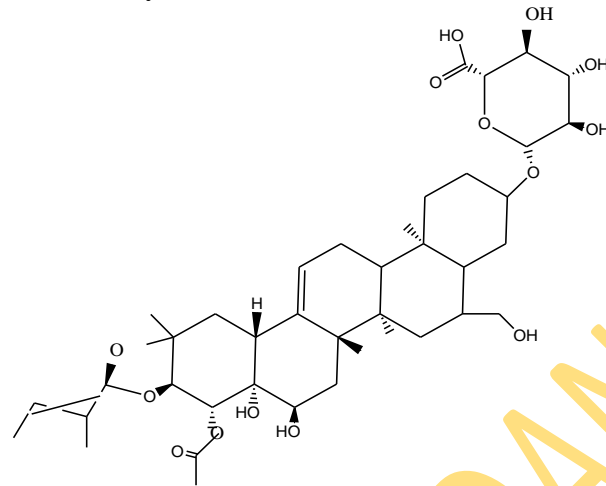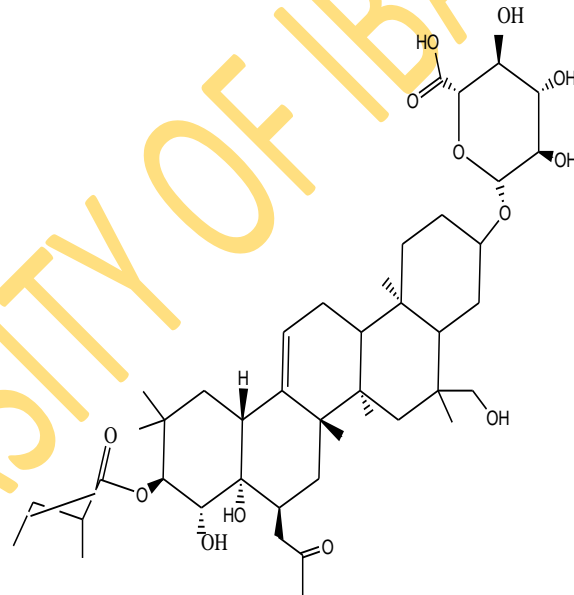
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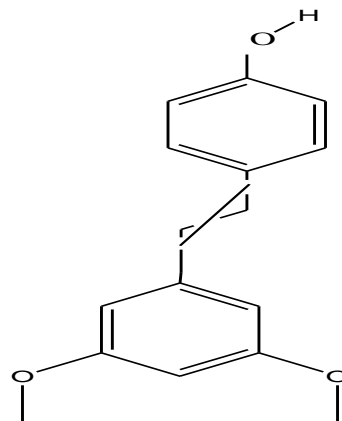
| *Lagersroemia speciosa* | Lythraceae | Corosolic acid | Klein *et al.,* 2007. |

| *Malpighia emarginata* DC. | Malpighiaceae | Cyanidin-3-α-O-rhamnoside. | Hanamura *et al*., 2005. |

| *Malpighia emarginata* DC | Malpighiaceae | Quercetin-3-O-rhamnoside | Hanamura *et al*., 2005. |
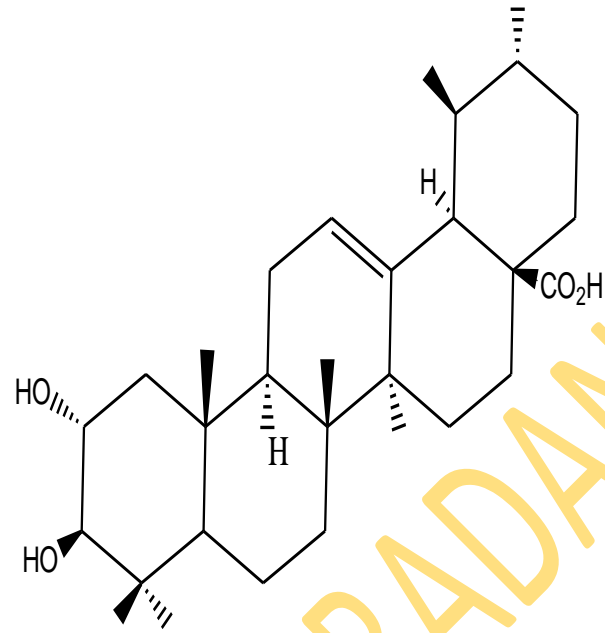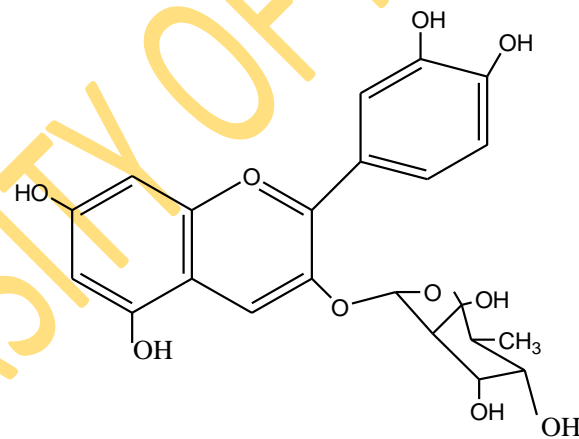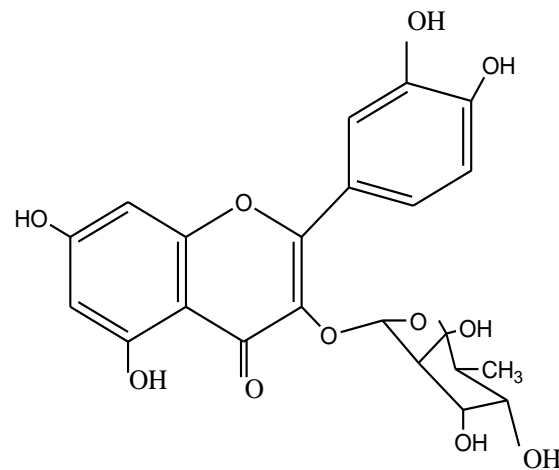
Malpighia emarginata DC.	Malpighiaceae	Pelargonidin-3-α-O-rhamnoside.	Hanamura *et al*., 2005.
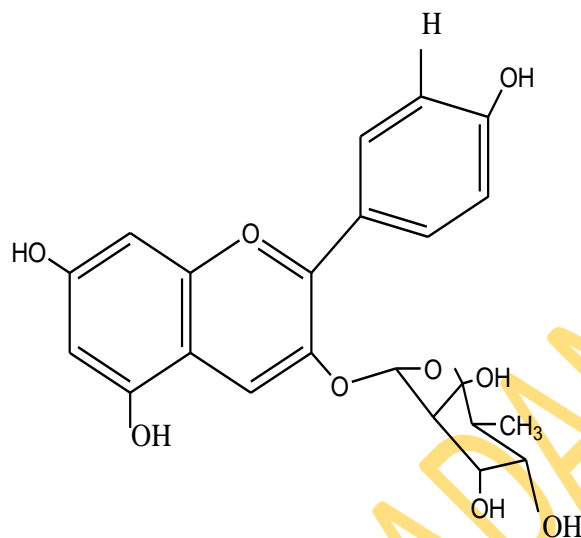
Olea europea	Oleaceae	Oleuropein	Khan *et al*., 2007.
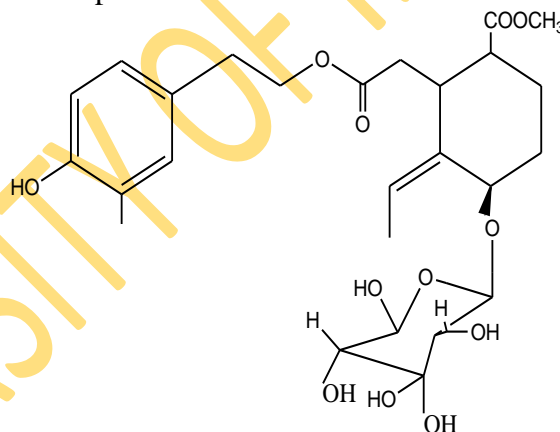
Olea europea	Oleaceae	Hydroxy tyrosol	Khan *et al*., 2007.
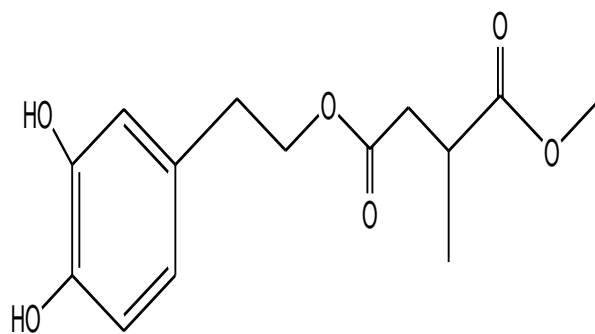
2.5.1.1 Alloxan- induced Diabetes

Alloxan (2,4,5,6-tetraoxypyrimidine-5,6-dioxy uracil) is a cyclic urea analog. It exerts its diabetogenic action when it is administered parenterally, intravenously, intraperitoneally or subcutaneously. The doses of alloxan required for inducing diabetes depends on the animal species, route of administration and nutritional status (Elzirik *et al.,* 1994). The most frequently used intravenous dose of this drug, to induce diabetes in rats is 65 mg/kg body weight (Gruppuso *et al.* 1990; Boylan *et al.,* 1992). Fasted animals are more susceptible to alloxan (Katsumata *et al.*, 1992; Szkudelski *et al.*, 1998).

2.5.1.2 Mechanism

The mechanism of alloxan action has been intensively studied *in vitro*. Using isolated islets and perfused rat pancreas (Kliber *et al.*, 1996), it was demonstrated that alloxan evokes a sudden rise in insulin secretion in the presence or absence of glucose. The sudden rise in blood insulin concentration was also observed *in vivo* just after alloxan injection to rats (Szkudelski *et al.* 1998). Alloxan-induced insulin release is, however, of short duration and is followed by complete suppression of the islet response to glucose even when high concentration (16.6 mM) of this sugar was used (Kliber *et al.*, 1996). The action of alloxan in the pancreas is preceded by its rapid uptake by the β-cells (Boquist *et al.*, 1983). Rapid uptake by insulin-secreting cells has been proposed to be one of the important features determining alloxan diabetogenicity (Szkudelski, 2001).

2.5.1.3 Draw backs:

- High mortality in rats

- Causes ketosis in animals due to free fatty acid generation.

- Diabetes-induced is reversible

- Some species like guinea pigs are resistant to diabetogenic action.

The range of the diabetogenic dose of alloxan is quite narrow and even light overdosing may be generally toxic causing the loss of many animals (Lenzen and Panten, 1988).

2.5.1.4 Streptozotocin-induced Diabetes

Streptozotocin STZ (2 – deoxy -2-(3-methy-1,3- nitrosourea)1-D-glucopyrase) is a broad spectrum antibiotic, which is produced from *Streptomyces achromogens*. Its diabetogenic property was first described by Rakieten *et al*., (1963).

2.5.1.5 Mechanism of β-cell damage:

- By process of methylation,

- Free radical generation and

- Nitric oxide production.

Streptozotocin induces diabetes in almost all species of animals. Diabetogenic dose varies with species and the optimal doses required in various species are: in rats 40-60 mg/kg, intraperitoneally (i. p.), or intravenously (Ganda *et al*., 1976), but higher doses are also used, mice (175- 200 mg/kg i. p. or i. v.) and dogs (15 mg/kg, for 3 days). The blood glucose level shows the same tri-phasic response as seen in the alloxan treated animals with hyperglycaemia at 2 h, with a concomitant drop in blood insulin. About six hours after, hypoglycaemia occurs with high levels of blood insulin.

Finally, hypoglycaemia develops and blood insulin levels decrease. These changes in blood glucose and insulin concentrations reflect abnormalities in β-cell function. Streptozotocin impairs glucose oxidation (Bedoya *et al*., 1996) and decrease insulin biosynthesis and secretion (Bolaffi *et al.*, 1987). It was observed that STZ at first abolished the β -cell response to glucose. Temporary return of responsiveness then appears which is followed by its permanent loss and the cells are damaged.

2.5.1.6 Advantages and Disadvantages

- Greater selectivity towards β –cells,

- Lower mortality rates and

- Longer or irreversible diabetes induction.

However, guinea pigs and rabbits are resistant to its diabetogenic action (Rakieten *et al*., 1963; Povoski *et al.,* 1993; Ar' Rajab and Ahren 1993; Chattopadhyay *et al*., 1997; Wright and Lacy 1998).

2.5.2 Hormone-induced Diabetes Mellitus

Dexamethasone, a long acting glucocorticoid is used to produce NIDDM. A NIDDM form of diabetes is produced when dexamethasone is administered at a dose of 2-5 mg/kg i. p. twice daily over a number of days in rats (Ogawa *et al*., 1992).

2.5.3 Insulin Antibodies- induced Diabetes

Giving bovine insulin along with CFA to guinea pigs produces anti-insulin antibodies. Intravenous injection of 0.25-1.0 mL guinea pig anti- insulin serum to rats induces a dose dependent increase in blood glucose levels up to 300 mg. This unique effect of guinea-pig anti-insulin serum is due to neutralization of endogenous insulin by the insulin anti-bodies. It persists, as long as the antibodies are capable of reacting with

insulin remaining in circulation. Slow intravenous infusion or intraperitoneal injection prolongs the effect for more than a few hours. However, large doses and prolonged administration are accompanied by ketonemia, ketonuria, glycosuria and acidosis and are total to the animal. At lower doses, the diabetic syndrome is reversible after a few hours (Moloney and Coval, 1955).

2.5.4 Diabetes induced by surgery

Induction of diabetes mellitus can be achieved through surgical removal of all or part of the pancreas. In partial pancreatectomy more than 90% of the organ must be removed to produce diabetes. Depending on the amount of intact pancreatic cells, diabetes may range in duration from a few days to several months. Total removal of pancreas results in an insulin-dependent form of diabetes, and insulin therapy is required to maintain experimental animals (Kaufmann and Rodriquez, 1984).

2.5.4.1 Disadvantages

- Surgical removal of pancreas results in loss of α- and δ-cells in addition to β-cells. This causes loss of counter-regulatory hormones, glucagon and somatostatin.

- There is a loss of the pancreatic enzymes necessary for proper digestion; therefore, the diet of pancreatomised animals must be supplemented with these pancreatic enzymes.

- The total resection of the pancreas in rat is very difficult to achieve and the development and severity of the diabetic state appear to be strain specific.

The use of pancreatectomy in combination with chemical agents, such as alloxan and STZ, produces a stable form of diabetes mellitus in animals, such as cats and dogs,

which does not occur when each procedure is used independently. Organ damage is also reduced (Kaufmann and Rodriguez, 1984).

2.6 Methods of Determining Blood Glucose

There are various methods employed in the determination of glucose in the blood. Some of these methods are enumerated below:

2.6.1 Urine

Urine is a waste product of the body secreted by the kidneys through a process of filtration from blood. Cellular metabolism generates numerous waste compounds, many of which are rich in nitrogen and require elimination from the blood stream. Urine provides an indirect measurement of glycaemia, it is least expensive and has been the mainstay of convenient assessment of diabetics. However, urine glucose readings taken are not much useful. This is because in properly functioning kidneys, glucose does not appear in urine until the renal threshold for glucose has been exceeded. This is substantially above any normal glucose level and so is an evidence of an existing hyperglycemic condition. Renal threshold varies from one individual to another and this makes it inaccurate (Peterson, 1982). Urine test strips are used.

2.6.2 Blood sugar

Physiologically, the term means only glucose in the blood, though other sugars are present, sometimes in more than trace amounts but only glucose serves as a controlling signal for metabolic regulation. Glucose can be measured in the whole blood, serum or plasma. Historically, blood glucose values were given in terms of whole blood, but now most laboratories measure and report serum glucose levels because serum has more water content and consequently more dissolved glucose than whole blood.

Finger-prickling lancets and automatic devices are available for use though more recent advances have led to the use of chemically impregnated strips which are designed to monitor whole blood glucose from drops of blood obtained from finger pricking (Peterson, 1982).

Different methods are employed in the measurement of blood glucose:

2.6.2.1 The Folin-Wu method

Here, glucose is determined in the serum (usually protein free blood filtrate is used) by reduction of alkaline copper sulphate using phosphomolybdic acid reagent to form a phosphomolybdenum oxide complex which is blue in colour. This is then estimated colorimetrically.

2.6.2.2 The Nelson-Somogyi method

Here, protein free blood is prepared with Zinc hydroxide (Zn (OH)), so as to remove most of the interfering reducing substances. Then the alkaline copper sulphate reaction by Asenomolybdic acid is carried out. An Asenomolybdenum oxide complex which is blue in colour is the end product which can be determined colorimetrically.

2.6.2.3 The ortho-toluidine method

This method makes use of aromatic amines and hot acetic acid. There is the formation of glycosylamine and Schiff's base which is emerald green in colour. This method unlike the previous two is specific for hexose-glucose, mannose and galactose which are normally present in very small concentrations. However, the reagent used is toxic.

2.6.2.4 The glucose-oxidase method

This enzymatic method is specific for blood glucose. The enzyme glucose-oxidase acts on the blood glucose thereby converting it to gluconic acid and hydrogen peroxide. The hydrogen peroxide oxidizes O-dianisidine (a chromogen) in the presence of a peroxidase to form a coloured product which is then estimated colorimetrically.

2.6.2.5 Glucometer

This is a home monitoring blood glucose assay method. Here, a glucometer uses a test strip impregnated with a glucose-oxidase reagent to which a blood sample is applied, and then inserted into the meter for reading. Test strip shapes and their exact chemical composition vary between meter systems and cannot be interchanged.

2.7 Alpha-amylase and alpha-glucosidase inhibitors from plants

The therapeutic strategies for the treatment of type 2 diabetes include the reduction of the demand for insulin, stimulation of endogenous insulin secretion, enhancement of the action of insulin at the target tissues and the inhibition of degradation of oligo and disaccharides (Funke and Melzing, 2006). Inhibition of α-amylase, an enzyme that plays a role in digestion of starch and glycogen, is considered a strategy for the treatment of disorders in carbohydrate uptake, such as diabetes and obesity, as well as, dental caries and periodontal diseases. Some inhibitors currently in clinical use are acarbose, miglitol and voglibose. They are known to inhibit a wide range of glycosidases such as α-glucosidase and α-amylase, but because of their non-specificity in targeting different glycosidases these hypoglycaemic agents have their limitations (Cheng and Fantus, 2005). Herbal medicines with anti-diabetic potential

have different mode of action - mimic insulin, act on insulin secreting beta cells or modify glucose utilization (Wadkar *et al*., 2008). Herbs which modify glucose utilization act by altering the viscosity of the gastrointestinal contents, delaying gastric emptying or delaying glucose absorption (Wadkar *et al*., 2008). Plants are an important source of chemical constituents with potential for inhibition of α-amylase and can be used as therapeutic or functional food sources (Paloma *et al*., 2012). There are a large number of plants with alpha-glucosidase inhibitor action (Benalla *et al*., 2010).

One therapeutic approach for treating type 2 diabetes mellitus is to decrease the postprandial glucose levels. This could be done by retarding the absorption of glucose through the inhibition of the carbohydrates-hydrolysing enzymes, α-glucosidase and α-amylase, present in the small intestinal brush border that are responsible for the breakdown of oligosaccharides and disaccharides into monosaccharides suitable for absorption (Goke and Herrmann-Rinke, 1998; Lebovitz, 1998; Inzucchi, 2002 and Laar *et al*., 2008). Inhibitors of these enzymes, like acarbose, delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise (Cheng and Fantus, 2005; Laar *et al*., 2008).

2.8 Anti-oxidants from Plants

Plants are an excellent source of chemical structures with a wide variety of biological activities including anti-oxidant and hypoglycaemic properties. Their phytochemical and biological investigations should be encouraged, especially in view of the urgent need to discover new bioactive isolated molecules with greater efficacy and fewer

side effects than existing drugs (Tundis *et al*., 2012). Interest in the search for new natural anti-oxidants has grown dramatically over the past years because reactive oxygen species (ROS) production and oxidative stress initiated by free radicals (Maxwell, 1995; Weisburger, 2002) have been shown to be linked to a large number of human degenerative diseases, such as cancer (Paz-Elizur *et al*., 2008), diabetes (Jain, 2006; Naito *et al*., 2006), inflammation (Mukherjee *et al*., 2007), Parkinson's disease (Chaturvedi and Beal, 2008) and acquired immunity deficiency syndrome (AIDS) (Sepulveda and Watson, 2002).

There is growing evidence supporting the observation that reactive oxygen species and free radicals are associated with certain diseases including atherosclerosis, coronary heart disease and even cancer (Stohs, 1995; Valavanidis *et al*., 1995). Hence intake of constituents from foods that have free radical scavenging activities is considered important in preventing such diseases or occurrence (Takayuki *et al*., 2004).

For modern biomedical researchers dealing with the cause of diabetes, anti-oxidants are becoming essential tools in investigating oxidative stress-related diabetic pathologies and therapies (Laight *et al*., 2000; Evans *et al*., 2002). Therefore, it is being realized that identification of molecular basis for the protection afforded by a variety of anti-oxidants against oxidant- induced damage might lead to the discovery of pharmacological targets for novel therapies to prevent, reverse or delay the onset of resultant pathologies (Evans *et al*., 2002).

Secondary metabolites, especially phenylpropanoid act as anti-oxidant to overcome the harmful effect of reactive oxygen species in addition to enzymes such as catalase, guaicol peroxidase, amylase and phosphatase. Patients with type 2 diabetes are

insulin resistant and often have a metabolic syndrome, a multifactorial intervention including aggressive treatment of arterial hypertension and dyslipidaemia (Stolar, 2010). During normal metabolic functions, highly reactive compounds called free radicals are created in the body. However, free radicals may also be introduced from the environment. These compounds are inherently unstable since they have an odd number of electrons. To balance the number of electrons, these free radicals will react with certain chemicals in the body, and in so doing; they interfere with the cell's ability to function normally. As such, development of ethnomedicines from plants with strong anti-oxidants properties has received much attention (Sharma and Prasad, 2012). Several non-volatile compounds such as carnosol, quercetine, caffeic acid and rosmarinic acid are known to be good scavengers of free radicals, but some volatile compounds from essential oils possess also the potential as natural agents for food preservation (Kulisica, 2004).

A potent anti-oxidant exhibits a significant peroxyl radical scavenging ability by the donation of its hydrogen atom to the radical species. Natural products with anti-oxidant activity could retard the oxidative damage of the tissue by increasing those defences in different degenerative diseases such as diabetes. A rapid, simple and inexpensive method to measure the anti-oxidant capacity involves the use of the free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Relatively stable organic radical DPPH has been widely used in the determination of the anti-oxidant activity of single compounds as well as the different plant extracts (Yen and Duh, 1994; Brand-Williams *et al*., 1995). The method is based on the reduction of alcoholic DPPH solutions in the presence of a hydrogen donating anti-oxidant. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) solutions show a strong absorption band at 517 nm appearing

as a deep violet colour. The absorption vanishes and the resulting decolourization is stoichiometric with respect to degree of reduction.

Free radicals are constantly generated due to environmental pollutants, radiation, chemicals, toxins, physical stress and the oxidation process of drugs and food. Many plant phenolics have been reputed to have anti-oxidant properties that are even much stronger than vitamins E and C. In addition, currently available synthetic anti-oxidants like butylated hydroxyl anisole (BHA), butylatedhydroxytoluene (BHT) and gallic acid esters have been suspected to cause or prompt negative health effects and hence the need to substitute them with naturally occurring anti-oxidants (Rao *et al*., 2006; Aqil *et al*., 2006) .

CHAPTER 3

MATERIALS AND METHODS

3.1   General Experimental Procedures

3.1.1 Materials

Materials used include 10 L aspirator bottle, 2.5 L Winchester bottles, glass funnel, cotton wool, Buchner funnel, weighing balance, round bottom flasks, beakers, separating funnel, Buchi® Rotary evaporator, Hammer mill, Vacutec Labconco freeze zone 6 freeze drier (Labconco, USA), liquid nitrogen, Methanol, Hexane, Ethyl acetate and distilled water.

3.1.2 Plant collection and authentication

Anthodeista djalonensis   A. Chev and *Anthocleista vogelii* Planch leaves, stem bark and whole root were collected along Ijebu Ode - Benin road in November, 2007. They were authenticated at the Forest Herbarium Ibadan (FHI) of the Forestry Research Institute of Nigeria (FRIN), with voucher numbers FHI 10907 and FHI 10906, respectively. The plant samples were also prepared and deposited at the Department of Pharmacognosy Herbarium, University of Ibadan.

Plant materials consisting of leaves, stem bark and whole roots were washed, chopped (into smaller bits with a knife), air dried and then oven dried at 40°C. They were powdered using a hammer mill (with a 5-KVA motor) and kept in amber coloured bottles until ready for extraction.

3.2 Extraction

Different weights of powdered samples of the leaves, stem bark and whole roots of *A. djalonensis* and *A. vogelii* were macerated in 80% aqueous methanol for a period of 72 h. The flask was shaken regularly at intervals of 6 h and kept in a dark cool cupboard overnight. The solvent was decanted and filtered every 24 h and replaced with fresh 80% aqueous methanol thrice for the duration of the extraction period. The filtrate collected each day was pooled and concentrated *in vacuo* at 37°C using a Buchi rotary evaporator and then freeze dried on a Labconco freeze zone 6 Freeze drier (Labconco, USA). The crude methanol extracts were weighed and stored in clean sample tubes in a refrigerator until when required for analysis. The crude extracts from each of the plant samples were then screened for alpha-amylase inhibition, anti-oxidant and *in vivo* rat model anti-diabetic activities.

3.3 Fractionation of Crude Extracts

The crude aqueous methanol extracts of both plants were weighed and suspended in H_2O: MeOH (4:1) and partitioned with hexane (3x100 mL), respectively in a separating funnel. Further partitioning into ethyl acetate (EtOAc) (3x100 mL) was done to give the ethyl acetate fraction. The ethyl acetate fractions were concentrated *in vacuo* at 37°C, kept in a desiccator for further drying and subsequently tested for alpha-amylase inhibition and anti-oxidant activity.

3.4 Preliminary Phytochemical Screening

To ascertain the phytochemical constituents of the plants samples of *A. djalonensis* and *A. vogelii,* the following preliminary tests were carried out on the powdered leaves, stem bark and whole roots of both plants.

3.4.1 Borntrager's Test for Anthraquinone Derivatives

3.4.1.1 Test for Combined Anthraquinones

To show the presence of combined anthraquinones, 1 g of powdered sample was boiled with 2 mL of 10% HCl for 5 min, filtered (while hot) and allowed to cool. The cooled filtrate was partitioned against equal volumes of chloroform (2 x) and then separated with the aid of a Pasteur pipette. An equal volume of 10% ammonia solution was added to the chloroform layer and then shaken vigorously. The presence of a delicate rose-pink colour on the aqueous layer was considered positive (Evans, 1997).

3.4.1.2 Test for Anthraquinone Derivatives

To show the presence of anthraquinone derivatives, 5 mL of chloroform was added to about 1 g of each powdered sample and shaken for about 5 min. The extract was filtered and shaken with equal volume of 10% ammonia. A bright pink colour in the aqueous layer was considered positive.

3.4.2 Tests for Saponin Glycosides

About 1g of powdered sample was boiled in 10 mL distilled water for 10 min, filtered while hot and cooled. The following tests were then performed on the cooled filtrate.

3.4.2.1 Demonstration of Frothing

About 2.5 mL of the filtrate was diluted to 10 mL with distilled water and shaken for about 2 min. Formation of persistent foam shows the presence of saponin glycosides.

3.4.2.2 Demonstration of Emulsifying Properties

To the solution obtained in 3.4.2 was added few drops of liquid paraffin and vigorously shaken for about 5 min. Formation of a fairly stable emulsion was considered positive.

3.4.3 Test for Cardiac Glycosides

Each powdered sample (1 g) was extracted with 10 mL of 80% alcohol for 5 min on a water bath. The filtrate was diluted with equal volume of distilled water. Few drops of lead acetate was added to the diluted filtrate (which turned milky) and then filtered. The filtrate was extracted with aliquots of chloroform (2 x); this was then combined and divided into two portions in clean Petri dishes A and B. Each portion was evaporated to dryness (Evans, 1997). The following tests were performed on the obtained residue.

3.4.3.1 Keller – Killiani Test (for 2-deoxy sugars)

Cooled filtrate from Petri dish A was dissolved in 3 mL $FeCl_3$ reagent (0.3 mL of 10% $FeCl_3$ in 50 mL glacial acetic acid) in a clean test tube. 2 mL H_2SO_4 was carefully poured down the side of the test tube. Formation of a purple reddish-brown 'ring' at the inter-phase and a green colour on the acetic acid layer was considered positive (Evans, 1997).

3.4.3.2 Kedde's Test (for unsaturated lactones)

The residue from Petri dish B was mixed with 1mL of 2% 3,5- dinitrobenzoic acid in ethanol. The resulting solution was made alkaline with 5% NaOH. The formation of a reddish-brown to brownish yellow colour was considered positive (Evans, 1997).

3.4.4 General Alkaloid Tests

About 1 g of the powdered sample extracted with 10 mL of 10% HCl, filtered and the filtrate adjusted to pH of about 6-7. The resulting solution was divided into 4 portions, and small quantities of the following reagents were added.

3.4.4.1 Dragendoff's Reagent (potassium bismuth iodide)

To the first portion of extract, 1 mL of Dragendroff's reagent was added. An orange-red precipitate indicates the presence of alkaloids (Harbone, 1998).

3.4.4.2 Hager's Reagent (saturated aqueous solution of picric acid)

To the second portion of extract, 3 mL of Hager's reagent was added. The formation of a yellow coloured precipitate indicates the presence of alkaloids (Persinos and Quimby, 1967; Harbone, 1998)

3.4.4.3 Wagner's Reagent (iodine in potassium iodide)

To the third portion of the plant extract, 2 mL of Wagner's reagent was added Reddish brown colored precipitate indicates the presence of alkaloids (Sofowora, 1982; Evans, 1997).

3.4.4.4 Mayer's Reagent (Potassium mercuric iodide solution)

To the fourth portion of extract, 1 mL of Mayer's reagent was added. Whitish or cream colored precipitate indicates the presence of alkaloids.

3.4.5 Test for Tannins

About 1g of each powdered sample was boiled in 10 mL of water for about 5 min, cooled and filtered. The volume was adjusted to about 20 mL and the following tests were performed on each extract.

3.4.5.1 Ferric Chloride Test

The original solution (1 mL) was diluted to 5 mL with water in a test tube. A few drops of ferric chloride (0.1%) solution were added. Formation of blue-black, green or blue-green coloured solution was considered positive.

3.4.5.2 Bromine Water Test

To 1 mL of the original solution was added a few drops of bromine water. The colour any precipitate formed was noted.

3.4.5.3 Potassium Dichromate Test

To 1 mL of the original solution, a few drops of strong aqueous potassium dichromate were added. The colour of the precipitate was noted.

3.4.6 Test for Flavonoids

The plant sample (1 g) was extracted with 5 mL of methanol. The extract was filtered and 3 drops of concentrated HCl and magnesium chips were added. An orange to pink colour is taken as positive.

3.5 Experimental Animals and Materials

3.5.1 Materials

Metal cages, intragastric cannulars, One Touch-Basic Glucometer and Test strips (Johnson and Johnson, California), syringes, cotton wool, methylated spirit, water, pelletized feed (Ladokun® feeds), Glibenclamide (Daonil®), Nigerian-German Chemicals Plc, Nigeria)

3.5.2 Experimental Animals

Adult albino Wistar rats of both sexes weighing between 150-200 g were obtained from the animal house, University of Ibadan and were housed in metal cages in a well ventilated room. The animals were fed on standard pelletized feed (Ladokun® feeds) and allowed water *ad libitum.*

3.6. Preparation of Materials for Bioassays

3.6.1 Materials

Weighing balance, Metler Toledo pH meter, UV/VIS 916 spectrophotometer (GBC Scientific Equipment, Pty), Water bath, Pasteur pipettes, Eppendorf pipettes, distilled water, Sodium hydroxide (NaOH) Sodium chloride salt (NaCl), Sodium di-hydrogen phosphate (NaH_2PO_4), Di-sodium hydrogen phosphate (Na_2HPO_4), standard buffer tablets (buffers 4, 7 and 9), soluble starch (Sigma-Aldrich, Dramstadt Germany), 3,5-dinitrosalicylic acid, α-amylase from *apergillus oryzae (*Sigma-Aldrich, Dramstadt Germany*)*, α-tocopherol (Sigma-Aldrich, Dramstadt Germany), 2, 2'-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, Dramstadt Germany), maltose, Acarbose (Glucobay 50®, Bayer Schering Pharma, Germany).

3.6.2 Alpha-amylase Enzyme (1% solution)

Alpha-amylase enzyme from *Aspergillus oryzae,* E.C.3.2.1.1 30 units/mg was used for the assay. It was prepared by dissolving 0.5 g of enzyme in 50 mL of sodium phosphate buffer pH 6.9.

3.6.3 Sodium Phosphate Buffer (pH 6.9)

Sodium phosphate buffer (0.02 M) at pH 6.9 was used with 0.006 M NaCl. 2.8392 g of di-sodium hydrogen phosphate (Na_2HPO_4) was dissolved in 1 L distilled water, 2.3996 g of Sodium di-hydrogen phosphate (NaH_2PO4) was also dissolved in 1 L distilled water to make 0.02 M. To make 0.006 M Sodium chloride (NaCl), 0.3915 g of NaCl was added to the mixture of the sodium phosphate salts. A metler Toledo pH meter was used to check the pH.

3.6.4 Starch (1% solution)

This was prepared by dissolving 1.0 g soluble starch, in 100 mL of 0.02 M sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride. It was brought to a gentle boil to dissolve. Thereafter, it was cooled and the volume brought to 100 mL with water (Benfield, 1951).

3.6.5 3,5-Dinitrosalicylic Acid (DNS) Colour Reagent (1% solution)

This was prepared by dissolving 10 g of Sodium hydroxide (NaOH) pellets, 20 g of sodium potassium tartarate tetrahydrate salt and 10 g of DNS acid in 1L distilled water (Benfield, 1951). The solution was heated for a few minutes to ensure complete dissolution. It was now stored in an amber coloured bottle in the dark.

3.6.6 Maltose Stock Solution

This was prepared by dissolving 180 mg maltose (MW 360.3) in 100 mL reagent grade water in a volumetric flask. This was used to prepare ten maltose dilutions ranging from 0.3-5.0 µmoles/mL (Benfield, 1951).

3.6.7 Inhibitors

Acarbose, (a known alpha-glucosidase inhibitor served as the positive control) and plant extracts were dissolved in the buffer to give different concentrations ranging from 0.1 mg/mL to 1.0 mg/mL.

3.6.8 2, 2'-Diphenyl-1-picrylhydrazyl (DPPH)

The 6×10^{-5} M methanol solution of DPPH was prepared by dissolving 5.9132 mg of DPPH in 250 mL of HPLC grade methanol (Brand-Williams *et al*., 1995).

3.6.9 Preparation of Plant Extracts for Anti-oxidant Assay

The plant extracts were prepared in methanol to give a concentration of 0.2 mg/mL to 1.0 mg/mL. Alpha-tocopherol was also prepared in the same concentration range to serve as the positive control.

3.7 Determination of Maltose Standard Curve

Using the maltose stock solution, a maltose standard curve was prepared as follows: In numbered tubes, 10 maltose dilutions ranging from 0.3-5.0 μmoles/mL were measured with the aid of a pipette. Two blank tubes with reagent grade water only were included. Into a series of corresponding numbered tubes, 1 mL of each dilution of maltose was pipetted. Dinitrosalicylic acid colour reagent (1 mL) was added and incubated in boiling water bath for 5 minutes and cooled to room temperature. Distilled water (5 mL) was added to each tube and mixed well. Absorbance of the different concentration of maltose was read at 540 nm versus micromoles maltose.

Enzyme assay: 0.5 mL of respective enzyme dilutions were pipetted into a series of numbered test tubes, a blank with 0.5 mL reagent grade water was included. All tubes

were incubated at 37°C for 3-4 minutes to achieve temperature equilibration. At pre-determined intervals, 0.5 mL starch solution (at 37°C) was added and incubated at exactly 3 minutes. At timed intervals, 1 mL dinitrosalicylic acid colour reagent was added to each tube. All tubes were incubated in a boiling water bath for 5 min, cooled to room temperature and 10 mL reagent grade water added. After mixing well, absorbance was read at 540 nm. Maltose released was determined from standard curve (in μM).

3.8 Biological Assays

3.8.1 Alpha-amylase Inhibitory (AAI) Assay

The α-amylase inhibitory activity was determined by slightly modifying the dinitrosalicylic acid method described by Benfield (1951) and Miller (1959).

The total assay mixture containing 200 μL of 0.02 M sodium phosphate buffer, 200 μL of enzyme (α-amylase E.C. 3.2.1.1), and the plant extracts in the concentration range 0.1-1.0 mg/mL were incubated for 20 min at 37°C. This was followed by addition of 200 μL of 1% starch in all the test tubes. The reaction was terminated with addition of 400 μL of 3,5-dinitrosalycylic acid colour reagent, placed in boiling water bath for 5 min, cooled to room temperature and diluted with 5 mL of distilled water. The generation of maltose was quantified by the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid. This reaction (corresponding to colour change from orange-yellow to red) was detected at 540 nm. In the presence of an alpha-amylase inhibitor less maltose would be produced and the absorbance value would be decreased.

The absorbance was measured at 540 nm using a UV-VIS 916 Spectrophotometer. The control samples were also prepared accordingly without any plant extracts and were compared with the test samples.

The percentage inhibition was calculated using the formula:

% Inhibition = $[(A_{(control)} - A_{(test)})/ A_{(control)}] \times 100$

Where $A_{(control)}$ = Absorbance of Control.

$A_{(test)}$ = Absorbance of inhibitor (plant extract or acarbose).

The crude aqueous methanol extracts of the leaves and stem bark of both plants were tested along with their ethyl acetate fractions. The fractions collected from the column chromatography of the ethyl acetate fraction were also tested. All determinations were performed in triplicates.

3.8.2 Anti-oxidant Activity (AA) Assay

The anti-oxidant activities of the extracts and fractions were measured in terms of hydrogen-donating or radical-scavenging ability using the stable radical 2, 2'-diphenyl-1-picrylhydrazyl (DPPH). The method adopted for this assay was that of Brand-Williams *et al*., (1995).

All the crude aqueous methanol extracts and subsequently ethyl acetate fractions corresponding to the crude extracts found active were also tested. Column fractions from the ethyl acetate fractions and finally isolated compounds from active fractions were tested.

Briefly, the method involved preparing a methanol solution of the extracts to be tested in the concentration range of 0.2-1.0 mg/mL. Then, 50 μL of each concentration was placed in a cuvette and 2 mL of 6×10^{-5}M of DPPH solution was added. Absorbance measurements commenced immediately. The decrease in absorbance at 517 nm was obtained at intervals between 0 min and 1 h for all samples. The absorbance of the DPPH radical without anti-oxidant, which is the negative control, was measured daily. A standard anti-oxidant, α-tocopherol was used as the positive control. All determinations were performed in triplicates. The percentage scavenging activity of the DPPH radical by the samples was calculated according to the formula of Yen and Duh (1994):

% Anti-oxidant activity $= [(A_{C(0)} - A_{A(t)})/ A_{C(0)})]\times 100$

Where $A_{C(0)}$ = Absorbance of control at t = 0 min

$A_{A(t)}$ = Absorbance of the anti-oxidant at t = 0 min to 1h.

3.8.3 Alloxan-induced Diabetes

The rats were divided into four groups A, B, C, and D with five rats in each group. Rats in groups B-D were fasted overnight, given a single intraperitoneal injection of 80 mg/kg of alloxan monolydrate (Abdel-Barry *et al*., 1997) in isotonic saline and allowed to rest for three days to stabilize their blood glucose level. The rats in group A were not injected and were allowed food and water *ad libitum.* Rats in groups C and D were treated as shown in section 3.8.4.

3.8.4 Animal Grouping

Group A: Five normal, untreated control rats.

Group B: Five alloxan-induced diabetic rats that were neither treated with extract nor the drug of reference, glibenclamide. These served as the negative control.

Group C: Five alloxan-induced diabetic rats that were treated with plant extract 1 g/kg body weight.

Group D: Five alloxan-induced diabetic rats that were treated with 2.5 mg/mL glibenclamide. These served as the positive control.

The animals were observed for 7 days. On the first day, the basal glucose level was taken at 8 am in the morning to serve as the 0 min reading. Subsequently, animals in groups C and D were treated with the extracts and glibenclamide, respectively. Blood glucose was then measured after 1 h, 2 h and 3 h. For days 2-7, the extracts and glibenclamide were administered orally at 8 am in the morning, repeated at 11 am and 1 pm. The blood glucose level was then measured at 3 pm with the aid of a One-touch basic glucomemeter.

3.9 Chromatography

3.9.1 Materials

Glass columns of various dimensions, conical flasks (250 mL), round bottom flasks 50-500 mL, dessicator, HPLC grade solvents -hexane, ethyl acetate, dichloromethane, acetone and methanol (Merck Germany), methanol solution of DPPH, silica gel (70-230 and 230-400 mesh), TLC plates ((20 cm × 20 cm, Merck Damstadt Germany), capillary tubes, a Buchi® rotary evaporator and UV lamp (P.W. Allen and

Co., Liverpool, London), Perkin Elmer 400 FT-IR spectrophotometer, NMR machine, Bruker Avance (600 MHz), deuterated solvents, chloroform ($CDCl_3$) and acetone ($(CD)_2CO$).

3.9.2 Thin layer chromatography

Pre-coated fluorescent (F_{254}) aluminium TLC plates were used for the thin layer chromatography. Various solvent systems as found appropriate were used as the mobile phases. The plates were viewed under UV both at 254 nm and 366 nm using a UV lamp and sprayed with a methanol solution of DPPH.

3.9.3 Column Chromatography

Column chromatography was performed in a glass column of internal diameter 3.5 cm with a column height of 28 cm packed with activated silica gel (Kieselgel G60, 70- 230 mesh, ASTM) for each of the ethyl acetate fractions of the leaves and stem bark   of both plants. Ethyl acetate fraction (5 g each) was loaded in the column which was eluted with a gradient solvent system of hexane and ethyl acetate starting with 100% hexane 200 mL, and end ending with 100% EtOAc, 200 mL. The column was then washed with 100% methanol. A total of 18 fractions were collected for each ethyl acetate fraction loaded on the column, which were then spotted on analytical TLC plates (silica gel 60 F_{254}) using appropriate solvent systems. The plates were viewed under UV/VIS lamp at 366 nm and 254 nm and sprayed with a methanol solution of DPPH. All fractions were subjected to the AAI assay as described in section 3.8.1 while those that gave instant yellow colour on spraying were subjected to AA assay as previously described in section 3.8.2.

3.10   Spectral Studies

3.10.1 Nuclear Magnetic Resonance (NMR)

NMR spectra, 1D and 2D, ^{1}H and ^{13}C were acquired in CDCL$_3$/TMS for the crystals isolated from the leaves and stem bark of *A. vogelii* while for the compounds isolated from *A. djalonensis,* (CD)$_2$CO/TMS was used.

3.10.2 Infrared Spectroscopy (IR)

IR spectra were acquired on a Perkin Elmer 400 FT-IR spectrophotometer. The crystalline compound was examined neat while other compounds were dissolved in deuterated acetone.

3.10.3 Gas Chromatography - Mass spectrometry (GC-MS)

GC-MS of isolated compounds were performed on an Agilent 6890N GC, 5973 MS detector at 70eV, the column was HP 5 medium polarity, 30 m in length with internal diameter of 250 microns and film thickness of 0.25 microns. The conditions were initial oven temperature was 40°C, Ramp 1 15°C/min to 200°C for 5 min, Ramp 2 20°C/min to 24°C for 5 min. Injector: 280°C Splitless 110.72 kPa. Detector temperatures: 150°C Quad, 230°C Source. Helium was used as the carrier gas.

3.11   Statistical Analysis

Alpha-amylase inhibitory activity, anti-oxidant activity and reduction in blood glucose levels were expressed as means $\pm$ SEM. Analysis of variance (ANOVA) was used to evaluate the level of significance between groups at $p < 0.05$ and $p < 0.001$.

3.12 Purification of Active Column Fractions from *Anthocleista djalonensis* Stem bark

The column fractions from *A. djalonensis* stem bark were analysed on TLC (silica gel 60 F$_{254}$) plate using EtOAc/Hexane/MeOH 7:2:1 as mobile phase. The plate was sprayed with a methanol solution of DPPH. Fractions 6, 7, 8, 9,13, 14 and 18 gave immediate bright yellow colour while fractions 10, 11, 12 and 15 gave yellow coloration 3 min later. Based on their TLC profiles, similar fractions (7, 8 and 9) were pooled together and renamed fraction C.

3.12.1 Purification of Column Fraction C of *Anthocleista djalonensis* Stem bark

Fraction C was subjected to both AA and AAI assays as described in sections 3.8.1 and 3.8.2. Column chromatography of fraction C (476.33 mg) was performed in a glass column of internal diameter 1.2 cm with a column height of 41 cm and packed with activated silica gel G60 230-400 Mesh (ASTM Merck). A column was run starting with Hex/EtOAc (10:0, 10 mL), Hex/EtOAc (9.8:0.2, 10 mL), Hex/EtOAc (9.6:0.4, 10 mL) till Hex/EtOAc (0:10, 10 mL) and the column was stopped. Fifty fractions were collected and analysed on TLC (silica gel 60 F$_{254}$), using EtOAc /Hex/MeOH (70:20:10) as mobile phase and viewed under UV/VIS at 366 nm and 254 nm. Fraction C 27 was a whitish substance weighing 3.8 mg. It was subjected to IR, GC-MS, 1D and 2D-NMR experiments to determine its structure.

3.12.2 Purification of Column Fraction 11 of *Anthocleista djalonensis* Stem bark

Fraction 11, a clear yellow liquid (299 mg) eluted with 100% EtOAc, It was subjected to both AAI and AA assays as described in sections 3.8.1and 3.8.2. Fraction 11 (246.78 mg) was loaded on a column packed with silica gel G60 230-400 Mesh, column height of 32.5 cm, internal diameter of 1 cm and eluted with Hexane/EtOAc (20:80,10 mL).

Thirty-four fractions were collected, monitored on TLC (silica gel 60 F_{254}) using DCM/EtOAc (2.6:7.4) as mobile phase. Based on their TLC profiles, similar fractions were pooled to give six fractions labelled A-F. Fraction 11E (48 mg) was the purest with one spot on TLC and it was subjected to the AAI assay as described. Thereafter, IR experiments were performed, while 1D and 2D-NMR spectra were recorded in CD_3COCD_3 to determine its structure and GC-MS experiments to determine its mass.

3.13 Purification of Active Fractions from *Anthocleista vogelii* Leaf

The ethyl acetate fraction (5 g) of *A. vogelii* leaf was subjected to column chromatography as earlier described. Twenty fractions were collected from the column and were monitored on analytical TLC (silica gel 60 F_{254}) plate developed in EtOAc/Hex/MeOH (7:2:1). Plates were visualised under UV lamp at 366 nm and 254 nm and then sprayed with a methanol solution of DPPH. Fractions 13-20 gave instant bright yellow colour on spraying with DPPH, while fractions 6-12 gave very faint yellow colour. All the fractions were subjected to the AA and AAI assays. Fraction 5 eluted with Hex/EtOAc (60:40) gave a crystalline copound weighing 65 mg. Fraction 16 eluted with 100% EtOAc, concentrated *in vacuo* and dried in a desiccator gave a shiny greenish powdery substance weighing 206 mg.

3.13.1 Purification of Column Fraction 5 of *Anthocleista vogelii* Leaf

Fraction 5 crystals (recrystallised in HPLC methanol) were hair like, yellowish green and weighed 35 mg. It was analysed on TLC plate using EtOAc/Hex/MeOH (7:2:1) as mobile phase and further tested on the AAI assay. IR, 1D and 2D-NMR and GC-MS experiments were carried out on it so as to determine its structure.

3.13.2 Purification of Column Fraction 16 of *A. vogelii* Leaf

Fraction 16 (206 mg), was purified further by subjecting it to preparative TLC. The plate was developed using DCM/Acetone/MeOH (4:5:1) as a mobile phase. Five bands were scraped off the plate, washed with acetone, concentrated *in vacuo* and kept in a desiccator to dry. The bands were spotted on a TLC plate using DCM/EtOAc/MeOH (4:5:1) as mobile phase and viewed under UV/VIS lamp at 366 nm and 254 nm. The plate was also sprayed with a methanol solution of DPPH with band 4 giving an instant yellow colour. It was subjected to the AA assay. Fraction 16 band 4, (26.8 mg) had the same ^{1}H NMR peak pattern as the original fraction 16. Thus, it was further purified on a preparative TLC plate, developed twice using DCM/EtOAc/MeOH (4:5:1) as a mobile phase with four bands being scraped off. They were washed with acetone, concentrated *in vacuo* and then kept in a dessicator. Band 4:4 (14 mg) had exact TLC profile and ^{1}H NMR peak pattern as the parent band 4. The anti-oxidant activity was evaluated and the band was subjected to IR, 1D and 2D-NMR and GC-MS experiments for elucidation of its structure.

3.13.3 Purification of Active Fractions from *Anthocleista vogelii* Stem bark

The ethyl acetate fraction of *A. vogelii* (5 g) was fractionated on a column using increasing gradient of Hexane/EtOAc. Eighteen fractions were collected, analysed with TLC developed in EtOAc/Hex/MeoH (7:2:1). The plate was visualised under UV lamp at 366 nm and 254 nm and then sprayed with methanol solution of DPPH. The fractions showed varying degrees of AAI activities. However fraction 5 (16.7 mg) eluted with Hex/EtOAc (60:40) was the same crystals that were eluted with the same solvent system from *A. vogelii* leaf. Also, fractions 11 and 12 eluted with 100% EtOAc (260.4 mg and

247.4 mg, respectively) had the same ^{1}H-NMR and ^{13}C-NMR profiles as *A. djalonensis* fractions 11 and 12, with similar AAI activity.

CHAPTER 4

RESULTS

4.1 Yield of Crude and Partitioned Extracts

Table 4.1. Yield of Crude Extracts

Plant specimen/part	Powdered sample (g)	Crude aqueous MeOH extract (g)	Yield (%)
A.djalonensis leaf	1,800	152.67	8.5
A.djalonensis stem bark	2,000	181.69	9.1
A.djalonensis root	1,500	120.00	8
A.vogelii leaf	2,400	122.18	5.1
A.vogelii stem bark	1,200	106.11	8.8
A.vogelii root	1000	87.00	8.7

Table 4.2. Yield of Fractions of *Anthocleista djalonensis* and *Anthocleista vogelii*

Plant part	Crude aqueous MeOH extract (g)	Hexane fraction (g)	Yield (%)	EtOAc fraction (g)	Yield (%)
A.djalonensis leaf	120	2.69	2.2	24.91	20.8
A.djalonensis stem bark	135	2.40	1.8	18.89	14.0
A.vogelii leaf	60	1.14	1.9	14.61	24.4
A.vogelii stem bark	65	1.0	1.5	7.76	11.9

4.2   Preliminary Phytochemical investigation of the leaves, stem bark and whole root of *A. djalonensis* and *A. vogelii*

Table 4.3. **Phytochemical Screening of *Anthocleista djalonensis* and *Anthocleista vogelii***

Plant part	Anthracene derivatives	Cardiac glycosides	Flavonoids	Saponin	Sapogenin	Tannins	Alkaloids
Anthocleista djalonensis leaf	+ + +	+ +	+ +	+	_	_	+ +
Anthocleista djalonensis stem bark	+ + +	+	_	+	+	_	+ +
Anthocleista djalonensis root	+ + +	+	_	+ +	+ +	_	+ +
Anthocleista vogelii leaf	+++	_	+	+	+	+ +	+ +
Anthocleista vogelii stem bark	++	+	+	++	++	+	+
Anthocleista vogelii root	++	_	+	++	+	+	++

Key   +   = Present in trace amount

      + +   = moderately present

    + + +   = abundantly present

       -    = absent

81
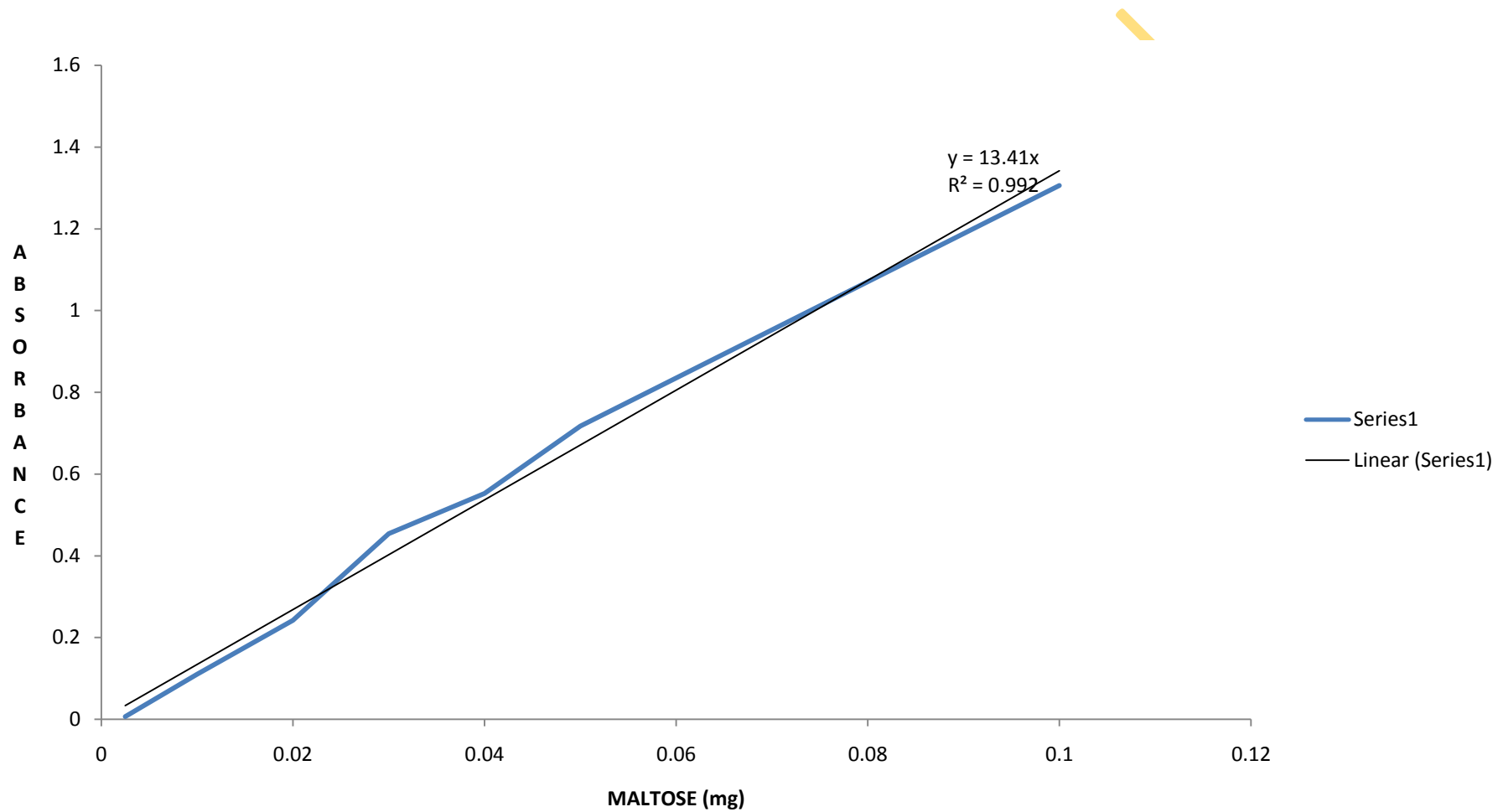
Fig. 4.1. Maltose standard curve using alpha-amylase (E.C. 3.2.1.1) from *Aspergillus oryzae*.
The concentration of enzyme in the reaction mixture is 1%. The plot is linear with a correlation coefficient for **y** on **x** to be 0.99. One unit of enzyme will liberate 1 mg of maltose from starch in 3 minutes at pH 6.9
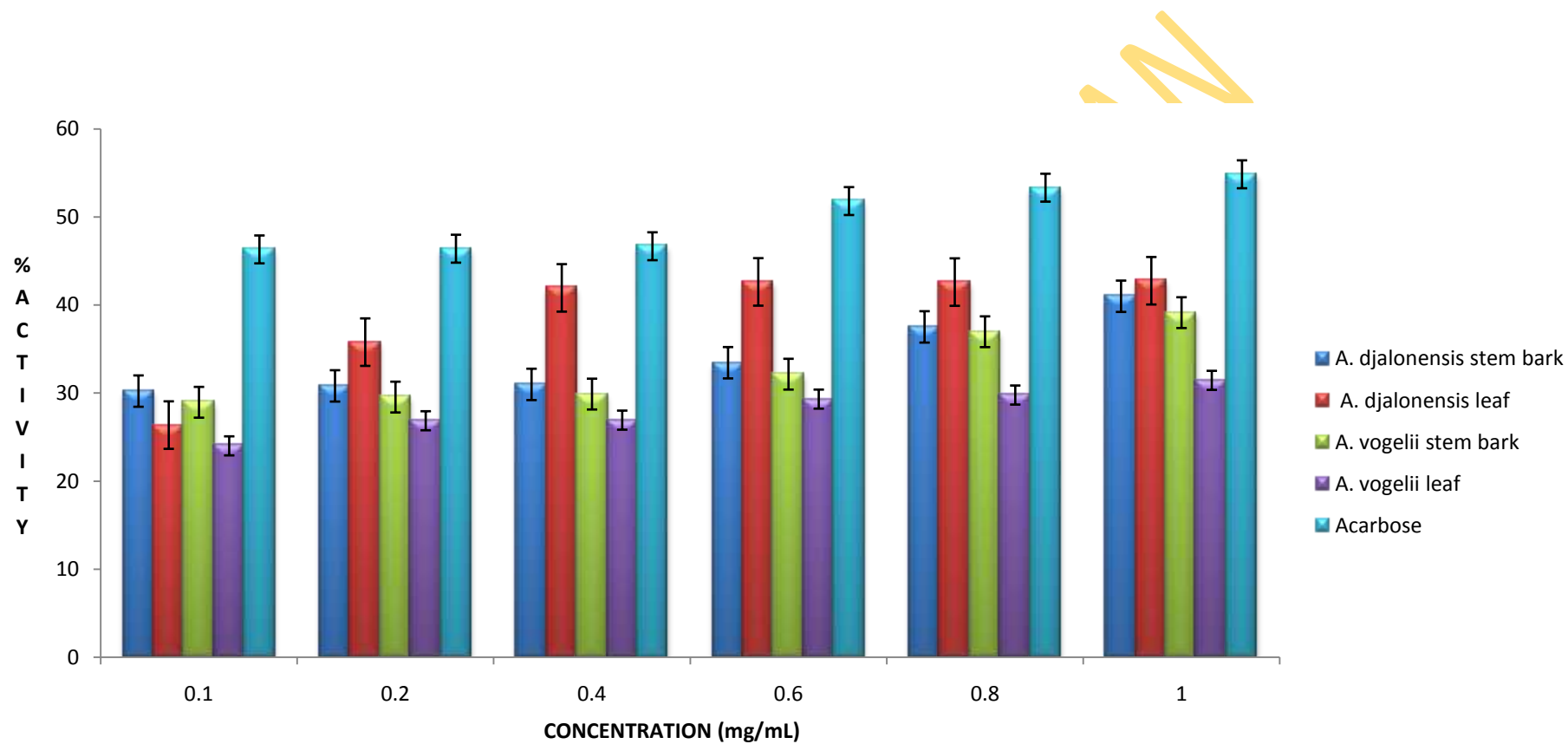
Fig. 4.2. Alpha-amylase inhibition of *Anthocleista djalonensis*, *Anthocleista vogelii* crude aqueous methanol extracts and acarbose

Values are mean ± SEM, n = 3 significantly different from control at P<0.05, Λ_{max} = 540 nm.
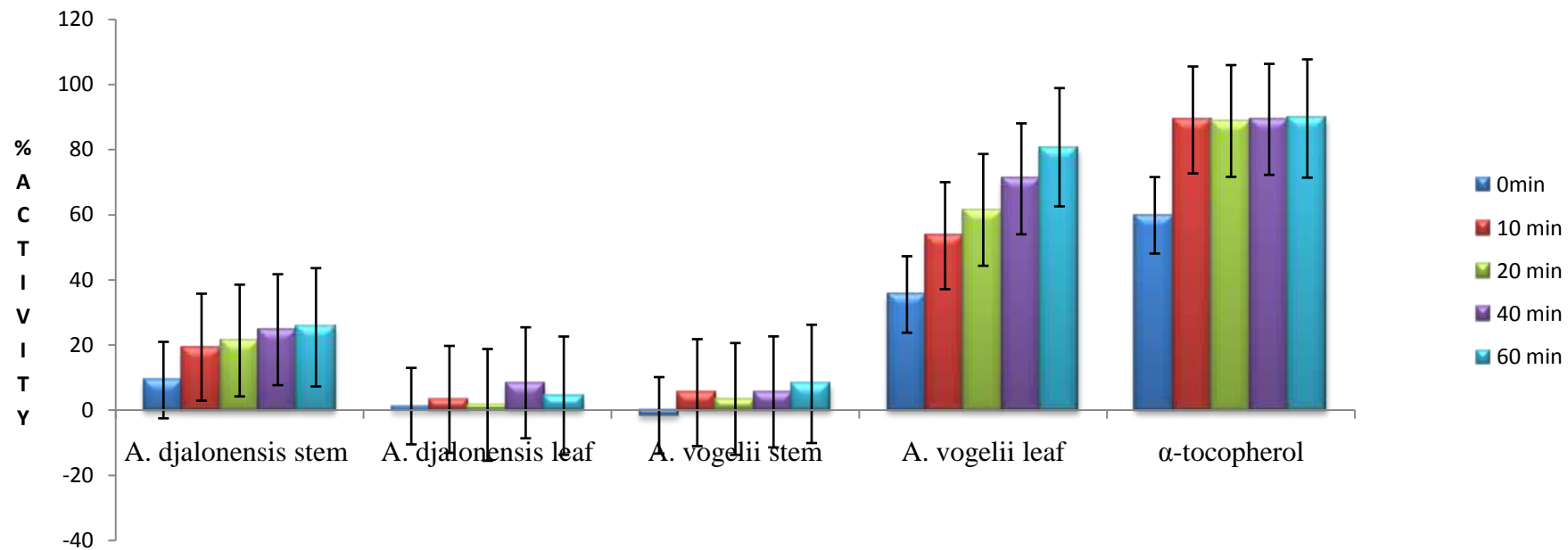
Fig. 4.3. Anti-oxidant activities of the crude aqueous methanol extracts of *Anthocleista djalonensis*, *Anthocleista vogelii* and alpha-tocopherol at 1 mg/mL

Values are mean ± SEM, n = 3 significantly different from control at P<0.05, Λ_{max} = 517 nm.

Table 4.4. Effect of *Anthocleista djalonensis* Crude Aqueous Methanol Leaf Extract (1 g/kg) on Blood Glucose Level (mg/dL) of Alloxan-induced Diabetic Rats

Groups	Day 1				Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	0 min	1 h	2 h	3 h						
Non-diabetic rats	41.3 ±0.67	41.3 ±0.67	43.7 ±2.33	40.3 ±0.33	83.7 ±11.29	56.7 ± 5.61	71 ± 5.51	55.3 4.63±	50.7 ±6.69	67 ±3.60
Diabetic Untreated	253.6 ±5.44	294.0 ±5.41	258.6 ±5.53	259.6 ±3.85	247.6 ±4.96	261.0 ±5.24	273.6 ±5.07	283.6 ±5.14	289.3 ±5.3	294.6 ±5.35
Diabetic treated (1 g/kg)	302.6 ±8.42	280.0 ±5.27	396.3 ±7.23	412.3 ±8.92	267.3 ±5.38	329.0 ±7.82	246.3* ±7.04	279.0* ±7.66	204.0* ±7.0	183.6* ±7.65
Glibencla-mide (2.5 mg/kg)	388.3 ±7.27	327.6 ±6.23	380.0 ±6.44	375.3 ±7.12	350.6 ±6.41	338.6 ±5.81	309.6 ±6.02	175.3* ±4.32	157* ±4.12	125.6* ±3.20

Values are mean ± SEM n=5, * significantly different from control at $P < 0.05$

Table 4.5. Effect of *Anthocleista djalonensis* Crude Aqueous Methanol Stem bark Extract (1 g/kg) on Blood Glucose Level (mg/dL) of Alloxan-induced Diabetic Rats

Groups	Day 1				Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	0 min	1 h	2 h	3 h						
Non-diabetic rats	41.3 ±0.67	41.3 ± 0.67	43.7 ±2.33	40.3 ±0.33	83.7 ±11.29	56.7 ± 5.61	71 ± 5.51	55.3 4.63±	50.7 ±6.69	67 ±3.60
Diabetic untreated	253.6 ±5.44	294.0 ± 5.41	258.6 ±5.53	259.6 ±3.85	247.6 ±4.96	261.0 ±5.24	273.6 ±5.07	283.6 ±5.14	289.3 ±5.3	294.6 ±5.35
Diabetic treated (1 g/kg)	217.6 ±3.13	159.6* ±5.0	225.3* ±9.40	259.3 ±3.80	137.0* ±3.44	125.0* ±4.23	147.6* ±6.63	86.0 * ±2.70	79.3 * ±2.61	98.3 * ±2.82
Glibenclamide (2.5 mg/mL)	388.3 ±7.27	327.6 ±6.23	380.0 ±6.44	375.3 ±7.12	350.6 ±6.41	338.6 ±5.81	309.6 ±6.02	175.* ±4.32	157 * ±4.12	125.6* ±3.20

Values are Mean ± SEM, n=5, * significantly different from control at $P < 0.05$

Table 4.6.   Effect of *Anthocleista djalonensis* Crude Aqueous Methanol Root Extract (1 g/kg), on Blood Glucose Level (mg/dL) of Alloxan-induced Diabetic Rats

Groups	Day 1				Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	0 min	1 h	2 h	3h						
Non-diabetic rats	41.3 ±0.67	41.3 ±0.67	43.7 ±2.33	40.3 ±0.33	83.7 ±11.29	56.7 ±5.61	71 ±5.51	55.3 4.63±	50.7 ±6.69	67 ±3.60
Diabetic untreated	253.6 ±5.44	294.0 ±5.41	258.6 ±5.53	259.6 ±3.85	247.6 ±4.96	261.0 ±5.24	273.6 ±5.07	283.6 ±5.14	289.3 ±5.3	294.6 ±5.35
Diabetic treated (1 g/kg)	256.0 ±4.54	279.3 ±6.70	202.6* ±4.92	286.3 ±4.3	196.3 * ±5.35	245.6* ±6.15	192.0* ±5.38	205.6* ±6.07	152.0* ±4.27	151.6 * ±4.31
Glibencla-mide (2.5 mg/mL)	388.3 ±7.27	327.6 ±6.23	380.0 ±6.44	375.3 ±7.12	350.6 ±6.41	338.6 ±5.81	309.6 ±6.02	175.* ±4.32	157 * ±4.12	125.6* ±3.20

Values are mean ± SEM n= 5, * significantly different from control at $P<0.05$
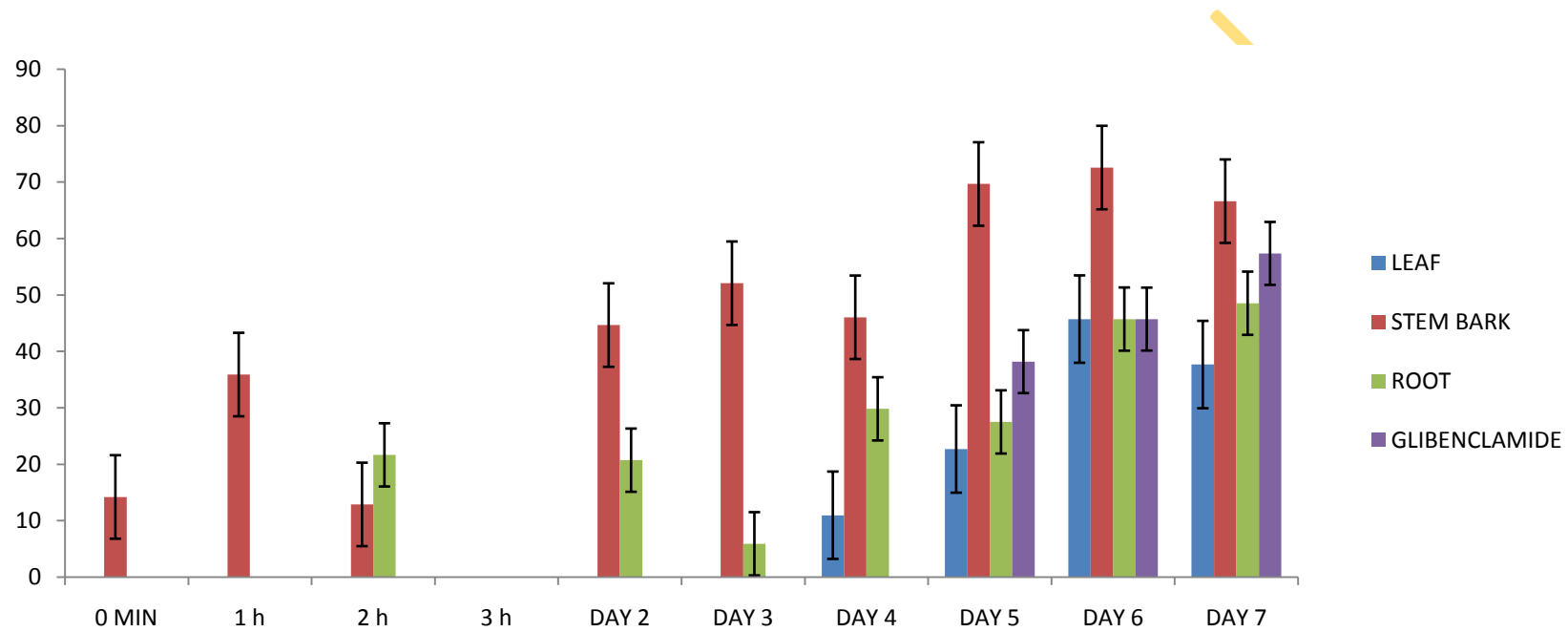
Fig. 4.4. Percentage reduction in blood glucose levels (mg/dL) of rats treated with *Anthocleista djalonensis* crude aqueous methanol extracts (1 g/kg)

Values are mean ± SEM, n= 5, * significantly different from control at P<0.05

Table 4.7. Effect of *A.vogelii* Crude Aqueous Methanol Leaf Extract (1 g/kg), on Blood Glucose Level (mg/dL) of Alloxan-induced Diabetic Rats

Groups	Day 1				Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	0 min	1 h	2 h	3 h						
Non-diabetic rats	41.3 ±0.67	41.3 ±0.67	43.7 ±2.33	40.3 ±0.33	83.7 ±11.29	56.7 ±5.61	71 ±5.51	55.3 4.63±	50.7 ±6.69	67 ±3.60
Diabetic untreated	255.4 ±6.60	288.2 ±16.52	266.3 ±11.20	273.9 ±10.65	285.1 ±28.68	294.5 ±35.18	274 ±44.39	284.2 ±45.61	289.8 ±48.62	295.1 ±49.74
Diabetic treated (1 g/kg)	302.6 ± 5.80	280 ± 5.77	264.5 ±11.06	239 ±17.68	265.3 ±42.17	216 ±55.24	181.3 ±44.48	119 * ±37.64	137.3* ±29.72	117 * ±12.22
Glibencl-amide (2.5mg/kg)	390.4 ±2.50	339.5 ±17.16	380 ±5.55	365.9 ± 5.13	284 ±18.15	272 ±34.31	243 ±24.70	175.3* ±28.35	150.3* ±22.58	122.7* ±22.70

Values are mean ± SEM n= 5, * significantly different from control at $P<0.05$

Table 4.8.   Effect of *Anthocleista vogelii* Crude Aqueous Stem bark Extract (1 g/kg), on Blood Glucose Level (mg/dL) of Alloxan-induced Diabetic Rats

Groups	Day 1				Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	0min	1 h	2 h	3h						
Non-diabetic rats	41.3 ±0.67	41.3 ±0.67	43.7 ±2.33	40.3 ±0.33	83.7 ±11.29	56.7 ±5.61	71 ±5.51	55.3 4.63±	50.7 ±6.69	67 ±3.60
Diabetic untreated	255.43 ±6.60	288.21 ±16.52	266.3 ±11.20	273.87 ±10.65	285.13 ±28.68	294.53 ±35.18	274 ±44.39	284.23 ±45.61	289.77 ±48.62	295.1 ±49.74
Diabetic treated (1 g/kg)	240.7 ±12.08	159.5* ± 5.77	225.3* ± 5.77	242.6 ± 8.76	137.1* ±20.61	125.1* ±31.00	111.2 ±39.42	86.2 * ±12.48	98 * ±10.44	91.8* ±10.98
Glibencl-amide (2.5mg/kg)	390.4 ±2.50	339.5 ±17.16	380 ±5.55	365.9 ± 5.13	284 ±18.15	272 ±34.31	243 ±24.70	175.3* ±28.35	150.3* ±22.58	122.7* ±22.70

Values are mean $\pm$ SEM n= 5, * significantly different from control at $P<0.05$

Table 4.9. Effect of *Anthocleista vogelii* Crude Aqueous Methanol Root Extract (1 g/kg), on Blood Glucose Level (mg/dL) of Alloxan-induced Diabetic Rats

| Groups	Day 1				Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	0 min	1 h	2 h	3 h						
Non-diabetic rats	41.3 ±0.67	41.3 ±0.67	43.7 ±2.33	40.3 ±0.33	83.7 ±11.29	56.7 ±5.61	71 ±5.51	55.3 4.63±	50.7 ±6.69	67 ±3.60
Diabetic untreated	255.3 ±6.60	288.2 ±16.52	266.3 ±11.20	273.9 ±10.65	285.1 ±28.68	294.5 ±35.198	274 ±44.39	284.2 ±45.61	289.8 ±48.62	295.1 ±49.74
Diabetic treated (1 g/kg)	254 ± 4.16	279.03 ± 5.81	202.* ± 5.74	247.7 ±10.83	192.6* ± 6.13	179.1* ± 19.56	192.5 ±50.13	162.2* ±34.13	185.3 ±21.11	151.9* ±32.15
Glibencl-amide (2.5mg/kg)	390.4 ±2.50	339.5 ±17.16	380 ±5.55	365.9 ± 5.13	284 ±18.15	272 ±34.31	243 ±24.70	175.3* ±28.35	150.3* ±22.58	122.7* ±22.70

Values are mean ± SEM n= 5, * significantly different from control at $P<0.05$
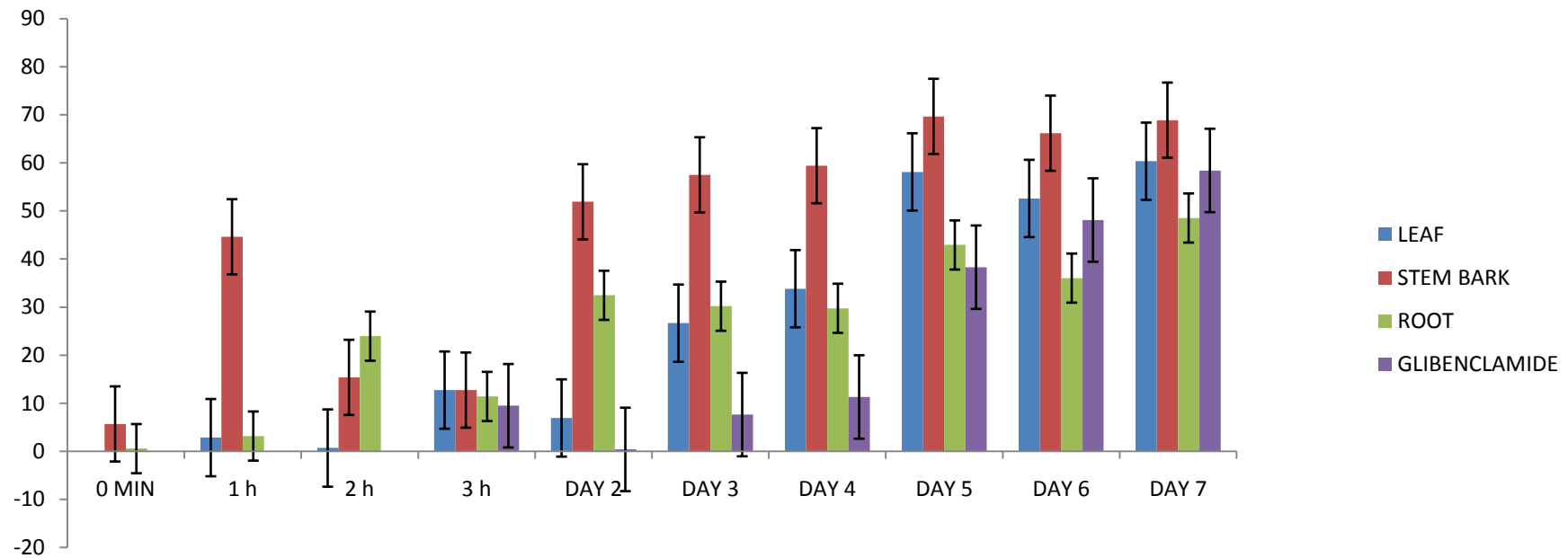
Values are mean ± SEM, n= 5, * significantly different from control at P<0.05

Fig. 4.5. Percentage reduction in blood glucose levels (mg/dL) of rats treated with *Anthocleista vogelii* crude aqueous methanol extracts (1 g/kg)
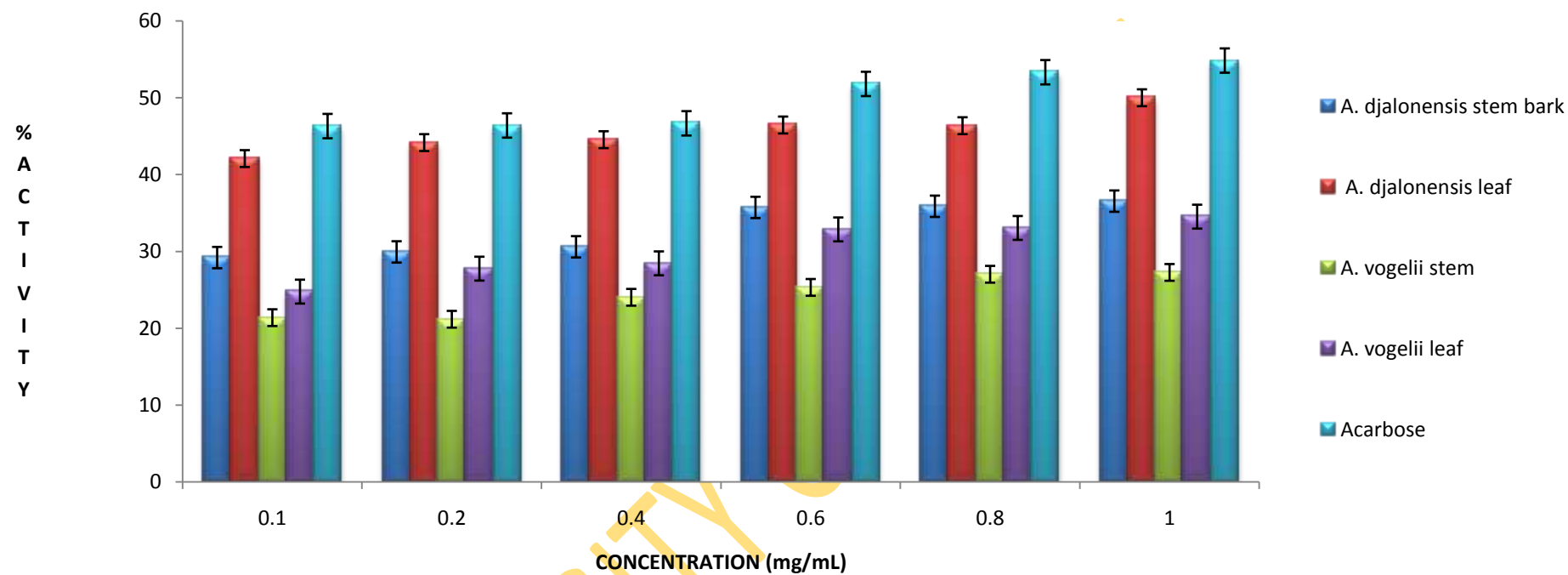
Fig. 4.6. Alpha-amylase inhibition of *Anthocleista djalonensis*, *Anthocleista vogelii* ethyl acetate fractions and acarbose

Values are mean ± SEM, n = 3 significantly different from control at P<0.05, $Λ_{max}$= 540 nm
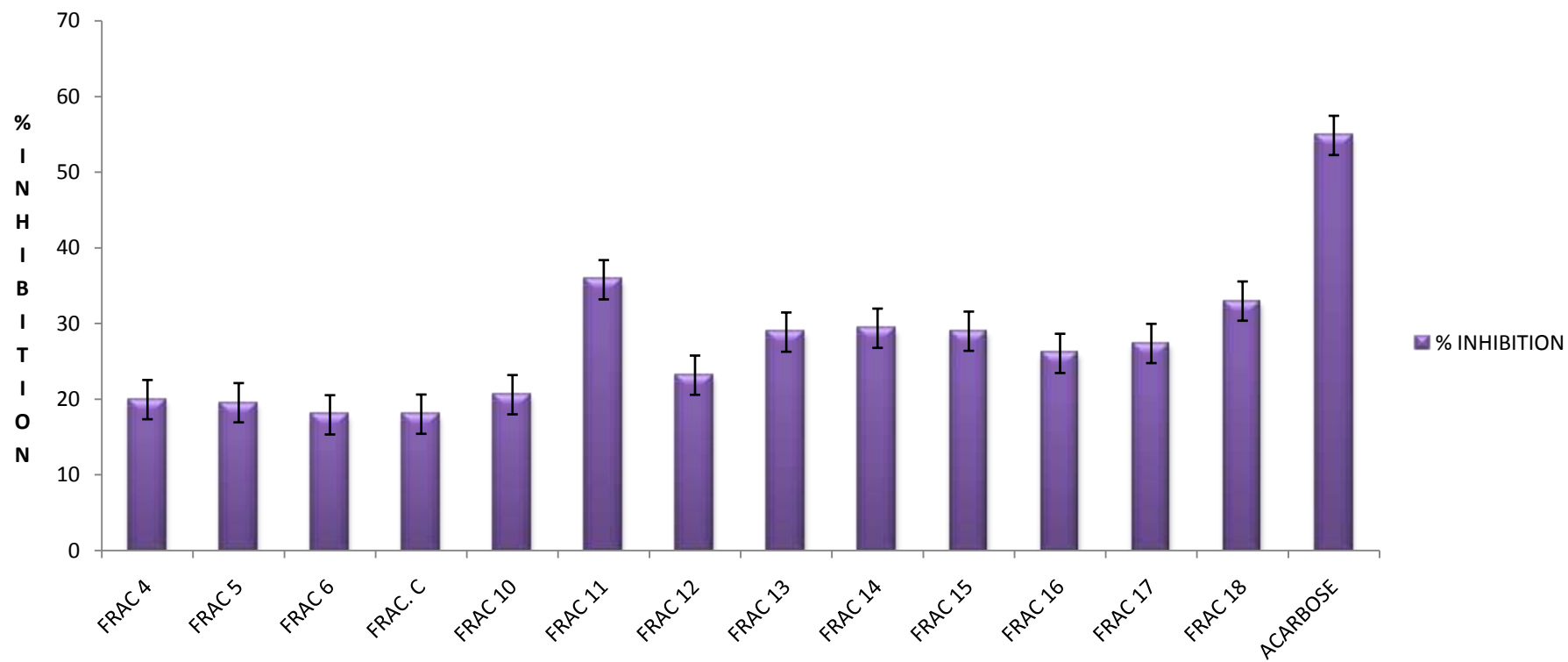
Fig. 4.7. Alpha-amylase inhibition of *Anthocleista djalonensis* stem bark column fractions and acarbose

Values are mean ± SEM, n = 3, significantly different from control at $P<0.05$, $\Lambda_{max} = 540$ nm
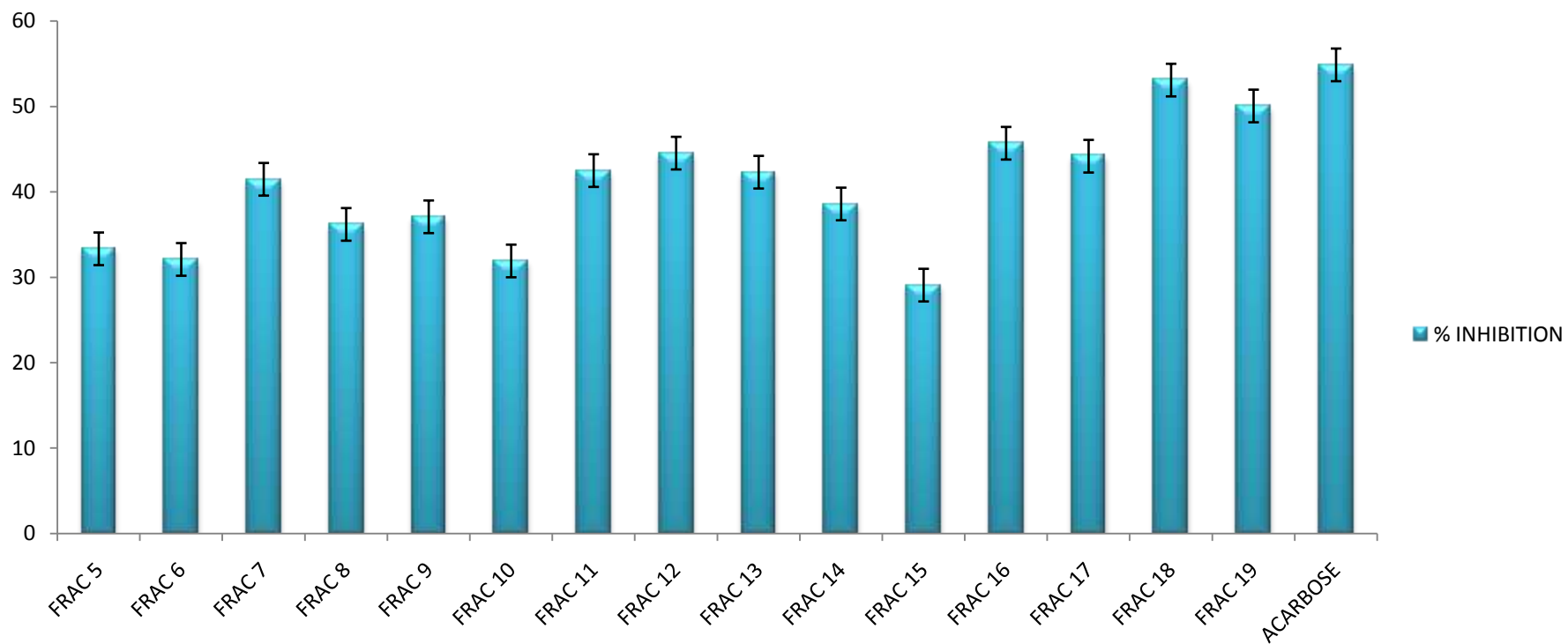
Fig. 4.8. Alpha-amylase inhibition of *Anthocleista djalonensis* leaf column fractions and acarbose

Values are mean ± SEM, n = 3, significantly different from control at P<0.05, $Ʌ_{max}$ = 540 nm
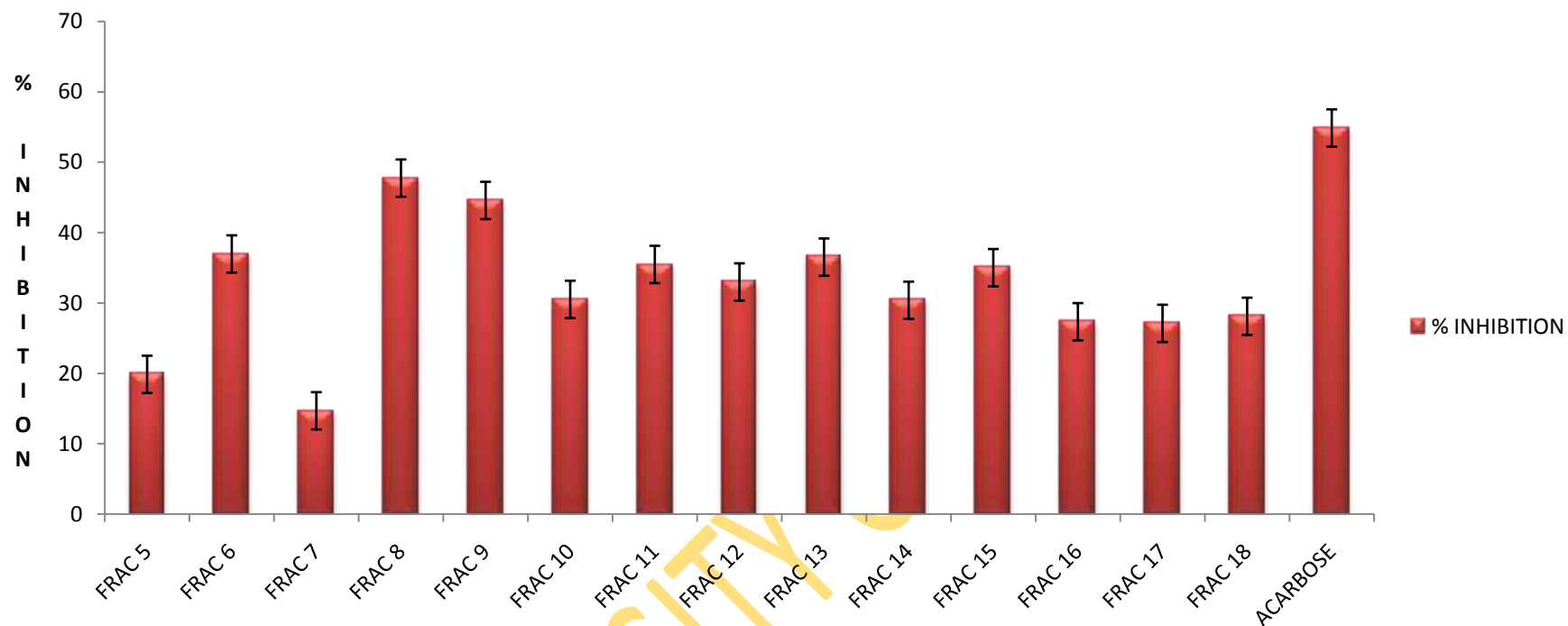
Fig. 4.9. Alpha-amylase inhibition of *Anthocleista vogelii* stem bark column fractions and acarbose

Values are mean ± SEM, n = 3, significantly different from control at P<0.05, $Λ_{max}$ = 540 nm
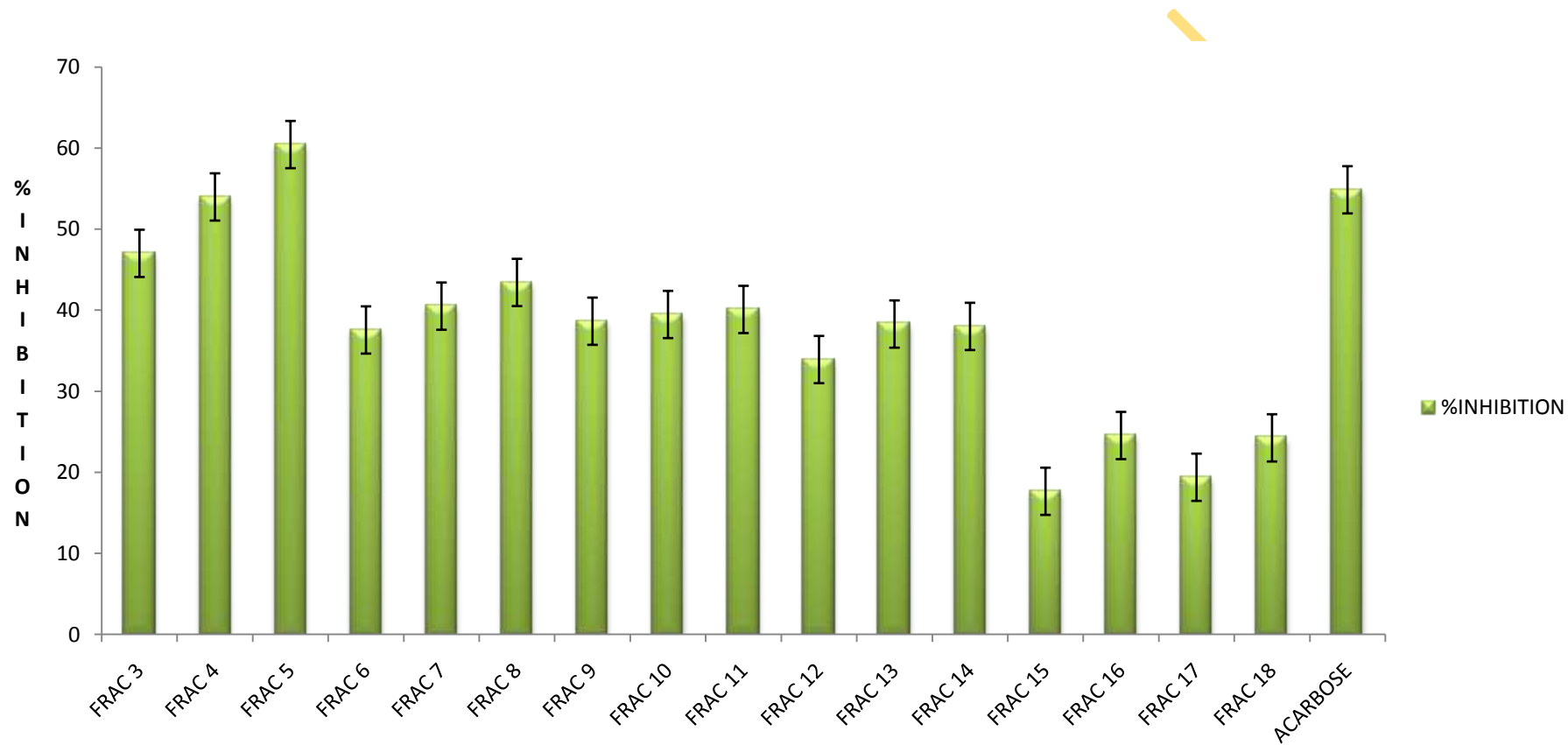
Fig. 4.10. Alpha-amylase inhibition of *Anthocleista vogelii* leaf column fractions and acarbose

Values are mean ± SEM, n = 3, significantly different from control at P<0.05, $Λ_{max}$ = 540 nm
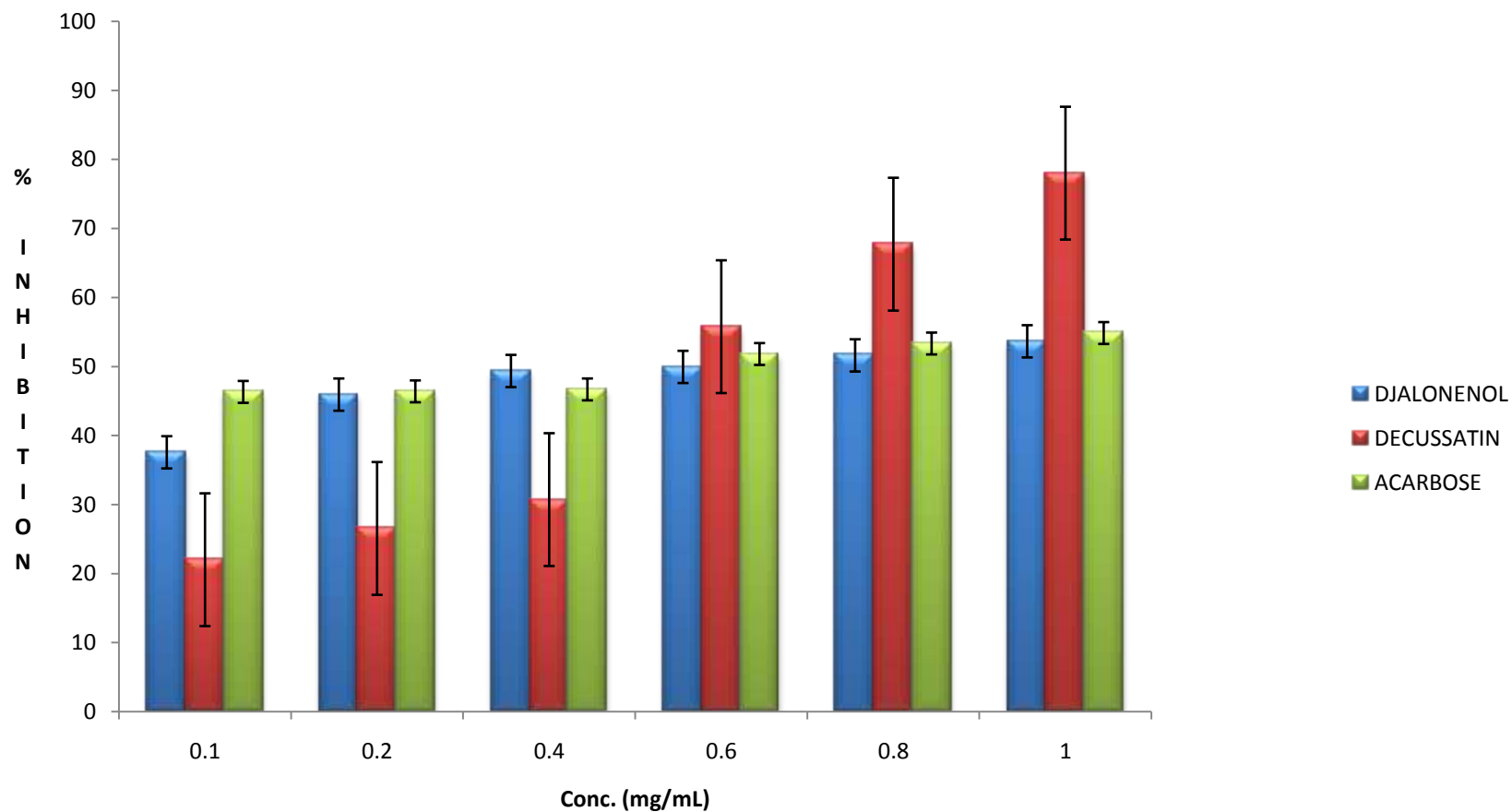
Fig. 4.11. Alpha-amylase inhibition of isolated compounds and acarbose at different concentrations

Values are mean ± SEM, n = 3 significantly different from control at P<0.001, Λ_{max} = 540 nm

Table 4.10. Alpha-amylase Inhibition of the Isolated Compounds Compared with Acarbose at 1 mg/mL

ISOLATED COMPOUNDS	X_1	X_2	X_3	MEAN X $\pm$ SEM	% INHIBITION	P<0.001
DJALONENOL	0.303	0.3188	0.3102	0.3107 ± 0.005	53.7	0.00000697
DECUSSATIN	0.1653	0.131	0.1225	0.1396 ± 0.013	78.0	0.00000913
ACARBOSE	0.3983	0.4096	0.4288	0.4122 ± 0.08	54.9	0.00023

X_1, X_2, X_3 = Absorbance values at Λ_{max} = 540 nm

Mean X = mean of absorbance values
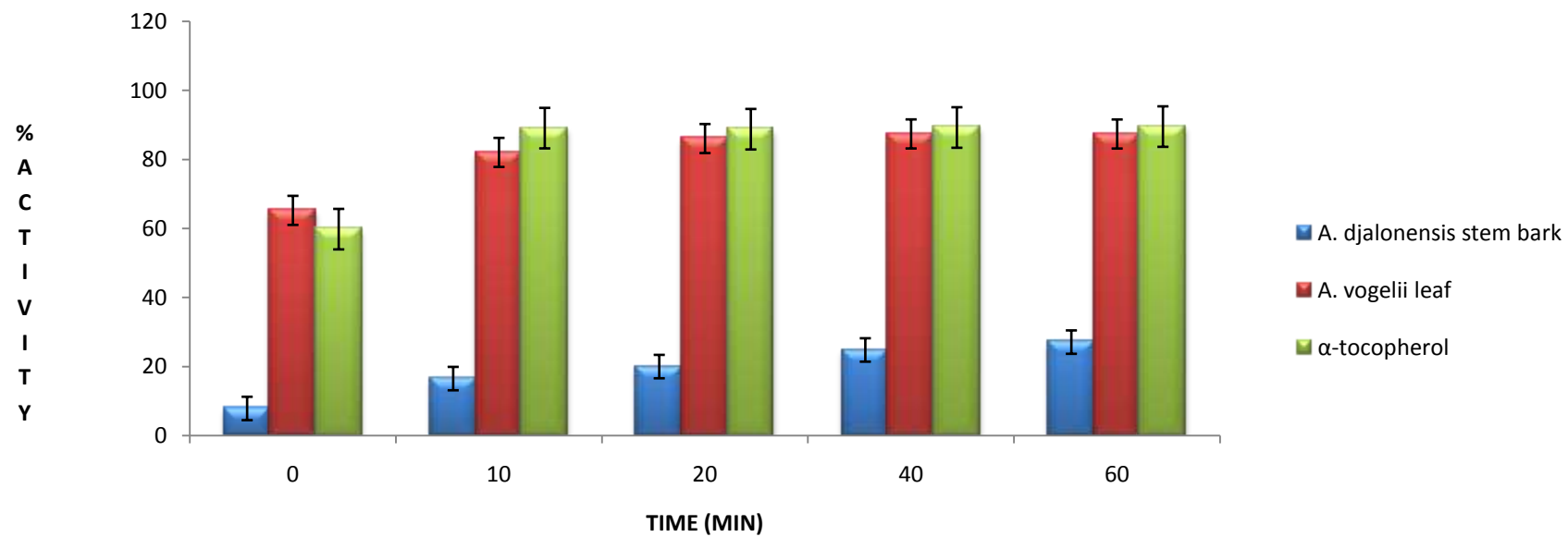
Fig. 4.12. Anti-oxidant activities of the ethyl acetate fractions of *Anthocleista djalonensis* stem bark, *Anthocleista vogelii* leaf and alpha-tocopherol at 1mg/mL

Values are mean ± SEM, n = 3 significantly different from control at P<0.05, Λ_{max} = 517nm
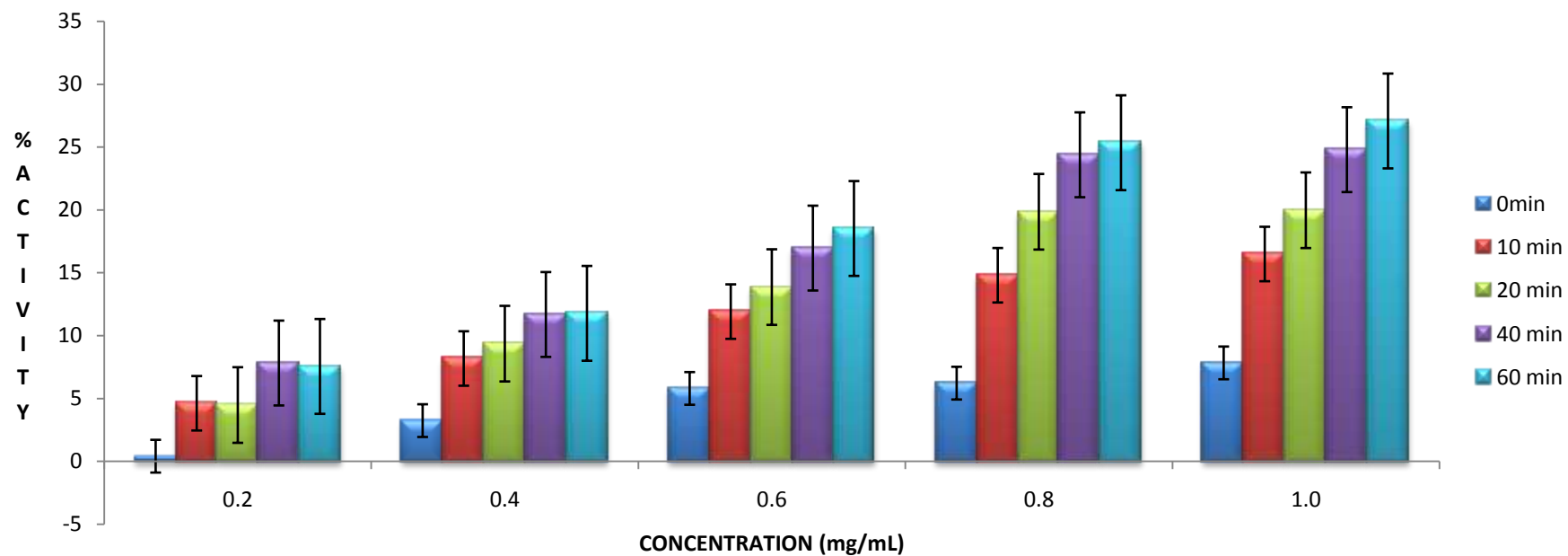
Fig. 4.13. Anti-oxidant activities of the ethyl acetate fraction of *Anthocleista djalonensis* stem bark, at different concentrations

Values are mean $\pm$ SEM, n = 3 significantly different from control at P<0.05, Λ_{max} = 517 nm
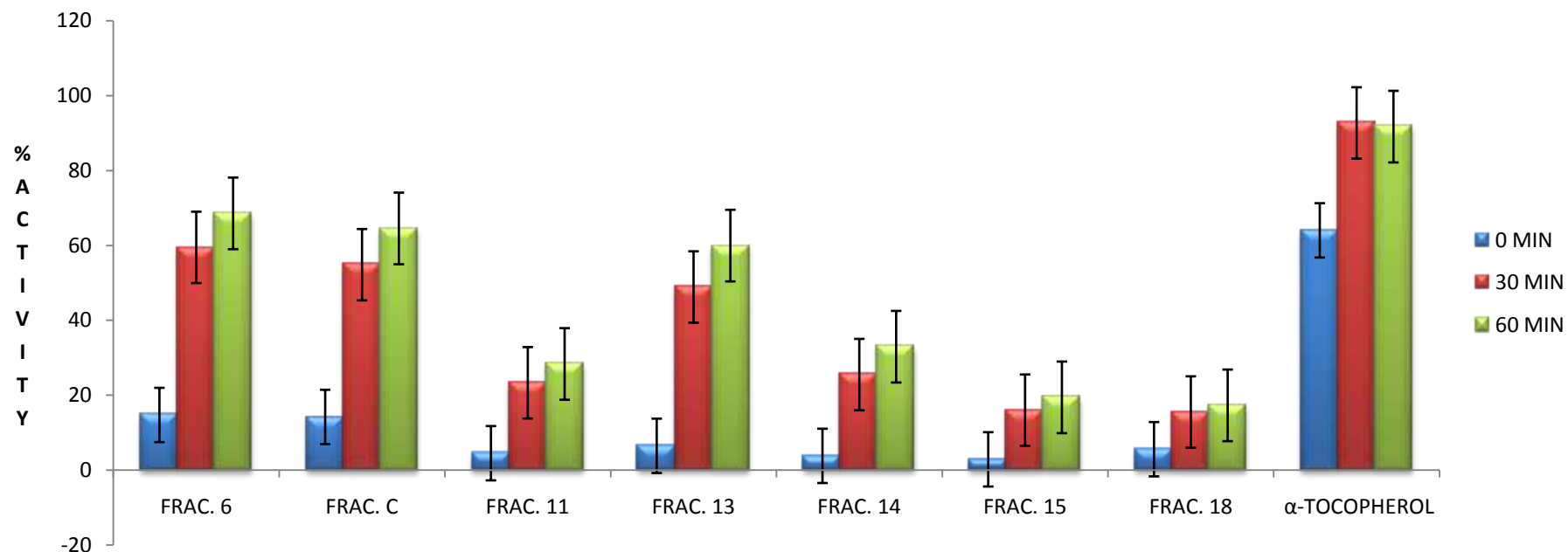
Fig. 4.14. Anti-oxidant activities of the column fractions of *Anthocleista djalonensis* stem bark and alpha-tocopherol, at 1 mg/mL

Values are mean ± SEM, n = 3 significantly different from control at P<0.05, Λ_{max} = 517nm
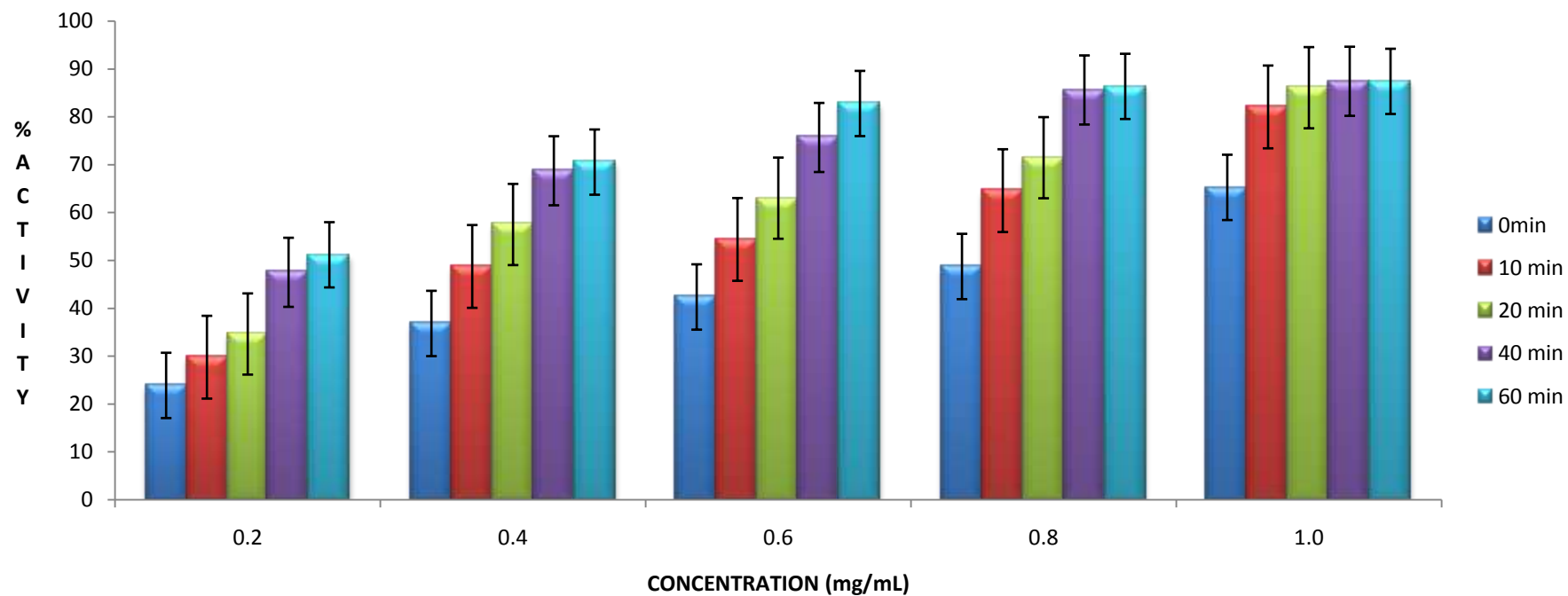
Fig. 4.15 Anti-oxidant activities of the ethyl acetate fraction of *Anthocleista vogelii* leaf, at different concentrations

Values are mean ± SEM, n = 3 significantly different from control at P<0.05, $Λ_{max}$ = 517nm
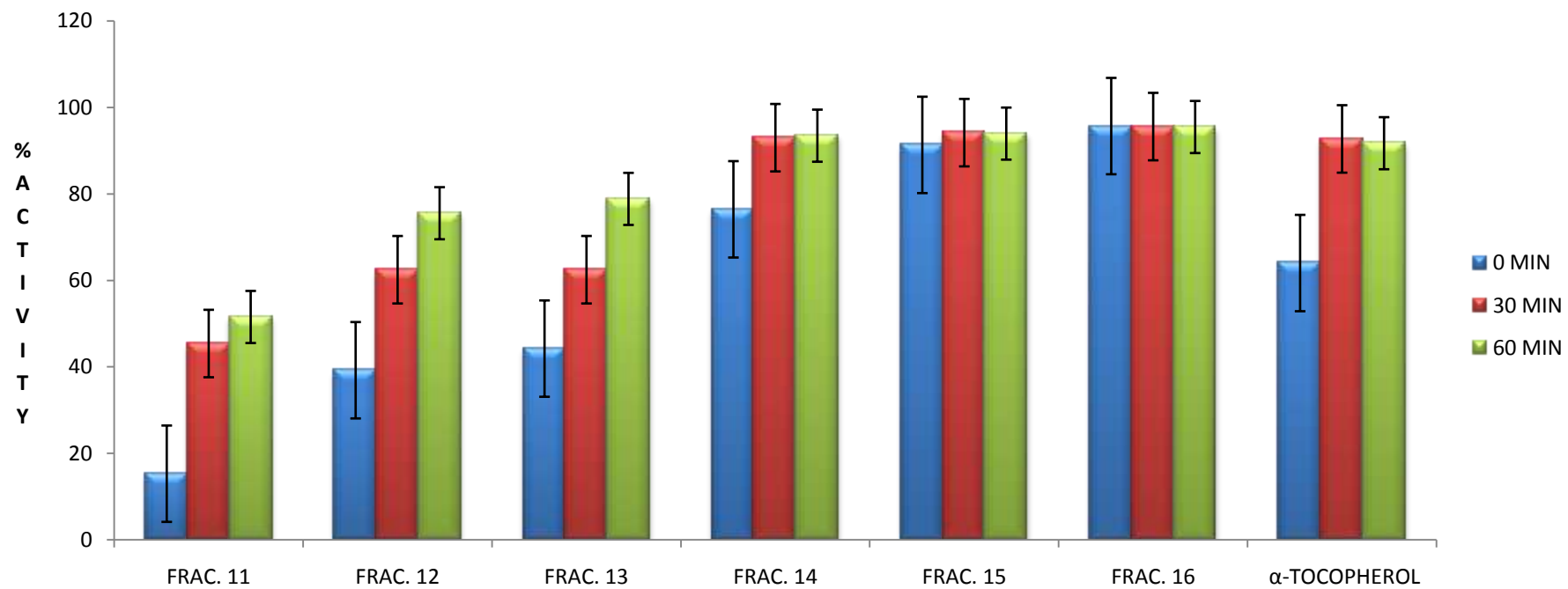
Fig. 4.16 Anti-oxidant activities of the column fractions of *Anthocleista vogelii* leaf, at 1 mg/mL

Values are mean ± SEM, n = 3 significantly different from control at P<0.05, $λ_{max}$ = 517nm
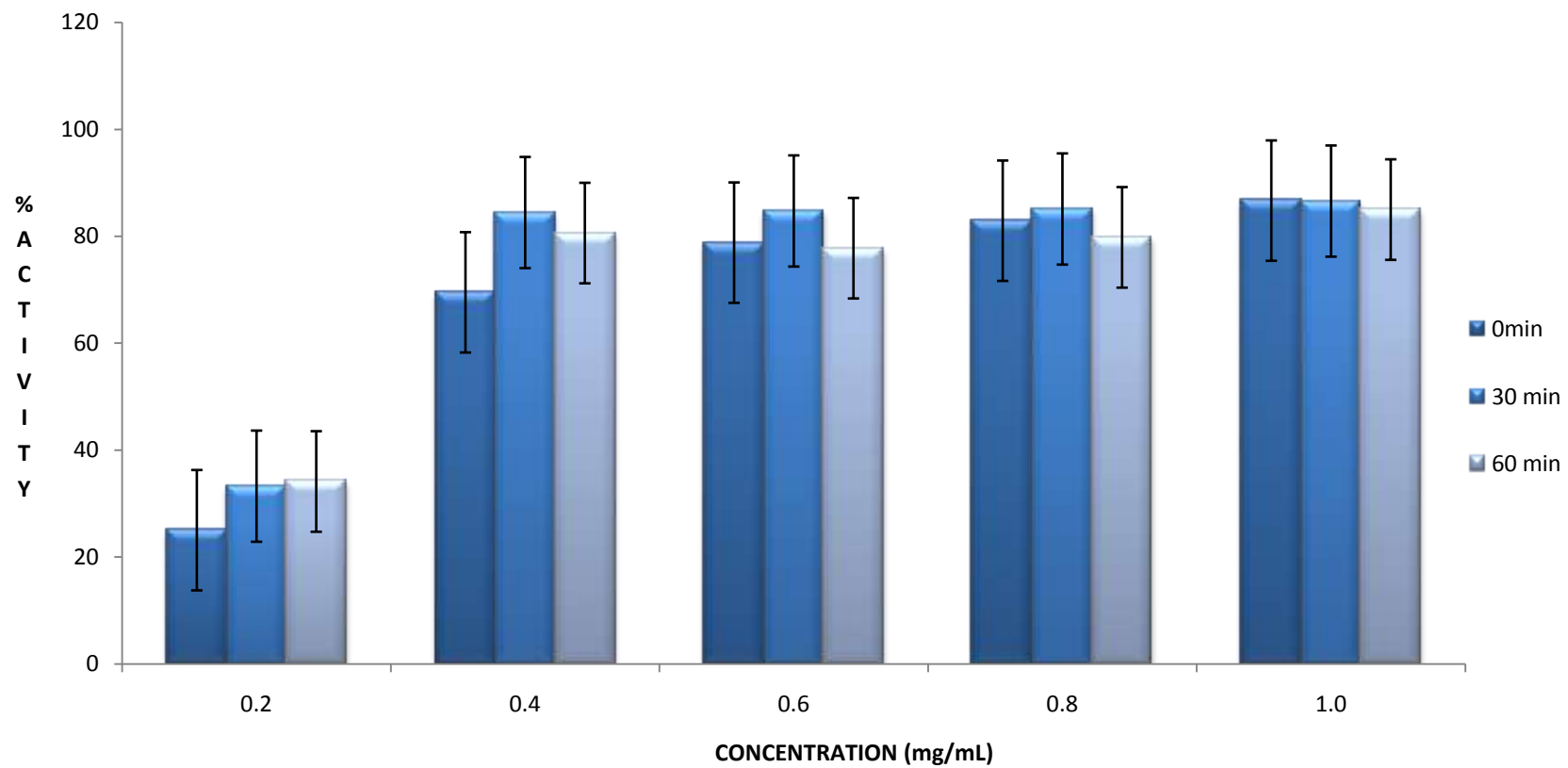
Fig. 4.17. Anti-oxidant activity of Eluate 16 of *Anthocleista vogelii* leaf, at different concentrations

Values are mean ± SEM, n = 3 significantly different from control at $P<0.05$, $\Lambda_{max} = 517$ nm
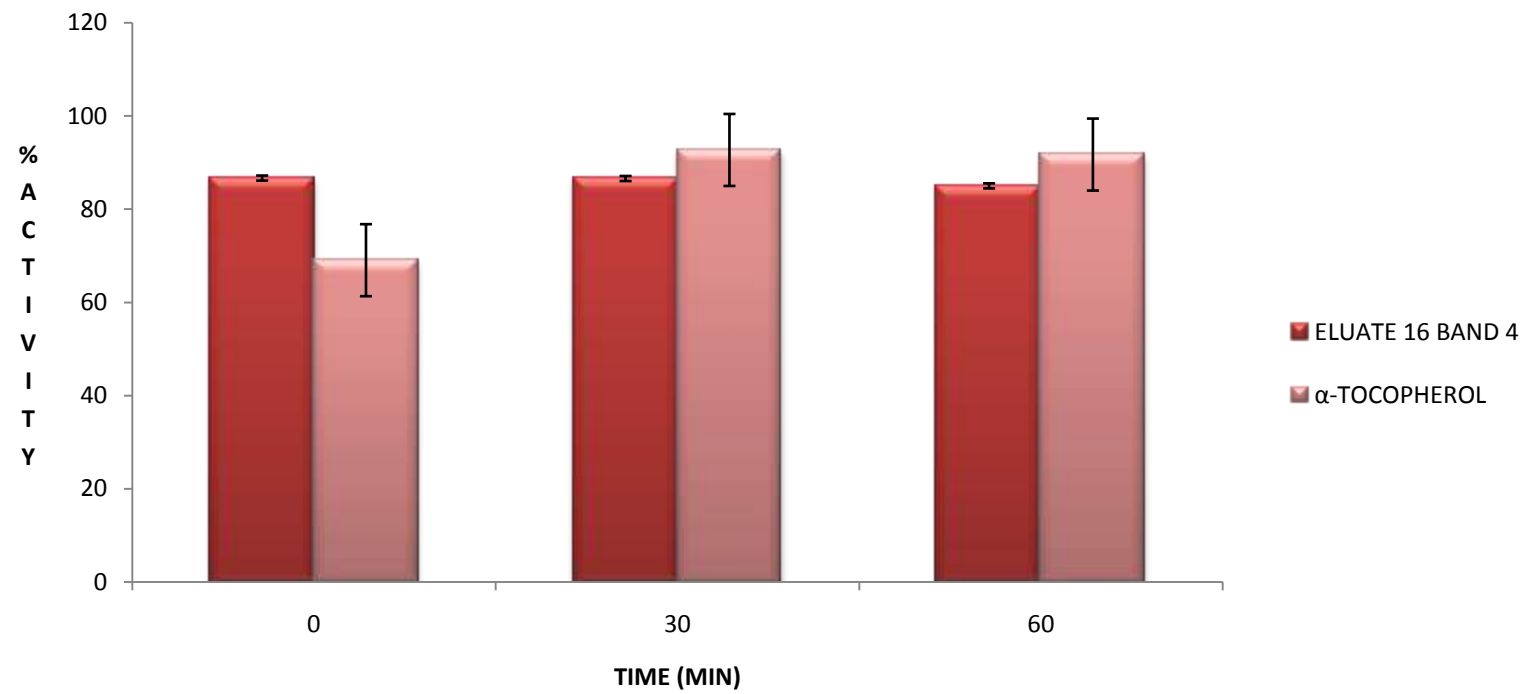
Fig. 4.18. Anti-oxidant activity of Eluate 16 band 4 of *Anthocleista vogelii* leaf and α-tocopherol at1 mg/mL.

Values are mean ± SEM, n = 3 significantly different from control at P<0.05, $Λ_{max}$ =517nm

4.3 Spectral Characterization of Isolated Compounds

4.3.1 Djalonenol: tetrahydro-4-(1-hydroxybut-3-en-2-yl)-3(hydroxymethyl)pyran-2-one.

This is a new alpha-amylase inhibitor from the stem bark *A. djalonensis*. It is a clear yellowish liquid which gave a 53.65% alpha-amylase inhibition (P=0.00000697) at 1 mg/mL. By comparing spectra with literature (Onocha *et al*., 1995), the structure of the compound was elucidated and identified as Djalonenol R_f=0.48 (silica gel, DCM:EtOAc:MeOH 4:5:1). It gave a pink colour in UV and faint yellow colour when sprayed with methanol solution of 2,2'-Diphenyl-1-picrylhydrazyl (DPPH).

The IR ν_{max} cm^{-1} data of the compound showed major peaks at 3380, 2923, 1710, 1477, 1404, 1265,1072 924, which were consistent with those observed by Onocha *et al*., 1995). The presence of characteristic stretching vibrations corresponding to C=O (1710cm^{-1}), O-H (3380 cm^{-1}), C-H (2923, 1477 and 1404 cm^{-1}) stretching and bending vibrations characteristic of sp^3 hybridized carbons, and C-O (1072 cm-^{1}) typical of alcohols, further helped in identifying the compound as djalonenol.

The GC-MS data were obtained using GC-EIMS. The mass spectrum of djalonenol ($C_{10}H_{16}O_4$, MW 200) exhibited ions at *m/z* 170 (loss of $H_2C = O$ due to McLafferty rearrangement) and *m/z* 152 (further loss of H_2O), 127, 79 and 41. This was consistent with the mass spectrum of djalonenol isolated from *Tachia grandiflora* by Silver *et al*. (2013).

^{1}HNMR and ^{13}CNMR data (600 MHz $(CD_3)_2CO$/TMS) of the compound compared well with that of Onocha *et al*. (1995) as shown in Table 4.11.

Table 4.11. Comparison of ^{1}H and ^{13}C NMR data of Djalonenol with literature

POSITION	^{1}H δ (ppm)	^{1}H δ (ppm)*	^{13}C δ (ppm)		^{13}C δ (ppm)*	
			C-2	172.88	C-2	176.2
H-3	1.96 (1H,m)	1.98 (1H,m)	C-3	46.79	C-3	48.2
H-4	2.35 (1H, m)	2.26 (1H,m)	C-4	33.90	C-4	34.9
H-5	1.74 (2H,m)	1.76 (1H, m)	C-5	26.08	C-5	27.1
H-6	4.30 (2H,m)	4.35 (2H,m)	C-6	67.05	C-6	68.9
H-7	5.81 (1H, m)	5.74 (1H,m)	C-7	138.36	C-7	138.5
H-8	2.70 (1H, m)	2.69 (1H,m)	C-8	49.58	C-8	50.9
H-9	5.13 (2H, dd)	5.13 (2H, dd)	C-9	116.67	C-9	118.5
H-10	3.80 (2H, dd)	3.72 (2H, dd)	C-10	62.28	C-10	63.2
H-11	3.94 (2H,dd)	4.07 (2H, dd)	C-11	63.04	C-11	63.9

*NMR data for djalonenol from Onocha *et al*., 1995.
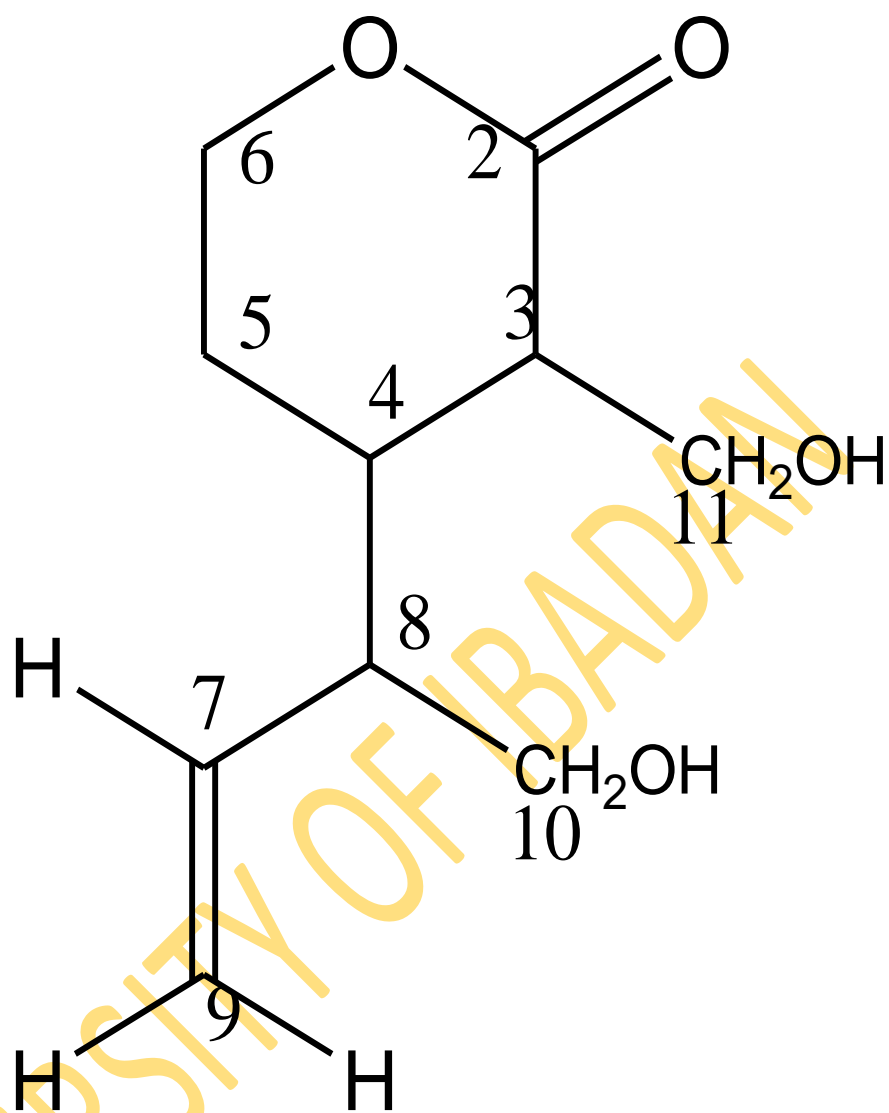
Fig.4.19: Djalonenol [tetrahydro-4-(1-hydroxybut-3-en-2-yl)-3(hydroxymethyl)pyran-2-one].

4.3.2 Benzoic Acid from Fraction C27 of *Anthocleista djalonensis* Stem bark

A whitish substance was isolated from fraction C27 of *Anthocleista djalonensis* stem bark, $R_f = 0.69$ (silica gel, DCM: EtOAc: MeOH 4:5:1). The TLC spot was pink under UV 254 nm and gave an instant yellow colour when sprayed with DPPH indicating that it had anti-oxidant properties.

The IR data of compound C27 showed major peaks at 3280, 2923.60, 1710, 1603.59, 1584.52, 1495.38, 1424.04, 1265.02, 1193.85, 1072.97 cm^{-1}. The peak at 3280cm^{-1} is characteristic of an O-H stretching vibration, the absorption band seen at 1710cm^{-1} is due to the presence of a C=O stretching vibration of an acid, while the two peaks observed at 1265.02 and 1072.97cm^{-1} are for the C-O stretching vibrations, finally the peaks corresponding to C-H stretch of a benzene ring are seen at 1603.59-1424.04 cm^{-1}. These IR data helped in identifying C27 as an aromatic acid.

^{1}HNMR and ^{13}CNMR (600 MHz $(CD_3)_2CO$/TMS) data were compared with that of a commercially available standard Benzoic acid 100134, Merck KGaA Darmstadt Germany. The δ (ppm) shifts values compared well with that of the standard thus, leading to the identification of compound C27 as benzoic acid.

Table 4.12. Comparison of ^{1}H NMR and ^{13}C NMR Data of Benzoic Acid with Literature

POSITION	^{1}H δ(ppm)	^{1}H δ(ppm)*	^{13}C δ (ppm)	^{13}C δ (ppm)*
H-1			C-1 129.5	C-1 129.54
H-2	8.04	8.06	C-2 130.7	C-2 130.56
H-3	7.51	7.52	C-3 128.4	C-3 128.43
H-4	7.63	7.64	C-4 132.8	C-4 132.88
H-5	7.51	7.52	C-5 128.4	C-5 129.43
H-6	8.04	8.06	C-6 130.7	C-6 130.56
H-7			C-7 166.7	C-7 166.78

* Benzoic acid 100134, Merck KGaA Darmstadt Germany.

The ^{1}H NMR and ^{13}C NMR data compared well with that of authentic benzoic acid purchased from Merck, Germany.
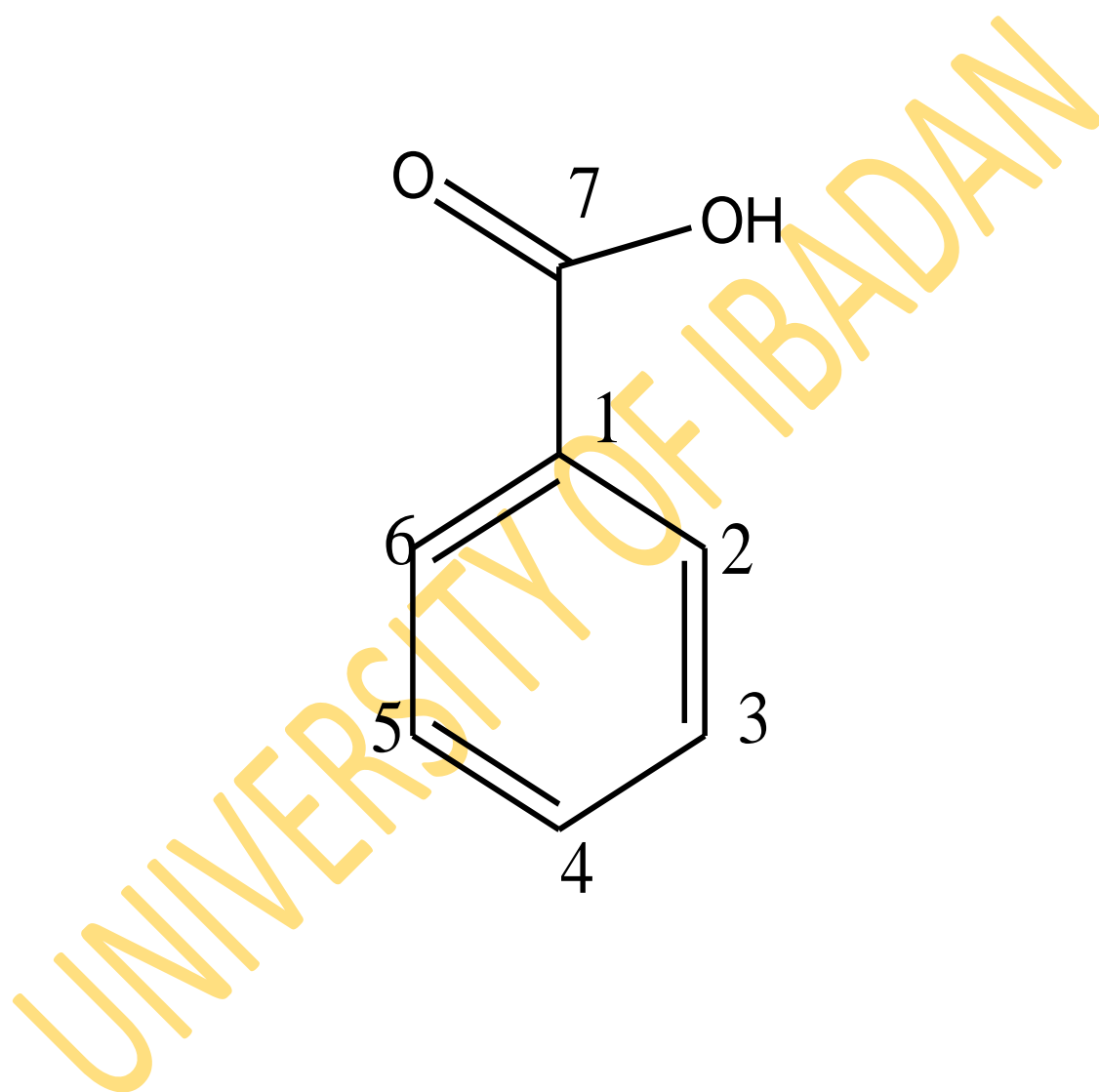
Fig. 4.20. Benzoic acid from *A. djalonensis* stem bark.

4.3.3 Decussatin Crystals from Fraction 5 of *Anthocleista vogelii* Leaf.

Fraction 5 of *A. vogelii* leaf eluted from the column as yellowish green needle-like crystals, which were further purified by recrystallization. Its melting point was 152.3^{o}C-153.9^{o}C [comparable to that given by Okorie (1976) as 152-154°C and Alaribe *et al*. (2012) as 150-154°C], R$_f$ =0.79 (silica gel, DCM: EtOAc: MeOH 4:5:1). TLC had an intense pink colour under UV 254 nm, faint yellow colour on spraying with methanol solution of DPPH and 78.0% alpha-amylase inhibition (P=0.00000913) at 1 mg/mL.

The IR ν_{max} cm^{-1} data showed absorption at 3300, 2916, 1603.2, 1655, 1481.65, 826.96 cm^{-1} for major peaks. The peak at 3300 cm^{-1} is consistent with a structure containing a phenolic O-H forming an intramolecular H-bond with the C=O of a xanthone at 1603 cm^{-1}. Also the sharp peak at 1481.65 cm^{-1} is suggestive of aromatic C-H stretching vibrations. These values compared well with those given by Okorie (1976) and Alaribe *et al*. (2012).

The GC-MS spectrum of Fraction 5 (C$_{16}$H$_{14}$O$_6$ MW 302) exhibited ions at m/z 302 [M$^+$] at run time 32.58 min and 287 [M$^+$ -15), base peak] 259, 227, 201,   171,   143, 122, 79, 51. These values were in agreement with Alaribe *et al*.   (2012) and Silver *et al*. (2013).

Comparing the ^{1}H NMR and ^{13}C NMR data (600 MHz (CDCl$_3$/TMS) with those in literature further helped in identifying the compound. The presence of the phenolic O-H at δ_H 13.28 , the three singlets CH$_3$O at δ_H 3.90-4.02, the two pairs of   aromatic doublets   at positions H3, H4, H5 and H7, with the δ_C at 1 81.17 consitent with that of a xanthone led to the identification of the compound as Decussatin.

Table 4.13. Comparison of ^{1}H NMR and ^{13}C NMR Data of Decussatin with Literature

POSITION	[1]H δ (ppm)	[1]H δ (ppm)*	[13]C δ (ppm)	[13]C δ (ppm)*
Phenolic-OH	13.28(1H,s)	13.24(1H,s)	C-1 151.04	C-1 148.9
			C-2 148.92	C-2 149.3
Ar H-3	7.32 (1H, d)	7.31 (1H,d)	C-3 120.50	C-3 120.5
Ar H-4	7.18 (1H, d)	7.15 (1H,d)	C-4 115.80	C-4 112.8
			C-4a 149.31	C-4a 151.0
Ar H-5	6.31 (1H,d)	6.31 (1H,d)	C-5 96.84	C-5 96.9
			C-5a 157.12	C-5a 157.1
			C-6 166.45	C-6 166.4
Ar H-7	6.34 (1H,d)	6.20 (1H,d)	C-7 92.06	C-7 92.1
			C-8 163.83	C-8 163.9
			C-8a 104.06	C-8a 104.1
			C-9 181.17	C-9 181.2
			C-9a 112.75	C-9a 115.8
OCH$_3$- 10	3.95	3.91 (3H,s)	C-10 61.28	C-10 61.28
OCH$_3$-11	3.90	3.86 (3H, s)	C-11 55.72	C-11 55.8
OCH$_3$-12	4.02	3.90 (3H, s)	C-12 57.2	C-12 57.2

* Alaribe *et al*. (2012).

The ^{1}H NMR and ^{13}C NMR data compared well with that of decussatin isolated by Alaribe *et al*. (2012) from *A. vogelii*.
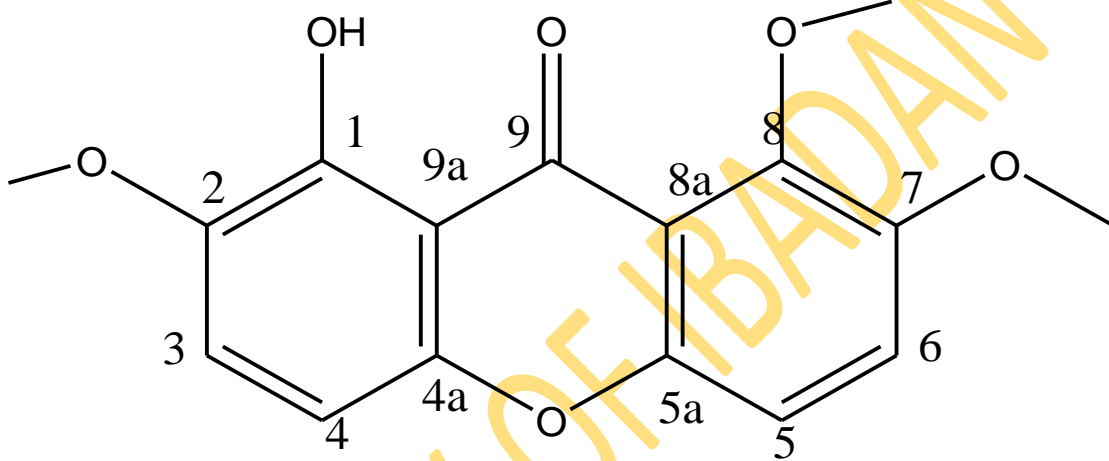
Fig. 4.21. Decussatin (1-hydroxy-2, 7, 8-trimethoxy-9*H*-xanthene-9-one) from *A. vogelii* leaf.

CHAPTER 5

DISCUSSION

The World Health Organization (WHO) estimated that 80% of the population living in the developing countries rely almost exclusively on traditional medicine for their primary health care needs (WHO, 2002). In almost all the traditional medicines, medicinal plants play a major role and constitute the backbone of the traditional medicine (Mukherjee, 2002; Biren, 2010). Diabetes is a major threat to global public health that is rapidly getting worse with the biggest impact on the adults of working age in developing countries (WHO, 2004).

Diabetes is a condition primarily defined by the level of hyperglycaemia giving rise to the risk of microvascular damage (retinopathy, nephropathy and neuropathy). It is associated with reduced life expectancy, significant morbidity due to specific diabetes related microvascular complications, increased risk of macrovascular complications (ischaemic heart disease, stroke and peripheral vascular disease), and diminished quality of life (WHO/IDF, 2006).

Although diabetes is often not recorded as the cause of death, globally, it is believed to be the fifth leading cause of death in 2000 after communicable diseases, cardiovascular disease, cancer and injuries (Roglic *et al*., 2005). The number of cases of non-insulin dependent diabetes mellitus (Type 2) has increased dramatically due to the changes in lifestyle, increasing prevalence of obesity, and ageing of populations.

One therapeutic approach for treating Type 2 diabetes is to decrease postprandial hyperglycaemia. The effective control of blood glucose is the key in preventing or reversing diabetic complications and improving the quality of life for both type 1 and

type 2 diabetic patients. Although different types of oral hypoglycaemic agents are available along with insulin for the treatment of diabetes mellitus, none offers complete glycemic control permanently (Jiang *et al.*, 2003).

Modern medicines such as biguanides, sulfonylureas, and thiozolidinediones are available for the treatment of diabetes. However, they also have undesired effects associated with their uses (Mitra, 2008). Other drugs like alpha-glucosidase inhibitors also called "diabetes pills" such as acarbose, miglitol and voglibose (Bailey, 2003) do not have a direct effect on insulin secretion or sensitivity. These agents which slow down the digestion of starch in the small intestine as well as some peptides analogues including incretins which are insulin secretagogues (Briones and Bajaj, 2006; Gallwitz, 2006) are all associated with adverse effects of weight loss, various gastrointestinal side effects such as abdominal pain, flatulence, nausea and diarrhoea.

Alternative medicines, predominantly herbal drugs are available for the treatment of diabetes. Common perceived advantages of herbal drugs are effectiveness, safety, and acceptability (Fowler, 2007). Some medicinal plants or natural products involve retarding the absorption of glucose by inhibiting the carbohydrate hydrolyzing enzymes, such as pancreatic amylase. The inhibition of this enzyme delay carbohydrate digestion and protract overall carbohydrate digestion time, resulting in the reduction in glucose absorption rate and consequently dulling the postprandial plasma glucose rise. Several indigenous medicinal plants have a high potential in inhibiting α-amylase enzyme activity (Valiathan, 1998).

The α–amylase enzyme is one of the major secretory products of the pancreas and salivary glands, playing a role in digestion of starch and glycogen and can be found in microorganisms, plants and higher organisms (Kandra, 2003). Alpha-Amylase

enzyme catalyses the initial step in hydrolysis of starch to mixture of oligosaccharides consisting of maltose, maltotriose, and branched oligosaccharides of 6–8 glucose units that contain both α-1,4 and α-1,6 linkages. These are further degraded to glucose by alpha-glycosidase which on absorption enters bloodstream. Rapid degradation of starch by alpha-amylase enzyme leads to elevated postprandial hyperglycaemia (PPHG). Thus, decreasing the degradation of starch to reducing sugar by inhibition of alpha-amylase enzyme plays a key role in the control of diabetes. Inhibitors of pancreatic alpha-amylase prevent starch breakdown and absorption thereby lowering postprandial glucose levels and also weight loss in humans (Bailey, 2003; Tarling *et al.*, 2008).

Interest in the search for new natural anti-oxidants has grown dramatically over the past years because reactive oxygen species (ROS) production and oxidative stress have been shown to be linked to a large number of human degenerative diseases, including cancer, cardiovascular diseases, inflammation and diabetes (Waris & Ahsan, 2006). It is generally accepted that free radicals play an important role in the development of tissue damage and pathological events. A potent anti-oxidant exhibits a significant peroxyl radical scavenging ability by the donation of its hydrogen atom to the radical species. Natural products with anti-oxidant activity could retard the oxidative damage of the tissue by increasing those defences in different degenerative diseases such as diabetes (Tundis *et al.*, 2012).

Anthocleista djalonensis and *Anthocleista vogelii* are plants used traditionally to treat diabetes and other diseases in some parts of Africa. Table 4.1 shows the yield of the different crude aqueous methanol extracts after maceration and it can be generally seen that the percentage yield is higher for the stem bark of both plants. Table 4.2

displays the yield of the ethyl acetate fractions with higher percentage yield values observed for the ethyl acetate fractions than the hexane fractions. Phytochemical investigations of the leaves, stem bark and whole roots of both plants showed the presence of alkaloids, cardiac glycosides, saponins, tannins, flavonoids and anthracene derivatives in various degrees Table 4.3.

The crude aqueous methanol extracts of both plants were subjected to alpha amylase inhibitory assay using a combination of the methods of Benfield (1951) and Miller (1959) with some modifications. The maltose standard curve result in Fig 4.1 shows a linear plot with a correlation coefficient of y on x axis to be 0.99. From the results in Fig. 4.2, all the crude extracts tested (stem bark and leaves of both plants) from 0.1 mg/mL-1.0 mg/mL, had alpha-amylase inhibitory activities measured at $\Lambda_{max} = 540$ nm. The lowest inhibition of 31.5% at 1 mg/mL was observed for the crude aqueous methanol extract of *A.vogelii* leaf while the corresponding *A. djalonensis* leaf at 1 mg/mL had the highest inhibition of 42.8% (P<0.05). These results compared well with that of acarbose, a known alpha-glucosidase inhibitor given to diabetes patients which gave a 54.85% inhibition at 1.0 mg/mL. Fig. 4.3 shows the anti-oxidant activity results for the crude extracts at 1.0 mg/mL observed at different time intervals from 0-60 min compared to alpha-tocopherol, a standard anti-oxidant. From the results, absorbance values (at $\Lambda_{max}=517$ nm) for *A. djalonensis* leaf and *A. vogelii* stem bark crude aqueous methanol extracts showed extremely low anti-oxidant activities (less than 10%) while *A. djalonensis* stem bark extract at 60 min had 25.4% and *A.vogelii* leaf had 80.7% which was significantly different from the control and comparable to that of alpha-tocopherol which was 89.5% at P<0.05. From these results, both plants

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are seen to possess alpha-amylase inhibitory potential and anti-oxidant properties in varying degrees.

The anti-diabetic activities of both plants were investigated *in vivo* using rat models. Tables 4.4-4.6 display the reduction in blood glucose levels of the alloxan-induced diabetic rats treated with the leaves, stem bark and whole roots at 1 g/kg body weight. Glibenclamide, a sulphonylurea anti-diabetic drug was used as a positive control. Significant reduction (P<0.05) in blood glucose level was observed from day-4 of treatment using the leaf extract while the stem bark and whole root extracts had significant reductions starting from day-1. For glibenclamide, the reduction was observed starting from day-5. This is because glibenclamide, like other sulphonylureas, is effective in mild diabetic state and ineffective in severe diabetic animals where pancreatic β-cells are completely destroyed (Qamar, 2011). Fig. 4.4 shows the percentage reduction in blood glucose levels for the *A. djalonensis* extracts. Peak reduction of 45.7% and 72.6% were observed for the leaves and stem bark respectively on day-6. Previous work by Olagunju *et al*. (1998) on the bioactivity of isosaline extracts of the leaves of *A. djalonensis* caused a 77.7% reduction by day-15 in fasting blood glucose levels of extracts treated diabetic rats when compared with untreated ones. The root and glibenclamide gave peak reductions of 48.5% and 57.4% on day 7 at P<0.05. This result was also in agreement with anti-diabetic activities of ethanol root extract/fractions of *A. djalonensis* evaluated in alloxan-induced diabetic rats for 14 days. A significant reduction in fasting blood glucose level (P<0.001) of the diabetic rats was observed both in acute study and prolonged treatment of 2 weeks, (Okokon *et al*., 2012).

The anti-diabetic activities of the extracts of *A. vogelii* are shown in Tables 4.7-4.9. Significant reductions were observed for the leaves, stem bark and roots as observed for those of *A. djalonensis*. However for the percentage reductions in blood glucose levels shown in Fig.4.5, *A. vogelii* leaf gave a peak reduction of 60.4% on day-7, the stem bark had 69.7%, and the root gave 48.5% while glibenclamide gave 58.43% all at $P<0.05$. The hypoglycaemic effect of roots of *A. vogelii* was studied in mice, rats and rabbits. The extract (100, 400 and 800 mg/kg) induced significant hypoglycaemic activity in a dose-related fashion at 2 h after oral administration in mice and rats with ED_{50} of 250 mg/kg and 350 mg/kg respectively. The extract (800 mg/kg, orally) similarly induced statistically significant lowering of blood glucose levels at 8 h in normoglycaemic rabbits (Abuh *et al*., 1990).

The alpha-amylase inhibitory activities of the ethyl acetate fractions of the leaves and stem bark of both plants were done at concentrations of 0.1-1.0 mg/mL. From the results displayed in Fig. 4.6, at 1 mg/mL, the ethyl acetate fraction of *A. vogelii* stem bark had the lowest alpha-amylase inhibition of 27.3% while the highest inhibition of 50.0% was recorded for *A. djalonensis* leaf. These results were comparable with that of acarbose which gave a 54.85% inhibition which was significantly different from control at $P<0.05$. Each of the ethyl acetate fractions were subjected to column chromatography and the resulting fractions tested on the assay. Figs. 4.7-4.10 showed the different inhibition percentages at 1 mg/mL. The percentage inhibition of *A.djalonensis* stem bark fractions is shown in Fig. 4.7 with fraction 11 giving the highest inhibition of 35.8% ($P<0.05$), Fig 4.9 showed the inhibition profile for *A.vogelii* stem bark with fraction 8 having the highest inhibition of 47.7% ($P<0.05$), while the results for *A.vogelii* leaf are displayed in Fig. 4.10 with fraction 5 giving a

60.4% inhibition significant at P<0.05. Fraction 11 of *A.djalonensis* stem bark and fraction 5 of *A. vogelii* leaf were further purified, and compounds isolated were then subjected to the alpha-amylase assay at different concentrations of 0.1-1.0 mg/mL and compared to acarbose. Fig. 4.11 shows the percentage inhibitions with Decussatin isolated from *A. vogelii* leaf giving the lowest inhibition of 22.0% at 0.1 mg/mL and rising gradually to 78.0% at 1.0 mg/mL which was statistically significant at P = 0.00000913 (Table 4.10). In Fig. 4.11, Djalonenol isolated from the stem bark of *A. djalonensis* gave a percentage inhibition of 37.6% at 0.1 mg/mL and rose to 53.7% at 1 mg/mL significant at P = 0.00000697. These values were comparable to acarbose which gave a 43.6% inhibition at 0.1mg/mL and 54.9% at 1.0 mg/mL which was significant at P = 0.00023.

Djalonenol is a monoterpene diol characterized as tetrahydro-4-(1-hydroxybut-3-en-2-yl)-3(hydroxymethyl) pyran- 2-one that had previously been isolated from *A. djalonensis* (Onocha *et al*., 1995) and also from *Tachia grandiflora* (Silva *et al*., 2013). Its ^{1}H NMR and ^{13}C NMR δ (ppm) shifts values compared well with those of Onocha *et al*., 1995 in Table 4.11. Its infra red major peaks 3380, 2923, 1710, 1477, 1404, 1265, 1072, 924 compared well also with those observed by Onocha *et al*. (1995). Its GC EIMS exhibited ions at m/z 170 (loss of $H_2C=O$ due to McLafferty rearrangement) and m/z 152 (further loss of H_2O), 127, 79 and 41, which was similar to the fragmentation pattern observed by Silva *et al*., 2013. This compound has been known to possess antimalarial activities (Silva *et al*., 2013), mild cytotoxicity, antimicrobial, antifungal activities, hepatoprotective, cytoprotective and central nervous depressant actions, (Onocha *et al*., 2003; Ateufack *et al*., 2007; Alaribe *et al*.,

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2012). We are reporting here for the first time, the alpha-amylase inhibitory activity of this compound isolated from the stem bark of *A. djalonensis*.

The anti-oxidant activities of the fractions of *A. djalonensis* and *A. vogelii* were investigated and the results in Fig. 4.12 showed the ethyl acetate partitioned fractions of *A. vogelii* leaf (1 mg/mL) had the highest anti-oxidant activity of 87.4% at 60 min while *A. djalonensis* stem bark had 27.1% at the same concentration. Fig.4.13 showed the anti-oxidant activity of *A. djalonensis* stem bark at different concentrations. In Fig. 4.14, fraction C is seen to show promising anti-oxidant properties of 64.5% which was comparable to 91.7% observed for α-tocopherol. Fraction C27, isolated as Benzoic acid was monitored on TLC R_F = 0.69, (silica gel, DCM/EtOAc/ MeOH 4:5:1) sprayed with a methanol solution of DPPH and it gave an instant yellow colour. The ^{1}H NMR and ^{13}C NMR spectral data compared well with that of commercially purchased Benzoic acid from Merck, Germany in Table 4.12. Its infra red data showed major peaks at 3000, 2849, 1685, 1323, 1292, 933 which were also comparable to the infra red data of the commercially purchased benzoic acid. This is the first report of its isolation from *A.djalonensis*. The fact that it also tested positive to the DPPH spray shows that it possesses anti-oxidant activities which is actually a contribution to the anti-diabetic activities shown by the stem bark.

Decussatin which is a known xanthone was characterized as (1-hydroxy-2, 7, 8-trimethoxy-9*H*-xanthene-9-one). It was purified as yellowish green needle-like crystals with m.p. 152.3°C-153.9°C. Its ^{1}H NMR and ^{13}C NMR δ (ppm) shifts values Table 4.13, compared well with those of Okorie (1972) and Alaribe *et al*. (2012). The infra red data showed 2916, 1655, 1599, 1481, 981 for major peaks which were consistent with peaks with xanthone like structures. The GC-MS data showed the M$^+$

peak at 302 and fragmentation pattern with peaks having m/z of 287, 259, 227, 201, 171, 143, 122, 79, and 51 which were similar to those recorded by Alaribe *et al.* (2012). Decussatin Fig. 4.21 has been previously reported by Silva *et al.* (2013) to possess antimalarial activities. Alpha-amylase inhibitory activities for decussatin is hereby reported for the first time.

Further anti-oxidant investigations were done for the leaves of *A. vogelii* as shown in Figs. 4.15-4.16. Fraction 16 promises to be a potent anti-oxidant in view of the observed high activity corresponding to 84.98% at 60 min . This fraction was further purified as earlier described but the structure is yet to be determined. Its presence in the plant as an anti-oxidant could be of benefit since anti-oxidants are becoming essential tools in investigating oxidative stress related diabetic pathologies and therapies (Laight *et al*., 2000; Evans *et al*., 2002).

CHAPTER 6

CONCLUSION AND RECOMMENDATION

The quest for natural remedies for the treatment of diabetes is on the increase as more people are becoming affected by the disease due to a change towards a Western lifestyle, while the main synthetic drugs used currently for diabetes have undesirable side effects. For patients suffering from Type 2 diabetes, controlling postprandial hyperglycaemia is of utmost importance in the management of the disease and in the prevention of complications usually caused by the generation of free radicals.

There is justification for the use of *Anthocleista djalonensis* and *Anthocleista vogelii* traditionally to treat diabetes and here scientific evidence is being provided to back up this ethnomedicinal usage. The presence of alpha-amylase inhibitors from these plants could mean that the plants exert their anti-diabetic effect through alpha-amylase inhibition as one of the probable mechanisms of action. Thus, these plants and compounds isolated are potent targets for natural remedies that could be explored for drug development. The presence of anti-oxidants in the plants is also good because this can be utilised in terms of prevention of micro and macro vascular complications.

We report here for the first time, the anti-diabetic activities of the stem bark of *A. vogelii* and the alpha-amylase inhibitory activities of Djalonenol and Decusatin isolated from the stem bark of *A. djalonensis* and the leaves of *A. vogelii* respectively. Also, the anti- oxidant activities of *A. djalonensis* and *A. vogelii* are reported for the first time with the isolation of Benzoic acid from the stem bark of *A.djalonensis*. The high anti-oxidant content of *A. vogelii* leaves is a good indication that the leaves could be a source of potent anti-oxidant compound(s) that

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could be useful in managing complications of oxidative stress related to diabetes. *Anthocleista djalonensis* and *Anthocleista vogelii* definitely have a lot to offer in terms of compounds that can still be isolated from them which will be beneficial to humanity. The roots of both plants have been shown in this and other studies to possess anti-diabetic activity, further bioassay work should be done on the extracts and fractions to know the mechanism by which they exert their anti-diabetic effect. Subsequently, the compounds responsible for such activities should be isolated.

Furthermore, some compounds previously isolated from these plants should be tested for their bioactivity.

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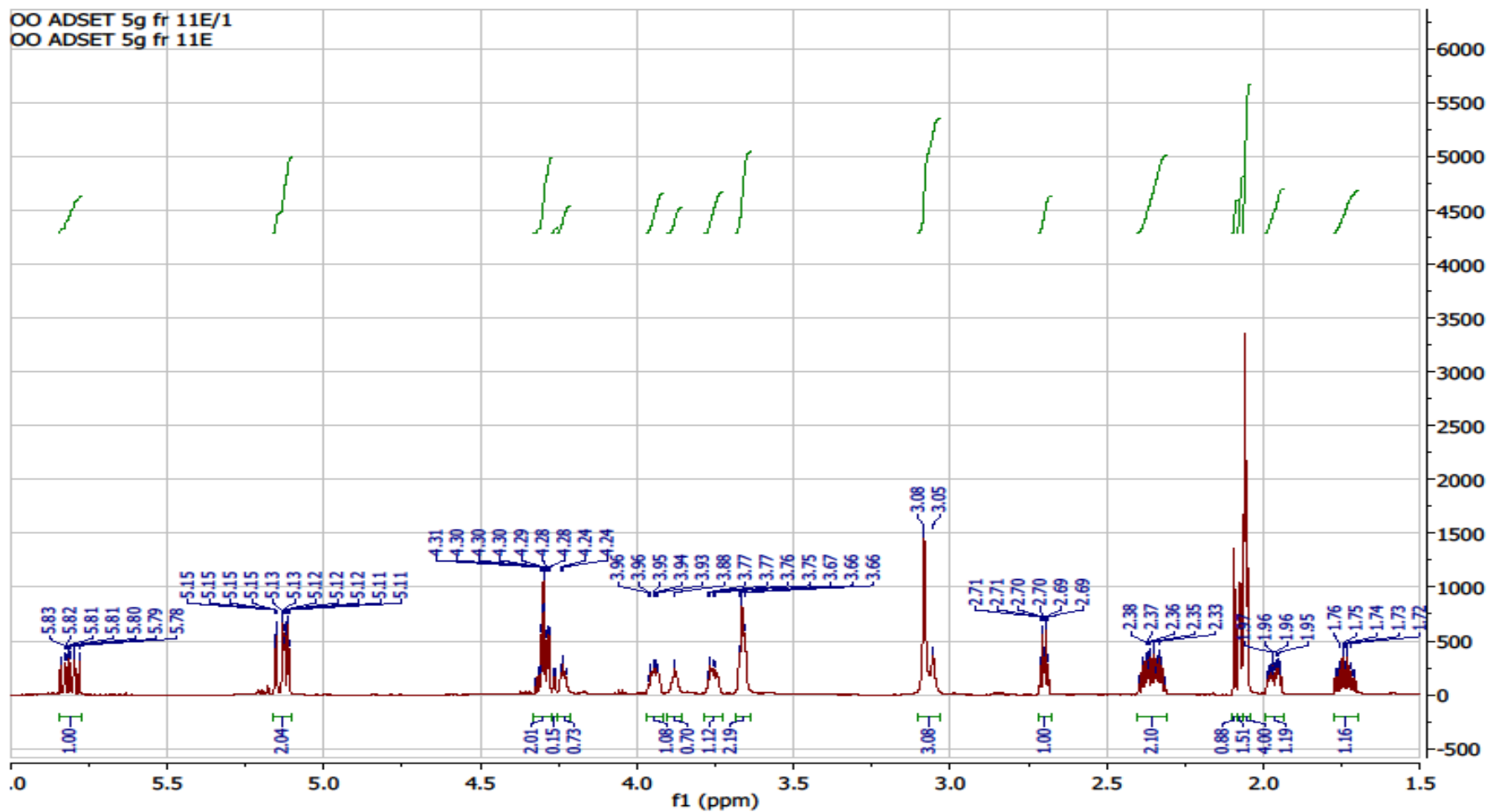
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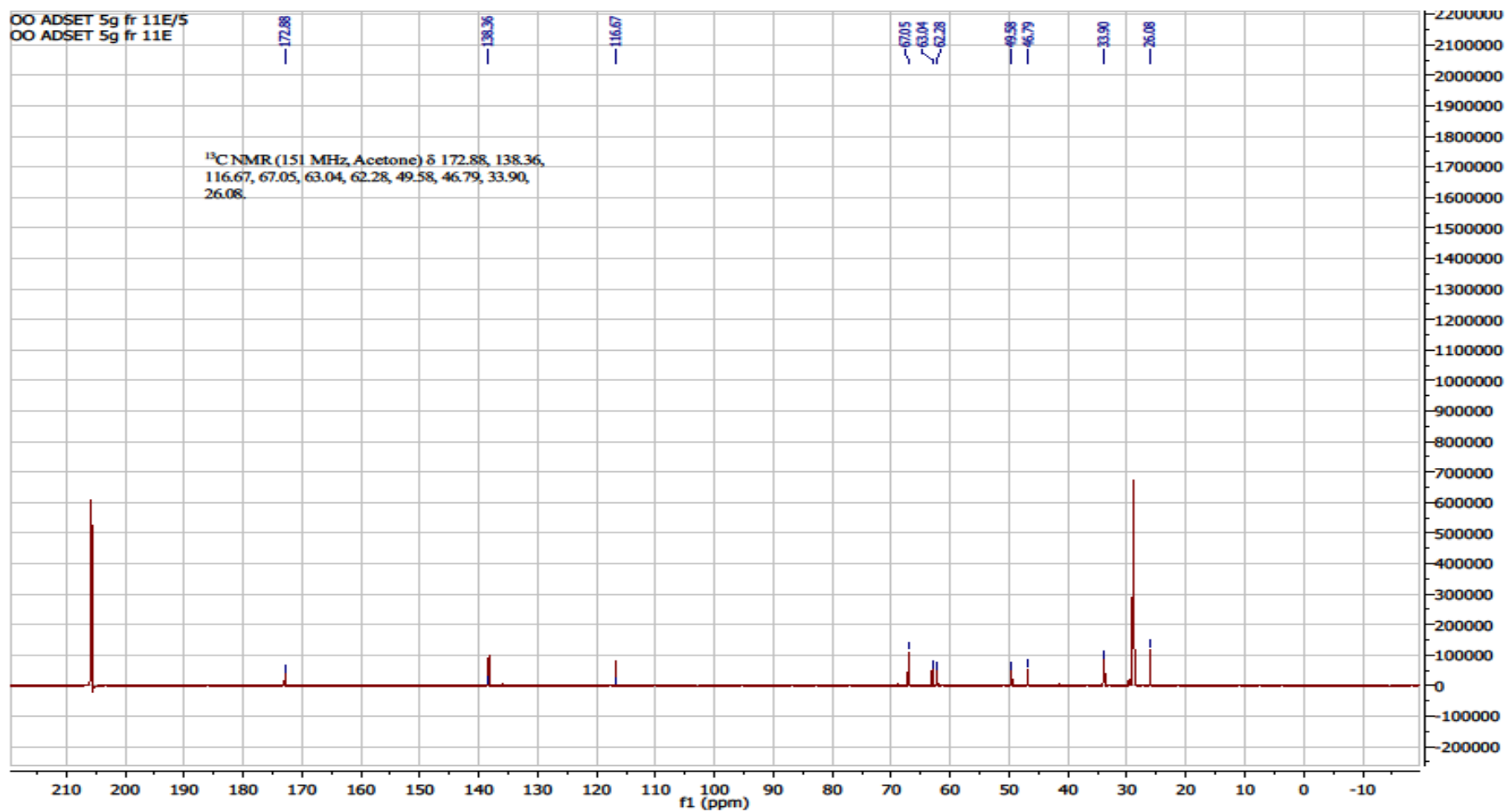
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APPENDIX 1 ^{1}H NMR Spectrum of Djalonenol from *Anthocleista djalonensis* Stem bark

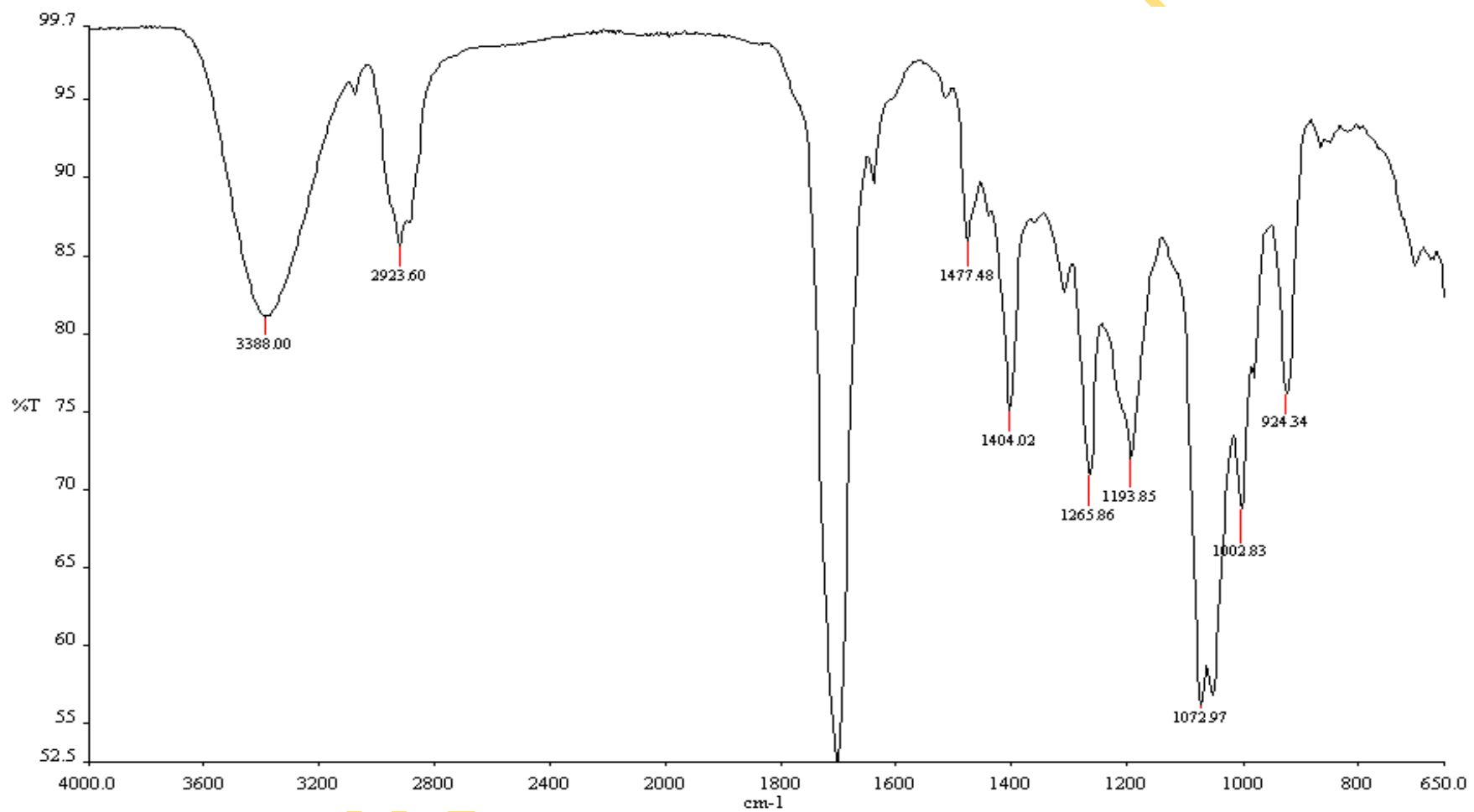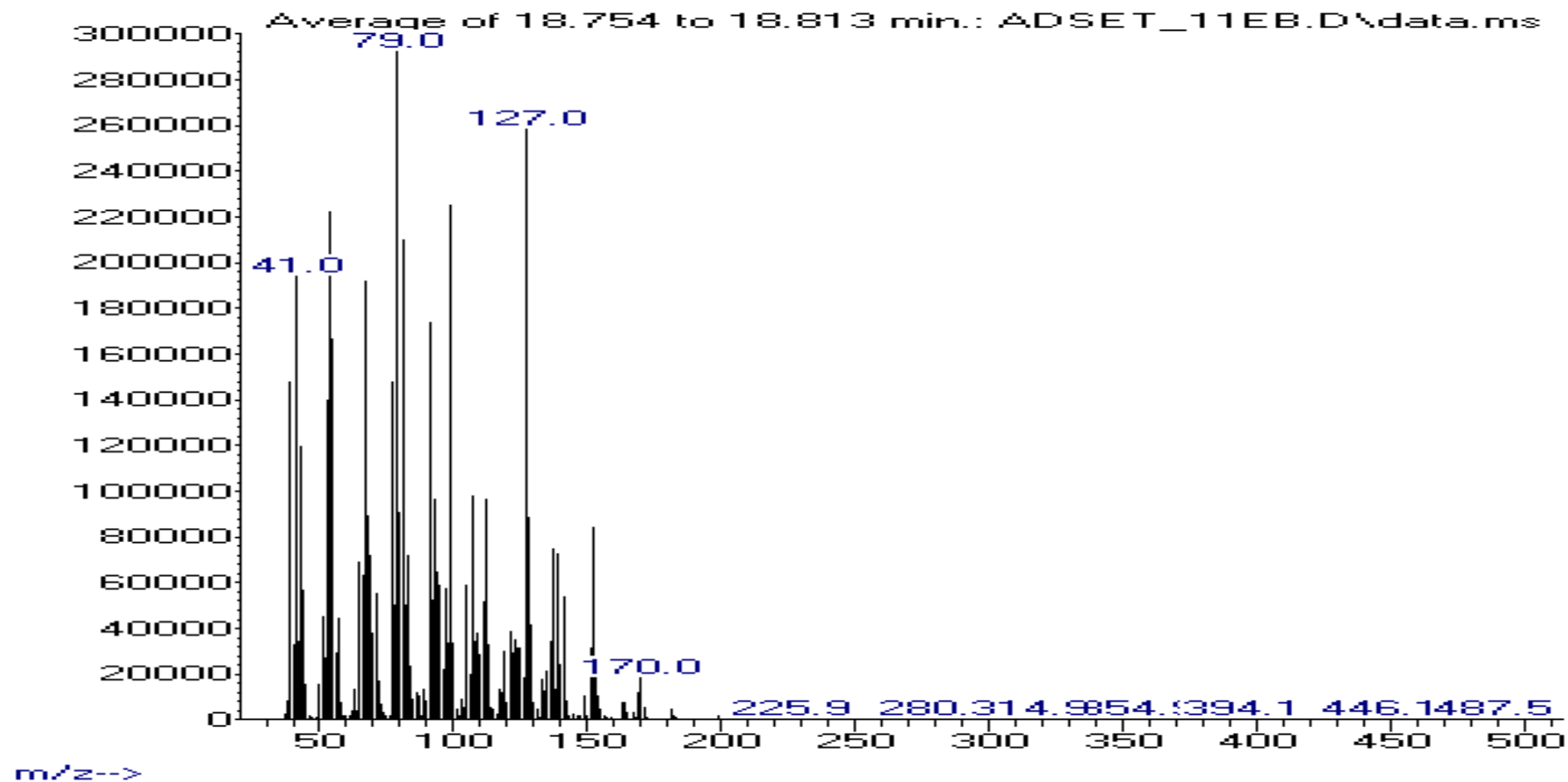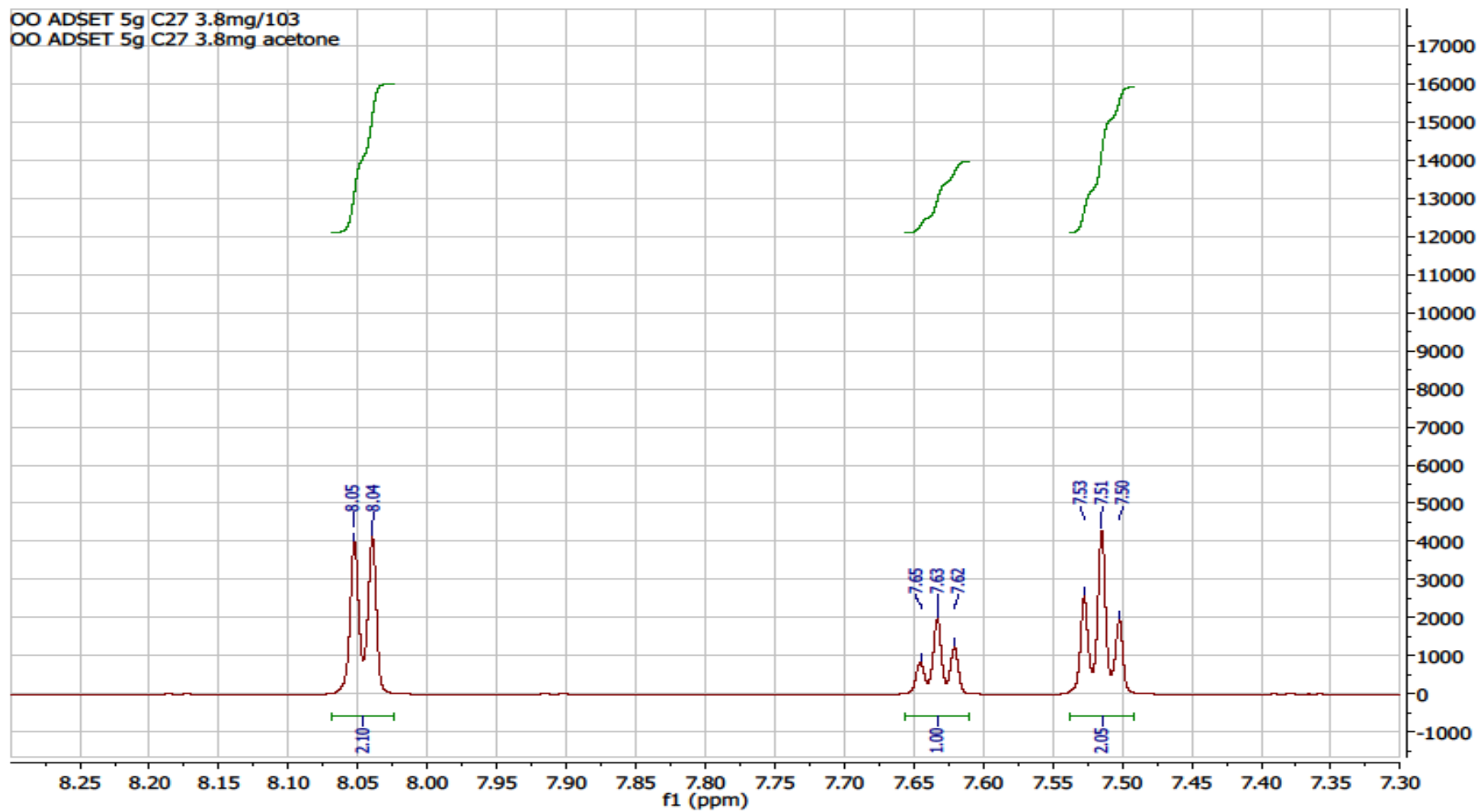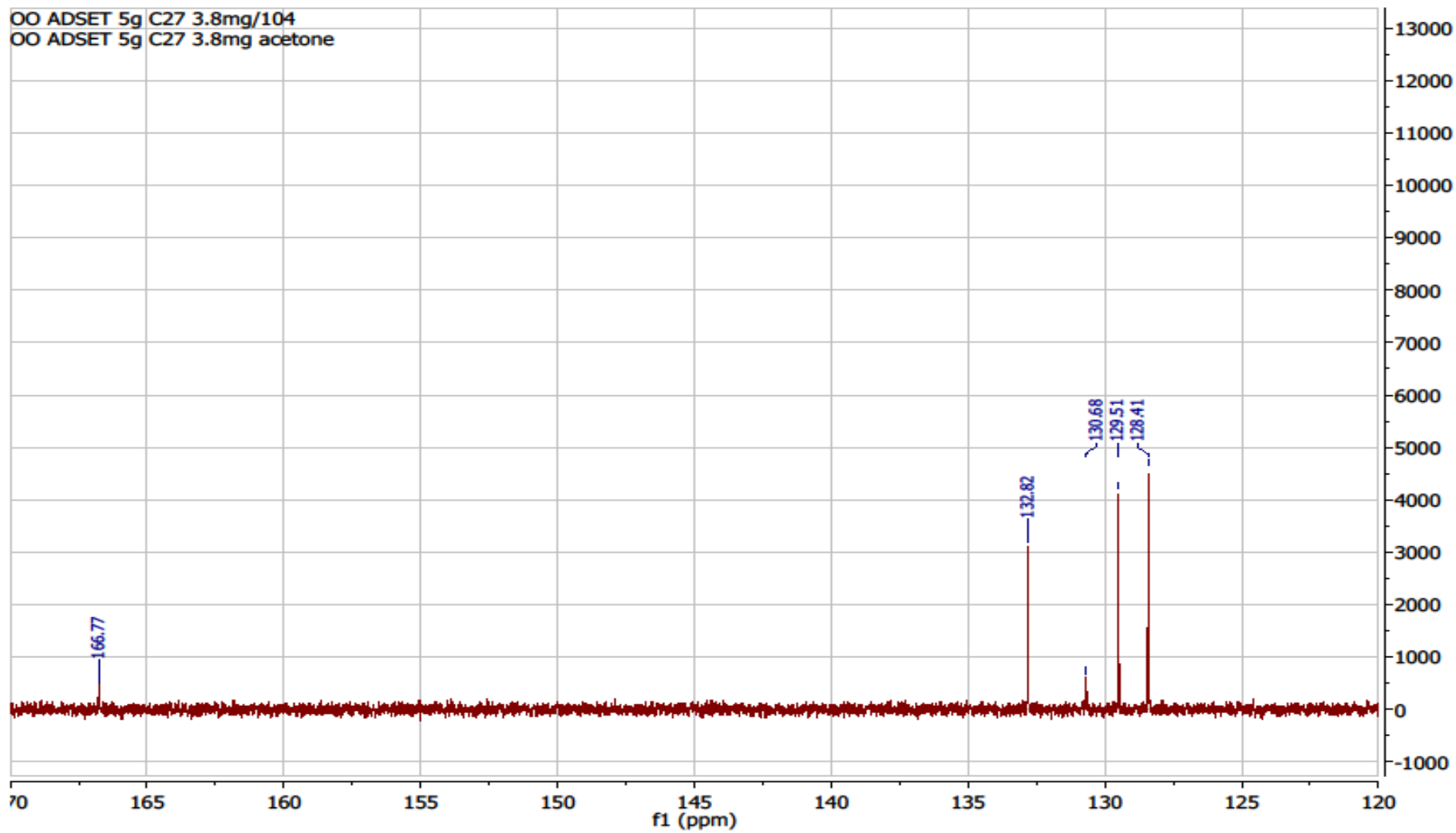**APPENDIX 2 ^{13}C NMR Spectrum of Djalonenol from *Anthocleista djalonensis* Stem bark**

OO ADSET 5g fr 11E/5
OO ADSET 5g fr 11E

^{13}C NMR (151 MHz, Acetone) δ 172.88, 138.36, 116.67, 67.05, 63.04, 62.28, 49.58, 46.79, 33.90, 26.08.

APPENDIX 3 DEPT 135 Spectrum of Djalonenol from *Anthocleista djalonensis* Stem bark

OO ADSE 5g fr 11E/10
OO ODSE 5g fr 11E

¹³C NMR (151 MHz, Acetone) δ 205.59,
172.87, 138.28, 135.90, 128.99, 116.79, 116.71,
68.87, 67.03, 63.01, 62.89, 62.30, 62.18, 49.53,
46.79, 41.63, 33.95, 26.08.
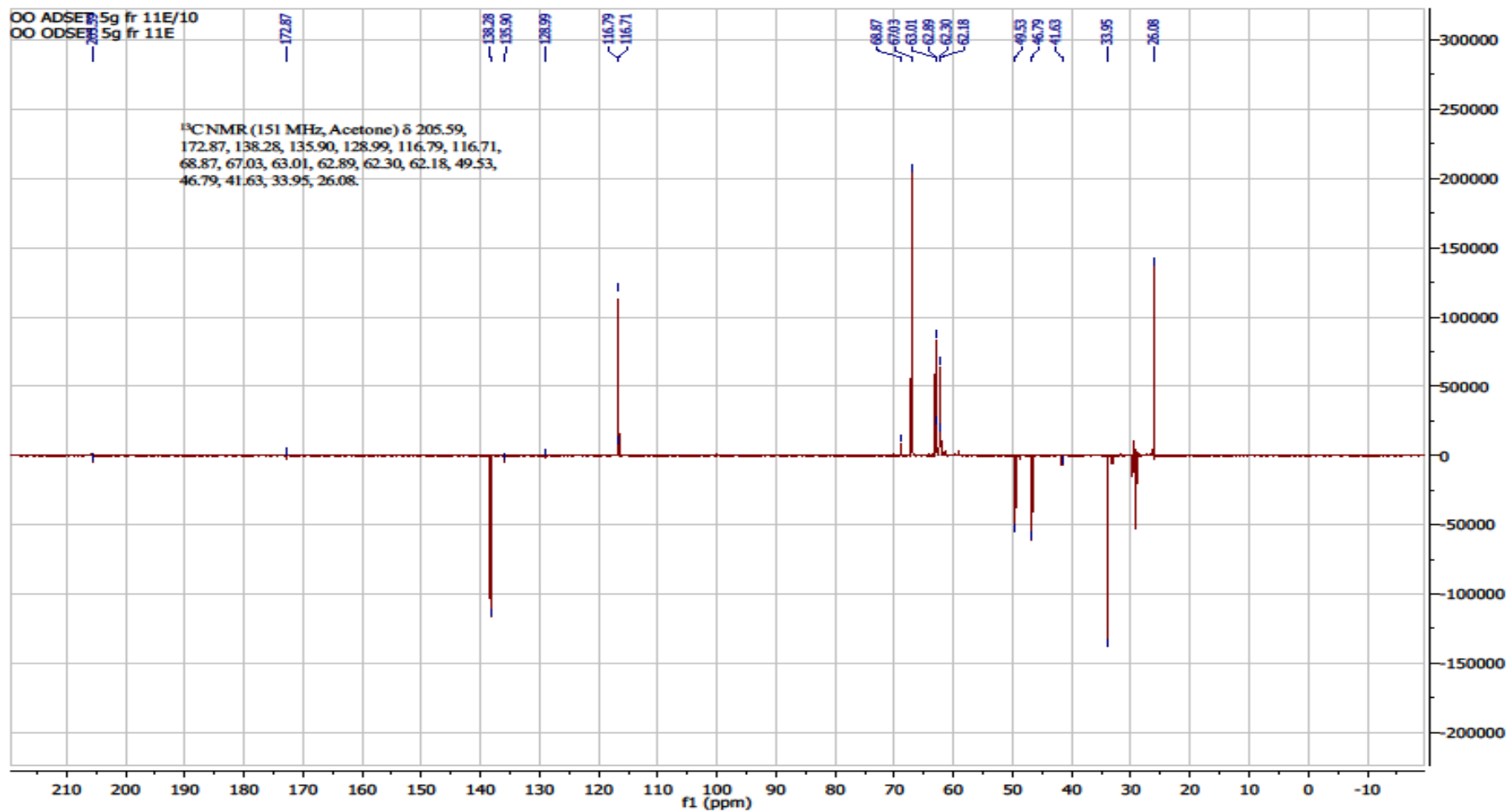
APPENDIX 4 H-H COSY Spectrum of Djalonenol from *Anthocleista djalonensis* stems bark

OO ADSET 5g fr 11E/6
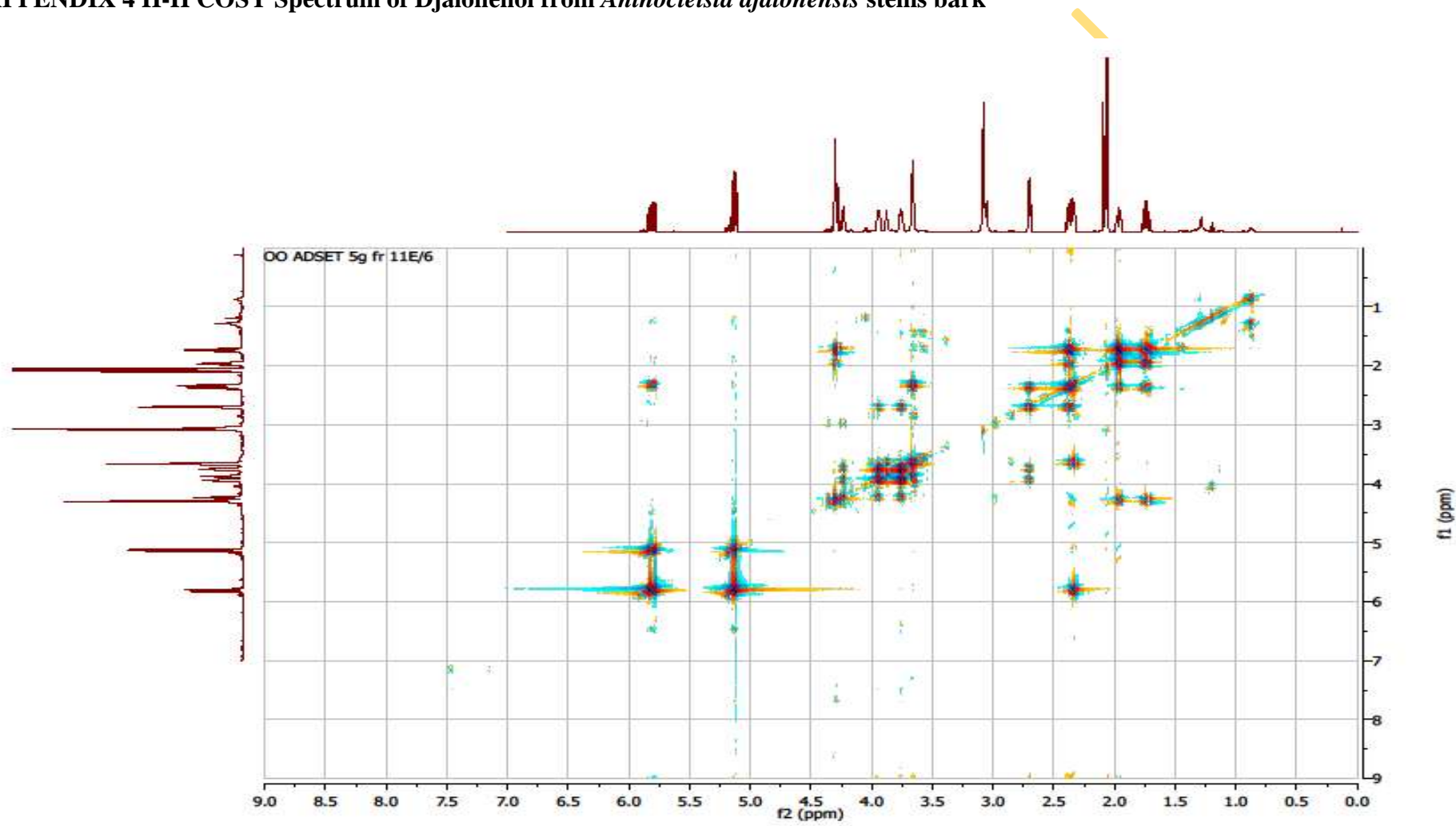
APPENDIX 5 HSQC Spectrum of Djalonenol from *Anthocleista djalonensis* Stem bark
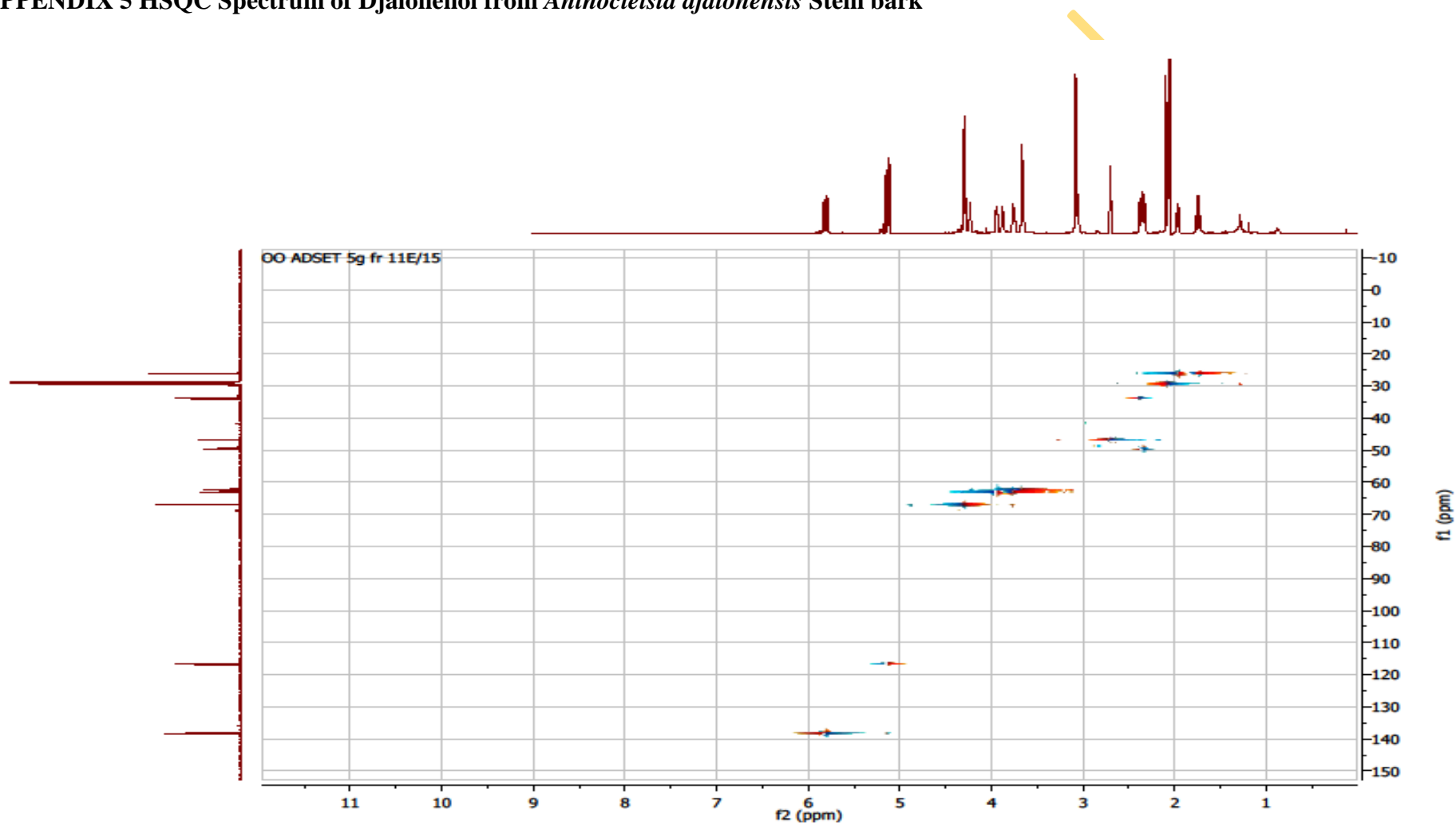
OO ADSET 5g fr 11E/15

OO ADSET 5g fr 11E/16
HMBC3F
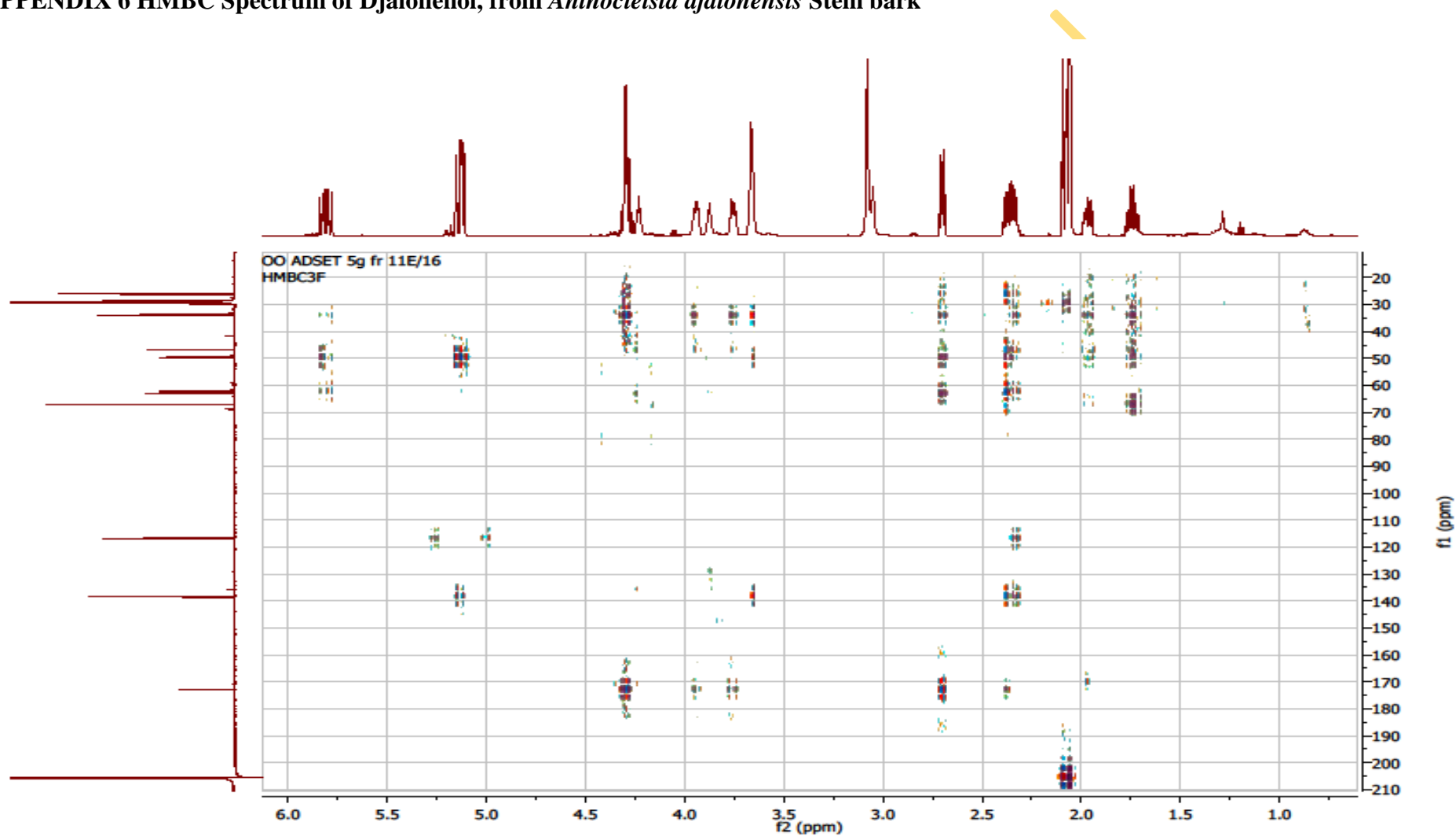
APPENDIX 7 NOESY Spectrum of Djalonenol from *Anthocleista djalonensis* stem bark

OO ADSET 5g fr 11E/17

f1 (ppm)

f2 (ppm)
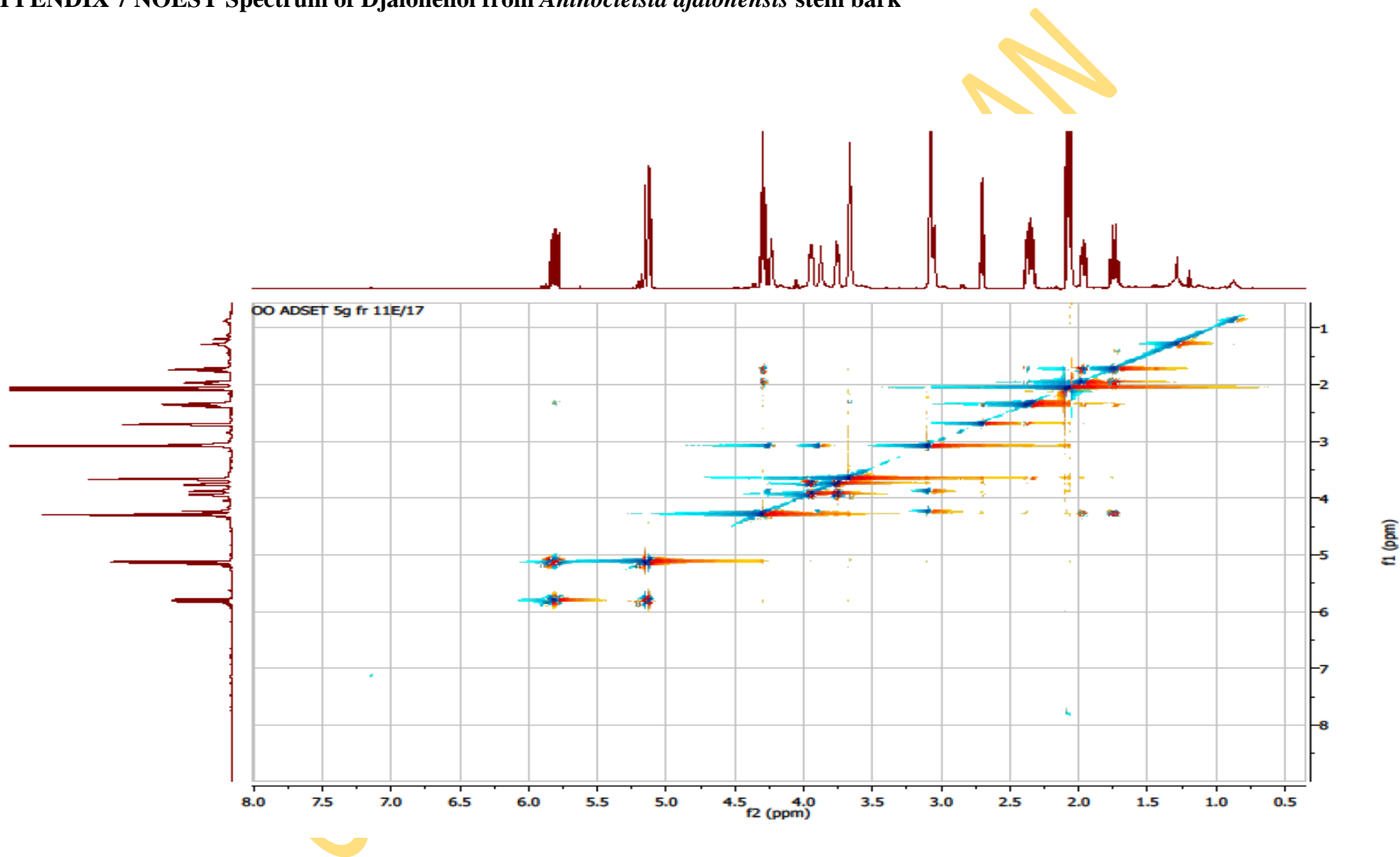
APPENDIX 8 IR Spectrum of Djalonenol from *Anthocleista djalonensis* Stem bark

APPENDIX 9 GC-MS Fragmentation Pattern of Djalonenol from *Anthocleista djalonensis* Stem bark

Abundance

Average of 18.754 to 18.813 min.: ADSET_11EB.D\data.ms

m/z-->

APPENDIX 10 ^{1}H NMR Spectrum of Benzoic acid from *Anthocleista djalonensis* Stem bark

OO ADSET 5g C27 3.8mg/103
OO ADSET 5g C27 3.8mg acetone

APPENDIX 11 ^{13}C NMR Spectrum of Benzoic acid from *Anthocleista djalonensis* Stem bark

OO ADSET 5g C27 3.8mg/104
OO ADSET 5g C27 3.8mg acetone

166.77
132.82
130.68
129.51
128.41

f1 (ppm)

APPENDIX 12 COSY Spectrum of Benzoic acid, from *Anthocleista djalonensis* Stem bark

OO ADSET 5g C27 3.8mg/109
OO ADSET 5g C27 3.8mg acetone (COSY)
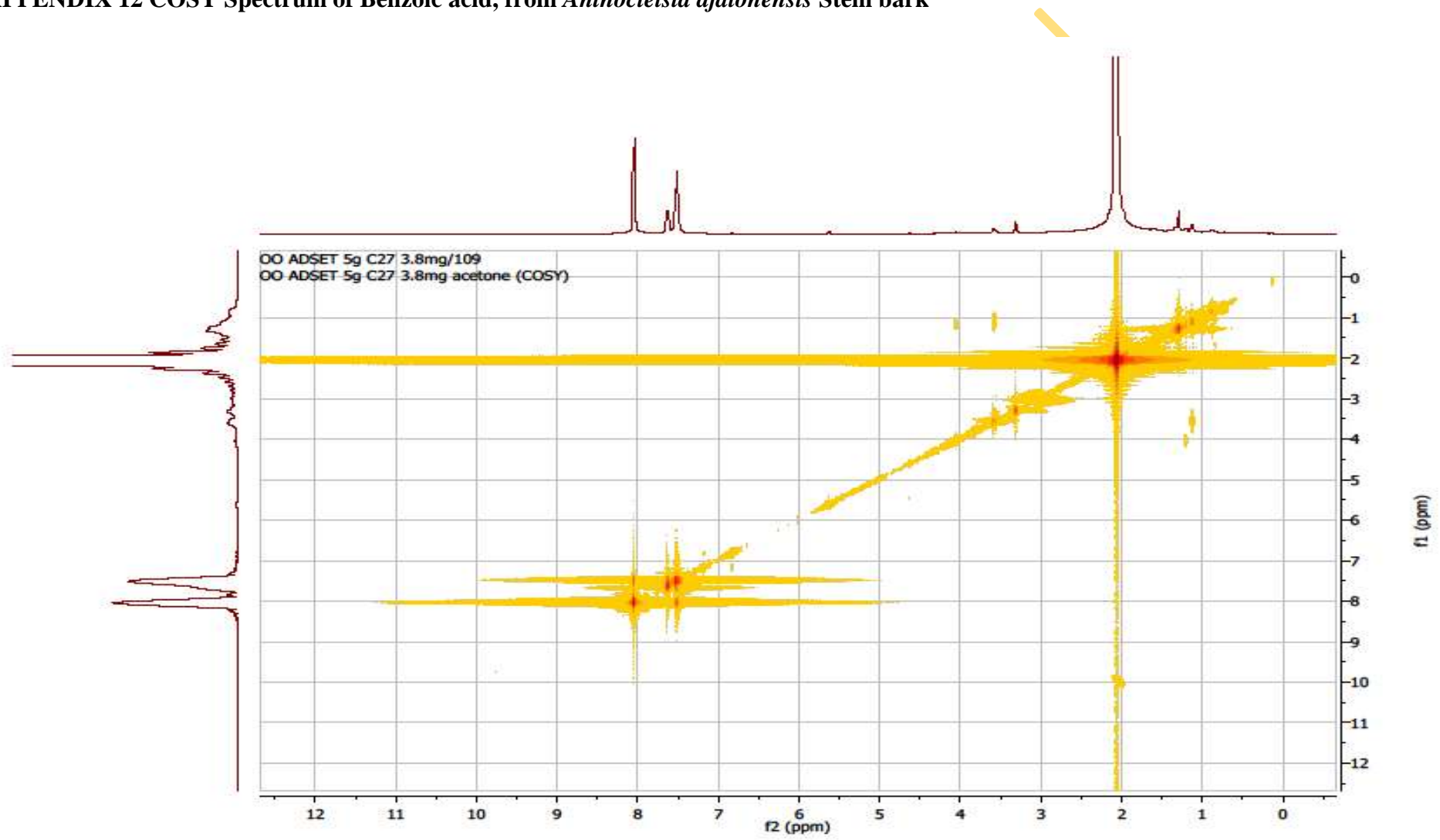
APPENDIX 13 HSQC Spectrum of Benzoic acid from *Anthocleista djalonensis* Stem bark

OO ADSET 5g C27 3.8mg/110
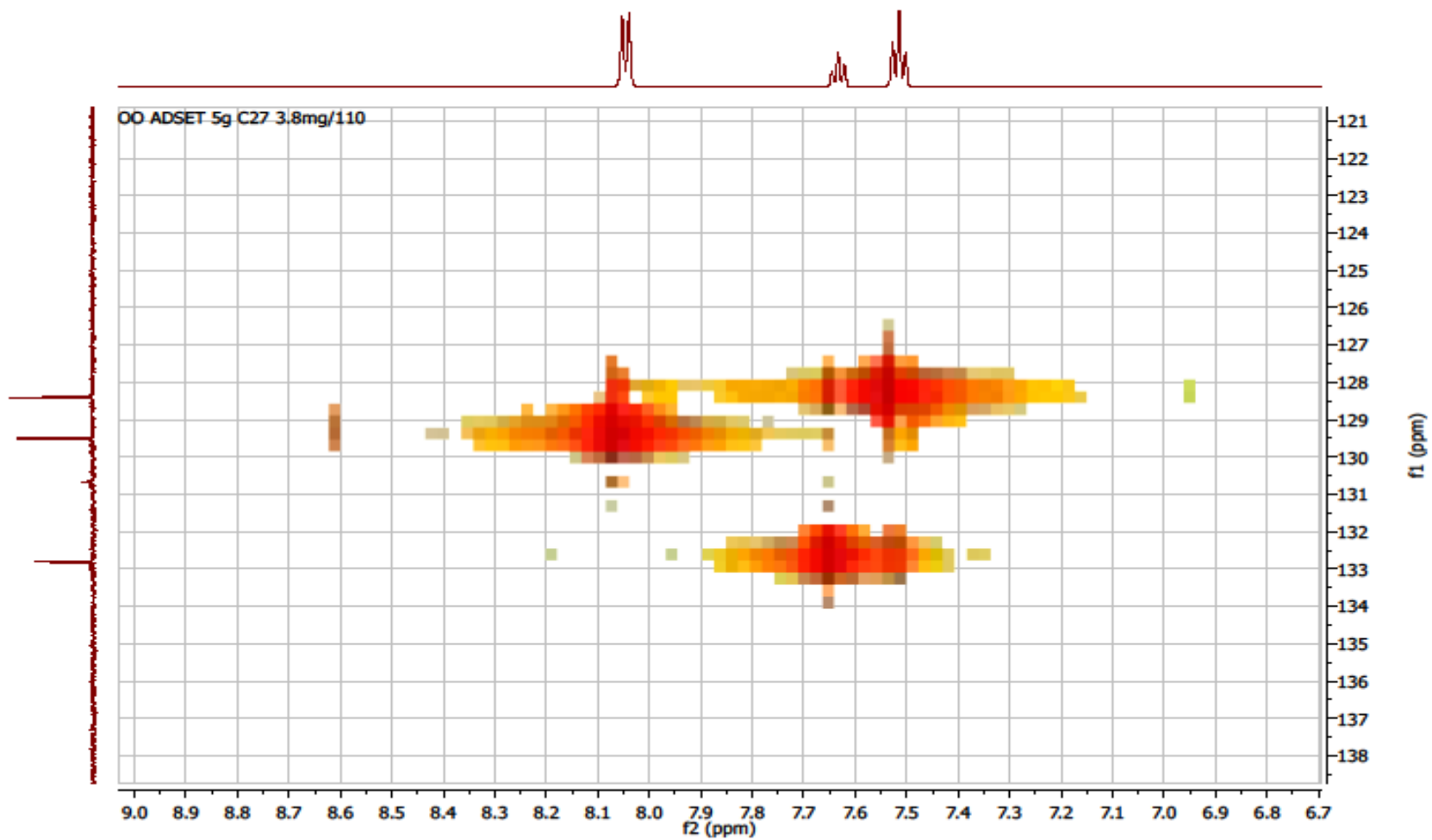
APPENDIX 14 HMBC Spectrum of Benzoic acid from *Anthocleista djalonensis* Stem bark

OO ADSET 5g C27 3.8mg/111
HMBC3F
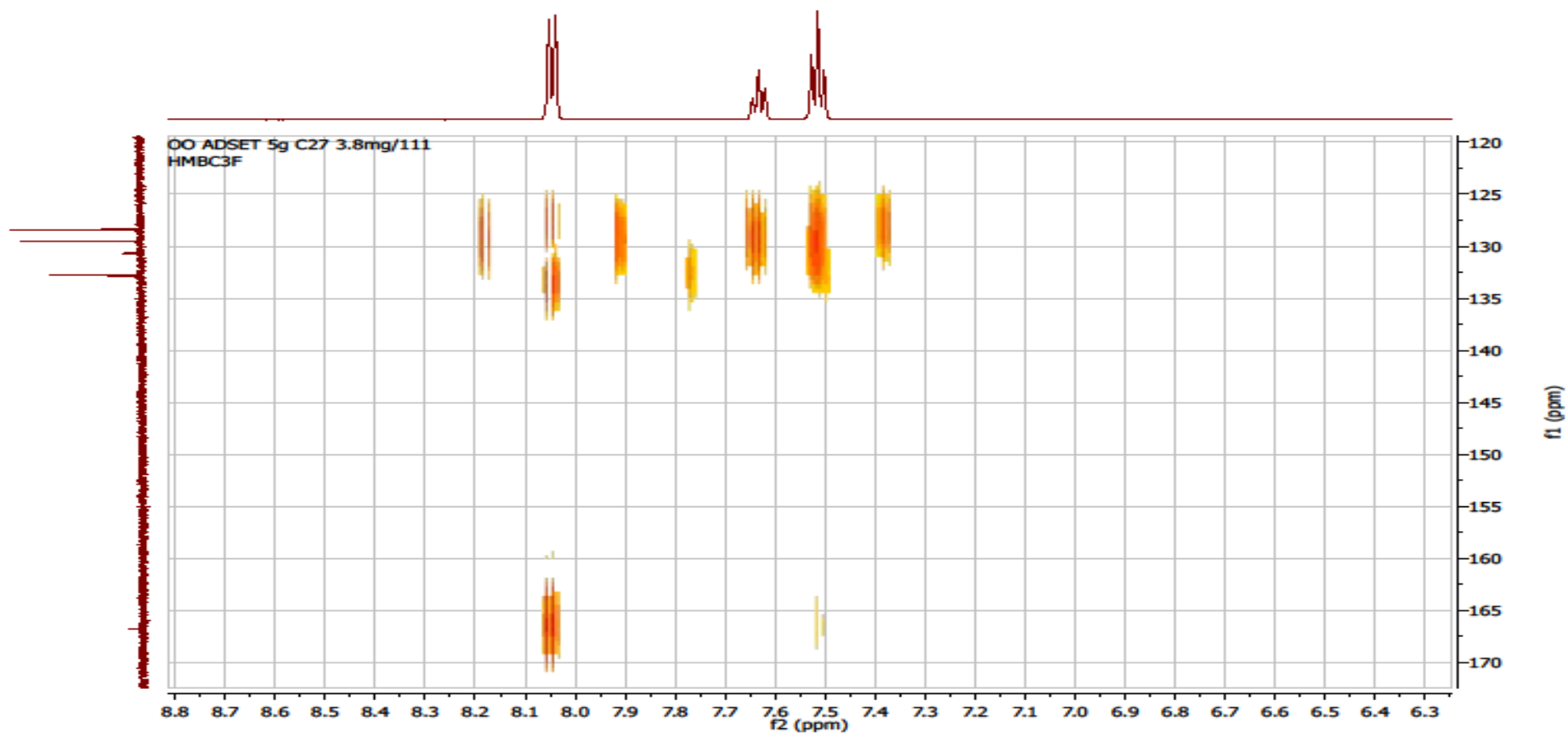
APPENDIX 15 IR Spectrum of Benzoic acid from *Anthocleista djalonensis* Stem bark
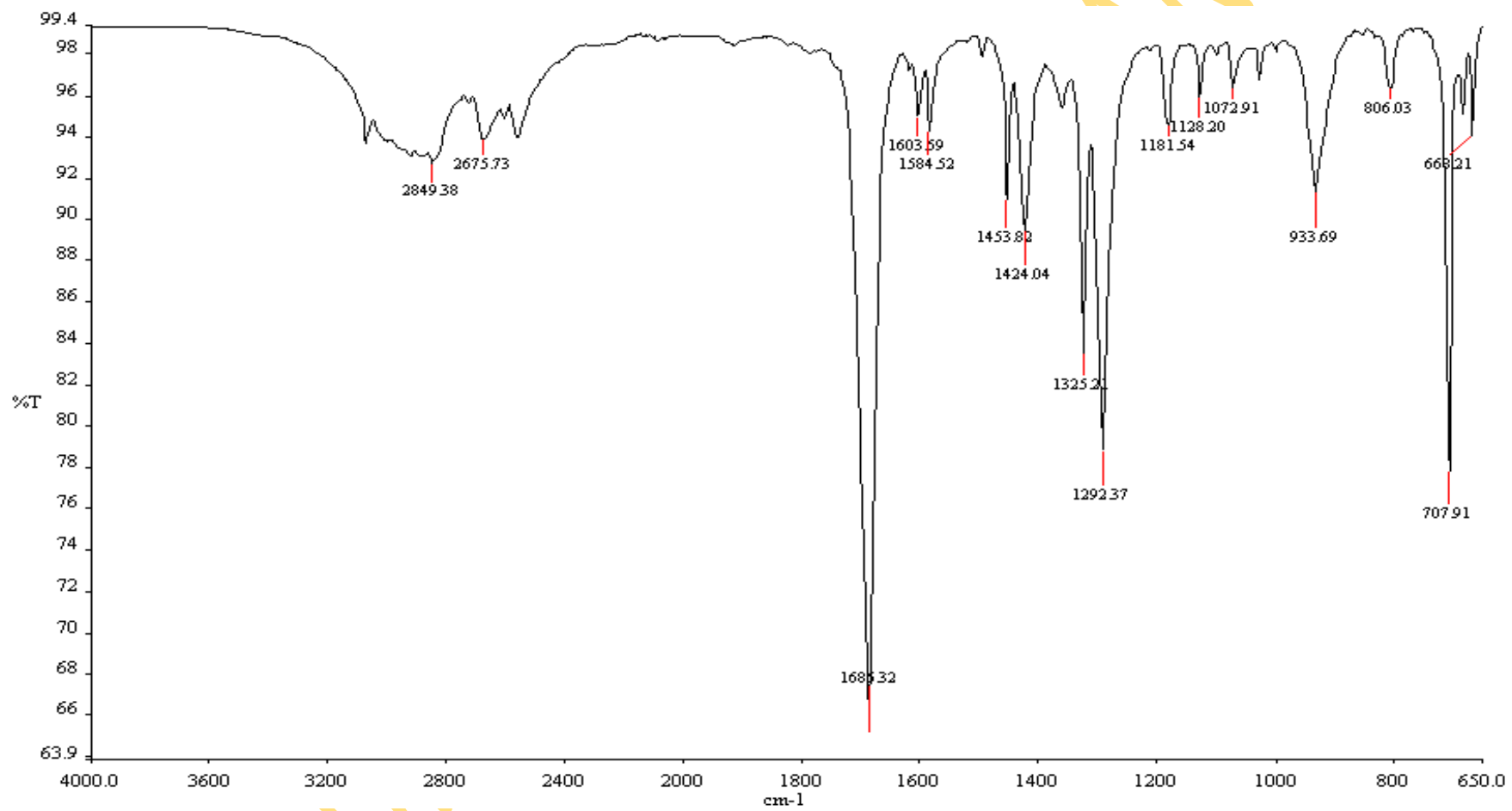
APPENDIX 16 Comparison of ^{1}H NMR Spectrum of Isolated Benzoic acid with Standard Benzoic acid 100134, Merck KGaA Darmstadt Germany
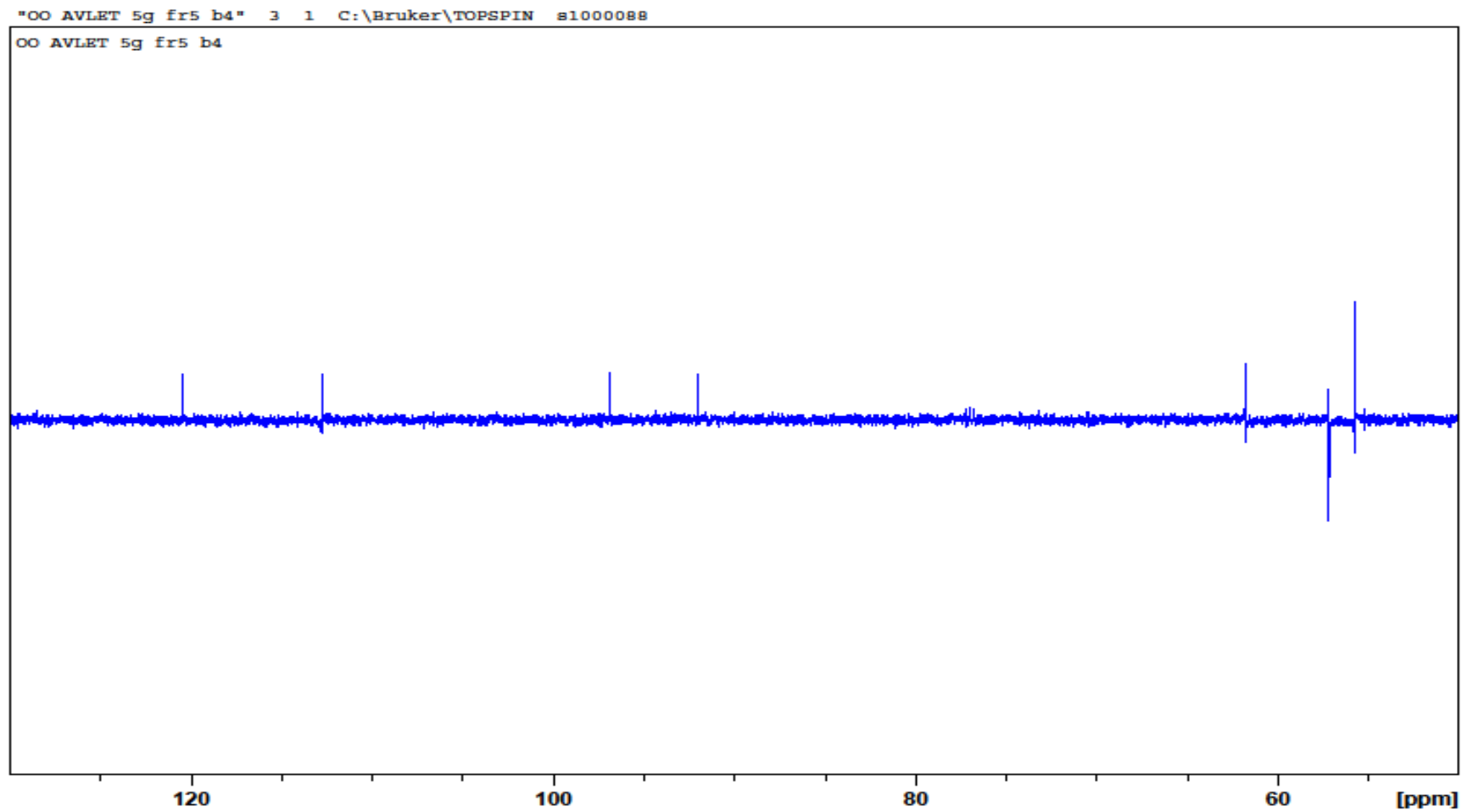

OO ADSET 5g C27 3.8mg/103
OO ADSET 5g C27 3.8mg acetone 2 2

benzoic acid standard oseyemi/1
Benzoic acid standard oseyemi 1 1

f1 (ppm)
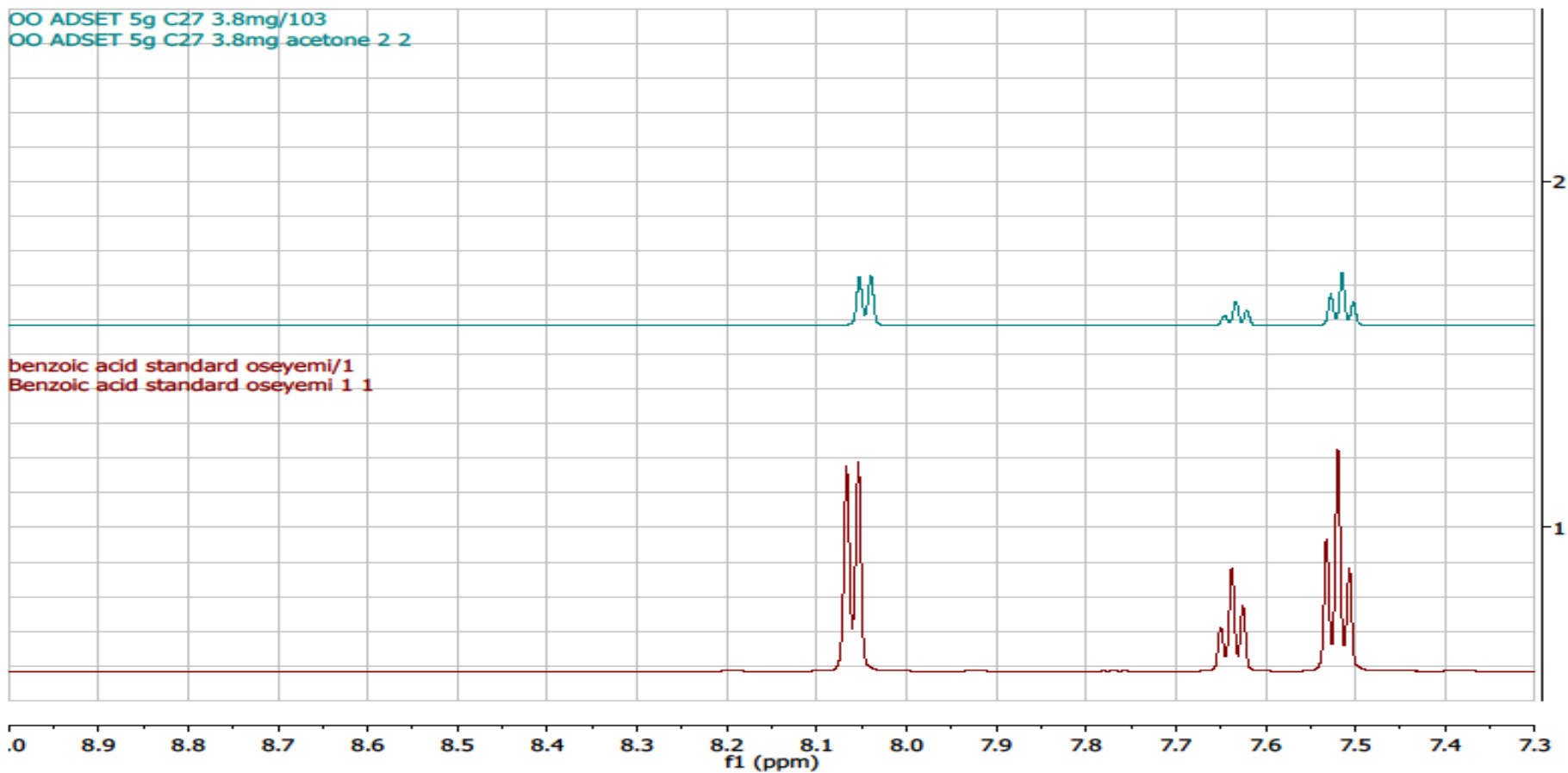
APPENDIX 17 ^{1}H NMR Spectrum of Decussatin from *Anthocleista vogelii* Leaf
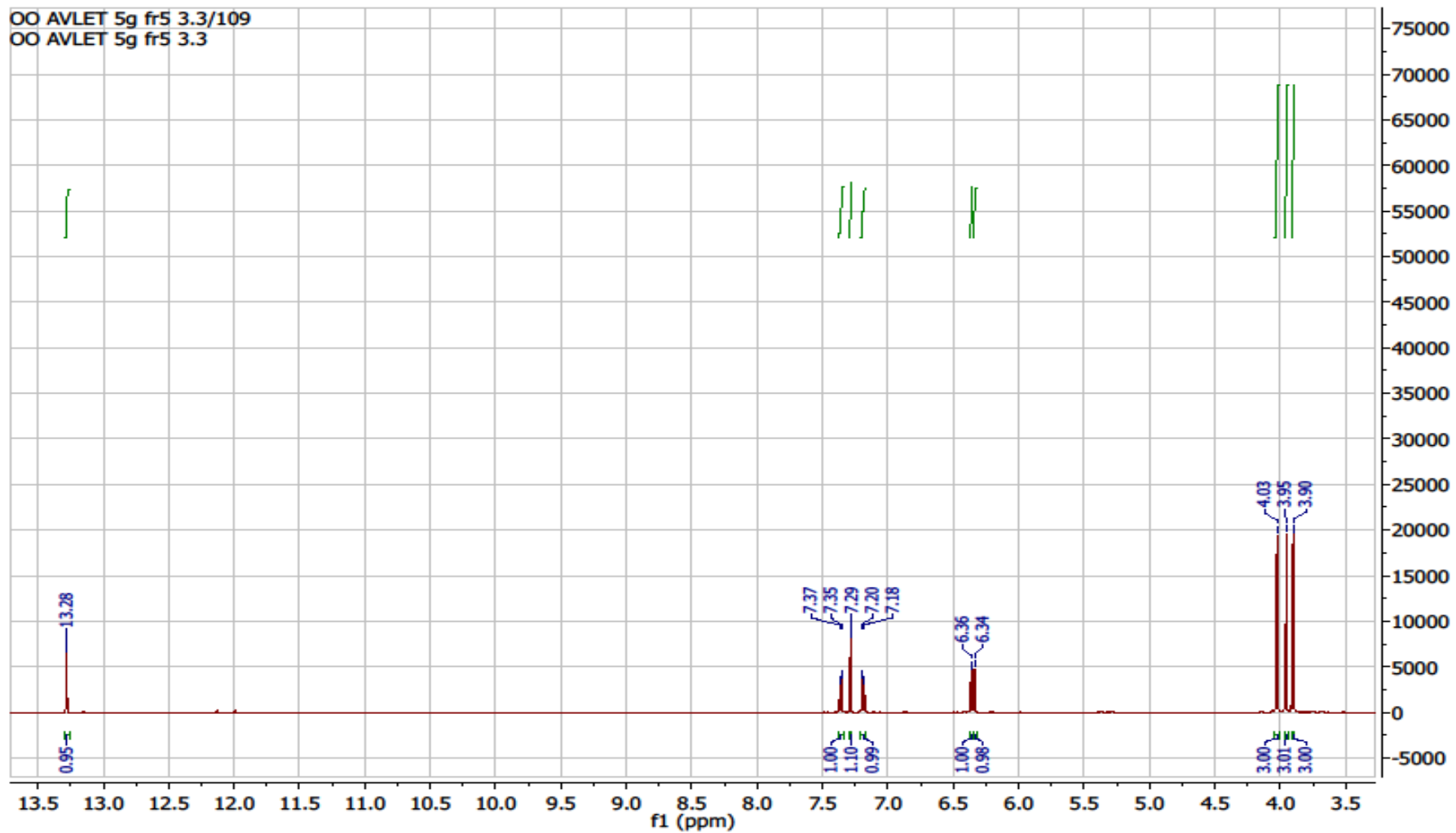
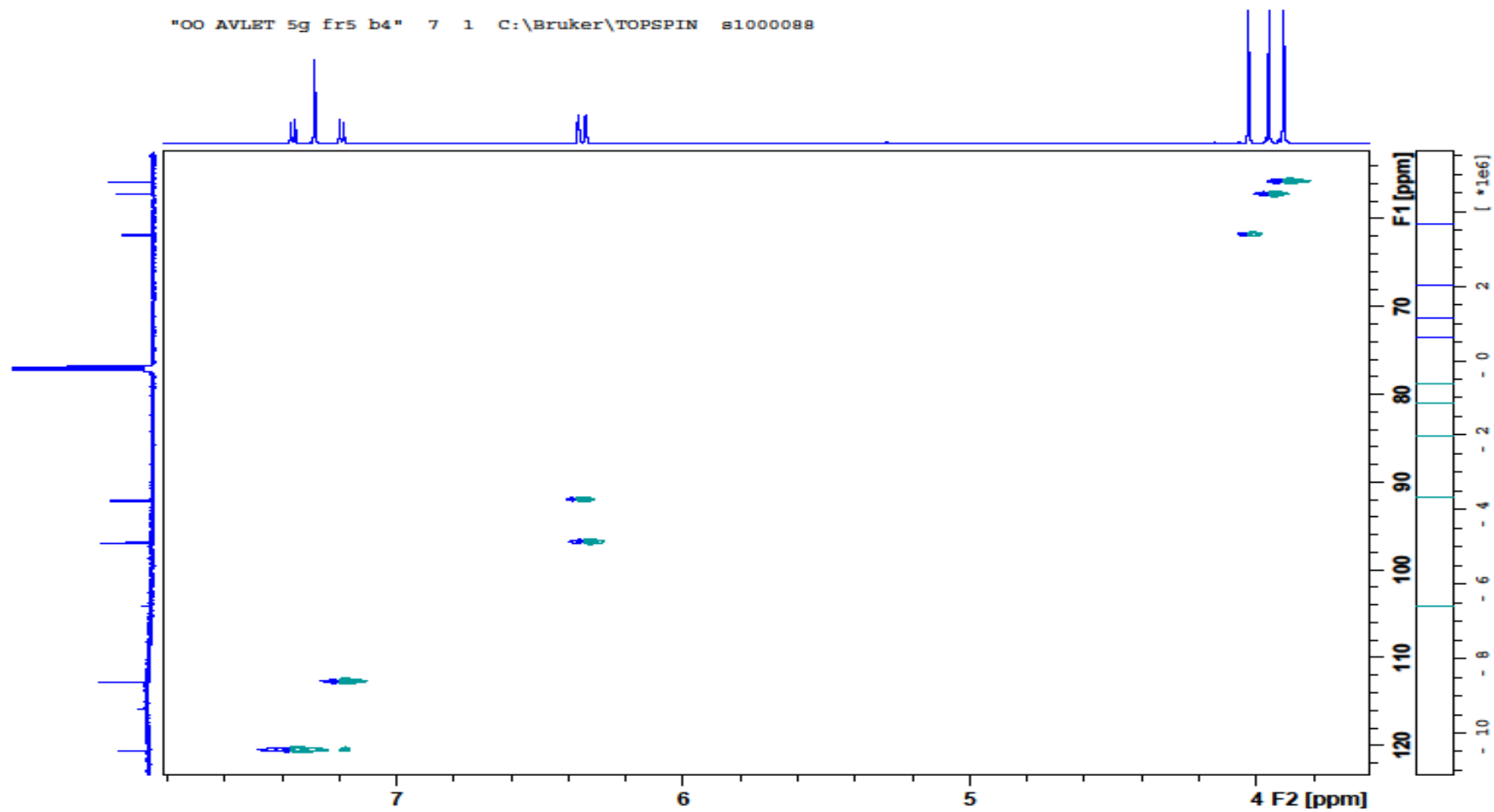# APPENDIX 18 ^{13}C NMR Spectrum of Decussatin from *Anthocleista vogelii* Leaf
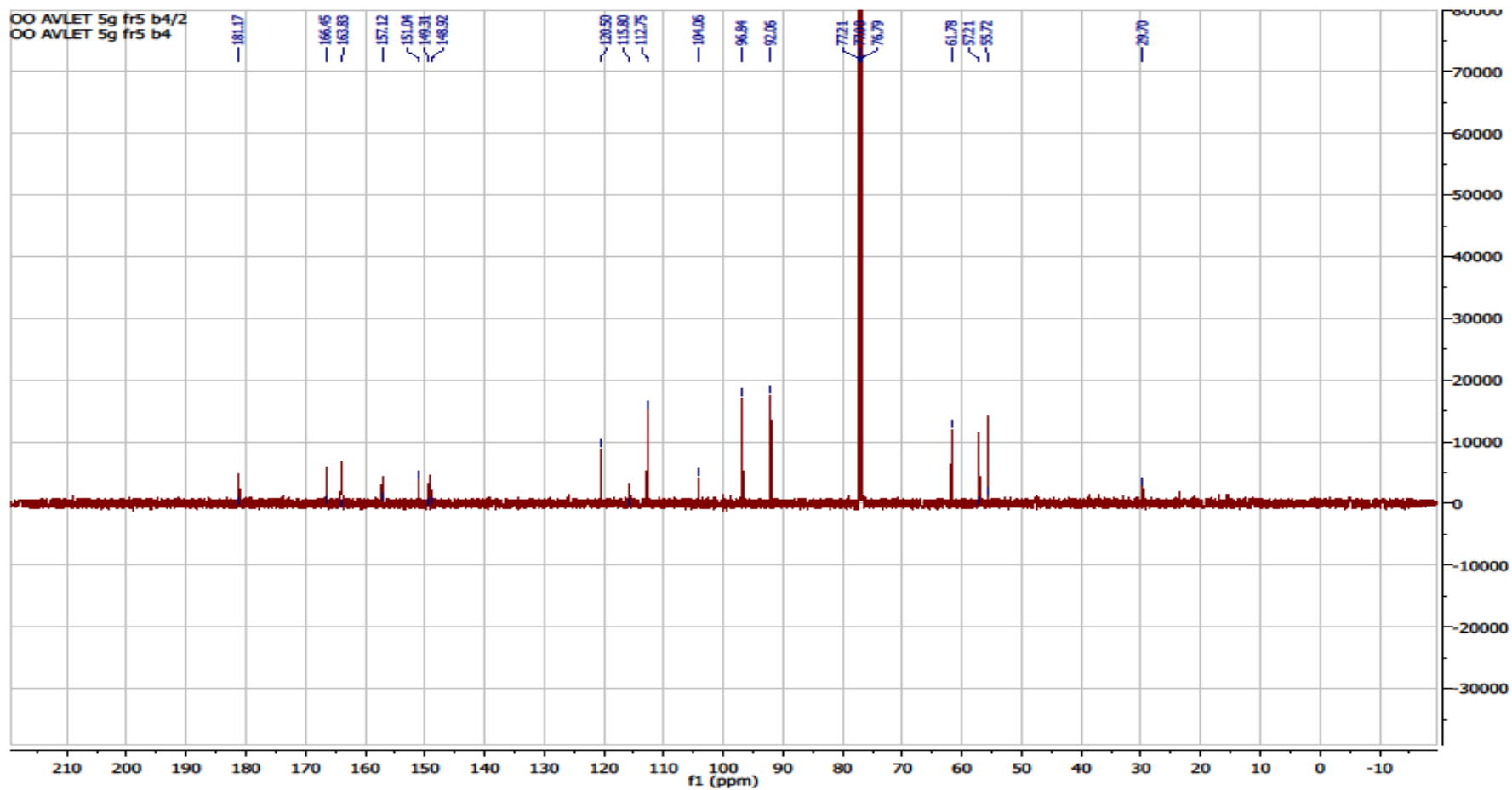
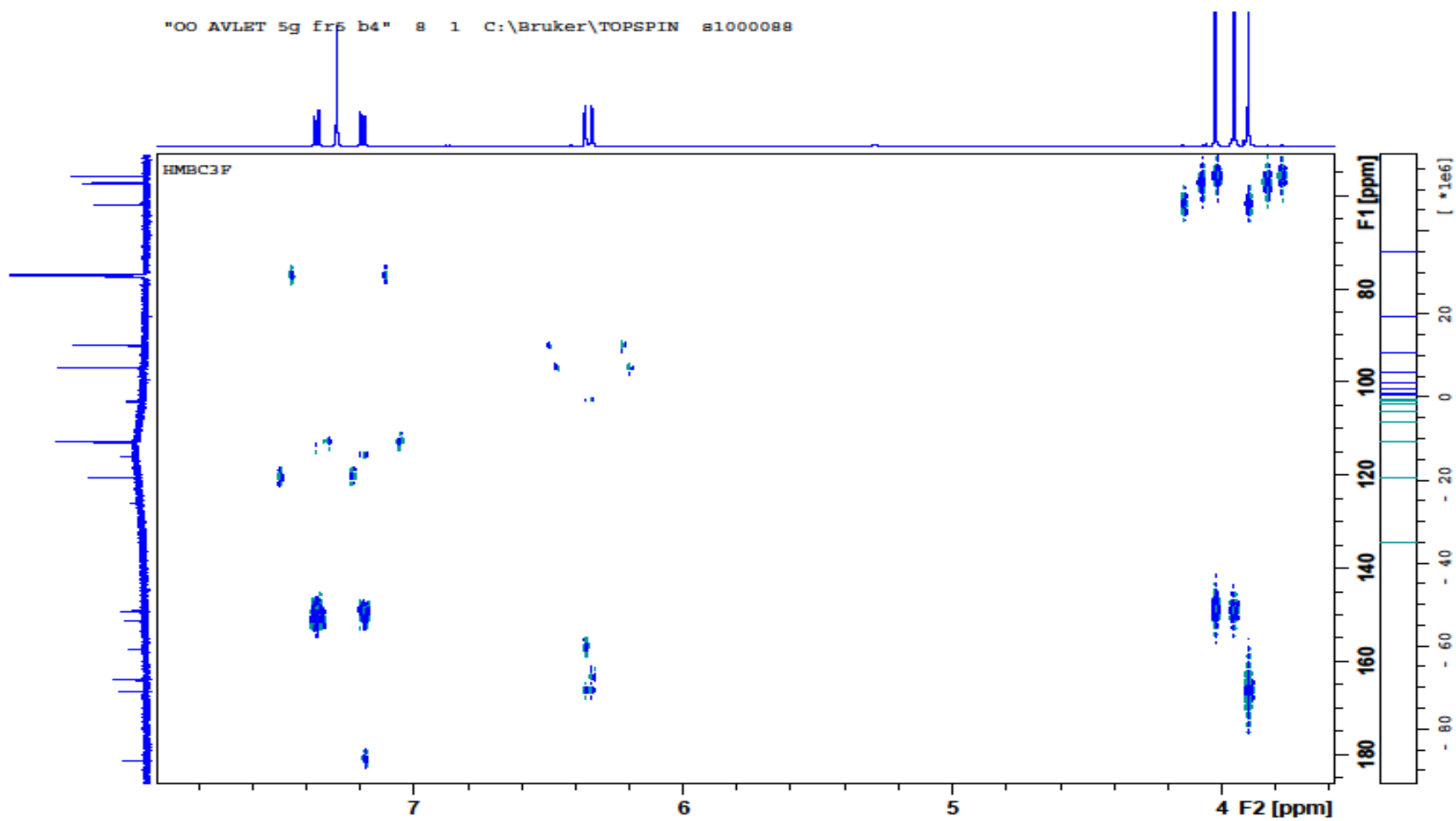**APPENDIX 19 DEPT 135 Spectrum of Decussatin from *Anthocleista vogelii* Leaf**

"OO AVLET 5g fr5 b4" 3 1 C:\Bruker\TOPSPIN s1000088

OO AVLET 5g fr5 b4

"OO AVLET 5g fr5 b4"   4   1   C:\Bruker\TOPSPIN   s1000088

OO AVLET 5g fr5 b4

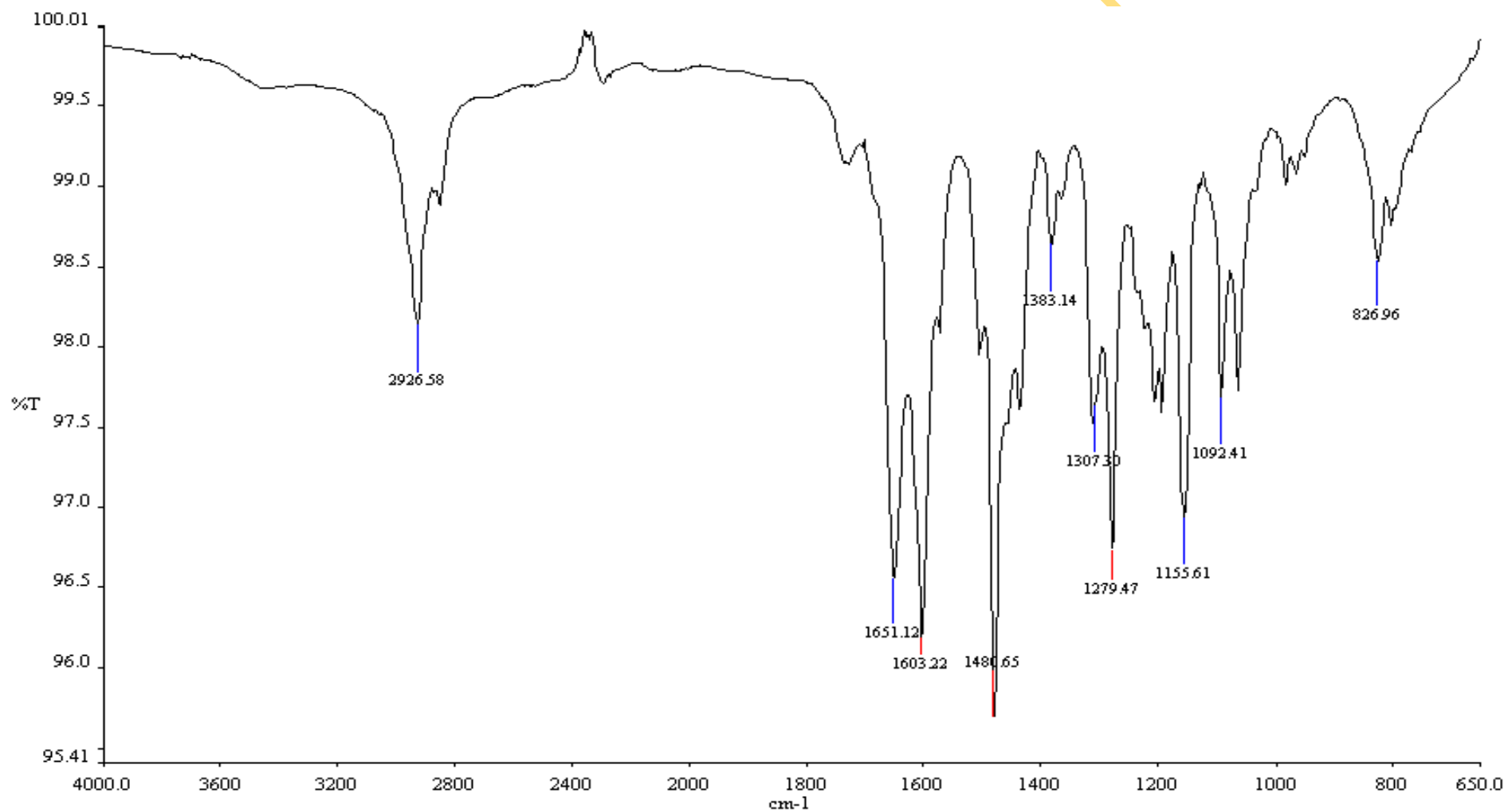"OO AVLET 5g fr5 b4"   7   1   C:\Bruker\TOPSPIN   s1000088
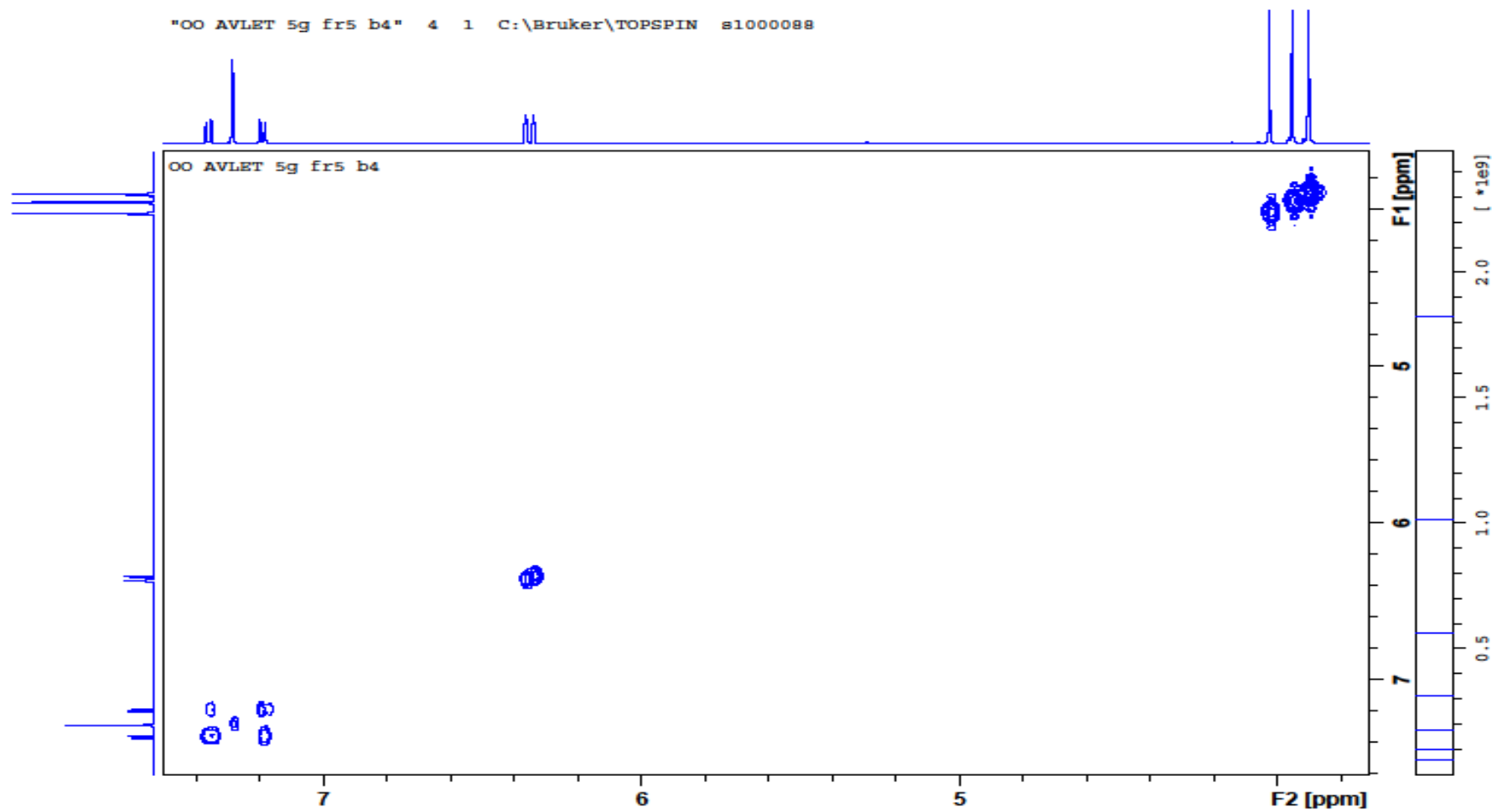
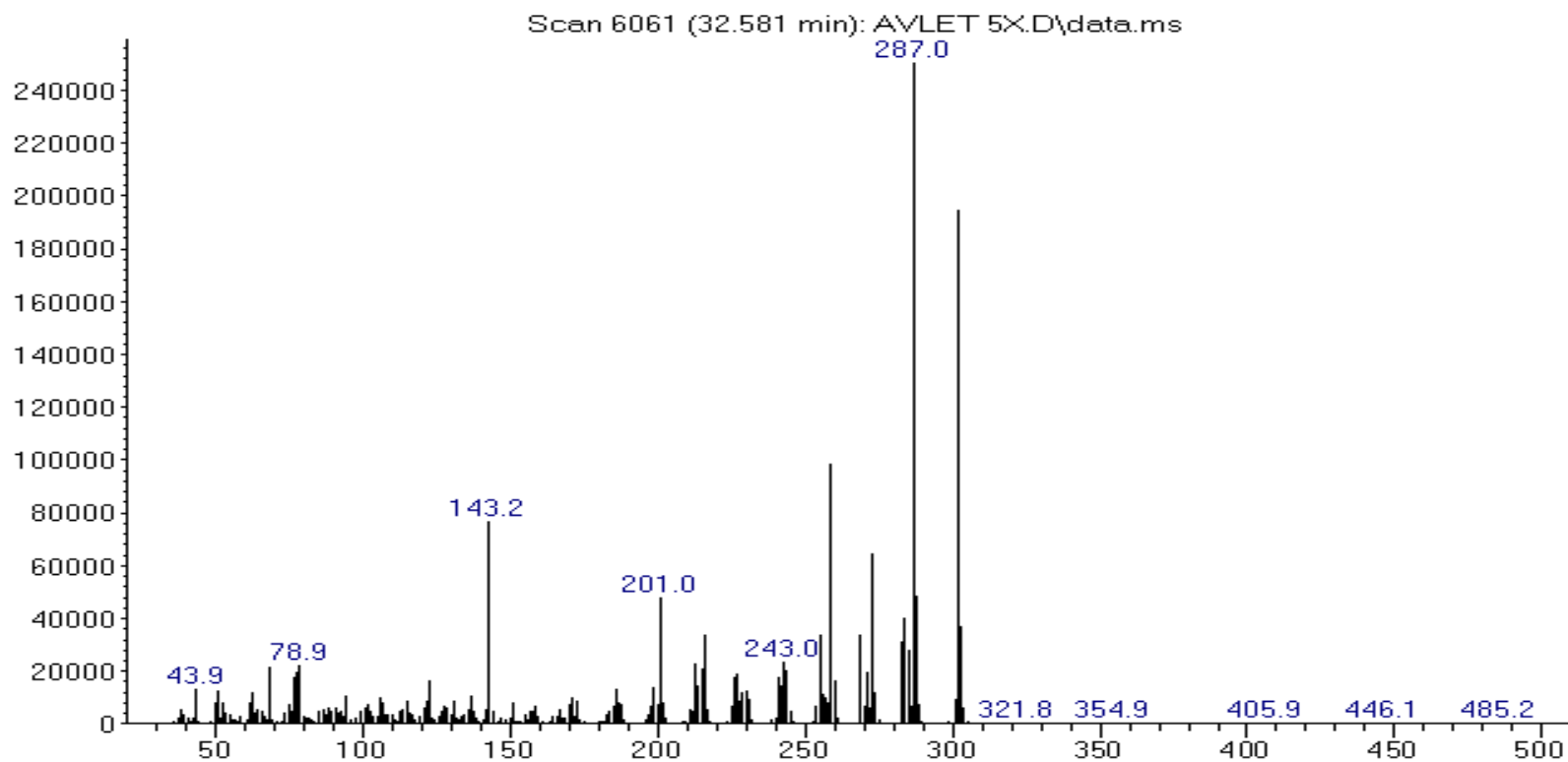**APPENDIX 22 HMBC Spectrum of Decussatin from *Anthocleista vogelii* Leaf**

APPENDIX 23 IR Spectrum of Decussatin from *Anthocleista vogelii* Leaf

APPENDIX 24 GC-MS Spectrum of Decussatin from *Anthocleista vogelii* Leaf

APPENDIX 25: Alpha-amylase inhibitory activity of two *Anthocleista* species and *in vivo* rat model anti-diabetic activities of

   Anthocleista   djalonensis extracts and fractions