PHYTOCHEMICAL ANALYSIS OF ACKEE (*BLIGHIA SAPIDA*) PODS

by

AINSLEY A. PARKINSON

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Date	Chair of Examining Committee Dr. Edward J. Kennelly, Lehman College, CUNY
Date	Executive Officer Dr. Richard L. Chappell
	Dr. Dwight T. Kincaid Lehman College, CUNY
	Dr. Miguel Cervantes-Cervantes Lehman College, CUNY
	Dr. Jack G. Valdovinos Lehman College, CUNY
	Dr. Linda S. Einbond Columbia University
	Dr. Eugene P. Mazzola University of Maryland

THE CITY UNIVERSITY OF NEW YORK

Abstract

PHYTOCHEMICAL ANALYSIS OF ACKEE (*BLIGHIA SAPIDA*) PODS

By

Ainsley Anthony Parkinson

Advisor: Dr. Edward J. Kennelly

Blighia sapida Köenig (Sapindaceae), known commonly as ackee or akee, is a plant indigenous to equatorial Africa and cultivated in the West Indies. The fruit aril of ackee is edible, and is an important ingredient in a popular Jamaican codfish dish. The pod and the seed of the fruit are discarded, and are a byproduct in the ackee canning industry, accounting for almost 70% (w/w) of the ackee fruit. Three new triterpene saponins, 3-*O*-[α-L-arabinopyranosyl-(1→4)-3-*O*-acetyl-*β*-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl] hederagenin (**1**) (blighoside A), 3-*O*-[α-L-arabinopyranosyl-(1→4)-3-*O*-acetyl-*β*-D-glucopyranosyl-(1→3)-α-L-rhamno-pyranosyl-(1→2)-α-L-arabinopyranosyl] oleanolic acid (**2**) (blighoside B), and 3-*O*-[4,6-*O*-diacetyl-*β*-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→4)-3,6-*O*-diacetyl-*β*-D-glucopyrano-syl-(1→3)-α-L-rhamnopyranosyl(1→2)-*β*-D-xylopyranosyl-(1→3)-*β*-D-xylopyranosyl] oleanolic acid (**3**) (blighoside C), and two known steroids, stigmasta-5,22-dien-3-ol and stigmasta-5,22-dien-3-*O*-glucopyranoside, were isolated from the EtOAc fractions of *B. sapida* pod. Blighosides A-C, which are monodesmosidic

saponins, exhibited significant cytotoxic effect on the growth of ER^- MDA-MB-453 (Her2 overexpressing) human breast cancer cells at IC_{50} = 6.9 μM (**B**), 10.0 μM (**C**), and 10.3 μM (**A**), as compared to actein (IC_{50} = 14.8 μM; 10 μg/ml). The *n*-butanol and EtOAc fractions of the ackee pod also exhibit inhibitory effects at IC_{50} = 20 μg/ml and 43 μg/ml, respectively.

Additionally, in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical assay, the EtOAc fractions of *B. sapida* pod and seed showed strong DPPH antioxidant activities, and high total phenolic and flavonoids content. Through activity-guided fractionation of selected fractions, six known polyphenolic antioxidants, methyl gallate (**1**), quercetin 3-*O*-*β*-glucopyranoside (isoquercitrin) (**2**), cyanidin 3-*O*-*β*-glucopyranoside (**3**), 3,4-dihydroxybenzoic acid (protocatechuic acid) (**4**), gallic acid (**5**), ellagic acid (**6**), and quercetin (**7**) were isolated and identified from the EtOAc fractions of the ackee pod for the first time. These polyphenolic antioxidants have strong radical scavenging properties and may contribute to the high activity of the ackee pod.

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IMPORTANT ABBREVIATIONS USED IN THE TEXT

^{13}C-NMR	Carbon-13 nuclear magnetic resonance
CHD	Coronary heart disease
CVD	Cardiovascular disease
COSY	Correlation spectroscopy
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle's medium
DPPH	1,1-diphenyl-2-picrylhydrazyl
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FRAP	Ferric reducing ability/antioxidant power
HMBC	Heteronuclear multiple bond correlation
^{1}H-NMR	Proton nuclear magnetic resonance
HPLC	High performance liquid chromatography
HRESIMS	High resolution electronspray ionization mass spectrometry
HSQC	Heteronuclear single quantum coherence
LDL	Low-density lipoprotein
LH-20	Sephadex LH-20
PDA	Photodiode array
QE	Quercetin equivalent
ROESY	Rotating frame Overhauser effect spectroscopy
ROS	Reactive oxygen species
SEM	Standard error of the mean

Si gel	Normal phase silica gel
TE	Trolox equivalent
TLC	Thin layer liquid chromatography
TOCSY	Total correlation spectroscopy
TOF-ESIMS	Time-of-flight electronspray ionization mass spectrometry
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
TPC	Total phenolic content
TFC	Total flavonoid content
UV-visible	Ultra-violet and visible spectra

CHAPTER 1

Introduction

1.1. *Phytochemical Antioxidants and Human Health*

There is considerable interest in the use of plant-derived chemicals, specifically phytochemical antioxidants, which may protect against coronary heart disease (CHD), cancer, and other diseases.[1-4 5,6] Certain phytochemical antioxidants have been found to be effective cardioprotective agents *in vivo* and *in vitro*. [7-9] These compounds are produced by plants in which their chemical properties provide protection against high ultraviolet (UV) exposure, oxidative stress cause by reactive oxygen species (ROS) such as superoxide anion, and attack by microorganisms and various herbivores.[10,11] Phytochemical antioxidants from fruits and vegetables have been given special consideration because many are ingested in the human diet and they may protect against free radicals that accelerate degenerative disease processes and pathogenesis.[12-14]

Polyphenols are the major non-vitamin nutritional antioxidants in food and have been widely investigated. Certain common Western fruits, such as blueberries and cranberries have been show to have high polyphenol content. Polyphenolics from fruits are associated with a reduced risk of CHD and other disease states.[13,15-17] Epidemiologic studies have shown that high consumption of fruits, vegetables, and whole grain foods are associated with a reduced risk of CHD.[18,19] Experimental studies in animal models have further supported the importance of a diet rich in fruits and vegetables for preventing cardiovascular disease (CVD) and cancer.[20,21] Other studies have demonstrated that consumption of fruits and vegetables that are especially high in polyphenolics content resulted in elevated levels of phytochemical antioxidants and their metabolites in plasma, and also an increase in plasma antioxidant capacity.[22-24]

1.2. *Polyphenolics as Antioxidants and Antiradical Agents*

Based on epidemiologic and clinical studies in the last 15 years, there is a strong agreement between nutritionists and other researchers that plant-derived compounds such as polyphenols are responsible for the protective effects of fruits and vegetables, due to their antioxidant, antiradical, and other biological properties.[25-27] Fruits are a good source of flavonoids and phenolic acids, and in some cases they are the major chemical constituents. Flavonoids are water-soluble pigments found in nearly all plants and are stored in the vacuole. They are known to have antioxidant and reducing abilities in vitro and in vivo, and have been shown to have many biological activities that are attributed to their radical-scavenging and metal-chelating properties.[24,27-29] Chemically, flavonoids are divided into at least 10 subclasses that include flavones, flavonols, and anthocyanins (Figure 1.1), and they may act as primary and secondary antioxidants. Their role as primary antioxidants can be attributed to their ability to prevent the initiation of new radicals, such as lipid peroxyl, hydroxyl, and superoxide radicals, and by trapping transition metal ions that are mutagenic.[30-32] Their role as secondary antioxidant is due to their capacity to dampen the chain propagation and amplification of lipid peroxidation by scavenging alkylperoxyl radicals.[33,34] For example, catechin, a common tea polyphenol, acts by scavenging ROS, leading to the regenerating oxidized antioxidants like α-tocopherol that are found in low-density lipoproteins (LDL), and promoting the preservation of erythrocyte polyunsaturated fatty acids from oxidation.[35]

In animal models studies, tea polyphenols were shown to increase the activities of the endogenous antioxidant enzymes cytochrome P_{450}, and catalase in rat liver, thereby reducing the risk of nutritionally linked cancers.[36,37] Humans acquire in significant levels

of polyphenols by consuming fruits and vegetables, which has been associated with a protective effect against lung and colorectal cancer, as well as decreasing CHD mortality rate.[38,39]

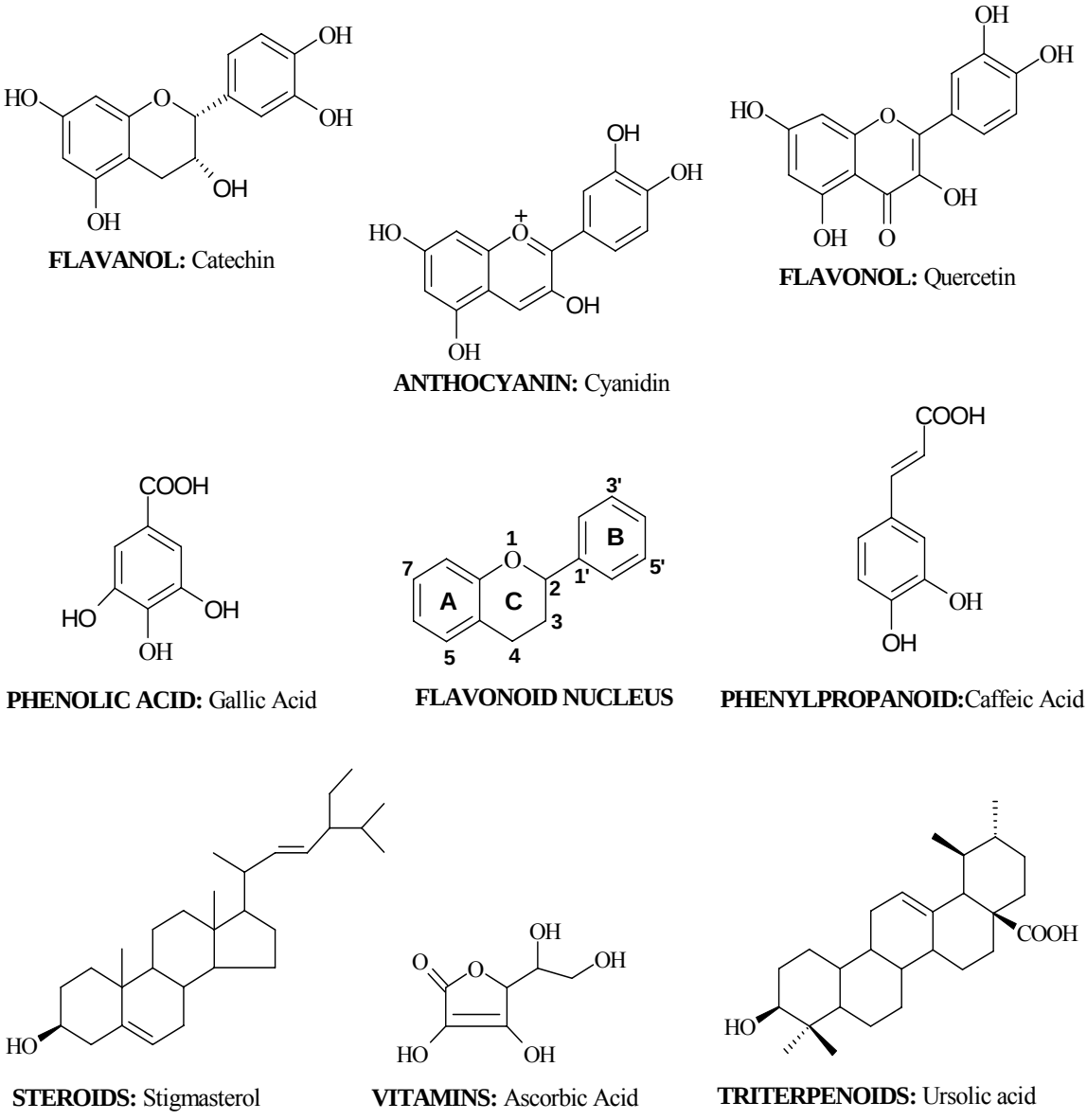


Figure 1.1. Chemical structure of selected classes of phytochemical antioxidants

However, some studies have shown that polyphenols may also have pro-oxidant activity, which is not beneficial because they contribute to the oxidation of the same molecules for which they act as antioxidants.[40,41] Pro-oxidant activity is observed if the polyphenol

contains a large number of hydroxyl substituents. However, out of the 6,000 structurally different flavonoids, there are only a few displaying pro-oxidant activity y.[40-42]

1.3. *Prevention of Lipid Peroxidation by Phytochemicals*

The oxidation of LDL by mutagenic free radicals or ROS give rise to lipid hydroperoxide, peroxyl and enals (α,β-unsaturated aldehydes), and is the first step in the pathogenesis of atherosclerosis and macrophage recruitment.[43-45] Modified LDLs, have atherogenic effects, and therefore accelerates all stages leading to CVD, including initiation of inflammatory events, endothelial damage, macrophage recruitment and unregulated uptake of atherogenic LDL by macrophage to form foam cells.[46,47]

A number of phytochemical and vitamin antioxidants inhibit LDL oxidation due to their chelating of metals, radical scavenging properties, and physiological induction, and they therefore supplement endogenous defense mechanism against oxidative damage.[43,48,49] This endogenous defense mechanism includes several enzymes such as catalase, gluthathione peroxidase, and superoxide dimutase.[50] Various studies and reviews have dealt with the scavenging activity of phytochemicals commonly found in fruits, reporting that these phytochemicals contain potent antioxidants that inhibit oxidation of LDL in plasma by reducing damaging reactive oxygen and nitrogen species or their precursors.[45,51-53] Phytochemicals such as polyphenolics, triterpenoids and steroids, and their glycosides, which possess antioxidant activities in vitro and in vivo, are preventative agents against LDL oxidation.[24,49,54,55] Some of these compounds, such as catechin, quercetin, and ursolic acid, may delay atherogenesis by reducing peroxidative reactions and cytotoxic oxidized LDL levels, and down-regulating inflammatory events that may lead to CHD.[54,56,57]

1.4. *Chemoprotective Agents from Fruits Grown in the Tropics*

Tropical fruits and vegetables are thought to be rich in chemoprotective agents and other secondary metabolites because plants have evolved phytochemical-based mechanisms to reduce damage by UV light, microorganisms, and predators.[58,59] These chemoprotective agents may be important to human health in preventing chronic pathologies and degenerative diseases.[14,60,61] Tropical fruits are known to be a good source of classical antioxidant nutrients such as vitamins E and C and β-carotenoid. For example, fruits of *Muntingia glabra* (the Barbados cherry) contain high amounts of the dietary antioxidant vitamin C.[62]

Fruits are also rich in non-vitamin antioxidants that are chemoprotective. Work performed in our laboratory in the past decade has resulted in the isolation of 78 plant-derived compounds which belong to four principal classes of phytochemicals, benzophenones, coumarins, flavonoids and phenolic acids (Table 1.1).[63-70] Among the 78 isolated compounds, 15% are new compounds demonstrating strong to moderate antioxidant and cytotoxic properties. Many of the isolated compounds exhibited cytotoxic effects against SW-480, HT-29, and HCT-116 human colon cancer cell lines.[70] Among the five classes of compounds isolated, 60% belonged to the flavonoids and phenolic acid groups that include flavonoids and phenolic acids.

Fruits grown in the tropics are rich in polyphenols, and consequently several studies have shown an association between Caribbean descendants' risk of CHD and high consumption of fruits and vegetables.[72-75]

There are many chemoprotective compounds from tropical fruits that have been examined for their antioxidant activity, antitumor and anticancer properties.[15,75,76] Some

of these compounds, such as quercetin, caffeic acid, and saponins, have been examined as cardioprotective agents.[38,76,77] Plant-based food products are also an excellent source of chemoprotective antioxidants and antiradical agents.[78-80] Consequently, research on unexplored tropical fruits may provide a new battery of chemoprotective agents.[7,14,25] The present work describes the investigation into the bioactivity of constituents from the edible tropical fruit *Blighia sapida*.

1.5. *Blighia sapida*: *Taxonomy, Phytochemistry and Ethnobotany*

1.5.1. Classification and Nomenclature: *Blighia sapida* Koënig is a member of the Sapindaceae, a family that consists of 147 genera and about 2,215 species. This family as well as its order (Sapindales) is monophyletic, as revealed by both morphological data, and *atp*B and *rbcL* nucleotide sequence analysis.[81] The Sapindales consists of 13 families and approximately 5800 species.[81] The classification of members within the Sapindales has been facilitated by using chemical evidences, leading to substantiate a more accurate relationship between the studied members. The chemical evidence includes the presence of compounds such as hypoglycins, which are cyclopropane amino acids, and are indicative of the members in the Sapindales.[81,82] For example, the occurrence of these compounds in the family Hippocastanaceae assured their placement in the same order as the Sapindales.[81]

Table 1.1. Compounds isolated from various fruits studied in our laboratory, and their bioactivity

Chemical Class	Compound Name	DPPH IC_{50} (μM)	Cytotoxicity IC_{50} (μM) [a]	References
Benzophenones	Aristophenone A	125	33.3[b]	63
	Alloathyriol	na	117[b]	
	Amentoflavone	184	111[b]	
	3,8"-Biapigenin	> 400	185[b]	
	(±)-Fukugetin	62	89[b]	
	(±)-Fukugiside	116	> 200[b]	
	Glutterone A	61	5.0[a]/5.0[b]	
	Guttiferone E	68	7.5[b]	
	Guttiferone H*	64	12.4[b]	
	Gluttiferone I*	62	5.0[a]/10.0[b]	
	Gambogenone*	39	188[b]	
	Isoxanthochymol + Cycloxantho-chymol	73	16.6 [b]	
	(±)-Volkensiflavone	298	185[b]	
	Xanthochymol	53	8.3[b]	
Coumarins	Mammea A/AA	135	25.9/25.1[b]	70
	Mammea A/AA cyclo D	na	32.2/38.4[b]	
	Mammea A/AA cyclo F	na	75.8/80.6[b]	
	Mammea A/AC cyclo D	na	32.0/38.9[b]	
	Mammea A/AD cyclo D	na	30.8/38.5[b]	
	Mammea B/BA	90	16.1/17.5[b]	
	Mammea B/BA cyclo F	na	46.4/48.9[b]	
	Mammea B/BA hydroxylcyclo F*	na	76.7/88.1[b]	
	Mammea B/BB	91	12.9/17.7[b]	
	Mammea B/BC	90	14.5/15.7[b]	
	Mammea B/BD	92	15.9/15.4[b]	
	Mammea E/BA	86	10.7/13.9[b]	
	Mammea E/BB	88	13.9/15.4[b]	
	Mammea E/BC*	88	11.5/14.2[b]	
	Mammea E/BD*	88	12.0/14.7[b]	
Flavonoids	(+)-Catechin	35	na	64
	Catechin-3-*O*-gallate	19	na	
	Cyanidin-3-glucoside	11	100/100[b]	65
	Delphinidin-3-glucoside	11	12.0/20.0[b]	
	2′,4′-dihydroxy-4-MDHC	38	na	
	Dihydromyricetin	31	na	66
	(−)-Epicatechin	38	na	
	(−)-Epigallocatechin	44	na	
	(+)-Gallocatechin	21	na	68
	Gallic acid	21	na	
	Hypolaetin 8-*O*-*β*-D-glucuronide	58	na	
	Kaempferol	90	na	69

Table 1.1. (*Continued*)

Chemical Class	Compound Name	DPPH IC_{50} (μM)	Cytotoxicity IC_{50} (μM)	References
Flavonoids	Isoscutellarein-8-*O*-glucuronide	68	na	65
	Isoscutellarein-8-*O*-glucuronide-6″-methyl ester	69 --	na --	
	Quercetin	40	na	66
	Quercetin 3-*O*-*β*-D-glucuronide	44	na	
	Quercetin-3-*O*-*β*-D-glucuronide-6″-methyl ester	42 --	na --	
	Quercimeritrin	na	na	68
	Quercitrin	74	na	
	Myricitrin + Isoquercitrin	56	na	
	Myricitrin	26	na	69
	Theograndin I*	341	205[a]/164[b]	
	Theograndin II*	120	143[a]/125[b]	
Phenolic Acids	Caffeic acid	58	na	64
	Cimicifugic acid A	12	na	
	Cimicifugic acid B	21	na	
	Cimicifugic acid D	na	na	
	Cimicifugic acid E	66	na	
	Cimicifugic acid F	152	na	
	Cimicifugic acid G*	37	10	
	p-Coumaric acid	na	na	65
	4,5-di-*O*-Caffeoylquinic acid	na	na	
	Ferulic acid	121	na	
	Ferulate-1-methyl ester	na	na	66
	Fukinolic acid	13	na	
	4-*O*-Galloylchlorogenic acid	24	154[a]/134[b]	
	Isoferulic acid	289	na	67
	1-Isoferuloyl-*β*-D-glucopyranoside	na	na	
	Jaboticabin*	51	65[c]	
	8-*O*-Methoxyjaboticabin	na	30[a]	68
	Methyl-4-*O*-Galloylchlorogenate*	13	190[a]/160[b]	
	Methyl chlorogenate	na	na	
	Methyl caffeate	na	na	
	Protocatechualdehyde	na	na	
	Protocatechuic acid	na	na	
Lignins	Actaealactone*	26	14	67

* = new compounds; [a] = HCT-116 cytotoxic human colon cancer (HCC) activity; [b] = SW-480 cytotoxic HCC activity; [c] = HT-29 cytotoxic HCC activity; [d] = MCF-7 cytotoxic activity; [e] = Il-8 cytotoxic activity

The genus *Blighia* consists of about seven species, all native to West Tropical Africa. To date only three members of the genus exist, namely *Blighia sapida*, *Blighia unijugata*, and *Blighia welwitschii*.[83,84] Plants in the *Blighia* genus, and other members within the Sapindaceae, are an excellent source of acetylated and non-acetylated saponins, thus the name soapberry family.[85-89] *Blighia* is closely related to the *Sapindus*, and to other genera such as *Jagera*, *Eriocoelum*, and *Radlkofera*, as confirmed by the presence of saponins (with hederagenin and oleanolic acid sapogenins), and sugar moiety consisting of arabinose, glucose, rhamnose and xylose.[83,90-92]

1.5.2. Botanical Description: *Blighia sapida* Koënig (ackee) is a medium-sized to large evergreen tree, reaching 10-50 m tall and usually with a short trunk, and native to Western Africa. The leaves are large, petiolate and compound with 3-5 pairs of leaflets that are cuneate-obovate to elliptic or oblong, and short-acuminate at its apex and 15-30 cm long. Each leaflet contains pinnately veined leaves that are bright green and glossy on the upper side but paler and dull on the underside. The inflorescence is axillary and racemiform, and the buds are subconical. The flower is polygamous, with male flowers borne in racemes that are about 7.5 to 18 cm long. The flowers are also creamed-colored to white, hairy, and fragrant, with 5 villous petals that are ovate-lanceolate. The 8 stamens are 0.5-0.6 cm long and inserted within a swollen disc. The ovary contains three locule and three carpels. The fruit is 3-locular, leathery, and large, containing a 3-lobed yellowish to red oblong-obovate capsule, 6-10 cm in length. The fully mature fruit splits open to reveal 3 cream to yellow-colored, fleshy and glossy arils, having 3 smooth and shiny black seeds. Some of the seeds may be small or not be present on the aril, The aril is attached to the inside of the pod (husk or jacket) by a red membrane.[84,93]

1.5.3. Abiotic Requirements, Cultivars, and Propagation: *Blighia sapida* Koënig is common in Jamaica where it was introduced in 1778. The trees grow in house compounds and abandoned fields, and are sometimes used as a shade trees or as fence posts. The plant is found growing from sea level to elevations of about 1.2 km but it grows best at lower elevations and it only fruits sporadically at higher elevation. B. sapida was also introduced in other Caribbean Islands such as Haiti and Puerto Rico, and also in Central America and South Florida.[83]

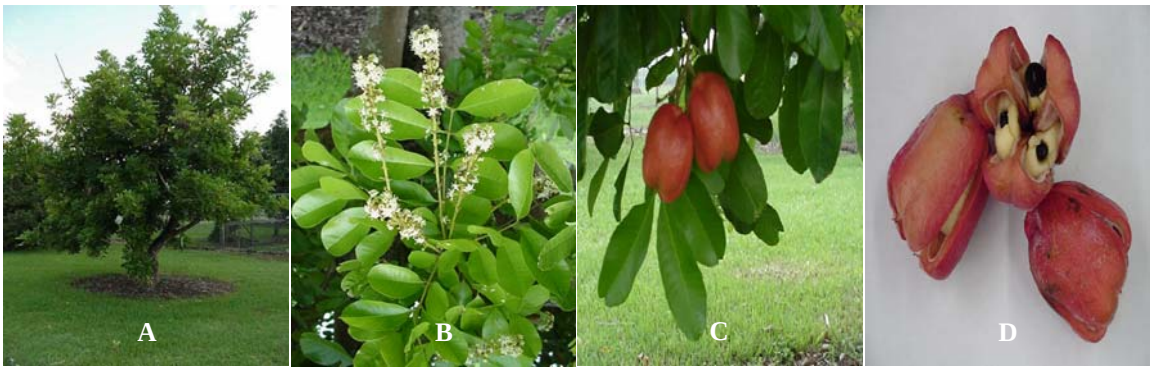


Figure 1.2. *B. sapida* in habitat (A), flower & leaves (B), fruits [unripe (C) and ripe (D)]

The ackee plant is very tolerant to drought, and grows in almost any soil types, but it grows best in alkaline and well-drained soils, on loams supplied with moisture, and in warm to moderately dry climatic conditions. Ackee has two cultivars, “butter”/soft and hard.[84,94] The hard variety is favored over the soft variety and is ideal for the canning industry because of its firmness, texture and palatability, with approximately 1,498-1,792 metric tons imported per year. The different ackee cultivars are grown from seedlings and these yields between 1,149-3,157 fruits/tree/year from the top producers in Jamaica. Trees are preferably grown from seedlings, instead of stem cuttings, take about three to four years to bear fruits. In order, to maintain the high market demands, ackee trees are grown from vegetative propagation via rooted branch tip cuttings, resulting in low

spreading trees that bore fruits within one or two years after planting. No appreciable success has been reported through grafting or budding, and in addition, plant tissue culture techniques for the propagation of ackee trees have now been explored for commercial purposes.[83]

Ackee can be harvested unopened for consumption, but the unopened fruit must be open within three days after harvest in order for it to be edible. If ackee is consumed while unopened (and if immature) it can lead to coma and/or death due to plant toxins, hypoglycin A and B.

Bruised ackee fruits are easily susceptible to attack by microorganisms such as *Botroyodiplodia theobrome*, *Rhizopus nigricans*, *Colletortrichum* sp., *Oidium erisyphe*, *Phyllositieta sp*., and others. The major anthropod pests affecting the fruits are beetles, aphids, thrips, whiteflies, mites and fruitflies.[83] Ackee fruits that were used for this study were devoid of pest and microorganisms, since the presence of microbes and pests may alter the composition of polyphenol and saponin constituents.

Ackee fruits have high levels of saponins, compounds which are important medicinally; they may act as natural defense compounds in plants due to their surface action on the membranes microbes, insects and other organism. To better understand the biological properties of these compounds, preliminary investigations of the ackee fruit's phytochemical constituents were accomplished, and the results are discussed in detail in chapters 2 and 3.

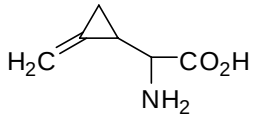
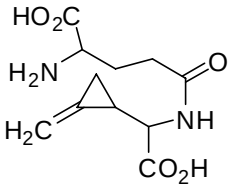
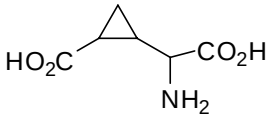
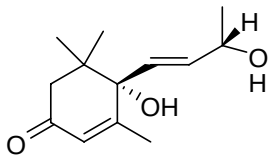
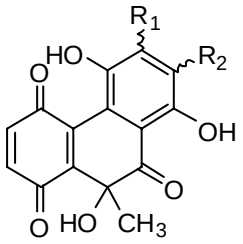
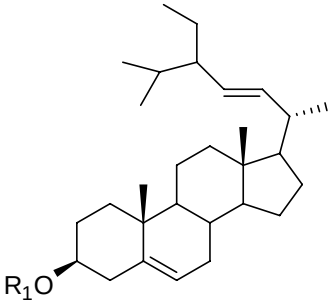
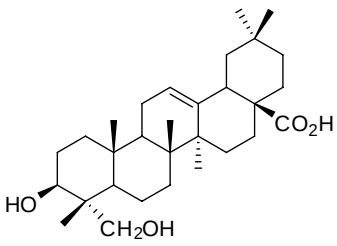
1.5.4. Ethnobotanical and Commercial Use of *Blighia sapida*: Very little is known about the ethnobotanical use of *B. sapida*, as hypoglycin A and B, have caused many deaths in the West Indies, including the "Jamaica vomiting sickness."[95,96] These

two unusual cyclopropanoid amino acids are abundant in the seeds, arils, and husks (pods) (Figure 1.2 D) of the immature ackee fruit. Only a few phytochemical investigations have been done to evaluate the chemoprotective potential of ackee in humans and other mammals, even though some important uses have been noted. For example, an infusion of ackee leaves is used in Jamaica to treat the common cold and fevers, and the leaf juice is given as eye drops to treat ophthalmia and conjunctivitis.[93,97] The immature fruit juice has been used as a rub to treat ringworm and some skin conditions in domesticated animals.[94] The pods and seeds are also used as fish poison, as well as in soap-making.[98] In Cuba, the fruit arils in combination with sugar and cinnamon are given as a febrifuge, and to treat dysentery.[93] The fruit aril is also used as a mordent to fix colors in fabric industry.[98] Despite all these uses for ackee very little research has been conducted over the last two decades to isolate the principles responsible for its biological activity, even though a few important classes of bioactive compounds have been isolated. Some of these compounds are highlighted below.

1.5.5. Phytochemical Constituents: Besides the usual occurrence of amino acids and fatty acids, several other classes of plant-derived phytochemicals, including the cyclopropane non-protein amino acids, polyphenols, quinones, sesquiterpenes, triperpenes, and steroids have been isolated (Table 1.2).[99-104] The aril and the pods of the ackee fruits contain steroids, triterpenoid, and saponins including sapogenins of stigmasterol, hederagenin and oleanolic acid. Triterpenoid saponins display many biological activities, and they have been implicated in the spasmolytic, molluscicidal and fish-poisoning properties of the ackee fruit.

Other isolated compounds from ackee include the quinine blighinone[102] and vomifoliol,[103] a sesquiterpene with abscisic acid-like properties. Additionally, small amounts of phytates and tannins have been reported.[104]

Table 1.2. Phytochemicals reported from of *B. sapida*

Compound Type	Chemical Structure	Compound Name
1. Cyclopropane amino acids		***Hypoglycin A***: α-amino-2-methylenecyclopropanepropanoic acid. Reference: 99
		***Hypoglycin B***: N-γ-glutamyl-3-(methylenecyclopropyl) alanine. Reference: 99
		*Trans*-(2*S*,1′*S*,2′*S*)-2-(2′-carboxy-cyclopropyl) glycine. Reference: 100
2. Sesquiterpene		***Vomifoliol***: 6,9-dihydroxy-4,7-mega-stigmadiene-3-one. Reference: 103
3. Quinone		***Blighinone***: Phenanthrenecarboxylic acid R_1=CO_2H, R_2= H or R_1= H, R_2= CO_2H Reference: 102
4. Steroid		***Stigmasterol:*** Stigmasta-5,22-dien-3-ol R_1= OH ***Saponin***: Stigmasterol-3-*O*-*β*-fructofuranoside; R_1= fructofuranoside Reference: 102
5. Triterpene		***Hederagenin***: 3β,23-di-hydroxyolean-12-en-28-oic acid. Reference: 101

1.6. *Bioactivity of Steroids, Triterpenoids, and their Glycosides (Saponins)*

1.6.1. Triterpenes: Triterpenes are C_{30} compounds that are formed from the cyclization of squalene 2,3 oxide (specifically, epoxy-3*S*-2,3-epoxy-2,3-dihydrosqualene), via from the head to tail condensation of two farnesol isoprenoid units, followed by oxidation via monooxygenase at C-2 and C-3 to form an epoxide. The 3-OH group, which is common in almost all triterpenes, is derived from the opening of the 2,3-oxide or the epoxide. Triterpenes differ from each other in their stereochemistry, which arises from the conformation (e.g. chair-boat-chair or chair-boat-boat) assumed by the squalene precursor, prior to ring formation (Figure 1.3). Subsequently, modification of the conformation assumed by squalene occurs via 1,2-proton and methyl shifts (due to the *trans*-antiparallel arrangement of the protons and methyl groups at C-17, C-13, C-14, and C-8) in the cation structure formed following cyclization; this results in the formation of the structurally homogenous tetra- and pentacyclic skeleton that characterizes the triterpene group (Table 1.2).[105,106]

There are over 4,000 isolated triterpenes, with over 40 different skeletons, whose variations are due to their stereochemistry and attached sugars moieties.[105] Of all the triterpenes isolated, oleanolic acid is the most widely distributed, and its pentacyclic structure is the most common triterpene form isolated from plants.

1.6.2. Steroids: Steroids are C_{25} to C_{27} secondary metabolites that are also derived from squalene oxide, but can be distinguished from triterpenes, because of their biosynthesis. Traditionally, steroids were distinguished from triterpenes by the presence of a methyl groups at C-4 and C-14. Steroids, like triterpenes, are synthesized from

acetate, via the mevalonate pathway, to squalene-2,3-oxide, but they differ from this point on.

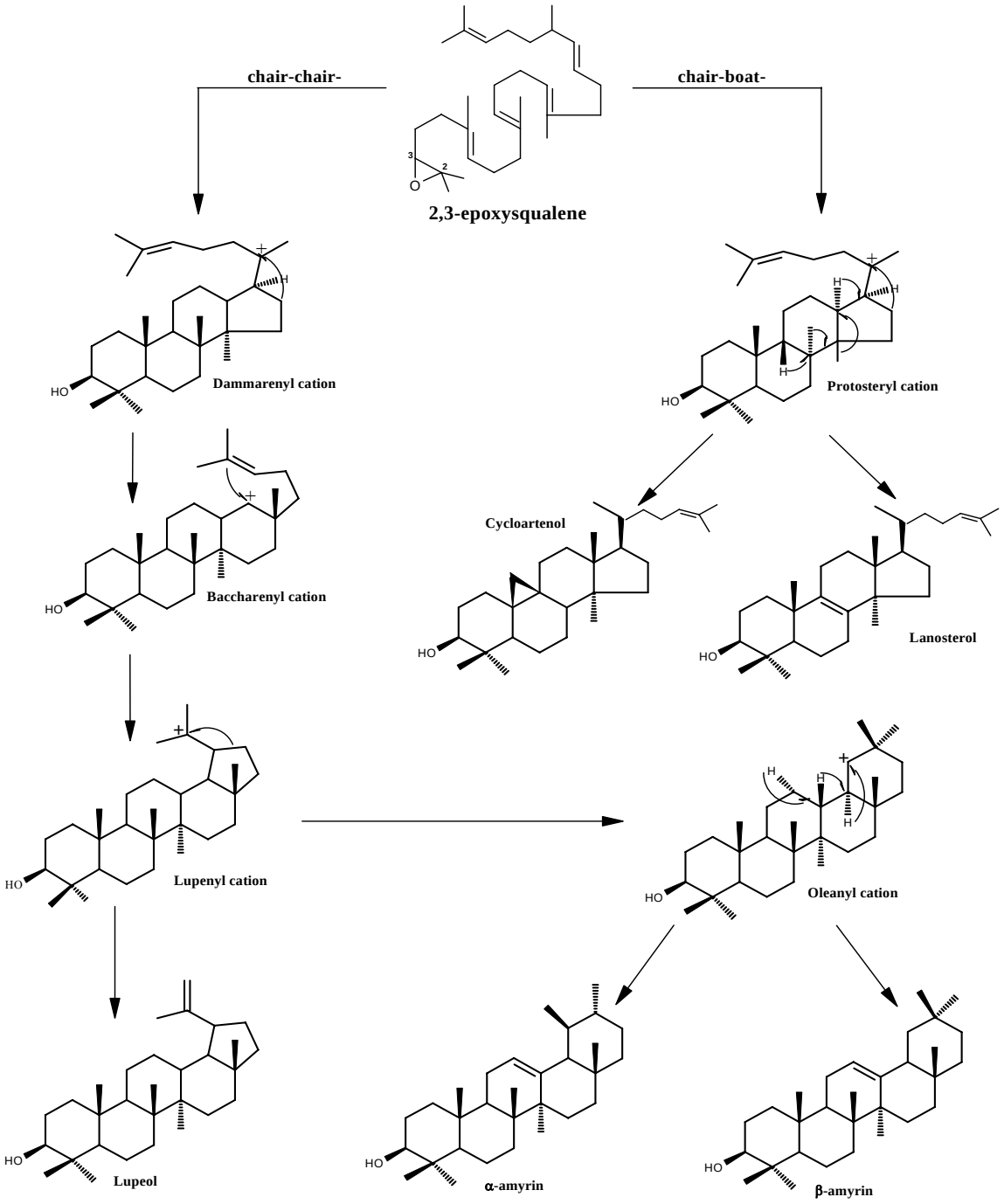


Figure 1.3. Cyclization of 2,3-oxidosqualene and triterpenoid biosynthesis[106]

The demethylation of the sterol precursor, cycloartenol, at C-4 and C-14 through a series of oxidation steps ($R\text{-}CH_3 \rightarrow R\text{-}CH_2OH \rightarrow R\text{-}CHO \rightarrow R\text{-}CO_2H \rightarrow R\text{-}H + CO_2$), and the opening of its 9,10-cyclopropane ring gives rise to cholesterol. Further modifications of the cholesterol structure, especially on the side chain, lead to other biologically important phytochemicals such as sterols and steroidal saponins. Sterols are important in the pharmaceutical industry and health professions because they are used as precursor moieties of contraceptives, and as anabolic and anti-inflammatory agents.[105,106]

1.6.3. Steroid and Triterpene Glycosides (Saponins): Saponins are glycosides with surfactant properties that form foamy solutions in water. It is this activity that makes them so attractive as detergents and/or hemolytic agent. Saponins are found in plants and other organisms, such as animals, marine organisms, fungi, and some bacteria.[82,106-108] They usually contain sugar residues such as arabinose, glucose, galactose, glucuronic acid, and methylpentose (rhamnose and fucose), linked to a lipophillic aglycone (sapogenin or genin) that may be a triterpene or steroid.[106,108] In the ackee fruit, the most prominent sugar residues described from the saponin rich extracts are arabinose, glucose, rhamnose, and xylose.[102] The oligosaccharide chain is normally attached at the C-3 position, but in some saponins attachment may be at additional positions, such as C-26 or C-28. The great structural diversity and complexity of saponins are due to the stereochemistry within the aglycone moiety, the pattern of the oligosaccharide side chains, and their attachment position. Over 10 years the scientific literature has highlighted the clinical interest and significance of these substances, and their important physiological, immunological and pharmacological properties.[76,109-110]

1.6.4. Role and Occurrence of Saponins in Plants: Saponins are widely found in many dicotyledonous plant species, but their physiological role in plants, and their biosynthesis is not fully understood because pathway intermediates are scarce, and the key enzymes in their biosynthesis are not thoroughly studied. Furthermore, in the biosynthetic pathways, the corresponding gene expression regulating these pathways are also not well understood either.[112-114] However, the genes encoding the key enzymes involved in cyclization of squalene 2,3-oxide, and of saponin glucosyltransferases have been cloned.[115] Triterpenoid saponins have been characterized in leguminous crops such as soybean (*Glycine max*) and alfalfa (*Medicago sativa*), and are particularly abundant in others like, licorice and ginseng. Steroidal saponins are common in monocotyledonous plants used as herbs due to their health-promoting properties, but also in some plant crops such as oats, capsicum peppers, asparagus, yam, and yucca.[116]

Many saponins are known to be antimicrobial, a few possessed antiviral properties, and others protect plants from insect attack as part of the these organisms' defense system.[10,117] For example, oat steroidal saponins (avenacosides) are activated by the plant's endogenous enzymes in response to tissue damage or pathogen attack.[118] Saponin content in plants is influenced by physiological conditions, age, environmental and agronomic factors. For example, saponin content has been shown to increase in soybean and pea (*Pisum sativum*) plants during its initial growth stages, but decrease in moth beans during this same stage.[80] Light exposure has been shown to affect the production of saponins in various plant tissues.[80]

1.6.5. Biological Activities of Saponins: Most saponins are monodesmosidic and bidesmosidic, and their biological properties have been ascribed to their influence on

biological membranes.[119,120] For most saponins their action on membrane activity is linked to the stereo-structure of their sugar side chain, the number of sugars, and the acidity of the aglycone. Some saponins are hemolytic, likely because of the affinity of the aglycone to membrane sterols.[121] Hemolytic properties may not be common to all saponins, and no such properties have been observed in saponin-rich extracts of ackee fruit, which contain both steroidal and triterpenoids saponins. Monodesmosidic saponins of both steroids and triterpenoids, possessing 2-3 or more sugar units than bidesmosidic saponins, have stronger hemolytic activity even if they have similar aglycone; and acidic and neutral saponins have weaker hemolytic properties than their corresponding esters. Some saponins have been found to improve the growth and health of ruminants because of their anti-protozoa activity.[122] Some are also toxic to soft membrane organisms, and to organisms which utilized gills for breathing, such as snails, mollusk, fish, and frogs due to their ability to induce hemolysis of respiratory cells, or membrane channel formation and/or modification. Consequently, plants containing them are traditionally used as active ingredients for fish and mollusk poison.[108,123] *Quillaja* saponins and *Blighia sapida* seed extract saponins are reported to have molluscicidal and fish poisoning properties.[124,125] The saponin extract of *B. sapida* is used in West Africa to control invasive fish and snail species in lakes and ponds that may a source of schistosomiasis.[126] Monodesmosidic triterpene saponins are more lethal against snails and mollusks than bidesmosidic saponins. This may explain the molluscicidal activity of *B. sapida* fruit extract, but to date only monodesmosidic saponins have been characterized. Besides the activities outlined above, saponins have been shown to reduce the amount of harmful LDL-cholesterol in human subjects, and they also have anti-inflammatory, antimutagenic,

antifungal, antiviral, antioxidant, cytotoxic, and apoptotic properties. Saponins they also induce the production of interleukins and interferons, and inhibit the growth of cancerous cells, *in vitro*.[76,110,127-129]

1.7. *Fruit Selection Based on Taxonomy and Chemical Evidences*

Chemotaxonomy, taxonomy, botany, and ethnobotanical information provide morphological, biochemical, physical, physiological, and other characteristics that allow the formal classification and the determination of phylogenetic relationships. Therefore, plants can be screened for their drug constituents based on reference to their taxonomy and/or phylogenetic relationship to other plants.[94] Chemical evidence is employed even at the highest levels of hierarchy, if it can substantiate accurate classification based on existing morphological data. At best, chemical evidence involving compounds such as polyphenolics, lignans, triterpenes, and steroids, provides supplementary evidence background towards classification and phylogenetic relationships already under study, and may be valuable in providing cues into other related species.[94]

With the development of rapid analytical technique screening of plant materials with similar chemical profiles can be accomplished in a short period. Today, it is easier for researchers to discover similar precursors for commercial synthetic drugs, in related group or species that are more efficacious in treating human ailments and diseases, due to these analytical methods. Such a rationale was employed in finding an alternative means of acquiring pacitaxel (Taxol®). Based on improved screening and analytical techniques an alternative plant source was devised based on chemical evidence criteria, and whose method of drug extraction was less harmful to the plant. This method of "information extrapolation" was allied to the evaluation of the *Blighia* genus, which is closely related

to the saponin-rich *Sapindus* genus, and which is poorly studied for its phenolic content. There are obvious limits to the information that chemical evidence and taxonomic relationships can supply; however literature searches and preliminary experiment results indicate that *B. sapida* warrants further investigation.

1.8. *Overview of Research Project*

This introductory chapter provides the rationale for the investigation of the beneficial effects, and health-promoting contributions of fruits grown in the tropics, specifically in the Caribbean. After extensive literature reviews and preliminary investigation of various tropical fruits, the focus is narrowed on one fruit species from the Sapindaceae family whose fruit is known to be an important food crop in Jamaica, namely *Blighia sapida*. No significant investigations into its polyphenolics and saponin constituents had been done prior to this work. The second chapter presents the preliminary work done on selected fruits grown in the Caribbean, the reason for the selection of the ackee fruit, and a detailed analysis into the polyphenol contents and constituents of the fruit, especially of the pod through various methods. Several polyphenols were isolated and characterized based on spectrometric methods. The third chapter describes further phytochemical analysis on the pod, but in this case, emphasizing the saponin content for which the family is known to possess in great abundance. Three new bioactive compounds were isolated and characterized by spectrometric methods, and two known compounds were also identified. The fourth and concluding chapter deals with a concise review into the studies taken on the ackee fruit and the results of numerous experiments, and future investigations on the other parts of the fruit.

CHAPTER 2

Polyphenolic Antioxidants from *Blighia sapida* (Ackee) Byproducts

1. Introduction

The plant species *Blighia sapida*, also known as ackee or akee, has a tropical to sub-tropical distribution, and is indigenous to equatorial Africa. It is cultivated in the West Indies, Central and South America, and Southern Florida for its fruits. The ackee fruit is obtuse or pear shaped, and either yellow, red or yellow-red in color and splits open longitudinally when exposed to sunlight during the mature stage.[100] The fruit aril of ackee is edible, and is an important ingredient in a popular Jamaican codfish dish. The fruit aril is given as a febrifuge to treat dysentery, and is used as a mordant to fix colors in fabric.[96,101] The leaves are used to treat colds and fevers, the leaf juice is used to treat ophthalmia and conjunctivitis, and when used as a rub, to treat ringworm and skin conditions in domesticated animal. The pods and seeds are used as a source of fish poisons and soaps due to their saponin content.[96,97,100,101]

Since the 1950's, ackee has typically been processed in the food industry; canned ackee is the most popular form sold today.[97] In the canning process, approximately 72% of the fruit is lost as industrial waste since only the aril is consumed as food.[97] Canned ackee arils in brine have been exported to the United States for years, but in 1972 the importation was banned by the Food and Drug Administration (FDA) because of the risk to consumers of unacceptable levels of the toxic peptides hypoglycin A and B. At that time, there was no suitable analytical method to measure the levels of the toxin in the fruit.[130] In the year 2000, the ban was lifted and canned aril is now imported to the United States from Jamaica, with an estimated value of US$10 million per year.[130] As part of the Jamaican Ministry of Agriculture's Fruit Tree Crop Project, 880 hectares of ackee

orchards are reserved for the production of ackee for export.[131] Producers in Mexico, Costa Rica, and Hawaii also export canned ackee to meet rising global demands. [131129]

In 2001, about 4,500 tons of pods and seeds were generated as a byproduct of the canning industry in Jamaica.[97,131] This large amount of byproduct presents a waste management issue with potential environmental concerns. Therefore, it will be helpful to find an effective way to utilize ackee byproducts, especially as a source for the isolation of bioactive plant-derived compounds such as polyphenols, steroids, triterpenes, and saponins.

Polyphenolics exhibit a wide range of pharmacological and biological properties including antioxidant, antibacterial, antithrombotic, antiinflammatory, anticarcinogenic, and vasodilatory actions.[15,42,132] They also modulate a wide range of molecular targets, such as gene expression which affects physiological functions, and may be beneficial to human health.[23,33] The chemoprotective properties of polyphenols have been supported in several cross-cultural epidemiological studies,[18,19] and are briefly introduced in Chapter 1. These chemoprotective compounds are found constitutively in fruits and their byproducts, which are consumed in the tropics and the Caribbean. For example, most Caribbean people in the UK have a reduced risk of CHD compared to Caucasians in UK. This phenomenon has been correlated to the high consumption of fruits and vegetables among these groups of people.[72,74] This reduced risk is also true for Caribbean-born immigrants living in the UK who practice traditional plant-based food preparation, compared to those who consume western foods.[73,74] Similar findings were observed in a study that examined dietary risk factors of people of African ancestry in the Harlem neighborhood of New York City, and found that Caribbean-born immigrants have diets

that place them in the lowest risk category for chronic diseases.[71] Caribbean-born immigrants diets are typically high in fruits and vegetables, and low in fats.

In our preliminary study, we screened 19 edible fruits grown in the Caribbean, and one cultivar from Southern Florida, for their antioxidant activity, and found that ackee pod contained one of the strongest antioxidant activities among all the fruits, and have high total phenolic content. Therefore, we decided to investigate the ackee pod for its antioxidants constituents in this study.

2. Materials and Methods

2.1. *Plant Material*

Fruits from eleven tropical plants, *Annona muricata*, *Annona squamosa*, *Artocarpus heterophyllus*, *Averrhoa bilimbi*, *Averrhoa carambola, Blighia sapida*, *Chrysophyllus cainito*, *Diospyros blancoi*, *Malpighia glabra, Manilkara zapota,* and *Muntingia calabura* were collected at the Fruit and Spice Park in Homestead, Florida, and transported to New York City by overnight carrier. *Annona squamosa* var. *kampong* fruit was obtained from the Kampong (Coconut Grove, Florida). Fruits from, *Artocarpus altilis, Hibiscus sabdariffa*, *Mangifera indica*, *Psidium guajava*, *Spondias dulcis, Spondias mombin*, and *Tamarindus indica* were purchased at local markets in Queens, and Bronx Counties, NY. *Blighia sapida* fruits from Jamaica were purchased at local markets on the day of departure, frozen, and delivered to New York City within 24 hours. All fruits were weighed, catalogued, and frozen at -20°C until analyzed.

2.2. *Chemicals and Supplies*

Solvents for chromatography, HPLC-grade acetonitrile, MeOH, formic acid, and HPLC-grade water, were obtained from J.T. Baker (Phillipsburg, USA), and GR-grade MeOH, EtOAc and acetone, from VWR Inc (Bridgeport, USA). Sephadex LH-20 (25-100 μm) was obtained from Pharmacia Fine Chemicals (Piscataway, USA); silica gel (230-400 μm mesh) from EM Science (Darmstadt, Germany); Diaion HP-20 from Supelco (Bellofante, USA), and reversed-phase C_{18} bonded-silica gel (40 μm) from J.T. Baker (Phillipsburg, USA). Silica gel 60 $F_{254,}$ and RP_{18} F_{254} thin-layer chromatography (TLC) plates were obtained from EM Science (Darmstadt, Germany). Cyanidin chloride, gallic acid, quercetin, quercetin 3-*O*-rutinoside, quercetin-3-*O*-rhamoside, ellagic acid, aluminum chloride hexahydrate, anhydrous sodium carbonate (Na_2CO_3), sodium acetate, sodium nitrite, Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxlic acid), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), ferric chloride hexahydrate, ferrous sulfate heptahydrate, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and Folin-Ciocalteu phenol were purchased from Sigma-Aldrich (St. Louis, MO., USA). Compounds were visualized on TLC plates by spraying with 10% conc. H_2SO_4 (v/v in 95% ethanol) and then heating it above 100°C.

2.3. *Extraction of Plant Material*

Whole fruits (60-100 g) were washed in distilled water to remove debris. After deseeding, the edible portions of all the fruits were homogenized with MeOH and extracted exhaustively at room temperature and filtered. The combined MeOH crude extracts were filtered and concentrated under reduced pressure at 37-40°C. The resulting concentrated crude MeOH extracts were suspended in H_2O and then partitioned

sequentially with hexane and EtOAc, yielding three samples: hexane, EtOAc, and aqueous. The hexane and EtOAc fractions were dried under reduced pressure, and the aqueous sample lyophilized. All samples were weighed and stored at –20°C until analyzed.

For the large-scale extraction of ackee pods (11.0 kg) were homogenized with MeOH and extracted exhaustively at room temperature (Figure 2.2). The combined MeOH extract was filtered and concentrated under reduced pressure at 37-40 °C. The resulting MeOH crude extract was partitioned three times, first with EtOAc, and then with butanol. The combined EtOAc fractions were dried under reduced pressure, and the butanolic fraction and the aqueous fraction were lyophilized. For comparison purposes, other aerial parts of ackee, as well as ackee pods from Jamaica, were also extracted as described above for the survey edible fruits; these parts include the aril, seed, stem, and leaf. These extracts were dried under reduced pressure, and the aqueous fractions were lyophilized.

2.4. *General Procedures*

HPLC analysis of fractions and purified compounds were perform using on a Waters 2695 Separations Module (Milford, USA) equipped with a Waters 996 photodiode array detector, and Waters Empower software, using a 4.6 × 250 mm (5 µm) Aqua C_{18} column (Phenomenex, Torrence, USA).

^{1}H and ^{13}C NMR spectra were determined on a JEOL GX-400 MHz spectrometer (Akishima, Japan) operating at 400 MHz and 100 MHz, respectively, or on a Bruker Avance AV-300 MHz spectrometer (Billerica, MA), operating at 300 MHz and 75 MHz, respectively. All ^{1}H and ^{13}C NMR spectra were obtained in CD_3OD, with chemical shifts

expressed in δ and coupling constants (J) in hertz (Hz). Electrospray ionization-mass spectrometry (ESI-MS) was recorded on a ThermoQuest Finnigan LCQ Mass Spectrometer (San Jose, CA) equipped with Xcalibur software. Samples were dissolved in HPLC grade MeOH and introduced by direct injection. The capillary voltage was 10 V; the spray voltage was 4.5 kV; and the tube lens offset was 0 V. The capillary temperature was 230°C. Nitrogen was used both as an auxiliary and sheath gas, with a flow rate of 30 and 80 (arbitrary units), respectively.

2.5. *Antioxidant and Antiradical Determination Assays*

2.5.1. Microtiter-based DPPH Assay: This assay is rapid, simple and inexpensive, and has been used extensively in many laboratories as an initial screening, of antioxidant capacity of plant extracts, fruits juices, phytochemical antioxidants and standards.[133,134] The DPPH method tests the ability of compounds to scavenge the stable DPPH radical in a 96-well microtiter-based assay by acting as proton and electron donors.

Briefly, all test samples (0.6-2.4 mg) were reconstituted in 4 ml dimethylsulfoxide (DMSO) to a final concentration (600 µg/ml). This stock solution was serially diluted from 120 µg/ml. DPPH at 400 µM in ethanol (95%) was combined with 50 µl test samples to a final well volume of 200 µl. DMSO was used as the negative control, and gallic acid and ascorbic acid were used as the positive controls. The reaction mixtures, in duplicate, were incubated for 30 min at 37 ºC, and absorbance measured at 517 nm. The activity of a test sample is determined as percent inhibition of DPPH radicals. Percentage inhibition is calculated by the formula %In = [(C-S)/C] x 100. The IC_{50} value is obtained

after interpolation of C at 50% inhibition. $IC_{50} < 50$ is very active; $50 \leq IC_{50} < 100$ is active; $100 \leq IC_{50} < 200$ is moderately active; $IC_{50} \geq 200$ is not active.

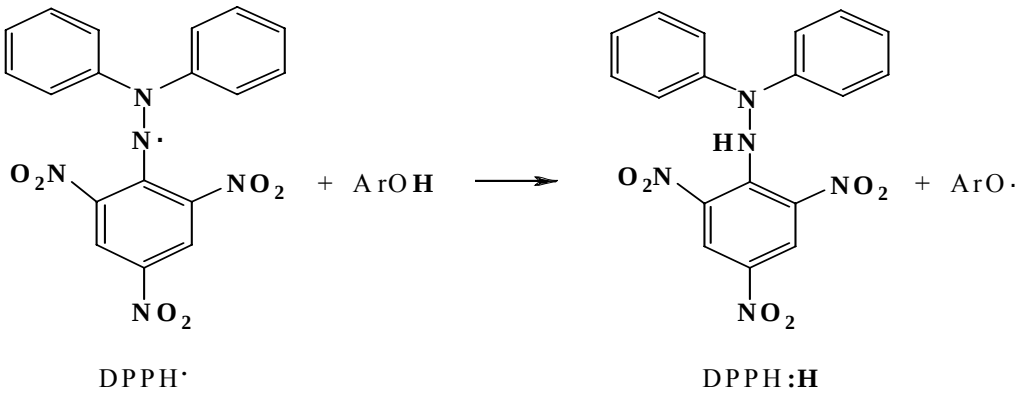


Figure 2.1. Scavenging of DPPH radical by an polyphenol antioxidant (ArOH)

2.5.2. Ferric Ion Reducing/Ability Power Assay: This assay is inexpensive, simple and can be completed in one day; it is especially for many solid or liquid samples. The FRAP reagent has to be prepared on the same day of usage. The assay is based on the original method developed by Benzie and Strain (1996), and it determines the total antioxidant (reducing) power of plant-based extracts, fractions and food products by its ability to reduce of Fe^{3+} (ferric ion)/tripyridyltriazine complex, present in stoichiometric excess, to Fe^{2+} (ferrous ion).[135] The assay has been automated, making it more efficient and faster, and it has also been modified for microtiter-based measurements.[136] The FRAP values are expressed as antioxidant (reducing) power (μM), antioxidant (reducing) power/dry weight and/or matter or fresh weight (μmol/g), or as standard (mostly Trolox), equivalent/dry weight and/or matter or fresh weight (μmol TE/g).

An aliquot of test sample (30 μl) was mixed with 990 μl FRAP reagent. For blank, 30 μl of distilled water and 990 μl FRAP reagent were mixed. An aliquot of 200 μl of the reaction mixture (sample and reagent) was added to a 96-well microtiter plate in

triplicate and the absorption (at 593 nm) of the sample, incubated at 37 °C was measured after 4 min. For calibration, $FeSO_4 \cdot 7H_2O$ and Trolox solutions (0.1-2.0 mM) were used. The reducing ability of the test samples was determined from a calibration curve of $FeSO_4 \cdot 7H_2O$ and Trolox standards. The reducing ability of the test samples was determined from a calibration curve of $FeSO_4 \cdot 7\ H_2O$, and relative reducing ability was expressed as Trolox equivalents (TE) and per g dry matter. A more detail explanation of this assay is provided in Appendix B.

2.6. *Polyphenolic Content Determination Assays*

2.6.1. Total Phenolic Content Assays: The total polyphenol content (TPC) of ackee extracts were determined by a modified Folin-Ciocalteu method adjusted for microscale amounts of material.[137,138] The assay, a microtiter-based assay, has been introduced in our laboratory and is useful to screen for the relative quality and quantity of polyphenols in plant extracts, fractions, and plant based food product. The total polyphenol content of the extracts and fractions were expressed as mg gallic acid (31.3 μg/ml to 0.5 mg/ml) equivalents per gram (mg GAE/g) of dry matter as determined from a calibration curve.

An aliquot of extract (100 μl, 0.6-10.0 mg/ml) dissolved in methanol/water (1:1) was mixed with 1 ml of 10% (v/v) Folin-Ciocalteu's phenol reagent and incubated for 5 min at room temperature. A 10% Na_2CO_3 solution (1 ml) was then added and allowed to stand for 1.5 hr. Aliquots of the reaction mixtures (200 μl) were pipetted into a 96-well microtiter plate in triplicate and the optical density of the blue-colored samples was then measured immediately at 765 nm. The total polyphenol content was determined from the regression equation of the calibration curve ($y = 3.164x + 0.0328$, $R^2 = 0.9885$). The

results of crude estimates of total polyphenol content of dried extracts and fractions are summarized as mean values and standard errors in Tables 2.2 and 2.3.

2.6.2. Total Flavonoid Content Assays: This assay is very inexpensive and can be done very rapidly; it is spectrophotometric and flavonoid content is measured by the formation of a complex with aluminum chloride ($AlCl_3$). The total flavonoid content (TFC) of ackee extracts and fractions were determined by the method previously described.[139,140] An aliquot of test sample (200 µl) or standard (quercetin) was mixed with 1 ml of distilled water followed by an addition of 60 µl of a 5% $NaNO_2$ solution. After 6 min, 120 µl of a 10% $AlCl_3 \cdot 6H_2O$ was added and allowed to react for 5 min more before 400 µl of 1 M NaOH was added. Distilled water (220 µl) was added and mixed thoroughly. The reaction mixtures (200 µl) were added to a 96-well plate, and their absorbance at 510 nm against a blank was measured after 15 min. The results are expressed as milligram quercetin per gram (mg QE/g) of fraction per dry weight.

2.7. *Isolation and Identification of Polyphenol Antioxidants*

Activity-guided fractionation on the EtOAc fraction was performed to isolate active antioxidant constituents. The EtOAc fraction was initially separated over two different stationary phases, Diaion HP-20 (HP-20) and normal-phase silica gel (Si gel), and later purified over reversed-phase silica gel (RP-18) and Sephadex LH-20 (LH-20) (Figure 2.2).

(**A**). The EtOAc residue (40 g) of ackee pods was redissolved in MeOH/H_2O (2:8) under sonication, filtered, and separated over HP-20 (600 g), and eluted sequentially with H_2O, H_2O/MeOH (1:1), MeOH, and acetone, producing four fractions (Fig. 2.2). Fraction H_2O/MeOH (1:1) (10 g; IC_{50} = 11.88 µg/ml) was chromatographed over LH-20

(200 g), and eluted isocratically with H_2O/MeOH (1:1), obtaining 23 fractions (AP-B_{1-23}) that were pooled into 10 fractions (B_1, B_2, B_3, B_4, $B_{5'}$, $B_{7'}$, $B_{9'}$, $B_{16'}$, $B_{20'}$, and $B_{22'}$) after analysis by Si gel and RP-TLC. Fraction $B_{5'}$ (0.5 g) was chromatographed over a RP-18 column (15 g) to give 130 subfractions, which were further combined in 30 fractions. Fractions $B_{5'}$-15′ (61 mg), $B_{5'}$-83 (10 mg), $B_{5'}$-108′ (6 mg), and $B_{5'}$-6a (9.7 mg) were active in the DPPH assay, and were further purified by column chromatography.

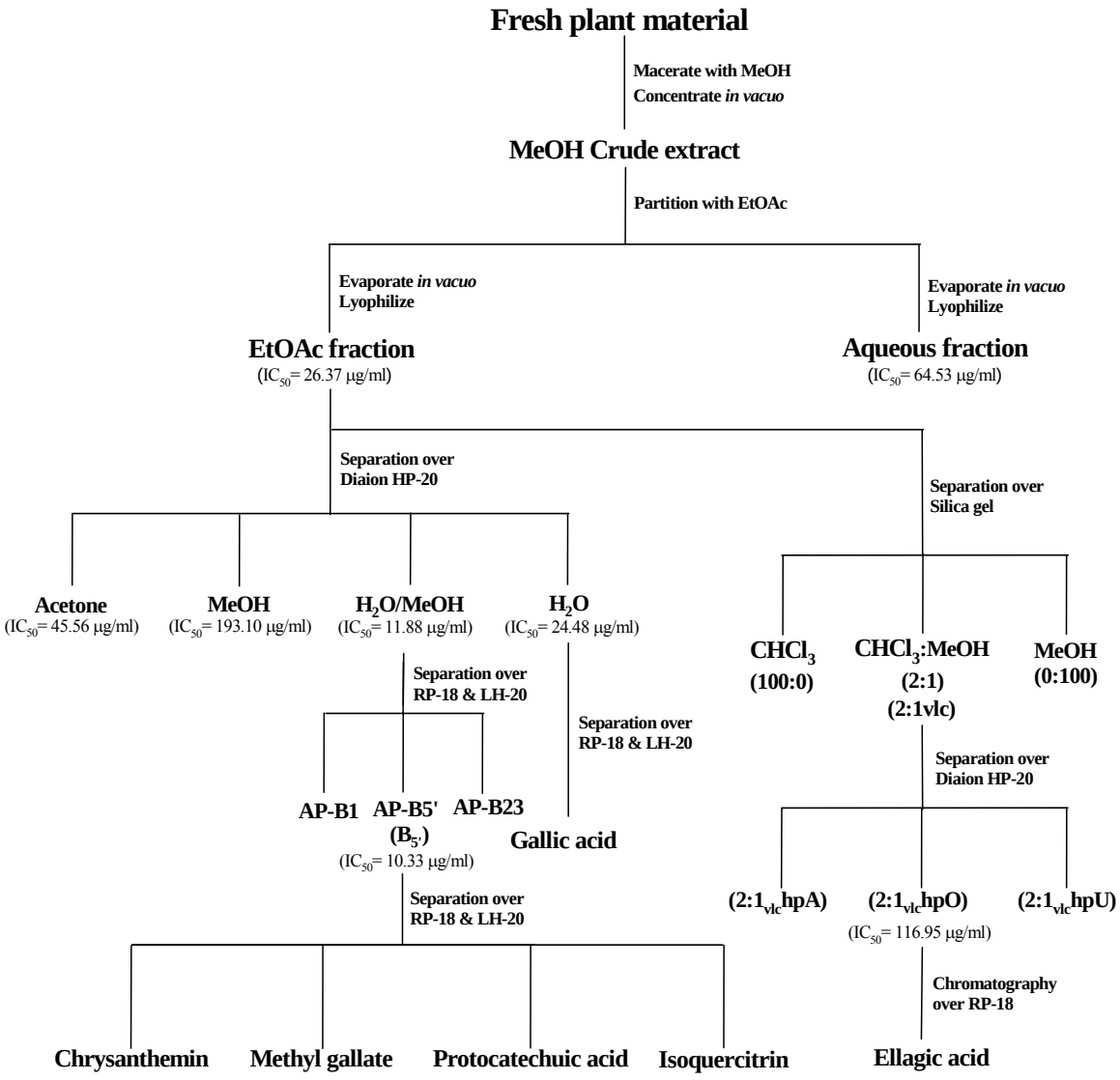


Figure 2.2. Partition and isolation scheme of polyphenols from *B. sapida* fruit pods

Fraction $B_{5'}$-15′ was purified over LH-20 to give five subfractions. The five subfractions were analyzed by RP-TLC and HPLC, and pooled to give $B_{5'}$-$15'_1$, $B_{5'}$-$15'_2$, and $B_{5'}$-$15'_4$. Fraction $B_{5'}$-$15'_4$ (59 mg) was purified further over RP-18 and afforded three fractions $B_{5'}$-$15'_4$-1, $B_{5'}$-$15'_4$-2, and $B_{5'}$-$15'_4$-3. Fraction $B_{5'}$-$15'_4$-1 (29.6 mg) was analyzed by Si gel-TLC and by HPLC. Compound $B_{5'}$-$15'_4$-1 was identified as methyl gallate (**1**) upon further analysis by ESI-MS and ^{1}H and ^{13}C-NMR.

Fraction $B_{5'}$-83 was purified over LH-20 with an isocratic gradient of H_2O/MeOH (1:1) to give 14 subfractions that were analyzed for purity by Si gel-TLC and HPLC analysis. Fraction $B_{5'}$-83_{13} (3.5 mg) was further analyzed by ESI-MS and NMR analysis. UV-vis spectra, and ^{1}H and ^{13}C NMR data are consistent with published data for isoquercitrin (**2**).

Fraction $B_{5'}$-108′ was purified over LH-20 (5 g) to give 10 subfractions. The 10 subfractions were analyzed by RP-TLC and HPLC, and pooled to give $B_{5'}$-108′-1, $B_{5'}$-108′-2, $B_{5'}$-108′-3′, $B_{5'}$-108′-5′, and $B_{5'}$-108′-8′. Fraction B_5'-108′-3′ (1.5 mg) was analyzed further by ESI-MS and HPLC-PDA. The HPLC solvent system was applied in a linear gradient from 100 % to 50 % A (10% v/v formic acid) over 15 min, followed by 5 min of 100 % B (acetonitrile) at a flow rate of 1 ml/min at room temperature. Peaks for individual compounds were evaluated by A_{280} and A_{344}. Fraction B_5'-108′-3′ was determined to be a cyanidin 3-*O*-*β*-glucopyranoside (**3**).

Fraction $B_{5'}$-6a was chromatographed over LH-20 to give 16 fractions, which were further analyzed by RP-TLC and Si gel-TLC, and then pooled to give B_5-$6a_1$, B_5-$6a_3$, B_5-$6a_6$, $B_{5'}$-$6a_9$, and $B_{5'}$-$6a_{11}$. Fraction $B_{5'}$-$6a_{11}$ (2.6 mg) was further purified over LH-20 to afford five subfractions which were pooled to give $B_{5'}$-$6a_{11}$-1′, $B_{5'}$-$6a_{11}$-4, and $B_{5'}$-

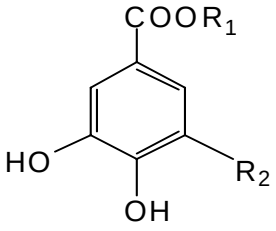
$6a_{11}$-5 after analysis by Si gel-TLC and HPLC. Fraction $B_{5'}$-$6a_{11}$-1′ (2.0 mg) was analyzed by ESI-MS and NMR, and was identified as 3,4-dihydroxybenzoic acid (**4**), also known as protocatechuic acid.

(**B**). A portion of aqueous residue (4 g) from the original HP-20 column described above, was resuspended in MeOH/H_2O (2:8) with sonication, filtered, and chromatographed over HP-20, and eluted sequentially with H_2O, H_2O/MeOH (1:1), MeOH, and acetone to give four fractions. They were analyzed by RP-18 and Si gel-TLC. Upon analysis, fraction G_{grn} (60.3 mg) was selected for further purification. Fraction G_{grn} (53.3 mg) was fractionated over RP-18 to give 27 subfractions, which were pooled and combined into eight fractions after Si gel-TLC analysis. Fractions 1-3 were combined to give, G_{grn}-1′ (8.4 mg), which was further purified over LH-20 to yield 40 fractions. These 40 fractions were analyzed by Si gel-TLC, pooled into five subtractions, G_{grn}-$1'_1$ (1-26), G_{grn}-$1'_{27}$ (27-31), G_{grn}-$1'_{32}$ (32-34), G_{grn}-$1'_{35}$ (35-37), and G_{grn}-$1'_{38}$ (38-40). Fraction G_{grn}-$1'_{32}$ (2.9 mg) was analyzed further by ESI-MS and NMR, and was identified as gallic acid (**5**).

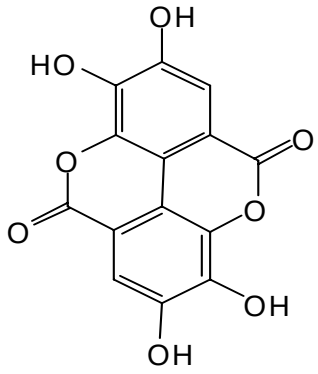
(**C**). A portion of the EtOAc residue (60 g) was redissolved in MeOH under sonication, filtered, and dried in Kieselgel 100 (180 g). The sample was applied to Si gel (600 g) placed in a sintered glass column and fractionated under vacuum, eluting with a discontinuous gradient of $CHCl_3$/MeOH from 100% $CHCl_3$ to 100%MeOH, producing 17 fractions. The 10^{th} fraction, designated $2{:}1_{vlc}$ (3 g), was further separated over HP-20 (60 g), eluting with 100% H_2O to 100% MeOH, acetone/MeOH (1:1), and 100% acetone, producing 21 fractions ($2{:}1_{vlc}$hpA - $2{:}1_{vlc}$hpU). Fraction $2{:}1_{vlc}$hpO (392 mg; IC_{50} = 116.95 µg/ml) was chromatographed over RP-18 to produce 126 subfractions. Subfraction 125

(2.5 mg), redesignated 2:1$_{vlc}$hpO-125, was analyzed further by ESI-MS and NMR, and identified as ellagic acid (**6**). Further identification was achieved through HPLC spiking experiments with authentic standards.

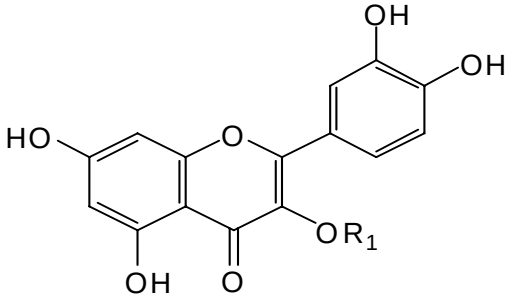
Quercetin (**7**) was also identified in the HP-20 fractions (B, above) by HPLC spiking and HPLC-PDA methods. All other isolated compounds were identified by the comparison of their ESI-MS, NMR, and UV-vis spectral data with those of published results.[141-143] The structures of the isolated compounds and are shown in Figure 2.3, and their radical scavenging activity is shown in Table 2.1.



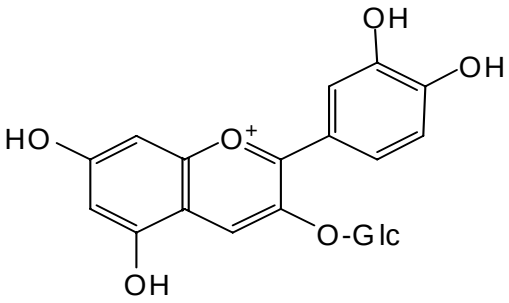
R_1= CH_3, R_2= OH, Methyl gallate (**1**)
R_1= H, R_2= H, 3,4-Dihydroybenzoic acid (**4**)
R_1= H, R_2= OH, Gallate acid (**5**)



Ellagic acid (**6**)



R_1= glucose, Isoquercitrin (**2**)
R_1= H, Quercetin (**7**)



Cyanidin-3-*O*-glucoside (**3**)

Figure. 2.3. Polyphenolic antioxidants isolated from ackee fruit pods

Methyl gallate (**1**): yellow powder; negative ESI-MS *m/z* 183 $[M - H]^-$; ^1H NMR (CD_3OD, 400 MH_Z) δ 7.08 (2H, s, H-2 and 6), 3.83 (3H, s, OCH_3); ^{13}C NMR (CD_3OD,

100 MH_Z) δ 167.7 (C-7), 138.4 (C-4), 145.2 (C-3, 5), 120.2 (C-1), 108.8 (C-2, 6), and 50.9 ($COOCH_3$).[144]

Isoquercitrin (**2**): is a yellow powder, with a negative ESI-MS *m/z* of 463.3 $[M - H]^-$ and 301.3 $[M - H–162]^-$, and MS^2 of *m/z* 301.4 $[M - H - 162]^-$; ^1H NMR (CD_3OD, 400 MH_Z) δ 7.73 (1H, d, *J* = 2.1 H_Z, H-2′), 7.61 (1H, dd, *J* = 8.4, 2.1 H_Z, H-6′), 6.90 (1H, d, *J* = 8.4 H_Z, H-5′), 6.41 (1H, d, *J* = 2.1 H_Z, H-8), 6.12 (1H, d, *J* = 2.1 H_Z, H-6), 5.29 (1H, d, *J* = 7.5 H_Z, H-1″), 4.24 (1H, brd, *J* = 5.7 H_Z, H-6a″), 3.74 (1H, dd, *J* = 9.6, 2.1 H_Z, H-6b″), and 3.66 ~ 3.35 (4H, m, H-2″, 3″, 4″, and 5″).[142]

Cyanidin 3-*O*-β-glucopyranoside (**3**): is a red glossy powder, with a negative ESI-MS *m/z* 447 $[M - H]^-$ and 285 $[M - H - 162]^-$. HPLC spiking experiment with an authentic standard was used to identify this compound.[142]

3,4-Dihydroxybenzoic acid (**4**): is a white powder, with a negative ESI-MS *m/z* of 153 $[M-H]^-$. ^1H NMR (CD_3OD, 300 MH_Z) δ 7.45 (1H, brs, H-2), 7.43 (1H, dd, *J* = 8.7, 2.1 H_Z, H-6), and 6.81 (1H, dd, *J* = 8.7 Hz, H-5); ^{13}C NMR (CD_3OD, 75 MHz) δ 169.0 (C-7), 150.0 (C-3), 144.6 (C-4), 122.4 (C-5), 122.0 (C-1), 116.3 (C-2), and 114.3 (C-6).[142,144]

Gallic acid (**5**): is a white powder, with negative ESI-MS *m/z* of 169 $[M - H]^-$; ^1H NMR (CD_3OD, 300 MH_Z) δ 7.07 (2H, s, H-2 and 6); ^{13}C NMR (CD_3OD, 75 MH_Z) δ 169.4 (C-7), 144.9 (C-3, 5), 138.0 (C-4), 121.2 (C-1), and 108.9 (C-2, 6). UV-vis spectra (λ_{max} = 272), and ^1H and ^{13}C NMR data are consistent with spectral data of an authentic standard and with published data.[142,144]

Ellagic acid (**6**): is a yellowish white powder, with a negative ESI-MS *m/z* of 301$[M-H]^-$. Compound **6** was identified by HPLC spiking.[141,142]

Quercetin (**7**): is a yellowish solid with a negative ESI-MS *m/z* of 301[M-H]$^-$. Compound **7** was identified by HPLC spiking, HPLC-PDA, and MS methods.[141,142]

2.8. *Statistical Analysis*

All DPPH and total polyphenol content experiments were conducted in triplicate, unless otherwise specified. Data are expressed as mean ± SEM obtained by using JMP ver. 5.1 Statistical Software (SAS Institute, Inc.; Cary, IN, USA).

Table 2.1. Antioxidant activity, spectral data of standards and purified polyphenolic compounds from *Blighia sapida*

COMPOUNDS	UV-Vis spectra (HPLC-PDA) λ_{max} (nm)	MS	MS/MS	DPPH Activity IC_{50} (μM)[a]
		$[M-H]^-$ ion	Fragment ion (*m/z*)	
Isolated polyphenolics				
Methyl gallate (**1**)	273	183		27.06 ± 0.18
Quercetin-3-*O*-glucoside (**2**)	263, 351	463, 301	301	81.08 ± 0.29
Cyanidin-3-*O*-glucoside (**3**)	280, 518	447, 285	285	223.49 ± 1.30
Protocatechuic acid (**4**)	257, 293	153		111.08 ± 0.42
Gallic acid (**5**)	272	169		24.06 ± 0.05
Ellagic acid (**6**)	253, 365	301		64.47 ± 0.05
Quercetin (**7**)	256, 362	301		45.13 ± 0.21
Standard – polyphenols and vitamins				
Quercetin 3-*O*-rutinoside	265, 294	609, 301	301	70.41 ± 1.44
Quercetin-3-*O*-rhamnoside	256, 349	301,447	301	58.71 ± 0.23
Cyanidin chloride	276, 527	321		19.81 ± 0.06
Delphinidin chloride	276, 532	337		28.60 ± 0.31
Ascorbic acid				152.87 ± 0.27

[a] Based on DPPH (400 μM) free radical-scavenging assay.

3. Results and Discussion

3.1. *Evaluation of Plant Material for Study*

As part of an ongoing study to isolate chemoprotective agents from tropical fruits, we surveyed 19 edible fruits grown in the Caribbean and southern Florida, and one cultivar from southern Florida (Table 2.2). The antioxidant activity values of the EtOAc fractions of the 19 fruits varied from a high IC_{50} value (19.97 μg/ml) for mabolo, to a low IC_{50} (186.47 μg/ml) for the ackee aril. Nine fruit fractions tested in the microtiter-based DPPH assay displayed strong antioxidant activity ($IC_{50} \leq 50$ μg/ml). Ackee pod, mabolo, mango, and sweetsop had $IC_{50} \leq 27.00$ μg/ml which are comparable to the IC_{50} value of vitamin C ($IC_{50} = 26.91$ μg/ml). The ackee pod, which is not edible, demonstrated high radical scavenging activity in our assay, an activity seven times higher than that of the edible ackee arils ($IC_{50} = 186.47$ μg/ml). The antioxidant activity of ackee pod is of interest since no previous phytochemical studies have been directed toward identifying the antioxidant constituents. Ackee aril is a good source of traditional dietary antioxidants such as vitamins A and C.[145] Since we tested the EtOAc soluble extracts, the antioxidant capability of the water-soluble vitamin C is not reflected in the IC_{50} values found in these experiments.

Moderate antioxidant activity (50 μg/ml $< IC_{50} \leq$ 100 μg/ml) was displayed in nine fruit fractions including, soursop, jackfruit, bilimbli, star fruit, star apple, sorrel, Barbados cherry, Spanish plum, and June plum. Of all the fruits with moderate antioxidant activity, Barbados cherry contained the highest reported levels of vitamin C, 3200 mg per 100 g edible portions. This plant is widely grown in the Caribbean.[145] Three fruit extracts, breadfruit ($IC_{50} = 104.30$ μg/ml), tamarind ($IC_{50} = 128.31$ μg/ml), and ackee

aril (IC_{50} = 186.47 μg/ml), displayed low antioxidant activity (100 μg/ml < IC_{50} ≤ 200 μg/ml).

Based on this preliminary analysis we found that ackee showed strong antioxidant activity and a high TPC value (Table 2.2). We proceeded to obtain ackee fruit from both Jamaica and Florida, in the immature and ripe stages. According to DPPH, FRAP, TPC, and TFC assays, the ackee fruits from both Florida and Jamaica showed similar antioxidant and polyphenolic levels (Table 2.3). Consequently, we decided to pursue the purification of the Florida plant material, because fruits from Florida were more readily available. Since the immature fruit is poisonous, it is not used in the food industry. Therefore our research focused on the mature ackee fruit, even though the immature ackee displayed higher levels of polyphenolics content than the mature fruit. Additionally, since the pods represent about 71-83% weight per fresh weight of the total fruit and the seed represents 7%, the pod was selected as the plant material for the investigation in this study. However, because of the high antioxidant activities, TFC and TPC values, further studies are needed to examine the antioxidant constituents from the seed in the future.

3.2. *Antioxidant Activity of Ackee Plant Parts and Fractions*

The EtOAc fractions of ackee demonstrated much higher antioxidant activities compared to the aqueous fractions (Table 2.2), indicating that most of the phenolic constituents of the crude methanolic extracts have been partitioned into the EtOAc fraction (Figure 2.2). Analysis of the EtOAc fractions of the different plant parts of ackee revealed that the pod (IC_{50} = 26.37 μg/ml) and the seed (IC_{50} = 28.22 μg/ml) have the best DPPH radical scavenging activities. The antioxidant activity of the other EtOAc fractions

decreases in the following order: stem (IC_{50} = 42.67 μg/ml) > leaf (IC_{50} = 66.61 μg/ml) > edible aril (IC_{50} = 186.47 μg/ml). This trend in DPPH scavenging activity was similar to that observed for the aqueous fractions.

To further verify the antioxidant activity, we used the FRAP assay, which evaluates the ability of antioxidants in reducing Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. This assay closely represents half reactions that occur under biological conditions; in contrast the DPPH is an endogenous free-radical scavenging assay. The results of the FRAP assay showed a similar trend as the DPPH assay (Table 2.3). The FRAP values were highest among the EtOAc fractions as compared to the aqueous fractions, except for the aqueous fractions from the immature ackee pod. The high FRAP value of this fraction may be an indication of an increased production of polyphenols, probably phenolic acids, during the fruit maturation stages. Several studies have shown that polyphenol levels may change during fruit ripening, and the type and quantity of individual polyphenols may vary during development and maturity.[144]

3.3. *Total phenolic and flavonoid content of ackee plant parts and fractions*

Total phenolic content (TPC) and total flavonoid content (TFC) of ackee fractions was tested using a modification of the Folin-Ciocalteu method and the aluminum chloride colorimetric assay, respectively, to further prioritize fractions for purification (Table 2.3-2.4). The TPC values of the EtOAc and aqueous fractions varied from 11.15 mg GAE/g in the aril aqueous fraction, to 666.25 mg GAE/g in the seed EtOAc fraction (Table 2.3). The highest levels of TPC were found in the EtOAc fraction of the seed, followed by pod, stem, leaf, and aril. The TFC values of the EtOAc and aqueous fractions varied from 0.66 mg QE/g in the aril aqueous fraction to 1513 mg QE/g in the seed EtOAc fraction. The

highest amount of TFC was found in the EtOAc fraction of the seed, followed by stem, leaf, pod, and aril, respectively. For both the TPC and TFC assays, values were highest for the EtOAc fractions with respect to the aqueous fractions, with the lowest TPC and TFC values present in the aqueous fraction of the edible ackee aril.

There is a strong correlation between TPC and TFC values and antioxidant activity. Fractions with high antioxidant capacity (low IC_{50} value for DPPH) generally have very high TPC values (Tables 2.2 and 2.3). The Folin-Ciocalteu's phenol reagent is not specific to polyphenols and also detects other substances; therefore, TPC values only represent an estimate of a sample's polyphenolic contents. Since antioxidant activity measurements using the DPPH assay may not accurately represent actual biological conditions due to exogenous stable free radicals,[138] the combination of different assays, such as the TPC, DPPH and FRAP, is useful to screen for the relative quality and quantity of polyphenols, and the antioxidant capacity of ackee fractions.

In our study, we found that the ackee pod polyphenols could be extracted efficiently with methanol, and could then be concentrated in the EtOAc partition fraction (Table 2.4). Subsequent fractionation of the EtOAc fraction over HP-20 provided optimal concentration of these polyphenolic compounds in three very active fractions, including acetone, water, and MeOH/H_2O (1:1) (Table 2.3). These three fractions had very high radical scavenging activity, as well as high TPC and FRAP values, indicating that ackee pod polyphenolic constituents were relatively polar and nonpolar. The ratio of total phenolic content to total flavonoid content for the pod EtOAc fractions was about 3:1, whether from the immature or mature fruits from either Florida or Jamaica. This ratio indicates that the EtOAc pod fraction is rich in phenolcarboxylic acid derivatives as

compared to flavonoids. The ratio of polyphenols (TPC/TFC) differs in the various plant part fractions (Table 2.4). In fact, for the ackee pod the ratio of polyphenols was higher in the other fractions, as compared to the EtOAc fractions. The ratio was similar for all fractions of the stem except for the aqueous fractions which was about 2:1 higher. All of the seed fractions showed similar ratio; however, these fractions contained higher amounts of flavonoids with respect to phenols.

3.4. *Scavenging Activity of Ackee Polyphenols and Chemoprotection*

3.4.1. Phenolic Acid Antioxidants: Compounds **1**, **4**, **5**, and **6** were identified as phenolic acids or phenolcarboxylic acid derivatives, possessing the catechol (ortho hydroxyl) or pyrogalloyl configuration. The antioxidant activities of these ackee phenolic compounds decreased as follows: gallic acid (IC_{50} = 24.06 μM) > methyl gallate (IC_{50} = 27.06 μM) > ellagic acid (IC_{50} = 64.47 μM) > protocatechuic acid (IC_{50} = 111.08 μM) (Table 2.1). Phenolic acids are widely distributed in higher plants, and are particularly abundant in many edible fruits and vegetables. Phenolic compounds have been shown to be chemoprotective in vitro and in vivo, reducing lipid autooxidation, scavenging free-radicals, inducing apoptosis, chelating divalent metals, increasing human phenolsulfotransferase (PST) activity, and inhibiting the activity of Type 1 5α-reductase.[147,148] Gallic acid and its methyl ester, methyl gallate, are the two major antioxidants (weight/weight) isolated from the ackee pod. These compounds have been reported to be strong antioxidants, and gallic acid has been shown to increase the activity of PST, an enzymes that plays an important role in the detoxification of endogenous compounds.[148] Gallic acid, in combination with other phenols, such as *p*-hydroxylbenzoic acid, enhances PST, exemplifying the synergistic effects of some phenols. Gallic acid is

also known to have peroxyl radical scavenging and anti-fungal activities.[149] Methyl gallate, which is found in many fruits, is reported to have in vitro activity in an Epstein-Bar virus early-antigen activation assay, as well as anticarcinogenic activity.[150] Protocatechuic acid, also present in ackee and in many edible fruits, is a strong DPPH radical scavenger and a potent chemoprotective agent in colon and oral carcinogenesis in animal models.[151] Ellagic acid, a dilactone and a dimeric condensation product of gallic acid, is also common in many fruits. It has been shown to exhibit antimutagenic, antitumor, anticancer, and antiviral.[152] Additionally, it has been shown to inhibit in vitro lipid oxidation in the erythrocyte membrane shuttle system and in rat liver microsomal assay.[153]

Numerous biological and pharmacological activities of phenolic acids have been attributed in part to their antioxidant activity due to the number and position of hydroxyl groups in their structures. Phenols tend to be stronger antioxidants and more effective radical scavengers than flavonoids and flavonoid glycosides.[154]

3.4.2. Flavonoid Antioxidants: Compounds **2** and **3** were identified as flavonoid glycosides, possessing the 3-*O*-*β*-glucopyranoside in the C-ring, the catechol or the ortho hydroxyl configuration within the B-ring, and the 5,7 hydroxylation in the A-ring. The antioxidant activity of the two isolated flavonoid glycosides, quercetin 3-*O*-glucopyranoside and cyanidin 3-*O*-glucopyranoside, were IC_{50} = 81.08 μM and IC_{50} = 223.49 μM, respectively. These IC_{50} values are comparable to those of known standards having structural similarities, and include rutin and quercitrin (quercetin 3-rhamnoside) (Table 2.1). Compound **3**, cyanidin 3-*O*-glucopyranoside, is an anthocyanin and, like phenolic acids, is widely distributed in higher plants. Anthocyanins are plant pigments

that give many sepals, petals, leaves, fruits, and vegetables their distinctive colors,[155] and have been studied widely for their pharmacological properties.[28] They are supplied through daily fruit consumption, as part of a balanced diet, and their bioavailability as chemoprotective agents in vivo and in vitro are important healthwise.[156] Anthocyanins are believed to play an important role in aging, modulating cancer incidence, and reducing radical induced malignancies, neurological diseases, and mortality.[60] The in vivo activity of anthocyanins depends on their subsequent absorption across the intestinal mucosa wall of the gut. Anthocyanins are more readily transported across the intestinal wall than quercetin glycosides.[157] In vivo, anthocyanins have been shown to display vasoactive properties, as well as inhibitory activity against HCT-15 intestinal cancer, human vulva carcinoma cells overexpressing epidermal growth factor receptor, and growth inhibition of HT-29 and HCT 116 colon cancer cells.[158,159]

Compound **2** is a glycoside of quercetin (**7**), which like anthocyanins possess similar ability in their antiradical and apoptotic properties.[160] Quercetin, one of the most common polyphenols in the flavonoid group, is found abundantly in the edible portion of fruits, and occurs widely as glycosides.[28] The degree of hydroxylation of flavonol glycosides is important in their radical scavenging property, their ability to inhibit radical-mediated LDL oxidation, and chelate metals.[161] Since compounds **2** and **3** possess the catechol configuration in their B-ring, their antiradical properties are similar to those of phenolic acids, but they are somewhat less effective DPPH scavengers. In our study, the presence of a free 3-hydroxyl group in the C-ring of flavonoids correlates with a significant increase in their antiradical property, as shown in Table 2.1. However, the presence of the bulky sugar moiety at the 3-position of the C-ring offsets the planarity of

the compound, as in isoquercitrin and cyanidin 3-*O*-glucopyranoside. This tends to decrease its antioxidant activity, as compared to the corresponding aglycone quercetin (IC_{50} = 43.15 μM).

3.5. *Industrial Potential for Ackee Byproducts*

Our activity-guided fractionation studies resulted in the isolation and identification of seven polyphenolic antioxidants, methyl gallate, isoquercitrin, cyanidin 3-*O*-*β*-glucopyranoside, protocatechuic acid, gallic acid, ellagic acid, and quercetin. The EtOAc fraction of the ackee pod is especially rich in phenolcarboxylic acid derivatives, such as gallic acid and methyl gallate, which showed strong antioxidant activity. The yield of six of the isolated polyphenolic antioxidants we identified from ackee pods decreased in the following order: methyl gallate (16.3 mg/kg fresh weight) >> gallic acid (3.64 mg/kg) > quercetin 3-*O*-glucopyranoside (1.86 mg/kg) > ellagic acid (1.62 mg/kg) > protocatechuic acid (1.06 mg/kg) > cyanidin 3-*O*-glucopyranoside (0.80 mg/kg). Since about 77% weight per fresh weight of the whole ackee fruit can be lost as by-product (pod and seed) during canning (4,821 tons in 2001), One may estimate that 71.2 kg, 1.59 kg, 8.13 kg, 7.08 kg, 4.63 kg, and 3.50 kg of methyl gallate, gallic acid, isoquercitrin, ellagic acid, protocatechuic acid, and cyanidin 3-*O*-glucopyranoside, respectively, are lost as pod waste annually.

Phenolic acids are widely distributed in higher plants, and are found abundantly in many edible fruits and vegetables. They possess chemoprotective properties in vitro and in vivo, and many biological and pharmacological activities of phenolic acids have been attributed in part to their antioxidant activity in turn due to the number and position of hydroxylation in their structures.[149-152] This study indicates that methyl gallate is present

in the highest concentration among the six isolated polyphenols from the ackee pods, with a yield is about 4.5, 10, and 15 times higher than the phenolcarboxylic acid derivatives gallic acid, ellagic acid and protocatechuic acid, respectively. These simple phenolic compounds are important because of their range of biological activities. Moreover, cyanidin 3-*O*-glucopyranoside, an important member of the class of anthocyanin compounds, is an expensive natural product with a price in 2007 of $95.90/mg. Anthocyanin compounds are also abundant in grape byproduct extracts, and are used in many industrial applications.[155,162,163] These polyphenol compounds could be used as additives and preservatives in food products, as anti-aging and photoprotective agents in cosmetics and pharmaceutical applications, and as antioxidants in dietary supplements.[60,158]

There are limitations in the processing of byproducts from different fruits or plant sources and extraction and processing methods vary depending on the plant material matrix.[164] The recovery of plant natural products from industrial byproducts, such as grape seeds and skins and citrus peels, has become more common as methods for extraction and recovery have improved. Consequently, the commercial recovery of antioxidants and other bioactive constituents from plant byproducts is a growing and promising enterprise, especially for the cosmetic, dermatology, and pharmaceutical industries.[165] The polyphenols from several commercially important plant sources can be efficiently extracted with ethanol, acetone, and methanol, as demonstrated in many extraction studies involving fruits, vegetables, and other plant byproducts.[163,166] However, extraction results may vary in many laboratories depending on the plant material used.

The recovery of bioactive phenolic compounds from plants, especially from industrial byproducts, has become an emerging interest, likely due to the growing demand for natural product conservation, and naturally occurring antioxidants for the preservation of food. Extraction methods for the processing of byproducts may be costly, but in the long-term, in addition to producing useful polyphenolics, it can be an environmentally sound strategy. Therefore, it is useful to develop a practical method for the exploitation and recovery of important bioactive constituents from ackee fruit byproducts.

Ackee pods could be an important source for some useful polyphenolics. However, caution is needed since the immature ackee fruit contains cyclopropyl peptide toxins, and even the mature fruits may contain low levels of these compounds.[167]

4. Conclusion

In this study, ackee fruit was selected from several tropical fruits due to high DPPH and TPC values. We proceeded to evaluate ackee plant and its various parts further by comparing their DPPH, FRAP, TFC, and TPC values. The ackee pod was selected for further fractionation work based on activity and quantity, and six polyphenolic compounds (**1**-**6**) were isolated from its EtOAc fraction after repeated chromatography. The yields of the six polyphenol antioxidants were determined to be 16.3, 3.65, 1.86, 1.62, 1.06, and 0.80 mg per kg fresh weight for methyl gallate (**1**), gallic acid (**5**), isoquercitrin (**2**), ellagic acid (**6**), protocatechuic acid (**4**), and cyanidin 3-*O*-glucopyranoside (**3**), respectively. Further work is needed to determine the utility of ackee byproducts as possible sources of polyphenol-enriched extracts that are suitable for

food, cosmetics, and pharmaceutical applications, and as a potential source of natural fiber and paper products.

Table 2.2. Antioxidant activity and total phenolic content of selected EtOAc fractions from Caribbean fruits assayed by DPPH and TPC method, respectively

Plant Name	Family	Vernacular Name	DPPH IC_{50} (μg/ml)[a]	TPC (mg GAE/g)[a]
Annona muricata	Annonaceae	Soursop	73.24 ± 1.44[b]	80.16 ± 0.24
Annona squamosa	Annonaceae	Sweetsop	22.43 ± 0.30	638.48 ± 0.30
Annona squamosa var. *kampong*	Annonaceae	Sweetsop	34.41 ± 0.23	444.64 ± 0.15
Artocarpus altilis	Moraceae	Breadfruit	104.30 ± 1.02	85.18 ± 0.24
Artocarpus heterophyllus	Moraceae	Jackfruit	61.79 ± 0.39[b]	165.42 ± 0.09
Artocarpus heterophyllus	Moraceae	Jackfruit w/seed	30.15 ± 1.26	366.12 ± 0.15
Averrhoa bilimbi	Oxalidaceae	Bilimbli	69.21 ± 0.31	130.42 ± 0.29
Averrhoa carambola	Oxalidaceae	Star fruit	62.11 ± 1.31	290.69 ± 0.08
Blighia sapida	Sapindaceae	Ackee, aril	186.47 ± 11.7	56.07 ± 0.28
Blighia sapida	Sapindaceae	Ackee, pod	26.37 ± 0.09	410.48 ± 0.69
Chrysophyllus cainito	Sapotaceae	Star apple	62.10 ± 1.30	nd
Diospyros blancoi	Ebenaceae	Mabolo	19.97 ± 0.16	418.30 ± 0.09
Hibiscus sabdariffa	Malvaceae	Sorrel	66.00 ± 0.33	89.49 ± 0.06
Malpighia glabra	Malpighiaceae	Barbados cherry	54.44 ± 1.39	110.29 ± 0.18
Mangifera indica	Anacardiaceae	Mango	22.57 ± 0.18	343.93 ± 0.80
Manilkara zapota	Sapotaceae	Sapodilla	19.60 ± 0.15	nd
Muntingia calabura	Elaeocarpaceae	Jamaica cherry	32.11 ± 0.32	284.37 ± 0.04
Psidium guajava	Myrtaceae	Guava	42.58 ± 1.52	155.54 ± 0.27
Spondias dulcis	Anacardiaceae	June plum	75.16 ± 0.17	68.90 ± 0.12
Spondias mombin	Anacardiaceae	Spanish plum	51.64 ± 0.95	140.26 ± 0.42
Tamarindus indica	Anacardiaceae	Tamarind	128.31 ± 3.98	47.26 ± 0.11
Standards				
Gallic acid			4.09 ± 0.05	
Ascorbic acid (Vitamin C)			26.31 ± 0.27	

GAE/g = gallic acid equivalent per gram dried fraction; nd = not determined

[a] Data are presented as mean ± standard error (n =3).

[b] DPPH value is presented as mean ± standard error (n =2).

Table 2.3. Antioxidant activities, and total flavonoid and phenolic content of various ackee EtOAc fractions assayed by DPPH and FRAP reduction, and TFC and TPC methods

PLANT PARTS	FRACTIONS	DPPH IC_{50} (μg/mL)[a]	FRAP (mmol/g)[a]	FRAP (mmol TE/g)[a]	TFC (mg QE/g)[a]	TPC (mg GAE/g)[a]
	Ackee plant parts					
Pod	EtOAc fraction from Florida (mature)	26.37 ± 0.88[b]	5.99 ± 0.00	2.15 ± 0.00	123.10 ± 0.27	410.48 ± 0.00
Pod	Aqueous fraction from Florida (mature)	64.53 ± 3.04	2.0 ± 0.00	0.065 ± 0.00	10.00 ± 0.14	60.31 ± 0.04
Pod	EtOAc fraction from Florida (immature)	11.50 ± 0.06	9.42 ± 2.79	3.12 ± 0.03	184.20 ± 0.27	612.96 ± 0.05
Pod	Aqueous fraction from Florida (immature)	95.36 ± 1.39[b]	12.25 ± 0.30	3.31 ± 0.03	24.45 ± 0.00	101.22 ± 0.30
Pod	EtOAc fraction from Jamaica (mature)	18.39 ± 0.08	5.19 ± 0.91	1.79 ± 0.09	142.55 ± 0.00	410.88 ± 0.05
Pod	Aqueous fraction from Jamaica (mature)	118.77 ± 6.07[b]	1.44 ± 0.14	0.38 ± 0.01	10.40 ± 0.32	83.54 ± 0.14
Aril	EtOAc fraction from Florida (mature)	186.47 ± 11.7[b]	0.58 ± 0.00	0.15 ± 0.00	46.72 ± 0.23	56.07 ± 0.28
Aril	Aqueous fraction from Florida (mature)	918.35 ± 150	0.42 ± 0.00	0.00 ± 0.00	00.66 ± 0.03	11.15 ± 0.10
Leaf	EtOAc fraction	66.61 ± 1.32[b]	2.61 ± 0.71	0.65 ± 0.01	155.21 ± 0.00	135.08 ± 0.71
Leaf	Aqueous fraction	413.41 ± 2.23	1.55 ± 0.04	0.15 ± 0.00	28.55 ± 0.27	38.33 ± 0.04
Seed	EtOAc fraction	28.22 ± 0.82[b]	25.66 ± 0.97	7.12 ± 0.01	1513.21 ± 0.27	666.25 ± 0.40
Seed	Aqueous fraction	288.71 ± 5.58	0.39 ± 0.10	0.039 ± 0.01	73.74 ± 0.00	51.04 ± 0.10
Stem	EtOAc fraction	42.67 ± 0.36[b]	4.50 ± 1.01	1.47 ± 0.01	298.26 ± 0.00	294.81 ± 0.19
Stem	Aqueous fraction	236.81 ± 6.61	0.88 ± 0.51	0.12 ± 0.03	67.99 ± 0.00	144.24 ± 0.07
	Selected activity-guided fractions					
Pod	H_2O fraction from HP-20 CC	24.48 ± 0.34	5.16 ± 0.48	1.76 ± 0.05	122.27 ± 0.91	655.77 ± 0.15
Pod	H_2O/MeOH fraction from HP-20 CC	11.88 ± 0.12	10.92 ± 1.30	3.78 ± 0.01	196.94 ± 1.21	731.19 ± 0.15
Pod	MeOH fraction from HP-20 CC	193.10 ± 0.12	0.68 ± 0.29	0.045 ± 0.00	1.92 ± 0.00	38.25 ± 0.29
Pod	Acetone fraction from HP-20 CC	45.56 ± 0.32	2.35 ± 0.44	0.51 ± 0.00	120.67 ± 0.91	104.85 ± 0.44
Pod	Fraction $B_{5'}$ from H_2O/MeOH	10.33 ± 0.09	10.82 ± 1.46	3.74. ± 0.02	317.97 ± 0.46	599.55 ± 1.46
Pod	Fraction $2{:}1_{vlc}$hpO from 2:1vlc	116.95 ± 7.19	1.32 ± 0.10	0.33 ± 0.01	11.90 ± 0.20	26.38 ± 0.10

TE and QE/g = trolox and quercetin equivalent per gram dried of fraction; nd = not determined;

[a] Data are presented as mean ± standard error (n =3)

[b] Data are presented as mean ± standard error (n = 2).

Table 2.4. Total flavonoid and phenolic content of partition fractions of various ackee plant parts by TFC and TPC assays

PLANT PART	FRACTIONS	TFC Assay (mg QE/g)[a]	TPC Assay (mg GAE/g)[a]	TPC/TFC Ratio
Pod	Methanol crude extract from Florida mature fruit	19.48 ± 0.00	81.10 ± 0.00	4.16
Pod	Fraction after hexane partition of crude extract	21.81 ± 0.14	109.9 ± 0.23	5.04
Pod	Ethyl acetate fraction	123.1 ± 0.27	410.5 ± 0.00	3.33
Pod	Aqueous fraction	10.00 ± 0.14	60.31 ± 0.04	6.03
Seed	Methanol crude extract	206.7 ± 0.05	97.60 ± 0.69	0.47
Seed	Fraction after hexane partition of crude extract	222.3 ± 0.00	101.4 ± 0.09	0.46
Seed	Ethyl acetate fraction	1513 ± 0.27	666.3 ± 0.40	0.44
Seed	Aqueous fraction	79.52 ± 0.27	51.04 ± 0.10	0.64
Stem	Methanol crude extract	93.30 ± 0.22	86.22 ± 0.58	0.92
Stem	Fraction after hexane partition of crude extract	71.30 ± 0.27	77.01 ± 0.38	1.08
Stem	Ethyl acetate fraction	298.3 ± 0.00	294.8 ± 0.19	0.99
Stem	Aqueous fraction	67.99 ± 0.00	144.2 ± 0.07	2.12

TFC and TPC = total flavonoids and phenolic content expressed as mg quercetin and gallic acid equivalents, respectively, per g of dry material from fractions.

[a] Values are presented as mean standard error of triplicate experiments (n = 3).

CHAPTER 3

Three New Bioactive Triterpene Saponins from *Blighia sapida*

1. Introduction

The genus *Blighia* belonging to the family Sapindaceae (soapberry family) and is composed of three species, *Blighia sapida*, *B. unijugata*, and *B. welwitschii*. *Blighia* is taxonomically related to the genus *Sapindus*, which is known to contain saponins of hederagenin and oleanolic type.[87] Of the three species, *Blighia sapida* is the most well known and it is cultivated in the West Indies, Central and South America, and Florida for its edible yellow fruit arils. The fruit and various parts of the plant have been used in many applications in folk medicine.[96,100] Previous phytochemical investigations on ackee has been minimal, except for a few reported compounds, including a quinone whose structure has not been completely determined, the steroid stigmasterol and stigmasterol fructoside, the triterpene hederagenin, and unidentified saponins.[102] Saponins are known to have antitumor, anticancer, anti-inflammatory, spermicidal, hemolytic, neurological and molluscicidal activities (Section 1.5.5).[108-112,168] In this chapter, the isolation and structural determination of three new monodesmosidic triterpene saponins, and two known compounds is described. The determination of cytotoxicity of these three new compounds and the effects on the growth of the estrogen-insensitive MDA-MB-453 (Her2 overexpressing) human breast cancer cell line are also described.

2. Material and Methods

2.1. *Plant Material*

Blighia sapida fruits were collected at the Fruit and Spice Park (Homestead, Florida), and transported to New York City by overnight carrier frozen. *B. sapida* fruits were weighed, catalogued, and frozen at -20°C until analyzed.

2.2. ***General Experimental Procedures***

Optical rotation experiments were measured at 29 ºC on an AutoPol III automatic polarimeter (Rudolph Research Analytical, Hackettstown, USA). HRESIMS was performed using a Q-Tof Ultima mass Spectrometer (Micromass). FAB-MS was performed with a JOEL Sx102a double-focusing, magnetic-sector instrument (Peabody, MA). Samples were prepared in a CsI "magic-bullet" matrix consisting of a mixture of sulfur-substituted 4-carbon sugars. All NMR spectra were recorded on either a Bruker 500 or 700 MHz spectrometer, equipped with a 5-mm, inversed-detection probe (90° ^{1}H pulse width, 9.5 μs; 90º ^{13}C decoupler pulse width, 9.4 μs). Spectra were recorded for samples in CD_3OD at a probe temperature of 25 ºC. Chemical shifts expressed in δ (ppm) and coupling constant (J) in hertz. Peak positions of the residual CD_2H signal (δ 3.30) and the $^{13}CD_3$ signal (δ 49.2) were used as secondary references relative to internal TMS ($(CH_3)_4Si$) at 0 ppm. Experiments were performed using standard Bruker software. ^{1}H and ^{13}C spectra were obtained with spectral windows of 5.5 and 184 ppm, respectively, 32,768 experimental points (zero-filled to 65,536), and 45° pulses. COSY spectra were acquired in the absolute-value mode with 5.5 ppm spectral widths along both axes; 1024 experimental data points and time increments (both zero-filled to 2048), 16 transients per increment, 256 increments, and a relaxation delay of 0.6 s. Pseudo-echo processing was used along both axes, followed by triangular folding. TOCSY and ROESY spectra were measured in the phase-sensitive mode using the same spectral windows and data points as for COSY, 16 transients per increment, 256 increments (linear predicted to 1024 with zero-filling to 2048), and a relaxation delay of 1.5 s. Isotropic mixing times for TOCSY spectra were 10 and 100 ms, and the ROESY spin-lock time was 150 ms. Gaussian

weighting was used for both spectra. HMBC spectra were determined with the same ^{1}H and ^{13}C spectral windows listed above, 1024 experimental points (zero-filled to 2048), 256 time increments, a 0.6-s relaxation delay, and 80 transients per increment. Standard HSQC spectra were recorded with the same ^{1}H spectral width and experimental data points interval as above, a ^{13}C spectral width of 135 ppm 256 increments, a relaxation delay of 2 s (to minimize decoupler heating), a BIRD nulling delay of 0.3 s, and 16 transients per increment. Coupled-HSQC spectra were determined with a ^{1}H spectral width of 1 ppm and a ^{13}C spectral width of 32 ppm, 2048 experimental data points (zero-filled to 8192) and 256 time increments. A BIRD nulling delay of 0.85 s was selected to minimize OH and CD_2H signals, with a relaxation delay of 0.5 s, and with 16 transients per increment.

All of the above 2D spectra were acquired and processed in the phase-sensitive mode except the HMBC spectra, which were processed in the mixed mode (phase-sensitive) along F_1 and absolute-value along F_2. Gaussian weighting was used in all cases, except for the absolute-value, HMBC F_2–axis, where a 1/3 shifted sine-bell was used. For standards (oleanolic acids and hederagenin), ^{1}H NMR, and ^{13}C NMR spectra were recorded in pyridine-d_5 using a Bruker AMX-300 MHz NMR spectrometer, operating at 300 and 75 MHz, respectively. APCI-MS was performed with a ThermoQuest Finnigan LCQ instrument (San Jose, CA) equipped with Xcalibur software. Samples were dissolved in MeOH and introduced by direct injection. The capillary voltage was 10 V, the spray voltage was 4.5 kV, and the tube lens offset was 0 V. TLC analyses were performed on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany), with compounds visualized by spraying with a vanillin solution (1 g vanillin in 10% conc.

H_2SO_4 ethanolic soln.). Sephadex LH-20 (25-100 μm; Pharmacia Fine Chemicals, Piscataway, NJ), C_{18} reversed-phase silica gel (40 μm; J. T. Baker, Phillipsburg, NJ), and Diaion HP-20 (Supelco, Bellefonte, PA) were used for column chromatography.

2.3. ***Extraction and Isolation***

Ackee pods were homogenized and prepared as described in Chapter 2 (Section 2.2.3). A portion of the EtOAc residue (15 g) was redissolved in MeOH/H_2O (8:2) under sonication, dried in Si gel (45 g), and the mixture packed on and fractionated over Si gel (600 g) placed in a sintered glass column under vacuum (vacuum liquid chromatography (VLC). Elusion of the column was performed by a discontinuous gradient of $CHCl_3$/MeOH from 100% $CHCl_3$ to 100% MeOH. Fraction 6:1vlc (named 6:1($CHCl_3$/MeOH); 770 mg) was selected after TLC analysis, reconstituted, diluted with deionized distilled H_2O, and partitioned with *n*-butanol (*n*-BuOH). A portion of the *n*-BuOH residue (6:1B; 330 mg) was chromatographed over RP-18 (20 g) by eluting with gradients of MeCN-H_2O (from 1:9 to 100:0 MeCN-H_2O) to give 30 subfractions (6:1B_1-6:1B_{30}). Subfraction 6:1B_{21} (70.0 mg) was pure and gave compound **1**, and fraction 6:1B_{24} (50.2 mg), was further separated isocratically over LH-20 (2 g) using solvent MeCN-H_2O (6:4) to give compound **2**. Fraction 8:1vlc (named 8:1$_{vlc}$ ($CHCl_3$/MeOH)) (0.54 g) was chromatographed over RP-18 (20 g) by eluting with gradients of MeCN-H_2O from 9:1 to 100:0 MeCN-H_2O to give 22 subfractions (8:1$_{vlc1}$-8:1$_{vlc22}$). Subfraction 8:1$_{vlc7}$ (113.0 mg) was chromatograph over LH-20 eluting with MeOH-H_2O (8:2) to produce 30 subfractions (8:1$_{vlc7\text{-}1}$-8:1$_{vlc7\text{-}30}$). Subfraction 8:1$_{vlc7\text{-}10}$ (25 mg) was separated further over LH-20 using solvent system MeOH-H_2O (8:2) to give 10 subfractions (8:1$_{vlc7\text{-}10\text{-}1}$ -8:1$_{vlc7\text{-}30\text{-}20}$). Subfractions 3 and 4 were combined (named 8:1$_{vlc7\text{-}10\text{-}3'}$; 15 mg))

and purified over LH-20 eluting with MeOH-H_2O (8:2) to give compound **3**. Additionally, two known steroid compounds, stigmasta-5,22-dien-3-ol and stigmasta-5,22-dien-3-*O*-*β*-glucopyranoside, were also identified in the EtOAc partition fraction. The identity of these two compounds, and saponins **1-3**, were elucidated on the basis of their spectroscopic evidence, and by comparison of published literature data. The relative purity of all fractions were tested by normal phase TLC analysis using a solvent system of either 8:1, 4:1 and/or 2:1 $CHCl_3$/MeOH.

2.4. *Cell culture Assay*

MDA-MB-453 (ER negative, Her2 over-expressing) human breast cancer cell was obtained from the ATCC (Manassas, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) at 37 °C, and 5% CO_2 (Gibco BRL Life Technologies, Inc., Rockville, USA).

2.5. *Cell Proliferation assay*

Cell proliferation was determined using the MTT [3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H tetrazolium bromide] (Dojindo, Tokyo, Japan) cell proliferation assay system, according to the manufacturer (Roche Diagnostic, Mannheim, Germany). Cells were seeded into a 24-well plate at a density of 1 x 10^4 cells and allowed to attach for 24 h. The medium was then replaced with fresh medium containing compounds to tested or DMSO (Sigma-Aldrich, St. Louis, USA). The cells were treated for 4 days after which they were incubated with MTT reagents and the absorbance read at 600 nm, as previously described.[169] Actein standard for this assay was obtained from ChromaDex (Laguana Hills, USA).

3. Results and Discussion

3.1. *Isolation of Steroid and Triterpene Constituents*

The ethyl acetate (EtOAc) fraction from *B. sapida* pod was prepared as described in the general procedures section. Two fractions, 6:1vlc and 8:1vlc, were selected after initial fractionation over Si gel. Fraction 6:1vlc was partitioned between *n*-BuOH, and a portion of this residue was purified over RP-18 to give fraction $6{:}1B_{21}$ and $6{:}1B_{24}$. Fraction $6{:}1B_{21}$was pure and gave compound **1,** and fraction $6{:}1B_{24}$ was further purified over LH-20 to give compound **2.** Fraction $8{:}1_{vlc}$ was purified after repeated chromatography over RP-18 and LH-20 to give compound **3**. Compounds **1-3** were new triterpene glycosides. Two known steroids were also identified in the EtOAc partitioned fraction. The isolated compounds were elucidated on the basis of their spectroscopic properties.

The most abundant of the three isolates, compound **1**, $[\alpha]_D^{29}$ +21.48º (MeOH, c = 0.0069M), was isolated as a white amorphous solid and assigned the molecular formula $C_{54}H_{86}O_{22}$ (requiring 12 units of unsaturation), as determined by high-resolution mass spectrometry (HRMS). Elemental analysis by TOF-ESMS (Appendix: A.9 (A)) in positive-ion mode showed a molecular ion fragment peak at *m/z* 1087.5693 $[M + H]^+$ (1087.5689 calcd.) and confirms the formula determined from 1D-NMR analysis. The positive APCI-MS of **1** showed a molecular ion peak at *m/z* 1087 $[M + H]^+$ and prominent fragments at 1043 $[M + H - 44]^+$ and 911 $[M + H - 44 - 132]^+$, due to the subsequent loss of an acetyl group and a pentose, *m/z* 749 $[M + H - 44 - 132 - 162]^+$ and 603 $[M + H - 44 - 132 - 162 - 146]^+$, due to subsequent loss of two hexoses, and at 471 $[M-H - 44 - 132 - 162 - 146 - 132]^+$, due to the loss of a pentose.

^{1}H, ^{13}C-NMR, and ^{13}C-DEPT (45, 90 and 135) NMR spectra of **1** suggested that it is a saponin and has four monosaccharide units. The structure of the aglycone moiety was determined before confirming the identity and linkage of the four sugar units. The identification of the aglycone is described in the preceding section.

3.2. *Identification of the Aglycone Moiety of Saponin* 1

The structure and stereochemistry of the aglycone unit were determined by a combination of the three 1D NMR experiments in addition to high-resolution HSQC and HMBC NMR experiments. The ^{1}H-NMR spectrum of the aglycone portion of **1** showed 11 methylenes, six 3H-singlets for the six tertiary methyl groups at δ 1.18 (3H, s), 0.98 (3H, s), 0.94 (3H, s), 0.91 (3H, s), 0.82 (3H, s), and 0.71 (3H, s) that were attributed to H-27, H-25, H-30, H-29, H-26, and H-24, respectively (Table 3.2), and two other methyl groups not associated with the aglycone, with one having an acetate functionality. Additionally, a characteristic olefinic proton at 5.25 (t, J = 3.8 Hz) was also observed. The ^{13}C-NMR spectra of the aglycone of **1** showed 30 signals that revealed six methyls, 11 methylenes, five methines, and eight quaternary carbons (Table 3.1). The ^{13}C-NMR spectra also showed one carbonyl signal at δ 182.0 (C-28), two sp^2 carbons at δ 123.7 (C-12) and 145.4 (C-13), and one oxymethylene signal at δ 64.7 (C-23). The signal of the six methyl protons displayed strong HMBC correlations, and the two- and three-bond correlations from these methyl groups, in addition to those from other critical protons, permitted identification of the aglycone as hederagenin, as described below.

The elucidation of the complete structure of the triterpene was achieved in the following fashion: two methyl groups (C-29 and C-30) and a methyl and CH_2OH group (C-24 and C-23, respectively) were shown by the HMBC spectrum to be geminal pairs,

and the CH_2OH and the six methyl groups exhibited the following connectivities: (i) δ 0.71 (H-24)/(δ 3.56 and 3.34) H-23AB to δ 82.6 (C-3), 44.1 (C-4), and 48.3 (C-5); (ii) δ 0.98 (H-25) to δ 39.8 (C-1), 48.3 (C-5), 49.1 (C-9), and 37.8 (C-10); (iii) δ 0.82 (H-26) to δ 33.6 (C-7), 40.7 (C-8), 49.1 (C-9), and 43.1 (C-14); (iv) δ 1.18 (H-27) to δ 40.7 (C-8), 145.4 (C-13), 43.1 (C-14), and 29.0 (C-15); (v) δ 0.91 (H-29)/δ 0.94 (H-30) to δ 47.4 (C-19), 31.8 (C-20), and 35.1 (C-21). These HMBC correlations, together with HSQC and COSY connectivities, allowed for the identification of 21 carbons, and the direct linking of 16 of these to the aglycone. The remaining nine carbons were identified and placed in the developing structure by means of their proton and carbon HMBC connectivities to portions of the structure that had been previously elucidated (Table 3.1-3.4).

The determination of the stereochemistry at C-3 and C-4 and their assignment as axial or equatorial and of virtually all the protons and methyl groups of **1** was accomplished in several ways. First, for certain methine protons, such as H-3, observation of a large (10-12 Hz) vicinal coupling requires that the proton be axial. Likewise, H-16A was determined to be axial by virtue of its two large couplings (13.5 Hz) exhibited in its ^{1}H NMR spectrum. Second, for other methine protons, such as H-5 and H-9, whose signals are located in congested regions of the ^{1}H-NMR spectrum, strong ROESY cross peaks between these protons and H-3 indicated that they too are axial. Similarly, strong HMBC cross peaks between H-3 and H-5 to C-24, and weak correlations between these protons and C-23 dictated that C-24 is axial and C-23 equatorial. Third, the assignments of C-29 as equatorial and C-30 as axial were determined on the basis of strong HMBC cross peaks between C-29 (δ 33.6) and H-19A (δ 1.70) and H-21A (δ 1.39), and weak correlations between the C-29 and these same protons. Likewise, H-2A (δ 1.87) was

determined to be equatorial by virtue of the two strong HMBC cross peaks between it and C-4 (δ 44.1) and C-10 (δ 37.8), as compared to the weak correlations between H-2B and these two carbons.

Finally, ROESY spectra were most helpful in determining the relative orientations of the methylene protons. For example, protons 1B (δ 0.97), 3, 5, 7A (δ 1.63), 9, 16A (δ 2.02), 19A (δ 1.70), 21A (δ 1.39), and H-27 displayed strong ROESY cross peaks and are situated on the α-face ("bottom") of **1**. Conversely, protons 2B (δ 1.75), 6B (δ 1.38), and 15A (δ 1.78) and methyls 24, 25, and 26 exhibited strong ROESY correlations and are, therefore, located on the β-face ("top") of **1**. Similarly, protons 18 and 22A (δ 1.75) and CH_3-30 are situated on the β-face of the E-ring of **1**. Therefore, the aglycone is the known triterpene 3β,23-dihydroxy-Δ^{12}-oleanen-28-oic acid (**5**), commonly called hederagenin, and one of the most common saponin aglycone.[108]

3.3. *Identification of the Monosaccharide Units of Saponin* 1

The absolute configuration of the cyclic monosaccharide units was determined by the fact that chair conformations of the cyclohexane-type pyranose structures in which the hydroxyl groups are in equatorial positions are the most stable and will be assumed by cyclic monosaccharide units. For glucose and xylose, the β-D-form; and for arabinose and rhamnose, the α-L-form, predominate in plants. The other configurations are rarely found in plant tissue and are minimal in most of the observed literature in which plant natural products are reported.[105,108] Based on these data, we concluded that the absolute configurations of the sugars were α-L-rhamnose, β-D-glucose, and α-L-arabinose. Additionally, ^{1}H, ^{13}C, and DEPT NMR spectra of **1** suggested that this compound is a saponin and confirmed the presence of a four sugar oligosaccharide.

Inspection of the ^{1}H, ^{13}C, and DEPT NMR spectra of **1** revealed the presence of four anomeric protons and carbons that suggested that **1** is a tetrasaccharide. The broadened singlet observed for the proton at δ 5.23, and the *ca*. 7-Hz doublets seen for those at 4.20-4.62 ppm indicated that these protons are equatorial and axial, respectively, and that **1** possesses one α- and three β-linkages. In order to determine which proton signals belonged to what sugar units, TOCSY spectra were recorded with 10-ms and 100-ms mixing times. Inspection of the cross-sections of the latter, through both the anomeric protons and the non-aglycone methyl protons (J = 6.2 Hz) doublet at δ 1.26, identified the ^{1}H-NMR signals associated with each anomeric proton and the methyl group. Analysis of the short TOCSY (10 ms) permitted the sequencing of protons in each monosaccharide unit. In this way, one α-linked and one β-linked hexasaccharide, and two β-linked pentasaccharides were identified.

The next step in elucidating the complete assignment of the protons of the monosaccharide was to determine the orientation of the remaining, non-anomeric, ring protons in each sugar unit. First, the three ring protons, H-3 and H-4 in one hexasaccharide and the H-4 in the other, were seen to be *ca*. 10-Hz triplets in both their ^{1}H-NMR and HSQC spectra and were accordingly recognized as axial protons that are, in turn, flanked by axial protons. However, such additional identification was essentially impossible from both types of spectra, due to severe signal overlap in the congested carbohydrate region of the ^{1}H-NMR spectrum and insufficient resolution in the HSQC spectra.

Fortuitously, high-resolution coupled-HSQC spectra, covering just the ^{1}H and ^{13}C spectral widths of the carbohydrate region (see experimental section above), permit the

assignment of orientation of these ring protons.[170] Coupled-HSQC spectra provide not only the magnitude of anomeric one-bond C-H couplings but also the H-H couplings. The one-bond C-H coupling information is useful in distinguishing axial from equatorial anomeric protons because the one-bond C-H couplings of the former are *ca*. 160 Hz while those of the latter are *ca*. 170-Hz (Table 3.5).[170] However, H-H coupling is difficult to obtain due to both the considerable H-NMR spectral overlap, at. 3.4-3.6 ppm, and the second-order nature of certain H-NMR signals in the carbohydrate region.

When protons on adjacent carbons have nearly identical chemical shifts, the determination of vicinal couplings, in both the 1D and 2D ^{1}H-NMR spectra, is greatly complicated due to the resulting strong coupling of these protons.[171] However, in a coupled-HSQC spectrum, ^{1}H-^{1}H couplings are determined for subunits of the type ^{1}H-^{13}C-^{12}C-^{1}H. Here the large, one-bond C-H coupling effectively results in a *weak* H-H coupling, analogous to the use of ^{13}C satellite signals in H-NMR spectra to determine couplings, between otherwise chemical shift equivalent nuclei, that are normally inaccessible in typical H-NMR spectra.[172] Information obtained from the coupled-HSQC spectra permitted identification of the four monosaccharide units of **1** as a rhamnose, glucose, and two arabinose units. Glucose was readily identified since H-2, H-3, and H-4 exhibit large, *anti*-vicinal couplings (*ca*. 10-Hz) indicating that all are axial. The anomeric proton appeared as a 7.8-Hz doublet, and is also axial. H-5 (δ 3.50) is inferred to be axial since H-4 is a 9.5-Hz triplet. In addition, the considerably downfield shift of H-3 (δ 5.04) suggested that the unassigned acetyl group was attached at C-3 (δ 76.8) of glucose. The attachment of the acetyl group to C-3 was confirmed by the existence of an HMBC cross peak between H-3 of glucose and the acetyl carbonyl carbon at δ 173.2.

The rhamnose unit was identified by both its equatorial anomeric proton, a broad singlet at δ 5.23, and its 6.2-Hz methyl doublet at δ 1.26. H-4 appeared as a large triplet (*ca*. J = 10 Hz) in both its H-NMR and coupled-HSQC spectra. These conditions indicate that H-3, H-4, and H-5 were axial. H-3 exhibited both a large and small doublet in its coupled-HSQC spectrum, indicating that it is adjacent to an equatorial proton (H-2).

The two remaining sugars were identified as arabinoses. The difficulty in the assignment of the arabinose units is the determination of which close-lying signals belong to each monosaccharide unit. There was sufficient signal dispersion at 700 MHz to accomplish the assignment of the signals to the appropriate arabinose units. The arabinose anomeric protons were observed as *ca*. 6.5-Hz doublets and are assigned as axial. Since the H-2s are *ca*. 8-Hz triplets, this indicates that they must also be axial and have two axial neighbors. The H-3s appeared as a doublet of doublets (*ca*. 8.5 and 3 Hz) and H-4s as broadened singlets. This observation indicates that the H-3s are axial and H-4s equatorial. The H-5As for both sugar units were determined to be equatorial on the basis of the large HMBC cross peaks that they displayed with C-1 and C-3 of both arabinose units.

The final step in the structural elucidation of **1** consisted of determination of the interglycosidic linkage of the monosaccharide units, and to the aglycone moiety. In the case of each interglycosidic link, complimentary pairs of three-bond HMBC connectivities were observed between (i) an anomeric proton and a carbon of a second monosaccharide unit and (ii) the corresponding carbinol proton of the second monosaccharide unit and anomeric carbon of the first unit. These results were further supported by ROESY correlations (Figure 3.2). Likewise, complimentary HMBC cross

peaks between the anomeric proton of arabinose-I (δ 4.49) and C-3 (δ 82.6 downfield shift) of the aglycone and H-3 of the aglycone (δ 3.62) and the anomeric carbon of arabinose-I (δ 105.1) established the point of attachment of the tetrasaccharide unit to the aglycone. The complete assignments based on all spectroscopic evidence outlined above are noted in the Tables 3.1-3.5, and compound **1** was determined to 3-*O*-[α-L-arabinopyranosyl-(1→4)-3-*O*-acetyl-*β*-D-glucopyranosyl-(1→3)-α-L-rhamno-pyranosyl-(1→2)-α-L-arabinopyranosyl] hederagenin and was named blighoside A.

3.4. *Identification and Structural Elucidation of Saponin* 2

Compound **2** was isolated as a white amorphous solid, $[\alpha]_D^{29}$ +10.30º (MeOH, c=0.0187M), was assigned a molecular formula $C_{54}H_{86}O_{21}$, as determined 1D- and 2D-NMR. High resolution mass spectrometry by TOF-ESIMS (Appendix: A.9 (B)) in positive-ion mode showed a molecular ion fragment peak at m/z 1071.5730 $[M + H]^+$ (1071.5740 calcd.) corresponding to the molecular formula $C_{54}H_{86}O_{21}$ and confirms the formula originally assigned by 1D-NMR. The negative APCI-MS of **2** showed a $[M - H]^-$ ion at *m/z* 1069. The ^{1}H-NMR spectral data of **2**, showed four anomeric protons identical to those of **1**. Further observation of the ^{1}H- and ^{13}C-NMR spectrum of the aglycone revealed the presence of an additional methyl signal at δ 1.04 (3H, s) and an upfield shift (+36.0 ppm) in the carbon resonance of C-23 (δ 28.7), corresponding to the loss of a carbinol methylene signal at δ 64.7 (C-23) as compared to **1** (Table 3.1). This result indicated that the C-23 is a methyl carbon, which was confirmed further through HMBC cross peaks at δ 1.04/δ 90.6 (H-23/C-3), C-4 (δ 40.3), and C-5 (δ 57.2). In addition, H-3 (δ 1.04 (-0.49 ppm)) and C-4 (δ 40.3 (-3.8 ppm)) of the aglycone experienced an upfield shift due to the presence of the geminal methyls, C-23 and C-24, while C-3 (δ 90.6)

experienced a downfield shift (+8 ppm) as compared to the C-3 (δ 82.6) of **1**. It was evident from the above spectra that **2** is the oleanolic acid (**4**) derivative of **1**. The above spectra also indicated that the oligosaccharide moiety of **2** is the same as that of **1**. HMBC (Tables 3.1-3.3) and ROESY spectra demonstrated that this is the case, and that **2** was a new saponin given the structure 3-*O*-[α-L-arabinopyranosyl-(1→4)-3-*O*-acetyl-*β*-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl] oleanolic acid and named blighoside B.

3.5. *Identification and Structural Elucidation of Saponin* **3**.

Compound **3** was isolated as a white amorphous solid, $[\alpha]_D^{29}$ + 25.71º (MeOH, c=0.0002M), and was assigned the molecular formula $C_{72}H_{112}O_{33}$, as determined by 1D-NMR. High resolution mass spectrometry by FAB-MS of **3** showed a weak molecular ion peak at *m/z* 1505 $[M + H]^+$.

The ^{1}H-NMR spectrum of the aglycone portion of **3** showed seven 3H-singlets for the seven tertiary methyl groups at δ 1.16 (3H, s), 1.03 (3H, s), 0.95 (3H, s), 0.94 (3H, s), 0.91 (3H, s), 0.85 (3H, s), and 0.81 (3H, s) that were attributed to H-27, H-23, H-25, H-30, H-29, H-24 and H-26, respectively (Table 3.3), and six other methyl groups not associated with the aglycone, with four having an acetate functionality. A characteristic olefinic proton at δ 5.25 (t, J = 3.6 Hz) was also observed for **3**, as with **1** and **2**. Further inspection of the ^{1}H, ^{13}C, and DEPT NMR spectra suggested that the aglycone of **3** was the similar to **2**, and the presence of six anomeric protons and carbons indicated that **3** has six monosaccharide units. The identity of the sugar moiety is described below.

The sugar portion of **3** exhibits 42 carbon signals, eight of which were assigned to four acetyl groups, having four carbonyl carbons at δ 172.7, 172.5 (x 2), and 171.9, and

four methyl groups at δ 20.9 (x 3) and 21.1. Six anomeric carbons at δ 105.8, 105.5, 105.4, 105.3, 101.5 and 101.4 were visible. The carbon resonances for the remaining 28 carbon signals also revealed four oxymethylenes, 22 oxymethines, and two methyls, belonging to two of the sugar units (Table 3.1). The broadened singlets observed for the anomeric protons at δ 5.18 and 5.19 and the *ca*. 5-8 Hz doublets seen for those at *ca*. 4.5-4.6 ppm indicated that these anomeric protons are equatorial and axial, respectively, and that **3** possesses two α- and four β-linkages. Inspection of the cross-sections of 100-ms mixing time TOCSY spectra, through both the anomeric protons and the CH_3-protons of the 6.2-Hz (non-aglycone) doublets at δ 1.23, identified those ^{1}H NMR signals associated with each anomeric proton and the methyl groups. Analysis of the COSY spectra permitted the sequencing of protons in each monosaccharide unit. The COSY and TOCSY spectra, together with one-bond ^{13}C-^{1}H couplings [*ca*. 172-Hz (2) and *ca*. 161-Hz (4)] (Table 3.5), indicted that **3** has two α-linked and two β-linked hexasaccharides and two β-linked pentasaccharides.

As with **1**, high-resolution coupled-HSQC spectra permitted the orientation of the glycosidic ring protons to be determined.[170] Like **1,** two glucose and two rhamnose units were readily identified. In addition, the considerably deshielded chemical shifts of protons 3 (δ 4.95), 6A (δ 4.41), and 6B (δ 4.22) in one glucose and protons 4 (δ 4.81), 6A (δ 4.21), and 6B (δ 4.09) in the other indicated that the four unassigned acetyl groups be placed at the 3,6- and 4,6-positions of the two glucose units.

The two remaining sugars were identified as xyloses. Their anomeric protons are observed as *ca*. 4.5-Hz doublets and are thus axial. Since the 2-protons are *ca*. 6-Hz doublets of doublets, they must also be axial and have two axial neighbors. The 3-protons

appear as 7-Hz triplets and must likewise have flanking axial neighboring protons. The 4-protons are seen as a triplet of doublets (7 and 2 Hz) and confirm that each has two axial neighbors. The 5B protons were determined to be axial on the basis of the large ROESY cross peaks that they displayed with protons 1 and 3 of both xylose units.

The final step in the structural elucidation of blighoside C consisted of determining the linkage sites both between the monosaccharide units and to the aglycone moiety. As was the case with **1**, complimentary pairs of 3-bond HMBC connectivities were observed between (i) an anomeric proton and a carbon of a second monosaccharide unit and (ii) the corresponding carbinol proton of the second monosaccharide unit and anomeric carbon of the first unit for each interglycosidic link. Likewise, complimentary HMBC cross peaks between the anomeric proton of xylose-I (δ 4.48) and C-3 of the aglycone (δ 90.62) and H-3 of the aglycone (δ 3.12) and the anomeric carbon of xylose-I (δ 105.34) established the point of attachment of the hexasaccharide unit to the aglycone. These and other HMBC connectivities are listed in Table 3.4. In addition, complimentary pairs of ROESY correlations were found between anomeric and carbinol protons on opposite sides of the interglycosidic linkages and H-3 of the aglycone. Based on complete spectral observations the structure of compound **3** was determined as a new saponin, 3-*O*-[4,6-*O*-diacetyl-*β*-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→4)-3,6-*O*-diacetyl -*β*-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl(1→2)-*β*-D-xylopyranosyl-(1→3)-*β*-D-xylopyranosyl] oleanolic acid, and was named blighoside C.

In addition to the three saponins two known steroids, stigmasta-5,22-dien-3-ol and stigmasta-5,22-dien-3-*O*-*β*-glucopyranoside,were also identified in the EtOAc fraction and verified through comparison of literature data based on spectroscopic evidences.

Saponins are known to have many biological properties (Chapter 1, Section 1.6.4). The EtOAc and *n*-BuOH fractions, saponins **1-3**, **5** (hederagenin, Table 3.1), and actein were evaluated for their ability to inhibit the proliferation of ER^- MDA-MB-453 (Her2 overexpressing) human breast cancer cells, and to determine any possible structure-activity relationships among the isolated saponins. The EtOAc and *n*-BuOH partitioned fractions demonstrated cytotoxic effects on the MDA-MB-453 cell line at IC_{50} = 43 μg/ml and 20 μg/ml, respectively. The cytotoxicity of the *n*-BuOH fraction was twice that of the EtOAc even though it had no significant antioxidant activity and polyphenol content (not shown), as compared with the EtOAc extracts (Table 2.3). This result may indicate that the cytotoxic effects of the *n*-BuOH fraction may be due to compounds other the polyphenol constituents.

In the same cytotoxicity assay, the isolated triterpenoid saponins (**1-3**) obtained from the EtOAc fraction, and hederagenin (**5**) exhibited cytotoxic effects at IC_{50} = 10.3 μM, 6.9 μM, 10.0 μM, and 69 μM (32 μg/ml), respectively, as compared to actein (14.8 μM; 10 μg/ml). Actein, a cycloartane-type triterpene arabinopyranoside, is found in black cohosh (*Actaea racemosa*) extracts and differs structurally from those of the isolated saponins (**1-3**).

Compounds **1-3** have oleanane aglycones; **1** possesses the hederagenin sapogenin, while saponins **2** and **3** have the oleanolic acid sapogenin. According to our results the oleanane-type saponins have similar cytotoxicity on the breast cancer cells as actein. Among the oleanane-type saponins, the oleanolic acid saponins (**2**) and (**3**) were more cytotoxic than the hederagenin saponin (**1**). A comparison between saponins **1** and **2** revealed that **1** was more cytotoxic against MDA-MB-453 cells despite having identical

oligosaccharide units. Additionally, saponin (**2**), having a tetrasaccharide unit, was more cytotoxic than **3**, which had a hexasaccharide sugar moiety.

Further observation indicates that the number and type of sugar units, and their attachment seems to influence their cytotoxicity. The tetrasaccharide saponin (**2**) is more cytotoxic than the hexasaccharide saponin (**3**), and that saponins with more than one sugar unit are more cytotoxic, as compared to actein which contains one sugar unit. According to studies involving saponins their biological activities depends on the type of aglycone, number of sugars, and the pattern and attachment of sugar units to the aglycone (Section 1.6).[108] In addition, to their cytotoxic activity against MDA-MB-453 human breast cancer cells, structurally speaking, saponins **1-3** may also exhibit other cytotoxic effects and antitumor activity against other cell lines due to their unique structural signature comprising a free carboxylic acid group at C-28 of the oleanane-type aglycone, and a sugar sequence α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl at the C-3 position of the aglycone; with the oleanolic acid more potent than the hederagenin sapogenin. [109,173]

4. Conclusion

The three new triterpene glycosides (**1-3**), along with two known steroid compounds were isolated from the EtOAc fraction of ackee pod. These three saponins (**1-3**) when tested with the EtOAc and the n-butanol fraction exhibit inhibitory effects on the proliferation of ER^- MDA-MB-453 (Her2 overexpressing) human breast cancer cells. Structurally, it appears that the oleanane-type saponins, and with more than one monosaccharide units are more cytotoxic than the cycloartane-type saponin, as exemplified by actein.

Table 3.1. ^{13}C NMR (CD_3OD, 125/175 MHz) spectral data of saponins **1-3**, and triterpenes **4-5**

	1	**2**	**3**	**4**[a]	**5**[a]			**1**	**2**	**3**			**3**	
C	δ_C	δ_C	δ_C	δ_C	δ_C	(DEPT)	**Sugar**	δ_C	δ_C	δ_C	(DEPT)	**Sugar**	δ_C	
1	39.8	40.0	39.9	39.4	39.2	(CH_2)	**Ara (Xyl-I)**					**Glc-I**		
2	26.8	27.1	27.1	28.5	28.1	(CH_2)	1	105.1	105.6	105.3	(CH)	1	105.5	(CH)
3	82.6	90.6	90.6	78.5	73.9	(CH)	2	76.9	76.9	76.4	(CH)	2	73.5	(CH)
4	44.1	40.3	40.3	40.2	43.3	(C)	3	74.2	73.5	73.6	(CH)	3	78.4	(CH)
5	48.3	57.2	57.1	56.2	48.6	(CH)	4	70.1	69.2	69.0	(CH)	4	69.9	(CH)
6	19.0	19.4	19.4	19.2	19.0	(CH_2)	5	67.8	67.7	64.8	(CH_2)	5	75.2	(CH)
7	33.5	34.1	34.0	33.7	33.4	(CH_2)						6	64.4	(CH_2)
8	40.7	40.6	40.6	39.8	40.2	(C)	**Rha (Xyl-II)**					**3**-CO**C**H_3	21.1	(CH_3)
9	49.1	49.1	49.1	48.5	49.1	(CH)	1	101.8	101.8	105.4	(CH)	**3**-**C**OCH_3	172.5	(C)
10	37.8	38.0	38.0	37.8	37.7	(C)	2	71.3	71.3	76.4	(C)	**6**-CO**C**H_3	20.9	(CH_3)
11	24.7	24.6	24.6	24.1	24.3	(CH_2)	3	83.0	82.9	73.7	(CH_2)	**6**-**C**OCH_3	172.7	(C)
12	123.8	123.5	123.7	123.0	123.0	(CH)	4	72.7	72.7	69.1	(CH)			
13	145.4	145.4	145.2	145.2	145.3	(C)	5	70.2	70.1	64.7	(CH/CH_2)	**Rha-II**		
14	43.1	43.0	42.9	42.6	43.3	(C)	6	18.3	18.1		(CH_3)	1	101.5	(C)
15	29.0	28.9	28.9	28.7	28.7	(CH_2)						2	71.5	(CH_2)
16	24.2	24.2	24.1	24.2	24.1	(CH_2)	**Glc (Rha-I)**					3	82.9	(CH)
17	47.8	47.8	47.7	47.1	47.1	(C)	1	105.7	105.6	101.5	(CH)	4	72.6	(CH)
18	42.9	42.9	42.8	42.4	42.4	(CH)	2	73.7	73.6	71.5	(CH)	5	70.0	(CH)
19	47.4	47.4	47.3	46.9	46.9	(CH_2)	3	76.8	76.7	82.5	(CH)	6	18.1	(CH_3)
20	31.8	31.7	31.7	31.4	31.4	(CH_2)	4	77.7	77.5	72.6	(CH)			
21	35.1	35.0	34.9	34.6	34.6	(CH_2)	5	76.8	76.7	70.0	(CH)	**Glc-II**		
22	34.0	33.9	33.9	33.6	33.6	(CH_2)	6	61.4	61.3	18.1	(CH_2/CH_3)	1	105.8	(CH)
23	64.7	28.7	28.7	29.2	64.4	(CH_3/CH_3)	**3**-CO**C**H_3	21.7	21.5		(CH_3)	2	75.3	(CH)
24	13.9	17.2	17.2	16.9	13.5	(CH_3)	**3**-**C**OCH_3	173.2	173.1		(C)	3	75.3	(CH)
25	16.6	16.0	16.0	15.9	16.4	(CH_3)						4	72.2	(CH)
26	17.9	17.8	17.8	17.8	17.9	(CH_3)	**Ara-II**					5	73.2	(CH)
27	26.6	26.4	26.4	26.7	26.6	(CH_3)	1	106.1	105.9			6	63.8	(CH_2)
28	182.0	182.3	181.9	180.6	180.6	(C)	2	72.9	72.8			**4**-CO**C**H_3	20.9	(CH_3)
29	33.6	33.6	33.6	33.6	33.6	(CH_3)	3	74.5	74.5			**4**-**C**OCH_3	171.9	(C)
30	24.1	24.1	24.0	24.1	24.1	(CH_3)	4	69.9	70.0			**6**-CO**C**H_3	20.9	(CH_3)
							5	65.9	64.8			**6**-**C**OCH_3	172.5	(C)

ax = axial; eq = equatorial; capital letter (eg. D, T) indicates large coupling and small letter indicates small coupling

[a] spectral data performed in pyridine-d^5 (75 MHz)

Table 3.2. ^{1}H-NMR spectral data and HMBC correlation for saponins **1-2** (CD_3OD, 700 MHz)

	1		**2**	
H	δ_H (int., multi., *J* (Hz))	HMBC Connectivity	δ_H (int., multi., *J* (Hz))	HMBC Connectivity
1A	1.61 (Dt,13.2,4) eq	3,5,25	1.62 (Dt,13.2,4) eq	3,5,25
1B	0.97 (DDd,13.9,13.2,4) ax		0.99 (DDd,13.9,13.2,3.2) ax	
2A	1.87 (Dddd,13.9,4.7,4,3) eq	1AB, 3	1.84 (Ddd,13.9,4.5,4,3) eq	1AB, 3
2B	1.75 (TDd,13.9,12,4) ax		1.72 (TDd,13.9,12,4) ax	
3	3.62 (Dd,12,4.7) ax	1AB,2AB,5,23,24,Ara-I-1	3.13 (Dd,12,4.5) ax	1AB,2AB,5,23,24,Ara-I-1
4		2AB,3,23AB,24		2AB,3,23AB,24
5	1.27 ax	1AB,3,7AB,9,23AB,24,25	0.79 (Dd,11.7,1) ax	1AB,3,7AB,9,23AB,24,25
6A	1.51 (Dt,13.2,4) eq	5,7AB	1.56 (Dt,13.2,4) eq	5,7AB
6B	1.38 (Td,13.2,3.4) ax		1.42 (TDd, 13.2,11.7,3.4) ax	
7A	1.63 (Td,13.2,4) ax	5,9,26	1.51 (Td,13.2,4) ax	5,9,26
7B	1.27 (Dt,13.2,4) eq		1.32 (Dt,13,4) eq	
8		6AB,11AB,26,27		6AB,11AB,26,27
9	1.65 ax	5,7AB,12,25,26	1.65 ax	5,7AB,12,25,26
10		2AB,6AB,25		2AB,6AB,25
11A	1.91 (14)	9,12	1.90 (14)	9,12
11B	1.89 (14)		1.88 (14)	
12	5.25 (t, 3.8)	11AB, 18	5.24 (t, 3.6)	11AB, 18
13		11AB,15B,18,19AB,27		11AB,15B,18,19AB,27
14		12,16AB,18,26,27		12,16AB,18,26,27
15A	1.78 (Td,13.8,4.3) ax	16AB, 27	1.79 (Td,13.8,4) ax	16AB,27
15B	1.08 (Dt,13.8,4) eq		1.07 (Dt,13.8,4) eq	

Table 3.2. (*Continued*)

	1			2	
H	δ_H (int., multi., ***J*** (Hz))	HMBC Connectivity		δ_H (int., multi., ***J*** (Hz))	HMBC Connectivity
16A	2.02 (Td,13.6,4) ax	18, 22A	16A	2.00 (Td,13.6,4) ax	18, 22A
16B	1.60 (Ddd,13.5,4.3,4) eq		16B	1.60 (Ddd,13.5,4.3,4) eq	
17		15AB,19B,21AB	17		15AB,19B,21AB
18	2.85 (Dd,13.8,4.5) ax	12, 16AB, 22AB	18	2.86 (Dd,13.8,4.5) ax	12, 16AB, 22AB
19A	1.70 (t,13.8) ax	21AB, 29, 30	19A	1.70 (t,13.8) ax	21AB, 29, 30
19B	1.13 (Ddd,13.8,4.5,2) eq		19B	1.13 (Ddd,13.8,4.5,2) eq	
20		18, 22B, 29, 30	20		18, 22B, 29, 30
21A	1.39 (Td,13.6,3.4) ax	19AB, 29, 30	21A	1.39 (Td,13.6,3.4) ax	19AB, 29, 30
21B	1.21(Dtd,13.6,3.4,3.4,2) eq		21B	1.21(Dtd,13.6,3.4,3.4,2) eq	
22A	1.75 (Td,13.6,3.6) ax	16AB	22A	1.74 (Td,13.6,3.6)ax	16AB
22B	1.54 (Dt,13.6,3) eq		22B	1.54 (Dt,13.6,4) eq	
23/23A	3.56 (11.2)	3 ,5, 24	23/23A	1.04 eq	3 ,5, 24
23/23B	3.34 (11.2)		23/23B		
24	0.71 ax	3, 5, 23AB	24	0.86 ax	3, 5, 23
25	0.98 ax	1B, 5, 9	25	0.98 ax	1B, 5, 9
26	0.82 ax	7A, 9	26	0.82 ax	7A, 9
27	1.18 ax	15A	27	1.18 ax	15A
28		19, 21A, 30	28		16A, 18, 22AB
29	0.91 eq	19, 21A, 30	29	0.91 eq	19, 21A, 30
30	0.94 ax	29	30	0.94 ax	29

ax = axial; eq = equatorial; capital letter (eg. D, T) indicates large coupling and small letter indicates small coupling

Table 3.3. ^{1}H-NMR spectral data and HMBC correlations for saponin **3** (CD_3OD, 500 MHz)

	3			**3**	
H	δ_H (int., multi.,***J*** (Hz))	HMBC Connectivity		δ_H (int., multi.,***J*** (Hz))	HMBC Connectivity
1A	1.63 (Ddd,13.8,4.2,3) eq	3,5,25	16A	2.01 (Td,13.6,3.7) ax	18,22A
1B	1.01 (Td,13.8,3) ax		16B	1.59 (Ddd,13.6,3.7) eq	
2A	1.84 (Dtd,13.8,3,3,2.7) eq	1AB, 3	17		15AB,19B,21AB
2B	1.72 (TDd,13.8,12,4) ax		18	2.85 (Dd,13.6,4) ax	12,16AB,22AB
3	3.12 (Dd,11.2,2.7) ax	1AB,2AB,5,23,24,Xyl-I-1	19A	1.69 (t,13.6) ax	21AB,29,30
4		2AB,3,23AB,24	19B	1.11 (Ddd,13.6,4,1.8) eq	
5	0.82 (Dd,10.4,2) ax	1A,3,7AB,9,23AB,24,25	20		18,22B,29,30
6A	1.56 (TDd,13.7,10.4,2.5) eq	5,7AB	21A	1.39 (Td,13.7,4) ax	19AB,29, 30
6B	1.39 (TDd,13.7,10.4,4) ax		21B	1.19 (Ddd,13.7,4,1.8) eq	
7A	1.50 (Td,13.7,2.5) ax	5,9,26	22A	1.74 (Td,13.7,4)ax	16AB
7B	1.31 (Ddd,13.7,4,2.5) eq		22B	1.53 (Dt,13.7,4) eq	
8		6AB,11AB,26,27	23	1.03 (d,3) eg	3,5,24
9	1.59 ax	5,7AB,12,25,26	24	0.85 (d,3) ax	3,5,23
10		2AB,6AB,25	25	0.98 ax	1B,5,9
11A	1.89 (14)	9,12	26	0.81 ax	7A,9
11B	1.89 (14)		27	1.16 ax	15A
12	5.24 (t, 3.5)	11AB, 18	28		16AB,18,22AB
13		11AB,15B,18,19AB,27	29	0.91 eq	19,21A,30
14		12,16AB,18,26,27	30	0.94 ax	29
15A	1.77 (Td,13.6,3.7) ax	16AB, 27			
15B	1.09 (Dt,13.6,3.7) eq				

ax = axial; eq = equatorial; capital letter (eg. D, T) indicates large coupling and small letter indicates small coupling

Table 3.4. ^{1}H-NMR spectral data and HMBC correlations for the sugar moiety of saponins **1-3** (CD_3OD, 500 and 700 MHz)

	1			**2**	
H	δ_H (int., multi.,*J* (Hz))	HMBC Connectivity		δ_H (int., multi., ***J*** (Hz))	HMBC Connectivity
Ara-I	(1→ 3)Aglycone		**Ara-I**	(1→ 3)Aglycone	
1	4.49 (d, 6.2) ax	3, Ara-I-5A	1	4.48 (d, 5.4) ax	3, Ara-I-5A
2	3.65 ax	Rha-1, Ara-I-4	2	3.70 ax	Rha-1, Ara-4
3	3.66 ax	Ara-I-5A	3	3.69 ax	Ara-I-5A
4	3.76 eq	Ara-I-3, Ara-I-5AB	4	3.77 eq	Ara-I-3, Ara-I-5AB
5A	3.83 (Dd,12.6, 2.3) eq	Ara-I-4	5A	3.83 (dd,12.6, 2.3) eq	Ara-I-4
5B	3.53 (dd,12.6, 2) ax		5B	3.52 (dd,12.6, 1.0) ax	
Rha	(1→ 2)Ara-I		**Rha**	(1→ 2)Ara-I	
1	5.23 (brs,) eq	Ara-I-2, Rha-2	1	5.15 (brs,) eq	Ara-I-2, Rha-2
2	4.23 (dd,3,2) eq	Rha-1, Rha-3	2	4.19 (dd, 3.0,2.0) eq	Rha-1, Rha-3
3	3.91 ax	Glc-1, Rha-1, Rha-2	3	3.86 ax	Glc-1, Rha-1, Rha-2
4	3.56 (t,9.6) ax	Rha-2, Rha-5, Rha-6	4	3.56 (t, 9.6) ax	Rha-2, Rha-5, Rha-6
5	3.93 ax	Rha-1, Rha-6	5	3.88 ax	Rha-1, Rha-6
6	1.26 (d,6.2) eq	Rha-5	6	1.24 (d, 6.2) eq	Rha-5
Glc	(1→ 3)Rha		**Glc**	(1→ 3)Rha	
1	4.60 (d,7.8) ax	Rha-3, Glc-2	1	4.61 (d,7.8) ax	Rha-3, Glc-2
2	3.48 ax	Glc-1, Glc-3	2	3.46 ax	Glc-1, Glc-3
3	5.04 (t, 9.5) ax	Glc-2, Glc-4	3	5.03 (t, 9.5) ax	Glc-2, Glc-4
4	3.74 (t, 9.5) ax	Ara-II-1, Glc-5	4	3.74 (t, 9.5) ax	Ara-II-1, Glc-5
5	3.50 (m) ax	Glc-4, Glc-6AB	5	3.47 (m) eq	Glc-4, Glc-6AB
6A	3.93 (9.2) ax	Glc-4, Glc-5	6A	3.93 (dd,12.2, 3.5) ax	Glc-4, Glc-5
6B	3.90 (9.2) eq		6B	3.86 (dd,12.2, 3.5) eq	
3-CO**C**H_3	2.10		**3**-CO**C**H_3	2.09	
3-**C**OCH_3		Glc-3, CH_3 (δ = 2.10)	**3**-**C**OCH_3		Glc-3, CH_3 (δ = 2.09)

Table 3.4. (*Continued*)

	1			**2**	
H	δ_H (int., multi.,*J* (Hz))	HMBC cross peaks	**H**	δ_H (int., multi., *J* (Hz))	HMBC cross peaks
Ara-II	(1→ 4)Glc		**Ara-II**	(1→ 4)Glc	
1	4.20 (d, 6.8) ax	Glc-4,Ara-II-5A	1	4.20 (d, 6.8) ax	Glc-4,Ara-II-5A
2	3.46 ax	Ara-II-1, Ara-II -4	2	3.46 ax	Ara-II-1, Ara-II -4
3	3.47 ax	Ara-II-5A	3	3.47 ax	Ara-II-5A
4	3.76 eq	Ara-II-3, Ara-II-5AB	4	3.75 eq	Ara-II-3, Ara-II-5AB
5A	3.84 (Dd,12.6,3.8) eq	Ara-II-4	5A	3.84 (Dd,12.3,4.4) eq	Ara-II-4
5B	3.52 (Dd,12.4,2) ax		5B	3.50 (Dd,12.3,2.5) ax	
	3			**3**	
Xyl-I	(1→ 3)Aglycone		1	5.19 (d, 1.4) eq	Xyl-II-2, Rha-I-2
1	4.48 (d,5.3) ax	3,Xyl-I-2,Xyl-I -5A	2	4.13 (d,3,1.4) ax	Rha-I-1, Rha-I-3
2	3.75 (dd,7,5.3) ax	Xyl-I-1, Xyl-I-3	3	3.85 (dd,9.5,3) ax	Glc-I-1, Rha-I-1,2,4
3	3.69 (t,7) ax	Xyl-II-1,Xyl-I-2,Xyl-I-4,Xyl-I-5A	4	3.55 (t,9.5) ax	Rha-I-2,5,6
4	3.76 (dt,7,2) ax	Xyl-I-3, Xyl-I-5AB	5	3.90 (d,9.5,6.2) eq	Rha-I-1, Rha-I-6
5A	3.84 (Dd,9.7,2) eq	Xyl-I-4	6	1.23 (d,6.2) eq	Rha-I-5
5B	3.48 (Dd,9.7,7) ax		**Glc-I**	(1→ 3)Rha-I	
Xyl-II	(1→ 3)Xyl-I		1	4.59 (d,7.8) ax	Rha-I-3, Glc-I-2
1	4.47 (d,5.3) ax	Xyl-I-3, Xyl-II-2, Xyl-II-5A	2	3.44 (dd,9.4,7.8) ax	Glc-I-1, Glc-I-3
2	3.76 (dd,7,5.3) ax	Rha-I-1,Xyl-II-1,Xyl-II-3	3	4.95 (t, 9.4) ax	Glc-I-2, Glc-I-4
3	3.70 (t,7) ax	Xyl-II-2,Xyl-II-4,Xyl-II-5A	4	3.45 (dd,9.8,9.4) ax	Glc-I-3, Glc-I-5, Rha-II-1
4	3.77 (dt,7,2) ax	Xyl-II-3, Xyl-II-5AB	5	3.59 (Ddd,9.8,9.4,1) eq	Glc-I-4, Glc-I-6AB
5A	3.84 (Dd,9.7,2) eq	Xyl-II-4	6A	4.41 (Dd,12.2,2.2,1) ax	Glc-I-5
5B	3.48 (Dd,9.7,7) ax		6B	4.22 (Dd,12.2,1) eq	
Rha-I	(1→ 2)Xyl-II				

Table 3.4. (*Continued*)

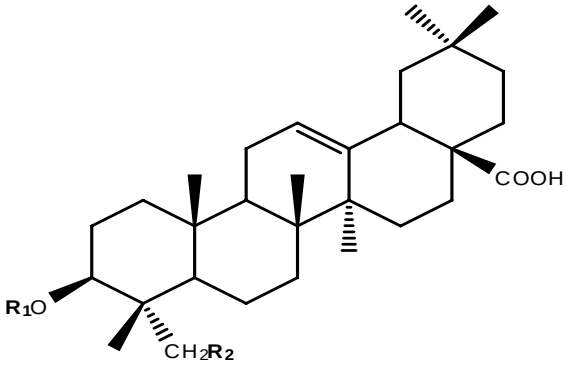
	3			**3**	
H	δ_H (int., multi.,*J* (Hz))	HMBC Connectivity	**H**	δ_H (int., multi., *J* (Hz))	HMBC Connectivity
3-CO**C**H_3	2.11		1	4.56 (d,7.8) ax	Rha-II-3, Glc-II-2
3-COCH_3		Glc-I-3, CH_3(2.11)	2	3.39 (dd,9,7.8) ax	Glc-II-1, Glc-II-3
6-CO**C**H_3	2.08		3	3.58 (dd, 9.7,9) ax	Glc-II-2, Glc-II-4
6-COCH_3		Glc-I-6, CH_3(2.08)	4	4.81 (dd,9.8,9.4) ax	Glc-II-3, Glc-II-5
Rha-II	(1→ 2)Glc-I		5	3.67 (Ddd,9.7,2.6,2) eq	Glc-II-4, Glc-II-6AB
1	5.18 (d, 1.4) eq	Glc-I-4, Rha-II-2	6A	4.21 (Dd,12.2,2.1.7) ax	Glc-II-5
2	4.13 (dd,3,1.4) ax	Rha-II-1, Rha-II-3	6B	4.09 (Dd,12.2,6) eq	
3	3.84 (dd,9.5,3) ax	Glc-II-1, Rha-II-1,2,4	**4**-CO**C**H_3	2.08	
4	3.57 (t,9.5) ax	Rha-II-2,5,6	**4-C**OCH_3		Glc-II-4, CH_3(2.08)
5	3.89 (dq,9.5,6.2) eq	Rha-II-1, Rha-II-6	**6**-CO**C**H_3	2.06	
6	1.23 (d,6.2) eq	Rha-II-5	**6-C**OCH_3		Glc-II-6, CH_3(2.06)
Glc-II	(1→ 3)Rha-I				

Ara = arabinopyranosyl; Glc = glucopyranosyl; Rha = rhamnopyranosyl; Xyl = Xylopyranosyl

Table 3.5. Carbon-proton coupling constants for anomeric protons of saponins **1-3** (CD_3OD; 500 and 700 MHz)

Saponin	Sugar I	Sugar II	Sugar III	Sugar IV	Sugar V	Sugar VI
	Ara-I	**Rha**	**Glc**	**Ara-II**		
1	168 (Hz)	178 (Hz)	162 (Hz)	161 (Hz)		
3	**Xyl-I**	**Xyl-II**	**Rha-I**	**Glc-I**	**Rha-II**	**Glc-II**
	162 (Hz)	162 (Hz)	171 (Hz)	161 (Hz)	170 (Hz)	160 (Hz)

Ara = arabinopyranosyl; Glc = glucopyranosyl; Rha = rhamnopyranosyl; Xyl = Xylopyranosyl



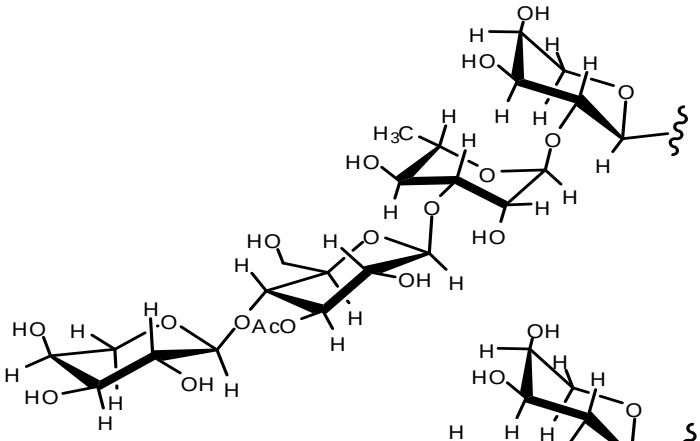
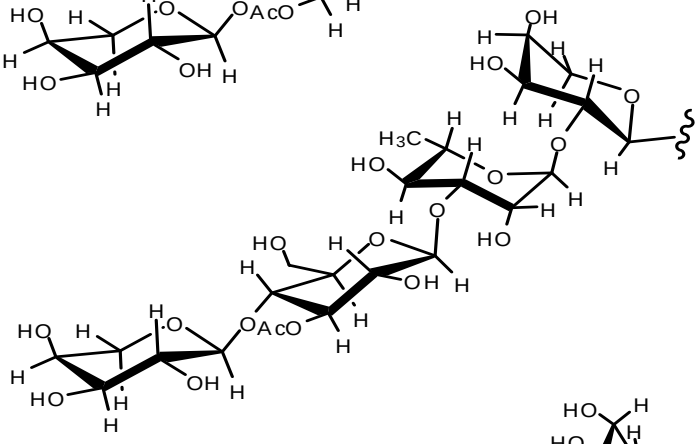
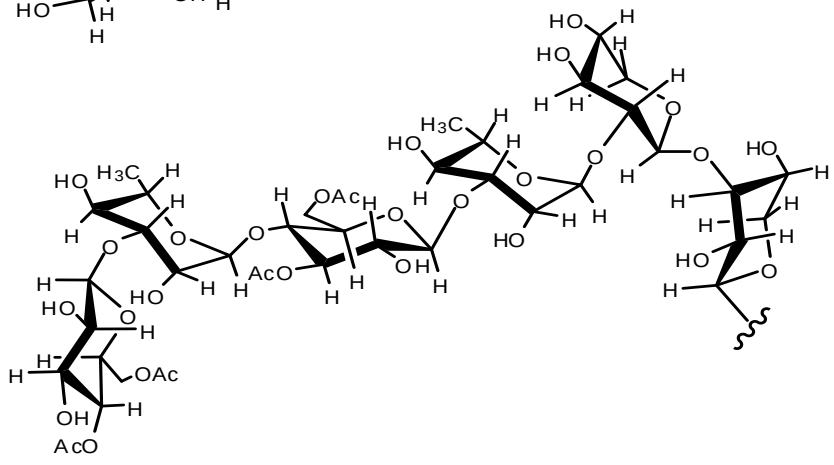
	R_1	R_2
1		**OH**
2		**H**
3		**H**

Figure 3.1. Chemical structures of blighosides A-C (**1-3**)

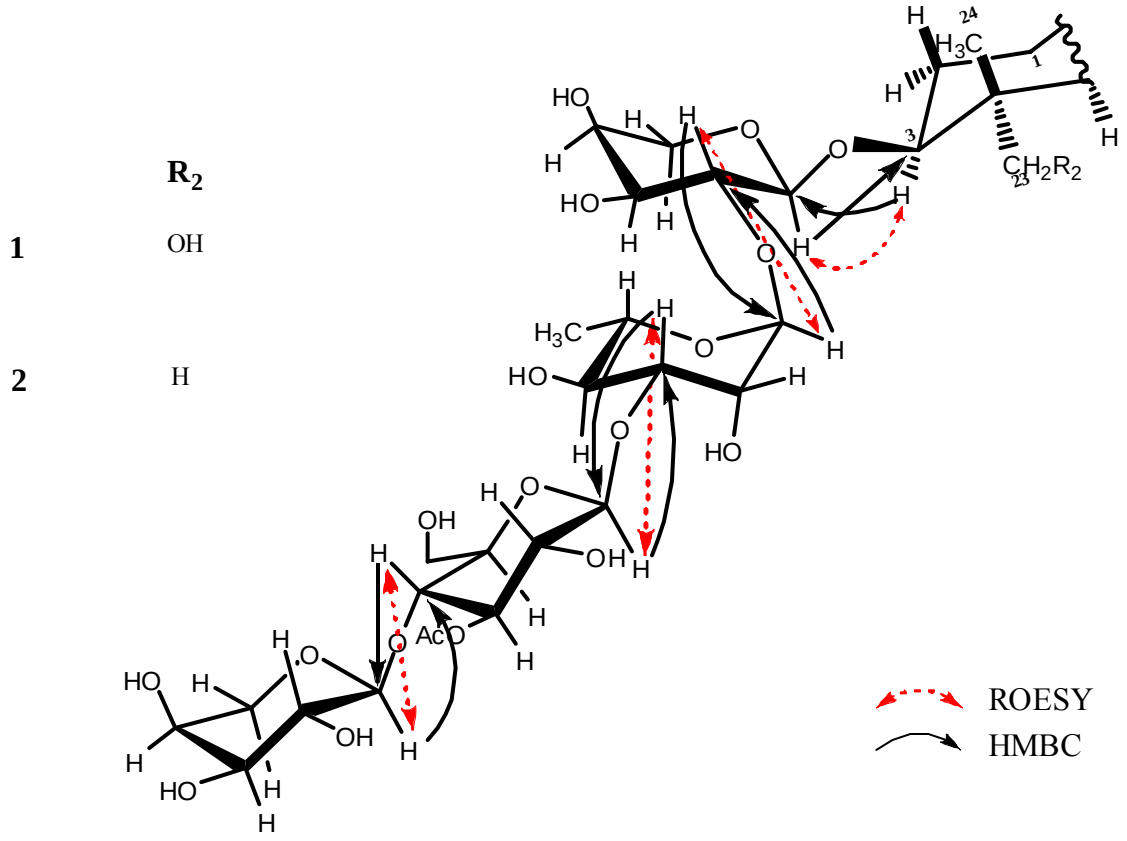


Figure 3.2. HMBC and ROESY correlations between sugar units of blighosides A and B (**1**and **2)**

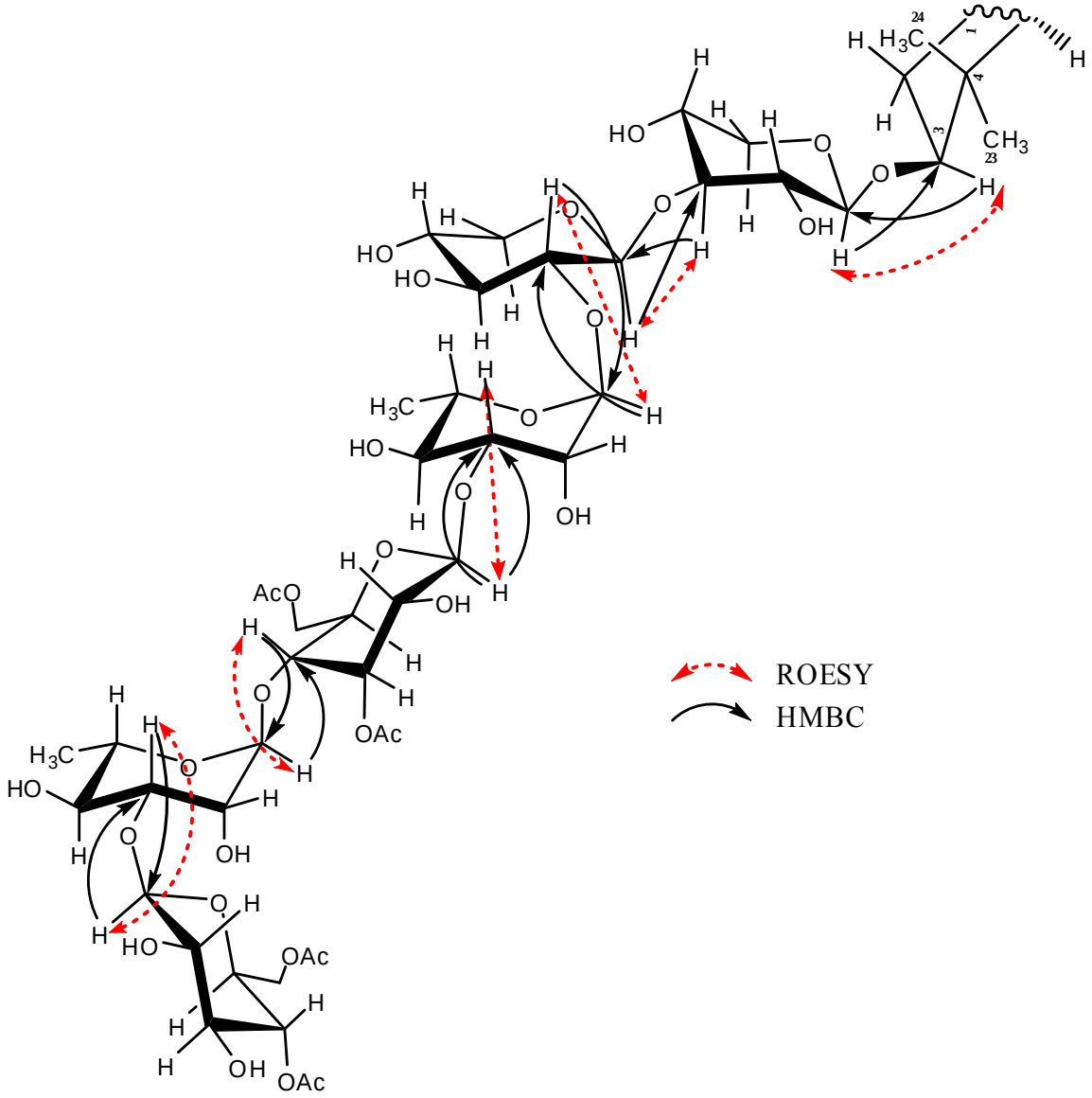


Figure 3.3. HMBC and ROESY correlations between sugar units of blighoside C (**3**)

CHAPTER 4

Conclusions

There has been a growing demand in recent years for more drugs to treat cancers and CVD. People are turning to natural food products, herbals, and dietary supplements to provide means for better health and longer life. Natural food sources such as fruits and vegetables are rich in bioactive compounds, and their consumption as part of a balanced diet is inversely CHD and certain types of cancer.[3,5,174] A number of bioactive plant compounds play an important role in drug development, especially in the health and pharmaceutical industries, where they are used to treat cancer and other diseases, and some are used as precursors in the manufacturing of synthetic drugs, thus providing significant contributions to the health, nutrition, and scientific communities. A well-known demonstration of the importance of dietary polyphenolic compounds was an epidemiological study conducted by Hertog *et al*. (1993,1995). The results of this study suggested that diets that are rich in plant-based products, and in fruits and vegetables correlate with a reduced risk in chronic diseases and certain types of cancer.[18,19]

This landmark study quickly resulted in an influx of new ideas, innovation and a movement to discover new and exciting compounds from edible plant sources and/or their byproducts. Over the last decade there have been significant contributions by our research team in the isolation of bioactive compounds from tropical fruits which have demonstrated antioxidant activity, and cytotoxic activity against various human cancer cell lines.[63-70]

This research project is based on our continuing efforts to isolate chemoprotective agents from edible tropical fruits, and those that are used in folklore medicine. In the preliminary investigation on the byproducts of the ackee fruit, the EtOAc partitioned fraction of the pod and seed was found to contain some important class of compounds

such as polyphenols and triterpenes, which prompted further studies into its chemical constituents. Subsequently, the testing of the EtOAc fractions of the pod and seed of ackee for their antioxidant properties revealed that they have strong DPPH radical-scavenging and FRAP reducing abilities. Additionally, the EtOAc fractions also contain high total amounts of phenolic and flavonoid compounds when tested in the TPC and TFC assay, respectively. From these preliminary studies the ackee pod, which constitute about 83% of the whole fruit, was chosen for further investigation because of its relative proportion (w/w) compared to aril and the seed, its high levels of saponins, and its comparable antioxidant activity and polyphenols content to the seed (Table 2.3).

Through activity-guided fractionation of the EtOAc partitioned fraction of ackee pod, followed by purification of selected subfractions (Table 2.3), resulted in the isolation and identification of three new saponins, blighosides A-C, and also two known steroids, namely, stigmasta-5,22-dien-3-ol and stigmasta-5,22-dien-3-*O*-glucopyranoside were identified through spectroscopic evidences, and verified by comparison with available published data.

Since saponins are known to have a variety of biological activities, and are of pharmacological importance, the three new saponins (blighoside A-C) were tested for their ability to inhibit the proliferation of ER^- MDA-MB-453 (Her2 overexpressing) human breast cancer cells. All three compounds showed cytotoxic activities on the MDA-MB-453 cell line that comparable to actein, a cycloartane glycoside that is found in black cohosh.[175]

Additionally, further activity-guided fractionation of selected subfractions (Table 2.3) resulted in the isolation and identification of seven bioactive polyphenolic

compounds, namely, methyl gallate (**1**), isoquercitrin (**2**; quercetin 3-*O*-*β*-D-glucopyranoside), cyanidin-3-*O*-β-glucopyranoside (**3**), 3,4-dihydroxybenzoic acid (**4**), gallic acid (**5**), ellagic acid (**6**), and quercetin (**7**). The relative amounts of six of the seven polyphenolic compounds were methyl gallate (16.3 mg kg^{-1} fresh weight) >> gallic acid (3.64 mg kg^{-1}) > quercetin 3-*O*-*β*-D-glucopyranoside (1.86 mg kg^{-1}) > ellagic acid (1.62 mg kg^{-1}) > protocatechuic acid (1.06 mg kg^{-1}) > cyanidin 3-*O*-glucopyranoside (0.80 mg kg^{-1}). Trace amounts of quercetin (**7**), a common flavonoid found in most fruits, were also identified in the EtOAc partitioned fraction, and verified by spectroscopic methods and comparison of literature data. These results are the first recorded work on the isolation of polyphenols from the ackee fruits despite its numerous ethnobotanical uses.

Based on this phytochemical investigation of *B. sapida*, we concluded that the ackee fruit byproducts, specifically the pod and the seed, could be a potential source for antioxidant and anticancer agents belonging to the polyphenol and saponin groups of bioactive compounds. Further investigation is needed on both the pod and seed to find and isolate additional chemoprotective agents.

APPENDIX A

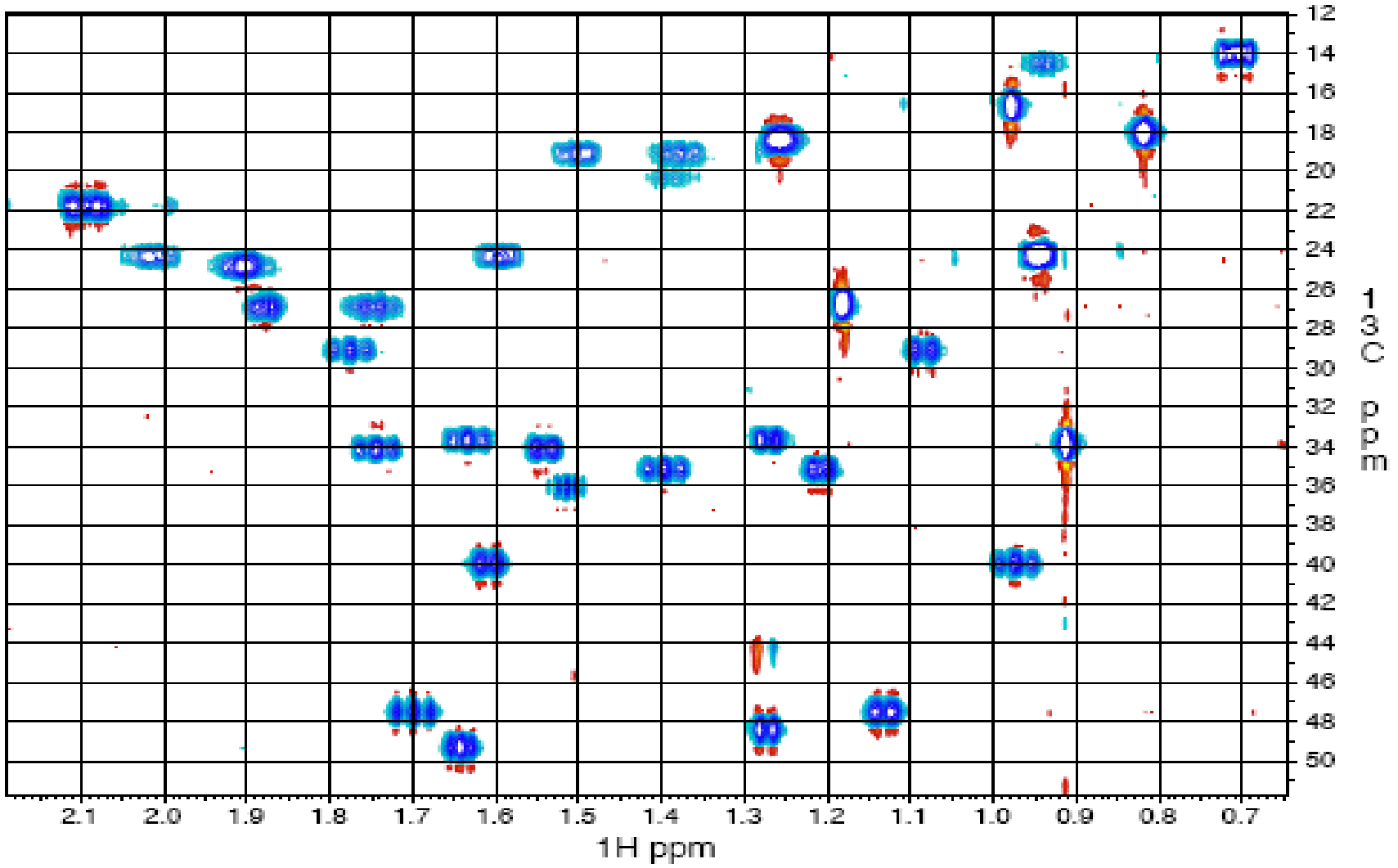


Figure A.1. HSQC spectrum of the triterpene portion of blighoside A recorded at 700 MHz in CD_3OD

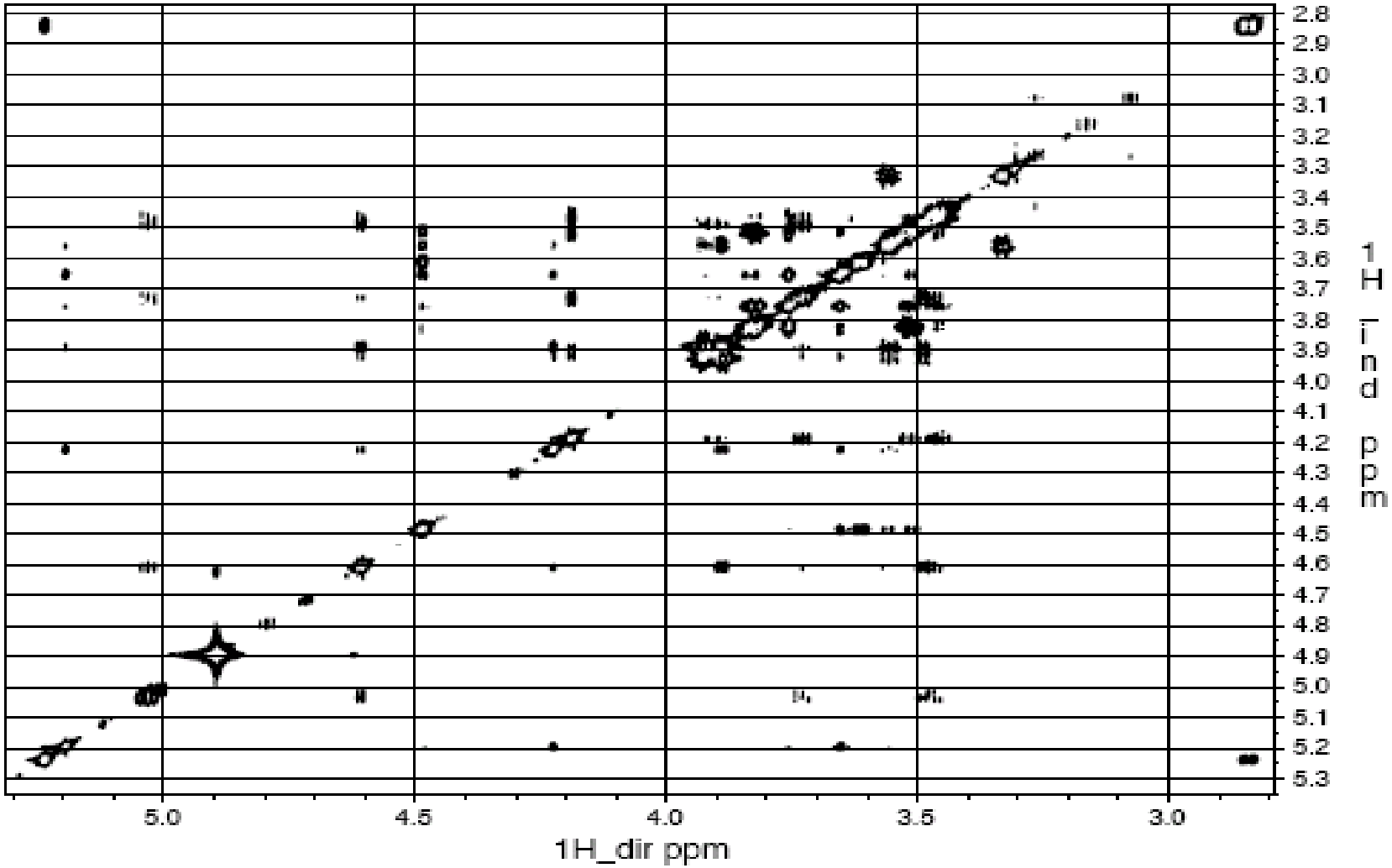


Figure A.2. ROESY spectrum of the oligosaccharide portion of blighoside A recorded at 700 MHz in CD_3OD

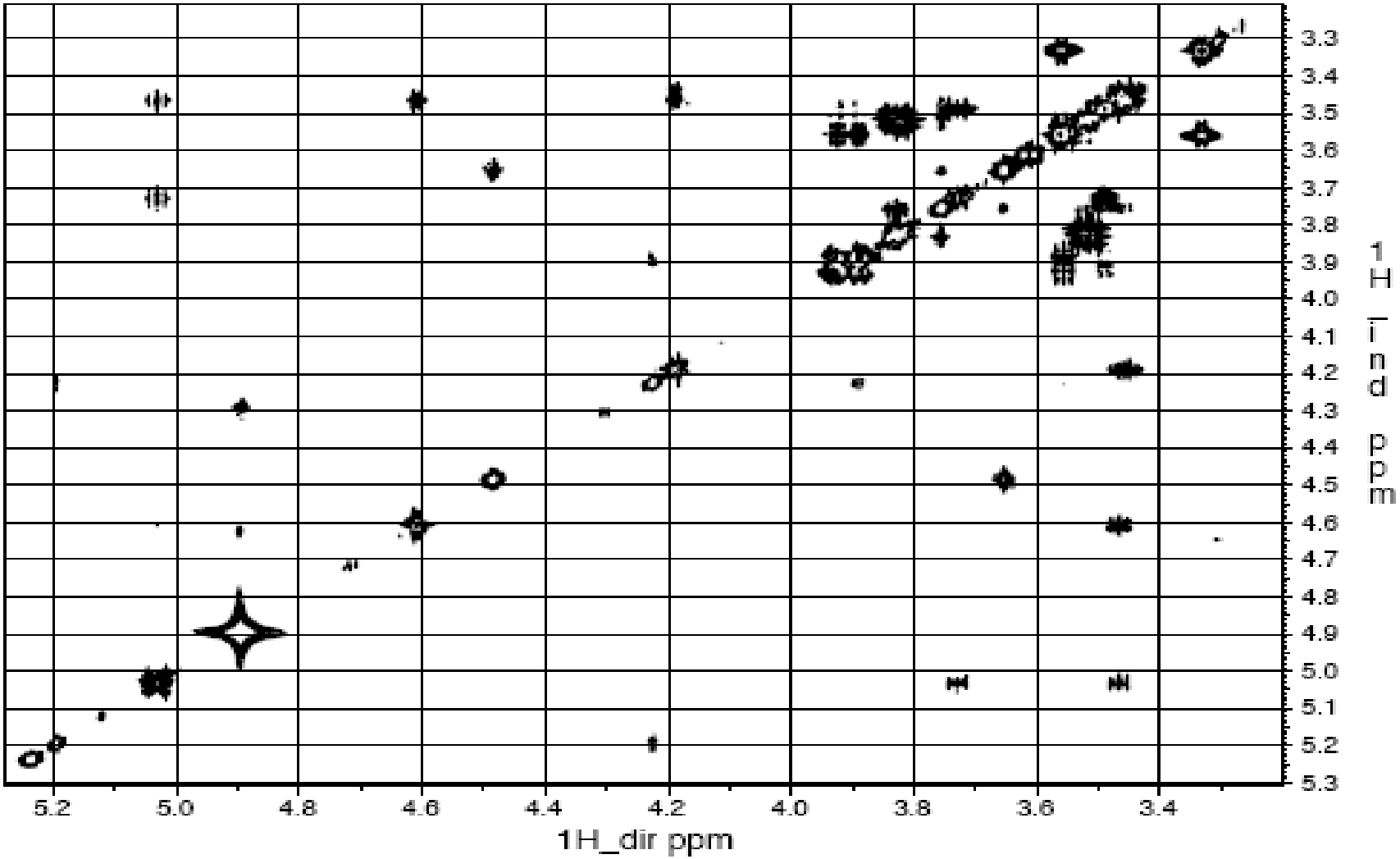


Figure A.3 TOCSY spectrum (10 ms) of the oligosaccharide portion of blighoside A recorded at 700 MHz in CD_3OD

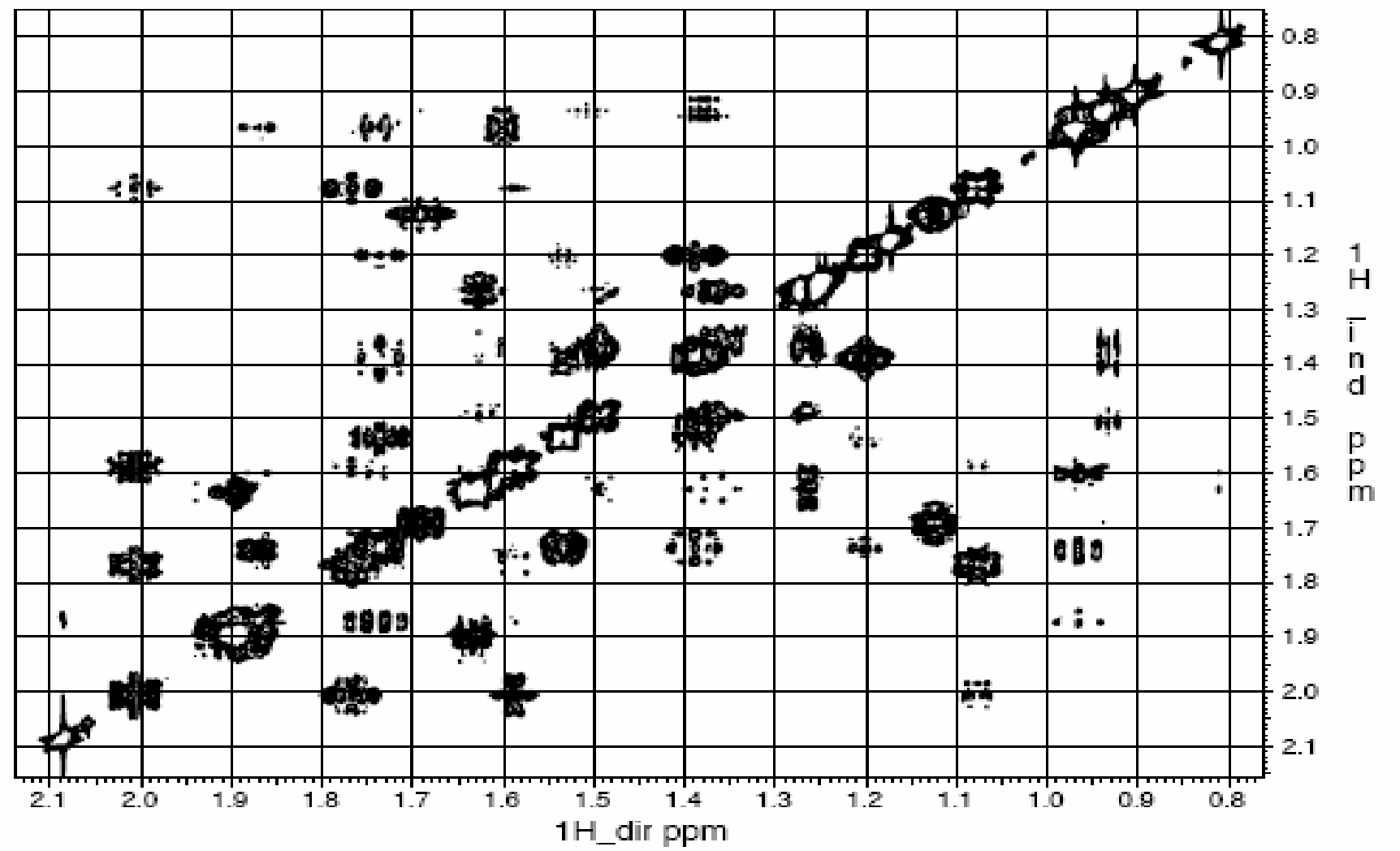


Figure A.4. TOCSY spectrum (10 ms) of the triterpene portion of blighoside A recorded at 700 MHz in CD_3OD

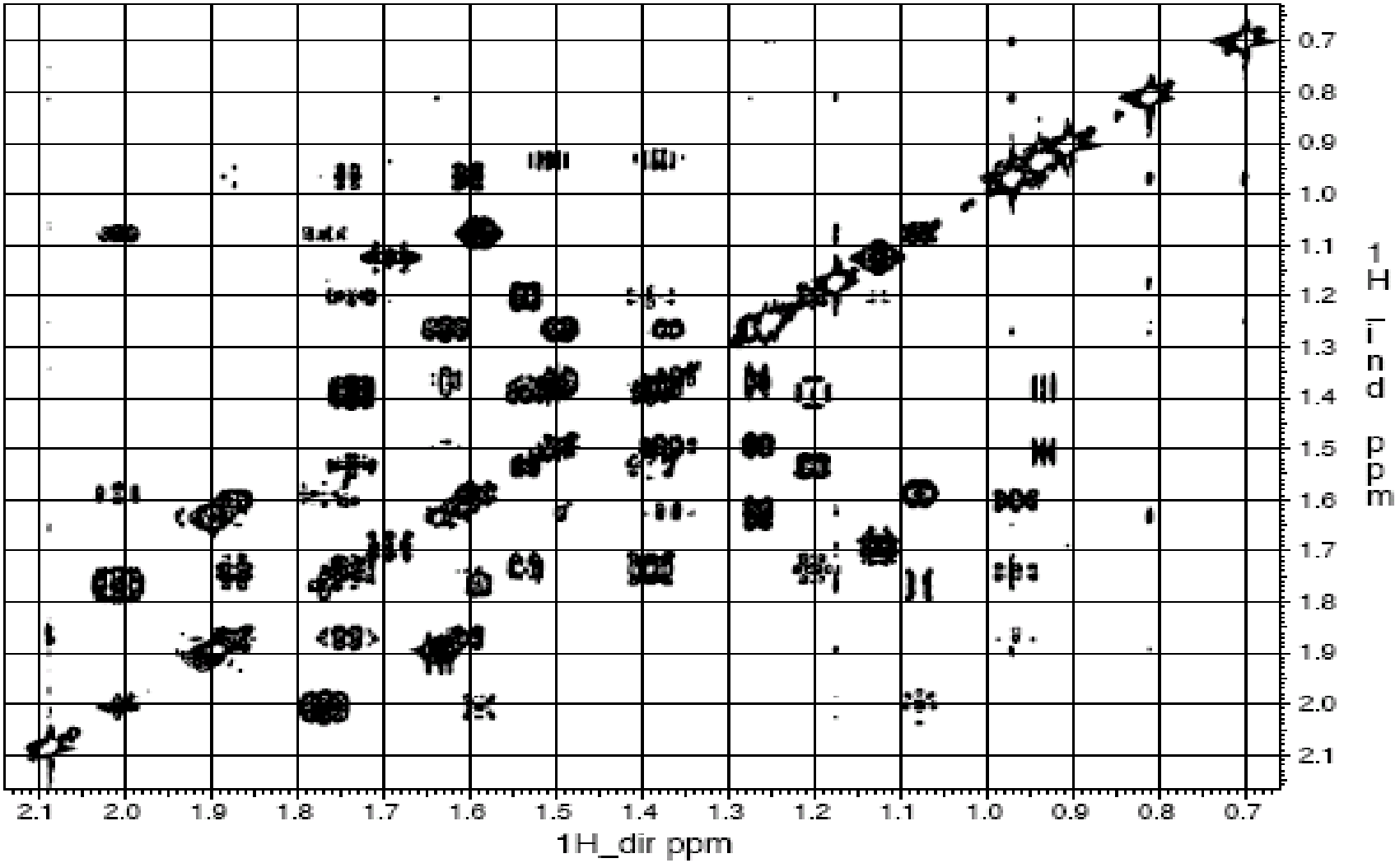


Figure A.5. TOCSY spectrum (100 ms) of the triterpene portion of blighoside A recorded at 700 MHz in CD_3OD

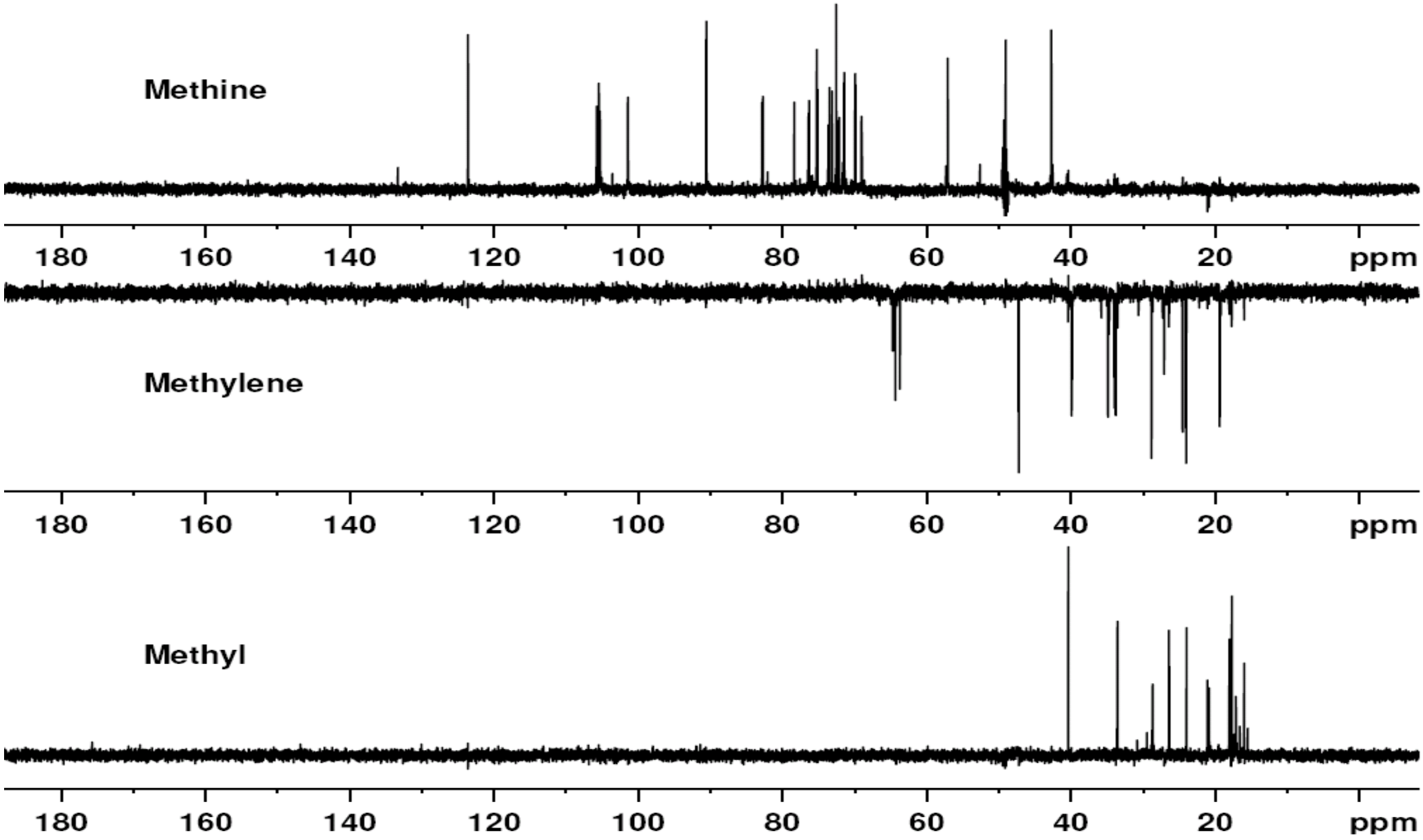


Figure A.6. ^{13}C-NMR DEPT spectrum of blighoside C recorded at 125 MHz in CD_3OD

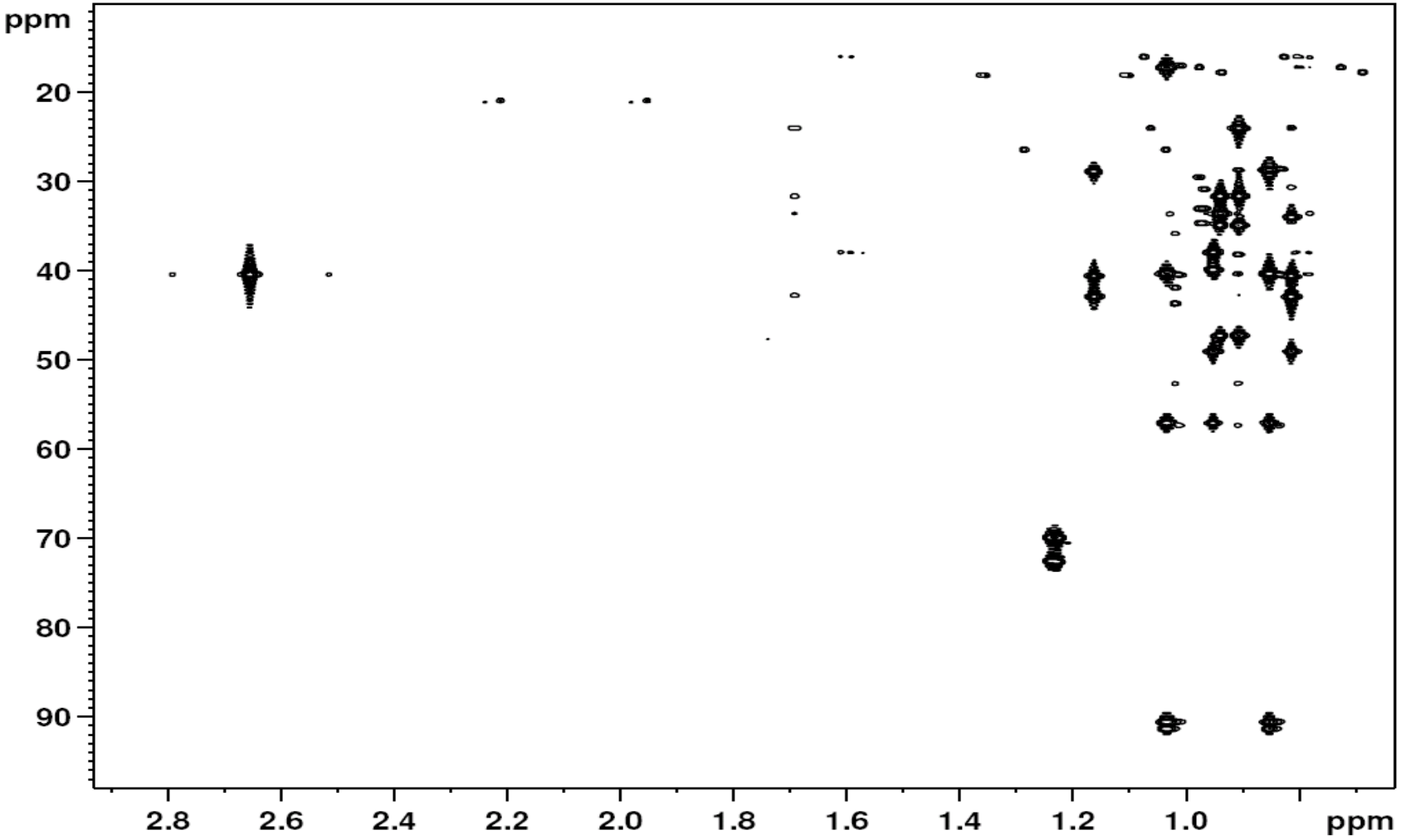


Figure A.7. HMBC spectrum of the triterpene portion of blighoside C recorded at 500 MHz in CD_3OD

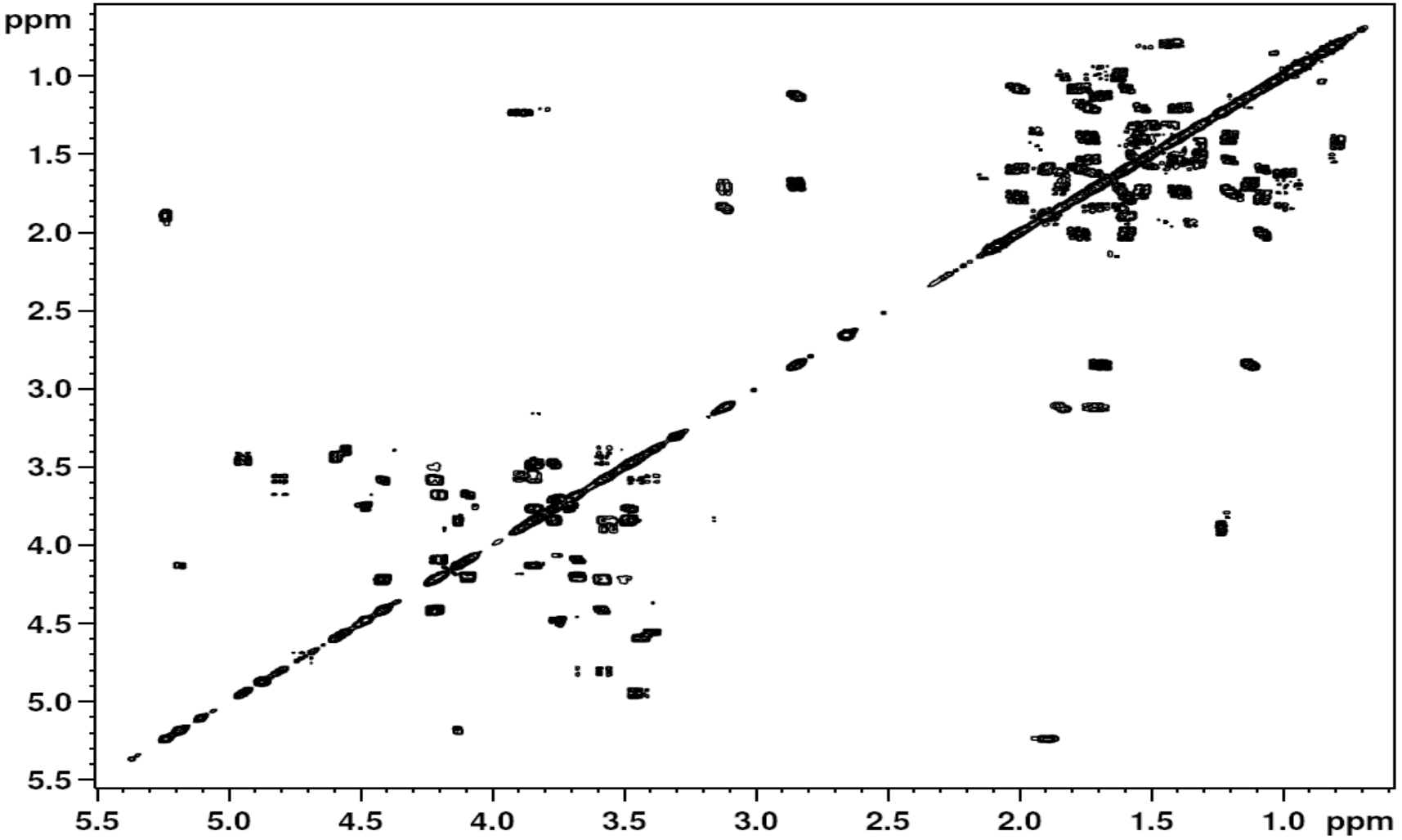
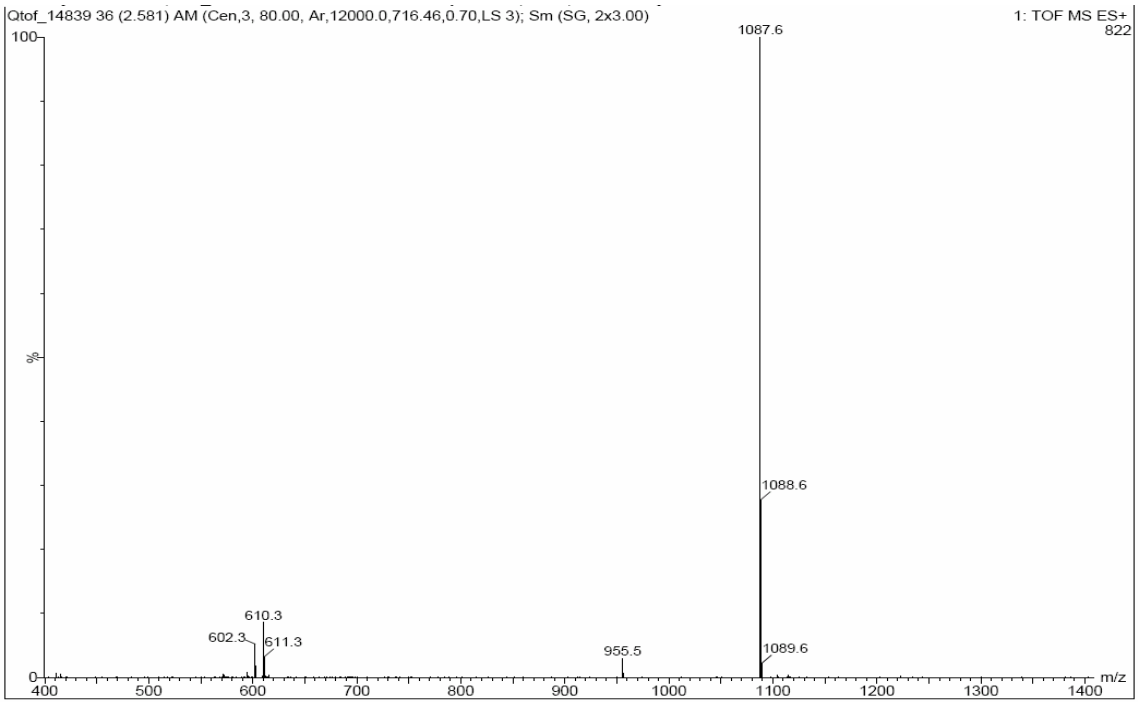


Figure A.8. COSY spectrum of blighoside C recorded at 500 MHz in CD_3OD

A.



B.

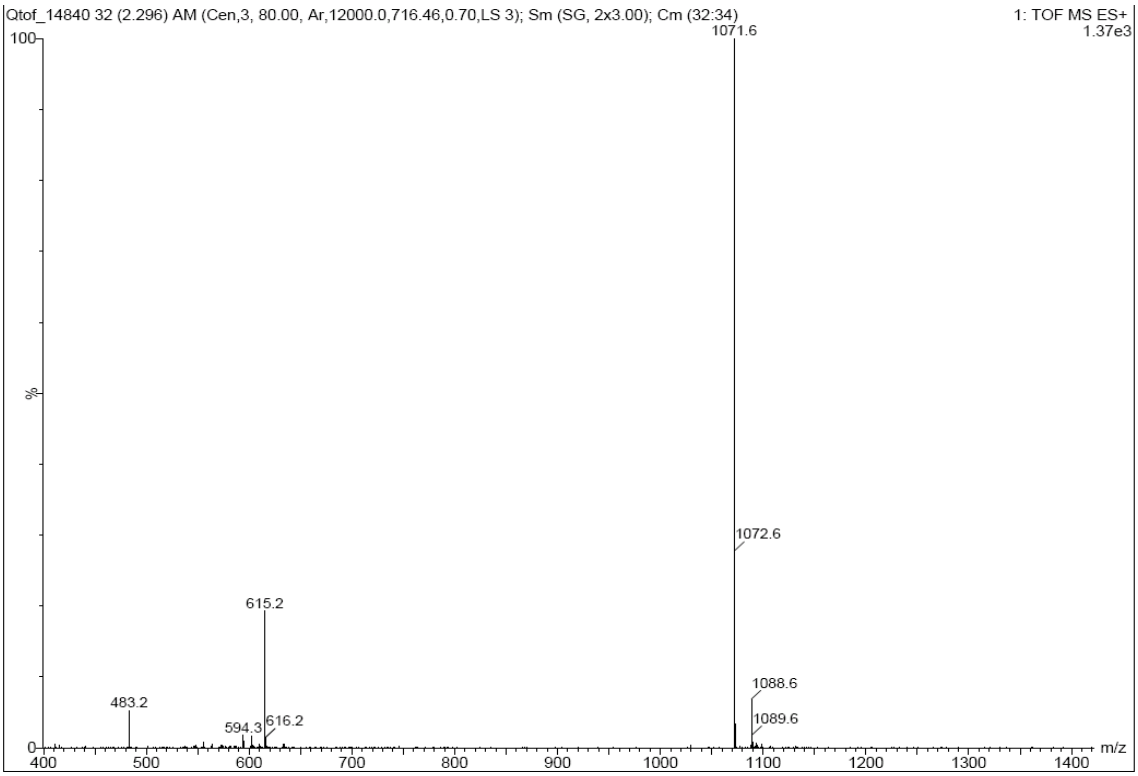


Figure A.9. HR-TOF-ESMS (positive mode) of blighoside A (**A**) and blighoside B (**B**)

APPENDIX B

Total Antioxidant Power Analysis

Using the Ferric Reducing Ability (Antioxidant) Power (FRAP) Method

Basic Method

This method of determining the total antioxidant (reducing) power of plant-based extracts is based on the original method developed by Benzie & Strain (1996), and later with other derivations published since its development.[135,136] The more recently published FRAP methods are fully automated with minor modifications in cases involving plant extracts. Solvents used in preparing plant extract and/or fractions to be tested are usually done in acetone. However, several recent methods have employed other extracting solvents such as methanol, ethanol, water, and 2-propanol. Different antioxidants in different solvents containing equal concentration of antioxidants gave only a small change to their FRAP values; therefore the FRAP assay is relatively insensitive to the type solvents (mention above) used, either as single or in combination.[136,138,176]

The FRAP assay is superior to the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC) assay of Miller & Rice-Evans (1996) and the oxygen radical absorbance capacity (ORAC) assay of DeLange & Glazer (1989),[177,178] in that the latter two assays are based on the antioxidant's ability to react with or neutralize free radicals generated in the assay systems; whereas the FRAP assay measures the reduction of Fe^{3+} (ferric iron)/tripyridyltriazine complex, present in stoichiometric excess, to Fe^{2+} (ferrous iron), which is blue-colored, in the presence of antioxidants (standards or antioxidant-containing extracts), resulting in an increase in

absorbance at 593 nm.[136] The Fe^{3+} to Fe^{2+} redox reduction occurs rapidly with all reductants having a half-reaction reduction potentials above that of Fe^{3+}/Fe^{2+}, thereby expressing the corresponding concentration of electron-donating antioxidants which is proportional to the change in absorbance (ΔA). The FRAP assay has been shown to have a limit of detection of < 2μM reducing/ability power and the precision, within- and between-runs, have CVs of < 0.5% and 1.0%, respectively, between 500 and 2000 μM (n > 8). FRAP values are expressed as antioxidant (reducing) power (μM), antioxidant (reducing) power/dry weight or fresh weight (μmol/g), or antioxidant (reducing) power/L of sample (μmol/L).

I have elected to use the FRAP analysis for four reasons. The FRAP assay is simple and is the only assay that directly measures antioxidants or reductants in a samples or extracts. The ORAC and the TEAC (and the DPPH) assays are more indirect because they measure the inhibition of reactive species (free radicals) generated (or present) in a reaction mixture system, and the results as compared to Cao et al (1996), Miller et al (2000) and Wang et al (1996), depend strongly on the type of reactive species (ROO, OH, Cu^{2+}) used.[178-181] Additionally, the FRAP assay employs antioxidants as reductants in a redox-linked colorimetric reaction. Lastly, the ORAC and TEAC assays used a lag phase type of measurement. This has been difficult to standardize in previous experiments and has generated various unexpected results in our laboratory. Additionally, the preceding calculations following data accumulation are very cumbersome.

Another positive involving the FRAP assay is that one prepared test samples can be readily analyzed, and stoichiometric factors are fairly constant and reproducibly for individual standard antioxidants; and the linearity is generally maintained over a wide

range, typically from 25 μM to 1000 μM. However, one disadvantage of the FRAP assay is its insensitivity to thiols. The FRAP reagents do not react with thiol-containing antioxidants such as glutathione because generally the reducing potential of most thiols are below that of the Fe^{3+}/Fe^{2+} half-reaction. However, since the target compounds for this thesis are directed towards polyphenols, triterpenes and steroids, and saponins, such possible disadvantages or shortcoming of the assay are not of any importance. Also, only limited amount of plant glutathione are absorbed in our diet, with the exception of a few dietary plants and plant-based products. Therefore, the FRAP method is suitable for assessment of total antioxidants in plants extracts and fractions.

In this study, we assessed the total antioxidants or reducing capacity of the aqueous and the EtOAc partitioned fractions of ackee pod, and standards by the FRAP method. FRAP values are expressed as the combined concentrations of all electron-donating reductants in a reaction mixture system. In some cases, test samples were diluted 5-40 fold due to there strong reducing power.

Preparation of Samples

Plant materials, fresh, dried or powdered, are dissolved in solvents such as tetrahydofuran (for lipophillic compounds), distilled water, ethanol, methanol, and/or in combinations as 1:1 (v/v). For example, 1 g of a sample can be dissolved in 100 ml of distilled water or any other solvent, and the test samples can be diluted, accordingly, so that data is within the appropriate range for accurate detection and limits of the assay. Standard solutions are dissolved in HPLC-grade solvents since they have less contaminant, and/or filtered distilled water.

Apparatus

Since our laboratory is not equipped with a Cobas Fara centrifugal analyzer that is commonly used to automate the assay, we employed instead a VersaMax tunable absorbance microtiter plate reader detector from Molecular Devices. The device is capable of reading 96-well microtiter plates, which will be used to perform the assay. Other supplies needed included a timer, 1000 ml volumetric flask, 7 ml and 20 ml vials, two water baths (set at 37 °C and 50 °C, respectively), and volumetric pipettes (50, 200 and 1000 μL).

Reagents

The reagents required antioxidant (reductant) standards, gallic acid (use in TPC assay) or quercetin (used in the TFC assay), 2,4,6-tri-pyridyl-s-triazine (TPTZ), sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$), ferrous sultfate heptahydrate ($FeSO_4 \cdot 7H_2O$), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), distilled water and HCl (11 M). The reagents are adjusted to a final volume of one liter for most solutions, as shown in the Table B.1 below, with the exception TPTZ solution, which required 10 ml of a 40 mM HCL solution.

A. Solution Preparation:

Standard antioxidant solutions of gallic acid or quercetin are prepared at concentrations, 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.01563 mg/ml, while standard ferrous sulfate heptahydrate solutions, in water or methanol, are prepared from a concentrated stock solution shown in Table B.2. To make smaller amounts of these and other reagents, such as trolox and ascorbic acid, refer to Table 3.

Table B.1. Show the amounts of reagents and solvent needed to make the desired FRAP solution

REAGENTS	Concentration	Amount of reagent (g)	Distilled water (mL)*
1. Ferric chloride hexahydrate	20 mM	5.407	1000
2. Ferric sulfate heptahydrate	2 mM	0.5562	1000
3. Sodium acetate trihydrate[a] + glacial acetic acid	300 mM	3.100 16 (mL)	984
4. Dilute hydrochloric acid (12M)	40 mM	1.3273 (mL)	1000
5. 2,4,6-tri-pyridyl-s-triazine[b]	10 mM	0.0310	10 (HCl)

*****distilled water amount is an approximate amount needed to bring to 1L in a volumetric flask with one exception, which is adjusted to 10 ml and requiring 10 ml of 40mM HCL solution.
[a] Sodium acetate trihydrate is part of a buffer system containing an additional 16 ml of glacial acetic acid (V_T = 1L).
[b] TPTZ solution must be keep in a 50 °C water bath.

B. Additional reagent and considerations:

Acetate buffer: The acetate buffer contained the amount (mg) of $NaC_2H_3O_2 \cdot 3H_2O$ in Table B.1, plus 16 ml of glacial acetic acid. The solution is adjusted to 1 liter and pH 3.6.

TPTZ (10 mM): The TPTZ reagent is dissolved in 10 ml diluted HCl solution (40 mM; Table B.1).

C. Ascorbic acid, Gallic Acid, Quercetin, and Trolox standards: 0.5 mg of these standards is dissolved in 1 ml water and methanol, respectively, to make a stock of 0.5 mg/ml. This concentration should be within the limit of linearity of the microtiter plate reader absorbance range. However, the linearity interval for the FRAP assay for 4 min

reaction time varies for each standard. For example, gallic acid and quercetin have an interval of 50-500 and 25–500 μM, respectively, whereas trolox and ascorbic acid have an interval of 50-1000 μM. The best standard to use in term of determining equivalents is trolox because its values in the FRAP remain relatively unchanged above the 4 min reaction time. Gallic acid and quercetin FRAP value increase above the 4 min reaction time period.

Standard ferrous sulfate solution can be store at –20 °C until ready to be used, and it has a linearity interval of 100–2000 μM in the assay. TPTZ reagent and the complete FRAP working solution must be made fresh. The acetate buffer can be store at 4 °C until use.

Procedures

1. The FRAP working solution required to run the assay is prepared by combining and mixing thoroughly the ingredients from Table B.1, and then preheating it in a water bath at 37 °C for immediate use. The FRAP working solution (240 ml; 10:1:1) is prepared fresh and is as follows:

(1). 200 ml or 25 ml of 0.3 M acetate buffer, pH 3.6
(2). 20 ml or 2.5 ml of 10 mM TPTZ (2,4,6-tri-pyridyl-s-triazine) in 40 mM HCl solution
(3). 20 ml or 2.5 ml of 20 mM $FeCl_3 \bullet 6H_2O$ in distilled water

2. Store the solution in **1**, and distilled water at 37°C and 50°C, respectively, in water baths.

3. Set the temperature control to 37°C, and the absorbance at wavelength (593 or 595 nm) for the microtiter plate reader.

4. Running set of blanks first, add 30 μL of water (50°C) to six wells on the microtiter plate reader, and then add 1 ml of FRAP working solution.

5. Record the absorbance of blanks after 4 minutes at 593 nm.

6. For samples and standards, always have sets of blanks. Follow the steps below for samples and standards (final dilution should be 1/33, 1/34 or 1/40). For blank solutions use step 4-5.

7. Add 30 μL (40 or 50 μL)of test sample to a 7 ml vial, then add 990 μL (1.32 or 1.65 ml) of FRAP working solution 1 min after adding the blank solution (200 μL) in the appropriate wells.

8. After 4 min record the absorbance of the blanks in wells, and then record the absorbance of the test solutions, in triplicate, at 5 min, and then every minute up to 16 min.

9. Repeat steps **7-8** for the remaining standard solutions and other test samples.

Note: If a microtiter plate is used and only a duplicate or triplicate experiments with each having a pipetted volume of 100-150 μL, then 10-20 μL of test sample can be mixed with 330-660 μL of FRAP working solution in the well, as in step 7 above.

10. Plot FRAP absorbance versus Fe^{2+} concentration (as $FeSO_4 \cdot 7H_2O$) to create a standard curve. Subtract the absorbance of the blank from each measurement of the standard or test sample (dried extract or fraction).

11. Using the equation for the linear line, calculate the antioxidant (reducing) power/g sample. Also, calculate the stoichiometric ratio for gallic acid and quercetin from plot of FRAP absorbance versus concentration of standards (gallic acid and quercetin).

Further Considerations

1. Initial concentrations of gallic acid (0.085 mg/ml) and quercetin (0.151 mg/ml) solutions, and subsequent dilutions, can be performed on the same plate as the Fe^{2+} determination (see below, for plate layout). The above concentrations are within the linear interval of the assay.

Table B.2. Possible layout of microtiter plate for FRAP assay analysis

	1	2	3	4	5	6	7	8	9	10	11	12
	2.0 mM	1.8 mM	1.6 mM	1.4 mM	1.2 mM		2.0 mM	1.8 mM	1.6 mM	1.4 mM	1.2 mM	1.0 mM
A	S1.1	S2.1	S3.1	S4.1	S5.1		GA 0.5	0.25	0.125	0.0625	0.0313	0.0156
B	S1.2	S2.2	S3.2	S4.1	S5.2		GA 0.5	0.25	0.125	0.0625	0.0313	0.0156
C	S1.3	S2.3	S3.3	S4.3	S5.3		GA 0.5	0.25	0.125	0.0625	0.0313	0.0156
D	S1.4	S2.4	S3.4	S4.4	S5.4		GA 0.5	0.25	0.125	0.0625	0.0313	0.0156
E	2.0 mM	1.8 mM	1.6 mM	1.4 mM	1.2 mM		2.0 mM	1.8 mM	1.6 mM	1.4 mM	1.2 mM	1.0 mM
F	blank1	blank2	blank3	blank4	Blank5		blank1	blank2	blank3	blank4	Blank5	Blank6
G	blank1	blank2	blank3	blank4	Blank5		blank1	blank2	blank3	blank4	Blank5	Blank6
H	blank1	blank2	blank3	blank4	Blank5		blank1	blank2	blank3	blank4	Blank5	Blank6

2. The amount of reagent solutions (in ml), as shown in Table B.1, can be adjusted to fit required needs, and an example for smaller amount (1 ml) of standard Fe^{2+} solution ($FeSO_4 \cdot 7H_2O$) rather than 2 ml is shown in Table B.3, below, with amounts in parenthesis. Standard iron solution (2 ml) preparation is also shown in Table B.3.

Table B.3. Amounts of standard reagents needed for the FRAP assay

Standard (μM)	$FeSO_4 \cdot 7 H_2O$ (x 10^3 μL)	Volume (x 10^3 μL) distilled water
100	0.1 (0.05)	1.9 (0.95)
200	0.2 (0.1)	1.8 (0.9)
400	0.4 (0.2)	1.6 (0.8)
600	0.6 (0.3)	1.4 (0.7)
800	0.8 (0.4)	1.2 (0.6)
1000	1.0 (0.5)	1.0 (0.5)
1200	1.2 (0.6)	0.8 (0.4)
1400	1.4 (0.7)	0.6 (0.3)
1600	1.6 (0.8)	0.4 (0.2)
1800	1.8 (0.9)	0.2 (0.1)
2000	2.0 (1.0)	0

Note: For 500 ml of standard $FeSO_4 \cdot 7H_2O$ use 0.278g.

Table B.4. Adjusted amount of reagents needed for the FRAP assay

REAGENTS	Concentration	Amount (g)	Distilled water (mL)*
Ferric chloride hexahydrate	20 mM	5.407	1000
		2.704	500
		1.352	250
		0.5407	100
Ferric sulfate heptahydrate	2 mM	0.2781	500
		0.1391	250
		0.05562	100
	3 mM	0.834	1000
		0.417	500
		0.208	250
2,4,6-tri-pyridyl-s-triazine	10 mM	0.310	100 (HCl)
Dilute hydrochloric acid (12M)	40 mM	0.6636 (mL)	500
		0.1327 (mL)	100
Sodium acetate trihydrate	300 mM		
+16 ml glacial acetic acid		3.10	984
+8 ml glacial acetic acid		1.55	500
+ 1.6 ml glacial acetic acid		0.310	100

APPENDIX C

APPENDIX

COLLABORATORS AND CONTRIBUTORS

Fruit collection locations:

1. Fruits and Spice Park

24801 SW 187th Avenue, Homestead, FL 33031

(305) 247-5727

Directors: Chris Rollins

http://www.fruitandspicepark.org/

2. The Kampong

4031 Douglas Road, Coconut Crove, FL 33133

(305) 442-7169

Director: Larry Schokman

http://www.ntbg.org/gardens/kampong.php

3. Rare Fruits and Vegetable Council of Broward County

5105 SW 208th Lane

Southwest Ranches, FL 33332

Contact: Margaret Basile, Ph.D

http://www.rfvbroward.org

Bio-Assay testing and NMR Analysis locations and collaborators:

1. Cytotoxicity Assays was performed by Linda Einbond, Ph.D

The Herbert Irving Comprehensive Cancer Center

College of Physicians and Surgeons

Columbia University

HHSC-1509, 701 W. 168th Street

New York, NY 10032

2. NMR analyses were performed by Darón Freedberg, Ph.D, and Eugene Mazzola, Ph.D.

The Joint Institute for Food Safety and Applied Nutrition

University of Maryland

College Park, Maryland 20742

3. Additional NMR Analysis and elucidation work was performed by Senior Research Fellow Bruce Coxon, Ph.D.

The National Institutes of Health-

National Institute of Child Health and Human Development (NICHD)

Bethesda, Maryland 20892

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