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CHEMICAL SCIENCES

Characterization of *Blighia sapida* seed extracts

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Abstract: *Blighia sapida*, commonly known as the ackee, is a member of the Sapindaceae family. The tree is native to the forests of West Africa and was brought to the Caribbean and later Florida, where it is cultivated as an orchard crop in some areas. Arilli of the fruit are processed to make canned ackee in brine whereas the seeds, raphe and pods are discarded. Physiochemical studies were conducted on extracts of the seed. Qualitative analysis detected the presence of phenolics and reducing sugars. Aqueous extracts of the seeds (ASE) exhibited free radical scavenging activity and had an inhibitory concentration of 2.59 mg/mL. Gas chromatography mass spectrometry led to the identification of several metabolites including amino acids and fatty acids. Hypoglycin B was isolated utilizing ion exchange chromatography. Fourier transform infrared spectroscopy of hypoglycin B detected a band resonating at 3070 cm^{-1} which may be attributed to the methylenecyclopropane moiety of hypoglycin B. The seeds had a lipid content of 5.72 ± 0.25 % (w/w). The ackee seed oil (ASO) had a saponification value of 152.07 ± 37 and a carotenoid content of 23.7 ± 1.8 mg/kg. The ackee seeds are a source of bioactive components.

Key words: ackee seed, *Blighia sapida,* free radical scavenging activity, hypoglycin B, lipids.

INTRODUCTION

The ackee (*Blighia sapida*) is found growing in tropical regions such as the Caribbean and Florida but has its origins in West Africa. The tree is evergreen and polygamous taking approximately 4 years before bearing fruit. In its early stages, the ackee fruit has thick green ovoid pods that gradually become yellow, changing to red as the fruit matures. Once ripe, the fruit splits, exposing three cream or yellow coloured fleshy arilli, with a pink raphe embedded in the center and black shiny seeds at the head. In rare instances, there may be four arilli within the fruit.

Industrial applications of the fruit are in the area of agro-processing. Jamaica is the primary producer and exporter of canned ackees. Agro-processing involves the production of canned arilli in brine. Except for callaloo and breadfruit, ackee is one of the only agro-processing raw materials sourced one hundred percent (100%) locally. Ackee has always managed to stay closely competitive with top Jamaican agricultural export products such as coffee. For the fiscal year 2018, it was reported that canned ackee exports outperformed coffee grossing US $15,673 while the coffee crop grossed US $14,624 (STATIN 2019). Jamaica's Ministry of Industry, Commerce, Agriculture & Fisheries (MICAF) has plans to revolutionize ackee farming in Jamaica by aggressively pursuing the development of ackee orchard farms thereby increasing raw material availability, stabilizing and increasing the abundance of ackee agro-processing activities in Jamaica (MICAF 2017).

Large quantities of waste are generated from this industry inclusive of the seeds, pods, raphe and some arilli of the fruit. A comprehensive analysis of the ackee seed was undertaken with the intent to explore potential commercial applications. This is the first comprehensive report on the chemical composition of aqueous and non-aqueous extracts of the ackee seed. In the current study, extracts of the ackee seed were characterized utilizing various spectroscopic techniques such as Nuclear Magnetic Resonance Spectroscopy, Fourier Transform Infra Red Spectroscopy (FTIR) and Gas Chromatography Mass Spectrometry (GC-MS). We report for the first time the free radical scavenging activity and inhibitory concentration of ackee seed extracts. A comparison is made of the chemical characteristics of ackee seed oil extracts obtained from different geographical locations. An alternative method for the isolation of hypoglycin B, a dipeptide present in the seed of the fruit is detailed utilizing ion exchange and size exclusion chromatography. FTIR absorption frequencies associated with the methylenecyclopropane ring moiety within the hypoglycin structure were identified.

MATERIALS AND METHODS

Ackee seed extracts

Ackee seeds were obtained from a local manufacturer of canned ackee in brine. Excess arilli were removed from the seeds which were then washed, drained and dried. Seeds were dried to a constant weight (96 °C, 18 h) in a Beckman oven (17210E053) and ground to a fine powder utilizing a coffee grinder (Brentwood, Vernon, CA, USA). Two extracts were prepared from the dried milled seeds. For the first extraction, milled seeds were extracted with n-hexane utilizing direct solvent extraction (24 h) and Soxhlet extraction (2 h). The resulting ackee seed oil (ASO) was concentrated *in vacuo* (BUCHI Rotavapour R-124). Percent crude oil was determined gravimetrically and reported on a dry weight basis. The second extract was prepared by extracting dried milled seeds with ethanol (80 %). The resulting ethanolic extract (ASE) was concentrated *in vacuo* (BUCHI Rotavapour R-124).

Phytochemical screening

The following phytochemical tests were conducted on (ASE) (Sheel et al. 2014):

Saponins/Foam Test: Extracts were diluted with water (5 mL) and shaken vigorously. Foam formation is indicative of the presence of saponins.

Reducing sugars: To ASE was added a mixture of Fehling's solution A and Fehling's solution B (5 mL). This was followed by boiling. The formation of a red precipitate due to the formation of cuprous oxide and a green suspension is indicative of the presence of reducing sugars.

Phenolics: To ASE was added ferric chloride (10%, w/v, 1 mL) and the mixture vigorously shaken. The formation of a green precipitate is indicative of the presence of phenolic compounds.

Carboxylic acids: Sodium bicarbonate solution (5%, w/v, 1 mL) was added to the extract. Effervescence due to liberation of carbon dioxide constitutes a positive test.

Tannins: Ferric chloride (0.1%, w/v) was added to seed extracts. A colour change to blue or green is indicative of a positive test result.

Terpenoids: The Salkowski test was performed using concentrated sulphuric acid (2 mL) and chloroform (2 mL). The formation of a red brown coloration at the interface of the mixture is indicative of a positive test.

Characterization of ASO

Total soluble solids (TSS)

The TSS of ASO was measured using a refractometer (Hanna HI 96801 Refractometer).

Acid value & percentage free fatty acid content

The acid value of ASO was determined utilizing the standard AOAC method (1990). ASO extract (1 g) was mixed with neutralized ethanol (10 mL) and titrated against NaOH (0.1 M) using phenolphthalein as indicator.

$$\text{Acid Value} = \frac{\text{Titer (mL of 0.100 M)} \times 5.61}{\text{mass of sample used}}$$

$$\%\ \text{FFA} = \frac{\text{Titer (mL of 0.100 M)} \times 10^{-4} \times \text{MW} \times 100}{\text{mass of sample used}},$$

FFA = Free fatty acid
MW = Molecular weight of the predominant fatty in the seed lipid extract (Gondoic acid, 310.51 g/mol).

Saponification value

ASO samples were mixed with KOH (10 mL) and refluxed. The resulting mixture was titrated against HCl (0.5 M).

$$\text{Saponification value} = \frac{(\text{Blank} - \text{Titer volume}) \times 0.02805 \times 1000}{\text{weight of sample}}$$

Theoretical iodine value (IV)

The iodine value of ASO was determined based on its fatty acid profile and calculated utilizing the formula:

$$\text{Predicted IV} = xC1 + yC2 + zC3$$

C1, C2 and C3 corresponds to the relative percentage concentrations of unsaturated fatty acids (one, two and three double bonds, respectively) whereas x, y, and z are coefficients (x = 1, y = 1.5, and z = 2.62) (Kyriakidis & Katsiloulis 2000).

Carotenoid content

A spectrophotometric assay was used to determine the carotenoid content of ASO (Dauqan et al. 2011). Samples (0.5 g) were weighed and transferred to a volumetric flask and n-hexane (25 mL) added. The absorbance of the solution was measured at 446 nm.

$$\text{Carotenoid content (ppm)} = [V \times 383 \times (A_s - A_b)] / (100 \times W)$$

Where:
V = volume used for analysis
383 = Extinction coefficient for carotenoids
A_s = sample absorbance
A_b = blank absorbance
W = sample weight (g)

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay and IC_{50} value

The DPPH assay was performed according to the method of Brand-Williams et al. (1995). Ackee seed extracts (ASO & ASE, 200 mg) were suspended in ethanol (80%, v/v) containing HCl (1%, v/v, 2 mL). Extracts were reacted with the stable DPPH radical in an ethanol solution. The reaction mixture consisted of ackee extract (500 µL), DPPH (0.214 M, 0.3 mL) and ethanol (3 mL). This was followed by incubation in the dark at room temperature (30 min). The absorbance was measured at 517 nm using a spectrophotometer (Helios Omega, Thermo Fisher Scientific). A mixture of ethanol (3.3 mL) and extract (0.5 mL) served as the blank. A control solution was prepared by mixing ethanol (3.5 mL) with the DPPH radical solution (0.3 mL). The data obtained was used to calculate the radical scavenging capacity according to the formula:

$$\%\ \text{Free radical scavenging} = (1 - A_1/A_0) \times 100$$

where:
A_1 = abs of sample

A_0 = abs of control

The IC_{50} value for ASE was calculated from the linear regression equation of a dose response curve.

Purification of hypoglycin B

Hypoglycin B was isolated from ASE utilizing ion exchange chromatography and size exclusion chromatography. Amberlite IRA-402 (Cl) was the selected resin for ion exchange chromatography. The resin bed (3 cm x 13 cm) was sequentially washed with sodium hydroxide (1 M), distilled water, hydrochloric acid (1 M), distilled water, sodium hydroxide (1 M) and distilled water (final eluent pH of 7) prior to use. The ASE was applied to the column and eluted with HCl (1 M). The hypoglycin fraction was further purified utilizing 2-(diethylamino)ethyl-Sephadex A-50 (DEAE). Gradient elution was employed utilizing sodium chloride (0.1 M to 0.7 M) in 0.01 M sodium acetate buffer at pH 6.0. The protein concentration of each fraction collected was measured spectrophotometrically at 280 nm.

Nuclear Magnetic Resonance (NMR) Characterization

^{1}H and ^{13}C NMR characterization were performed on a Bruker BioSpin 500 MHz at 500 MHz. ASO was analyzed in deuterated chloroform ($CDCl_3$) with tetramethylsilane as the internal standard whereas ASE was analyzed in deuterated methanol (CD_3OD) at 25 °C. In reporting ^{1}H NMR the following terms were used: singlet (s), doublet of doublet (dd), triplet (t), multiplet (m).

Fourier Transform Infrared Spectroscopy (FTIR)

ASO and hypoglycin B were analyzed utilizing a Bruker Vector 22 Fourier Transform Infrared (FTIR) spectrometer. Samples were applied directly to the crystal of the instrumentation and the FTIR spectrum recorded between 4000 and 600 cm^{-1}. The spectrum was obtained by averaging 20 scans recorded at a resolution of 2 cm^{-1}. The spectrum was baseline-corrected. OPUS software was used to acquire and manipulate the spectral data.

Data analysis

Samples were analyzed in triplicate. Means and standard deviations of the data are presented. Data analysis was conducted utilizing Microsoft Excel.

Gas Chromatography Mass Spectrometry (GC-MS)

ASE (10.0 mg/mL) was silylated with N-tert-butyldimethylsilyl-N-methyl-trifluoroacetamide (MTBSTFA, 0.1 mL). Silylated samples (1 µL) were chromatographed on an Agilent HP6890 series Gas Chromatograph interfaced with a HP5972 Mass Selective detector (Santa Clara, CA, USA). Silyl derivatives were eluted with helium carrier gas (flow rate 1.2 cm^3/min) through a DB-1701 column (30 m × 0.25 mm i.d. × 0.25 µm film thickness, Agilent, Santa Clara, CA, USA) in an oven programmed at 80 °C for 2 min and increased at a ramp rate of 20 °C/min up to 280 °C for 10 min. Samples were injected at 250 °C while the detector was maintained at 280 °C. Constituents were identified by utilizing the National Institute of Science and Technology (NIST) library of mass spectra (match quality >90 %).

RESULTS AND DISCUSSION

Ackee seeds are a byproduct of the canned ackee in brine industry. Currently there are limited applications of the seed commercially. Several papers have detailed the chemical and nutritional content of the ackee arilli (Howélé et al. 2010, Grande-Tovar et al. 2019 & Sybron et al. 2019). There is however less documentation on the chemical composition of the seeds of the fruit. Recommendations have been made for the use of the seeds in animal feed and food for

human consumption due to its starch (44 %, w/w) and protein (22 %, w/w) content (Djenontin et al. 2009). This however should be approached with caution due to the toxic components hypoglycin A (L-α-amino-β-methylenecyclopropane propionic acid) and hypoglycin B (γ-L-glutamyl-α-amino-β-methylenecyclopropyl propionic acid), present in the seeds of the fruit (Bowen-Forbes & Minott 2009). The toxins are water soluble and should be removed prior to utilization in any food applications. The seeds have a high potash content and have been utilized in soap making (Omobuwajo et al. 2000). Preliminary phytochemical screening of seed extracts detected the presence of reducing sugars and phenolic compounds. Kaempferol-3-O-rutinoside was identified as the predominant phenolic compound present in ackee seed extracts (Sybron et al. 2019).

Oil extraction

Edible oils have numerous applications in the food industry. They serve as a heat transfer medium in frying and are components of various food products such as salad dressings, margarines and mayonnaise. Traditional sources of vegetable oil include soybean oil, cottonseed oil, corn oil and olive oil. Ackee seeds were evaluated for their oil content. The seeds were dried, and the oil extracted via direct solvent extraction and Soxhlet extraction. Higher yields of ASO was obtained by Soxhlet extraction (5.72 ± 0.25 %, w/w) as compared to direct solvent extraction (1.54 ± 0.38 %, w/w). The Soxhlet extractor is more efficient as it facilitates the continuous percolation of the sample with hot solvent. The percentage oil yield was lower than that reported for ackee seeds from Benin (22 %) and Nigeria (15 %) (Djenontin et al. 2009, Omobuwajo et al. 2000) and appears to be impacted by geographical location. Soxhlet extraction was employed for seeds obtained from Benin whereas direct solvent extraction with vigorous shaking (30 min) was utilized for seeds from Nigeria. Both extractions utilized hexane as solvent. The arilli of the mature fruit contains much higher levels of lipids containing over 50 % (w/w) on a dry weight basis (Goldson-Barnaby et al. 2018). ASO was further characterized. Parameters evaluated included the total soluble solids, acid value, free fatty acid content, saponification value, iodine value and carotenoid content (Table I).

Table I. Characterization of ackee seed oil (ASO).

Parameter	Values
Lipid content	5.72 ± 0.25 %
Total soluble solids	70.3°
Acid value	4.73 ± 0.43 mgKOH/g
Free fatty acid	2.62 ± 0.24 %
Saponification value	152.07 ± 37
Theoretical iodine value	62
Carotenoid content	23.7 ± 1.8 ppm

Total soluble solids (TSS)

A refractometer is routinely utilized to test the total soluble solids (TSS) content of beverages. It may however be utilized to assess the purity of oils and other ingredients. The TSS of ASO (70.3°) was similar to that of arilli oil (69°) (Goldson-Barnaby et al. 2018). Coconut and soybean oil had values of 66° and 73° respectively (Goldson-Barnaby et al. 2018). As observed, different oils exhibit different TSS values. TSS may therefore be utilized as one of the parameters to determine sample authenticity.

Acid value and percentage free fatty acid content

The acid value and percentage free fatty acid (FFA) are indicators of oil quality. Oil quality may

be reduced due to hydrolysis, oxidation and polymerization reactions. The acid value is the quantity of potassium hydroxide required to neutralize FFAs in 1 g of oil. Sodium hydroxide is used in oil refineries during the neutralization process. The oil is treated with sodium hydroxide and FFAs are converted to insoluble soaps. The oil is subsequently separated from the soaps via centrifugation and washing.

The acid value of ASO was 4.73 ± 0.43 mgKOH/g which is higher than values normally observed in other edible oils (Negash et al. 2019). This suggests the presence of higher levels of free fatty acids within the oil which is expected as the oil is unrefined. Ackee arilli oil exhibited a lower acid value of 1.3 mgKOH/g (Goldson-Barnaby et al. 2018). Corn oil has acid values ranging between 0.1 – 5.72 mgKOH/g (Bi et al. 2010, Moreau et al. 1996).

Free fatty acids (FFAs) indicate the processing or storage conditions of an oil. There is an inverse relationship between % FFA and oil quality. In the refining of oils FFAs are removed during neutralization. The FFA of refined edible oil must be ≤ 0.05 % of the oil's weight. Frying oils with a % FFA > 2 % are discarded or used in conjunction with fresh oil to reduce the % FFA. The % FFA of the ackee seed oil was 2.62 ± 0.24 %. This high value is as a result of the oil being unrefined.

Saponification value (SAP)

The saponification value (SAP) is dependent on the molecular weight and fatty acid composition of an oil. It is defined as the number of mg of potassium hydroxide required to saponify 1 g of fat. The larger the SAP value, the better its soap making ability. Larger SAP values are related to higher proportions of short chain fatty acids. Coconut oil which is an example of a saturated oil consists primarily of lauric acid (C12) and has a SAP value of 250 (Gopala et al. 2010). Avocado oil and sunflower oil which contains long chain fatty acids such as oleic acid (C18) and linoleic acid (C18:1) have lower SAP values of 201.65 and 188.17 respectively (Dymińska et al. 2017). ASO had a saponification value of 152.07 ± 37 which is close to the value of 145 reported by Djenontin et al. (2009). The low SAP value observed in ASO is due to the presence of the long chain fatty acids, gondoic acid (C20:1), arachidic acid (C20:1) and oleic acid (C18) (Goldson-Barnaby & Williams 2017). A higher SAP value of 245.43 mgKOH/g was reported for seeds from Nigeria (Omosuli 2014). Lipid extracts of the seeds have been utilized for soap making (Omobuwajo et al. 2000).

Table II. Fatty acid (% w/w) profile of ackee seeds (Goldson-Barnaby & Williams 2017).

[1]FAME		Seed
Palmitic acid	C16:0	4.50 ± 3.86
Stearic acid	C18:0	3.84 ± 1.86
Oleic acid	C18:1	13.65 ± 4.05
Linoleic acid	C18:2	*0.54
Arachidic acid	C20:0	28.72 ± 5.33
Gondoic acid	C20:1	48.36 ± 9.09
Behenic acid	C22:0	1.25 ± 0.27

[1]FAME: Fatty acid methyl ester. *Detected in 1 sample.

Iodine value (IV)

The iodine value (IV) measures the level of unsaturation in an oil. This value was calculated based on the fatty acid profile of ASO (Table II) reported by Goldson-Barnaby & Williams (2017). ASO had an IV of 62 which correlates well with the value of 66 reported by Djenontin et al. (2009) who also utilized the fatty acid composition of ASO to calculate the IV. Much lower values were reported for oil extracts of seeds from Nigeria (22.8) (Omosuli 2014). Gondoic acid (48 %, C20:1) is the main unsaturated fatty acid present in oil

extracts of the seed followed by oleic acid (14 %, C18) whereas arachidic acid (29 %, C20) is the major saturated fatty acid (Goldson-Barnaby & Williams 2017). Similar findings were reported by Djenontin et al. (2009) with gondoic acid (48 %), arachidic acid (20 %) and oleic acid (15 %) being the predominant fatty acids present. In the study conducted by Goldson-Barnaby & Williams (2017), ASO was methylated with methanol and acetyl chloride followed by GC-MS analyses. Djenontin et al. (2009) methylated ASO with sodium methanolate and HCl which was subsequently analyzed utilizing capillary gas chromatography. ASO is expected to be stable due to the absence of polyunsaturated fatty acids such as linoleic acid. The fatty acid composition of ackee seeds from Nigeria appears to be completely different from that reported for ackee seeds harvested in Jamaica and Benin. Oleic acid (39 %) and linoleic acid (35 %) were identified as the major fatty acids present in ackee seeds from Nigeria (Omosuli 2014). This variation in the reported fatty acid profile of ackee seeds from Nigeria could account for differences in the IV and SAP values observed in ASO from this location. ASO obtained from trees growing in Nigeria were reported as having a smoke point of 135 °C and a flash point of 170 °C (Omosuli 2014).

Carotenoid content

Carotenoids are fat soluble compounds contributing to the red, orange and yellow colours observed in carrots, pumpkin, mangoes, pineapples and tomatoes. They also contribute to the colour of various oils and are often used in the food, pharmaceutical, cosmetics, and animal feed industries as a natural colorant and antioxidant. Most carotenoids are tetraterpenoids (C40) and are derived from mevalonic acid. Crude palm oil is a rich source of carotenoids containing 500 – 700 mg/kg (Chiu et al. 2009). The main carotenoids present are α and β carotenes. Other palm fruits that contain carotenoids include *Astrocaryum vulgare* (tucumã), *Oenocarpus bacaba* (bacaba), *Mauritia flexuosa* (buriti), *Bactris gasipaes* (pupunha) and *Maximiliana maripa* (inajá) (Santos et al. 2015). Tucumã had the highest carotenoid content (1222 mg/kg) followed by buriti (540 mg/kg). Bacaba had the lowest carotenoid content (13 mg/kg). ASO was yellow in colour and had a carotenoid content of 23.7 ± 1.8 mg/kg which is comparable to that of ackee aril oil (21 ± 0.2 mg/kg) (Goldson-Barnaby et al. 2018).

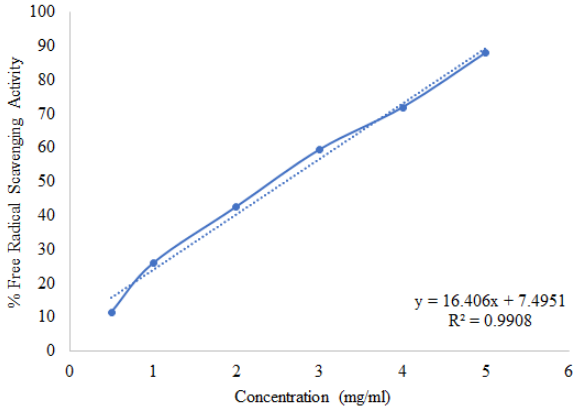

Figure 1. Free radical scavenging activity of aqueous seed extracts.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay and IC_{50} activity

Plant extracts are known to exhibit free radical scavenging activity (FRSA). This is due to the presence of secondary metabolites which aid in plant defense mechanisms. ASO and ASE exhibited FRSA with IC_{50} values of 75.7 mg/mL and 2.59 mg/mL, respectively (Figure 1). FRSA may be due to the presence of phenolic compounds. Stigmasterol (780 ppm) and β-sitosterol (420 ppm) have been reported as the major sterols in ASO (Djenontin et al. 2009). These phenolic compounds were also detected in ASE utilizing GC-MS. Campesterol (160 ppm) and Δ5-avenasterol (60 ppm) are present at

lower concentrations (Djenontin et al. 2009). A total tocopherol content of 338 ppm has been reported with α-tocopherol being present in the highest concentration (147 ppm) followed by γ-tocopherol (122 ppm) and δ-tocopherol (70 ppm) (Djenontin et al. 2009).

Olea europaea (olive) oil contains the phenolic compounds hydroxytyrosol and oleuropein that are potent scavengers of superoxide radicals (Visioli et al. 1998). *Punica granatum* L. (pomegranate) seed oil exhibited significant free radical-scavenging activity utilizing the DPPH and 2,2'-azinobis-(3-ethylbenz-thiazoline-6-sulfonic) diammonium salt (ABTS) radical assays (Liu et al. 2012). The antioxidant property of pomegranate seed oil is due to the presence of tocopherols. Kernel oil from *Torreya grandis*, *Carya cathayensis* and *Myrica rubra* exhibited free radical scavenging activity with IC_{50} values ranging from 0.060 mg/mL to > 0.120 mg/mL depending on the geographical region from which they were harvested (Ni & Shi 2014). While the FRSA of ackee seed extracts is lower than that reported in the literature, the data suggests that the seeds are a source of bioactives which can be further explored.

Purification of hypoglycin B

Ion exchange chromatography is widely utilized for the purification of proteins (Jungbauer & Hahn 2009). Amberlite IRA-402 (Cl) a strongly basic anion exchange resin, was used in the purification of hypoglycin B. This resin has a crosslinked polystyrene structure with lower cross linkages (6 %) allowing for better diffusion rates. It is utilized in water treatment, deionization and removal of amino acids. ASE extract was chromatographed (Amberlite IRA-402) and the fractions eluted with HCl. Fraction 4 contained the highest protein content as determined by UV spectrophotometry. This fraction was further purified using size exclusion chromatography

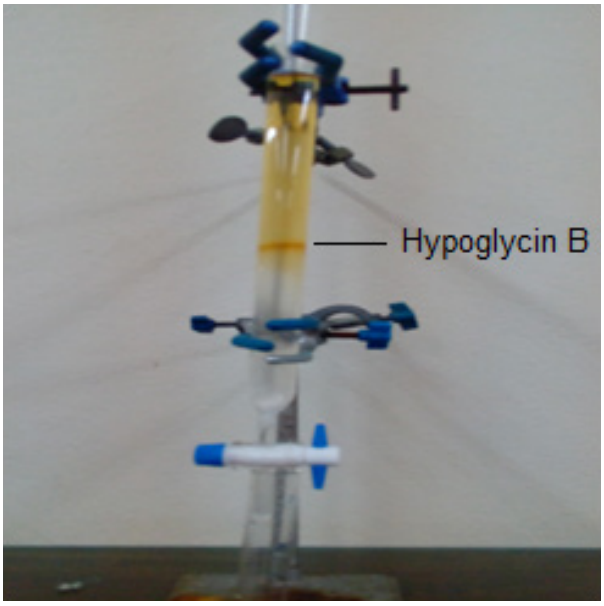

Figure 2. Purification of Hypoglycin B using DEAE Sephadex.

(DEAE Sephadex A50) (Figure 2) utilizing NaCl (0.1 M) as eluant. The desired compound eluted in fraction 3 (0.48 %, w/w).

In a prior study, hypoglycin B was purified utilizing a strongly acidic cation exchange resin [Dowex 50, (H+)] which was eluted with pyridine (1 M) followed by a strongly basic anion exchange resin (Dowex 1), which was eluted with acetic acid (Bowen-Forbes & Minott 2009). Purification of hypoglycin B with Amberlite IRA-402 (Cl) and DEAE Sephadex A50 eliminated the need for the use of pyridine.

Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopic analysis of ASO showed peaks characteristic of the presence of triacylglycerols. In the ^{1}H NMR, terminal methyl groups from fatty acid side chains was observed at δ 0.82 and methylene groups at δ 1.20. The protons on the glycerol backbone of the triacylglycerol were observed as a doublet of doublets (δ 4.10 and δ 4.27) and a multiplet (δ 5.21). Unsaturation within sides chains of the triacylglycerols was evident

from a peak resonating at δ 5.27 (Table III) which is due to the presence of gondoic acid and oleic acid. The ^{13}C NMR had peaks resonating between δ 13.99 and δ 34.07 due to alkyl side chains within the triacylglycerols (Table IV). Carbons on the glycerol backbone of the triacylglycerol were observed at δ 61.99; δ 68.86, δ 172.64 and δ 173.05. Unsaturation was indicated by peaks resonating at δ 129.66; δ 129.77 and δ 129.85.

The ^{1}H NMR spectroscopic data of hypoglycin B correlated with that reported in the literature (Bowen-Forbes & Minott 2009). Protons from the exocyclic double bond (C7) present in hypoglycin B resonated as a singlet at δ 5.36 and δ 5.40. The proton attached to C2 resonated downfield as a multiplet (δ 4.28) due to its close proximity to the carboxylic carbon and amine functionality. The proton at C2' resonated at δ 3.80. Methylene protons were observed at δ 1.67, δ 1.86 (C3), δ 2.14 (C3') and δ 2.54 (C4'). Protons on the cyclopropyl ring resonated at δ 1.03 and δ 1.32 (C6) and δ 1.45 (C4).

Fourier Transform Infra Red (FTIR) spectroscopy

FTIR analysis of ASO revealed significant absorption bands at 2922 cm^{-1}, 2852 cm^{-1}, 1743 cm^{-1} and 1165 cm^{-1} (Figure 3 and Table V). The sharp band at 1743 cm^{-1} was due to the carbonyl stretching (carboxyl ester functionalities) present in triacylglycerols (El-Bahy 2005, Poiana et al. 2015). Aliphatic stretching vibrations due to the acyl side chains of triacylglycerols were demonstrated by bands occurring at 2852 cm^{-1} and 2922 cm^{-1} (Figure 3). Methylene (CH_2) bending vibrations were observed at 1165 cm^{-1} (El-Bahy 2005, Poiana et al. 2015). Olefinic carbons due to the presence of gondoic and oleic acids were observed at 721 cm^{-1} (Poiana et al. 2015).

A characteristic feature of hypoglycin is the presence of a methylenecyclopropane ring. Detailed assignment of the methylenecyclopropane moiety may be beneficial in detecting the presence of hypoglycin in ackee samples. Research conducted by Bertie and Norton characterized the infrared, Raman spectra and vibrational assignment of methylenecyclopropane in which two regions of absorption were reported for CH stretching vibrations (Bertie & Norton 1970). These included a methylenic stretch (2996 cm^{-1}, 2999 cm^{-1}, 3070 cm^{-1})

Table III. ^{1}H Nuclear magnetic resonance spectroscopy of ackee seed lipid extracts.

Proton	Functionality	Seed δ ppm
CH_3	Terminal methyl	0.82 (m)
CH_2	Methylene	1.20 (s)
CH_2-CH_2-COO	Acyl chains	1.55 (m)
CH_2-COO	All acyl chains	2.25 (t)
	Diacylglycerol	3.60 (s)
CH_2O(α)	Glycerol (triglycerides)	4.10 (dd)
		4.27 (dd)
CHO(β)	Glycerol (triglycerides)	5.21 (m)
CH=CH	Olefinic protons	5.27 (m)

m: multiplet; s: singlet; dd: doublet of doublet, t: triplet.

Table IV. ^{13}C Nuclear magnetic resonance spectroscopy of ackee seed lipid extracts.

Carbon	Assignment	Seed δ (ppm)
α-CH_3	Acyl chains	13.99 (CH_3)
β-CH_3	Acyl chains	22.61 (CH_2)
C3	Acyl chains	24.77 (CH_2)
C8–11 (oleyl)	Allylic	27.12 (CH_2)
CH_{2n}	Acyl chains	29.04-29.69
β-C2	Acyl chains	34.07 (CH_2)
α-CH_2O	Glycerol moiety	61.99 (CH_2)
β-CH_2O	Glycerol moiety	68.86 (CH)
β-C9	Oleyl	129.53 (CH)
C11	Gondoyl	129.66 (CH)
α-C12	Gondoyl	129.77 (CH)
α-C10	Oleyl	129.85 (CH)
α-C1	Glycerol moiety	172.64 (C)
β-C1	Glycerol moiety	173.05 (C)

Table V. FTIR spectral data for ackee seed oil.

Functionality	Frequency cm^{-1}
C-H stretching (aliphatic) (asymmetric stretching vibration of C–H of aliphatic CH_2 group)	2922 (s)
Symmetric stretching vibration of C–H of aliphatic CH_3 group	2852 (s)
C=O stretching (triacylglycerols) (stretching vibration of ester carbonyl functional groups of triacylglycerides)	1743 (s)
C=O stretching vibration of free fatty acid carbonyl group	1712 (sh)
CH_2 bending (acyl chain) and/or CH_3 deformation (bending vibration of C–H of CH_2 and CH_3 aliphatic groups)	1458 (m)
CH_3 bending (bending symmetric vibration of C–H bonds of CH_2 groups)	1379 (w)
CH_2 bending (methylene)	1165 (m)
C-O-C stretching (esters) stretching vibration of C–O ester groups	1095 (w)
–HC=CH-cis (olefinic carbons) overlapping of CH_2 rocking vibration and the out-of-plane vibration of cis –HC=CH- group of disubstituted olefins	721 (m)

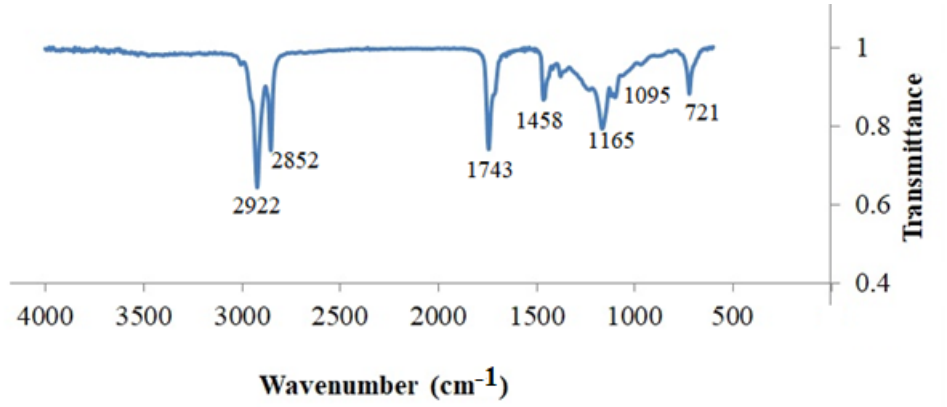

Figure 3. FTIR spectrum of ackee seed oil.

and ethylenic stretch (3008 cm^{-1}, 3086 cm^{-1}). The methylene (C=C) stretching vibration was observed at 1742 cm^{-1} with CH_2 deformation vibrations occurring at 1411 cm^{-1} and an ethylenic wag at 890 cm^{-1} (Bertie and Norton 1970). Three ring vibrations were reported, namely, the ring breathing mode (1034 cm^{-1}), the ring deformation mode (723.8 cm^{-1}) and the ring stretching mode (895 cm^{-1}) (Bertie & Norton 1970).

In the infrared spectral data reported for hypoglycin B (Table VI) the band at 3070 cm^{-1} was assigned as an amino acid (NH^{3+}) (Bowen-Forbes & Minott 2009). Based on the assignments by Bertie and Norton (1970) this band may be due to the CH stretching vibration of methylenecyclopropane. Bands indicative of the presence of the methylenecyclopropyl moiety of hypoglycin B were observed at 725, 897, 1047, 1425, 1744, 2993, 3070 and 3086 cm^{-1} (Table VI). Other pronounced bands observed were due to the carbonyl moiety of hypoglycin B (1724 cm^{-1}) and a band at 3429 cm^{-1} which is due to an amide functionality.

Gas Chromatography Mass Spectrometry (GC-MS)

ASE was silylated and analyzed by GC-MS. Metabolites identified in the extract included amino acids, lipids, phenolics, sugars and

Table VI. FTIR spectral data for hypoglycin B.

Assigned Functionality	Hypoglycin B cm $^{-1}$
Ring vibrations	630, 725,766
Olefin (CH_2=C)	862, 924
Ring vibrations	897, 1014, 1047
CN stretching, NH bending	1229
CH_2 Deformation	1425
Amino acid CO^{-2}	1427
Amino acid NH_3, CN stretching, NH bending	1582
Amide, Carbonyl, C=O stretching	1693
Carbonyl	1724
Methylene	1744
NH and/or OH	2943
Methylenic stretch	2993
Amino acid NH^{3+}	2996
Methylenic stretch	3070
Ethylenic stretch	3086
Amide, NH stretching	3269-3429

carboxylic acids. Amino acids detected in seed extracts included isoleucine, valine, glutamine, tyrosine, proline, aspartic acid, alanine, threonine and phenylalanine (Table VII). Prior studies have confirmed the presence of these amino acids with glutamic acid (3.9 g/100 g), arginine (2.5 g/100 g), aspartic acid (2.3 g/100 g) and leucine (2.1 g/100 g) being present in the highest concentration (Djenontin et al. 2009). Other amino acids detected by Djenontin et al. (2009) included serine, threonine, glycine, alanine, proline, valine, methionine, isoleucine, phenylalanine, cysteine, lysine, histidine and tyrosine. Golden et al. (2002) reported the presence of hydroxyproline, glutamic acid, serine, glycine, threonine, alanine, histidine, arginine, tyrosine, valine, methionine, isoleucine, phenylalanine and lysine. The dipeptide hypoglycin B was present in the highest concentration (106.0 per 100 g) followed by histidine (44.5 mg per 100 g) (Golden et al. 2002). We were unable to detect

Table VII. Metabolites identified in *Blighia sapida* seed ethanolic extracts.

Amino acids	**Percentage (%)**
Isoleucine	1.01 ± 0.38
Valine	0.80 ± 0.45
Glutamine	0.76 ± 0.09
Tyrosine	0.74 ± 0.07
Proline	0.71 ± 0. 0
Aspartic acid	0.47 ± 0.11
Alanine	0.49 ± 0.14
Threonine	0.42 ± 0.25
Phenylalanine	0.41 ± 0.04
Pyroglutamic acid	26.9 ± 7.38
Fatty acids	**Percentage (%)**
Hexadecanoic acid (palmitic acid)	13.30 ± 6.60
Octadec-9-enoic acid (oleic acid)	1.81 ± 0.30
Octadecanoic acid (stearic acid)	1.76 ± 1.41
9,12-Octadecadienioic acid (linoleic acid)	1.00 ± 0.71
Dodecanoic acid (lauric acid)	0.71 ± 0.03
Eicosanoic acid (arachidic acid)	0.67 ± 0.25
Carboxylic acids	**Percentage (%)**
Butanedioic acid (succinic acid)	1.70 ± 0.42
Malic acid	0.94 ± 0.16
Benzoic acid	0.39 ± 0.08
2-Butenedioic acid (fumaric acid)	0.27 ± 0.04
Trans cinnamic acid	0.24 ± 0.08
Phenolic compounds	**Percentage (%)**
β-sitosterol	3.06 ± 0.0
Stigmasterol	1.19 ± 0.0
Carbohydrates	**Percentage (%)**
4-oxo pentanoic acid (levulinic acid)	11.4 ± 5.06
Myo-inositol	0.84 ± 0.54
Glucose	0.49 ± 0.12

the presence of hypoglycin B in ASE utilizing GC-MS as this compound is not present in the NIST library utilized for the analysis. High levels of pyroglutamic acid (27 %) were detected in seed extracts. At elevated temperatures pyroglutamic acid is formed by the cyclization of glutamic acid or glutamine to form a lactam. Trace levels of the fatty acids, palmitic acid, oleic acid, stearic acid, myristic acid, lauric acid, linoleic acid and arachidic acid were detected (Table VII). The carboxylic acids, malic acid, benzoic acid, *trans* cinnamic acid, 2-butenedioic acid (fumaric acid) and butanedioic acid (succinic acid) were identified. Malic acid exhibits antimicrobial activity against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* (Kim et al. 2016). Another analyte detected in ASE was 4-oxo pentanoic acid (levulinic acid). Levulinic acid is formed from starch under acidic conditions. Ackee seeds are a rich source of starch (Djenontin et al. 2009, Goldson-Barnaby & Williams 2017). Glucose, a reducing sugar, myo-inositol and the phenolic compounds stigmasterol and β-sitosterol were also detected in ethanolic extracts of the seed (Table VII). Myo-inositol plays a role in the storage of phosphates in seeds (Mitsuhashi et al. 2008).

CONCLUSION

Ackee seed extracts are a source of bioactive components exhibiting free radical scavenging activity. Hypoglycin B was successfully isolated and may be detected by FTIR spectroscopy, a rapid and non-destructive method of analysis.

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