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Effect of the Fractions of the Hexane Bark Extract and Stigmast-4-en-3-one Isolated from *Anacardium occidentale* on Blood Glucose Tolerance Test in an Animal Model

[1]Ruby Lisa Alexander-Lindo, [1]Errol Y.St.A. Morrison,
[2]Muraleedharan G. Nair and [3]Donovan A. McGrowder
[1]Department of Basic Medical Sciences (Biochemistry),
University of the West Indies, Mona, Kingston 7, Jamaica
[2]Department of Horticulture and Bioactive Natural Products and Phytoceuticals,
Michigan State University, East Lansing, Michigan
[3]Department of Pathology (Chemical Pathology), University of the West Indies, Mona, Kingston 7, Jamaica

Abstract: The study was undertaken to investigate the possible effect(s) of the hexane extract of the bark of *Anacardium occidentale* (cashew) and the fractions collected at different stages of the purification process on glucose tolerance in normoglycaemic dogs. The possible hypoglycaemic effect of stigmast-4-en-3-one isolated from the hexane extract of the bark of *Anacardium occidentale* and cholest-4-en-3-one was also investigated. The hexane extract of the bark of *Anacardium occidentale* was administered at 300 mg kg^{-1} Body Weight (BW) to normoglycaemic dogs followed by an oral glucose tolerance test. There was significant reduction in the fasting and postprandial blood glucose concentrations especially at the 1.0 and 2.0 h time points ($p<0.05$). Subsequent fractions of the hexane extract of the bark of *Anacardium occidentale* showed a hypoglycaemic effect on fasting and postprandial blood glucose concentrations. A hypoglycaemic assay guided extraction, isolation and structure elucidation produced stigmast-4-en-3-one. Stigmast-4-en-3-one administered at 3 mg kg^{-1} BW produced significant reductions in the postprandial blood glucose concentrations especially at the 1.5 h time point ($p<0.05$). Cholest-4-en-3-one at 3 mg kg^{-1} BW (i.v.) was found to be more potent than stigmast-4-en-3-one at the 1.5 h time point ($p<0.05$). The results indicate that stigmast-4-en-3-one isolated from hexane extract of the bark of *Anacardium occidentale* possesses hypoglycaemic activity and this lends credence to the suggested use of this herb in the control and/or management of type 2 diabetes mellitus in Jamaica and other Caribbean islands.

Key words: Diabetes mellitus, *Anacardium occidentale*, hexane extract, blood glucose

INTRODUCTION

For the past twenty years diabetes mellitus has been among the leading cause of death in the Caribbean. The point prevalence of diabetes mellitus in the Jamaican population is 17.9% which is set to double in the next two decades (Ragoobirsingh *et al*., 1995). The practice of traditional medicine in the Caribbean is currently an interest among biochemists, pharmacologists as well as botanists. The medicinal values of many plants typical of this tropical region are being investigated. Among these tropical plants *Anacardium occidentale*, or Cashew as it is commonly known, is of particular interest. It has been used in folklore medicine for treating diabetes mellitus. Preliminary work lends credence to the folkloric use of this plant in the management and/or control of type 2 diabetes mellitus (Morrison and West, 1982).

Anacardium occidentale Linn sp. Pl. 1: 383 (1753) is grown in countries such as Brazil and Jamaica (Adams, 1972). The different compounds produced by this tree have a wide range of applications. The bark and leaves of the tree are used medicinally. They are a rich source of tannins which have been found to exert anti-inflammatory and astringent effects in rats (Mota *et al*., 1985). Methanolic bark extracts exhibited *in vitro* anti-microbial activity in a number of micro-organisms (Akinpelu, 2001). In addition *Anacardium occidentale* is known to exert hypotensive effect in urethane anaesthetized normotensive Sprague-Dawley rats (Garg and West, 1994).

Aqueous extracts of *Anacardium occidentale* was reported to have a protective role against the diabetogenic action of streptozotocin in rats (Kamtchouing *et al*., 1998). Laboratory evaluation using

Corresponding Author: Ruby Lisa Alexander-Lindo, Department of Basic Medical Sciences (Biochemistry),
University of the West Indies, Mona, Kingston 7, Jamaica

stem-bark extracts showed that the hypoglycaemic effect of the methanolic plant extract was more pronounced than the aqueous extract in normoglycaemic and streptozotocin-induced diabetic rats (Ojewole, 2003). Stem-bark aqueous and methanolic extracts of *Anacardium occidentale* contain a diverse mixture of chemical compounds. The hyperglycaemic effect may be due to terpenoids, coumarin or any other compounds. Therefore it is difficult to draw any logical conclusion about the specific compound(s) within the extracts that may cause the hypoglycaemic effect (Ojewole, 2003).

Cholesterol functions as a precursor in the biosynthesis of a variety of plant steroids but does not occur much in plants (Singh *et al*., 1969). Cholesterol oxidase is a monomeric flavoenzyme that catalyzes the oxidation and isomerization of cholesterol to cholest-4-en-3-one (Coulombe *et al*., 2001). Cholest-4-en-3-one has been found as an intermediate in the conversion of cholesterol to 3β-cholestanol in potato leaves (Johnson *et al*., 1964). Stigmast-4-en-3-one may be an intermediate in the biosynthesis of β-sitosterol and biosynthesis of spirostanols such as tigogenin and gitogenin from cholesterol (Stohs and El Olemy, 1971). In addition, the biosynthesis of tigogenin and gitogenin from cholesterol passes through cholest-4-en-3-one (Tschesche *et al*., 1968). Cholest-4-en-3-one and stigmast-4-en-3-one both possess a double bond between C-4 and C-5 and a carbonyl group at C-3.

The aim of this study was to investigate the possible effect(s) of the hexane extract of the bark of *Anacardium occidentale* (cashew) and the fractions collected at different stages of the purification process on blood glucose tolerance in normoglycaemic dogs. In addition the possible hypoglycaemic effect of stigmast-4-en-3-one isolated from the hexane extract of the bark of *Anacardium occidentale* and cholest-4-en-3-one was also investigated.

MATERIALS AND METHODS

Preparation of plant extract: The bark of *Anacardium occidentale* (cashew) was collected, air-dried, ground to a fine powder and stored at room temperature. The dried milled bark (200 g) was extracted sequentially with hexane, ethyl acetate and methanol (3×800 mL) at room temperature over the course of 24 h for each solvent. The extract was filtered and the solvent evaporated under reduced pressure using a rotary evaporator (Buchi RE III). The yield of crude extracts from hexane, ethyl acetate and methanol were 1.56 g (0.78%), 6.42 g (3.3%) and 52.60 g (26.3%), respectively. The extracts were kept refrigerated till hypoglycaemic screening using the Oral Glucose Tolerance Test (OGTT), purification and identification of active components were carried out.

Purification of hypoglycaemic hexane extract: The purification of the hexane extract is documented in a publication by Alexander-Lindo *et al*. (2004). Briefly, the yellow-brown oily residue of the hexane extract of the bark of *Anacardium occidentale* showed hypoglycaemic activity. The hexane extract (13.39 g) was fractionated by Vacuum Liquid Chromatography (VLC) and Column Chromatography (CC) using silica gel (Analtech Silica Gel 60 A pore size, 35-75 μm diameter) as absorbent and using hexane and hexane/acetone mixtures as mobile phases. Four fractions of 50 mL (PI to PIV) were collected and analysed by Thin Layer Chromatography (TLC). When PI to PIV were assayed for hypoglycaemic activity, only PII showed significant hypoglycaemic activity. Therefore, fraction PII was further purified by repeated VLC on silica gel using hexane (100%, 600 mL), hexane: acetone (6: 1, 300 mL) and hexane: acetone (4: 1, 800 mL) as solvent systems (Alexander-Lindo *et al*., 2004). Three major fractions PIIA, PIIB and PIIC were obtained and assayed for hypoglycaemic activity. The fraction PIIB was found to possess hypoglycaemic activity and further purified by medium pressure liquid chromatography (MPLC) on silica gel and eluted with hexane (100%, 600 mL), hexane: acetone (10: 1, 300 mL), hexane: acetone (4:1, 5 00 mL) and finally acetone (100%, 600 mL) (Alexander-Lindo *et al*., 2004). Three major fractions PIIB1, PIIB2 and PIIB3 were obtained and assayed for hypoglycaemic activity. The active fraction PIIB2 was further purified by repeated MPLC. This resulted in the biologically active PIIB2c which was further purified by preparative TLC using a pentane: diethyl ether (6: 1) solvent system.

Stigmast-4-en-3-one was identified by spectral and chemical methods including Melting point, Ultra-Violet (UV), Infra-red (IR) and Nuclear Magnetic Resonance (NMR) Spectroscopy. Melting point was determined using a Thomas Model 40 micro hot-stage apparatus and is uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Varian VXR 300 and 500 MHZ spectrophotometers in deuterio-chloroform solutions (ca 10%). Chemical shifts are given δ units. EIMS and FABMS were recorded on JEOL JMS-AX505 and JEOL JMS HX110 mass spectrometers (Alexander-Lindo *et al*., 2004).

Animals: The protocol was conducted in accordance with guidelines of the University of the West Indies Animal Committee. In these experiments, normal healthy male and female dogs of an average weight of 10.0 kg were obtained

from the Pre-Clinical Animal House at the University of the West Indies. The animals were maintained in the animal house under supervision of the attendants and a veterinary consultant. They were all treated with KO de-worming medicine before use and maintained on multivitamin supplements, a diet of Purina Laboratory Chow (Purina, St. Louis, MO, USA) and water *ad libitum.*

Oral glucose tolerance test: Dogs were starved for approximately 18 h prior to each experiment. They were first weighed then anaesthetized using sodium pentobarbitone (30 mg mL^{-1} in water) which was given intravenously at a concentration of 1 mL kg^{-1} Body Weight (BW) of the animal. A cuffed endo-tracheal tube was inserted and connected to a Palmer's pump respirator to ensure adequate breathing of the animal throughout the experiment. A gastric tube was also inserted to enable the administration of the glucose load, 1.75 g kg^{-1} BW dissolved in water.

Each dog was used as its own control. The extract or the carrier (in the case of the control) was given immediately after a fasting blood sample was taken (F1). Blood samples were taken from the vein of the animal's leg. The blood samples (0.1 mL) were immediately pipetted into a centrifuge tube containing 2.5 mL of distilled water to avoid clotting. This was then later used in the glucose determination.

Samples taken at 30 and 60 min after anaesthetization were named as F2 and 0 h, respectively. The glucose load was then given via the gastric tube immediately after the 60 min blood sample was taken. Postprandial blood samples were then taken at 30 min intervals for the next 2 h. These samples were named 0.5, 1, 1.5 and 2 h, respectively. A total of 7 samples were taken and treated similarly to prevent clotting. The blood glucose concentration in all samples was determined using the glucose-oxidase method (Huggertt and Nixon, 1957). Absorbance was measured at 420 nm using Milton Ray Company Spectronic 20D. The centrifuge used during the hypoglycaemic screening was Dynac II Centrifuge, Clay Adams.

The crude hexane extract was administered orally at a concentration of 300 mg kg^{-1} BW and intravenously at a concentration of 30 mg kg^{-1} BW using ten (10) dogs for each concentration. Stigmast-4-en-3-one and cholest-4-en-3-one (Sigma Chemicals, St. Louis, MO, USA) were administered intravenously at 3 mg kg^{-1} BW using ten (10) dogs for each compound. The carrier solvent used was dimethyl sulfoxide (DMSO) which showed no effect on fasting and postprandial blood glucose concentrations.

Statistical analysis: Results are reported as Mean±SEM Blood glucose concentrations at different time points were compared by using the unpaired student's t-test for controls and test experiments. Further statistical comparison was made using analysis of variance (ANOVA) at the 95% confidence limits, followed by Benferroni multiple comparison test (LeFloch *et al*., 1990) to examine differences in blood glucose concentrations at each time interval during the OGTT. Analysis of the data was done using the Sigma Plot and Sigma Statistics software packages (Jandel Scientific). A probability value of less than 0.05 was considered to indicate significance in all cases.

RESULTS

Controls treated with corn oil exhibited a normal glucose tolerance curve (Fig. 1). The postprandial blood glucose concentration increased to 6.67±0.18 mmol L^{-1} at the 0.5 h time point and decreased below the fasting level at the 2 h time point (5.33±0.06 mmol L^{-1}). The efficacy of the crude hexane extract of the bark of *Anacardium occidentale* on the fasting blood glucose was significant. Crude hexane extract at 300 mg kg^{-1} BW caused a

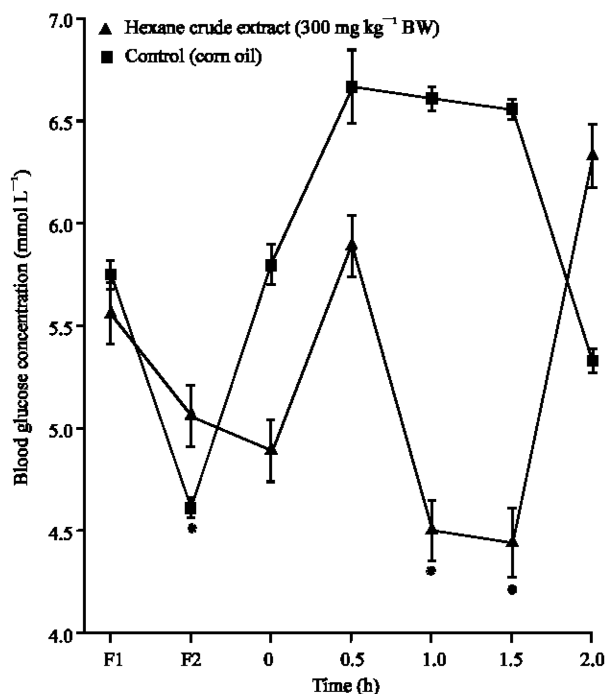

Fig. 1: Oral glucose tolerance test graphs of control dogs administered with corn oil (n = 10) and those treated orally with hexane crude extract at 300 mg kg^{-1} BW (n = 10). Statistical significant differences is shown by *$p<0.05$

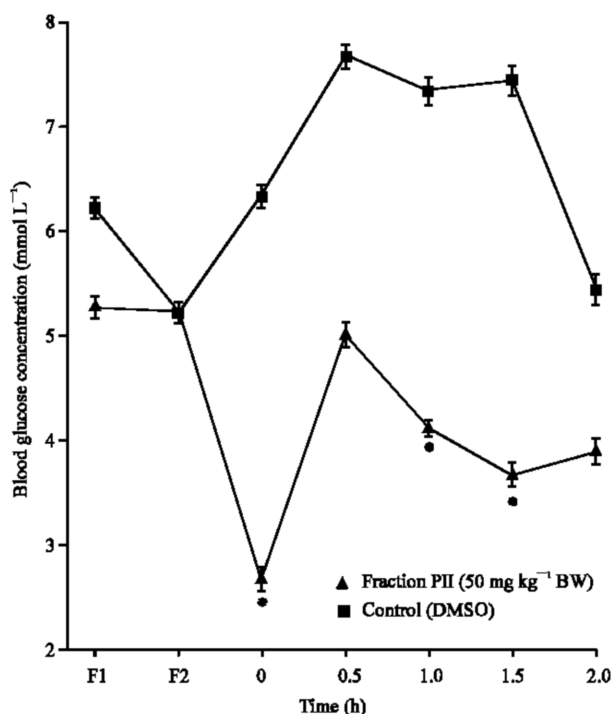

Fig. 2: Oral glucose tolerance test graphs of control dogs with DMSO (n = 10) and those treated with fraction PII at 50 mg kg^{-1} BW (n = 10) by i.v. administration. Statistical significant differences is shown by *$p<0.05$

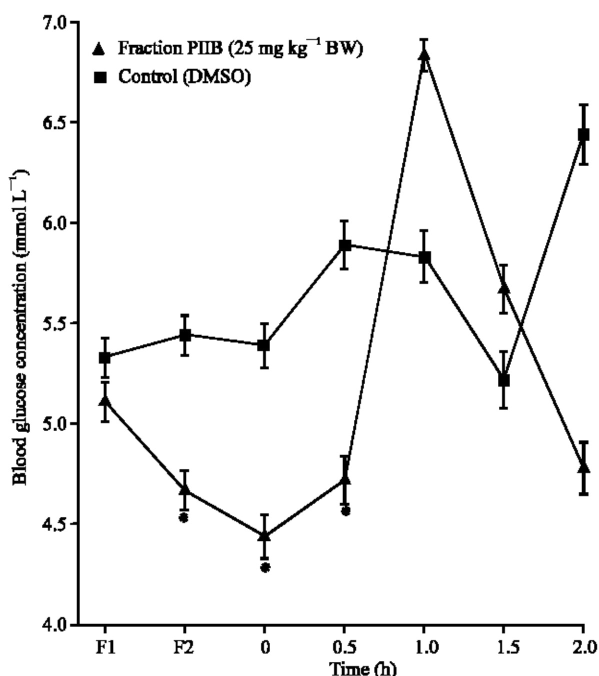

Fig. 3: Oral glucose tolerance test graphs of control dogs administered with DMSO (n = 10) and those treated with fraction PIIB at 25 mg kg^{-1} BW (n = 10) by i.v. administration. Statistical significant differences is shown by *$p<0.05$

significant reduction in fasting blood glucose concentration from 5.56±0.15 mmol L^{-1} at F1 to 4.89±0.11 mmol L^{-1} at 0 h time point. This was followed by an increase to 5.89±0.17 mmol L^{-1} at 0.5 h due to the oral administration of a glucose load. There was a significant reduction of postprandial blood glucose concentration to 4.50±0.20 mmol L^{-1} at 1.0 h ($p<0.05$) and 4.44±0.18 mmol L^{-1} at 1.5 h ($p<0.05$), thus exhibiting a hypoglycaemic effect.

The initial purification of the hexane crude extract yielded fraction PII. PII lowered both fasting and postprandial blood glucose concentrations (Fig. 2). PII significantly lowered the fasting blood glucose concentration from 5.27±0.10 mmol L^{-1} at F1 to 2.67±0.11 mmol L^{-1} at 0 h ($p<0.05$). This was followed by an increase of postprandial blood glucose concentration to 5.00±0.15 mmol L^{-1} at 0.5 h and significant lowering at 1.0 h (4.11±0.08 mmol L^{-1}) and 1.5 h (3.67±0.13 mmol L^{-1}; $p<0.05$) time points.

The fraction PIIB was administered at a dose of 25 mg kg^{-1} BW and resulted in a significant decrease in fasting blood glucose concentration at F2 (4.67±0.10 mmol L^{-1}) and 0 h (4.44±0.15 mmol L^{-1}) time points ($p<0.05$; Fig. 3). However, a significant increase in the postprandial blood glucose concentration was observed at 1.0 h (6.83±0.08 mmol L^{-1}) followed by a decrease (4.78±0.13 mmol L^{-1}) at the 2.0 h time point, indicating a secondary hypoglycaemic effect ($p<0.05$). All three values of blood glucose concentrations at F2, 0 and 0.5 h were considerably below the control values indicating the presence of hypoglycaemic principle component(s) in fraction PIIB which is/are responsible for reducing the fasting blood glucose concentration and the delayed response to the glucose load.

The fraction PIIB2 was administered at a dosage of 15 mg kg^{-1} BW (Fig. 4). The blood glucose concentration varied considerably with reduction of fasting at the 0 h time point (3.94±0.12 mmol L^{-1}) and of postprandial at time points 1.0 h (3.92±0.10 mmol L^{-1}) and 2.0 h (3.69±0.14 mmol L^{-1}; $p<0.05$). There were mild elevations of postprandial blood glucose concentrations at the 0.5 h (4.67±0.15 mmol L^{-1}) and 1.5 h (4.81±0.14 mmol L^{-1}) time points. Generally, the blood glucose concentrations were below those of the control indicating the presence of the active component responsible for the hypoglycaemic activity. The fraction PIIB2c administered at a lower dosage of 10 mg kg^{-1} BW was found to be active as shown in Fig. 4. The significant reduction in fasting

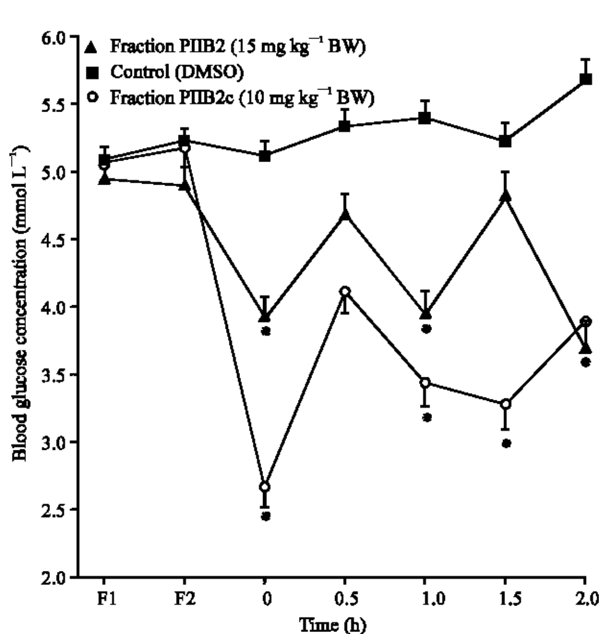

Fig. 4: Oral glucose tolerance test graphs of control dogs administered with DMSO (n = 10) and those treated with fraction PIIB2 at 15 mg kg^{-1} BW (n = 10) and fraction PIIB2c at 10 mg kg^{-1} BW (n = 10) by i.v. administration. Statistical significant differences is shown by *p<0.05

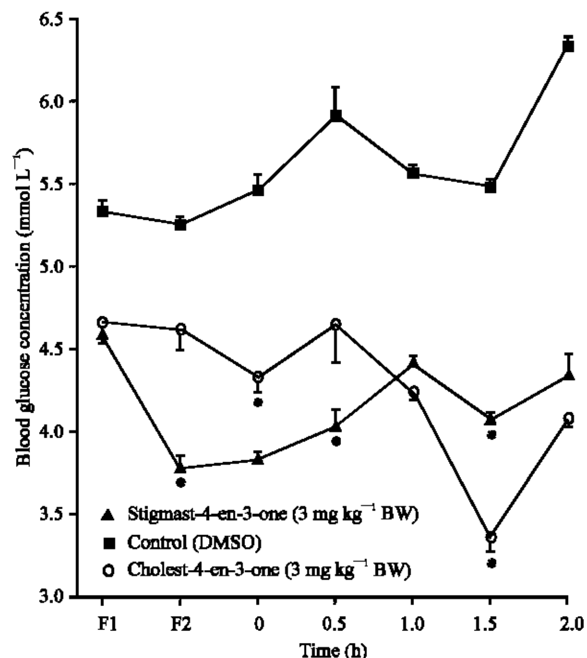

Fig. 5: Oral glucose tolerance test graphs of control dogs administered with DMSO (n = 10) and those treated with stigmast-4-en-3-one at 3 mg kg^{-1} BW (n = 10) and cholest-4-en-3-one at 3 mg kg^{-1} BW (n = 10) by i.v. administration. Statistical significant differences is shown by *p<0.05

blood sugar concentration was observed at 0 h (2.67±0.13 mmol L^{-1}; $p<0.05$) and there were also significant reductions in the postprandial values at the 1.0 h (3.44±0.15 mmol L^{-1}), 1.5 h (3.28±0.13 mmol L^{-1}) and 2.0 h (3.89±0.12 mmol L^{-1}) time points ($p<0.05$). In general, all of the blood glucose concentrations were lower than the control and indicated that this fraction has an anti-diabetic activity.

Stigmast-4-en-3-one at 3 mg kg^{-1} BW exhibited significant hypoglycaemic effect (Fig. 5). Significant reductions in fasting blood glucose concentrations of dogs treated with stigmast-4-en-3-one occurred at the F2 time point (3.78±0.08 mmol L^{-1}; $p<0.05$) and the 1.5 h time point (4.07±0.10 mmol L^{-1}; $p<0.05$). In addition, there was no significant elevation of the blood glucose concentration after the glucose load was administered at the 0 h time point, with maximum value of 4.41±0.30 mmol L^{-1} at the 1.0 h time point. The blood glucose concentration at 0.5 h (4.03±0.11 mmol L^{-1}) was significantly lower than that of the controls ($p<0.05$).

Cholest-4-en-3-one at a dosage of 3 mg kg^{-1} body weight exhibited a significant decrease in fasting blood glucose concentration at the 0 h time point (4.33±0.10 mmol L^{-1}; Fig. 5). There was a slight increase in the postprandial blood glucose concentration at the 0.5 h time point (4.65±0.24 mmol L^{-1}) followed by significant reduction at the 1.5 h time point (3.40±0.12 mmol L^{-1}; $p<0.05$).

DISCUSSION

The hexane extract of the bark of *Anacardium occidentale* contains a number of chemical compounds each of which is capable of producing definite biological activities via different mechanisms. The result of this experimental animal study indicates that the crude hexane extract of the bark of *Anacardium occidentale* and the fractions produced using purification by methods of VLC and TLC contained a number of compounds which possessed both hypoglycaemic and hyperglycaemic properties. One of the compounds present in the bark of *Anacardium occidentale* was identified as stigmast-4-en-3-one by comparison of spectral results obtained with spectral analysis from the literature (Greca *et al*., 1990; Takayuki and Shigeharu, 1974). Stigmast-4-en-3-one has been isolated from the stem of *Denbrobium clavatum* var. *aurantiacum* (Chang *et al*., 2001), *Harrisonia abyssinica* (Balde *et al*., 2000), chloroform extract of *Parkia speciosa* empty pods (Jamaluddin *et al*., 1995) and *Diospyros rhodocalyx* (Sutthivaiyakit *et al*., 1995). Stigmast-4-en-3-

one may be an intermediate in the biosynthesis of β-sitosterol and the biosynthesis of spirostanols such as tigogenin and gitogenin from cholesterol (Stohs and El Olemy, 1971). In addition, the biosynthesis of tigogenin and gitogenin from cholesterol passes through cholest-4-en-3-one (Tschesche *et al*., 1968) which also has been found to be an intermediate in the conversion of cholesterol to 3β-cholestanol in potato leaves (Johnson *et al*., 1964).

Stigmast-4-en-3-one appears to act promptly and markedly on both fasting and postprandial blood glucose concentrations. It exhibited significant hypoglycaemic activity especially at the 1.5 h time point. The presence of the double bond between C-4 and C-5 and a carbonyl group at C-3 may be responsible for the observed hypoglycaemic activity (Thomas and Lloyd, 1968). Cholesterol oxidase is a monomeric flavoenzyme that catalyzes the oxidation and isomerization of cholesterol to cholest-4-en-3-one (Coulombe *et al*., 2001). Cholest-4-en-3-one also possesses a double bond between C-4 and C-5 and a carbonyl group at C-3. Stigmast-4-en-3-one exhibited greater hypoglycaemic effect on fasting blood glucose concentration (F2 and 0 h) but cholest-4-en-3-one produced more significant reductions in postprandial blood glucose concentrations especially at the 1.5 h time point. The difference in hypoglycaemic activity may be related to their structure where there is the absence of an ethyl group at C-24 in cholest-4-en-3-one but present in stigmat-4-en-3-one (Tschesche *et al*., 1968).

The mechanism of the hypoglycaemic effect of stigmast-4-en-3-one and cholest-4-en-3-one could be similar to pancreatic hypoglycaemic mechanism of the sulphonylurea glibenclamide, which involves direct stimulation of β-pancreatic cells and subsequent insulin secretion and inhibition of glucagon release. This results in increased glucose uptake into muscle possibly by increasing glucose transporter activity (GLUT 1 and GLUT-4) and increased utilization by peripheral tissues (Gilman *et al*., 1990; Sandouk *et al*., 1993). In addition, extra-pancreatic hypoglycaemic mechanisms of these compounds could possibly be due to: an increase number of insulin receptors and insulin receptor binding affinity, an increase insulin sensitivity of peripheral tissues mediated by post-receptor mechanisms (Mimura *et al*., 1994) and a decrease hepatic glucose production and hepatic glucose output by decreasing gluconeogenesis (by blocking the enzyme pyruvate carboxylase) and glycogenolysis (Horikoshi *et al*., 1990).

A study by Ojewole and colleagues has found that methanolic stem-bark extract of *Anacardium occidentale* exhibited a hypoglycaemic effect in normoglycaemic rats (Ojewole, 2003). Two other studies have documented the protective quality of the leaf extract of *Anacardium occidentale* against steptozotocin-induced diabetes (Swanston-Flatt *et al*., 1989; Kamtchouing *et al*., 1998). In addition, stigmast-4-en-3-one isolated from chloroform extract of *Parkia Speciosa* empty pods has been found to possess hypoglycaemic activity in normal and alloxan diabetic rats and is therefore an oral hypoglycaemic agent occurring naturally in food (Jamaluddin *et al*., 1995).

The next step in our experimental studies will be to establish the mechanism(s) of the hypoglycaemic activity of stigmast-4-en-3-one and cholest-4-en-3-one. This will be carried out by investigating the hypoglycaemic effect of the compounds with glibenclamide in a type 2 diabetic model and examine any differences in cellular binding of insulin to its receptors on the cell membranes of adipocytes and muscle cells.

In conclusion, the results of this study provide evidence of the hypoglycaemic effects of stigmast-4-en-3-one isolated from the bark of *Anacardium occidentale* which may offer promise in the treatment of type 2 diabetes mellitus. This lends credence to its use in the treatment and/or management of Type 2 diabetes mellitus in Jamaica and other Caribbean islands.

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