FEASIBILITY OF GROWING BITTER MELON (*MOMORDICA CHARANTIA* L.) IN TEXAS AND ITS POTENTIAL HEALTH PROMOTING PROPERTIES

A Dissertation

by

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ABSTRACT

In the United States, bitter melon (*Momordica charantia* L.), is an underutilized horticultural crop that has been traditionally used to manage diabetes and various inflammatory conditions. In this study, cultivation of five bitter melon cultivars grown under field conditions in College Station, Texas, was evaluated. Fruit quality was measured by the levels of phytochemicals such as vitamin C, amino acids and total phenolics. The yield for the various cultivars was comparable to other bitter melon growing regions. The highest levels of total ascorbic acid were shown in the Japanese Spindle, while the lowest levels were expressed in the Hong Kong Green. The levels of total phenolics and amino acids were also evaluated. Ultrahigh performance liquid chromatography-high resolution mass spectrometry was used to identify 15 phenolic and 46 triterpenoids in various bitter melon extracts. Total phenolic levels were highest in methanolic extracts of the inner tissue of the Indian Green cultivar, which also correlated to the highest DPPH radical scavenging activity. Differential inhibition of α-amylase and α-glucosidase activity was observed in response to extract polarity, cultivar and tissue type. Furthermore, we evaluated the postharvest changes of three household processing techniques on the levels of vitamin C, amino acids, total phenolics, and antioxidant activity of bitter melon during storage at various temperatures for up to 8 weeks. The largest decrease of vitamin C was observed in blended bitter melon samples stored at 10 °C for one week, while the highest levels of vitamin C were observed in chopped fruit stored at 2 °C and -20 °C. Total phenolics and DPPH activity drastically increased in chopped fruit stored at -20 °C while this was not the case in whole fruit. During maturation, lutein and β-

carotene levels fluctuated depending on the tissue type and the levels of trans-lycopene were found to be high in aril tissues. Additionally, various carotenoid esters and *cis*-lycopene isomers have also been identified in bitter melon for the first time. Lastly, bitter melon extracts and compounds were found to influence on the expression of genes involved in the formation of the NLRP3 inflammasome complex in RAW 264.7 macrophage cells.

DEDICATION

This dissertation is dedicated:

to my wife, Mirna, whose love and unwavering support has been instrumental in the completion of my degree. During times of exasperation, my wife always consoled and encouraged me to keep moving forward. At one point, during my degree I was faced with the decision to have to move with my job and continue my education. A decision that entailed uprooting my wife from her job, friends, family, and home; all while she was half-way through her graduate education. Her support during those times and the endless lab hours is only one of the many reasons why I am grateful to God for putting her in my life.

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All work conducted for this dissertation was completed by the student independently.

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1. INTRODUCTION

Bitter melon (*Momordica charantia* L.) is a member of the *Cucurbitacea*e family. The characteristic bitter taste is viewed as desirable and popular in some countries, but in the United States bitter melon is not appreciated and it is considered an underutilized horticultural crop. Beside the use of bitter melon for nutritional purposes, bitter melon has also been used in traditional medicine to alleviate various health ailments. As in all plants, bitter melon has a wide array of secondary metabolites that protect the plant from both biotic and abiotic stress. The levels of these metabolites are known to fluctuate in response to both endogenous and exogenous stimuli experienced by the plant. In recent years, some of these metabolites have been found to have human health benefits. Among the compounds that have been reported in bitter melon, are cucurbitane-type triterpenoids, polyphenolic compounds, vitamin C and carotenoids. These compounds have all been reported to be bioactive against various chronic diseases such as cancer, cardiovascular disease and diabetes. All of which have inflammation as an underlying factor.

Variation in levels of bioactive compound can be manifested in response to genotypic, cultivation, and postharvest factors. Furthermore, advances in analytical techniques now give us the capabilities to more accurately profile the composition of metabolites present in bitter melon. In order to advance our knowledge on how bitter melon can potentially ameliorate inflammatory disease, a comprehensive study on the variation of these bioactive compounds is critical. With the accurate profiling of these metabolite we can continue to enhance our knowledge on how bitter melon may be exerting its various biological properties. With this in mind we developed this research program to add valuable

information to the current body of knowledge involving bitter melon production and health benefits.

The objectives of the investigation presented here were:

1) To evaluate the growth performance of five bitter melon cultivars under Texas field conditions to determine the feasibility of commercial production.

2) To determine the metabolic profiles of bitter melon metabolites of two commercial cultivars present in the market.

3) To determine the influence of postharvest storage time, temperature and processing on the phytochemical profile of bitter melon.

4) To evaluate changes in carotenoid content in bitter melon tissues during fruit ripening for the possible utilization of material considered postharvest loss for carotenoid supplement development.

5) To evaluate the anti-inflammatory effects of bitter melon extracts and purified compounds by interactions involving the NF-κB transcription factor and the formation of the NLRP3 inflammasome complex.

2. LITERATURE REVIEW[1]

Bitter melon is a monoecious species native to Southeast Asia and is characterized by multilobed leaves and numerous yellow flowers. Flowers typically appear 30-50 days after planting. Anthesis happens early morning between the hours of 2:00 to 7:00 am with pollen losing viability as the day progresses[1]. Bitter melon grows well under tropical and sub-tropical conditions but can grow well under a wide range of environmental conditions.

There are several common names for bitter melon throughout the world such as bitter gourd, kugua, balsam pear, cundeamor, karela, ampalaya, to name a few. Bitter melon fruit has a typical ovoid to oblong shape with characteristic pericarp covered in triangular tubercles or smooth warts. Bitter melon fruit is typically harvested off vines typically two weeks after anthesis when the fruit is still green and has a creamy white pulp, this timeframe depends on specific cultivars. The fruits are considered marketable once they obtain an optimal color or size depending on the cultivar. Typically, marketable bitter melon fruits have a green or white color with a creamy white pulp containing 5-15 seeds. Once the fruit matures, it begins to lose moisture and becomes bright orange. Additionally, the pulp undergoes a dramatic change and the seed membranes become scarlet red. At this point, the fruit become undesirable to consumer and hence unmarketable.

After harvest, fruits continue to have a relatively high respiration rate, hence bitter melon has a relatively short shelf-life. At 10-12 °C, bitter melon can last 7-12 days.

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[1] Part of this chapter is reprinted with permission from *Separation of cucurbitane-type triterpenoids from bitter melon*, by J.L. Perez, G. K. Jayaprakasha, B.S. Patil, in Instrumental Methods for the Analysis and Identification of Bioactive Molecules, ed. by G. K. Jayaprakasha, B.S. Patil, F. Pellati, 2013, American Chemical Society, Washington, DC, USA. Copyright [2013] American Chemical Society.

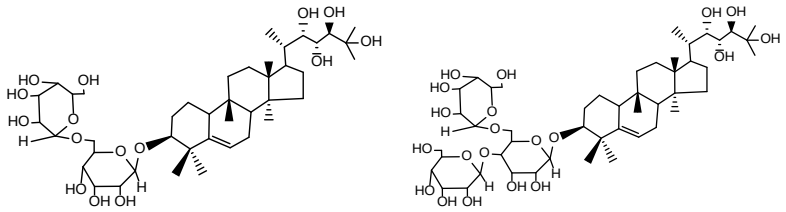
Unfortunately, bitter melon is extremely sensitive to chilling injury hence storage at lower temperature is not feasible for fresh produce production.

2.1. Health benefits of bitter melon

In traditional medicine, bitter melon has been used as a natural way to manage several diseases, including type 2 diabetes [2 3]. Recently, several studies have evaluated these claims using various animal, cell and enzymatic assays, particularly illustrating promising antidiabetic and anti-cancer activities. Animal studies using various bitter melon extracts have reported bitter melon to suppress prostate cancer progression, enhanced insulin signaling, and favorably influenced blood glucose and blood pressure regulation [4-6]. Furthermore, various cell culture models have reported bitter melon extracts to induce apoptosis of human pancreatic carcinoma cells, have reparative effects on pancreatic β-cell, inhibit adipocyte differentiation, and increase insulin sensitivity in muscle cells [7-10]. With recent accumulation of data correlating bitter melon and positive health benefits, an increase interest in identifying the possible bioactive components has emerged.

Studies have reported bitter melon to contain several health benefiting compounds such as vitamin C, carotenoids, flavonoids and other polyphenols, but current interest is focused on the isolation and purification of cucurbitane-type triterpenoids found in the various parts of the bitter melon plant [11-14]. These cucurbitane-type triterpenoids are typically found in plants belonging to the *Cucurbitaceae* family, in which bitter melons are included [15]. Cucurbitane-type triterpenoids are characterized as having a tetracyclic nucleus, principally 9β-methyl-19-nor-lanosta-5-ene, with various oxygenation functionalities throughout its structure [15]. Furthermore, cucurbitane-type triterpenoids may be present in various aglycone and glycosidic forms leading to the vastly diverse pool of compounds

found in bitter melon. To date, over 250 cucurbitane type molecules have been identified from various parts of the bitter melon plant [16].



A B

Figure 2-1. Momordicoside A and B

2.2. Cucurbitane-type triterpenoids

Momordicosides (A and B) were first isolated in 1980 from bitter melon seeds [17]. Other cucurbitane-type triterpenoids from other fruits have been previously reported but the novelty in these molecules was the absence of oxygen at the C 11 position (Figure 2-1). The molecules were identified as the 3-*O*-β-gentiobioside and 3-*O*-β-D-xylopyranosyl(1→4)[β-D-glucopyranosyl(1→6)]-β-D-glucopyranoside of cucurbit-5-ene-3β, 22(*S*), 23(*R*), 24(*R*), 25-pentol. In the following publications, an additional twenty–one momordicosides (C-X) were identified from fruits, seeds, vines, and roots of the bitter melon plant [18-27]. Similarly, several other triterpenoids have been identified and named from the bitter melon plant, such as momordicine (I-VIII), [28-34], goyaglycosides (A-H) and goyasaponins (I-III) [35], karavilagenin (A-F) and karavilosides (I-XII) [36-38], charantosides (I-VIII, and A-G) [39-42], kuguacin (A-S) [43, 44], kuguaglycosides A-H [45], neokuguaglucoside [46], kuguaosides (A-D) [47], kuguasaponins (A-H)[48], and octonorcucurbitacins (A-D) [49]. Additionally, two novel pentanorcucurbitane triterpenes, 22-hydroxy-23,24,25,26,27-

pentanorcucurbit-5-en-3-one and 3,7-dioxo-23,24,25,26,27-pentanorcucurbit-5-en-22-oic acid, together with a new trinorcucurbitane triterpene, 25,26,27-trinorcucurbit-5-ene-3,7,23-trione, were isolated from bitter melon stem methanolic extracts [50]. Selected structures of these triterpenoids are presented in Figure 2-1, 2-2, and 2-3. Additionally, a summary of named cucurbitane-type triterpenoids and related information is presented in Table 2-1.

Table 2-1. Various triterpenoids, molecular formulas and exact masses reported from bitter melon.

Name	Source	Formula	Exact Mass	ref
Charantosides				
I	Fruit	$C_{37}H_{58}O_8$	630.4131	39
II	Fruit	$C_{38}H_{62}O_9$	662.4393	39
III	Fruit	$C_{36}H_{56}O_7$	600.4026	39
IV	Fruit	$C_{36}H_{56}O_7$	600.4026	39
V	Fruit	$C_{37}H_{60}O_8$	632.4288	39
VI	Fruit	$C_{37}H_{60}O_8$	632.4288	39, 41
VII	Fruit	$C_{36}H_{54}O_8$	614.3818	39
VIII	Fruit	$C_{38}H_{62}O_9$	662.4393	39
A	Fruit	$C_{37}H_{60}O_9$	648.4237	40
B	Fruit	$C_{36}H_{58}O_9$	634.4080	40
C	Fruit	$C_{37}H_{58}O_9$	646.4080	40
D	Fruit	$C_{37}H_{59}O_9$	647.4165	42
E	Fruit	$C_{37}H_{59}O_9$	647.4165	42
F	Fruit	$C_{37}H_{59}O_7$	615.4266	42
G	Fruit	$C_{36}H_{55}O_8$	615.3891	42
Goyaglycosides				
A	Fruit, Leaves	$C_{37}H_{61}O_9$	648.4237	35, 51
B	Fruit, Leaves	$C_{37}H_{60}O_9$	648.4237	35, 36, 40, 41, 47, 51
C	Fruit	$C_{38}H_{62}O_9$	662.4393	35, 36, 39, 52
D	Fruit	$C_{38}H_{62}O_9$	662.4393	35, 36, 39, 41, 47, 52
E	Fruit	$C_{42}H_{68}O_{13}$	780.4659	35, 53
F	Fruit	$C_{42}H_{68}O_{13}$	780.4659	35, 53
G	Fruit	$C_{43}H_{70}O_{14}$	810.4765	35
H	Fruit	$C_{42}H_{70}O_{15}$	814.4714	35, 53
Goyasaponins				
I	Fruit, Seed	$C_{65}H_{102}O_{31}$	1378.6405	35, 54
II	Fruit, Feed	$C_{70}H_{110}O_{35}$	1510.6827	35, 54
III	Fruit	$C_{49}H_{76}O_{19}$	968.4980	35
Karavilagenin				
A	Fruit	$C_{32}H_{54}O_3$	486.4072	36
B	Fruit	$C_{31}H_{52}O_3$	472.3916	36
C	Fruit	$C_{31}H_{52}O_3$	472.3916	36
D	Leaves, Vines, Fruit	$C_{30}H_{46}O_4$	470.3396	37, 44, 51, 55
E	Fruit	$C_{30}H_{48}O_3$	456.3603	37
F	Leaves, Stems	$C_{31}H_{50}O_5$	502.3647	38

Table 2-1 (Cont.)

Name	Source	Formula	Exact Mass	Ref
Karavilosides				
I	Fruit	$C_{38}H_{64}O_8$	648.4601	36, 39
II	Fruit	$C_{38}H_{64}O_8$	648.4601	36, 40, 56
III	Fruit, Root	$C_{37}H_{62}O_8$	634.4420	36, 45, 56
IV	Fruit	$C_{37}H_{62}O_9$	650.4393	36
V	Fruit, Root	$C_{43}H_{72}O_{14}$	812.4922	36, 45
VI	Leaves, Fruit	$C_{37}H_{58}O_9$	646.4080	37, 51
VII	Fruit	$C_{37}H_{60}O_8$	632.4288	*37*
VIII	Vine, Leaves, Fruit	$C_{36}H_{58}O_9$	634.4080	*37, 57*
IX	Fruit	$C_{42}H_{68}O_{14}$	796.4609	*37*
X	Vine, Leaves, Fruit	$C_{42}H_{68}O_{14}$	796.4609	*37, 57*
XI	Fruit, Root, Vine, Leaves	$C_{36}H_{60}O_{10}$	652.4186	24, 37, 45, 57
XII	Leaves, Stems	$C_{38}H_{60}O_9$	660.4237	38
XIII	Leaves, Stems	$C_{36}H_{58}O_8$	618.4123	38
Kuguacin				
A	Roots	$C_{30}H_{46}O_4$	470.33961	43
B	Roots	$C_{30}H_{48}O_3$	456.360345	43
C	Roots	$C_{27}H_{42}O_3$	414.313395	43
D	Roots	$C_{27}H_{40}O_4$	428.29266	43
E	Roots	$C_{27}H_{42}O_4$	430.30831	43, 44
F	Leaves, Vines	$C_{30}H_{42}O_5$	482.303225	44
G	Leaves, Vines	$C_{30}H_{44}O_6$	500.31379	44
H	Leaves, Vines	$C_{30}H_{44}O_5$	484.318875	44
I	Leaves, Vines	$C_{31}H_{46}O_5$	498.334525	44
J	Leaves, Vines	$C_{30}H_{46}O_3$	454.344695	44
K	Leaves, Vines	$C_{25}H_{34}O_6$	430.23554	44
L	Leaves, Vines	$C_{25}H_{36}O_4$	400.261359	44
M	Leaves, Vines	$C_{22}H_{28}O_4$	356.19876	44
N	Leaves, Vines	$C_{30}H_{46}O_4$	470.33961	44, 49
O	Leaves, Vines	$C_{30}H_{42}O_4$	466.30831	44
P	Leaves, Vines	$C_{27}H_{40}O_4$	428.29266	44
Q	Leaves, Vines	$C_{29}H_{44}O_5$	472.318875	44
R	Leaves, Stems	$C_{30}H_{48}O_4$	472.35526	38, 44
S	Leaves, Vines	$C_{30}H_{44}O_4$	468.32396	44

Table 2-1 (Cont.)

Name	Source	Formula	Exact Mass	Ref
Kuguaglycosides				
A	Root	$C_{37}H_{62}O_8$	634.4444	45
B	Fruit, Root	$C_{37}H_{62}O_8$	634.4444	45, 58
C	Leaves, Fruit, Root	$C_{36}H_{56}O_8$	616.3975	41, 45, 48, 51, 59
D	Root, Leaves, Vines	$C_{36}H_{60}O_9$	636.4237	45, 57
E	Root	$C_{42}H_{70}O_{14}$	798.4765	45
F	Root	$C_{43}H_{72}O_{13}$	796.4972	45
G	Root	$C_{42}H_{70}O_{13}$	782.4816	7, 45, 60
H	Root	$C_{48}H_{80}O_{18}$	944.5344	45
Kuguaoside				
A	Fruit	$C_{36}H_{58}O_8$	618.4131	47
B	Fruit	$C_{37}H_{60}O_9$	648.4237	47
C	Fruit	$C_{36}H_{58}O_9$	634.4080	47
D	Fruit	$C_{37}H_{60}O_9$	648.4237	47
Kuguasaponins				
A	Fruits	$C_{36}H_{56}O_8$	616.3975	48
B	Fruits	$C_{38}H_{62}O_9$	662.4393	48
C	Fruits	$C_{38}H_{62}O_9$	662.4393	48
D	Fruits	$C_{38}H_{62}O_9$	662.4393	48
E	Fruits	$C_{38}H_{62}O_9$	662.4393	48
F	Fruits	$C_{42}H_{66}O_{14}$	794.4452	48
G	Fruits	$C_{42}H_{69}O_{13}$	782.4810	48
H	Fruits	$C_{36}H_{60}O_9$	636.4237	48
Momocharaside				
A	Seed	$C_{42}H_{72}O_{15}$	816.4871	54
B	Seed, Fruit	$C_{36}H_{62}O_{10}$	654.4343	24, 54
Momordicine				
I	Leaves, Vine, Fruit, Ro	$C_{30}H_{48}O_4$	472.3552	7, 28, 30, 31, 33, 43, 44, 48, 61
II	Leaves, Vine Fruit	$C_{36}H_{58}O_9$	634.4080	7, 28-31, 57, 61, 62
III	Leaves, Vine	$C_{36}H_{56}O_{10}$	648.3873	28
IV	Leaves	$C_{36}H_{58}O_9$	634.4080	57, 62, 63
V	Leaves	$C_{39}H_{60}O_{12}$	720.4084	34
VI	Leaves, Stem	$C_{31}H_{50}O_4$	486.3709	38
VII	Leaves, Stem	$C_{34}H_{52}O_7$	572.3703	38
VIII	Leaves, Stem	$C_{35}H_{54}O_7$	586.3862	38

Table 2-1 (Cont.)

Name	Source	Formula	Exact Mass	Ref
Momordicosides				
A	Seed, Fruit	$C_{42}H_{72}O_{15}$	816.4871	17, 24, 35, 40, 52, 53, 56
B	Seed, Fruit	$C_{47}H_{80}O_{19}$	948.5293	17, 24, 56
C	Seed, Fruit	$C_{42}H_{72}O_{14}$	800.4922	18, 35, 40, 54
D	Seed	$C_{42}H_{70}O_{13}$	782.4816	18, 53
E	Seed	$C_{37}H_{60}O_{12}$	696.4080	18, 54
F1	Fruit	$C_{37}H_{60}O_{8}$	632.4288	19, 35, 36, 39, 40, 47, 52
F2	Fruit	$C_{36}H_{58}O_{8}$	618.4130	19, 36, 39, 40, 47, 52, 53
G	Fruit, Leaves	$C_{37}H_{60}O_{8}$	632.4288	19, 36, 40, 51, 52
I	Fruit	$C_{36}H_{58}O_{8}$	618.4130	19, 35, 36, 40, 47, 53
K	Fruit, Leaves	$C_{37}H_{60}O_{9}$	648.4237	19, 35, 36, 41, 45, 47, 51, 52, 57, 62
L	Fruit, Leaves	$C_{36}H_{58}O_{9}$	634.4080	19, 23, 36, 47, 51, 52, 56, 57
M	Fruit	$C_{42}H_{68}O_{14}$	796.4609	23, 40, 56
N	Fruit	$C_{42}H_{68}O_{14}$	796.4609	23, 56
O	Fruit	$C_{42}H_{68}O_{15}$	812.4558	23
P	Fruit	$C_{36}H_{58}O_{9}$	634.4080	27
Q	Fruit	$C_{36}H_{60}O_{10}$	652.4186	24
R	Fruit	$C_{42}H_{70}O_{15}$	814.4714	24
S	Fruit	$C_{48}H_{82}O_{20}$	978.5399	24, 56
T	Fruit	$C_{53}H_{90}O_{24}$	1110.5822	24
U	Fruit	$C_{38}H_{64}O_{9}$	648.4237	25, 47
V	Fruit	$C_{36}H_{58}O_{8}$	618.4131	25
W	Fruit	$C_{36}H_{59}O_{9}$	634.4080	25
X	Whole Plant	$C_{36}H_{58}O_{9}$	634.4080	26
Octonorcucurbitacin				
A	Stem	$C_{22}H_{30}O_{3}$	342.2189	49
B	Stem	$C_{22}H_{30}O_{3}$	342.2189	49
C	Stem	$C_{22}H_{30}O_{3}$	342.2189	49
D	Stem	$C_{23}H_{32}O_{5}$	388.2240	49
Taiwacin				
A	Stem, Fruit	$C_{44}H_{68}O_{14}$	820.4609	64
B	Stem, Fruit	$C_{25}H_{36}O_{4}$	400.2613	64

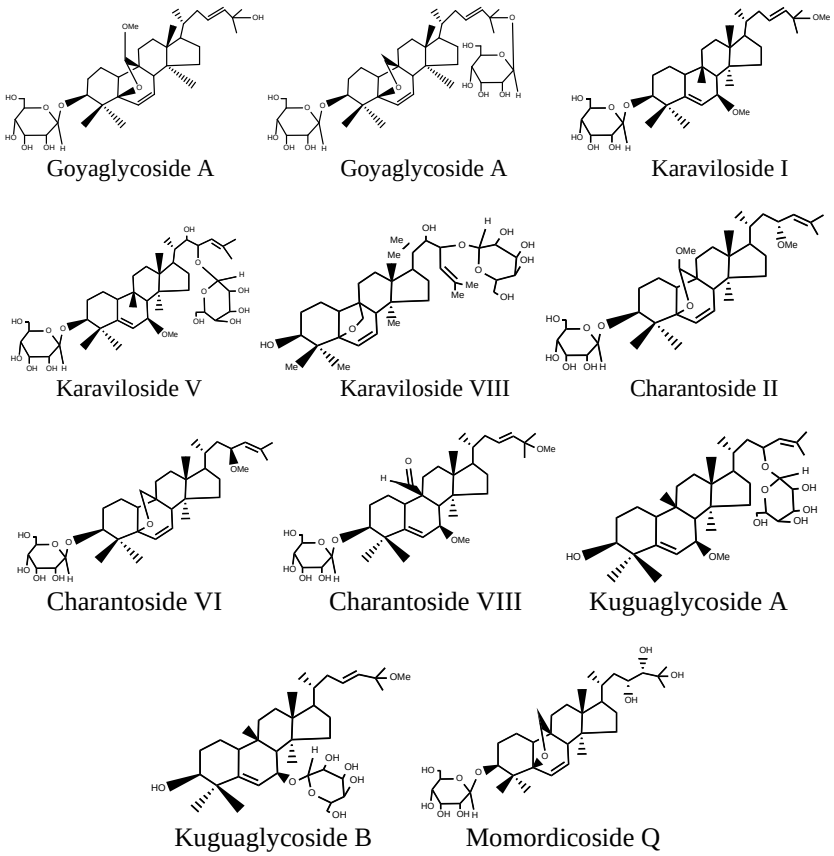


Figure 2-2. Structures of triterpenoid glucosides isolated form bitter melon

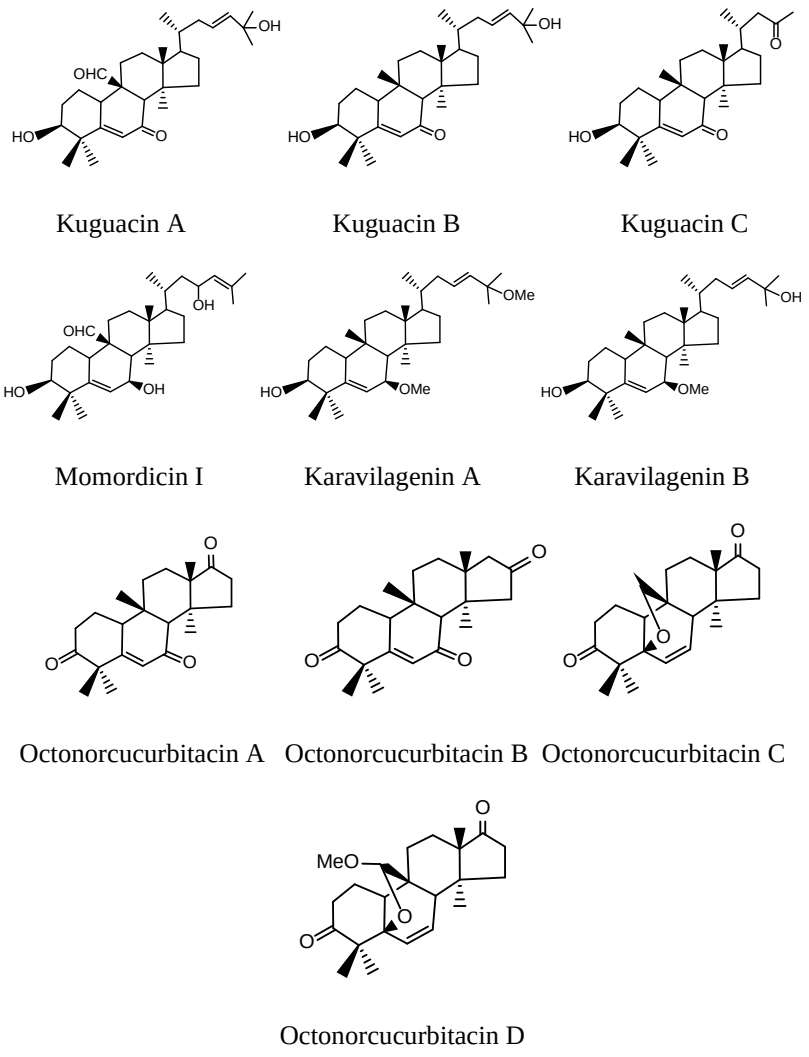


Figure 2-3. Structures of selected cucurbitane-type triterpenoid aglycones reported from bitter melon

2.3. Additional cucurbitane-type triterpenoids reported from bitter melon

2.3.1. Fruits

In addition to the above-mentioned molecules several other compounds, principally triterpenoids, have been identified from various parts of the bitter melon plant. For example, in 2005, Kimura et al. isolated three new cucurbitane-type triterpenoids from fruits of Japanese bitter melon. The newly identified molecules were (19*R*,23*E*)-5β,19-epoxy-19-methoxycucurbita-6,23,25-trien-3β-ol, (23*E*)-3β-hydroxy-7β-methoxycucurbita-5,23,25-trien-19-al, and (23*E*)-3β-hydroxy-7β,25-dimethoxycucurbita-5,23-dien-19-al [65]. Several studies have also identified several cucurbitane-type triterpenoids from Chinese bitter melon fruit. Methanolic fruit extracts were used to isolate three new triterpenes [58]. Other cucurbitane-type triterpenoids isolated from Chinese bitter melon include (23*E*)-5β,19-epoxycucurbita-6,23,25-triene-3β-ol, (19*R*, 23*E*)-5β,19-epoxy-19-ethoxycucurbita-6,23-diene-3β,25-diol and 5β,19-epoxy-cucurbita-6,22*E*,24-trien-3β-ol [66, 67]. Additional CTMs isolated from Taiwanese wild bitter melon were identified as cucurbita-6,22(*E*),24-trien-3β-ol-19,5β-olide, 5β,19-epoxycucurbita-6,22(*E*),24-triene-3β,19-diol, 3β-hydroxycucurbita-5(10),6,22(*E*),24-tetraen-19-al, 19-dimethoxycucurbita-5(10),6,22(*E*),24-tetraen-3β-ol, and 19-nor-cucurbita-5(10),6,8,22(*E*),24-pentaen-3β-ol [68]. Other studies have also identified cucurbitane-type triterpenoids isolated from fruit material, but the origin of the fruit material is not clearly stated. Among the cucurbitane-type triterpenoids identified are 3β,25-dihydroxy-7β-methoxycucurbita-5,23(*E*)-diene, 3β-hydroxy-7,25-dimethoxycucurbita-5,23(*E*)-diene, and 3-*O*-β-D-allopyranosyl-7β,25-dihydroxycucurbita-5,23(*E*)-dien-19-al, 7β,25-dimethoxycucurbita-5(6),23(*E*)-dien-19-al 3-*O*-β-D-allopyranoside, and 25-methoxycucurbita-5(6),23(*E*)-dien-19-ol 3-*O*-β-D-

allopyranoside [52, 69]. While the exocarp of the bitter melon fruit is commonly consumed, one study evaluated the content of the fruit pulp for the content of cucurbitane-type triterpenoids and identified two novel compounds as 25-methoxycucurbita-5,23(*E*)-diene-3β-19-diol and 7β-ethoxy-3βhydroxy-25-methoxycucurbita -5,23(*E*)-din-19al [70].

2.3.2. Leaves

Similarly, several cucurbitane-type triterpenoids have been isolated from leaves of the bitter melon plant. For example, 3β,7β,23-trihydoxycucurbita-5,24-diene-7-*0*-β-D-glucoside, 3β,7β,25-trihydroxycucurbita-5,23(*E*)-dien-19-al, were identified from leaves of bitter melon, along with previously identified momordicin and momordicosides [71]. Triterpenoids isolates from leaves of bitter melon plant have also been found to have potent biological activities. For example, Japanese bitter melon methanolic leaf extracts yielded 11 previously isolated and six new compounds, (23E)-3β,25-dihydroxy-7β-methoxycucurbita-5,23-dien-19-al, (23S*)-3β-hydroxy-7β,23-dimethoxycucurbita-5,24-dien-19-al, (23R*)-23-O-methylmomordicine IV, (25ξ)-26-hydroxymomordicoside L, 25-oxo-27-normomordicoside L, and 25-O-methylkaravilagenin D [51]. In another study, lanost-5, 23(Z)-diene-3β,7β,25-triol-30a-carbaldehyde, known as charantal, was isolated from leaves of bitter melon and was shown to have strong antitubercular activity [72]. CTMs of similar structural characteristics, such as (19*R*,23*E*)-5β,19-epoxy-19,25-dimethoxycucurbita-6,23-dien-3β-ol and (19*R*,23*E*)-5β,19-epoxy-19-methoxycucurbita-6,23-diene-3β,25-diol, were also identified in other *Momordica* species [73].

Recently, sixteen cucurbitane-type triterpenoids were isolated from leaves and vines of bitter melon, of which six were new compounds. These novel compounds were identified as (3β,7β,15β,23E)-3,7,15,25-tetrahydroxycucurbita-5,23-dien-19-al, (3β,7β)-3,7,22,23-

tetrahydroxycucurbita-5,24-dien-19-al, (3β,7β)-3,7,23,24-tetrahydroxycucurbita-5,25-dien-19-al, (3β,7β,23*S*)-3,7,23-trihydroxycucurbita-5,24-dien-19-al 7-β-D-glucopyranoside, (3β,7β,23E)-cucurbita-5,23-diene-3,7,19,25-tetrol 7-β-D-glucopyranoside, and (3β,7β,23E)-3,7-dihydroxy-25-methoxycucurbita-5,23-dien-19-al 3-β-D-allopyranoside [57].

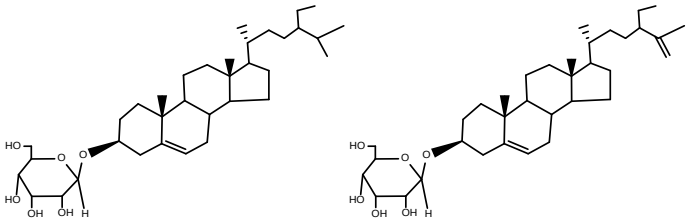
2.3.3. Stems

Further studies, focusing on the identification of cucurbitane-type triterpenoids from bitter melon ethanolic stem extracts led to the discovery of several new molecules. In a study by Chang et al., five new cucurbitane-type triterpenes, (23*E*)-25-methoxycucurbit-23-ene-3β,7β-diol, (23*E*)-cucurbita-5,23,25-triene-3β,7β-diol, (23E)-25-hydroxycucurbita-5,23-diene-3,7-dione, (23*E*)-cucurbita-5,23,25-triene-3,7-dione, and (23*E*)-5β,19-epoxycucurbita-6,23-diene-3β,25-diol were isolated bitter melon stems and characterized along with a previously identified molecule [74]. Similarly in 2008, stems were used for isolation of four new compounds cucurbita-5,23(*E*)-diene-3β,7β,25-triol, 3β-acetoxy-7β-methoxycucurbita-5,23(*E*)-dien-25-ol, cucurbita-5(10),6,23(*E*)-triene-3β,25-diol, and cucurbita-5,24-diene-3,7,23-trione [75]. Additional isolation efforts yielded triterpenoid from stems, identified as 3β-hydroxymultiflora-8-en-17-oic acid, cucurbita-1(10),5,22,24-tetraen-3α-ol and 5β,19β-epoxycucurbita-6,22,24-trien-3a-ol, which were also shown to possess antioxidant properties [76].

2.4. Sterols

Several acylglucosyl sterol have been isolated from the immature *Momordica charantia* fruit, but many were in such low concentrations that their characterization was not performed. The major acylglucosyl sterol was 3-O-[6′-O-palmitoyl-β-d-glucosyl]-stigmasta-5,25(27)-diene while the minor component was 3-O-[6′-O-stearyl-β-d-glucosyl]-

stigmasta-5,25(27)-diene [77]. Another group of sterols that received considerable attention was a group of molecules known as charantin (Figure 2-4). Charantin is composed of a 1:1 mixture of sitosteryl glucoside and stigmasteryl glucoside. Early studies have proposed charantin to be attributing to bitter melon's antidiabetic properties [78]. Additional sterols have been identified from fruits including 24(R)-stigmastan-3β,5α,6β-triol-25-ene 3-*O*-β-glucopyranoside, 25ξ-isopropenylchole-5,(6)-ene-3-*O*-β-D-glucopyranoside and 7-oxo-stigmasta-5,25-diene-3-*O*-β-D-glucopyranoside [41, 58, 79].



A B

Figure 2-4. Charantin a mixture of sitosteryl glucoside (A) and stigmasteryl glucoside(B)

2.5. Ascorbic acid

Ascorbic acid is ubiquitous in fruits and vegetables and is widely accepted as one of the principal antioxidants for aiding in human health. Compared to other popular fruits and vegetables, relatively little attention has been dedicated to determining the levels of ascorbic acid in bitter melon. The range of ascorbic acid levels in bitter melon varies depending on cultivar, cultivation methods, and processing. For example, the use of 1-methylcyclopropene slowed down the loss of ascorbic acid during storage [80] . Variation in ascorbic acid content is influenced by genotype and fruit maturity with levels ranging from 12 to127 mg/100g [81, 82] . Bitter melon leaves are also a rich source of ascorbic acid with levels ranging from 20 to 122 mg/100g depending on maturity and drying methods used [83].

Additionally the levels of ascorbic acid in minimally processed irradiated bitter melon were observed to be relatively stable throughout storage [84] .

2.6. Polyphenolics

Phenolic compounds have also been reported to be present in various tissues of the bitter melon plant. Studies have correlated bitter melon phenolic content with its widely reported antioxidant capacity. Phenolic compounds such as gallic acid, protocatechuic acid, gentisic acid, catechin, vanillic acid , chlorogenic acid, syringic acid, epicatechin, *p*-coumaric acid, ferulic acid, sinapic acid, benzoic acid, *o*-coumaric acid and *trans*-cinnamic acid have all been reported in bitter melon fruit[85]. The levels of these phenolic compounds have been reported to fluctuate in response to cultivar, maturity, tissue and drying methods [83, 86, 87]. In addition of the presences of phenolic compounds in bitter melon reproductive tissues (fruit and seeds), phenolic compounds have also been reported in vegetative plant tissues such as stems, leaves and roots[88, 89].

2.7. Carotenoids

As the bitter melon fruit matures it undergoes dramatic physical and physiological changes. The fruit pericarp changes from emerald green or white to bright orange when fully ripe. Furthermore, the membrane surrounding the seeds changes to white to bright orange. These changes correspond to the changes in the levels of various carotenoids found in bitter melon tissues. Lycopene, lutein and β-carotene are carotenoids that have been correlated to anti-cancer, anti-oxidant and anti-inflammatory activities [90]. The composition of carotenoids has been characterized before, but recent advances in analytical methods now allow for more accurate determination of carotenoids and their bioactive isomers. The change in color of bitter melon from green to orange has been correlated to the degradation

of chlorophyll and accumulation of β-carotene. β-carotene levels in bitter melon have been reported to range from 15 to 300 µg/g [91-93] . Additionally, the levels of lutein have also been reported in bitter melon ranging from 10 to 206 µg/g. Furthermore, the bright red color of the bitter melon seed aril has been reported to be in response to high levels of lycopene in the tissues, mainly *trans*-lycopene. Levels of *trans*-lycopene in bitter melon and other *Momordica* species range from around 200 to over 6000 µg/g [87, 94]. There are several health benefits related to *trans*-lycopene but the bioavailability is much higher for *cis*-lycopene isomers. To date there is no record of *cis*-isomer levels in bitter melon.

2.8. Diabetes

Currently, the incidence of diabetes, principally type-2 diabetes, is growing at an alarming rate in developed and developing nations. It is projected that by 2050, if the current trends persist, one in three Americans will be diabetic [95]. Type-2 diabetes is a metabolic disorder characterized by sub-optimal regulation of glucose in the body, in response to the malfunction of several tissues in the body, such as the liver, muscle, adipose, and pancreatic tissues [96]. The etiology of diabetes is very complex. Under normal homeostatic conditions glucose is metabolized or utilized by the actions of pancreatic hormones such as glucagon and insulin. When the release of these hormones is hindered or the signaling is no longer effective, the homeostatic balance of glucose is affected. These imbalances affect the function of glucose utilizing tissues such as adipose, muscle, liver and pancreatic tissues. Imbalances are typically caused by over-nutrition, poor-nutrition, and a sedentary life style, in addition to genetic and other physiological factors. Two major components of diabetes are the failure to release insulin in response to high levels of glucose and the resistance of tissues to insulin.

Insulin release is a response that is carried out by pancreatic β-cells in response to high glucose levels. Defective insulin secretion has been attributed to glucotoxicity, lipotoxicity, oxidative stress, endoplasmic reticulum (ER) stress and the alteration of gut microbiota [97]. All of these conditions are associated with the inflammatory response. Increase in glucose levels in response to over-feeding results in increased metabolic stress resulting in the generation of reactive oxygen species (ROS). The increase in glucose levels result in an increase in demand for insulin leading to ER stress. Additionally, free fatty acids in circulation due to high fat diet and the resulting change in gut microbiota also lead to the production or release of molecules that can stimulate an inflammatory response. These activities typically result in the production of proinflammatory cytokines such as TNF-α and IL-6 [98]. Pancreatic β-cells are sensitive to ROS production as a result of inflammation and lead the eventual malfunction and cell death.

Insulin resistance, also associated with the inflammatory response, results from cells accumulating excessive nutrients which in turn lead various tissues not reacting to circulating insulin [99]. The excessive nutrients in adipose cells leads to ER stress and hypertrophy. These conditions result in the initiation of the inflammatory response. At this time macrophage migrate towards the site of inflammation in adipose tissue leading to hypoxia and the induction of further inflammatory responses[100]. Additionally, glucose transporters in muscles cells become insensitive to constant presence of insulin the blood. Both deficient insulin secretion and resistance lead to a hyperglycemic condition.

Current management strategies include the supplementation of insulin to make up for insufficient levels, preventing the absorption of glucose by the inhibition of glucose metabolizing enzymes and to stimulate the uptake of glucose in to various tissues.

Recently, different approaches have been geared toward the protection of tissues from the underlying cause of all of these aliments, inflammation. Many studies, have reported that routine consumption of fruits and vegetables is inversely correlated to the development of several chronic diseases, such as cancer or diabetes [101-103]. As such, bitter melon has the potential to become the next superfruit.

2.9. Anti-diabetic bitter melon studies

Bitter melon has been reported to have several health promoting properties. Among these are anti-cancer, anti-bacterial, anti-obesity, anti-oxidant, anti-inflammatory and anti-diabetic. The anti-diabetic properties of bitter melon have been the focus of several research programs for the past several decades. Many studies have been published regarding the effect of bitter melon on the various aspects of diabetes etiology and diabetic complications. Accumulating evidence indicates that bitter melon is indeed a potential antidiabetic agent.

2.9.1. Clinical studies

Early clinical trial evaluated the effects of bitter melon preparations and extracts on diabetic patients. The results of these studies indicated that bitter melon significantly reduces blood glucose levels, reduces post-prandial serum glucose, and improved glucose tolerance [104-106]. Recent studies have reported improved glycemic control with various bitter melon treatments [107-112]. Treatment with bitter melon has been shown to reduce glucose level in the blood and the reduction of fasting glucose levels [113-116]. The lowering of glucose levels may be due to the reported increase in insulin levels also correlated to bitter melon treatments [117, 118].

2.9.2. *In vivo* studies

In vivo studies involving rodents (mice/rats) have also evaluated bitter melon's anti-diabetic potential. Treatments with bitter melon extracts in animal models have concluded that bitter melon has hypoglycemic effects and improved fasting glucose levels [5, 119-121]. Animal studies demonstrated that some possible ways bitter melon was exerting its hypoglycemic effect was by increasing glucose uptake in tissues, increasing insulin sensitivity, and decreasing gluconeogenesis in the liver [122-124]. Purification and screening of specific compounds extracted from bitter melon have also yielded promising results. High molecular weight molecules extracted from bitter melon have been reported to reduce blood glucose levels in diabetic mice, but the molecules were not identified in the study [125]. Similarly, A compound identified as "C2", resulted in decrease glucose levels in diabetic mice [126]. In the same study, C2C12 myocytes were used to elucidate the possible mechanism of action. Here it was concluded that this compound resulted in the translocation of glucose transported to the membrane, hence increasing glucose uptake. The use of *in vitro* studies can help in elucidating the possible mechanisms of action carried out by bitter melon extracts and purified compounds from *in vivo* studies.

2.9.3. In vitro studies

The main cell type used for investigating diabetes *in vitro* are β-cell, muscle cells, adipose cells and liver cells. Pancreatic β-cells produce insulin in response to high circulating glucose levels. The malfunction of pancreatic cells leads to insufficient release of insulin. The treatment of β-cells with bitter melon *in vitro* has resulted in the protection and repair from inflammatory damage and increase insulin secretion[127-129]. Bitter melon extracts and compounds have been found to stimulate glucose uptake and increase insulin

sensitivity in muscle cells in culture [130] [131]. Furthermore, bitter melon has been shown to influence adipocyte differentiation, hypertrophy, lipogenic gene expression, and adiponectin secretion[132-134]. Lastly, bitter melon compounds were found to have a hypoglycemic effect on FL83B hepatocytes[135, 136]. Results of all of the above studies illustrate how bitter melon has potential to be a useful antidiabetic agent by affecting various aspects of diabetes.

2.10. Inflammasome

Inflammasomes are cellular multiprotein complexes that are responsible for sensing and reacting to pathogen and danger associated molecular patters (PAMPs and DAMPs). The end result in the activation of inflammasome complexes is the cleavage of pro-IL-1β and pro-IL-18 to produce proinflammatory cytokines IL-1β and IL-18. The inflammasome complex can be triggered by a wide range of stimuli ranging from bacteria, fungi, viruses, silica, asbestos, ATP and uric acid crystals (to name a few), but ultimately all trigger the production of IL-1β, IL18 and in some cases IL-33. These are the only cytokines reported, to date, to be produced by the NLRP3 inflammasome because of the presence of the caspase subunit of the inflammasome, namely caspase-1[137, 138] . Caspase-1 has only a few know substrates, principally of IL-1β, IL18 and possibly IL-33. There are other less characterized inflammasomes that have other caspase types in their structure but they have also been reported to produce IL-1β and IL 18. Inflammasome were initially thought to be found solely in macrophages and dendritic cells, but recent studies had reported their occurrence in many cells such as endothelial cells and adipose cells. Most studies involving the inflammasome typically utilize macrophages for inflammasome research. There have been several inflammasome classes reported to date but the most widely

studied is the NLRP3 inflammasome complex, due to its ability of react to a wide range of molecular signals. The signals are recognized by nod-like receptors (NLRs) which then recruits the apoptosis-associated speck-like protein, containing a C-terminal caspase recruitment domain (CARD) named ASC, and caspase-1. The activated caspase -1 in turn cleaves pro-IL-1β and pro-IL-18 into their active forms. This activation leads to the release of inflammatory cytokines, and enhanced inflammatory response and pyroptosis[139, 140] .

The activation and formation of inflammasomes are broadly lumped into two general steps, which of course is a much more complicated process. The first step involves the priming of recognition of specific signals by TLRs membrane proteins, TLR4 specifically. The ligation between a signal (LPS or IL-1β, to name a few) and TLR4 in turn leads to the activation of NF-κB resulting in the transcription of the components NLPR3 and pro-IL-1β. The second phase involves the sensing of endogenous cell signals that leads to the formation of the inflammasome complex such as ROS. The ligation or stimulus of these signals cause a conformational change in the NLRP3 domain that allow the recruitment of the ASC domain and lastly the recruitment of caspase-1 through the CARD domain of ASC. The end result is the maturation of pro-IL-1β to IL-1β. The production of IL-1β in turn lead to the activation of toll like receptors and inflammasomes which in turn may lead to the polarization of macrophages to the M1 phenotype [141].

3. EVALUATION OF BITTER MELON (*MOMORDICA CHARANTIA*) CULTIVARS GROWN IN TEXAS AND LEVELS OF VARIOUS PHYTONUTRIENTS[2]

3.1. Introduction

Bitter melon (*Momordica charantia*) is currently an under-utilized horticultural crop in the United States, interestingly it is commonly grown and consumed in many Asian and South American countries. Bitter melon is a monoecious plant typically flowering about 30-50 days after germination. Marketable fruit is produced 15-20 days after fruit set[142-144]. Bitter melon grows well in hot and humid areas with well drained sandy loam soils. As such, the development of bitter melon is sensitive to prolonged water logging.[143] Bitter melon is typically grown on trellis systems for support and healthy growth of the fruit. Several cultivars have been developed differing in fruit size, shape, color and texture, such as round or triangular tubercles[143]. Diverse rheological profiles have also resulted from such differences, such as bitterness, texture and overall consumer preference.

Despite its characteristic bitter taste, bitter melon is regularly consumed in many cultures due to its reported potential health benefits attributed to the presence of health promoting compounds such as ascorbic acid, polyphenols and triterpenoids[86, 88, 144, 145] . Various parts of the bitter melon plant, such as the fruit exocarp, seeds, leaves, roots and vines, have all been reported in traditional medicine to possess health promoting properties[51, 146]. Studies have reported on the anti-viral, anti-microbial, anti-cancer and

[2] This chapter is reprinted with permission from *Evaluation of bitter melon (Momordica charantia) cultivars grown in Texas and levels of various phytonutrients*, by J.L. Perez, G.K. Jayaprakasha, K. Crosby, B. S. Patil, 2019, Journal of the Science of Food and Agriculture, 99, 379-390. Copyright [2019] John Wiley & Sons, Ltd.

most prominently on its anti-diabetic properties[147-151]. *In vitro* studies have concluded that bitter melon extracts and bioactive compounds regulate glucose uptake, stimulate insulin secretion from pancreatic cells, protect pancreatic β-cells from inflammatory insults, reduce lipid accumulation in adipose cells[47, 53, 152, 153]. Furthermore, *in vivo* studies determined that bitter melon exhibits significant hypoglycemic properties by acting as an insulin secretagogue, displaying insulin mimetic activities, lowering blood glucose levels and the percentage of glycosylated hemoglobin, and modulating carbohydrate metabolizing intestinal enzymes[154-156].

Currently, diabetes rates in the United States are growing at a phenomenal rate[157, 158]. By establishing its optimal grown condition and promoting their potential health benefits, bitter melon production as a specialty crop may be profitable to producers. Currently, limited data is available regarding optimal growing requirements of bitter melon in the United States. Bitter melon producers in the United States consist primarily of small acreage farmers. It is evident from terminal market data that there is a need for more bitter melon production in the United States[159]. Production in the United States mainly serves local and in limited cases of state-wide markets, while the rest of the demand is satiated by importation from neighboring countries, such as Mexico and Guatemala. Therefore, it is imperative that cultivation practices and varietal differences are evaluated in Texas, which is logistically located to possibly serve the bitter melon needs for the United States. Furthermore, it is crucial to evaluate the levels of the antioxidant phytonutrients found in bitter melon to provide consumers reliable information for making intelligent and healthy food choices. To date there is a void in information regarding cultivation practices for

bitter melon, especially for Texas. Furthermore, there is no information regarding which cultivar may perform better under Texas growing conditions. The data presented in this study adds valuable information for growers looking to expand in to the bitter melon specialty market.

3.2. Material and Methods

3.2.1. Plant material

The seeds for the cultivars evaluated in this study were purchased from Evergreen Seed Company (Anaheim, CA). The cultivars consisted of 'Indian Green', 'Indian White', 'Japanese Spindle', 'Hong Kong Green', and 'Large Top'. Seeds were sown on May 4, May 3, and May 2 for years 2013, 2014, 2015 respectively, into black poly seeding trays containing sunshine potting mix #4 and a superficial layer of vermiculite. To facilitate germination, the seed coat was mechanically removed before sowing. The daily addition of reverse osmosis water maintained moisture during the early germination phase. The seeds were placed in the greenhouse for germination and the temperature was maintained at 32 °C until plants attained the recommended size (5-6 true leaf stage) for transplanting into the field.

3.2.2. Experimental site

The cultivation conducted at College Station, TX. The 2013 trial was carried out in Texas A&M Horticulture Farm Lat 30° 36'34.4"N 96°21'16.70"W. Trials for 2014 and 2015 were forced to relocate due to construction efforts at the initial test plot to Lat 30° 37'52.09"N 96°22'15.31"W. Climatic data, such as temperature, precipitation, and solar radiation was obtained from a weather station located at close proximity to the test site

(CR21XL Micrologger; Campbell Scientific, Inc., North Logan, UT). Growing degree-days (GDD) were calculated using the following equations: GDD daily = $(T_{max} + T_{min})/2 - T_{base}$, where T_{max} is the maximum daily temperature, T_{min} is the minimum daily temperature, and T_{base} is the base temperature where bitter melon growth and development is not deemed to occur (16 °C) [143]. Cumulative GDD's were calculated by summing daily GDD's that were above 16 °C. Soil analysis was performed for each test plot and before each growing season at the Soil, Water and Forage Testing Laboratory at Texas A&M University, College Station, TX.

3.2.3. Field preparation and planting

The experimental field was prepared by disking the test plot until the soil was of a fine texture. Subsequently, three rows of raised beds (70 cm wide, 20 cm high) were prepared. The experiment was laid out in a randomized complete block design with 5 cultivars and 3 blocks (replicates). Fifteen individual trellises (4.2 m in length) were built for individual cultivars and replicates (blocks). A 3.0 m spacing was inserted between trellises within the same row. The amount of irrigation required was calculated based on the evapotranspiration (ETo) monthly (mm $month^{-1}$) averages for College Station, Texas reported by the Texas ET Network and the crop coefficient for cucurbits reported by the FAO [160]. The crop coefficient for specific growth stages used were $K_{c\ ini} = 0.5$, $K_{c\ mid} = 0.95$, and $K_{c\ end} = 0.75$. Irrigation was applied via an above ground drip irrigation system (Chapin drip tape-BTF; Jain Irrigation, Inc, Watertown, NY). The drip irrigation tape had emitters spaced at 30 cm and a flow rate of 1.13 L h^{-1} per dripper. Bitter melon seedlings were transplanted when plants attained a length between 10-20 cm. After an initial irrigation of the test plots, seedlings were transplanted into the field at a spacing of 0.6 m

with in row and 2.1 m between rows. The amount irrigation applied was calculated from drip tape flow rate, number of irrigation events and duration of irrigation event. The total fertilizer applied was 166N-0P-0K kg ha^{-1}, 191N-127P-79K kg ha^{-1}, 161N-127P-106K kg ha^{-1} through fertigation during the 2013, 2014 and 2015 seasons, respectively.

3.2.4. Fruit harvest and yield

Bitter melon fruits were harvested once marketable size and color were attained, every 3-4 days throughout the growing season. The harvesting season for the years evaluated consisted of 38 DAP (days after planting) to 146 DAP (2013), 36 DAP to 153 DAP (2014), 32 DAP to 142 DAP (2015). At each harvest, fruits were counted and weighed. Yields were presented in grams per plot, total yield of fruit per season and the yield was extrapolated for yield per hectare (kg ha^{-1}). Non-marketable fruits were not counted towards the total yield.

3.2.5. Water use efficiency

The total water used during the growing season was determined as the sum of the total irrigation water applied to the field and rainfall received during the growing season. Water Use Efficiency (WUE) was determined as the yield of marketable fruit (kg ha^{-1}) per millimeter of total water applied.

3.2.6. Ascorbic acid analysis

Fresh bitter melon pericarp from individual fruits was utilized for ascorbic acid analysis. Fruits were harvested and samples were taken on the same day and stored in -80 °C until extraction. Ascorbic acid, dehydroascorbic acid (DHA) and total ascorbic acid were determined according to our previously reported methods.[161] Briefly, 10 g of bitter melon

pericarp was homogenized in 20 mL of 3% meta-phosphoric acid using a polytron homogenizer (Brinkman, Instrument, Westbury, New York, USA) for 30 s at medium speed followed by a 10 s vortex agitation. Additionally, each sample was sonicated three times for 30 min using a sonic dismembrator (Model F60, Thermo Fisher Scientific, Pittsburgh, PA). The samples were again vortexed and a 2-mL aliquot was transferred into micro-centrifuge tubes. The sample was then centrifuged at 4000 x g for 10 min. The resulting supernatant was used for ascorbic acid analysis.

For analysis of DHA, tris (2-carboxy ethyl) phosphine hydrochloride (TCEP) was used for the reduction of DHA to ascorbic acid. After centrifugation of extracted samples, 0.5 mL of extract was treated with 0.5 mL of 5 mmol TCEP. The mixture was vortexed and incubated for 30 min at 25 °C. Following the incubation period, the samples were analyzed by HPLC. The DHA content in the sample was calculated as the difference between total ascorbic acid (ascorbic acid after reduction with TCEP) and ascorbic acid (ascorbic acid level before reduction with TCEP). For quantification of ascorbic acid levels in bitter melon, a calibration curve was constructed using standard levels ranging from 0.07 to 10 µg per injection. The resulting regression equation was utilized for the quantification of ascorbic acid in bitter melon samples. Three biological replicates were prepared for each sample and each sample was injected three times for HPLC analysis.

3.2.7. Chromatographic conditions for ascorbic acid analysis

Chromatographic separation of ascorbic acid was performed using Alliance 2695 high performance liquid chromatograph (HPLC) system equipped with a 2996 photodiode array detector (Waters, Milford, MA, USA). The separation of ascorbic acid was carried out using a Phenomenex Gemini C_{18} column (4.6 mm× 260 mm, 5 µm particle size)

maintained at 27 °C. The chromatographic separation was performed using an isocratic mobile phase composed of 3 mM phosphoric acid at a flow rate of 1ml per min. A 10 µL sample was injected into the HPLC system for analysis and elution profiles were detected at λ_{254} nm using the photodiode detector. Ascorbic acid peaks were identified by the comparison of retention times and UV spectra. Additionally, mass spectral analysis was performed to further validate the presence of ascorbic acid.

3.2.8. Amino acid extraction

Extraction of free amino acids from bitter melon pericarp was performed according to previously published methods with slight modifications.[162] A 500-mg aliquot of freeze dried bitter melon pericarp was homogenized in 5 mL of 8:2 (v/v) methanol: water (80% MeOH) for 30 s (3 times at 10 s intervals). After homogenization, glass beads were added to the mixture and tubes were placed in a boiling water bath for 20 min to extract free amino acids. The mixture was cooled to room temperature and centrifuged at 4000 x g for 10 min. The resulting supernatant was then filtered through glass wool and rinsed with 1 mL of 80% MeOH. The remaining pellet was re-extracted two additional times to ensure complete extraction of amino acids. The pooled filtrates were concentrated and adjusted to a final volume of 5 mL with 80% MeOH.

3.2.9. Derivatization for amino acid analysis by HPLC

A 2 mL-aliquot of extract was reduced to dryness using SPD 1010 SpeedVac (Thermo Savant, Holbrook, NY, USA) and the residue was taken-up in 500 µL of 20 mmol HCL and filtered through 0.2 µm filters. The filtrates containing the extracted amino acids were derivatized using Waters AccQ-Tag following their protocol as described previously.[163] The derivatized amino acids were separated on an AccQ Tag (4 µm particle size) (3.9 mm

× 150 mm) column fitted in Waters Alliance 2695 HPLC system equipped with a 2996 photo-diode array detector. The chromatography was monitored at 254 nm. The chromatographic conditions were maintained exactly as described in the "AccQ-Fluor Reagent Kit" manufacturer's specification.

Regression curves using standard compounds were developed for each amino acid following the same derivatization and separation techniques described above. Concentrations of individual amino acids in the extracts were calculated using regression equation. The amounts of individual free amino acids are reported as nmole per mg of freeze-dried tissue.

3.2.10. Extraction of organic and phenolic acids

Five grams of freeze-dried pericarp was extracted with 80% MeOH in 50 mL tubes. After the addition of the solvent, the solution was vortexed for two minutes and homogenized for an additional minute. The tubes were then placed in an ultrasonication bath and sonicated for 10 min. The samples were centrifuged and the resulting supernatant was recovered. This process was repeated two additional times, after which, the resulting extracts were pooled with the previous. The combined extracts were concentrated under vacuum and freeze dried. All samples were subsequently stored at -80°C until analysis.

3.2.11. Measurement of total phenolics

The total phenolic content of each extract was determined by the Folin- Ciocalteu (FC) method and adjusted for a 96-well format as presented our previous publications.[164] The results were presented as catechin equivalents (CE) mg per g of freeze-dried material.

3.2.12. LC/QTOF-MS

All bitter melon extracts were analyzed by an ultrahigh performance liquid chromatography –electrospray ionization high resolution time of flight mass spectrometer (UHPLC-ESI-HR-QTOFMS) analysis was performed on a maXis impact mass spectrometer (Bruker Daltonics, Billerica, MA) coupled to a 1290 Agilent Rapid Resolution LC system (Agilent, Santa Clara, CA) using a electrospray ionization source (Bruker Daltonics, Billerica, MA). Metabolites were separated on an Ascentis Express C_{18} column (10cm x 2.1mm, 2 µm) (Supelco, Bellefont, PA) and maintained at 70 °C. The column was eluted at a flow rate of 0.2 mL/ min and a gradient consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Initial composition of gradient was comprised of 100% A, linearly decreased to 95% A in one min, further decreased to 40%A in 5.5 min and lastly to 5% A in 2.5 min. The mobile phase condition was returned to 100% A in one min and maintained at those conditions for an additional one min before the start of the next injection. The samples were monitored at 210 and 254 nm during the chromatographic run. The mass spectrometer conditions were the following: ionization mode ESI (+); MS scan range 50–1000 m/z; end plate offset −500 V; capillary −3000 V, nebulizer gas (N2) 0.4 bar; dry gas (N2) 4 L/min; dry temperature: 180 °C; ion transfer conditions funnel RF: 200 Vpp; multiple RF: 200 Vpp; quadruple low mass 55 m/z; collision energy 5.0 eV; collision RF 600 Vpp; ion cooler RF 50–250 Vpp ramping; transfer time 121µs; pre-pulse storage time 1 µs. Calibration was done before each run through a loop injector of 20 µL of 10 mM sodium formate.

Mass spectral data obtained was processed with the Data Analysis (version 4.1) and TargetAnalysis™ software packages. An in-house database was constructed comprising of

several phenolic compounds and organic acids. The database consisted of the monoisotopic masses, elemental composition, and retention times. These programs searched for targeted masses of metabolites in extracts based on the factors defined above and compared them to previously measured standards. Extracted ion chromatograms of the expected $[M+H]^+$ ion of standards in the database were created with a narrow mDa m/z range (± 10 mDa). Compounds were positively identified when a mass tolerance of < 50 ppm and a retention time window of ± 0.1 min were achieved.

3.2.13. Quantification of organic and phenolic acids

Chromatographic separation of organic and phenolic acids phenolic compounds present in 80% MeOH bitter melon pericarp extracts was performed by means of a Waters Alliance 2695 HPLC system (Waters, Milford, MA) equipped with a 2996 photo-diode array detector using a Phenomenex Gemini C_{18} Column (4.6 mm× 260mm, 5 µm particle size) maintained at 27 °C, and eluted at 1mL/min. Organic and phenolic acids were identified by matching peak retention times (t_r) and the UV spectra of known standards and extracts. For quantitative measurement of different compounds in the extracts, regression curves were developed using known standard compounds.

3.2.14. Statistical analysis

Data for fruit yield per hectare, average yield per plot, average fruit number, average number of fruits per plant, average fruit weight and water use efficiency, were taken from three replicates per cultivar and each replicate consisted of seven plants. All chemical analysis was performed in triplicate. Statistical analysis was performed using the JMP 11 software (SAS Institute, 2014). The ANOVA platform was used to evaluate the differences in yields, WUE and chemical analysis. The Tukey's test of significance between means

was used for illustration of significance. Significance was reported if the P-value was at least $P < 0.05$. The Fit Model platform was utilized to evaluate the effects of cultivar, season and cultivar & season using the effects test. To evaluate the effect of various environmental parameters on yield the multivariate platform was used.

3.3. Results and Discussion

3.3.1. Evaluation of growth of bitter melon under field conditions

Bitter melon is a monoecious plant with reproductive and growing characteristics that vary with climatic conditions, cultural practices and cultivar.[143] Bitter melon is well adapted to tropical and subtropical areas and is typically grown in the spring and summer seasons. Cultivation during the winter time is also possible in some subtropical areas, but this is not a common practice due to its susceptibility to frost. In this study, bitter melon was grown in College Station, Texas in 2013, 2014 and 2015. Climatic conditions and soil nutrients were monitored to evaluate their effects on bitter melon growth performance. The monthly maximum, minimum and average temperatures along with growing degree days for each season are presented in **Table 3-1**. Additionally, average daily photoperiod and radiation for each month and season are presented in **Table 3-2**. Bitter melon performs well in a variety of soil types and environmental conditions. The soil profile for each of the test plots is presented in **Table 3-3**. Optimally, bitter melons should be grown in well drained sandy loam with a pH ranging from 6.0 to 6.7[165]. Bitter melon yields for the three growing seasons were evaluated (**Figure 3-1 and Table 3-4**). Compared to 2013, the yields in the 2014 and 2015 growing seasons were drastically reduced in all varieties evaluated. The reduction in yields for 2014 and 2015 were significant except when comparing yields from the 2013 and 2015 in the ‘Large Top’ cultivar. Even though the

difference in yield was noticeable, the large standard error mean between replicates deemed the yields were similar.

Table 3-1. Average monthly minimum, maximum and mean temperatures for the 2013, 2014, and 2015 growing season. Additionally growth degree days (GDD) are also presented.

	Min Temp. (°C)			Max Temp (°C)			Mean Temp (°C)			GDD[a]		
	2013	2014	2015	2013	2014	2015	2013	2014	2015	2013	2014	2015
June	18.9	19.4	22.2	41.1	34.4	34.4	29.5	28.1	27.9	324.3	289.8	285.1
July	20.0	20.6	21.7	39.4	36.1	38.3	29.4	28.4	29.4	415.9	386.2	418.1
August	20.6	20.6	21.1	40.0	37.2	41.1	30.7	29.3	29.9	455.6	412.0	432.6
September	16.7	15.6	16.7	37.8	36.7	36.1	28.4	26.9	27.6	374.0	329.0	347.2
October	11.1	11.1	11.7	32.8	33.9	35.6	23.8	24.2	24.7	94.0	98.0	104.1

[a]GDD calculated by the following equation: $(T_{max}+T_{min})/2-T_{base}$. T_{max} =maximum daily temperatre, T_{min}= minimum daily temperature, T_{base}= 16°C (temperature at which growth and development is not deemed to occure

Table 3-2. Average daily photoperiod and radiation for each month during the 2013, 2014, and 2015 growing seasons.

	2013		2014		2015	
	Av. Daily Photoperiod (h)	Av. Daily Radiation (KW/m^2)	Av. Daily Photoperiod (h)	Av. Daily Radiation (KW/m2)	Av. Daily Photoperiod (h)	Av. Daily Radiation (KW/m2)
June	1406.8	8.1	1406.8	6.9	1406.8	6.6
July	1366.5	7.2	1368.1	7.1	1368.4	7.7
August	1307.6	7.1	1304.1	7.2	1304.4	7.2
September	1214.3	5.9	1214.7	5.7	1214.6	5.9
October	1141.2	5.4	1141.7	5.2	1142.0	5.2

Table 3-3. Pre-plant physical and chemical soil parameters for the 2013, 2014, and 2015 growing seasons.

	2013	2014	2015
pH	7.7	6.3	6.1
Conductivity (umho/cm)	137	34	109
Nitrate-N (ppm)	7	5	11
Phosphorus (ppm)	298	44	44
Potassium (ppm)	153	75	62
Calcium (ppm)	2162	611	424
Magnesium (ppm)	300	84	46
Sulfur (ppm)	15	3	6
Sodium (ppm)	212	36	22
Sand (%)	69	86	35
Silt (%)	20	11	34
Clay (%)	11	3	31
Text Class	Sandy Loam	Loamy Sand	Clay loam
Organic Matter (%)	3.02	0.61	0.58

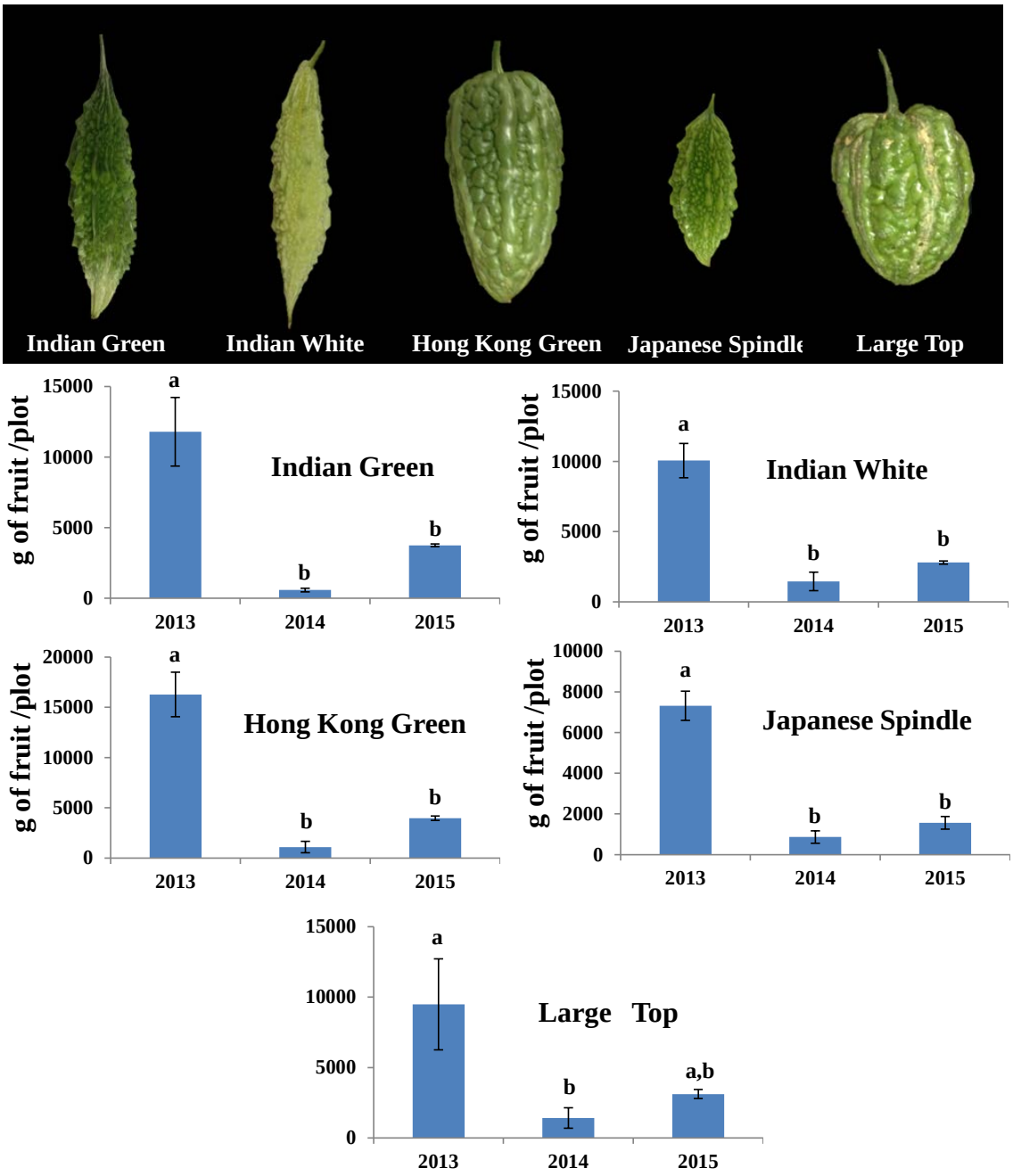


Figure 3-1 Average yield in grams of marketable fruit per plot for each cultivar during the 2013, 2014, and 2015 growing seasons. Similar lettering above bars depict statistical similarities. Bars represent the standard error mean. Difference in letters above bars depict a statistical difference of at least $p<0.05$

Table 3-4. Total yield in kilograms, number of fruits, fruits per plant, average fruit weight in grams extrapolated yield per hectare in kilograms and water use efficiency (WUE) for each cultivar evaluated during 2013, 2014 and 2015. Difference in letters within column of growing season depict a statistical difference of at least p<0.05.

	Total Yield (Kg)	Av. Number of Fruits per Plot	Fruits Plant^{-1}	Average Fruit Weight (g)	Kg ha^{-1}	WUE
			2013			
Indian Green	35.4	83.6 ± 23.7b	11.9 ± 3.3b	140.1 ± 17.5c	15116.4 ± 5368.2 ab	36.1 ± 12.9ab
Indian White	30.1	71.3 ± 2.3bc	10.1 ± 0.3bc	141.5 ± 32.9c	12882.8 ± 2700.4 ab	30.9 ± 6.5ab
Hong Kong Green	48.8	58.0 ± 15.3c	8.29 ± 2.2c	282.2 ± 17.3b	20839.4 ± 4915.9 a	50.0 ± 11.8a
Japanese Spindle	21.9	146.0 ± 38.9a	20.8 ± 5.5a	52.1 ± 14.8d	9371.5 ± 1590.6 b	22.5 ± 3.8bc
Large Top	28.4	29.0 ± 18.1d	4.1 ± 2.5c	345.5 ± 173.3a	12149.4 ± 7173.5 b	29.2 ± 17.2b
			2014			
Indian Green	1.8	9.0 ± 3.0a	1.3 ± 0.4a	64.4 ± 4.55bc	750.5 ± 292.1a	1.8 ± 0.7a
Indian White	4.3	21.3 ± 9.4a	3.0 ± 1.3a	63.8 ± 28.9bc	1857.7 ± 1475.5a	4.5 ± 3.5a
Hong Kong Green	3.3	6.6 ± 5.5a	0.9 ± 0.7a	156.1 ± 17.4a	1374.2 ± 1263.2 a	3.4 ± 3.0a
Japanese Spindle	2.6	18.6 ± 6.6a	2.6 ± 0.9a	44.6 ± 17.4c	1108.5 ± 670.6 a	2.7 ± 1.6a
Large Top	4.2	8.0 ± 7.2a	1.1 ± 1.0 a	117.7 ± 101.9ab	1806.8 ± 1621.2a	4.3 ±3.9a
			2015			
Indian Green	11.3	57.0 ± 8.8a	8.1 ± 1.3a	67.1 ± 12.4b	4806.7 ± 204.6 a	11.5 ± 0.5a
Indian White	8.4	37.3 ± 8.5bc	5.3 ± 1.21bc	76.9 ± 12.7b	3588.1 ± 250.6a	8.6 ± 0.6a
Hong Kong Green	11.9	26.3 ± 0.5cd	3.8 ± 0.1cd	150.7 ± 13.8a	5086.9 ± 480.7 a	12.2 ± 1.2a
Japanese Spindle	4.6	49.6 ± 11.0ab	7.1 ± 1.6ab	30.9 ± 4.1b	1999.8 ± 693.4a	4.8 ± 1.6a
Large Top	9.3	21.0 ± 8.7d	3.0 ± 1.2d	163.8 ± 56.7a	3989.5 ± 696.3 a	9.6 ± 1.7a

Several factors, individually or compounded, may have influenced the difference in yield among the years evaluated. First of all, the 2013 growing season was conducted at the Texas A&M University Horticulture Farm, College Station, TX. The soil profile for this location is presented in **Table 3-3**. The texture of the soil at this location was classified as sandy loam with a pH of 7.7 and organic matter of 3.02%. While the pH of this location is slightly alkaline, bitter melon can very well tolerate soil pH up to 8.0[165]. For the following years of the field evaluations, cultivation was relocated to another location of Texas A&M University due to university construction efforts. The soil profiles for the subsequent evaluations **(Table 3-3**) illustrate a change in soil texture and organic matter.

When comparing 2013 yield data to the subsequent evaluation years, it is important to note the drastic change in organic matter content in the soil profile. Soil organic matter is known to influence plant growth [166] . The low levels of organic matter in the field for 2014 and 2015 may have played an important role in the significant decrease in yield levels in bitter melon. While initial nutrient and micronutrient profiles were different, levels were equilibrated for optimal cucurbit growth.

In addition to differences in soil profile, the several climatic conditions among the growing years may have contributed to the drastic difference in bitter melon's growth performance. Temperature profiles for College Station, Texas for each of the growing seasons are presented in **Table 3-1**. Minimum temperatures did not fluctuate drastically for each of the growing season except for the month of June, which ranged from 18.9°C in 2013 to 22.2°C in 2015. Interestingly, maximum temperatures during the evaluated years seem to have played an important role in bitter melon growth. For the 2013 growing season, temperatures were typically higher than the 2014 and 2015 growing season, which was reflected in the mean monthly average (**Table 3-1**). A subtle difference was also observed while comparing growth degree days for each of the seasons. The growth degree day (GDD) was calculated using these means and the threshold growing temperature for cucurbits (16 °C) which resulted in a total of 1663, 1515, and 1587 GDD for 2013, 2014, and 2015, respectively. While several factors influence the growth and productivity of bitter melon, it is important to consider the possible role temperature played in this field evaluation concerning differences in yield.

For each of the cultivars evaluated in this study, the yield, number of fruits per plant, and fruit weight obtained in the 2013 season were significantly higher than the 2014 and

2015 season. In the 2013 growing season, maximum temperatures were usually higher than the 2014 and 2015 growing seasons. Similarly, the GDD was higher in the 2013 growing season compared to 2014 and 2015. Optimal growth temperatures for bitter melon production range from 25 °C to 30 °C[143]. Higher GDD in 2013 may be one of the factors that contributed to higher yields in 2013 compared to the two subsequent years. For the 2013 growing season, the 'Japanese Spindle' cultivar had significantly higher number of fruits per replicate plot (146) and plant (20.8) compared to the other evaluated cultivars. In 2014, there was no statistical difference in number of fruits per plot among cultivars, while in 2015 the highest number of fruits was observed in the 'Japanese Spindle' (49.6) and 'Indian Green' (57) cultivars. For 2013, the highest average fruit weight was 345.5g in the 'Large Top' cultivar. In 2014 both the 'Large Top' (117.7 g) and 'Hong Kong Green' (156.1 g) cultivars had the largest fruit weights. The extrapolated yield per hectare in 2013 was the highest in the 'Hong Kong Green' cultivar, yielding 20839.4 kg ha^{-1}. The yield per hectare of the 'Hong Kong Green' cultivar was statistically similar to 'Indian Green' and 'Indian White' cultivars, 15,116.4 and 12,882.8 kg ha^{-1} respectively. In 2014 and 2015 there was no significant difference in yield among all the cultivars. Similarly, water use efficiencies for each cultivar mirrored the yield in kg ha^{-1} differences for 2013 2014 and 2015. Water use efficiency was drastically higher in all cultivars evaluated in the 2013 growing season compared to 2014 and 2015.

Water requirements and availability also play a crucial role in fulfilling the plant's need for fruit production. The deficit water requirement for the bitter melons grown in this study was fulfilled via drip irrigation. The total amount of water applied (irrigation + rainfall) is presented in **Table 3-5**. The overall water levels were held constant for each of

the trials. It is important to note that the amount of rainfall in 2014 was almost double (414.53 mm) the amount of rainfall for the 2013 (213.11 mm) and 2015 (218.95 mm) growing seasons. In July 2014, College Station, TX experienced high rainfall activity (169.42 mm) of rainfall compared to 36.57 mm and 7.87 mm for 2013 and 2015 respectively. Ample rainfall in the July 2014 season led to some water stagnation in the field and subsequently to several plants displaying various disease symptoms. The principle type of growth during most of July is 'vegetative' so the stagnation of water and the incidence of disease affected the growth and reproduction for the remainder the 2014 growing season. Leaf size, branching and flowering were severely reduced compared to the other growing seasons.

Table 3-5. Total rainfall and supplemental irrigation for the 2013, 2014, and 2015 growing seasons.

	Rainfall (mm)	Irrigation (mm)	Total (mm)
2013	213.11	203.44	416.55
2014	414.53	40.82	455.35
2015	218.95	195.96	414.91

Another factor influencing growth and development of plants is the amount of solar radiation received during various growth phases. Higher solar radiation levels are conducive to higher potential fruit yield [167]. During this study, photoperiod and solar radiation was recorded (**Table 3-2**). The average daily radiation values varied throughout the growing seasons. In the initial part of the growing season (June) for 2013 the average daily radiation was 8.1 KW/ m^2 compared to 6.9 and 6.6 in 2014 and 2015, respectively. Solar radiation values did not change for the remaining months of the bitter melon growing

season. The dose of solar radiation during June 2013 may have triggered more photosynthetic activity early during the plants vegetative growth leading to vigorous vine growth and higher yield. The Pearson's correlation for yield its relation to the various agronomic parameters is presented in **Table 3-6**.

Table 3-6. Pearson's correlation coefficients of yield and agronomical parameters

	Yield	WUE	GDD	Prec	OM
Yield	1	0.99***	0.87***	-0.57***	0.90***
WUE		1	0.87***	-0.57***	0.90***
GDD			1	-0.84***	0.87***
Prec				1	-0.46***
OM					1

WUE=water use efficiency, GDD=growth degree days, Prec= precipitation, OM= organic matter. ***-indicates significance at $p<0.001$.

3.3.2. Variation of ascorbic acid

The levels of ascorbic acid were determined by HPLC and confirmed by mass spectral data. The levels of ascorbic acid are presented in three forms; ascorbic acid, dehydroascorbic acid and total ascorbic acid (**Figure 3-2**). Ascorbic acid is readily oxidized under physiological condition in plant and animal tissue to form dehydroascorbic acid. The use of 3% meta-phosphoric acid and TCEP is typically used to reduce dehydroascorbic acid back to ascorbic acid. The levels of these two are used to determine the levels of total ascorbic acid. When comparing the various ascorbic acid levels, the 'Japanese Spindle' cultivar grown in the 2013 season had the highest level of total ascorbic acid (162.97 mg/100 g fresh fruit) amongst all cultivars evaluated over the three-year study. Conversely, the lowest levels of total ascorbic acid were found in the 'Hong Kong Green' (42.69 mg/100g fresh fruit) and 'Large Top' (44.72 mg/100g fresh fruit) cultivars

for the same cultivation year (2013). While the 'Japanese Spindle' cultivar was observed to have the highest total ascorbic acid levels in 2013, the levels observed in the subsequent cultivation years decreased. The levels of ascorbic acid for all cultivars followed the similar proportional trend as the total ascorbic acid levels suggesting that ascorbic acid was the major form of vitamin C present in the fruit matrix. Interestingly, the levels of dehydroascorbic acid were consistently higher in all bitter melon cultivars harvested in 2013. It is also important to note the at the levels of dehydroascorbic acid was lowest in the 'Hong Kong Green' and 'Large Top' cultivars harvested in 2015. The differences in level among the various cultivars and seasons are presented in **Table 3-7.**

Ascorbic acid is critical in maintain healthy immune system and reducing chronic inflammatory diseases[168]. The ascorbic acid content varies among fruits and vegetables[169]. Furthermore, variations have also been observed in crops in response to genotype and preharvest conditions, such as UV-light, irrigation, and temperature[170]. For example levels of ascorbic acid have been reported to change in response to high growing temperatures and soil nutrients such as nitrogen[169, 171]. In our study, the main difference in total vitamin C content was typically genotypic in nature. One important distinction to note is that in the 2013 growing season the level of dehydroascorbic acid was typically higher that in the other growing seasons. Dehydroascorbic acid is the oxidation product of ascorbic acid. The higher level of dehydroascorbic acid observed in 2013 may have been in response to higher GDD during that particular growing season.

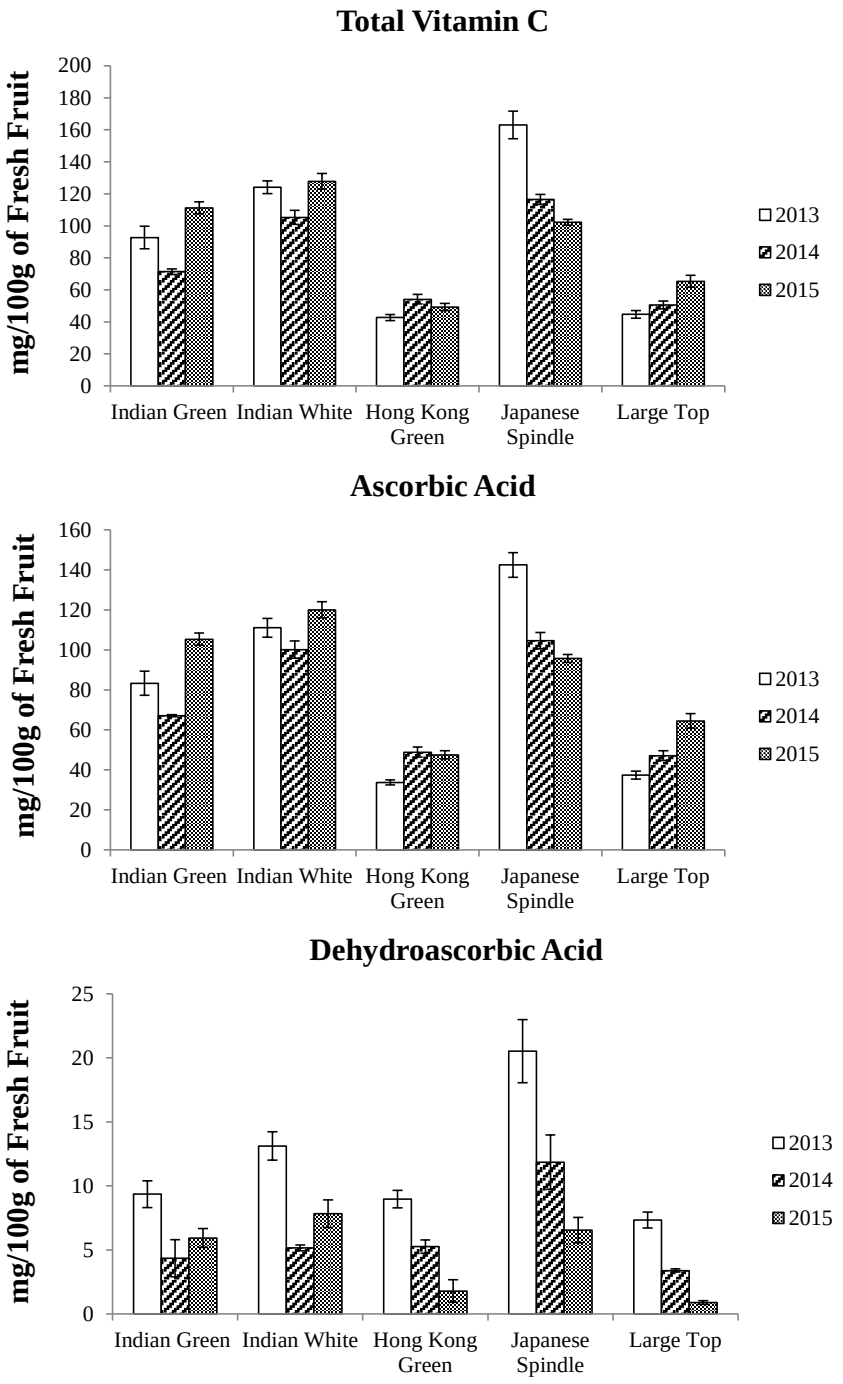


Figure 3-2. Comparison of total ascorbic acid, ascorbic acid and dehydroascorbic acid for each cultivar during the 2013, 2014, and 2015 growing seasons. Values are presented in as ascorbic acid mg/ 100g of fresh fruit ± standard error mean.

Table 3-7. ANOVA statistical analysis of total ascorbic acid, ascorbic acid and dehydroascorbic acid. JS-Japanese Spindle, IW- Indian White, LT- Large Top, IG-Indian Green, and HK – Hong Kong Green.

	Total Ascorbic Acid														
	JS 2013	JS 2014	JS 2015	IW 2013	IW 2014	IW 2015	LT 2013	LT 2014	LT 2015	IG 2013	IG 2014	IG 2015	HK 2013	HK 2014	HK 2015
JS 2013	-	***	***	***	***	***	***	***	***	***	***	***	***	***	***
JS 2014	***	-	NS	NS	NS	NS	***	***	***	NS	***	NS	***	***	***
JS 2015	***	NS	-	*	NS	**	***	***	***	NS	***	NS	***	***	***
IW 2013	***	NS	*	-	NS	NS	***	***	***	***	***	NS	***	***	***
IW 2014	***	NS	NS	NS	-	*	***	***	***	NS	***	NS	***	***	***
IW 2015	***	NS	**	NS	*	-	***	***	***	***	***	NS	***	***	***
LT 2013	***	***	***	***	***	***	-	NS	*	***	**	***	NS	NS	NS
LT 2014	***	***	***	***	***	***	NS	-	NS	***	*	***	NS	NS	NS
LT 2015	***	***	***	***	***	***	*	NS	-	***	NS	***	*	NS	NS
IG 2013	***	NS	NS	***	NS	***	***	***	***	-	**	NS	***	***	***
IG 2014	***	***	***	***	***	***	**	*	NS	**	-	***	***	NS	*
IG 2015	***	NS	NS	NS	NS	NS	***	***	***	NS	***	-	***	***	***
HK 2013	***	***	***	***	***	***	NS	NS	*	***	***	***	-	NS	NS
HK2014	***	***	***	***	***	***	NS	NS	NS	***	NS	***	NS	-	NS
HK 2015	***	***	***	***	***	***	NS	NS	NS	***	*	***	NS	NS	-
	Ascorbic Acid														
	JS 2013	JS 2014	JS 2015	IW 2013	IW 2014	IW 2015	LT 2013	LT 2014	LT 2015	IG 2013	IG 2014	IG 2015	HK 2013	HK 2014	HK 2015
JS 2013	-	***	***	***	***	**	***	***	***	***	***	***	***	***	***
JS 2014	***	-	NS	NS	NS	NS	***	***	***	**	***	NS	***	***	***
JS 2015	***	NS	-	NS	NS	***	***	***	***	NS	***	NS	***	***	***
IW 2013	***	NS	NS	-	NS	NS	***	***	***	***	***	NS	***	***	***
IW 2014	***	NS	NS	NS	-	*	***	***	***	NS	***	NS	***	***	***
IW 2015	**	NS	***	NS	*	-	***	***	***	***	***	NS	***	***	***
LT 2013	***	***	***	***	***	***	-	NS	***	***	***	***	NS	NS	NS
LT 2014	***	***	***	***	***	***	NS	-	NS	***	*	***	NS	NS	NS
LT 2015	***	***	***	***	***	***	***	NS	-	*	NS	***	***	NS	NS
IG 2013	***	**	NS	***	NS	***	***	***	*	-	NS	**	***	***	***
IG 2014	***	***	***	***	***	***	***	*	NS	NS	-	***	***	*	*
IG 2015	***	NS	NS	NS	NS	NS	***	***	***	**	***	-	***	***	***
HK 2013	***	***	***	***	***	***	NS	NS	***	***	***	***	-	NS	NS
HK2014	***	***	***	***	***	***	NS	NS	NS	***	*	***	NS	-	NS
HK 2015	***	***	***	***	***	***	NS	NS	NS	***	*	***	NS	NS	-
	Dehydroascorbic Acid														
	JS 2013	JS 2014	JS 2015	IW 2013	IW 2014	IW 2015	LT 2013	LT 2014	LT 2015	IG 2013	IG 2014	IG 2015	HK 2013	HK 2014	HK 2015
JS 2013	-	***	***	**	***	***	***	***	***	***	***	***	***	***	***
JS 2014	***	-	NS	NS	**	NS	***	***	***	NS	***	*	NS	**	***
JS 2015	***	NS	-	**	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS
IW 2013	**	NS	**	-	***	NS	*	***	***	NS	***	***	NS	***	***
IW 2014	***	**	NS	***	-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
IW 2015	***	NS	NS	NS	NS	-	NS	NS	**	NS	NS	NS	NS	NS	*
LT 2013	***	***	NS	*	NS	NS	-	NS	**	NS	NS	NS	NS	NS	NS
LT 2014	***	***	NS	***	NS	NS	NS	-	NS	*	NS	NS	NS	NS	NS
LT 2015	***	***	*	***	NS	**	**	NS	-	***	NS	NS	***	NS	NS
IG 2013	***	NS	NS	NS	NS	NS	NS	*	***	-	NS	NS	NS	NS	***
IG 2014	***	***	NS	***	NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS
IG 2015	***	*	NS	***	NS	NS	NS	NS	NS	NS	NS	-	NS	NS	NS
HK 2013	***	NS	NS	NS	NS	NS	NS	NS	***	NS	NS	NS	-	NS	**
HK2014	***	**	NS	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	-	NS
HK 2015	***	***	NS	***	NS	*	NS	NS	NS	***	NS	NS	**	NS	-

Significance dipicted by the following: *-p<0.05, **-p<0.01, ***-p<0.001, NS-not significant.

3.3.3. Variation of phenolic metabolites

Alongside ascorbic acid, phenolic compounds have also been known to have various potential health benefits. The total phenolic levels in bitter melon cultivars evaluated in this study ranged from 4.8 – 12.5 mg CE/ g of freeze dried (FD) pericarp. The total phenolic levels were similar in all three seasons in the 'Indian Green' and 'Indian White cultivars' (**Figure 3-3**). In the 'Japanese Spindle' and 'Large Top' cultivars the levels of total phenolics were higher in 2013 compared to the 2014 and 2015 growing seasons. The total phenolic levels in the 'Hong Kong Green' cultivar was the highest in the 2014 growing season. Overall the levels were consistently the highest in the 'Indian White' cultivar ranging from 10.6 to 12.5mg CE/g of FD material. In order to elucidate the phenolic compounds found within the bitter melon pericarp, HR-LCMS was used for identification. Extracts from fruits harvested from each of the seasons and from each cultivar were subject to HR-LCMS analysis. The chromatographic data was then compared to an in-house library to compare retention times and mass spectral data. The results from the analysis are presented in **Table 3-8**. Results indicated the presences of quinic acid, gallic acid, succinic acid, vanillic acid, ferrulic acid and salicylic acid among the tissues analyzed. Standard curves were developed for the quantification of organic and phenolic acids present in the various bitter melon samples. Overall the quinic acid was found in higher levels and in most of the bitter melon cultivars evaluated with levels ranging from 9.6 to 117 mg/g of freeze-dried bitter melon pericarp. Gallic acid was also present in most of the samples analyzed. The levels of gallic acid in bitter melon pericarp ranged from 0.003 to 0.021 mg per gram freeze dried pericarp. Quinic and gallic acid both showed noticeable differences in 2013 compared to 2014 and 2015. The levels of quinic acid were

typically the highest in the 2013 growing season and decreased in the subsequent growing seasons. However, gallic acid levels where higher in the 2013 season and decreased during in the later years of evaluation. The inverse was noted for gallic acid, with levels in 2014 and 2015 being higher that in the samples obtained during the 2013 growing season. Furthermore, the presence and level of other identified compounds do not follow a particular trend. The levels and of all the identified and quantified organic and phenolic acids are present in **Table 3-8**.

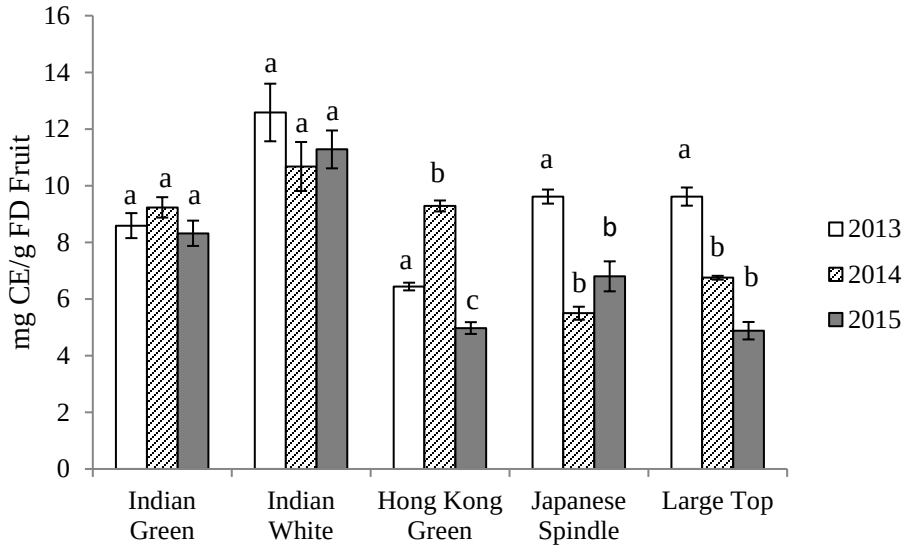


Figure 3-3. Total phenolic (mg of catechin equivalents (CE)/g) levels of each bitter melon cultivar evaluated for the 2013, 2014, and 2015 growing seasons. Significant differences among seasons were noted as differences in lettering at P<0.001.

As previously discussed, the levels of secondary metabolites have been reported to fluctuate in response to various factors such as genotype, environmental and cultural practices. In our study no clear trend was observed in relation to the level of total phenolics with respect to growing season. There was however a genotypic influence in the levels of total phenolics, with higher levels observed in the 'India White' cultivar. This is in agreement with the results reported by Horax et al in which the 'India White' cultivar had higher levels to total phenolics compared to the 'India Green' cultivar [85]. The total phenolic levels in fruits and vegetables has been positively correlated to the antioxidant activities and other human health benefits, as such the phenolic metabolite information presented here may be a selective factor for bitter melon breeding activities.

3.3.4. Amino acid analysis

The levels of various amino acids were analyzed by HPLC for each of the cultivars for different growing seasons. The levels of total amino acids in bitter melon ranged from 5.42 -31.67nmols/mg of freeze-dried material. The 'Large Top' cultivar had the highest level of amino acids in the 2013 growing season (31.67 nmol/mg) and decreased to 10.76 nmols/mg in 2015. Higher levels of amino acids were also measured in the 'Hong Kong Green' cultivar ranging from 11.71-25.84 nmols/mg. The measured amino acid levels for all cultivars and seasons are presented in **Table 3-9**. It is important to note that the level of the total amino acids was lowest for each variety in the 2015 growing season. More detailed studies are required to elucidate the possible effects environmental cues and their role in amino acid synthesis in bitter melon. The levels of individual amino acids vary depending on the individual amino acid, cultivar and season. The summary of differences and interactions are presented in **Table 3-10**. The highest measured amino acid was

threonine measuring 15.05 nmols/mg in the ‘Large Top’ cultivar samples harvested in 2013. There was no clear prominent amino acid for a particular cultivar, but alanine and aspartic acid were typically the most prominent amino acids from the cultivars analyzed. Similarly, no amino acid was consistently the lowest, but glycine was regularly the measure as the lowest in many of the bitter melon cultivars.

The correlation of free amino acid levels and bitterness has been reported in several studies[172]. Food with higher levels of free amino acids has been reported to have a lower bitter taste threshold, including bitter melon[173]. According to Kawai et al., individual amino acids vary in gustatory sensation in humans[174]. As such, the amino acid composition of bitter melon cultivars could be an important factor in the selection of lines for breeding bitter melons with reduced levels of particular free amino acids.

The levels of total ascorbic acid, total amino acids and total phenolics were also used to evaluate the differences in levels among the different varieties tested. The level of total ascorbic acid was highest in the ‘Japanese Spindle’ and ‘Indian White’ cultivar and lowest in the ‘Hong Kong Green’ and ‘Large Top’ cultivar (**Table 3-11**). Conversely, the highest levels of total amino acids were observed in the ‘Large Top’ and the ‘Hong Kong Green’ cultivars and the lowest in the ‘Indian White’ and ‘Indian Green’ cultivars. Lastly the ‘Indian White’ cultivar displayed the largest levels of total phenolics and the ‘Hong Kong Green’ cultivar had the lowest. The effect of cultivar, season and the interaction between both are presented in **Table 3-12**.

Table 3-8. Targeted screening report for the different cultivars evaluated for each of the growing seasons. Postively identified compounds were quantified and presented as µg/g of freeze-dried pericarp (FD)± standard error mean. Significance was noted as differences in lettering within row at P< 0.001.

		ΔRT			ΔmDa			Δppm			Concentration (µg/g of FD pericarp)		
		2013	2014	2015	2013	2014	2015	2013	2014	2015	2013	2014	2015
Quinic Acid	Indian Green	-0.1	-0.1	-0.1	-6.3	-7.4	-2.6	-32	-38.3	-13.5	73.79± 16.6 a	19.11±0.75b	13.26±0.06 b
	Indian White	ND	-0.1	-0.1	ND	-6	-1.7	ND	-31.3	-8.7	ND	103.07±6.16 a	8.94± 0.52 b
	Hong Kong Green	-0.1	-0.1	ND	-7.4	-8.6	ND	-38.4	-44.5	ND	117.69±9.59 a	10.91±2.78 b	ND
	Large Top	-0.1	-0.1	-0.1	-7.5	-3.2	-5.2	-39	-16.4	-32.1	53.91± 2.12 a	23.50± 0.37 b	16.86± 1.11 c
	Japanese Spindle	-0.1	-0.1	-0.1	-1.6	-0.1	-1.6	-8.2	-0.4	-8.2	51.00±1.16 a	9.65±0.04b	10.65±1.09 b
Gallic Acid	Indian Green	-0.1	0	0	-3.2	-5.5	1.3	-18	-32.6	7.5	6.59±0.24 a	12.11± 0.29 b	17.63±0.17 c
	Indian White	0	-0.1	0	-0.7	-3.2	2.3	-4.3	-19	13.2	11.47± 1.56 a	23.66±0.17 b	17.58±0.41c
	Hong Kong Green	-0.1	0	-0.1	-4.8	-6.2	-7.5	-28.1	-36.2	-44.1	11.10±0.22 a	14.74± 1.44 a	11.87±0.13 a
	Large Top	-0.1	0	-0.1	-5.4	3.3	-2.6	-31.3	19.1	-15	3.09±0.01 a	15.94±.0.03 b	21.76±0.15 c
	Japanese Spindle	0	0	-0.1	1.1	3.9	0.2	6.5	23.1	1.4	9.31±0.09 a	17.48±0.70 b	9.32±0.01 a
Succinic acid	Indian Green	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Indian White	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Hong Kong Green	ND	ND	-0.1	ND	ND	-6.3	ND	ND	37.4	ND	ND	trace
	Large Top	0.1	ND	ND	-4.1	ND	ND	-34.8	ND	ND	47.65±1.36	ND	ND
	Japanese Spindle	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Vanillic Acid	Indian Green	ND	-0.1	-0.1	ND	-6.4	1.5	ND	-38	8.7	ND	20.46±1.68 a	26.42±0.03 b
	Indian White	ND	-0.1	-0.1	ND	-5.1	0.2	ND	-30.2	1.3	ND	25.95±2.93 a	23..29±0.34 a
	Hong Kong Green	-0.1	ND	ND	-6.1	ND	ND	-35.9	ND	ND	20.55±0.79	ND	ND
	Large Top	-0.1	-0.1	-0.1	-6.2	-1.4	-2.8	-36.8	-8.5	-16.3	11.47±0.78 a	23.73±1.41 b	34.92±0.77 b
	Japanese Spindle	-0.1	-0.1	-0.1	0.1	0.8	-2.9	0.5	4.5	-17	26.59±2.33 a	18.85±0.33 b	19.67±0.05 b
Ferulic Acid	Indian Green	-0.1	-0.1	-0.1	-5.1	-6.8	1.8	-26	-35.1	9.4		26.47±1.76 a	58.21±0.88 b
	Indian White	-0.1	-0.1	-0.1	-4.4	-6.9	0.6	-22.6	-35.5	2.9	4.06±0.63 a	12.62±0.86 b	30.28± 3.25 c
	Hong Kong Green	-0.1	-0.1	ND	-6.2	-9.4	ND	-31.6	-48	ND	15.53±1.12 a	15.01±0.17 a	ND
	Large Top	-0.1	-0.1	-0.1	-8.2	0.9	-2.8	-42.2	-1.1	-14.5	3.18±1.68 a	16.63±7.63 b	23.43±5.74 b
	Japanese Spindle	ND	-0.1	ND	ND	0	ND	ND	-0.1	ND	ND	16.25±2.64	ND
Salicylic Acid	Indian Green	ND	ND	-0.1	ND	ND	1.7	ND	ND	12.4	ND	ND	14.53±0.57
	Indian White	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Hong Kong Green	-0.1	-0.1	ND	-3.3	-6.3	ND	-23.5	-45.4	ND	1.10±0.60 a	19.87±1.18 b	ND
	Large Top	ND	0	ND	ND	0.9	ND	ND	6.5	ND	ND	6.87±2.40	ND
	Japanese Spindle	-0.1	-0.1	-0.1	-0.1	-0.1	-1.5	-3.1	-0.5	-10.5	10.57±1.09 a	10.25±0.88 a	7.58±0.18 a
Caffeic Acid	Indian Green	ND	ND	-0.1	ND	ND	1.9	ND	ND	10.6	ND	ND	19.24±6.21
	Indian White	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Hong Kong Green	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Large Top	ND	ND	-0.1	ND	ND	-2.5	ND	ND	-13.7	ND	ND	12.49±4.11
	Japanese Spindle	ND	ND	-0.1	ND	ND	-3.1	ND	ND	-17.3	ND	ND	36.98±11.88

ND- value not determined. ΔmDa and Δppm were derived from measured *m/z* and the calculated *m/z* values for a pseudomolecular ion $[M+H]^+$, generated from the molecular formula.

Table 3-9. Free amino acid and total amino acid concentrations (nmole/mg of freeze dried pericarp ± standard error mean) in five bitter melon cultivar produced in the 2013, 2014 and 2015 growing season

		Asp	Ser	Glu	Gly	His	Arg	Thr	Ala	Pro	Cys	Tyr	Val	Lys	Phe	Total
Indian White	2013	2.88 ± 0.16^{A}	1.67 ± 0.10^{A}	1.63 ± 0.10^{A}	0.24 ± 0.01^{A}	2.26 ± 0.09^{A}	0.54 ± 0.03^{A}	2.55 ± 0.10^{A}	1.80 ± 0.11^{A}	1.26 ± 0.04^{A}	0.61 ± 0.02^{A}	0.59 ± 0.02^{A}	1.04 ± 0.03^{A}	1.13 ± 0.04^{A}	0.76 ± 0.02^{A}	19.00 ± 0.55^{A}
	2014	1.08 ± 0.04^{B}	1.31 ± 0.03^{B}	2.80 ± 0.10^{B}	0.59 ± 0.02^{B}	0.21 ± 0.00^{B}	1.18 ± 0.02^{B}	1.31 ± 0.02^{B}	1.22 ± 0.04^{B}	1.57 ± 0.07^{B}	0.40 ± 0.01^{B}	0.61 ± 0.01^{A}	1.35 ± 0.02^{B}	0.78 ± 0.02^{B}	0.32 ± 0.00^{B}	13.14 ± 1.69^{B}
	2015	1.43 ± 0.08^{B}	0.69 ± 0.07^{C}	0.65 ± 0.06^{C}	0.22 ± 0.01^{A}	0.76 ± 0.06^{C}	0.26 ± 0.04^{C}	0.77 ±0.03^{C}	1.61 ± 0.07^{A}	1.23 ± 0.04^{A}	0.27 ± 0.03^{C}	0.24 ± 0.02^{B}	1.04 ± 0.06^{A}	0.43 ± 0.04^{C}	0.24 ± 0.02^{C}	8.80 ± 1.10^{C}
Indian Green	2013	1.42 ± 0.05^{A}	1.48 ± 0.03^{A}	1.71 ± 0.04^{A}	0.37 ± 0.02^{A}	1.94 ± 0.03^{A}	0.93 ± 0.05^{A}	2.60 ± 0.28^{A}	1.82 ± 0.05^{A}	1.37 ± 0.07^{A}	0.40 ± 0.00^{A}	0.40 ± 0.01^{A}	0.46 ± 0.01^{A}	0.88 ± 0.01^{A}	0.62 ± 0.02^{A}	16.46 ± 0.28^{A}
	2014	2.30 ± 0.02^{B}	2.17 ± 0.04^{B}	1.27 ± 0.04^{B}	0.29 ± 0.01^{B}	0.35 ± 0.01^{B}	0.31 ± 0.02^{B}	0.50 ± 0.01^{B}	3.91 ± 0.03^{B}	0.89 ± 0.01^{B}	0.36 ± 0.02^{A}	0.50 ± 0.02^{B}	0.32 ± 0.01^{B}	0.95 ± 0.04^{A}	0.29 ± 0.01^{B}	14.47 ± 0.30^{A}
	2015	0.94 ± 0.15^{C}	0.59 ± 0.08^{C}	0.51 ± 0.07^{C}	0.22 ± 0.01^{C}	0.41 ± 0.01^{B}	0.17 ± 0.01^{B}	0.50 ± 0.01^{B}	1.14 ± 0.17^{C}	1.05 ± 0.01^{B}	0.21 ± 0.00^{B}	0.21 ± 0.01^{C}	0.52 ± 0.00^{C}	0.35 ± 0.01^{B}	0.19 ± 0.01^{C}	5.45 ± 1.22^{B}
Hong Kong Green	2013	1.95 ± 0.05^{A}	1.74 ± 0.02^{A}	1.78 ± 0.04^{A}	0.52 ± 0.00^{A}	4.48 ± 0.06^{A}	2.00 ± 0.02^{A}	8.24 ± 0.09^{A}	0.85 ± 0.01^{A}	0.83 ± 0.03^{A}	0.43 ± 0.01^{A}	0.68 ± 0.01^{A}	0.29 ± 0.00^{A}	1.37 ± 0.01^{A}	0.91 ± 0.01^{A}	23.61 ± 2.74^{A}
	2014	3.59 ± 0.03^{B}	2.53 ± 0.09^{B}	1.89 ± 0.01^{A}	0.38 ± 0.02^{B}	2.64 ± 0.06^{B}	0.95 ± 0.03^{B}	2.48 ± 0.09^{B}	2.86 ± 0.06^{B}	1.51 ± 0.01^{B}	0.90 ± 0.03^{B}	1.23 ± 0.02^{B}	0.77 ± 0.01^{B}	3.00 ± 0.05^{B}	1.66 ± 0.04^{B}	25.84 ± 0.73^{A}
	2015	1.53 ± 0.04^{C}	0.53 ± 0.01^{C}	0.59 ± 0.01^{B}	0.22 ± 0.01^{C}	1.30 ± 0.02^{C}	0.34 ± 0.01^{B}	2.91 ± 0.13^{C}	1.35 ± 0.03^{C}	1.24 ± 0.08^{C}	0.28 ± 0.01^{C}	0.34 ± 0.03^{C}	0.46 ± 0.01^{C}	0.59 ± 0.03^{C}	0.35 ± 0.02^{C}	11.71 ± 0.43^{B}
Large Top	2013	2.44 ± 0.10^{A}	2.61 ± 0.13^{A}	1.24 ± 0.04^{A}	0.88 ± 0.01^{A}	3.35 ± 0.04^{A}	1.50 ± 0.02^{A}	15.05 ± 0.24^{A}	0.88 ± 0.06^{A}	0.86 ± 0.02^{A}	1.30 ± 0.01^{A}	1.22 ± 0.03^{A}	ND	2.28 ± 0.07^{A}	1.96 ± 0.04^{A}	31.67 ± 4.01^{A}
	2014	5.12 ± 0.05^{B}	2.36 ± 0.08^{A}	1.63 ± 0.03^{B}	0.36 ± 0.01^{B}	1.62 ± 0.04^{B}	0.76 ± 0.03^{B}	2.31 ± 0.11^{B}	3.37 ± 0.04^{B}	1.71 ± 0.01^{B}	0.60 ± 0.02^{B}	1.15 ± 0.07^{A}	0.42 ± 0.01^{A}	2.44 ± 0.08^{A}	0.94 ± 0.05^{B}	24.76 ± 0.46^{A}
	2015	1.36 ± 0.01^{C}	0.93 ± 0.03^{B}	0.54 ± 0.01^{C}	0.29 ± 0.01^{C}	1.03 ± 0.04^{A}	0.42 ± 0.03^{C}	1.26 ± 0.05^{C}	1.62 ± 0.03^{C}	1.43 ± 0.01^{C}	0.26 ± 0.01^{C}	0.58 ± 0.02^{B}	ND	0.68 ± 0.02^{B}	0.31 ± 0.01^{C}	10.76 ± 0.29^{B}
Japanese Spindle	2013	3.04± 0.02^{A}	1.50 ± 0.04^{A}	1.95 ± 0.01^{A}	0.36 ± 0.01^{A}	2.06 ± 0.04^{A}	0.33 ± 0.02^{A}	1.05 ±0.03^{A}	2.35 ± 0.04^{A}	2.28 ± 0.03^{A}	0.39 ± 0.01^{A}	0.50 ± 0.01^{A}	0.64 ± 0.01^{A}	0.86 ± 0.02^{A}	0.47 ± 0.01^{A}	15.85 ± 1.99^{A}
	2014	1.33 ± 0.02^{B}	2.19 ± 0.03^{B}	1.48 ± 0.01^{B}	0.41 ± 0.03^{A}	1.90 ± 0.02^{B}	0.92 ± 0.02^{B}	1.38 ± 0.01^{B}	2.88 ± 0.10^{B}	1.64 ± 0.02^{B}	0.67 ± 0.01^{B}	1.10 ± 0.01^{B}	1.15 ± 0.01^{B}	2.22 ± 0.03^{B}	0.77 ± 0.01^{B}	17.88 ± 2.24^{A}
	2015	1.51 ± 0.02^{C}	1.06 ± 0.02^{C}	0.67 ± 0.01^{C}	0.18 ± 0.00^{B}	0.94 ± 0.01^{C}	0.33 ± 0.02^{A}	0.76 ± 0.02^{C}	1.15 ± 0.04^{C}	1.22 ± 0.05^{C}	0.30 ± 0.01^{C}	0.21 ± 0.01^{C}	0.82 ± 0.01^{C}	0.38 ± 0.00^{C}	0.30 ± 0.00^{C}	8.79 ± 1.10^{B}

Letters within columns indicate differences (P<0.01) between seasons from each cultivar and individual amino acid.

Table 3-10. Summary of ANOVA for different sources of varience.

Source	Asp	Ser	Glu	Gly	His	Arg	Thr	Ala	Pro	Cys	Tyr	Val	Lys	Phe	Total
Season (S)	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Cultivar (C)	***	***	***	***	***	***	***	***	***	***	***	ND	***	***	***
S x C	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***

Significance depicted by ***-p<0.001, ND-not determined.

Table 3-11. Averaged levels of total ascorbic acid, total amino acids and total phenolics for each cultivar evaluated. The averages were obtaind from pooled level for each year and variety

Cultivar	Total Ascorbic Acid (mg/100g)	Total Amino Acids (nmols/mg)	Total Phenolics (mg CE/g)
Indian Green	91.77±4.11 b	12.59±0.93 c	8.70±0.42 b
Indian White	119.09±3.13 a	14.17±0.93 c	11.50±0.55 a
Hong Kong Green	48.72±1.62 c	20.39±1.52 ab	6.89±0.40 c
Large Top	53.53±2.36 c	23.26±2.00 a	7.09±0.47 bc
Japanese Spindle	127.23±5.90 a	15.94±0.87 bc	7.29±0.43 bc
P-value	<0.0001	<0.0001	<0.0001

Letters within columns indicate differences (P<0.0001) between cultivars.

Table 3-12. ANOVA for total ascorbic acid, total amino acids and total phenolics relation to cultivars, year and the interaction between cultivar and season .

Source	df	F-ratio	P
Total Ascorbic Acid			
Cultivar (C)	4	238.8	< 0.0001
Year (Y)	2	16.3	< 0.0001
(C) X (Y)	8	20.8	< 0.0001
Total Amino Acid			
Cultivar (C)	4	74	< 0.0001
Year (Y)	2	270.2	< 0.0001
(C) X (Y)	8	16.1	< 0.0001
Total Phenolics			
Cultivar (C)	4	26.1	< 0.0001
Year (Y)	2	12.8	< 0.0001
(C) X (Y)	8	6	< 0.0001

In conclusion, the production of bitter melon in Texas has potential with yields that are comparable to yields in traditionally producing locations, but a careful study over climate and soil profiles is still required. Bitter melon is a crop that is rich in several beneficial phytonutrients and as such could be presented and marketed to the current health-conscious consumers. Currently, bitter melon is an underutilized horticultural crop in the United States. The bitter principles of bitter melon may be the determining factor in preventing its acceptance among consumers. The elucidation of the phytonutrients imparting health benefits and gustatory profiles can server as a valuable tool for future breeding efforts of a nutritious, less bitter cultivar to offer to consumers.

4. METABOLITE PROFILING AND IN VITRO BIOLOGICAL ACTIVITIES OF TWO COMMERCIAL BITTER MELON (*MOMORDICA CHARANTIA* LINN.) CULTIVARS[3]

4.1. Introduction

Bitter melon (*Momordica charantia* L.) is commonly cultivated in many Asian and South American countries. In traditional medicine, bitter melon has been utilized as a natural way to manage several diseases, including type 2 diabetes [3]. Recently, several studies have evaluated these claims using various animal, cell and enzymatic assays, particularly illustrating promising anti-diabetic and anti-cancer activities. Animal studies using bitter melon extracts reported the ability to favorably influence blood glucose and blood pressure regulation [5]. Furthermore, various cell culture models have reported bitter melon extracts to have reparative effects on pancreatic β-cell, inhibit adipocyte differentiation, and increase insulin sensitivity in muscle cells [8, 175]. With recent accumulation of data correlating bitter melon and positive health benefits, an increased interest in identifying the potential bioactive components has emerged.

Vitamin C, carotenoids, flavonoids and other polyphenols are among the bioactive compounds identified from various bitter melon tissues, but current interest is focused on the identification and purification of cucurbitane-type molecules [176, 177]. Cucurbitane-type molecules are typically found in plant belonging to the *Cucurbitaceae* family. These molecules are characterized as having a tetracyclic nucleus, principally 9β-methyl-19-nor-lanosta-5-ene, with various oxygenation functionalities throughout its structure [15].

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Furthermore, cucurbitane type molecules may be present in various aglycone and glycosidic forms leading to a diverse pool of compounds. To date, over 200 cucurbitane type molecules have been identified from various parts of the bitter melon plant, such as momordicosides, goyaglycosides, karavilagenins, and kuguacins [178].

The precise identification of phytochemicals within food matrices can be a complex task, especially for compounds with structural characteristics of the cucurbitane-type molecules. Several analytical methods have been reported for the identification of secondary metabolites in bitter melon such as the use of HPLC fitted with various types of detectors [47]. For example, liquid chromatography coupled to mass spectrometry has become the gold standard for separating and identifying metabolites from complex food and plant matrices. The use of quadrupole time-of-flight mass spectrometry is a widely-used tool in the field of metabolomics that yields high mass accuracy and elucidates the elemental composition of compounds [179-181]. High resolution mass spectral data yields valuable information relating to the unknown metabolites by screening the masses of the secondary metabolites present in the sample matrix. This screening procedure is a powerful tool because the identification of compounds can be carried out without actual reference standards. Exact masses of compounds in the sample are matched to theoretical exact masses reported in the literature, making this a valuable tool when reference standards are costly or not commercially available. Additionally, Bruker's TargetAnalysis reinforce the identification of compounds by comparing retention times, isotope pattern, mass error and qualifying ions. In the present study, comprehensive analysis of 10 different polar extracts from two commercially available bitter melon cultivars by UPLC-high resolution mass

spectrometry for the first time. Additionally, *in vitro* antioxidant activity and hypoglycemic potentials of all these extracts were evaluated.

4.2. Materials and methods

4.2.1. Chemicals

α-Amylase from porcine pancreas, α-glucosidase from *Saccharomyces cerevisiae,* Folin-Ciocalteu reagent, (+)-catechin, diosgenin, ascorbic acid, 2,2-diphenyl-1-pricrylhydrozyl (DPPH), 3,5-dinitrosalicylic acid (DNSA), *p*-nitrophenyl-α-*D*-glucopyranoside were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2. Plant material and extraction

Fruits of the 'Indian Green' and 'Chinese Green' cultivars were purchased from a local grocery store in College Station (TX). The inner tissue (pulp and seed) was manually separated from the pericarp. The material was then placed in a freezer at -20 °C in preparation for freeze drying. The lyophilized components were processed into a fine powder using a Waring laboratory blender. Bitter melon pericarp and inner tissue powder was extracted using a Soxhlet type extractor with a 1:3 plant material: extraction solvents ratio. The plant material was sequentially extracted using hexane, chloroform, acetone, MeOH, and MeOH/H_2O (8:2) for 16h each. The extracts were then filtered and concentrated under vacuum at 30 °C separately. Concentrated extracts were subsequently lyophilized and stored at -20 °C prior to analysis.

4.2.3. Sample preparation

4.2.3.1. Total phenolics and saponins

To determine the level of total phenolics in the lyophilized extracts, an aliquot (10 mg/mL) was dissolved in either acetone or MeOH/H_2O mixture. The samples were

vortexed and sonicated until all extract was uniformly dissolved. Total saponins were measured using 5 mg/mL aliquots dissolved in solvents corresponding to extraction method.

4.2.3.2. Biological activities

The determination the various *in vitro* biological activities were carried out by dissolving a known amount of lyophilized extract in DMSO. For the determination of DPPH, and α-glucosidase 10 mg/mL aliquots were prepared, while a 5 mg/mL aliquot was prepared for α-amylase inhibition.

4.2.3.3. Sample preparation for UPLC-high resolution Mass spectral studies

Hexane, chloroform, acetone, methanol, and MeOH/H_2O (8:2) lyophilized extracts were dissolved in the respective extraction solvents and filtered through 0.45-micron filters before injecting to LCMS for identification of cucurbitane type triterpenoids. For target analysis of phenolic and organic acids, lyophilized plant material was extracted with MeOH/H_2O (8:2) in a hot water bath (70 °C) for 1h followed by 30 min of sonication. The resulting mixture was then centrifuged and the supernatant was transferred to a storage container. This process was repeated twice with the resulting supernatant being pooled each time. The pooled extracts were then concentrated under vacuum and final volume made to 5 mL. The extracts were all stored in -80 °C until analysis.

4.2.4. Estimation of total phenolics content

The levels of total phenolics present in the various extracts were determined by the Folin-Ciocalteu (FC) method adjusted for a 96 well format as presented in our previous publication [182]. A standard curve was developed using various concentration of (+)-catechin (0.5, 1, 2, 3, 4, 5, and 7.5 µg/mL). The estimation of the total phenolic levels in

all bitter melon extracts was carried out in triplicates and the mean results were presented as (+)-catechin equivalents (CE) mg/g of freeze-dried material.

4.2.5. Estimation of total saponin content

Total saponin content from bitter melon extracts was carried out by UV/Vis spectrophotometry according to the method described by Liu et al [183]. Briefly, diosgenin was used to prepare a standard curve at various concentrations (0, 50, 100, 150, 200, and 250 µg/mL). The reaction consisted of the addition of 250 µL of bitter melon extracts to 250 µL of vanillin solution (8g/100mL ethanol). After vortexing, 2.5 mL of 72% sulfuric acid was added to the reaction mixture followed by incubation at 60 °C in a water bath for 10 min. The sample was then removed from the bath and allowed to cool to room temperature. The absorbance of the cooled samples was read at 544 nm. The total saponin content of extracts was expressed as diosgenin equivalent (DE) mg/g of freeze-dried extracts.

4.2.6. DPPH free radical scavenging activity

The methodology for the measuring of DPPH radical scavenging potential was performed according to our previously published method [184]. The radical-scavenging activity was expressed as ascorbic acid equivalents (AAE) mg / gram of extract.

4.2.7. Evaluation of α-amylase inhibitory activity

Briefly, aliquots (10 µL) of bitter melon extracts were pipetted into a 96 well micro plate and the final volume was made up to 140 µL with 1 % saline solution [185]. The reaction was carried out by the addition of 45 µL of 1 % starch solution and 45 µL of α-amylase (1 unit /mL) enzyme solution. The reaction mixture was then incubated for 40 minutes at 25°C. Following the initial incubation, 50 µL of DNSA (1% 3,5-dinitrosalicylic

acid in 20% Rochelle salt) was added to the reaction mixture and incubated an additional 60 min at 50°C after which the plate was read a 540 nm. The absorbance of reaction mixtures were compared to blanks that consisted of all the above mentioned components except in the addition of 1% starch. Various concentration of dextrose (0, 25, 50, 75, 100, 125, 150, 200 µg/mL) were used to develop a standard curve to evaluate the level of starch degradation by α-amylase in reaction wells. Acarbose (80µg/mL) was used as a positive control.

4.2.8. Evaluation of α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity was determined using methods adapted from the literature with minor modifications [186]. Four concentrations of each extract were assessed inhibitory properties on α-glucosidase activities. The sample was diluted to the appropriate concentration by the addition of 1M sodium phosphate buffer, (pH 6.8). The extract was then mixed with 100 µL of α-glucosidase solution (0.5 units/mL) and incubated at room temperature for 10 min. After the pre-incubation phase, 50 µL of *p*-nitrophenyl-α-*D*-glucopyranoside (2.5 mM) solution was added and the mixture was incubated for an additional 10 min at room temperature. The absorbance of the reaction mixture was recorded at 410 nm and the α-glucosidase inhibitory activity (%) was calculated in relation to a blank. Acarbose (80µg/mL) was used as a positive control.

4.2.9. Chromatographic and MS conditions

Chemo-profiling of bitter melon extract was performed by Agilent 1290 UPLC instrument (Foster City, CA) coupled to a time-of-flight-mass spectrometer (UHPLC-QTOF-MS). Separation of bitter melon compounds was carried out on a Supelco Ascentis Express C_{18} column (10cm x 2.1mm, 2µm) maintained at 70°C. The mobile phase

consisted of 0.1% formic acid in water (solvent A) and 100% acetonitrile (solvent B). The gradient elution was as follows: 100-95% A from 0-1 min, 95-40%A from 1-6.5 min, 40-5% A in from 6.5 to 9 min, 5-100%A from 9-11 min and equilibrated for one min before the next injection. The applied flow rate throughout the chromatographic run was 0.2 mL/ min and the peak separation was monitored at 210 and 254 nm. Mass spectral analysis was performed using high resolution mass spectrometer (maXis impact, Bruker Daltonics, Bellerica, MA) equipped with an electrospray ionization interface (ESI-Q-TOF) in positive ionization mode. The rest of mass spectrometer conditions were maintained same according to our recent publication [177]. The exact mass of cucurbitane-type triterpenoids obtained from mass spectral data was compared to the theoretical values obtained from the literature [178]. The accuracy between measure data and theoretical was reported as Δ (ppm). The ppm is calculated as follows: $[(\text{mass}_{exp} - \text{mass}_{calc})/ \text{mass}_{exp}]*10^{6}$, where mass_{exp}- is the experimental mass and the mass_{calc}- is the mass calculated from molecular formulas obtain from the literature. Lower ppm values indicate a higher probability of the measured compound being a positive match to a previously reported triterpenoid found in bitter melon.

4.2.10. Targeted analysis of phenolic acids and organic acids

Data acquired by UHPLC-QTOF-MS was processed with the DataAnalysis software version 4.1. Criteria for positive identification were defined and an in-house database was constructed comprising of several phenolic compounds and organic acids believed to be found within the bitter melon matrix. The database consisted of the monoisotopic masses, elemental composition, and experimental retention times. The psuedomolecular ions $[M+H]^{+}$ were accurately calculated and the extracted ion chromatograms were archived in

the database at a tolerance of 15 mDa. Targeted screening was carried out using the accurate mass as well as the retention times of standard compounds. Data points from each run were analyzed by the TargetAnalysis™1.2 software (Bruker Daltonik, Billerica, MA) for accurate determination of molecules. These factors were then utilized for comparison of bitter melon MeOH/H_2O (8:2) extracts to standard compounds. The calculated and experimental value of the targeted compounds in the bitter melon extracts were compared to mass error (ppm), mSigma valves and retention time (min). Δ Rt is a measure of the change in retention time between the measured standard found in the in-house database and the extract being evaluated, the smaller the Δ Rt the higher the possibility of the compound being a positive match. Since many other compounds may have similar retention times the accuracy between measured and theoretical mass data (mDa and ppm) values add certainty for positive identification. The mass accuracy (ppm) of the analyte compared to the calculated exact mass of the molecules of interest is an important piece of information that adds assurance to the identification of metabolites.

4.2.11. Statistical analysis

Each replicate extract was analyzed in triplicate, statistical analysis on the means of replicate experiments were performed using the ANOVA procedure of the InStat® software, 230 version 3.0 (GraphPad, San Diego, CA). The Tukey's test of significance between means. The correlation between total phenolics and total saponins was analyzed using JMP 13 statistical software using the multivariate parameters along with the pairwise correlation test of significance. Significance was determined if values were of at least $P \leq 0.05$ in all analysis.

4.3. Results and discussion

To date, reports have focused primarily on the utilization of bitter melon extracts obtained from polar solvents, such as methanol, ethanol and water, to assess bioactivity, while nonpolar extracts have remained relatively unexplored. Therefore, we explored extracts derived from the pericarp and inner tissues of bitter melon with a broad range of polarities (**Table 4-1**). MeOH extracts comprised 7.1-12.6% of the weight of the freeze-dried pericarp of both cultivars. With regards to the inner tissue, the MeOH extracts ranged from 8-9% of freeze-dried material. While methanolic extracts constitute the bulk of the extractible material, potential beneficial compounds may be found in acetone, chloroform, and hexane extracts. Previous studies have concluded that bitter melon has health benefitting properties, yet all have been carried out using polar (aqueous/ alcohol) extracts, leaving a large amount of potentially beneficial material unexplored. One study reported an increase of glucose uptake by muscle cells treated with bitter melon chloroform extracts, suggesting that non-polar extracts also have potential health benefits [187]. Unfortunately, the chemical constituents of the chloroform extract were not elucidated. In our current study, we screened and evaluated a wide range bitter melon extracts for their chemical constituents and *in vitro* biological activities.

4.3.1. Phenolic and organic acids

Bitter melon has been reported to possess several phenolic compounds [85, 88]. The levels of total phenolics were compared among acetone, MeOH, and MeOH/H_2O (8:2, v:v) extractions of the Chinese and Indian bitter melon tissues (**Table 4-1**). Our results indicate that the acetone extracts of the Indian pericarp had the highest levels of total polyphenols (26.85 ±1.64 mg CE/ g of FD extract) compared to the Chinese pericarp extracts (18.93 ±

0.46 mg CE / g). In contrast, the level of total phenolics in MeOH extracts (22.90 ± 0.62 and 24.31 ± 1.19 mg CE/ g) and MeOH/H_2O (8:2) (10.31 ± 0.27 and 16.16 ± 2.16 mg CE/g) was not significantly different between both cultivars, respectively.

Table 4-1. Yield of bitter melon extracts, total phenolics, DPPH free radical scavenging activity, total saponin content and α-amylase inhibition

		Extract Yield (g/100g of FD)	Total Phenolics (mgCE/g of FD)	DPPH (mg AAE/g of FD)	Total Saponins (mg DE/g of FD)	α-Amylase Inhibition (%)*
	Acarbose	-	-	-	-	88.02 ± 1.74
Chinese Pericarp	Hexane	0.51	ND	19.24 ± 1.28 ^{A}	51.29 ± 2.16 ^{A}	75.90 ± 6.50 ^{A}
	Chloroform	1.06	ND	14.53 ± 0.17 ^{B}	76.14 ± 2.53^{B}	42.72 ± 6.07 $^{B+}$
	Acetone	4.92	18.93 ± 0.46 ^{A}	13.86 ± 1.04 ^{C}	45.77± 5.75^{C}	92.60 ± 1.08 ^{C}
	MeOH	7.19	24.31 ± 1.19 ^{B}	20.40 ± 0.19 ^{D}	22.97 ± 2.58 ^{D}	77.46 ± 4.97 ^{D}
	MeOH/H_2O (8:2)	12.46	10.31 ± 0.27 ^{C}	14.69 ± 1.54 ^{E}	8.47 ± 4.01 ^{E}	87.51 ± 11.40 ^{E}
Indian Pericarp	Hexane	0.73	ND	15.15 ± 0.31 ^{a}	42.47 ± 5.82 ^{a}	39.54 ± 7.31 $^{a+}$
	Chloroform	2.16	ND	13.24 ± 0.91 ^{B}	41.91 ± 6.35 ^{b}	71.18 ± 1.60 $^{b+}$
	Acetone	6.21	26.85 ± 1.64 ^{a}	14.11 ± 0.62 ^{C}	64.05 ± 6.31 ^{c}	93.67 ± 1.25 ^{C}
	MeOH	12.61	22.90 ± 0.62 ^{B}	19.25 ± 0.20 ^{D}	14.64 ± 6.87 ^{d}	71.14 ± 1.14 $^{D+}$
	MeOH/H2O (8:2)	9.16	16.16 ± 2.16 ^{C}	10.84 ± 1.11 ^{e}	8.10 ± 2.38 ^{E}	37.88 ± 4.44 $^{e+}$
Chinese Inner Tissue	Hexane	4.31	ND	7.47 ± 1.69 ^{F}	37.01 ± 6.21 ^{F}	46.95 ± 2.93 $^{F+}$
	Chloroform	1.97	ND	9.58 ± 0.73 ^{G}	50.71 ± 6.35 ^{G}	53.79 ± 6.77 $^{G+}$
	Acetone	3.74	28.45 ± 0.37 ^{E}	12.96 ± 2.88 ^{H}	19.98 ± 2.32 ^{H}	94.62 ± 1.68 ^{H}
	MeOH	8.09	53.79 ± 1.57 ^{F}	27.94 ± 1.84 ^{I}	19.97 ± 3.92 ^{I}	78.54 ± 5.68 $^{I+}$
	MeOH/H2O (8:2)	4.75	26.62 ± 2.97 ^{G}	13.87 ± 2.66 ^{J}	6.84 ± 1.68 ^{J}	59.53 ± 5.68 $^{J+}$
Indian Inner Tissue	Hexane	8.22	ND	5.93 ± 2.17 ^{F}	22.34 ± 1.35 ^{f}	49.40 ± 1.42 $^{F+}$
	Chloroform	1.89	ND	7.34 ± 1.24 ^{G}	29.18 ± 2.64 ^{g}	66.41 ± 6.51 $^{G+}$
	Acetone	3.26	24.87 ± 1.97 ^{E}	11.20 ± 1.15 ^{H}	30.39 ± 11.64^{h}	69.41 ± 4.77 $^{h+}$
	MeOH	9.00	57.28 ± 1.02 ^{F}	30.48 ± 2.49 ^{I}	16.01 ± 2.25 ^{I}	91.58 ± 2.48 ^{i}
	MeOH/H2O (8:2)	4.19	35.37 ± 2.91 ^{g}	19.18 ± 1.51 ^{j}	9.07 ± 3.94 ^{J}	48.83 ± 3.13 $^{i+}$

Differences between varieties ($P<0.05$) are indicates within column as capitalize and lower-case letters. + indicates a significant difference from acarbose.Results are represented by the means ± standard deviation. Test were carried out in nine replicates.
Phenolics expressed as catechin equivalents (CE), DPPH activity expressed as ascorbic acid equivalents (AAE), total saponin content expressed as diosmin equivalents (DE) and α-amylase inhibition (%). *****α-Amylase inhibition was tested using 175 µg/mL of extract.

Regarding the inner tissue extracts, the highest level of total phenolics was displayed in the MeOH extracts of the Indian cultivar (57.28 ± 1.02 mg CE/g) while similar levels were displayed for the Chinese cultivar (53.79 ± 1.57 mg CE/g). The levels of the total phenolics in the MeOH/H_2O (8:2) extract were significantly higher in the Indian versus the Chinese cultivar (35.75 ± 2.91 mg vs 26.62 ± 2.97 mg CE/g, respectively). The total phenolic levels in the acetone extracts of the inner tissues of the Chinese and Indian varieties were not significantly different.

Plant phenolic compounds are known to fluctuate in response to several stimuli both biotic and abiotic. Furthermore, phenolic metabolites have been reported to vary in response to genotypic difference as well as plant maturity. Variation in total phenolic levels in bitter melon has been observed among cultivars and various processing methods such as drying, boiling, blanching and roasting [188, 189]. Our results reflected results from other studies in that the total phenolic levels do vary between cultivars. Additionally, our results also observe a difference in the levels of total phenolics in different tissues of bitter melon cultivars.

4.3.2. Targeted analysis of phenolic acids and organic acids

Elucidation of the possible phenolics compounds found in the various tissues was conducted using the targeted analysis approach by LCMS. Phenolic and organic acids were extracted with MeOH/H_2O (8:2) and subjected to chromatographic analysis. The extracts were used for the elucidation of the targeted compounds by LCMS and confirmed by TargetAnalysis™ 1.2 software (Bruker Daltonik). The results of the metabolite screening are presented in **Table 4-2**. Overall, the pericarp material yielded a wider range of compounds compared to the inner tissues. The presence of chlorogenic and ascorbic acid

was confirmed in our study and is corroborated by previous reports [86, 190]. Several aromatic carboxylic acids, such as trans-cinnamic, ferulic, protocatechuic, benzoic, salicylic, gallic and vanillic acids, were identified in the various tissues analyzed. Additionally, several acyclic carboxylic acids, such as malonic, succinic, malic, fumaric, and tartaric acids, were also identified that have not been previously reported in bitter melon. Lastly, two cyclitol compounds, quinic and shikimic acids, were identified by our targeted analysis strategy.

Variation of phenolic and organic acids in response to environmental conditions, cultivation strategies and cultivar genotypic differences in the plants is well documented. Our study showed variations in the various bitter melon tissues analyzed. Proper analytical procedures and equipment are essential for the study of plant metabolites. The use of HRMS allowed us to obtain the accurate mass spectra of the various metabolites we reported. The comparison of the experimental accurate mass and retention times with theoretical values resulted in a precise identification of phenolic metabolites.

Table 4-2. Targeted analysis of phenolic acids and organic acids identified* in bitter melon pericarp and inner tissue.

	Chinese Pericarp			Chinese Inner Tissue			Indian Pericarp			Indian Inner Tissue		
Compounds Identified	Δ RT	ΔmDa[a]	Δppm[a]	Δ RT	ΔmDa[a]	Δppm[a]	Δ RT	ΔmDa[a]	Δppm[a]	Δ RT	ΔmDa[a]	Δppm[a]
Ascorbic Acid	-0.1	5.2			-0.1	6.9	39					
Benzoic Acid				0.1	1.4	11.6	0.1	-0.2	-1.5	0	2	16
Caffeic Acid	0	4.8	26.3	0	-6.1	-33.9	0	7	38.6	-0.1	-3	-16.5
Chlorogenic Acid	-0.1	7.8	22.3	0.1	-2.6	-7.3	0.1	12.3	34.6	0.1	-3.4	-9.6
t-Cinnamic Acid	0.1	2.9	19.6	-0.1	-1.7	-11.5	0	4.4	29.4	0	1.5	10.3
Citric Acid	-0.1	5.2	29.1				0.1	7.4	38.6	0	4.1	21.2
Ferulic Acid	0.1	4.4	22.6	0.1	3.1	16	0.1	8	40.8	0.1	2.3	11.7
Fumaric Acid	0.1	-0.1	-0.8				0.1	5	42.7			
Gallic Acid	0	5.9	34.4									
Malic Acid	-0.1	1.6	11.8	-0.1	6.7	49.7				-0.1	2.9	21.6
Maleic Acid							-0.1	4.8	41.4			
Malonic Acid	0.1	2.9	19.6									
Protocatechuic Acid	0	5.2	33.4							-0.1	2	12.9
Quinic Acid	-0.1	5.1	26.6				-0.1	4.8	41.4	0	6.7	34.8
Salicylic Acid	0	6.7	48.4	0.1	6.7	48.3	0.1	6.1	44.1	-0.1	1.8	13.3
Shikimic Acid	-0.1	3	17.1	0	-6.4	36.5	0	5.6	32	-0.1	1.8	13.3
Succinic Acid	0.1	0.6	5	0.1	-3.1	-25.8	0	0.5	3.9	0.1	0.5	4.5
Tartaric Acid							0	-4.1	27.2	0	-4.1	-27.4
Vanillic Acid				0.1	4.4	25.9						

*Identification is scored with respect to measured and theoretical values for each compound

[a] Deviation of measured m/z from calculated m/z values for a pseudomelecular ion $[M+H]^+$, generated from the molecular formula.

4.3.3. Total saponins

Saponins have been reported to have several anti-diabetic activities, including mimicking the effect of insulin on cells [152]. The levels of total saponins in the pericarp extracts ranged from 6.84 ± 1.68 to 76.14 ± 2.53 mg DE/g of freeze dried material (**Table 4-1**). The highest level of total saponins was observed in the chloroform pericarp extracts of the Chinese cultivar, which was also significantly higher than the levels of saponins from the chloroform extract from Indian cultivar pericarp (41.91 ± 6.35 mg DE/g). A similar trend, while not as pronounced, was observed in the hexane and MeOH pericarp extracts. Conversely, acetone extracts of the Indian pericarp showed significantly higher (64.05 ± 6.31 mg DE/g) levels of total saponins compared to the Chinese cultivar (45.77 ± 5.75 mg DE/g) while total saponin levels of MeOH/H_2O (8:2) extracts were similar in both cultivars ranging from 8.10 ± 2.38 - 8.47 ± 4.01 mg DE/g. The total saponin levels of the inner tissue of the two bitter melon varieties are presented in Table 4-1. The highest total saponin levels were displayed by the chloroform extracts of the inner tissue of the Chinese cultivar (50.71 ± 6.35 mg DE/g), which was significantly higher than the corresponding inner tissue extract of the Indian cultivar (29.18 ± 2.64 mg DE/ g). A similar trend was observed in the levels of total saponins of inner tissue hexane extracts when compared between cultivars, with significantly higher levels (37.01 ± 6.21 mg DE/g) observed in the Chinese cultivar extract. The total saponin levels for the acetone extracts were significantly higher for the Indian cultivar versus the Chinese. No significant differences were observed in MeOH and MeOH/H_2O (8:2) inner tissue extracts between both cultivars.

The levels of total saponins measured for each of the extracts resembled levels reported by Tan et al. of water extracts of greenhouse-grown bitter melon [191]. Interestingly, Tan et al. evaluated whole fruit extracts (pericarp and inner tissues). While our levels for each type of extract individually are lower compared to this previous study, all extracts combined for each variety additively yield significantly higher total saponin level than reported by Tan et al. This reinforces our argument of the importance of evaluation of individual parts of bitter melon and the full spectrum of compound polarity. The chemical nature of the cucurbitane triterpenoids reported from bitter melon make their routine screening by traditional HPLC coupled with PDA expensive and in some cases inaccurate. The use of the total saponin method for the screening of bitter melon extracts, is a quick and reproducible way to identify extracts for evaluation of biological activities.

4.3.4. Mass spectral identification of cucurbitane type triterpenoids

Qualitative analysis of the cucurbitane type triterpenoids found in bitter melon extracts was performed using HRMS. LC/MS was utilized to detect and characterize 46 triterpenoids in the various extracts analyzed (**Tables 4-3 and 4-4**). All the metabolites detected by this method were tentatively characterized based on their mass spectral data and the interpretation of such data in accordance to literature available to date. The exact mass of the reported compounds was compared to the measured exact mass to generate a ppm value. If the error between the theoretical and measured exact mass was found to be a value between ±50 ppm the compound was considered to be a positive match. The exact masses were calculated based on the possible proton and sodium adducts under positive

ionization. Due to the low UV absorption properties of these compounds and the scarce availability of standard compounds found in the market, this is a suitable method for the identification of cucurbitane type molecules found in bitter melon. The distribution of triterpenoids in the various bitter melon matrices did not display a specific pattern except for kuguaglycocides and charantosides which were found to be specific to the Chinese and Indian bitter melon tissues, respectively **(Figure 4-1**). The structural characteristics of these compounds have been previously reviewed in our previous publication [178].

Table 4-3. Cucurbitane –type triterpenoids identified in bitter melon pericarp extracts by LC/MS

	Compound	Formula	Theoretical (*m/z*)	Experimental (*m/z*)	Adduct	Δ (ppm)[a]
		Chinese Pericarp				
Hexane	22-hydroxy-23,24,25,26,27-pentanorcucurbit-5-en-3-one	$C_{35}H_{40}O_2$	395.2914	395.2745	M+Na	-42.80
Chloroform	Momordicoside F1 aglycone	$C_{31}H_{50}O_3$	471.3827	471.3922	M+H	20.20
	(23*E*)-7β-methoxycucurbita-5,23,25-trien-3β-ol	$C_{31}H_{50}O_2$	455.3878	455.3938	M+H	13.22
	(23*E*)-3β-Hydroxy-7β,25-dimethoxycucurbita-5,23-dien-19-al	$C_{32}H_{52}O_4$	523.3753	523.3933	M+Na	34.48
	Karavilagenin B	$C_{31}H_{52}O_3$	473.3983	473.4092	M+H	23.07
Acetone	Kuguaglycoside F	$C_{42}H_{73}O_{13}$	797.5040	797.5437	M+H	49.81
	Goyaglycoside E	$C_{42}H_{68}O_{13}$	781.4727	781.5073	M+H	44.30
	Karaviloside I	$C_{38}H_{64}O_8$	649.4668	649.4868	M+H	30.83
	5β,19-epoxy-19,25-dimethoxy-cucurbita-6,23-diene-3β-ol	$C_{32}H_{52}O_4$	523.3753	523.3873	M+Na	23.02
MeOH	25,26,27-trinorcucurbit-5-ene-3,7,23-trione	$C_{27}H_{40}O_3$	413.3044	413.3260	M+H	52.31
	Momordinol	$C_{29}H_{46}O_2$	427.3565	427.3473	M+H	-21.40
	(23*E*)-Cucurbita-5,23,23-triene-3α,7α-diol	$C_{31}H_{48}O_{11}$	597.3264	597.3349	M+H	14.27
$MeOH/H_2O$ (8:2)	---------					
		Indian Pericarp				
Hexane	Kuguacin C	$C_{27}H_{42}O_3$	415.3201	415.3374	M+H	41.71
	Momordicin	$C_{31}H_{51}O_3$	471.3827	471.3671	M+H	-33.04
Chloroform	7β-Ethoxy-3β-hydroxy-25-methoxy-cucurbita-5,23(*E*)-dien-19-al	$C_{33}H_{54}O_4$	515.4089	515.3991	M+H	-18.96
Acetone	Taiwacin A	$C_{44}H_{68}O_{14}$	821.4675	821.4710	M+H	4.26
MeOH	Charantoside III	$C_{36}H_{56}O_7$	601.4092	601.4258	M+H	27.60
$MeOH/H_2O$ (8:2)	Charantoside IV	$C_{36}H_{56}O_7$	601.4092	601.4157	M+H	10.80

[a] Deviation of measured m/x from calculated m/z values for a pseudomelecular ion generated from the molecular formula

Table 4-4. Cucurbitane –type triterpenoids identified in bitter melon inner tissue extracts by LC/MS.

	Compound	Formula	Theoretical (*m/z*)	Experimental (*m/z*)	Adduct	Δ (ppm)[a]
		Chinese Inner Tissue				
Hexane	Kuguacin J	$C_{30}H_{46}O_3$	455.3514	455.3367	M+H	-32.23
Chloroform	25ξ-Isopropenylcholest-5(6)-ene 3-O-β-D-glucopyranoside	$C_{36}H_{60}O_6$	589.4457	589.4571	M+H	19.38
	3β,7β,25-Trihydroxycucurbita-5,23(E)-dien-19-al	$C_{30}H_{48}O_4$	495.3439	495.3578	M+Na	28.01
	Kuguacin R	$C_{30}H_{48}O_4$	495.3439	495.3549	M+Na	22.16
Acetone	Charantagenin D	$C_{37}H_{60}O_9$	671.4123	671.4033	M+Na	-13.43
	Kuguaglycoside A	$C_{37}H_{62}O_8$	657.4331	657.4274	M+Na	-8.70
	Kuguacin B	$C_{30}H_{48}O_3$	457.3670	457.3885	M+H	47.06
Methanol	Momordicoside Q	$C_{36}H_{60}O_{10}$	653.4254	653.4522	M+H	41.05
	Kuguaglycoside A	$C_{37}H_{62}O_8$	657.4331	657.4276	M+Na	-8.39
	Kuguaglycoside B	$C_{37}H_{62}O_8$	657.4331	657.4262	M+Na	-10.52
MeOH/H_2O (8:2)	Kuguaglycoside A	$C_{37}H_{62}O_8$	657.4331	657.4333	M+Na	0.27
		Indian Inner Tissue				
Hexane	Kuguacin J	$C_{30}H_{46}O_3$	455.3514	455.3405	M+H	-23.88
Chloroform	Octanorcucurbitacin A	$C_{22}H_{30}O_3$	365.2081	365.2051	M+Na	-8.27
	Momordicinin	$C_{30}H_{46}O_2$	439.3565	439.3709	M+H	32.82
	19-dimethoxycucurbita-5(10),6,22(*E*),24-tetraen-3β-ol	$C_{32}H_{50}O_3$	505.3646	505.3678	M+Na	6.29
	23*E*-3β-hydroxy-7β,25 dimethoxycucurbita 5,23-dien-19 al	$C_{32}H_{52}O_3$	507.3802	507.3813	M+Na	2.12
	cucurbita-1(10)5,22,24-tetraen-3α-ol	$C_{30}H_{46}O$	423.3616	423.3817	M+H	47.53
Acetone	3β,7β,23-trihydroxycucurbita-5,24-diene-7-*O*-β-D-glucoside	$C_{36}H_{60}O_8$	643.4174	643.4262	M+Na	13.63
MeOH	Karavilagenin E	$C_{30}H_{48}O_3$	457.3670	457.3688	M+H	3.98
	Charantoside III	$C_{36}H_{56}O_7$	601.4093	601.4108	M+H	2.54
	Momordicoside A	$C_{42}H_{72}O_{15}$	817.4938	817.4996	M+H	7.12
	Charantoside IV	$C_{36}H_{56}O_7$	601.4093	601.4176	M+H	13.84
	23*E*-3β-hydroxy-7β,25 dimethoxycucurbita 5,23-dien-19 al	$C_{32}H_{52}O_3$	507.3802	507.3738	M+Na	-12.65
	Momordicoside L	$C_{36}H_{58}O_9$	657.3967	657.4065	M+Na	14.87
	3β,7β,25-Trihydroxycucurbita-5,23(*E*)-dien-19-al	$C_{30}H_{48}O_4$	495.3439	495.3514	M+Na	15.09
	Cucurbita-5,24-diene-3β,23(*R*)-diol 7-*O*-β-D-glucopyranoside	$C_{36}H_{60}O_8$	643.4174	643.4216	M+Na	6.49
	5β,19-Epoxycucurbita-6,22,24-trien-3β-ol	$C_{30}H_{46}O_2$	439.3565	439.3687	M+H	27.82
MeOH/ H_2O (8:2)	Karaviloside III	$C_{37}H_{62}O_8$	659.4493	659.4555	M+H	9.40

[a] Deviation of measured m/x from calculated m/z values for a pseudomelecular ion generated from the molecular formula

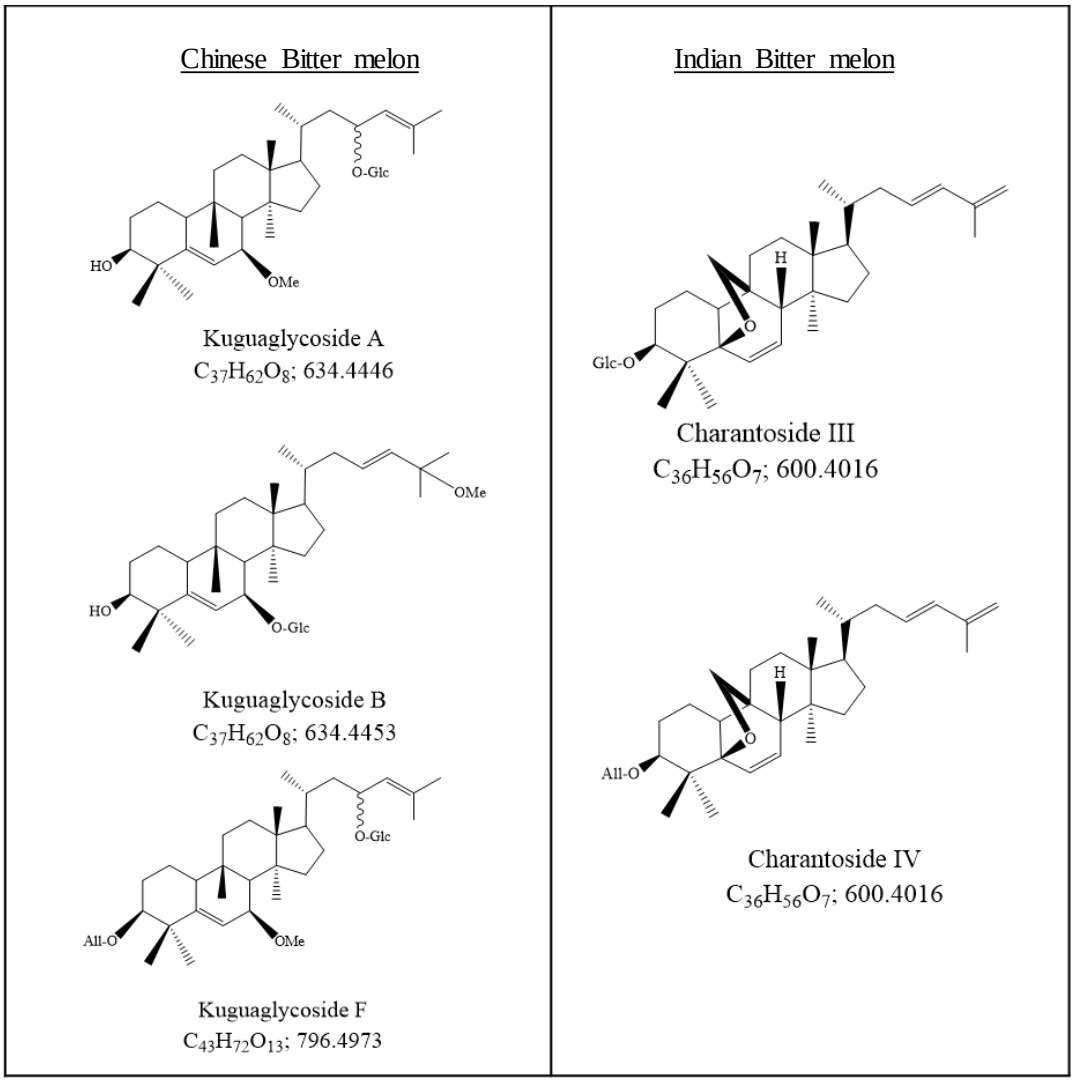

Figure 4-1. Structures of cucurbitane-type triterpenoids specific to bitter melon cultivars (acronyms Glc: glucose, All: allose)

4.3.5. DPPH free radical scavenging activity

The radical scavenging potential of bitter melon extracts was evaluated by the DPPH method. DPPH – radical scavenging activity varied depending on the tissue type, cultivar and extract polarity (**Table 4-1**). The highest DPPH activity was observed in MeOH extracts of inner tissues of both cultivars evaluated ranging from 27.94 ± 1.84 to 30.39 ± 11.64 mg AAE/ g of freeze-dried material. This may have been attributed to the fact that those extracts also had the highest levels of total phenolic compounds of all of the extracts evaluated. **Table 4-5** illustrates the positive correlation between total phenolic levels and DPPH activity. The hexane and MeOH/H_2O (8:2) pericarp extracts of the Chinese cultivar displayed significantly higher DPPH activity compared to the Indian cultivar. The rest of the pericarp extracts did not show significant differences between cultivars. Similarly, hexane, chloroform, acetone and MeOH extracts of the inner tissues did not show significant differences between the two cultivars. Higher DPPH activity was observed in the inner tissue MeOH/H_2O (8:2) extract of the Indian cultivar (19.18 ± 1.51 mg AAE/g) versus the Chinese, again possibly correlated to the total phenolic levels of those extracts (**Table 4-5**).

Table 4-5 . Pairwise correlation values between levels of total phenolics and saponins with *in vitro* bioassays.

	DPPH	α-Amylase Inhibition	α-Glucosidase Inhibition
Total Phenolics	0.7349^{+++}	0.1289	0.0299
Total Saponins	-0.2021	-0.044	0.5229^{+++}

Statistical significance between a pair of variables is presented as $^{+++}$ representing $p< 0.001$.

Reactive oxygen species have been linked to several chronic health conditions such as obesity and diabetes [192]. Under oxidative stress, glucose uptake by muscle cells is impaired and insulin secretion by pancreatic β-cells is greatly reduced [193]. A constant presences of reactive oxygen species has been reported to damage and interfere with the metabolic process of pancreatic β-cells, leading to diabetes [194]. Recent studies have evaluated the current anti-diabetic medication for their antioxidant activities and have proposed the use of antioxidants as tools for managing diabetes [195]. Differences in the chemical nature of the various extracts evaluated expressed differential DPPH activity. As illustrated in **Table 4-5**, there is a strong correlation between levels of total phenols and DPPH. Interestingly, hexane extracts also showed strong DPPH activity. This may be due to lipophilic antioxidants such as fatty acids, sterols, tocopherols, and carotenoids potentially extracted with nonpolar solvents like hexane. Bitter melon has been reported to contain all of these classes of compounds [87]. As such, the antioxidant properties of bitter melon extracts may possibly be a factor that affords bitter melon its antidiabetic activity.

4.3.6. In vitro hypoglycemic activities

A typical screening method for evaluating the antidiabetic potential of molecules is by measuring their capacity to inhibit enzymes that metabolize carbohydrates, such as α-amylase and α-glucosidase. These enzymes are responsible for the breakdown of polysaccharides into absorbable monosaccharide units. α-Amylase is secreted by salivary glands and the pancreas to hydrolyze the glucosidic linkages that bind starches and other polysaccharides together. Additionally, α-glucosidase is secreted by intestinal cells to further breakdown disaccharides into simple carbohydrates that are absorbable by the

intestinal epithelia. Hence, inhibition of these enzymatic activities lowers blood glucose levels by preventing the absorption of carbohydrates.

Several vegetables, including bitter melon, have been found to affect the action of α-amylase. While studies have evaluated the use of bitter melon extracts, and in some cases isolated compounds, to explore the inhibitory effects on polysaccharide degrading enzymes such as α-glucosidase, relatively few studies have explored the inhibitory effect on α-amylase. Most of the studies that have reported on bitter melon's α- amylase inhibitory activity evaluated water extracts, and interestingly in some cases not reporting significant inhibitory activity [196]. Another study, reported on the successive extraction of leaves, fruit and seeds, ranging from cold water extracts to cyclohexane [197]. Several of their polar fraction demonstrated potent inhibitory effects against α- amylase, but the nonpolar extracts were disregarded because of their inhibitory values less than 50 %. While we did observe such values in hexane extracts of the Indian pericarp material and the inner tissues, our inhibitory values for the Chinese pericarp hexane extracts was ~75% (**Table 4-1**), slightly lower than the value obtained from the acarbose standard (88%) These values are significantly higher than that observed in the Indian pericarp (39%), demonstrating the importance of evaluating bitter melon cultivars. The highest inhibitory activity was observed in the acetone extracts of the inner tissue of the Chinese cultivar (94%), which was significantly higher than the inhibition activity of acarbose and the inner tissue acetone extracts of the Indian cultivar (69%). Acetone extracts of the pericarp material showed inhibition of more than 90% in both the cultivars. In the case of chloroform extracts, both the Indian pericarp and inner tissue extracts showed inhibitory values around 70%. The inhibitory activity of the MeOH extracts of both varieties were not significantly different

from each other ranging from 71-77% inhibition; however, the MeOH extracts of the inner tissues of the Indian cultivar displayed significantly higher inhibitory activity (91%) compared to the Chinese (78 %). Lastly, Chinese bitter melon pericarp and inner tissue extracted with MeOH/H_2O (8:2) showed significantly higher levels of inhibitory activity compared to the corresponding tissues of the Indian bitter melon cultivar.

Furthermore, several concentrations of extracts were evaluated for inhibitory properties against α-glucosidase. The inhibition of α-glucosidase activity was assessed *in vitro* by the release of p-nitrophenol from p-nitrophenyl-α-D-glucopyranoside. A dose dependent increase in inhibitory activity was observed in the various bitter melon extracts analyzed in this study. All Chinese pericarp extracts displayed similar inhibitory properties in the lowest concentration evaluated (50 µg/mL), ranging from 24-33% inhibition of α-glucosidase (**Table 4-6**). The levels of inhibition within the 100 µg/mL group increased slightly from the previous extract concentration, but only the MeOH extract was significantly higher ($p< 0.01$). All of the 100 µg/mL Chinese pericarp extracts displayed similar inhibitory activity ranging from 36-43 % inhibition. The inhibitory activity of the Chinese pericarp extracts at 200 µg/mL significantly inhibited α-glucosidase activity, particularly in the hexane extract (80.8% enzyme inhibition) which was significantly higher ($p<0.001$) than all other extracts at that concentration level. The level of inhibition was similar to the acarbose standard. At the highest concentration of Chinese pericarp extracts evaluated (250 µg/mL), inhibition also increased significantly from the previous concentration levels. The hexane extract at this level continued having the highest inhibitory activity (101.5%). The level of inhibition at this concentration was significantly higher than the acarbose standard. The chloroform, acetone, methanol and 80% methanol

extracts all had similar inhibitory activities ranging from 75-88% inhibition. Even though the level of the chloroform extracts was lower, (88%) it was still considered as having similar inhibitory properties as the hexane extracts according to statistical analysis. Regarding the various extracts from the Indian bitter melon pericarp, hexane extract in all concentration were more active in inhibiting α-glucosidase activity versus the MeOH and MeOH/H_2O (8:2) extracts. In most cases, chloroform and acetone extracts of the Indian bitter melon pericarp were inhibitory at similar levels as hexane extracts. Furthermore, the chloroform extracts at the 250 μg/mL level displayed the highest inhibitory activity in this study (102%).

Table 4-6. Pair-wise comparison of α-glucosidase between cultivars and tissues for each of the evaluated extract concentrations

		Pericarp		Inner Tissue		Chinese		Indian	
	Conc. (µg/mL)	Chinese	Indian	Chinese	Indian	Pericarp	Inner Tissue	Pericarp	Inner Tissue
Hexane	50	33.5 ±9.6[a]	40.8±16.2[a]	54.7±25.0[a]	47.1±12.8[a]	33.5 ±9.6	54.7±25.0[++]	40.8±16.2	47.1±12.8
	100	43.0±7.8[a]	57.3±21.1[a]	73.2±3.5[+]	50.5±7.5[a]	43.0±7.8	73.2±3.5[+++]	57.3±21.1	50.5±7.5
	200	80.9±6.4	91.5±6.9[++]	82.1±2.01[+++]	57.7±1.0[a]	80.9±6.4	82.1±2.01	91.5±6.9[+++]	57.7±1.0
	250	101.6±6.7[b]	92.2±8.0	86.1±21.8	77.2±10.4	101.6±6.7[+]	86.1±21.8	92.2±8.0[+]	77.2±10.4
Chloroform	50	24.4±3.4[a]	38.6±18.9[a]	36.7±7.2[a]	33.2±15.5[a]	24.4±3.4	36.7±7.2	38.6±18.9	33.2±15.5
	100	36.9±2.5[a]	47.7±11.7[a]	45.5±5.2[a]	40.9±10.9[a]	36.9±2.5	45.5±5.2	47.7±11.7	40.9±10.9
	200	54.4±3.0[a]	93.0±8.4[+++]	49.8±5.3[a]	57.1±6.3[a]	54.4±3.0	49.8±5.3	93.0±8.4[+++]	57.1±6.29
	250	88.9±6.7	102.0±2.4[b]	75.1±5.4[+]	77.6±3.9	88.9±6.7	75.1±5.4	102.0±2.4[+++]	77.6±3.9
Acetone	50	36.4±10.4[a]	33.8±7.4[a]	28.6±4.3[a]	37.7±4.7[a]	36.4±10.4	28.6±4.3	33.8±7.4	37.7±4.7
	100	42.4±10.9[a]	40.4±7.5[a]	39.4±2.6[a]	52.3±7.7[a]	42.4±10.9	39.4±2.6	40.4±7.5	52.3±7.7
	200	57.9±11.8[a]	63.6±8.4[a]	50.0±0.1[a]	55.7±0.5[a]	57.9±11.8	50.0±0.1	63.6±8.4	55.7±0.5
	250	79.9±10.2	82.0±8.2	76.3±10.7	73.3±2.8	79.9±10.2	76.3±10.7	82.0±8.2	73.3±2.8
MeOH	50	25.9±7.4[a]	21.5±2.8[a]	29.2±6.1[a]	23.1±5.8[a]	25.9±7.4	29.2±6.1	21.5±2.8	23.1±5.8
	100	40.3±6.4[a]	33.3±5.1[a]	43.5±1.3[a]	42.9±3.7[a]	40.3±6.4	43.5±1.3	33.3±5.1	42.9±3.7
	200	58.6±5.9[a+++]	45.0±7.0[a]	59.2±3.2[a]	54.9±2.2[a]	58.6±5.9	59.2±3.2	45.0±7.0	54.9±2.2
	250	75.0±9.6[+]	60.0±4.8[a]	67.3±9.8	76.5±3.6	75.0±9.6	67.3±9.8	60.0±4.8	76.5±3.6
MeOH/ H_2O	50	26.2±6.7[a]	14.5±1.4[a]	26.9±4.7[a]	28.6±7.7[a]	26.2±6.7	26.9±4.7	14.5±1.4	28.6±7.7
(8:2)	100	36.5±4.1[a]	33.2±2.9[a]	38.6±6.4[a]	37.8±4.2[a]	36.5±4.1	38.6±6.4	33.2±2.9	37.8±4.2
	200	50.4±3.4[a]	54.9±0.2[a]	56.6±2.2[a]	62.4±5.3[a]	50.4±3.4	56.6±2.2	54.9±0.2	62.4±5.3
	250	77.4±2.7	70.1±3.1	74.3±5.2	79.8±7.3	77.4±2.7	74.3±5.2	70.1±3.1	79.8±7.3
Acarbose	80	81.9± 3.0	81.9± 3.0	81.9± 3.0	81.9± 3.0	-	-	-	-

The inhibitory action of the various extracts are presented as % inhibition ± S.E.

Statistical significance between a pair of values is presented as [+], [++], or [+++] representing $p< 0.05$, 0.01, or 0.001, respectively. Superscript [a] and [b] indicate inhibition significantly lower or higher compared to acarbose, respectively

The α-glucosidase activity was also evaluated for the various extracts obtained from the inner tissue of the Chinese and Indian bitter melon cultivars. Hexane extracts from the inner tissue of the Chinese bitter melon consistently showed the highest inhibitory activity, in all levels evaluated when compared to the Indian cultivar extract. Interestingly, the Chinese inner tissue hexane extract displayed the highest inhibitory activity of all the inner tissue extracts in this study. Hexane extract inhibition of α-glucosidase ranged from 54% in the lowest level to 86% at the highest level evaluated. The inhibitory activity of the hexane, chloroform, and acetone extracts of the Indian bitter melon inner tissues were of similar levels, ranging from 33-47% inhibition while MeOH extract inhibition was lower at 50 µg/mL levels of extract. When those same extracts were evaluated at the 250 µg/mL level, the MeOH/H_2O (8:2) extract had the highest inhibitory activity (79%) while the remaining extracts ranged from 73-77% inhibition. **Table 4-6** presents the pair-wise comparison of the inhibition of α-glucosidase activity between various tissue and cultivar combinations at the different concentration of extracts evaluated.

The pairwise correlation presented in **Table 4-5** indicates a positive correlation between the levels of total saponins and α-glucosidase inhibition. Saponins from various plant species, including bitter melon, have been reported to have anti-diabetic properties [198]. In vitro and in vivo studies have concluded that saponins play a role counteracting hyperlipidemia, oxidative stress, obesity and hyperglycemia. One strategy for the management of hyperglycemia is the inhibition of α-amylase and α-glucosidase, which break down polysaccharides into absorbable monosaccharides. Our results are congruent with previous studies indicating the inhibition of α-glucosidase by saponins found in other plant species [199].

Furthermore, previous studies have demonstrated a strong correlation between plant polyphenolic compounds and the inhibition of metabolic activities of α- amylase and α-glucosidase [200]. Our results indicate that inhibition of these enzymes was observed in medium polar to polar extracts such as acetone, MeOH and MeOH/H_2O (8:2) extracts, yet the inhibition of α-glucosidase and α-amylase was more pronounced in the non-polar extracts. In the case of α-glucosidase higher inhibition was typically observed in hexane and chloroform extracts, while the inhibition of α-amylase was typically found in medium polar extracts. These results are indicative that bitter melon's hypoglycemic properties may be due to both individual and synergistic relationships occurring between different classes of compounds. Our analysis of bitter melon extracts indicates that there are several compounds found within its matrix that may have these potential biological activities. Previous studies have individually evaluated the inhibitory activities of several phenolic compounds such as trans-cinnamic acid, ferulic acid, vanillic acid and caffeic acid, all reported as potent inhibitors both α-glucosidase and α-amylase [201, 202] . Similarly, the inhibitory activity of saponins or cucurbitane-type triterpenoids has also been evaluated from various plant sources, reported to be potent inhibitors of α-glucosidase and α-amylase activities [203, 204]. The inhibition of α-glucosidase and α-amylase may help regulate glucose uptake from the intestinal lumen leading to homeostatic blood glucose levels. While the inhibition of α-amylase was not correlated to the levels of total phenolic and saponin, it is possible other metabolites present in the bitter melon matrix may be playing a role.

4.4. Conclusion

Diabetes is a complex chronic disease whose incidence is increasing every year. Among the factors that contribute to the development of diabetes are the effect of reactive oxygen

species and hyperglycemia. Traditionally, bitter melon has been used to manage diabetic symptoms and complications. However, the mechanisms of action have not been clearly understood. Bitter melon is rich in various bioactive compounds that play an important role in regulation several biological processes in human health. The present study reports two commercial varieties commonly found in the market for their metabolite content and biological activities. The results of this investigation suggest that bitter melon extracts have chemical constituents with differential antioxidant and anti-hyperglycemic activities. It would be erroneous to promote one extract or cultivar over another. Our data shows that while one cultivar may have higher antioxidant properties, another may influence other metabolic enzymes. More in-depth studies are required to elucidate the actual mechanisms in which bitter melon may promote homeostatic well-being.

5. POSTHARVEST STORAGE CONDITIONS AFFECT ASCORBIC ACID LEVELS, AMINO ACIDS, AND ANTIOXIDANT CAPACITIES IN BITTER MELON (*MOMORDICA CHARANTIA* L.) FRUIT

5.1. Introduction

Over the past decade, increasing emphasis has been placed on the consumption of fruit and vegetables that are rich in antioxidants and other health-promoting phytonutrients. Unfortunately, these molecules of interest fluctuate in most horticultural crops in response to pre- and postharvest factors [205, 206] . For example, the levels of fertilizer, irrigation schedule, UV radiation, and pests (insects, bacteria, and fungi) have modify the levels of flavonoids and ascorbic acid [207-209]. Postharvest handling of horticultural crops is also a major factor influencing the levels of beneficial compounds [210]. Factors such processing techniques, packaging, and storage of fruit and vegetables drastically alter the levels of health-promoting compounds in various crops [211-213].

Bitter melon (*Momordica charantia* L.) is a horticultural crop that has been reported to have several health-beneficial properties. Several classes of compounds have been identified within the bitter melon matrix and the biological activities of these compounds have been reported [214]. Bitter melon has been used in traditional medicine as a hypoglycemic agent, anti-malarial, anti-oxidant, and anti-inflammatory treatment [214, 215]. Even though it is widely known and used in many Asian and South American countries, it is relatively unknown and underutilized in the United States.

Historically, bitter melon has been grown and sold locally due to limited demand; however, with the increase of ethnic diversity, the demand for various specialty crops such as bitter melon, has steadily increased over time, prompting studies on the feasibility of growing these crops in the United States. For example, we recently grew five cultivars of bitter melons in Texas [216]. To meet the recent increases in demand, bitter melon has been imported from neighboring countries. During this process, considerable fruit loss occurs due to various postharvest conditions. Bitter melon is a climacteric fruit, maturing rapidly at ambient temperature. To prevent rapid maturation, storage at low temperatures is typically employed to transport and store fruit before they reach the consumer market. Unfortunately, bitter melon is also prone to chilling injury. Bitter melon quality is maintained when fruit is stored between 10 and 12.5 °C, but storage below 10 °C leads to chilling injuries such as surface discoloration and pitting [217].

Vitamin C (ascorbic acid and dehydroascorbic acid) is a naturally-occurring essential vitamin that has various health benefitting properties. Vitamin C levels are affected by postharvest handling due to both enzymatic and non-enzymatic factors. For example, one study revealed that severe chilling injury was observed in fresh cut fruit stored at 7.5 °C, yet chilling injury and quality parameter changes, such as ascorbic acid content, were not as pronounced when fruit was stored at 2 °C [218]. Recently, we have reported the levels of ascorbic acid in five cultivars of bitter melon [216], showing that bitter melon is a rich source of ascorbic acid. Therefore, it is important to explore the effects of fruit processing and storage on total ascorbic acid content.

Similarly, other metabolites such phenolic compounds and amino acids fluctuate in response to biotic and abiotic factors. Phenolic compounds have potent antioxidant activities; hence, it is important of understand how these fluctuate in response to postharvest conditions, such as storage time, temperature, and processing. These conditions also affect levels of amino acids, which serve as building blocks for important biological molecules, and including some potent bioactive molecules [219-221]. For example, γ-aminobutyric acid (GABA) is a non-protein amino acid that has been reported to have anti-hypertensive, anti-cancer, anti-diabetic, and neurological effects [222]. Furthermore, arginine, leucine and alanine have been reported to play an important role in the prevention of diet-induced obesity [220, 221]. The levels of individual amino acids can fluctuate under different storage conditions and food preparation methods, but that change depends on the individual amino acid [223, 224]. In general, relatively few studies have evaluated the change of amino acids during storage and processing.

In recent years, consumers have become more conscious of the health-promoting properties and accurate profiling of bioactive compounds present in the food obtained from the supermarket. Additionally, consumers have now taken on a more active role in the preservation of the levels of these bioactive compounds at the home setting. Nutrition clearly influences the onset and progression of several chronic diseases. As population diversity increases over time, foods considered traditional or ethnic, such as bitter melon, will begin to gain interest from consumers. However, compared to other fruit and vegetables, relatively little information is available regarding postharvest effects on bitter melon bioactive

compounds. The objective of this study is to simulate household processing techniques to understand the changes in vitamin C, total phenolics, amino acids, and antioxidant activities in bitter melons stored at different temperatures for 8 weeks. To the best of our knowledge, this is the first report on postharvest changes in amino acid levels in bitter melons.

5.2. Materials and methods

5.2.1. Plant materials

Bitter melons were purchased from a local market (BCS Market, College Station, TX). The cultivar used in this study was identified as India Green according to the information furnished by the producer's label.

5.2.2. Minimal processing of bitter melons

Bitter melon fruit used for processing were disinfected, sliced, and seeds removed before allocating the fruit pericarp to a specific processing technique. Whole melons were disinfected and separated into storage groups. In addition to the evaluation of whole fruit, two processing procedures were evaluated in this study. First, sliced fruit was chopped into small cubes which were placed in 50-mL conical tubes to be stored for a predetermined time period (1, 2, 4, and 8 weeks) and at specific storage temperatures (25 [room temperature, RT], 10, 2, and -20 °C). The second processing procedure consisted of blending sliced bitter melon pericarp and aliquoting the puree into 50-mL conical tubes before storage under the conditions previously described. Each temperature and storage treatment included three individual biological replicates. After the storage time was attained, the

samples were subjected to sample preparation for the analysis of the metabolites of interest.

5.2.3. Vitamin C analysis

Immediately after removing samples from storage, ascorbic acid, dehydroascorbic acid (DHA) and total ascorbic acid levels were determined according to our previously reported method [225]. Briefly, 5 g of bitter melon pericarp was homogenized in 10 mL of 3% meta-phosphoric acid using a polytron homogenizer (Brinkman, Instrument, Westbury, New York, USA) for 30 s at medium speed followed by a 10 s vortex agitation. Additionally, each sample was sonicated three times for 30 s using a sonic dismembrator (Model F60, Thermo Fisher Scientific, Pittsburgh, PA). The samples were again vortexed and 1-mL aliquots were transferred into 1.5-mL micro-centrifuge tubes. The sample was then centrifuged at 4000 x g for 10 min. After centrifugation the supernatant was filtered through a 0.2 µm filter and used for ascorbic acid analysis by HPLC [225].

For analysis of DHA, tris (2-carboxy ethyl) phosphine hydrochloride (TCEP) was used for the reduction of DHA to ascorbic acid. After centrifugation and filtration of the extracted samples, 0.5 mL of extract was treated with 0.5 mL of 5 mM TCEP. The mixture was vortexed and incubated for 30 min at 25 °C. Following the incubation period, the samples were analyzed by HPLC. The DHA content in the sample was calculated as the difference between total ascorbic acid (ascorbic acid after reduction with TCEP) and ascorbic acid (ascorbic acid level before reduction with TCEP). For quantification of ascorbic acid levels in bitter melon, a calibration curve was constructed using standard ascorbic acid ranging

from 0.07 to 10 µg per injection. The resulting regression equation was used for the quantification of ascorbic acid in bitter melon samples. Each bitter melon sample was injected in triplicate for HPLC analysis.

5.2.4. Extraction of amino acids

The extraction of free amino acids from bitter melon pericarp was performed according to previously published methods with slight modifications [162]. Stored bitter melon (2 g) was homogenized with 5 mL of 8:2 (v/v) methanol: water for 30 s (3 times at 10 s intervals). After homogenization, glass beads were added to the mixture and tubes were placed in a boiling water bath for 20 min to extract free amino acids. The mixture was cooled to room temperature and centrifuged at 4000 x g for 10 min. The resulting supernatant was then filtered through glass wool. The remaining pellet was re-extracted two more times to ensure complete extraction of amino acids. The pooled filtrates were concentrated and adjusted to a final volume of 5 mL with 80 % MeOH.

5.2.5. Derivatization for amino acid analyses by HPLC

A 700 µL aliquot of each sample extract was transferred to an amber vial followed by the addition of 250 µL of dansyl chloride (10 g L^{-1} in acetone). Sodium borate buffer (700 µL; 5 mM) was the added to the mixture and vortexed for 1 min followed by a 30 min incubation in a heated water bath maintained at 60 °C. Following the incubation period, the samples were removed from the heated water bath and 60 µL of 2 N acetic acid was added, then the mixture was vortexed for 30 s to stop the derivatization reaction. The reaction mixture was then centrifuged for 5 min at 4000 x g and the supernatant was transferred to an amber

HPLC vial for analysis. The derivatized amino acids were separated on an Eclipse XDB-C8 column (5 μm particle size) (4.6 mm × 150 mm) connected to Perkin Elmer Series HPLC system using 1% formic acid (Solvent A) and acetonitrile with 1 % formic acid and 1 % TEA (Solvent B). The HPLC system was equipped with a FLD detector. The detection of the derivatized amino acids was carried out at 293 nm for excitation and 492 nm for emission.

Regression curves for 17 standard amino acids were developed by injecting different concentration of amino acid acids (0.03, 0.06, 0.12, 0.25, 0.50, and 1.01 mg L^{-1}) to obtain the peak areas. Concentrations of individual amino acids in the extracts were calculated using regression equations and dilution factor. The amounts of individual free amino acids are reported as mg kg^{-1} of sample.

5.2.6. Bioassays

5.2.6.1. Sample preparation

Bitter melon samples were removed periodically from their respective storage temperatures and weighed (2 g) into 15-mL tubes. Phenolic compounds were extracted with 10 mL of 80 % MeOH by 30 s of homogenization, and sonicated for 30 min and vortexed for 1 min. The samples were then centrifuged at 4,000 x g for 5 min. The resulting supernatant was then decanted into a 50-mL conical tube and the extraction procedure was repeated twice on the remaining pellet. The pooled supernatants were then concentrated to a final volume of 20 mL and stored at -20 °C until analysis.

5.2.6.2. Total phenolics

The quantification of the total phenolic levels in bitter melon extracts was carried out in accordance with our previously published method using a 96-well microplate reader [226]. The reaction consisted of 20 µL of bitter melon extract adjusted to 200 µL using nano-pure water. Folin-Ciocalteu reagent (20 µL, 1 M) was added to each well and incubated for 10 min at 25 °C. After the incubation period, saturated sodium carbonate (40 µL) was added to each reaction well and incubated for an additional 20 min at 25 °C and absorbance was measured at 760 nm, using a microplate reader (BioTek Instruments, Inc.). The levels of total phenolics in bitter melon extracts were expressed as mg of gallic acid equivalents per kg of fresh sample.

5.2.6.3. DPPH scavenging activity

The 2,2-diphenyl-1-picryhydrazyl (DPPH) radical-scavenging activity of bitter melon extracts was assayed according to our previously published method [210]. Bitter melon extracts (20 µL) were adjusted to 100 µL using methanol, 180 µL of DPPH solution (0.1 mM) was added each well and incubated for 20 min in dark. The absorbance of the reaction mixture was then measured at 515 nm using a microplate reader. Assay blanks consisted of 20 µL of samples with 280 µL of methanol. The difference between the absorbance values of the blank and the sample absorbance was used to determine the DPPH radical scavenging activity. Various concentrations of ascorbic acid were used to prepare a calibration curve and results were expressed as ascorbic acid equivalents per gram of fresh weight (mg kg^{-1}).

5.2.6.4. 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The ABTS reagent and ascorbic acid standard (0.05 g L^{-1}) were freshly prepared before the initiation of assays according to our previously published method [226]. The ABTS scavenging activity was expressed as ascorbic acid equivalents per fresh weight (mg kg^{-1}).

5.2.7. Statistical analysis

All experiments were performed in triplicate. For HPLC analysis, each sample was analyzed by triplicate injections. Statistical analysis on the means of triplicate experiments were performed using the ANOVA procedure with InStat software, version 3.0 (GraphPad, San Diego, CA). Tukey's test of significance between means was used for identification of significance. Amino acids data sets were analyzed using Metaboanalyst online software (https://www.metaboanalyst.ca/). Probabilistic quotient normalization was carried out using initial values as a reference group and followed by a log normalization of data. Normalized data was used to carry out partial least squares-discriminate analysis (PLS-DA). Variable importance in projection (VIP) scores were used for the illustration of important feature of the PLS-DA.

5.3. Results

5.3.1. Levels of ascorbic acid at different storage times and temperatures

5.3.1.1. Chopped fruit

The levels total ascorbic acid decreased during storage regardless of the temperature, with the exception of chopped fruit that was stored for one week at 2

°C (1.06 g kg^{-1}) compared to initial total ascorbic acid values (1.16 g kg^{-1}) (**Table 5-1**). Total ascorbic acid sharply declined when chopped fruit was stored for one week at room temperature, and thereafter values were not obtained due to fruit spoilage. Bitter melon fruit lasted for two weeks at 10 °C storage temperature. Significant decreases were observed throughout the two-week storage time, ending at 0.37 g kg^{-1}. A similar decrease was observed for samples stored at 2 °C, which lasted up to four weeks in storage under these conditions. While the level of total ascorbic acid was lowest after four weeks of storage, the levels were not significantly lower than the samples stored for two weeks at 2 °C. Lastly, chopped bitter melon samples stored at -20°C lasted through the whole 8-week evaluation period. The levels of total ascorbic acid at the end of the 8-week evaluation at -20 °C were significantly lower than levels measured at the other time periods. While the levels at the 1-, 2-, and 4-week storage time at this temperature were lower than the initial values, they remained relatively similar during this time, although they decreased significantly at the 8-week mark. It is important to note that the decrease in levels of total ascorbic acid in chopped fruit stored at -20 °C was much slower than whole and blended fruit stored at the same temperatures (**Table 5-1**). The levels of ascorbic acid followed a similar trend as total ascorbic acid. The largest decrease in ascorbic acid levels was measured in bitter melon samples stored at 10 °C for two weeks (0.23 g kg^{-1}).

The levels of dehydroascorbic acid in chopped bitter melon remained similar to initial levels or increased slightly during the first week of evaluation, except for samples stored at room temperature, which sharply decreased after one week of

storage (0.03 g kg^{-1}) (**Table 5-1**). Dehydroascorbic acid levels in samples stored at 10 °C remained similar to initial levels throughout the 2-week storage period. Samples stored at 2 °C had similar levels to initial values after one week of storage. Subsequently, the level of dehydroascorbic acid increased significantly after two and four weeks of storage when compared to initial values. Levels of dehydroascorbic acid in bitter melon samples stored at -20 °C significantly increased after one week of storage. Those levels remained constant when fruit stored for two and four weeks were evaluated. The level of dehydroascorbic acid after storage for 8 weeks reverted to initial levels.

Table 5-1. Changes in levels of total ascorbic acid, ascorbic acid, and dehydroascorbic acid (g kg^{-1} ± SEM) in bitter melon stored at room temperature (25 °C), 10 °C, 2 °C, and -20 °C.

		Storage time				
	Temp (°C)	Initial	1-Week	2-Weeks	4-Weeks	8-Weeks
Chopped						
Total Ascorbic Acid	25	1.16 ± 0.08^{a}	0.35 ± 0.04^{b}	ND	ND	ND
	10	1.16 ± 0.08^{a}	0.64 ± 0.04^{b}	0.37 ± 0.01^{c}	ND	ND
	2	1.16 ± 0.08^{a}	1.06 ± 0.01^{ab}	0.86 ± 0.04^{bc}	0.81 ± 0.03^{c}	ND
	-20	1.16 ± 0.08^{a}	0.91 ± 0.07^{b}	0.93 ± 0.01^{b}	0.87 ± 0.02^{b}	0.62 ± 0.04^{c}
Ascorbic Acid	25	1.02 ± 0.07^{a}	0.32 ± 0.04^{b}	ND	ND	ND
	10	1.02 ± 0.07^{a}	0.45 ± 0.03^{b}	0.23 ± 0.02^{c}	ND	ND
	2	1.02 ± 0.07^{a}	0.86 ± 0.01^{a}	0.65± 0.04^{b}	0.59 ± 0.03^{c}	ND
	-20	1.02 ± 0.07^{a}	0.68 ± 0.06^{b}	0.73 ± 0.009^{b}	0.63 ± 0.01^{bc}	0.48 ± 0.05^{c}
Dehydroascorbic Acid	25	0.13 ± 0.009^{a}	0.03 ± 0.003^{a}	ND	ND	ND
	10	0.13 ± 0.009^{a}	0.18 ± 0.008^{a}	0.13 ± 0.02^{a}	ND	ND
	2	0.13 ± 0.009^{a}	0.20 ± 0.01^{a}	0.21 ± 0.01^{a}	0.21 ± 0.01^{a}	ND
	-20	0.13 ± 0.009^{a}	0.22 ± 0.08^{a}	0.20 ± 0.01^{a}	0.23 ± 0.01^{a}	0.13 ± 0.004^{a}
Blended						
Total Ascorbic Acid	25	1.16 ± 0.08^{a}	ND	ND	ND	ND
	10	1.16 ± 0.08^{a}	0.11 ± 0.002^{b}	ND	ND	ND
	2	1.16 ± 0.08^{a}	0.63 ± 0.02^{b}	0.64 ± 0.004^{b}	0.58 ± 0.009^{b}	ND
	-20	1.16 ± 0.08^{a}	0.68 ± 0.01^{b}	0.65 ± 0.01^{b}	0.61 ± 0.004^{b}	0.70 ± 0.01^{b}
Ascorbic Acid	25	1.02 ± 0.07^{a}	ND	ND	ND	ND
	10	1.02 ± 0.07^{a}	0.003 ± 0.001^{b}	ND	ND	ND
	2	1.02 ± 0.07^{a}	0.25 ± 0.01^{b}	0.40 ± 0.006^{c}	0.18 ± 0.004^{b}	ND
	-20	1.02 ± 0.07^{a}	0.21 ± 0.01^{b}	0.28 ± 0.02^{bc}	0.20 ± 0.004^{b}	0.39 ± 0.004^{c}
Dehydroascorbic Acid	25	0.13 ± 0.009^{a}	ND	ND	ND	ND
	10	0.13 ± 0.009^{a}	0.11 ± 0.002^{a}	ND	ND	ND
	2	0.13 ± 0.009^{a}	0.38 ± 0.007^{b}	0.23 ± 0.003^{c}	0.39 ± 0.008^{b}	ND
	-20	0.13 ± 0.009^{a}	0.46 ± 0.005^{b}	0.37 ± 0.01^{c}	0.41 ± 0.004^{d}	0.31± 0.01^{e}
Whole						
Total Ascorbic Acid	25	1.16 ± 0.08^{a}	ND	ND	ND	ND
	10	1.16 ± 0.08^{a}	0.74 ± 0.03^{b}	0.70 ± 0.01^{b}	ND	ND
	2	1.16 ± 0.08^{a}	0.57 ± 0.02^{b}	0.53 ± 0.004^{b}	ND	ND
	-20	1.16 ± 0.08^{a}	0.77 ± 0.002^{b}	0.64 ± 0.03^{bc}	0.54 ± 0.02^{c}	0.38 ± 0.02^{d}
Ascorbic Acid	25	1.02 ± 0.07^{a}	ND	ND	ND	ND
	10	1.02 ± 0.07^{a}	0.60 ± 0.05^{b}	0.27 ± 0.01^{c}	ND	ND
	2	1.02 ± 0.07^{a}	0.36 ± 0.01^{b}	0.45 ± 0.01^{b}	ND	ND
	-20	1.02 ± 0.07^{a}	0.62 ± 0.009^{b}	0.34 ± 0.005^{c}	0.47 ± 0.03^{bc}	0.27 ± 0.01^{c}
Dehydroascorbic Acid	25	0.13 ± 0.009^{a}	ND	ND	ND	ND
	10	0.13 ± 0.009^{a}	0.13 ± 0.02^{a}	0.43 ± 0.02^{b}	ND	ND
	2	0.13 ± 0.009^{ab}	0.20± 0.01^{a}	0.07 ± 0.009^{b}	ND	ND
	-20	0.13 ± 0.009^{a}	0.14 ± 0.01^{a}	0.29 ± 0.03^{b}	0.06 ± 0.01^{a}	0.10 ± 0.003^{a}

$^{a\ b,c,d,e}$ –indicate statistical significance in values within rows. Differences were determined if P-values were at least $P<0.05$. ND – indicate that values were not determined due to fruit maturity or spoilage.

5.3.1.2. Blended fruit

Blended bitter melon samples were stored under the conditions described above and evaluated for total ascorbic acid, ascorbic acid, and dehydroascorbic acid. Blended samples stored at room temperature degraded before the 1-week evaluation period hence the determination of ascorbic acid was not performed for that series. Similarly, we only obtained 1-week storage data for samples stored at 10 °C, due to microbial spoilage of samples stored for two weeks. At this time point the level of total ascorbic acid was 0.11 g kg^{-1}, the largest decrease in total ascorbic acid in this study (**Table 5-1**). The levels of total ascorbic acid in samples stored at 2 °C significantly decreased during storage, yet the levels were similar at the 1-, 2- and 4-week evaluation times (0.58-0.64 g kg^{-1}). Again, sample at the 8-week storage time was no longer viable for analysis. A similar trend was observed for blended samples stored at -20 °C, in which the levels of total ascorbic acid drastically decreased during storage, but the levels remained similar throughout the 8-week study.

The changes of ascorbic acid levels reflected the changes in the levels of total ascorbic acid. The lowest level of ascorbic acid was measured in blended fruit that was stored at 10 °C for one week. For samples stored at 2 °C, the levels of ascorbic acid were drastically lower than the level at the initiation of the study. The level of ascorbic acid after one week of storage (0.25 g kg^{-1}) was similar to the levels measured after four weeks of storage (0.18 g kg^{-1}). The level of ascorbic acid decreased during storage at -20 °C. Interestingly, after eight weeks of storage, the

levels increased significantly (0.39 g kg^{-1}) compared to levels of ascorbic acid after one week of storage (0.21 g kg^{-1}).

The levels of dehydroascorbic acid were similar between the initial evaluation (0.13 g kg^{-1}) and after one week of storage at 10 °C (0.11 g kg^{-1}). The level of dehydroascorbic acid significantly increased during storage at 2 °C and remained higher than initial levels throughout the four weeks of evaluation. Similarly, the levels of dehydroascorbic acid remained significantly higher than initial levels throughout the eight weeks of storage at -20 °C (**Table 5-1**).

5.3.1.3. Whole fruit

Whole fruit was also evaluated for changes of level of ascorbic acid under the above-mentioned storage conditions. Whole-fruit levels were not reported for samples stored at room temperature due to rapid fruit maturation under these storage conditions. After one week of storage, whole melons were completely orange, an attribute that renders the fruit unusable by consumers. The levels of total ascorbic acid in fruit stored at 10 °C were lower (0.74 g kg^{-1}) than the levels at the beginning of the study (1.16 g kg^{-1}), but they remained similar through the two weeks of storage. A similar trend was observed for samples stored at 2 °C. Levels of total ascorbic acid continually decreased throughout the eight weeks of evaluation, decreasing to 0.38 g kg^{-1} under -20 °C storage conditions. The level of ascorbic acid significantly decreased over two weeks of storage at 10 and 2 °C. The levels of ascorbic acid in samples stored at -20 °C continually decreased during storage with the lowest levels measured in samples stored for eight weeks (0.27 g kg^{-1}).

Dehydroascorbic acid levels remained similar to initial values (0.13 g kg^{-1}) during the first week of storage regardless of storage temperature (**Table 5-1**). After two weeks of storage, the levels of dehydroascorbic acid significantly increased under all storage temperatures ranging from 0.13–0.20 g kg^{-1}. The levels of dehydroascorbic acid stored at -20 °C remained similar to initial levels with the exception of samples stored for two weeks. Here the levels increased to 0.29 g kg^{-1}, after which they returned to initial levels (**Table 5-1)**.

5.3.2. Variation of amino acids by processing, storage time and temperature

5.3.2.1. Chopped fruit

The levels of phenylalanine, threonine, alanine, tryptophan, and glutamine remained relatively unchanged during storage, with a few exceptions (**Table 5-2**). Phenylalanine levels increased significantly after one week of storage at room temperature and after two weeks of storage at -20 °C (16.20 and 24.25 mg kg^{-1}, respectively). While the levels increased significantly after two weeks of storage at -20 °C, the levels dropped back to initial levels after four and eight weeks of storage. A similar pattern was observed for glutamine and tryptophan. Alanine levels remained similar to initial levels (15.56 ± 2.30 mg kg^{-1}) over storage time and most of the storage temperatures evaluated. An increase in alanine levels was observed in chopped bitter melon samples stored at 2 °C with levels significantly increasing after four weeks of storage (32.04 ± 1.12 mg kg^{-1}).

Amino acids that were generally observed to increase in levels in response to storage were isoleucine, leucine, valine, serine, aspartic acid, proline, and β-alanine. Exceptions were observed in levels of aspartic acid, proline, and β-alanine.

While the levels of aspartic acid generally increased during storage at room temperature and 10 °C, the levels were similar to initial values in samples stored at 2 °C (383.37 ± 88.13 mg kg^{-1}). Proline levels also reverted back to initial levels in samples stored at 10 and -20 °C at the end of the storage period (0.79 ± 0.13 mg kg^{-1}). β-alanine levels typically increased during storage at room temperature, 10 °C, and 2 °C, but samples stored at -20 °C remained similar to initial values (**Table5-2**). The levels of L-citrulline, GABA, asparagine, glutamic acid, and arginine differentially fluctuated in response to storage time and temperature.

Multivariate statistical analysis of the effect of storage temperature and time on amino acid levels is presented in **Figure 5-1**. PLS-DA revealed that a total described variation of 69.7 %, 84 %, 90.6 %, and 96.4 % for chopped bitter melon stored for 1, 2, 4, and 8 weeks, respectively.

In these projections there are clear separations in response to storage temperatures. VIP scores were produced from PLS-DA analysis to distinguish amino acid profiles of importance for each storage time and temperature. Amino acids with VIP scores above 1 are considered variables of importance (**Figure 5-1**). For week one, isoleucine, leucine, β-alanine, and arginine were the amino acids with the highest VIP scores. The relative importance of each of these amino acids in response to storage temperature is presented as color gradients to the right of the VIP score chart. As in week one, branched-chain amino acids (isoleucine and leucine) seem to be variables of importance throughout the storage periods.

Table 5-2. Changes in amino acid levels (mg kg^{-1} ± SEM) in chopped bitter melon stored for 1–8 weeks at room temperature (25 °C), 10 °C, 2 °C, and -20 °C.

Amino acids	Temp (°C)	Storage time				
		Initial	1 Week	2 Weeks	4 Weeks	8 Weeks
Arg	25	15.38 ± 2.74[a]	34.87 ± 4.62[b]	ND	ND	ND
	10	15.38 ± 2.74[a]	51.2 ± 1.70[b]	23.56 ± 1.56[a]	ND	ND
	2	15.38 ± 2.74[a]	9.09 ± 0.83[a]	24.03 ± 4.71[a]	58.05 ± 4.16[b]	ND
	-20	15.38 ± 2.74[a]	35.21 ± 4.29[b]	36.32 ± 5.71[b]	31.21 ± 1.15[ab]	39.63 ± 1.34[b]
Ile	25	0.41 ± 0.06[a]	3.93 ± 0.24[b]	ND	ND	ND
	10	0.41 ± 0.06[a]	5.24 ± 0.41[b]	5.02 ± 0.41[b]	ND	ND
	2	0.41 ± 0.06[a]	3.48 ± 0.17[b]	4.42 ± 0.40[bc]	5.05 ± 0.29[c]	ND
	-20	0.41 ± 0.06[a]	4.71 ± 0.30[b]	7.26 ± 0.53[c]	4.71 ± 0.08[b]	5.07 ± 0.25[b]
Leu	25	1.14 ± 0.19[a]	5.53 ± 0.36[b]	ND	ND	ND
	10	1.14 ± 0.19[a]	8.43 ± 1.29[b]	7.46 ± 0.76[b]	ND	ND
	2	1.14 ± 0.19[a]	5.22 ± 0.25[b]	7.78 ± 1.16[b]	7.13 ± 0.39[b]	ND
	-20	1.14 ± 0.19[a]	8.31 ± 0.51[b]	12.08 ± 1.37[c]	6.48 ± 0.16[b]	8.12 ± 0.72[b]
Phe	25	9.97 ± 1.30[a]	16.20 ± 1.84[b]	ND	ND	ND
	10	9.97 ± 1.30[a]	14.81 ± 1.20[a]	13.83 ± 0.79[a]	ND	ND
	2	9.97 ± 1.30[a]	10.18 ± 0.73[a]	11.71 ± 0.88[a]	13.99 ± 0.73[a]	ND
	-20	9.97 ± 1.30[a]	12.63 ± 0.63[a]	24.25 ± 1.50[b]	11.60 ± 0.36[a]	13.01 ± 0.74[a]
Thr	25	34.14 ± 5.73[a]	31.64 ± 1.75[a]	ND	ND	ND
	10	34.14 ± 5.73[a]	37.40 ± 1.35[a]	31.87 ± 2.00[a]	ND	ND
	2	34.14 ± 5.73[a]	31.07 ± 1.61[a]	32.25 ± 1.79[a]	32.04 ± 1.28[a]	ND
	-20	34.14 ± 5.73[a]	34.74 ± 1.56[a]	43.89 ± 2.07[a]	37.90 ± 0.65[a]	35.61 ± 0.94[a]
Val	25	15.72 ± 2.39[a]	46.96 ± 2.39[b]	ND	ND	ND
	10	15.72 ± 2.39[a]	35.57 ± 1.37[b]	34.80 ± 1.95[b]	ND	ND
	2	15.72 ± 2.39[a]	32.04 ± 1.12[bc]	29.98 ± 0.69[b]	39.25 ± 1.35[c]	ND
	-20	15.72 ± 2.39[a]	33.29 ± 1.89[b]	37.82 ± 1.69[b]	38.11 ± 2.57[b]	31.73 ± 0.95[b]
Ala	25	15.56 ± 2.30[a]	10.77 ± 0.72[a]	ND	ND	ND
	10	15.56 ± 2.30[a]	14.46 ± 0.96[a]	11.62 ± 0.81[a]	ND	ND
	2	15.56 ± 2.30[a]	24.67 ± 1.28[b]	19.77 ± 0.36[ab]	32.04 ± 1.12[c]	ND
	-20	15.56 ± 2.30[a]	14.74 ± 0.71[a]	14.30 ± 0.47[a]	18.40 ± 3.06[a]	14.57 ± 0.35[a]
Ser	25	8.00 ± 1.37[a]	15.19 ± 0.39[b]	ND	ND	ND
	10	8.00 ± 1.37[a]	12.17 ± 0.56[b]	13.59 ± 0.45[b]	ND	ND
	2	8.00 ± 1.37[a]	15.62 ± 0.27[b]	15.27 ± 0.12[b]	17.09 ± 0.58[b]	ND
	-20	8.00 ± 1.37[a]	13.44 ± 0.54[b]	15.02 ± 0.40[b]	15.53 ± 1.28[b]	15.59 ± 0.54[b]
GABA	25	106.35 ± 17.02[a]	62.11 ± 4.61[b]	ND	ND	ND
	10	106.35 ± 17.02[a]	80.97 ± 5.33[b]	76.16 ± 3.22[b]	ND	ND
	2	106.35 ± 17.02[a]	126.23 ± 5.40[a]	105.31 ± 2.85[a]	107.50 ± 3.97[a]	ND
	-20	106.35 ± 17.02[a]	62.76 ± 1.41[b]	98.95 ± 3.30[a]	67.87 ± 5.25[b]	97.25 ± 3.91[a]
Trp	25	6.44 ± 1.22[a]	7.09 ± 0.49[a]	ND	ND	ND
	10	6.44 ± 1.22[a]	7.37 ± 0.25[a]	6.02 ± 0.47[a]	ND	ND
	2	6.44 ± 1.22[ab]	4.86 ± 0.19[a]	5.21 ± 0.26[ab]	7.70± 0.30[ab]	ND
	-20	6.44 ± 1.22[a]	4.95 ± 0.24[a]	11.20 ± 0.48[b]	8.11 ± 1.19[ac]	6.01 ± 0.19[ac]
Gln	25	25.13± 4.74[a]	18.46 ± 1.55[a]	ND	ND	ND
	10	25.13 ± 4.74[a]	39.58 ± 1.60[a]	28.97 ± 0.84[a]	ND	ND
	2	25.13 ± 4.74[a]	24.41 ± 0.90[a]	21.04 ± 0.51[a]	27.98 ± 1.14[a]	ND
	-20	25.13 ± 4.74[a]	23.39 ± 0.66[a]	30.48 ± 1.54[a]	46.26 ± 5.92[b]	18.20 ± 0.26[a]
Cit	25	10.70 ± 1.76[a]	3.98 ± 0.60[b]	ND	ND	ND
	10	10.70 ± 1.76[a]	4.67 ± 0.35[b]	2.16 ± 0.11[b]	ND	ND
	2	10.70 ± 1.76[a]	1.64 ± 0.14[b]	3.80 ± 0.51[b]	8.35 ± 0.34[a]	ND
	-20	10.70 ± 1.76[a]	3.78 ± 0.94[b]	12.65 ± 0.28[a]	3.97 ± 0.21[b]	4.21 ± 0.30[b]

[a] –indicates values are similar to initial values within column. Subsequent lettering ([b,c]) indicate differences in values within a row. Differences were determined if p-values were at least $p<0.05$. ND – indicate that values were not determined due to fruit maturity or spoilage.

Table 5-2 (Cont).

Amino acids	Temp (°C)	Storage time: Initial	1 Week	2 Weeks	4 Weeks	8 Weeks
Asn	25	84.23 ± 14.07[a]	86.84 ± 12.86[a]	ND	ND	ND
	10	84.23 ± 14.07[a]	54.24 ± 7.42[ab]	34.78 ± 2.92[b]	ND	ND
	2	84.23 ± 14.07[a]	45.48 ± 3.01[b]	47.07 ± 2.42[b]	69.26 ± 2.43[ab]	ND
	-20	84.23 ± 14.07[a]	48.62 ± 3.70[b]	144.19 ± 5.46[c]	42.19 ± 2.55[b]	56.99 ± 1.49[ab]
Glu	25	199.69 ± 26.47[a]	26.12 ± 2.73[b]	ND	ND	ND
	10	199.69 ± 26.47[a]	32.07 ± 3.54[b]	57.94 ± 7.97[b]	ND	ND
	2	199.69 ± 26.47[a]	172.18 ± 12.73[ab]	133.73 ± 10.77[b]	204.53 ± 13.62[a]	ND
	-20	199.69 ± 26.47[a]	90.53 ± 15.44[b]	181.33 ± 17.88[a]	190.01 ±11.62[a]	272.89 ± 9.56[c]
Asp	25	383.37 ± 88.13[a]	1781.54 ± 85.85[b]	ND	ND	ND
	10	383.37 ± 88.13[a]	1139.01 ± 12.88[b]	1086.75 ± 78.03[b]	ND	ND
	2	383.37 ± 88.13[a]	545.98 ± 17.87[a]	628.44 ± 55.22[a]	490.41 ± 28.89[a]	ND
	-20		1059.94 ± 78.95[b]	913.87 ± 43.28[b]	1176.49 ± 40.92[b]	619.73 ± 48.06[a]
β-Ala	25	0.79 ± 0.14[a]	2.26 ± 0.14[b]	ND	ND	ND
	10	0.79 ± 0.79[a]	0.68 ± 0.06[ab]	0.36 ± 0.05[b]	ND	ND
	2	0.79 ± 0.79[ab]	1.14 ± 0.14[a]	0.66 ± 0.04[ab]	1.43 ± 0.13[b]	ND
	-20	0.79 ± 0.79[a]	0.52 ± 0.03[a]	0.90 ± 0.02[a]	0.76 ± 0.04[a]	0.69 ± 0.04[a]
Pro	25	0.78 ± 0.13[a]	1.18 ± 0.09[b]	ND	ND	ND
	10	0.78 ± 0.13[a]	1.03 ± 0.06[a]	0.71 ± 0.03[a]	ND	ND
	2	0.78 ± 0.13[a]	1.21 ± 0.06[b]	1.12 ± 0.02[ab]	1.32 ± 0.05[b]	ND
	-20	0.78 ± 0.13[a]	0.79 ± 0.07[a]	1.44 ± 0.13[b]	0.96 ± 0.04[a]	0.96 ± 0.04[a]

[a] –indicates values are similar to initial values within column. Subsequent lettering ([b,c]) indicate differences in values within a row. Differences were determined if p-values were at least $p<0.05$. ND – indicate that values were not determined due to fruit maturity or spoilage.

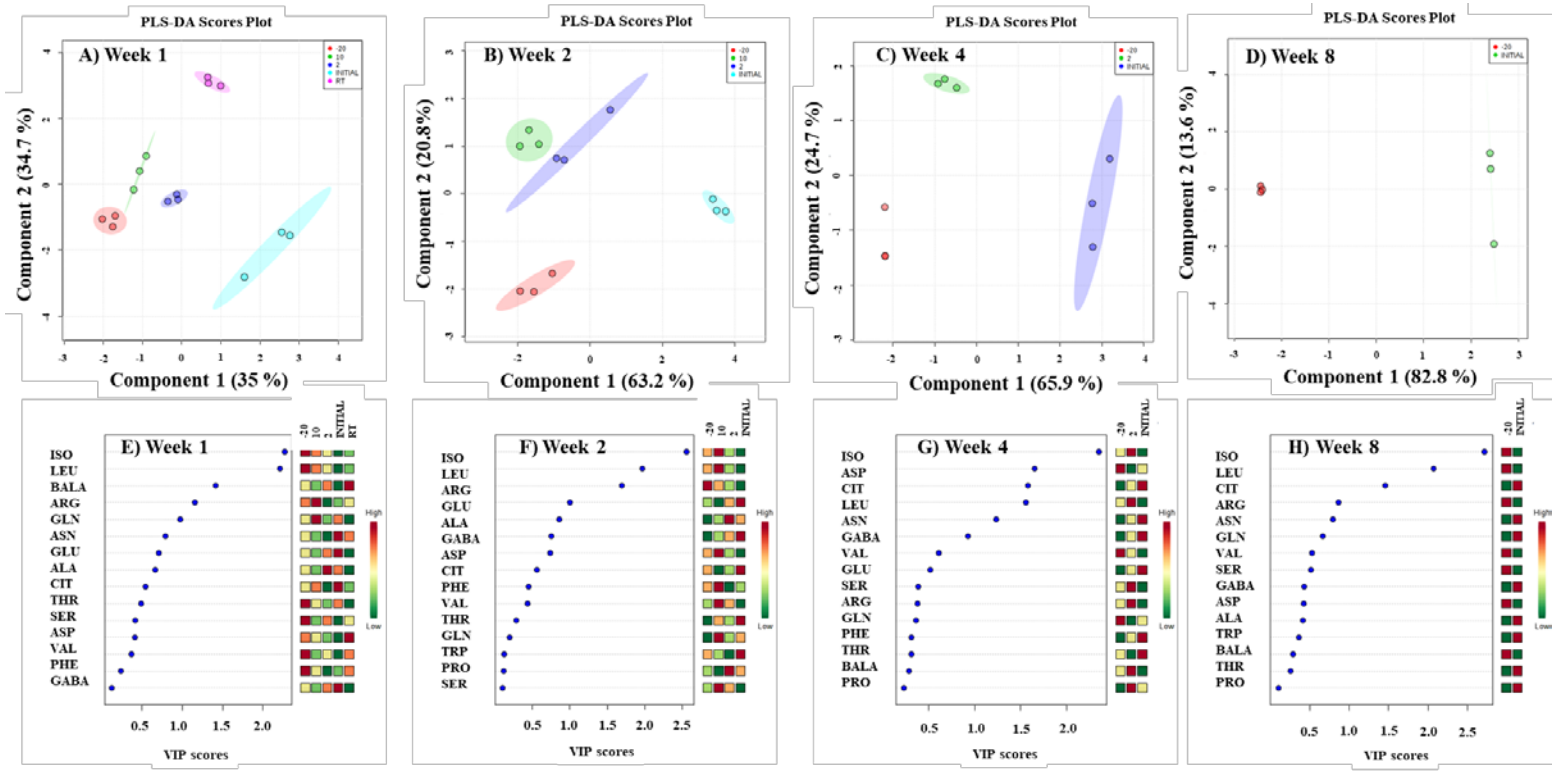

Figure 5-1. Multivariate analysis of chopped bitter melon. Partial least square discriminate analysis (PLS-DA) score plots for bitter melon stored for 1 (A), 2 (B), 4 (C) and 8 (D) weeks, discriminated between storage temperature (room temperature [RT, 25 °C], 10 °C, 2 °C, and -20 °C). Variable of importance on projection scores (VIP) for chopped bitter melon stored for 1 (E), 2 (F), 4 (G), and 8 (H) weeks from PLS-DA.

5.3.2.2. Blended fruit

The fluctuation in the levels of the various amino acids in blended fruit are presented in **Table 5-3**. The amino acids that were observed to increase over storage time were arginine isoleucine, leucine, phenylalanine, aspartic acid, β-alanine, and valine. Some exceptions were noted in response to temperature. Arginine levels remained statistically similar to initial levels throughout storage at 2 °C (15.38 ± 2.74 mg kg^{-1}), but varied in samples stored at 10 °C for two weeks and -20 °C for 1 and 4 weeks. Similarly, phenylalanine levels remained unchanged throughout storage at 10 °C (9.97 ± 1.30 mg kg^{-1}), with increases scattered throughout storage temperatures and durations. Valine levels generally remained similar to initial levels for blended bitter melon stored at 10 °C and -20 °C (15.72 ± 2.39 mg kg^{-1}). β-alanine levels were also unaffected by storage at -20 °C.

GABA, asparagine, L-citrulline, and glutamic acid showed a general decreasing trend. GABA typically decreased during storage but reverted to initial levels at the end of the storage period. Glutamic acid levels were observed to decrease regardless of temperature and duration of storage. GABA levels in blended fruit stored at room temperature for one week significantly increased. L-Citrulline levels were generally lower when blended fruit was stored at 10 °C and 2 °C but fluctuated under -20 °C storage conditions. Asparagine levels decreased notably at room temperature, 10 °C, and 2 °C, while levels remained generally similar to initial values when stored at -20 °C. Threonine, proline, tryptophan, and glutamine remained similar to initial levels. Threonine and tryptophan levels were mostly similar to initial values with the exception of samples stored for two weeks at -20 °C. Both amino acids drastically decreased under these conditions but the returned to initial levels.

Table 5-3. Changes in amino acid levels (mg kg^{-1} ± SEM) in bitter melon that has been blended and stored for 1–8 weeks at room temperature (25 °C), 10 °C, 2 °C, and -20 °C.

Amino acids	Temp (°C)	Storage time				
		Initial	1 Week	2 Weeks	4 Weeks	8 Weeks
Arg	25	15.38 ± 2.74[a]	20.81 ± 2.68[a]	ND	ND	ND
	10	15.38 ± 2.74[a]	29.90 ± 0.95[ab]	36.69 ± 5.99[b]	ND	ND
	2	15.38 ± 2.74[a]	27.44 ± 5.86[a]	26.42 ± 5.34[a]	35.11 ± 5.50[a]	ND
	-20	15.38 ± 2.74[a]	39.00 ± 1.29[b]	17.17 ± 3.76[a]	44.38 ± 6.84[b]	56.29 ± 3.59[b]
Ile	25	0.41 ± 0.06[a]	9.39 ± 1.60[b]	ND	ND	ND
	10	0.41 ± 0.06[a]	2.85 ± 0.07[ab]	3.59 ± 0.30[b]	ND	ND
	2	0.41 ± 0.06[a]	2.69 ± 0.22[ab]	4.02 ± 0.05[b]	5.05 ± 0.21[b]	ND
	-20	0.41 ± 0.06[a]	3.57 ± 0.28[bc]	1.17 ± 0.33[ab]	3.94 ± 0.49[c]	4.71 ± 0.27[c]
Leu	25	1.14 ± 0.19[a]	23.75 ± 4.68[b]	ND	ND	ND
	10	1.14 ± 0.19[a]	3.93± 0.11[a]	5.53 ± 0.53[a]	ND	ND
	2	1.14 ± 0.19[a]	3.88 ± 0.64[ab]	6.76 ± 0.10[ab]	10.52 ± 0.5[b]	ND
	-20	1.14 ± 0.19[a]	5.57 ± 0.43[a]	1.89 ± 0.29[a]	4.97 ± 0.80[a]	6.75± 0.56[a]
Phe	25	9.97 ± 1.30[a]	24.10 ± 4.06[b]	ND	ND	ND
	10	9.97 ± 1.30[a]	10.70 ± 0.35[a]	12.37 ± 0.95[a]	ND	ND
	2	9.97 ± 1.30[a]	13.76 ± 2.32[ab]	18.02 ± 1.15[b]	18.25 ± 0.97[b]	ND
	-20	9.97 ± 1.30[a]	19.18 ± 1.31[b]	7.09± 0.75[a]	17.73 ± 1.29[b]	20.05 ± 0.55[b]
Thr	25	34.14 ± 5.73[a]	34.55 ± 4.16[a]	ND	ND	ND
	10	34.14 ± 5.73[a]	21.21 ± 1.34[a]	29.96 ± 1.74[a]	ND	ND
	2	34.14 ± 5.73[a]	28.22 ± 3.71[a]	32.49 ± 1.89[a]	27.77 ± 1.38[a]	ND
	-20	34.14 ± 5.73[a]	34.28 ± 2.16[a]	15.52 ± 0.53[b]	35.36 ± 2.55[a]	39.27 ± 0.54[a]
Val	25	15.72 ± 2.39[a]	67.43 ± 9.34[b]	ND	ND	ND
	10	15.72 ± 2.39[a]	20.08 ± 1.55[a]	25.45 ± 1.63[a]	ND	ND
	2	15.72 ± 2.39[a]	18.73 ± 1.54[ab]	26 27± 0.66[ab]	33.95 ± 1.66[b]	ND
	-20	15.72 ± 2.39[a]	23.47± 1.82[a]	12.01 ± 0.79[a]	20.28 ± 2.78[a]	26.66 ± 1.17[a]
Ala	25	15.56 ± 2.30[a]	35.61 ± 2.96[b]	ND	ND	ND
	10	15.56 ± 2.30[a]	12.44 ± 1.38[a]	30.14 ± 1.87[b]	ND	ND
	2	15.56 ± 2.30[a]	15.71 ± 1.10[a]	18.19 ± 1.91[a]	43.13 ± 4.48[b]	ND
	-20	15.56 ± 2.30[a]	9.04 ± 0.70[a]	5.76 ± 0.31[a]	9.06 ± 0.60[a]	13.28 ± 1.24[a]
Ser	25	8.00 ± 1.37[a]	1.25 ± 0.12[b]	ND	ND	ND
	10	8.00 ± 1.37[a]	6.65 ± 0.56[a]	9.24± 0.71[a]	ND	ND
	2	8.00 ± 1.37[a]	6.57 ± 0.59[a]	10.52 ± 0.45[ab]	10.94 ± 0.64[ab]	ND
	-20	8.00 ± 1.37[a]	9.47 ± 1.06[a]	5.49 ± 0.52[ab]	7.42 ± 0.57[ab]	10.65 ± 0.61[a]
GABA	25	106.35 ± 17.02[a]	211.96 ± 12.22[b]	ND	ND	ND
	10	106.35 ± 17.02[a]	69.95 ± 8.78[a]	87.04 ± 4.21[a]	ND	ND
	2	106.35 ± 17.02[a]	61.78 ± 4.19[b]	79.82 ± 5.79[ab]	102.76 ± 13.57[ab]	ND
	-20	106.35 ± 17.02[a]	85.75 ± 5.83[ac]	33.40 ± 3.14[b]	59.21 ± 3.61[bc]	92.39 ± 6.89[ac]
Trp	25	6.44 ± 1.22[a]	8.57 ± 1.11[a]	ND	ND	ND
	10	6.44 ± 1.22[a]	4.95 ± 0.16[a]	5.52 ± 0.42[a]	ND	ND
	2	6.44 ± 1.22[a]	5.07 ± 0.14[a]	4.41 ± 0.21[a]	5.92 ± 0.60[a]	ND
	-20	6.44 ± 1.22[a]	6.36 ± 0.35[a]	2.48 ± 0.18[b]	6.00 ± 0.32[a]	6.49 ± 0.59[a]
Gln	25	25.13 ± 4.74[a]	2.80 ± 0.35[b]	ND	ND	ND
	10	25.13 ± 4.74[a]	17.26 ± 0.73[ab]	15.16 ± 0.68[b]	ND	ND
	2	25.13 ± 4.74[a]	25.03 ± 2.66[a]	28.14 ± 1.67[a]	24.42 ± 1.26[a]	ND
	-20	25.13 ± 4.74[a]	31.81 ± 2.87[a]	15.84 ± 0.66[ab]	29.08 ± 1.60[a]	25.64 ± 0.52[a]
Citr	25	10.70 ± 1.76[a]	11.59 ± 1.42[a]	ND	ND	ND
	10	10.70 ± 1.76[a]	5.53 ± 0.29[b]	6.41 ± 0.87[b]	ND	ND
	2	10.70 ± 1.76[a]	5.38 ± 0.80[b]	4.31 ± 0.20[b]	6.13 ± 0.55[b]	ND
	-20	10.70 ± 1.76[a]	7.23 ± 0.35[ac]	2.96 ± 0.41[bc]	5.71 ± 0.38[c]	6.90 ± 0.19[ac]

[a] –indicates value are similar to initial values within column. Subsequent lettering ([b,c]) indicate differences in values within row. Differences were determined if p-values were at least $p<0.05$. ND /– indicate that values were not determined due to fruit maturity or spoilage.

Table 5-3 (Cont.).

Amino acids	Temp (°C)	Storage time: Initial	1 Week	2 Weeks	4 Weeks	8 Weeks
Asn	25	84.23 ± 14.07[a]	16.79 ± 2.80[b]	ND	ND	ND
	10	84.23 ± 14.07[a]	39.02 ± 2.35[b]	38.99 ± 4.31[b]	ND	ND
	2	84.23 ± 14.07[a]	49.29 ± 7.99[b]	49.58 ± 2.68[b]	61.80 ± 5.77[ab]	ND
	-20	84.23 ± 14.07[a]	63.44 ± 4.65[a]	24.03 ± 2.19[b]	64.25 ± 5.25[a]	65.31 ±3.82[a]
Glu	25	199.69 ± 26.47[a]	49.06 ± 6.24[b]	ND	ND	ND
	10	199.69 ± 26.47[a]	49.74 ± 6.57[b]	44.65 ± 2.43[b]	ND	ND
	2	199.69 ± 26.47[a]	45.80 ± 7.48[b]	52.98 ± 3.91[b]	56.21 ± 3.09[b]	ND
	-20	199.69 ± 26.47[a]	93.70 ± 8.76[b]	74.07 ± 3.98[b]	103.79 ± 4.91[b]	84.24 ± 8.93[b]
Asp	25	383.37 ± 88.13[a]	2820.87 ± 353.17[b]	ND	ND	ND
	10	383.37 ± 88.13[a]	858.48 ± 27.93[b]	953.08 ± 36.62[b]	ND	ND
	2	383.37 ± 88.13[a]	775.69 ± 51.78[b]	776.91 ± 50.76[b]	870.33 ± 13.32[b]	ND
	-20	383.37 ± 88.13[a]	662.92 ± 67.62[a]	337.16 ± 26.52[a]	568.48 ± 62.26[a]	852.36 ± 33.06[b]
β-Ala	25	0.79 ± 0.14[a]	5.30 ± 0.41[b]	ND	ND	ND
	10	0.79 ± 0.14[a]	1.77 ± 0.12[a]	2.58 ± 0.16[b]	ND	ND
	2	0.79 ± 0.14[a]	0.56 ± 0.02[a]	0.89 ± 0.13[a]	2.19 ± 0.59[b]	ND
	-20	0.79 ± 0.14[a]	0.62 ± 0.02[a]	0.36 ± 0.03[a]	0.65 ± 0.05[a]	0.68 ± 0.08[a]
Pro	25	0.78 ± 0.13[a]	7.19 ± 1.10[b]	ND	ND	ND
	10	0.78 ± 0.13[a]	0.79 ± 0.08[a]	1.67 ± 0.20[a]	ND	ND
	2	0.78 ± 0.13[a]	1.15 ± 0.09[a]	1.71 ± 0.14[ab]	3.05 ± 0.41[b]	ND
	-20	0.78 ± 0.13[a]	1.17 ± 0.04[a]	0.31 ± 0.03[a]	1.24 ± 0.30[a]	1.21 ± 0.12[a]

[a] –indicates values are similar to initial values within column. Subsequent lettering ([b,c]) indicate differences in values within a row. Differences were determined if p-values were at least $p<0.05$. ND – indicate that values were not determined due to fruit maturity or spoilage.

Multivariate analysis grouped blended bitter melon samples stored under refrigeration (10 °C, 2 °C and -20 °C) after one week of storage and clearly discriminated samples stored at room temperature (**Figure 5-2**). PLS-DA reveals a total described variation for week one as 87.5 % in the first two components. After two weeks of storage, samples stored at 10 °C and 2 °C still grouped, but samples stored at -20 °C were clearly separated from the group. Clear distinctions were observed after four and eight weeks of storage.

VIP scores indicate that branched-chain amino acids (isoleucine, and leucine) are responsible for driving these discriminations after two weeks of storage.

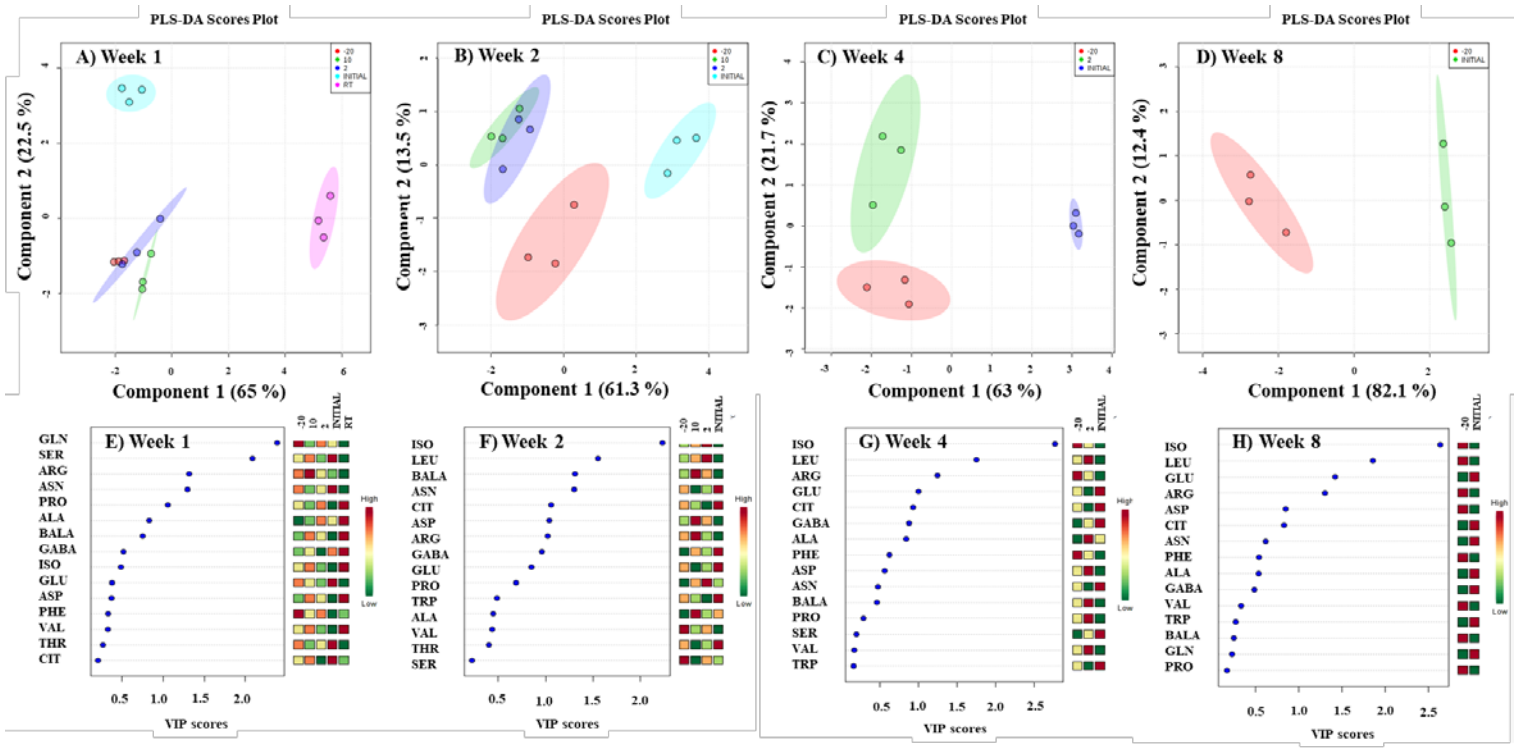

Figure 5-2. Multivariate analysis of blended bitter melon. Partial least square discriminate analysis (PLS-DA) score plots for bitter melon stored for 1 (A), 2 (B), 4 (C) and 8 (D) weeks, discriminated between storage temperature (room temperature [RT, 25 °C], 10 °C, 2 °C, and -20 °C). Variable of importance on projection scores (VIP) for chopped bitter melon stored for 1 (E), 2 (F), 4 (G), and 8 (H) weeks from PLS-DA.

5.3.2.3. Whole fruit

Due to the rapid ripening of bitter melon, whole fruit samples stored at room temperature were not available for analysis. At the 1-week storage interval, whole bitter melon fruit had completely ripened and at this point the fruit is no longer desirable for consumers, hence measurement of the levels of the various amino acids was not carried out. Generally, the levels of arginine, isoleucine, leucine, phenylalanine, valine, serine, aspartic acid, and proline increased during storage. For example, arginine levels more than doubled, compared with the initial concentration (15.38 ± 2.74 mg kg^{-1}) at the end of each storage period, increasing to ~47 mg kg^{-1} (**Table 5-4**). Conversely, the levels of alanine and L-citrulline significantly decreased during storage. An interesting trend was observed in the levels of threonine, GABA, tryptophan, glutamine, asparagine, glutamic acid and β-alanine. The levels of these amino acids initially all decreased after one week of storage and reverted to initial levels at subsequent interval evaluations.

Table 5-4. Changes in amino acid levels (mg kg^{-1} ± SEM) in bitter melon stored whole for 1–8 weeks at room temperature (25 °C), 10 °C, 2 °C, and -20 °C.

Amino acids	Temp (°C)	Storage time				
		Initial	1 Week	2 Weeks	4 Weeks	8 Weeks
Arg	25	15.38 ± 2.74[a]	ND	ND	ND	ND
	10	15.38 ± 2.74[a]	20.27 ± 0.49[a]	47.17 ± 0.70[b]	ND	ND
	2	15.38 ± 2.74[a]	18.54 ± 0.57[a]	44.22 ± 1.42[b]	47.73 ± 2.03[b]	ND
	-20	15.38 ± 2.74[a]	18.94 ± 0.26[b]	46.33 ± 1.81[c]	44.84 ± 1.57[c]	47.49 ± 2.30[c]
Ile	25	0.41 ± 0.06[a]	ND	ND	ND	ND
	10	0.41 ± 0.06[a]	2.03 ± 0.06[b]	4.69 ± 0.24[c]	ND	ND
	2	0.41 ± 0.06[a]	1.71 ± 0.19[b]	4.74 ± 0.19[c]	5.18 ± 0.20[c]	ND
	-20	0.41 ± 0.06[a]	1.91 ± 0.03[b]	4.70 ± 0.16[c]	4.82 ± 0.14[c]	5.26 ± 0.27[c]
Leu	25	1.14 ± 0.19[a]	ND	ND	ND	ND
	10	1.14 ± 0.19[a]	3.94 ± 0.12[b]	10.05 ± 0.55[c]	ND	ND
	2	1.14 ± 0.19[a]	3.31 ± 0.22[a]	8.61 ± 0.62[b]	11.98 ± 1.02[c]	ND
	-20	1.14 ± 0.19[a]	3.29 ± 0.09[a]	7.38 ± 0.29[b]	8.85 ± 0.78[b]	9.06 ± 0.73[b]
Phe	25	9.79 ± 1.30[a]	ND	ND	ND	ND
	10	9.79 ± 1.30[a]	7.26 ± 0.27[a]	17.44 ± 0.27[b]	ND	ND
	2	9.79 ± 1.30[a]	6.30 ± 0.30[b]	15.74 ± 0.49[c]	18.13 ± 0.62[c]	ND
	-20	9.79 ± 1.30[a]	6.82 ± 0.13[b]	15.63 ± 0.53[c]	15.77 ± 0.48[c]	17.61 ± 0.83[c]
Thr	25	34.14 ± 5.73[a]	ND	ND	ND	ND
	10	34.14 ± 5.73[a]	15.35 ± 0.60[b]	38.72 ± 0.46[a]	ND	ND
	2	34.14 ± 5.73[a]	13.74 ± 0.35[b]	34.74 ± 1.44[a]	39.12 ± 1.26[a]	ND
	-20	34.14 ± 5.73[a]	13.25 ± 0.41[b]	35.62 ± 1.33[a]	34.26 ± 1.01[a]	40.16 ± 2.15[a]
Val	25	15.72 ± 2.39[a]	ND	ND	ND	ND
	10	15.72 ± 2.39[a]	14.72 ± 0.40[a]	33.84 ± 1.02[b]	ND	ND
	2	15.72 ± 2.39[a]	13.03 ± 0.23[a]	30.25 ± 1.00[b]	36.69 ± 1.91[b]	ND
	-20	15.72 ± 2.39[a]	13.29 ± 0.27[a]	30.58 ± 1.14[b]	29.09 ± 0.64[b]	33.51 ± 1.45[b]
Ala	25	15.56 ± 2.30[a]	ND	ND	ND	ND
	10	15.56 ± 2.30[a]	5.52 ± 0.23[b]	12.43 ± 0.37[a]	ND	ND
	2	15.56 ± 2.30[a]	4.75 ± 0.13[b]	10.88 ± 0.41[c]	12.05 ± 0.51[c]	ND
	-20	15.56 ± 2.30[a]	4.70 ± 0.13[b]	10.47 ± 0.30[c]	9.86 ± 0.21[c]	11.76 ± 0.60[c]
Ser	25	8.00 ± 1.36[a]	ND	ND	ND	ND
	10	8.00 ± 1.36[a]	5.95 ± 0.16[a]	12.14 ± 0.57[b]	ND	ND
	2	8.00 ± 1.36[a]	5.12 ± 0.17[a]	12.29 ± 0.41[b]	13.79 ± 0.42[b]	ND
	-20	8.00 ± 1.36[a]	5.33 ± 0.05[a]	12.63 ± 0.48[b]	12.02 ± 0.33[b]	13.43 ± 0.65[b]
GABA	25	106.35 ± 17.02[a]	ND	ND	ND	ND
	10	106.35 ± 17.02[a]	54.42 ± 1.99[b]	119.38 ± 3.82[a]	ND	ND
	2	106.35 ± 17.02[a]	46.04 ± 1.21[b]	108.80 ± 3.59	113.65 ± 4.00[a]	ND
	-20	106.35 ± 17.02[a]	46.83 ± 1.75[b]	105.86 ± 3.27[a]	102.88 ± 2.57[a]	122.56 ± 6.86[a]
Trp	25	6.44 ± 1.22[a]	ND	ND	ND	ND
	10	6.44 ± 1.22[a]	2.69 ± 0.07[b]	6.25 ± 0.09[a]	ND	ND
	2	6.44 ± 1.22[a]	2.54 ± 0.05[b]	6.31 ± 0.20[a]	5.91 ± 0.23[a]	ND
	-20	6.44 ± 1.22[a]	2.69 ± 0.05[b]	6.72 ± 0.25[a]	6.15 ± 0.13[a]	6.74 ± 0.33[a]
Glu	25	25.13 ± 4.74[a]	ND	ND	ND	ND
	10	25.13 ± 4.74[a]	11.89 ± 0.30[b]	24.63 ± 1.28[a]	ND	ND
	2	25.13 ± 4.74[a]	10.97 ± 0.35[b]	27.46 ± 0.94[a]	29.35 ± 0.95[a]	ND
	-20	25.13 ± 4.74[a]	11.25 ± 0.17[b]	29.16 ± 1.34[a]	30.01 ± 0.83[a]	26.21 ± 1.38[a]
Cit	25	10.70 ± 1.76[a]	ND	ND	ND	ND
	10	10.70 ± 1.76[a]	2.00 ± 0.03[b]	5.09 ± 0.04[c]	ND	ND
	2	10.70 ± 1.76[a]	1.91 ± 0.04[b]	4.74 ± 0.15[c]	5.16 ± 0.17[c]	ND
	-20	10.70 ± 1.76[a]	1.99 ± 0.04[b]	4.86 ± 0.25[c]	4.77 ± 0.12[c]	4.86 ± 0.27[c]

[a] –indicates values are similar to initial values within column. Subsequent lettering ([b,c]) indicate differences in values within row. Differences were determined if p-values were at least $p<0.05$. ND – indicate that values were not determined due to fruit maturity or spoilage.

Table 5-4 (Cont.).

Amino acids	Temp (°C)	Storage time: Initial	1 Week	2 Weeks	4 Weeks	8 Weeks
Asn	25	84.23 ± 14.07[a]	ND	ND	ND	ND
	10	84.23 ± 14.07[a]	27.49 ± 1.09[b]	58.95 ± 3.93[c]	ND	ND
	2	84.23 ± 14.07[a]	24.84 ± 1.49[b]	63.33 ± 3.50[a]	68.95 ± 2.90[a]	ND
	-20	84.23 ± 14.07[a]	28.50 ± 1.11[b]	65.69 ± 3.87[a]	61.82 ± 2.49[a]	75.33 ± 2.81[a]
Glu	25	199.69 ± 26.47[a]	ND	ND	ND	ND
	10	199.69 ± 26.47[a]	74.00 ± 4.85[b]	155.38 ± 12.77[a]	ND	ND
	2	199.69 ± 26.47[a]	87.03 ± 1.85[b]	186.39 ± 8.68[a]	202.35 ± 11.26[a]	ND
	-20	199.69 ± 26.47[a]	86.68 ± 1.55[b]	197.47 ± 9.69[a]	191.65 ± 2.91[a]	197.64 ± 7.49[a]
Asp	25	383.37 ± 88.13[a]	ND	ND	ND	ND
	10	383.37 ± 88.13[a]	343.82 ± 20.92[a]	803.22 ± 25.67[b]	ND	ND
	2	383.37 ± 88.13[a]	263.28 ± 16.91[a]	635.91 ± 22.10[b]	705.65 ± 24.22[b]	ND
	-20	383.37 ± 88.13[a]	279.37 ± 5.96[a]	625.84 ± 34.06[bc]	552.79 ± 14.30[b]	725.52 ± 38.50[c]
β-Ala	25	0.79 ± 0.14[a]	ND	ND	ND	ND
	10	0.79 ± 0.14[a]	0.35 ± 0.02[b]	0.74 ± 0.07a	ND	ND
	2	0.79 ± 0.14[a]	0.30 ± 0.01[b]	0.69 ± 0.02a	0.71 ± 0.03a	ND
	-20	0.79 ± 0.14[a]	0.32 ± 0.01[b]	0.79 ± 0.07a	0.68 ± 0.03a	0.76 ± 0.06a
Pro	25	0.78 ± 0.13[a]	ND	ND	ND	ND
	10	0.78 ± 0.13[a]	0.72 ± 0.05[a]	1.81 ± 0.10[b]	ND	ND
	2	0.78 ± 0.13[a]	0.64 ± 0.04[a]	1.32 ± 0.05[a]	2.18 ± 0.24[b]	ND
	-20	0.78 ± 0.13[a]	0.51 ± 0.02[a]	1.01 ± 0.06[ab]	0.98 ± 0.06[ab]	1.61 ± 0.35[b]

[a] –indicates values are similar to initial values within column. Subsequent lettering ([b,c]) indicate differences in values within a row. Differences were determined if p-values were at least $p<0.05$. ND – indicate that values were not determined due to fruit maturity or spoilage.

Compared to the multivariate analysis of chopped and blended bitter melon, PLS-DA scores plot for week one of whole fruit showed no clear distinctions between fruit stored at 10 °C, 2 °C, and -20 °C. Similarly, in samples evaluated after two weeks of storage, the refrigerated samples remained in close proximity to one another. As in chopped and blended bitter melon samples, storage temperatures of whole fruit after four and eight

weeks were clearly segregated from each other. For each of these comparisons, the first component alone explained over 80 % of the variation observed (**Figure 5-3**).

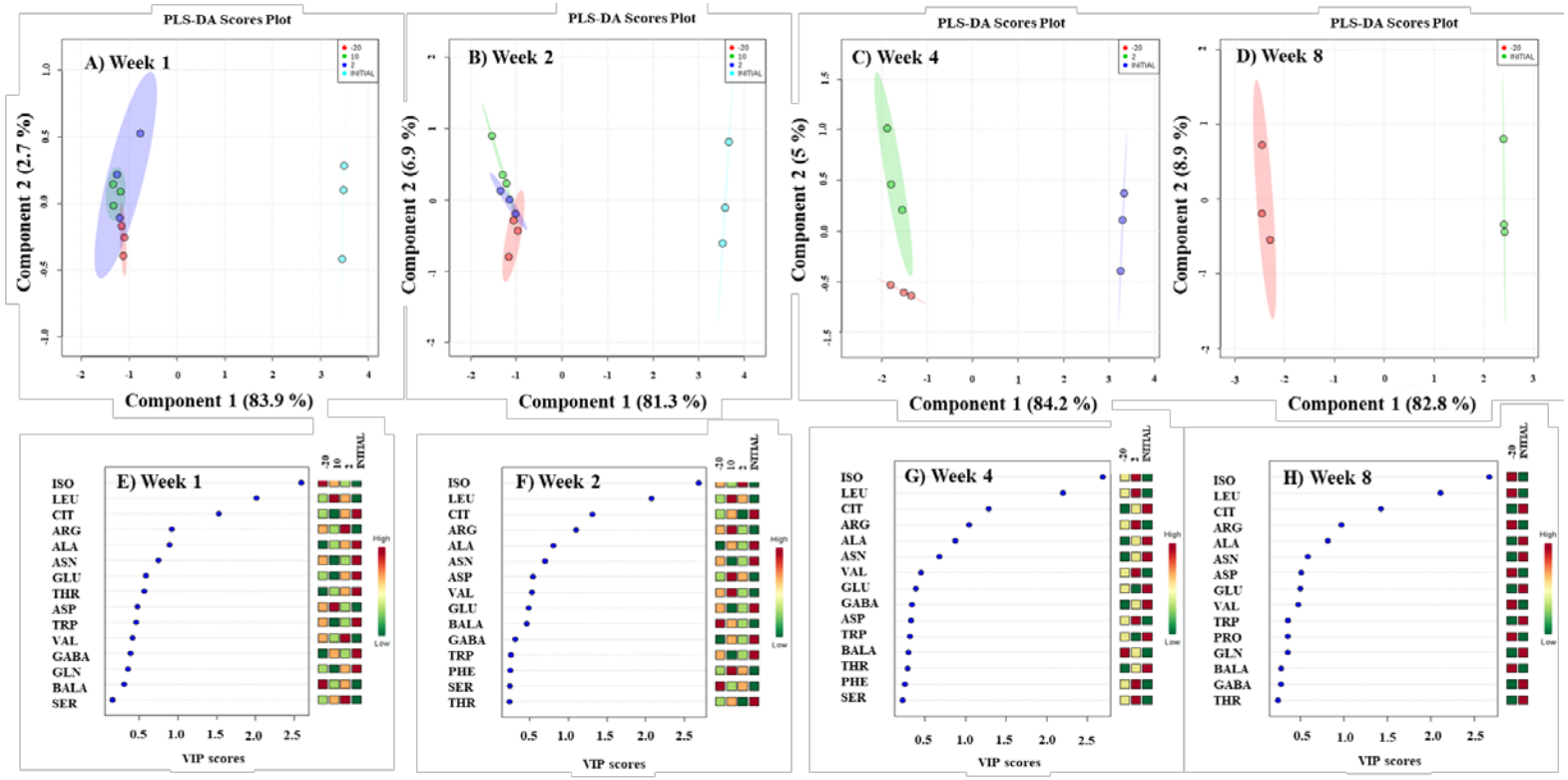

Figure 5-3. Multivariate analysis of whole bitter melon. Partial least square discriminate analysis (PLS-DA) score plots for bitter melon stored for 1 (A), 2 (B), 4 (C), and 8 (D) weeks, discriminated between storage temperature (room temperature [RT, 25 °C],10 °C, 2 °C, and -20 °C). Variable of importance on projection scores (VIP) for chopped bitter melon stored for 1 (E), 2(F), 4 (G), and 8 (H) weeks from PLS-DA.

Interestingly, VIP scores indicated that isoleucine, leucine, L-citrulline, and arginine were the highest scoring variables at each of the evaluation times of whole bitter melon fruit.

5.3.3. Total phenolic levels and antioxidant activities are differentially affected by processing, storage time and temperature

5.3.3.1. Chopped fruit

Chopped bitter melon stored at room temperature lasted up to one week of storage. During that one week of storage, the level of total phenolics significantly increased from 468.8 ± 13.62 to 776.8 ± 27.06 mg kg^{-1} GAE (**Figure 5-4**). Similarly, DPPH scavenging activity increased during the one week of storage from 182.81 ± 7.86 to 316.8 ± 8.84 mg kg^{-1} AAE. ABTS activity also increased significantly in chopped bitter melon samples during one week of storage at room temperature. The ABTS activity increased from 191.36 ± 6.76 to 249.68 ± 6.76 mg kg^{-1} AAE.

Under 10 °C storage conditions, total phenolics in chopped bitter melon also increased during the first week of storage but drastically decreased after two weeks. A similar pattern was observed for DPPH and ABTS scavenging activities, but the ABTS activity after one week of storage under these conditions remained similar to initial levels (228.20 ± 6.50 mg kg^{-1} AAE). For samples stored at 2 °C, total phenolics levels significantly increased during storage, with levels ranging from 641.8 ± 25.57 to 739.4 ± 13.48 mg kg^{-1} GAE. After one week of storage at 2 °C, the level of DPPH scavenging activity increased significantly but returned to the initial levels after that point. By contrast, the level of ABTS activity remained similar to the initial level after one and two weeks of storage but increase significantly after four weeks of storage at 2 °C (**Figure 5-4**).

Bitter melon samples stored at -20 °C generally had higher levels of total phenolics in this study, with the exception of the samples stored for 4 weeks. The highest level of total phenolics overall was observed in samples stored at -20 °C for 8 weeks (1068.4 ± 53.36 mg kg^{-1} GAE). Similarly, the highest values for ABTS and DPPH scavenging activity was found in these samples, 399.1 ± 18.26 and 342.7 ± 18.22 mg kg^{-1} AAE, respectively.

5.3.3.2. Blended fruit

In blended bitter melon fruit pericarp samples stored at RT, the levels of total phenolics, DPPH, and ABTS after one week of storage remained similar to initial levels. A similar trend was observed in samples stored at 10 °C after one week of storage. The levels of total phenolics of blended bitter melon fruit stored at 2 °C significantly increased after one week of storage and steadily declined back to initial levels at the end of the storage period (376.48 ± 50.79 mg kg^{-1} GAE). A similar trend was observed in the DPPH level in that storage group, but the increases were not significant. Similarly, the levels of ABTS scavenging activity remained similar to initial levels (191.36 ± 6.76 mg kg^{-1} AAE) throughout the storage period in fruit stored at 2 °C. In samples stored at -20 °C, the levels of total phenolics were significantly higher after one and eight weeks of storage, while the levels after two and four weeks were similar to initial levels. The highest level of DPPH scavenging activity was observed after one week of storage while the activity remained similar to initial levels after two, four, and eight weeks of storage. The highest ABTS scavenging activity of samples stored at -20 °C was measured in samples that were stored for eight weeks (307.61 ± 7.47 mg kg^{-1}AAE). Similarly, significantly higher ABTS

scavenging activity was measured in samples stored for one, two, and four weeks, 256.22, 287.45, and 264.29 mg kg^{-1}, respectively.

5.3.3.3. Whole fruit

Whole bitter melon samples stored at room temperature rapidly matured and decayed hence no total phenolic, DPPH and ABTS scavenging activity measurements were obtained from these samples. A significant increase in total phenolics, DPPH, and ABTS levels was observed only in fruit stored for eight weeks at -20 °C. The levels in the remaining treatments groups all resembled the initial total phenolics, DPPH, and ABTS levels (**Figure 5-4**).

5.4. Discussion

Bitter melon is commonly consumed in many Asian and South American countries. In addition to its various nutritional benefits, it is used to manage diabetes and diabetic complications. These beneficial properties have been linked to the wide array of phytonutrients in many fruit and vegetable, including bitter melon. Various reports have measured these metabolites, but their levels are not static. Levels of various phytonutrients vary depending on cultivar, cultivation practices, environment, biotic stress, maturation, postharvest handling, and processing. Bitter melon has a short shelf life due to the production of ethylene, which leads to the rapid maturation of fruit stored at room temperature. Refrigerated storage is a viable option for prolonging the shelf life of fruit and vegetables, but bitter melon is susceptible to chilling injury if stored below 4 °C [217]. The optimal storage condition for bitter melon has been reported to be 10 °C [80]. Even under these conditions, fruit and vegetables still undergo metabolic changes that may be driven

by enzymatic activity or innate chemical reactions, resulting in a change in the phytochemical profile of fruit and vegetables. Low or even freezing temperatures slow or arrest some of these reactions, but chemical reactions, such as oxidation, have still been observed under freezing conditions. Therefore, there is a void in the available information regarding the effect of various storage temperatures over time in bitter melon. Additionally, there is also a lack of information on how processing technique affect the levels of various bitter melon metabolites such as vitamin C, amino acids, and other antioxidants.

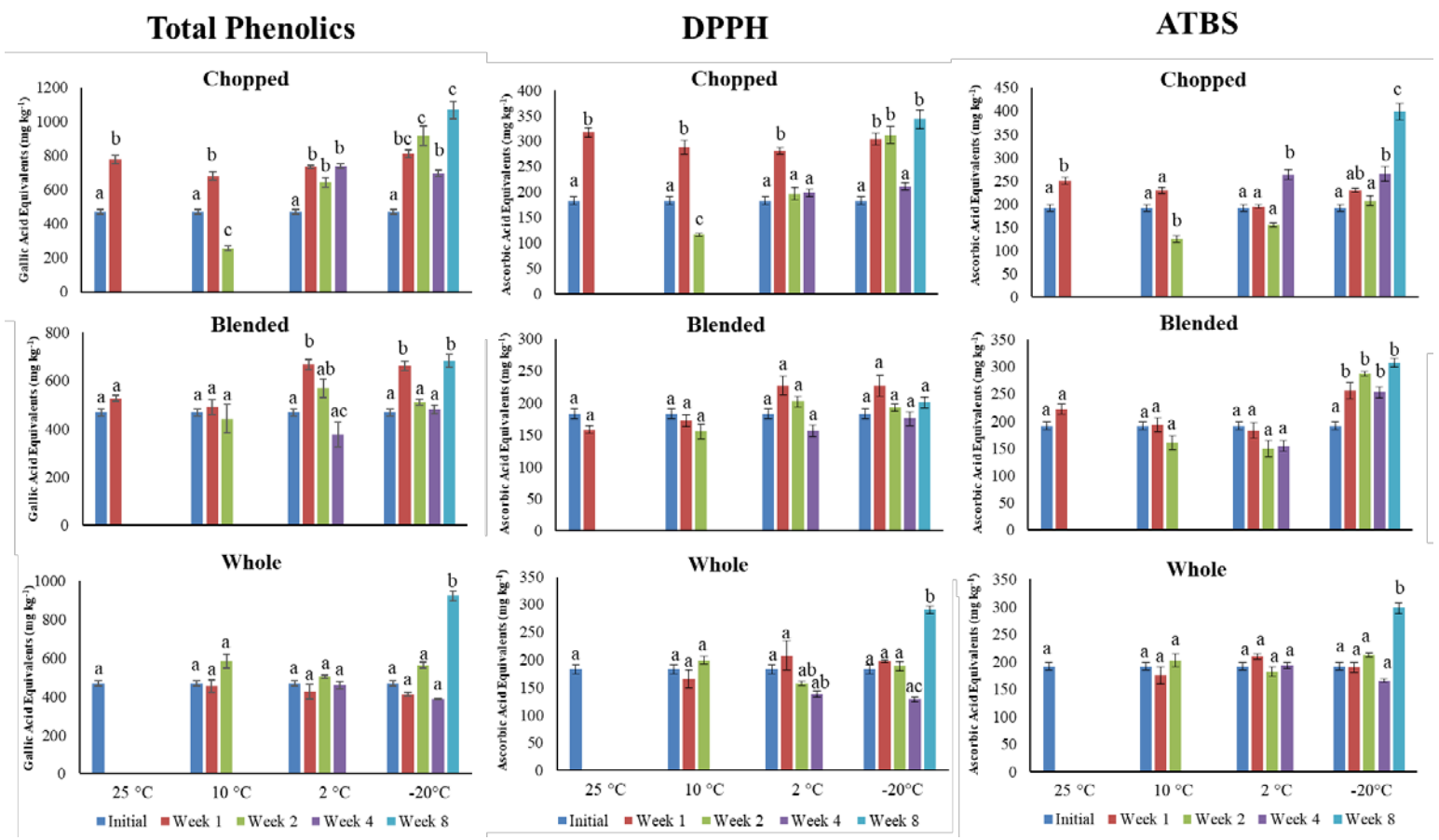

Figure 5-4. Total phenolic (mg kg^{-1} of gallic acid equivalents), free radical scavenging activities (mg kg^{-1} of ascorbic acid equivalents) from methanol extracts of bitter melon samples stored under different temperatures and storage times. Results are expressed as mean ± SEM. Differences are depicted with different letters over storage time for a particular temperature group ($p \leq 0.05$).

5.4.1. Loss of vitamin C

The levels of ascorbic acid, amino acids, total phenolics, and anti-oxidant activities were measured in bitter melon (chopped, blended, or whole) stored at room temperature, 10 °C, 2 °C, and -20 °C for one, two, four, and eight weeks. Ascorbic acid levels in fruit and vegetable are known to fluctuate in response to various postharvest factors such as processing and storage. Bitter melon has an extremely short postharvest life, so freezing may seem to be a feasible option to store bitter melon. Unfortunately, conventional freezing is not enough to fully arrest enzymatic reactions, senescence, and microbial growth [227]. As such, the loss of vitamin C continues during cold or frozen storage, as indicated by our results. The loss of vitamin C in stored foods may occur through chemical and enzymatic processes. Ascorbic acid is unstable in aqueous solutions, quickly oxidizing to dehydroascorbic acid. Total irreversible loss of ascorbic acid occurs when ascorbic acid is converted to 2,3-ketogluconic acid, typically in response to heat, reactive oxygen species, and breakdown of cellular tissues. [228]. Slow freezing of fruit and vegetables, as occurs in household freezers, results in irreversible structural damage to cell walls and internal cell organelles due to the formation of large ice crystals [229]. Additionally, slow chilling or freezing promotes a slow transition from a fluid membrane phase to a solid-gel phase resulting in changes in membrane permeability and increases in oxidative stress [230]. The loss of total ascorbic acid observed during storage was evident in all processing procedures we evaluated. The greatest loss overall was observed in blended fruit stored at 10 °C for one week (0.11 g kg^{-1}). The observed loss is most likely due to the blending procedure releasing endogenous enzymes from cells and their activity being favored by the storage temperature. Similarly, the greatest loss in chopped fruit was

observed in fruit stored at room temperature for one week. Surprisingly, processed fruit (chopped or blended) stored at -20 °C had higher levels of total ascorbic acid than whole fruit after eight weeks of storage. This may be due to facilitated freezing of the food matrix in response to increased surface area and quicker arrest or retardation of enzymatic activities. Quick-freezing strategies have been implemented at an industrial scale, but this is not a feasible option for the consumer.

5.4.2. Fluctuation of amino acid levels

Several factors including proteolysis and amino acid interconversions differentially affect levels of individual amino acids [223]. In certain instances, levels of several amino acids initially increased during the early portion of the storage study and returned to initial levels by the end of the evaluation. This observation may be explained by the initial breakdown if proteins during proteolysis thereby increasing the levels of free amino acids. The subsequent decrease in levels consequently may be in response to the interconversion of amino acids to secondary metabolites to respond to the increase level of reactive oxygen species produced during storage. For example, the increased level of arginine in blended and whole fruit during storage may be due to increased proteolytic activity in response to chilling temperatures. Arginine is a precursor of putrescene, which accumulates in plants under cold or freezing temperatures [231, 232]. Additionally, the accumulation of arginine may also be a response to increased reactive oxygen species in response to chilling injury. Arginine metabolism has been widely reported to be involved in abiotic stress responses [233].

Generally, threonine concentrations in blended samples decreased as storage time progressed; inversely, isoleucine concentrations increased. Threonine is a substrate in the biosynthesis of isoleucine [234]. Furthermore, isoleucine is a branched chain amino acid that is involved in the biosynthesis of various plant volatiles. Isoleucine accumulation has been reported in tomato fruit as a response to chilling injury leading to decreased production of characteristic volatiles. The accumulation of isoleucine is possibly due to the downregulation or arrest of branched chain aminotransferase activity [235]. Additionally, the increase in levels of leucine, isoleucine, and valine during storage may be due to the fact that these amino acids are major nitrogen components of membrane proteins. Damage to cell membranes during postharvest senescence would in turn result in the liberation of these amino acids from proteins [236]. GABA concentrations were differentially affected by storage time and temperature. GABA levels in tomato have been reported to accumulate as fruit maturation occurs and decline at the ripening stages [237]. GABA is a non-proteinaceous amino acid, so proteolytic enzyme activity should not affect GABA levels. In this study, a general decrease in GABA levels was observed throughout storage regardless of storage time and temperature. This may have been due to the production of reactive oxygen species during storage and the continuation of fruit maturation.

As expected, there is no clear trend encompassing the general pool of free amino acids. Different amino acids are precursors to a variety of nucleotides, phytohormones, and secondary metabolites; therefore, the physiological conditions in plants under different storage and processing conditions will have different effects on individual amino acid concentrations [238]. Additionally, β-alanine levels were differentially influenced by fruit

processing, storage time and temperature. This is the first report of β-alanine in bitter melon. β-alanine has been reported to mitigate cognitive deterioration in patients with Alzheimer's disease. Supplementation of diet with β-alanine increases carnosine levels, which have a protective role in the brain against oxidative stress [239]. β-alanine is typically nutritionally available in various meat products, but bitter melon can be an additional source of β-alanine in vegetarian diets.

5.4.3. Total phenolic and antioxidant activities

Bitter melon is a good source of natural antioxidants, which could potentially counteract the effects of harmful reactive oxygen species. In general, the highest total phenolic levels were observed in chopped bitter melon samples stored at -20 °C. The high levels of total phenolics of bitter melon stored at -20 °C seem to be related to the high DPPH radical scavenging activity of that same storage group. Additionally, regardless of the processing method, the measured total phenolics was the highest in samples stored at -20 °C for eight weeks. The increase in total phenolics in chopped bitter melon samples stored at -20 °C may be due to the increased surface area susceptible to freezing conditions. During storage, phenolics, possibly bound to cell membranes or proteins, may have been steadily unbound explaining the higher levels at these storage conditions. Furthermore, frozen storage slows enzymatic activity, so higher accumulation of these metabolites should be expected in frozen samples. Regarding blended samples, some increases in the total phenolic levels were observed in samples stored at 2 °C after one and two weeks of storage. Thereafter, the levels fell back to initial values. Similarly, in blended samples stored at -20 °C, samples stored for one week had higher total phenolic levels compare to initial measured

amounts. The during the second and fourth week of storage at this temperature, the levels were similar to initial values and followed by an increase of total phenolic levels after eight weeks. Blended samples stored at -20 °C, in general, had higher ABTS scavenging activity during storage compared with the initial levels. Lastly, the levels of total phenolics in whole fruit did not change dramatically during storage with the exception of samples stored at -20 °C for eight weeks. Similar results were observed in ABTS scavenging activity. The lack of change in the total phenolic levels in whole fruit may be due to the integrity of cells in the whole fruit. The drastic increase in the total phenolic levels of samples stored at -20 °C for eight weeks may be due to the release of bound phenolic components of cellular structures.

5.5. Conclusions

In this study, a various storage condition differing in duration and temperature were evaluated for bitter melon fruit that was chopped, blended and left whole. Vitamin C levels decreased during storage regardless of the storage temperature, duration, and processing techniques that could be used by consumers. The decrease of vitamin C was slower in chopped fruit stored at -20 °C, which may be a viable option for consumers to store bitter melon for periods of low availability. Similarly, amino acid levels, total phenolics, and antioxidant activity of bitter melon was differentially affected by processing technique and storage temperature. The information resulting from this study may be of great use for consumers searching for ways to maximize the contents of phytonutrients during storage of bitter melon.

6. DEVELOPMENTAL CHANGES IN LEVELS OF CAROTENOIDS, INTRASPECIFIC VARIATION AND IN VITRO BIOACCESSIBILITY OF ARIL LYCOPENE IN *MOMORDICA CHARANTIA*: A POTENTIAL SOURCE FOR CIS-LYCOPENE ISOMERS AND CAROTENOID ESTERS FROM POSTHARVEST WASTE

6.1. Introduction

Bitter melon (*Momordica charantia*) is commonly consumed in many Asian, African and South American countries. The popularity of bitter melon is due to various health benefits that are associated with its consumption, such as the reduction of blood sugar levels [240]. Bitter melon fruits undergo dramatic physiological changes during ripening, that in turn are translated to dramatic physical changes. Typically, bitter melon is consumed at the immature (green) stage, while ripe bitter melon is characteristically bright orange and soft. Characteristics that render the fruit unmarketable. The color of marketable bitter melon fruit ranges from white to dark green, clearly due to the levels of chlorophyll in the pericarp of these fruits [80, 241]. Interestingly, the pericarps of all bitter melon cultivars change to bright orange as they mature and ripen. Similarly, the internal pulp material and seed membranes /arils undergo a dramatic color change from white to bright red. The red coloration of mature arils has been attributed to high concentration of lycopene in bitter melon and other *Momordica* species [242]. At this point, the fruit is considered non-marketable or non-desirable by the end consumer and is considered a postharvest loss or waste. The drastic changes in coloration are due to the changes in levels of various

carotenoid, such as lycopene, found in the various plant tissues [243]. As such bitter melon "waste" products may be a valuable source of carotenoids.

As in other fruits and vegetables, the levels of bitter melon metabolites fluctuate in response to agronomic practices, biotic stressors and cultivar [244]. Intraspecific variation of many fruits and vegetables lead to changes in flavor profiles, disease resistance, and physical features such as color [245]. Variation in coloration among cultivars is a common occurrence and is, in some cases, the primary objective of breeding programs. Furthermore, other *Momordica* species been reported to possess high levels of carotenoid, making bitter melon a potential source of carotenoids nutritionally and for the nutritional supplement industry. As such, it is important to clearly delineate the developmental changes and varietal differences of carotenoids in bitter melon cultivars.

Currently, few studies have evaluated the levels of carotenoids in bitter melon varieties and at different physiological stages [241, 243, 246]. Early studies measure carotenoid levels by using spectrophotometric measurements which may be susceptible to reaction with interfering artifacts [81, 91, 243, 246]. Recent studies have evaluated bitter melon tissues using high performance liquid chromatography (HPLC) indicating that they may be a rich source of health promoting carotenoids [93, 94, 241, 247, 248]. While these studies present valuable data, a holistic evaluation is still lacking for the precise evaluation of bitter melon carotenoids. To the best of our knowledge, this is the first study to compare levels of carotenoids during development, the intraspecific variation and assess seed aril lycopene bioaccessibility, as well as biostability, after *in vitro* digestion. Furthermore, this is the first report to identify various lycopene isomers and carotenoid esters in bitter melon. Knowledge of the

carotenoid content and cultivars will be useful to nutritional experts for the selection of nutrient rich foods and proper dietary recommendations. Ripe bitter melon fruits are usually considered non-desirable for the fresh market. Hence, the quantitative data we report can potentially make ripe bitter melon fruits and seed arils an effective source of carotenoids for the nutraceutical and nutritional supplement markets.

6.2. Materials and methods

6.2.1. Sample collection

Analysis of intraspecific variation in bitter melon cultivars was carried out on fruits grown in College Station, Texas. The analysis of carotenoids during development was performed on 'India Green' bitter melon purchased at a local market (BCS Food Market, College Station, TX). Developmental stages were categorized as follows (**Figure 6-1A**): Stage 1- green pericarp with immature seeds; Stage 2 - green pericarp with fully developed seeds surrounded by white pulp/seed membrane; Stage 3- green pericarp with fully mature seeds with pink coloration around the seed membrane; Stage 4- light green pericarp with red coloration of seed membrane and degrading mesocarp; Stage 5- pericarp showing yellow/orange pigmented seed arils red and completely degraded mesocarp; Stage 6 - orange pericarp and bright red arils. Additionally, five bitter melon cultivars were evaluated for the levels of carotenoids. The cultivars consisted of 'India Green', 'India White', 'Japan Spindle', 'Hong Kong Green', and 'Large Top'. Fruits were harvested after attaining marketable stage or once the fruit became ripe, typically between 2-3 weeks after fruitset (AF) and 3-4week AF, respectively. After harvest, fruits were washed milliQ water and dried, followed by the separation of the fruit pericarp and seed material (**Figure 6-1 B**

and C). The seed material was further separated into seed and seed membrane/arils. The aril and pericarp material were stored at -80 °C until analysis of carotenoids.

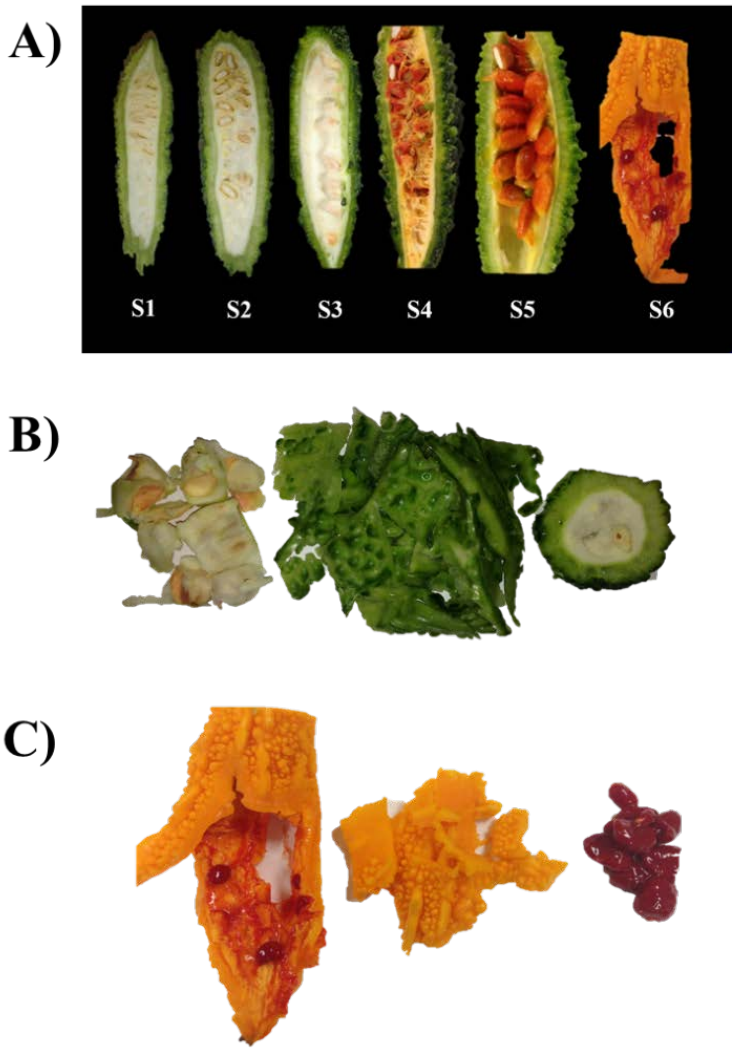

Figure 6-1. Developmental stages of bitter melon A). S1- green pericarp with immature seeds; S2 - green pericarp with fully developed seeds surrounded by white pulp/seed membrane; S3- green pericarp with fully mature seeds with pink coloration around the seed membrane; S4- light green pericarp with red coloration of seed membrane and degrading mesocarp; S5- pericarp showing yellow/orange pigmented seed arils red and completely degraded mesocarp; S6 -orange pericarp and bright red (arils). B). Immature (marketable) bitter melon mesoderm, pericarp and cross section, (C). Ripe bitter melon split open with pericarp and seed arils separated.

6.2.2. Extraction of carotenoids

Carotenoids of bitter melon pericarp, seed membranes and arils were extracted by a series of solvent extractions and under orange light to prevent the degradation of carotenoids. The fruit material (5 g) was extracted in a 50 mL conical tube using 10mL of acetone containing BHT (0.2%). The mixture was homogenized for one min using a polytron homogenizer, centrifuged (TJ-6, Beckman Coulter, Indianapolis, US) 20 min at 5000 x g. Following centrifugation, the supernatant was decanted and later pooled with subsequent extractions. The remaining pellet was then extracted two additional times with 10 mL a (1:1 v:v) hexane : acetone and 10 mL of hexane following the same process as described above. The extracts were pooled, dried over anhydrous sodium sulfate and evaporated to dryness using a rotary evaporator at 30 °C (Büchi Labortechnik AG, Australia). The resulting residue was dissolved in methanol/ tert-butyl methyl ether (TBME) (7:3 v:v) to a final volume of 10 mL for HPLC analysis.

6.2.3. Quantification of carotenoids

Carotenoid were analyzed using normal phase HPLC using according to our published method [226]. A 15 µL samples was injected for HPLC quantification of carotenoids in bitter melon extracts. The carotenoids were separated in using an Alliance 2695 high performance liquid chromatograph (HPLC) system equipped with a 2996 photodiode array detector (Waters, Milford, MA, USA). The separation of carotenoids was carried out using a YMC Carotenoid column (4.6 mm× 260 mm, 5 µm particle size) (Milford, MA, USA) maintained at 27 °C. The carotenoids were chromatographed using a bi-solvents system consisting of methanol (A) and methanol: tert-butyl methyl ether: water (17:80:3 v:v:v)

(B). The carotenoids were eluted at a flow rate of 0.6 mL per min and using the following mobile phase gradient: 0-2 min, 75% B; 2-16 min, 40% B; 16-17 min the gradient reached 100% A and was maintained at that condition for 12 min. The mobile phase combination was returned to the initial condition and equilibrated for 5 min before the following injection. The carotenoids were detected at 450 nm wavelength. The calibration curves for the three carotenoids quantified (lutein, β-carotene and lycopene) were prepared by injecting seven serial dilutions ranging from 0.05-3.75 µg/15 µL injection. Each sample was injected three times and the levels were presented as µg/gram on a fresh weight (FW) basis. Selected samples were used to verify the presence of carotenoids by UHPLC/ ESI-HR-QTOFMS.

6.2.4. Identification of carotenoid esters by atmospheric pressure chemical ionization

UHPLC/APCI-HR-QTOFMS was utilized to identify and confirm carotenoid peaks. The instrument consists of maXis impact mass spectrometer (Bruker Daltonics, Billerica, MA, USA) coupled to a model 1290 Rapid Resolution LC system (Agilent, Santa Clara, CA, USA) using an atmospheric pressure chemical ionization (APCI) source (Bruker Daltonics). Separation of carotenoids was carried out on a C30 column (3 µm, 50 mm× 2 mm, (YMC America, Allentown, PA, USA). The linear mobile phase gradient consisted of (A) MeOH: MTBE: water (80:15:5) and (B) MeOH: MTBE: water (15:80:4) at a flow rate of 0.35 mL/min. The gradient program was as follows 0-5 min 2%B; 2% -56% B (5 min); isocratic for 2 min (B); 56% - 65% B (6 min); 65% - 80 % B (3 min); isocratic for 2 min and returned to initial condition for 2 min. The post-run equilibrium time was 2 min.

Mass spectral analysis was performed using APCI in the positive ionization mode under the following parameters: APCI (+); MS scan range 50–2000 m/z; end plate offset 500 V; capillary 2500 V, nebulizer gas (N2) 1.6 bar; dry gas (N2) 4 L/min; capillary temperature 200 °C, vaporizer temperature 400 °C; ion transfer conditions funnel RF: 400 Vpp; multiple RF: 300 Vpp; quadruple low mass 75 m/z; collision energy 10eV; collision RF 1500 Vpp; transfer time 120 µs; pre-pulse storage time 8 µs. Calibration was done high-precision calibration (HPC) mode using APCI tune solution. Data acquired by UHPLC-HR-QTOF-MS was processed with the DataAnalysis software version 4.3.

6.2.5. In vitro bioaccessibility and biostability of lycopene

An *in vitro* bioaccessibility and biostability of lycopene was conducted using bitter melon samples. The assay was conducted according to our recently published method [179] with minor modifications.

6.2.5.1. Oral digestion

A 100 mg sample of bitter melon seed aril was mixed with 0.1 mL of DMSO and 0.1 mL of simulated salivary fluid containing 0.31 mg of α-amylase. The sample was then vortexed and placed in a shaking water bath (180 rpm) maintained at 37°C for 5 min. The product of this digestion phase represents the “bolus” that will be further processed by gastric digestion.

6.2.5.2. Gastric digestion

Gastric digestion was simulated by subjecting the previously prepared bolus to conditions resembling the stomach environment. The bolus pH was adjusted to 2 with 1N HCl and 60 µL of pepsin buffer (200 µg of pepsin in 1mL of 0.1M HCl) added to convert

the bolus mixture into “chyme”. The mixture was vortexed and placed in a preheated (37 °C) in a shaking water bath for 1h. This procedure simulates the conditions and time the food material is exposed to gastric conditions. Then, the pH was adjusted to 6.8 using 1N NaOH for intestinal digestion and absorption.

6.2.5.3. Intestinal digestion

Once the proper pH was attained 0.5 mL of pancreatin (6.25 mg/mL in 50 mM phosphate buffer) was added to the mixture. For assessing the bioaccessibility of lycopene, one set was samples was processed without bile acids (0.4 mL of 50 mM phosphate buffer) and a second set was processed with a mixture of bile acids (0.4 mL). The bile acid mixture consisted of 2.54 mM sodium deoxycholate, 12.08 mM sodium cholate, 13.22 mM sodium deoxycholate, and 5.14 mM sodium glycochenodeoxycholate. The mixture was vortexed and incubated in a preheated (37 °C) shaking water bath from 3 h. Then, the overall in vitro digestion process was arrested by inactivating digestive enzymes at 78 °C in a water bath for 7 min, centrifuged for 30 min at 2,500 x g The supernatant was decanted into separate tube and the pellet was rinsed with nano-pure water to remove bile acids. Bioaccessible lycopene was the extracted from the supernatant and remaining pellet was used to assess the biostability of lycopene. Bioaccessibility and biostability was calculated according to our published method.

6.2.6. Lycopene analysis

Lycopene was extracted from digestion fractions by chloroform using separating funnel twice. The combined chloroform layer was concentrated by a rotary evaporator at 30 °C. The resulting residue was then dissolved in methanol/ tert-butyl methyl ether

(TBME) (7:3 v:v) to a final volume of 1 mL for HPLC analysis. Lycopene was quantified by HPLC as described earlier.

6.2.7. Statistical analysis

The level of each carotenoid was quantified from three replicates per cultivar. Statistical analysis was performed using the JMP 11 software (SAS Institute, 2014). The ANOVA platform was used to evaluate the differences carotenoid content from each cultivar and individual excised tissues. The Tukey's test of significance between means was used for illustration of significance. Significance was reported if the P-value was at least $P < 0.05$.

6.3. Results and discussion

Bitter melon pericarp goes through strikingly characteristic color changes as it transitions from immature to ripe stages. Marketable fruits are characteristically green or white in color and have a firm texture. Ripe or over-ripe bitter melon pericarp becomes bright orange in color and soft in texture. The change in color is due to changes in level of carotenoids within the plant matrix [243]. Furthermore, the levels of these carotenoids tend to vary with cultivation practices, plant stress and genotype [249, 250]. It is of vital importance to identify and quantify the levels of phytonutrients in bitter melon fruit during maturation. The presence of lutein, β-carotene and lycopene in bitter melon tissues were confirmed by high-resolution mass spectrometry data. Furthermore, various carotenoid isomers and esters were identified for the first time in bitter melon.

6.3.1. Levels of carotenoids in pericarp

The levels of various carotenoids were assessed during the development of 'India Green' bitter melon pericarp. The levels of lutein in the pericarp tissue at stage 1 was 24.13 ± 2.95 µg/g (FW) (Table 1). Lutein level increased significantly at stages 2 and 3 (42.47 ± 5.07 and 52.52 ± 11.49 µg/g FW, respectively) followed by a constant decrease through stage 6, a trend previously reported in others studies [91, 92]. β-carotene levels in the developing pericarp slowly increase from the stage 1 to the stage 3, 3.68 ± 0.03 to 22.16 ± 3.42 µg / g respectively. The levels of β-carotene decrease at the stage 4 and stage 5 followed by a sharp increase at the stage 6 (12.06 ± 1.60 µg/g). This increase at the stage 6 is expected because of the notable characteristic change in fruit coloration upon ripening. Conversely, lycopene was not detected in bitter melon pericarp at the stage 1 and traces or minute quantities were found through development (**Table 6-1**).

The pericarps of five cultivars were evaluated for the levels of lutein, β–carotene, lycopene, and lycopene isomers both at the immature (marketable stage) and the ripe stage (Table 2). The level of lutein in the immature pericarp material was the highest (3.69 ± 0.36 µg/g) in the 'Large Top' cultivar followed by the 'Hong Kong Green' (3.56 ± 0.40 µg/g), 'Japan Spindle' (2.16 ± 0.19 µg/g), and the 'India Green' (1.78 ± 0.28 µg/g) cultivar. The lowest level of lutein was measured in the 'India White' cultivar (0.12 ± 0.04 µg/g). The highest levels of β-carotene in the immature pericarp was found in the 'Large Top' (39.38 ± 6.32 µg/g) cultivar but the level was statistically similar to the 'Hong Kong Green' cultivar (35.47 ± 5.83 µg/g). The 'Japan Spindle', 'India White', and 'India Green' cultivars had significantly lower levels of β-carotene than the 'Large Top'

cultivar. The overall lowest levels of β-carotene were found in the 'India White' cultivar (2.67 ± 0.35 µg/g). *Trans*-lycopene was present in a wide range of levels in the immature bitter melon pericarp. Levels ranged from 1.79 ± 0.36 to 54.43 ± 8.45 µg/g in the 'Large Top' and 'Hong Kong Green' cultivars, respectively. The wide range in lycopene level may be due to the lack of a truly standardized method to determine the maturity of fruit at harvest. Again, outlining the importance of understanding the chemical profiles of these fruits during development.

Regardless of the cultivar, all bitter melon fruits undergo a drastic color change to bright orange at fully ripe stage. Lutein was observed at very low levels in the ripe pericarp in all cultivars. Lutein levels have been reported to decrease in bitter melon as the fruit matures, an observation that is corroborated by our results [87, 92]. β- Carotene was found in high levels in the ripe pericarp material of all bitter melon cultivars evaluated. The most significant difference was observed between the 'Japan Spindle' and 'India Green' cultivars.

The 'Japan Spindle' cultivar had the largest difference in the levels of β-carotene (76.76 ± 9.33 µg/g), while the lowest levels were 42.57 ± 3.38 µg/g was measure in the 'India Green' cultivar, but overall the levels were statistically similar in all cultivars evaluated (**Table 6-2**). Interestingly, low levels or trace levels of 5-*cis*-lycopene were also measured in bitter melon pericarp. Chlorophyll a and b are the major pigments in immature bitter melon fruits; as such they are thought to mask the visibility of other carotenoids and flavonoids [251]. As fruits undergo ripening, ethylene is produced and the breakdown of chlorophyll begins [252]. Decrease in chlorophyll levels allows the visualization of other

colored compounds. The accumulation of carotenoids during fruit maturation has been well documented [253]. Interestingly, relatively few reports have documented the changes in carotenoids in bitter melon, particularly of known bioactive carotenoids such as β-carotene and lycopene. The level of β-carotene has been reported to be higher in mature bitter melon fruit compared to immature fruit, while the level of lycopene increases drastically at the later maturation stage [92].

Table 6-1. Content of carotenoids in bitter melon pericarp and seed membrane/ arils during fruit maturation.

		Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
Pericarp	Lutein	24.13 ± 2.95a	42.47 ±5.07b	52.52 ± 11.49b	1.45 ± 0.16 c	0.80 ± 0.26c	0.71 ± 0.04c
	β-carotene	3.68 ± 0.03a	20.41 ± 0.15b	22.16 ± 3.42b	2.04 ± 0.18a	4.85±0.0.06a	12.06 ± 1.60c
	15-*cis*-lycopene	nd	nd	nd	nd	nd	nd
	13-cis-lycopene	nd	nd	nd	nd	nd	nd
	9-cis-lycopene	nd	nd	nd	nd	nd	nd
	7-cis-lycopene	nd	nd	nd	nd	nd	nd
	trans-lycopene	nd	tr	tr	0.13 ± 0.08a	3.04± 1.75a	2.90 ± 1.07a
	5-*cis*-lycopene	nd	nd	nd	nd	nd	nd
Seed Membrane / Arils	Lutein	5.68 ± 0.90a	3.03 ± 1.19 a	2.65 ± 0.40a	51.34 ± 6.00b	18.07 ± 2.25c	4.77 ± 1.42a
	β-carotene	2.03 ± 0.02a	1.72 ± 0.57a	5.81 ± 0.79a	13.76 ± 1.11b	24.19 ± 2.95c	22.15 ± 2.84c
	15-*cis*-lycopene	nd	nd	nd	17.23 ± 1.94a	29.18 ± 4.90b	36.35 ± 3.07b
	13-cis-lycopene	nd	nd	nd	3.64 ± 1.94a	1.74 ± 0.85a	tr
	9-cis-lycopene	nd	nd	nd	nd	nd	21.83 ± 8.79
	7-cis-lycopene	nd	nd	nd	nd	nd	tr
	trans-lycopene	nd	6.09 ± 0.42a	2.99 ± 0.61a	2260.15 ± 100.44b	1272.91 ± 221.87c	1456.67 ± 390.32c
	5-*cis*-lycopene	nd	nd	tr	28.49 ± 1.29a	9.25 ± 2.44b	43.49 ± 10.78a

The values of carotenoids (µg/g FW) are presented as means ± standard error of replicate determinations (n=9). Differences in lettering within rows represent significant difference at a level of $p < 0.05$. Values were presented as (nd) and (tr) in cases where the particular metabolite was not present in the sample or only traces of the metabolite were found in samples, respectively.

6.3.2. Identification of carotenoids esters in ripe bitter melon pericarp.

The presence of various carotenoids in bitter melon has been previously reported but there are no reports on the presence of carotenoid esters. We identified 9 carotenoid esters in the pericarp tissues of mature bitter melon using UHPLC/APCI-HR-TOFMS in positive ionization mode. **Table 6-3** presents the retention time (tR), experimental and theoretical accurate mass, mass error and fragments of identified carotenoids esters. The UHPLC-UV chromatogram, extracted ion chromatograms (EICs) and their respective UV spectra were presented in **Figure 6-2**. The positive ion APCI-QTOF mass spectra of carotenoids esters were shown in **Figure 6-3**.

A peak eluted at retention time (tR) 11.8 min displayed a precursors ion at m/z 763.6456 $[M+H]^+$ with a mass error 8.9 ppm. The precursor ion loss a myristic acid $[M+H-228]^+$ to give product ion peak at m/z 535.4288 $(Y_0)^+$ (mass error -1.9 ppm). Thus, the present peak was identified as β-cryptoxanthin myristate. Likewise, a peak eluted at tR 12.8 min identified a β-cryptoxanthin palmitate having a precursors ion signal at m/z 791.6791 $[M+H]^+$ (mass error 6.7) which further loss of palmitic acid (-256 Da) gave a characteristic product ion at m/z 535.4281 $(Y_0)^+$ having a mass error -3.2 ppm. Positive APCI mass spectrum of a minor peak eluted at tR 13.8 min shown a $[M+H]^+$ ion at m/z 1033.8582 (mass error 6.7 ppm).

Table 6-2. The intraspecific content carotenoids in bitter melon pericarp and seed membrane/arils at immature and ripe stages.

Immature		Lutein	β-Carotene	*15-cis-L*ycopene	13-*cis*-lycopene	9-cis-lycopene	trans- lycopene	5-cis- lycopene
Pericarp	IG	1.78 ± 0.28a	20.22 ± 1.90a	nd	nd	nd	25.03 ± 9.71a	0.96 ± 0.40a
	IW	0.12 ± 0.04b	2.62 ± 0.35b	1.09 ± 0.38a	nd	nd	4.15 ± 1.50ac	nd
	HK	3.56 ± 0.40c	35.47 ± 5.83ac	tr	nd	nd	54.43 ±8.45b	tr
	JP	2.16 ± 0.19a	17.98 ± 4.93a	nd	nd	nd	5.63 ± 2.16c	nd
	LT	3.69 ± 0.36c	39.38 ± 6.32c	Nd	nd	nd	1.79± 0.36c	nd
Seed Membrane / Arils	IG	0.48 ±0.06a	6.25 ± 1.09a	0.93 ± 0.36a	nd	nd	18.40 ± 4.91a	1.01 ± 0.43a
	IW	0.59 ± 0.15ac	6.80 ± 2.21ab	0.85 ± 0.55a	nd	nd	30.70± 10.19a	1.76 ± 0.50a
	HK	1.67 ± 0.13b	11.07 ± 1.81ab	nd	nd	nd	91.80 ± 18.43b	4.10 ± 0.59b
	JP	1.64 ± 0.21b	8.84 ± 1.20ab	nd	nd	nd	1.34 ± 0.48c	nd
	LT	1.06 ± 0.10bc	12.50 ± 0.32b	nd	nd	nd	29.60 ± 6.98a	1.58 ± 0.32a
Ripe		Lutein	β-Carotene	*15-cis-L*ycopene	13-*cis*-lycopene	9-cis-lycopene	trans-lycopene	5-cis-lycopene
Pericarp	IG	0.09 ± 0.01a	42.55 ± 9.33a	nd	nd	nd	3.66± 0.92a	nd
	IW	0.48 ± 0.06b	52.46 ± 9.11a	nd	nd	nd	5.20 ± 0.75a	nd
	HK	0.15 ± 0.03ab	54.49 ± 8.42a	nd	nd	nd	28.08 ±0.92b	0.44± 0.08a
	JP	0.05 ± 0.02a	76.76 ± 9.11a	nd	nd	nd	13.15 ± 2.21c	tr
	LT	0.06 ± 0.01c	56.71 ± 6.46a	nd	nd	nd	3.55 ± 0.86a	nd
Seed Membrane / Arils	IG	0.46 ±0.07a	23.06 ± 2.36a	26.72 ± 6.06a	2.64 ± 1.05a	2.31 ± 0.61a	604.97± 39.24a	29.72 ± 5.52a
	IW	0.09 ± 0.01b	29.68 ± 0.43b	45.21 ± 1.55ac	3.65 ± 0.91a	3.12± 0.10a	993.77 ± 64.66b	32.36 ± 2.471a
	HK	0.57 ± 0.04a	15.05 ± 0.75c	16.83 ± 1.60ad	0.77 ± 0.09a	1.21 ± 0.09a	562.17 ± 95.63a	18.23 ± 2.20a
	JP	tr	21.85 ± 0.46a	22.92 ± 1.68ad	1.14 ± 0.06a	1.70 ± 0.14a	866.09 ± 110.69ab	42.87 ± 9.77a
	LT	0.59 ± 0.12a	42.61 ± 0.77d	88.07 ± 8.27b	2.67 ± 1.13a	1.80 ± 0.84a	765.83 ± 92.92ab	32.03 ± 2.69a

The values of carotenoids (µg/g FW) are presented as means ± standard error of replicate determinations (n=6). Differences in lettering within columns represent significant difference at a level of $p < 0.05$. Values were presented as (nd) in cases where the particular metabolite was not present in the sample.

Zeaxanthin laurate-myristate was also identified, the precursor ion was observed at m/z 961.8043 $[M+H]^+$ (mass error 3.7 ppm). The product ions m/z 761.6190 $[M+H-200]^+$ and 533.4223 $(Y_0)^+$ were yielded with a loss of lauric acid (m/z -200 Da) and myristic acid (m/z -228 Da) from zeaxanthin. Likewise, peaks eluted at tR 14.9 min and 15.2 min were identified as lutein dimyristate and zeaxanthin dimyristate with a precursor ion at m/z 989.8372 (mass error 5.2 ppm) and 989.8362 (mass error 4.2 ppm), respectively. Both precursor ions underwent sequential losses of two molecule of myristic acid and gave a prominent product ion at m/z 533.4137 $(Y_0)^+$ and m/z 533.4139 $(Y_0)^+$ with a mass error -0.7 ppm and -0.3 ppm, respectively.

A peak eluted at tR 16.6 min corresponded to lutein 3-O-myristate-3′-O-palmitate which represents a $[M+H]^+$ at m/z 1017.8651 (mass error 1.7 ppm). The precursor ion loss a molecule of myristic acid (-228 Da) and palmitic acid (-256 Da) and display a product ion peak at m/z 533.4126 $[M+H-228-256]^+$ ((Y0)+; mass error -2.8 ppm). Likewise, another peak at tR 18.6 min exhibited an accurate mass value at m/z 1045.8919 $[M+H]^+$ with a mass error -2.6 ppm. It also displays a prominent product ion signal at m/z 789.6569 $[M+H-256]^+$ and m/z 533.4121 $[M+H-256-256]^+$ $(Y_0)^+$ with corresponded to the loss of two molecules of palmitic acid. Thus, the present peak assigned as lutein diplamitate. A peak at tR 20.5 min displayed a precursor ion at m/z 1073.9230 $[M+H]^+$ (mass error -2.7 ppm). The product ions at m/z 818.6861 $[M+H-256]^+$ and at m/z 789.6601$[M+H-284]^+$ represent a loss of palmitic acid and stearic acid, respectively.

Table 6-3. **Carotenoid esters identified from the pericarp of fully ripe bitter melon**

	Rt	Compound	Experimental Mass	Theoretical Mass	Δppm	APCI (+) -MS $[M+H]^+$
1	11.8	β-Cryptoxanthin myristate	763.6456	763.6387	8.9	763, 535 $[M+H-228]^+$
2	12.8	β-Cryptoxanthin palmitate	791.6754	791.6700	6.7	791, 535 $[M+H-256]^+$
3	13.8	Zeaxanthin laurate-myristate	961.8043	961.8007	3.7	961, 761$[M+H-200]^+$ 733$[M+H-228]^+$, 533$[M+H-228-200]^+$
4	14.9	Lutein dimyristate	989.8372	989.8320	5.2	989, 761$[M+H-228]^+$, 533$[M+H-228-228]^+$
5	15.2	Zeaxanthin dimyristate	989.8362	989.8320	4.2	989, 761$[M+H-256]^+$, 533$[M+H-256]^+$
6	16.6	Lutein 3-*O*-palmitate-3′-*O*-myristate or Lutein 3-*O*-myristate-3′-*O*-palmitate	1017.8651	1017.8633	1.7	1017, 789$[M+H-228]^+$, 761$[M+H-256]^+$, 533$[M+H-228-256]^+$
7	18.6	Lutein dipalmitate	1045.8919	1045.8946	-2.6	1045, 789$[M+H-256]^+$, 533$[M+H-256-256]^+$
8	20.5	Lutein 3-*O*-palmitate-3′-*O*-stearate or Lutein 3-O-stearate-3′-O-palmitate	1073.9230	1073.9259	-2.7	1073, 817$[M+H-256]^+$, 789$[M+H-284]^+$, 533 $[M+H-256-284]^+$
9	21.4	Lutein distearate	1101.9503	1101.9572	-6.2	1101, 817$[M+H-284]^+$, 533$[M+H-284-284]^+$, 441 $[M+H-284-284-92]^+$

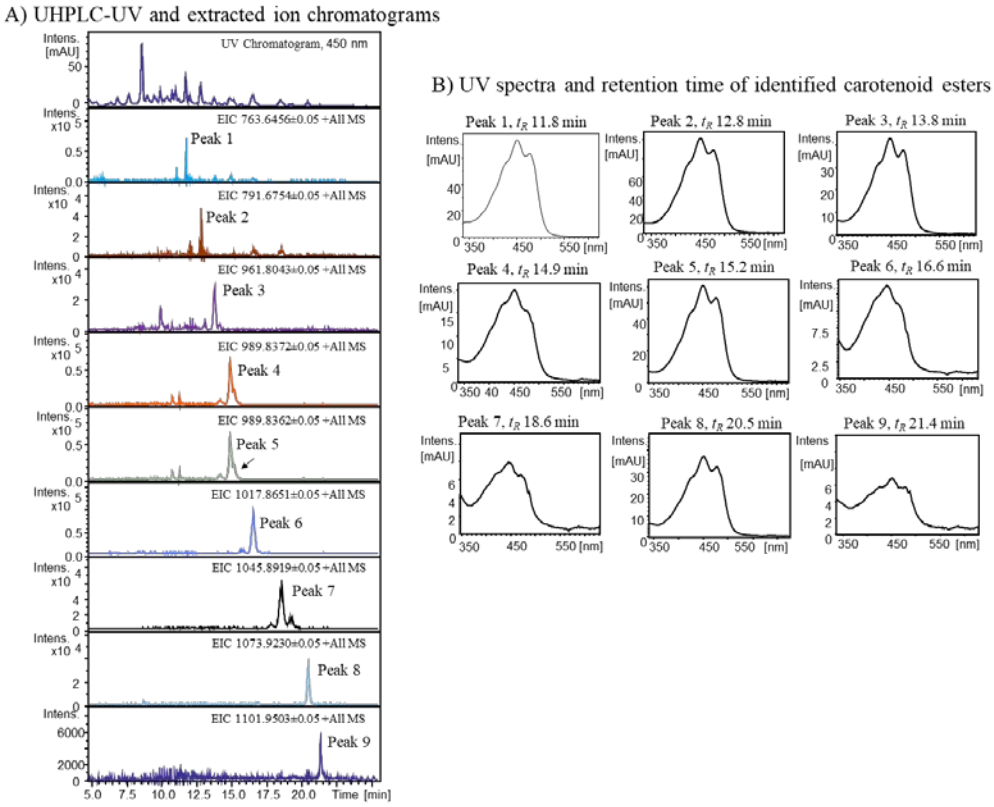

Figure 6-2. A) UHPLC-UV at 450 nm and extracted ion chromatograms; B) UV-spectra and retention time (t_R) of bitter melon carotenoids esters obtained from HR-APCI-QTOFMS analysis in positive ionization mode.

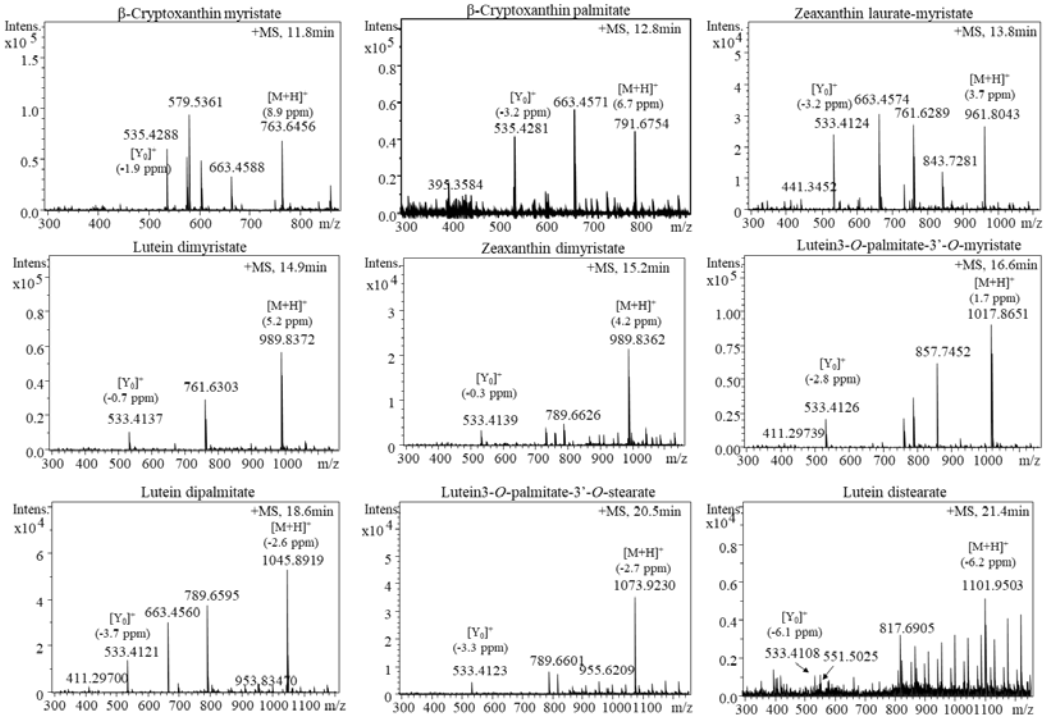

Figure 6-3. Positive ion HR-APCI-QTOF Mass spectra of carotenoids esters from bitter melon.

The mass spectra shows a prominent product ion at m/z 533.4123 $(Y_0)^+$ $[M+H-256-284]^+$ having a mass error -3.3 ppm. Thus, the present peak identified as lutein 3-O-palmitate-3′-O-stearate. Likewise, a minor peak (tR 21.4) exhibited a precursor ion at m/z 1101.9500 [M+H]+ (mass error -6.2 ppm) which loss a two molecules of steric acid and showed the characteristic product ion at m/z 817.6905 [M+H-284]+ and m/z 533.4108 [M+H-284-284]+ $((Y_0)^+$; mass error -6.1 ppm). Thus on the basis of APCI-MS spectrum and literature, the present peak identified as lutein distearate [254, 255].

Xanthophyll ester are present in plants of the following families: Anacardiaceae, Arecaceae, Caricaceae, Clusiaceae, Cucurbitaceae, Elaeagnaceae, Ericaceae, Malpighiaceae,

Malvaceae, Poaceae, Rosaceae, Rutaceae, Sapindaceae, Sapotaceae, and Solanaceae [254]. The conversion of free xanthophylls to xanthophyll esters is characteristic of various maturing fruits. These changes are directly linked to the transformation of chlorophyll to chromoplast leading to a gradual decrease in free xanthophylls [256]. The levels of lutein presented in **Table 6-1** display this type of change. Initially the levels of lutein increase followed by a drastic decrease in free lutein at stages 3, 4 and 5 of maturity. The accurate profiling of carotenoid esters in fruits and vegetables is important because the chemical properties of esterified carotenoid are different that free carotenoids. For example, esterified carotenoids are more lipophilic than their free forms. The property favors their integration into mixed micelles and enhances their bioavailability. Furthermore the esterification of carotenoids enhances their stability by facilitating their integration into cellular membranes resulting in protection against degradation[254]. Additionally, the esterification of xanthophylls has also been reported to affect their antioxidant properties compared to free xanthophylls[257, 258]. Esterified xanthophylls are rarely found in the human serum circulation, as such the de-esterification of these compounds must be occurring during absorption. In this regard, esterified xanthophylls may be guarded from degradation by digestive processes leading to higher levels of xanthophylls begin effectively absorbed.

6.3.3. Seed Membrane/ Aril Carotenoids.

During development, the level of lutein in the seed membrane/aril tissue increased during development up to the stage 4 (51.34 ± 6.00 μg/g) and ending back at initial levels (4.77± 1.42 μg/g) (Table 1). Conversely, the levels of β-carotene and lycopene generally increased during development. The levels of β-carotene increased from 2.03 ± 0.02 and 22.15 ± 2.84 μg/g

during the stage 1 to stage 6, respectively. The most drastic change observed during the development of bitter melon seed membrane/aril was in the levels of trans-lycopene. The levels of *trans*-lycopene were not detectable in stage 1. The *trans*-lycopene levels increased significantly from S2 to S6 stage, 6.09 ± 0.42 to 1456.67 ± 390.32 µg/g respectively. Furthermore, the levels of five *cis*-lycopene isomers (15-*cis* lycopene, 13-*cis* lycopene, 9-*cis* lycopene, 7-*cis* lycopene and 5-*cis*-lycopene) were measured for the first time in bitter melon. The levels of 15-*cis*-lycopene were observed to increase from stage 3 to stage 6 (17.23± 1.74 – 36.35 ± 3.07 µg/g, respectively). The levels of 13-*cis*-lycopenewere only observed in stages 4 and 5 ranging from 1.74 to 3.64 µg/g. Regarding 9-*cis* lycopene, 21.82 ± 8.79 µg/g were measured in stage 6, while only trace levels were observed of 7-*cis* lycopene were found at that same stage. The levels of 5-*cis* lycopene fluctuated between stages. Only trace levels were found in stage 3 while levels increased to 28.49 ± 1.29 µg/g at stage 4. The levels of 5-*cis* lycopene decreased significantly at stage 5 to 9.25 ± 2.44 µg/g and drastically increasing again at stage 6 (43.49 ± 10.78 µg/g).

Intraspecific variation was also observed in the levels of carotenoid in seed membrane/aril material. The level of lutein was highest in the immature seed membrane material of the 'Japan Spindle' and 'Hong Kong Green' cultivars, 1.64 ± 0.21 µg/g and 1.67 ± 0.13 µg/g, respectively (**Table 6-2**). The level of lutein in the 'Large Top' cultivar was 1.06 ± 0.10 µg/g, while the lowest levels were observed in the 'India Green' (0.48 ± 0.06 µg/g) and 'India White' (0.59 ± 0.15 µg/g) cultivars. The level of β-carotene in the immature seed membrane ranged from 6.25- 12.50 µg/g (Table 2). Interestingly, the level of lycopene in the immature seed membrane was relatively high in 'Hong Kong Green' cultivar (91.80 ± 18.43 µg/g), while only

negligible levels were observed in the ‘Japan Spindle’ cultivar (1.34 ± 0.48 µg/g) (**Table 6-2**). The wide range in *trans*-lycopene levels may have been due to differences in rate of ripening among cultivars. It was observed in our field studies (unpublished data) that the ‘Japan Spindle’ cultivar reached physiological maturity earlier than the other cultivars evaluated. As such the accumulation of *trans*-lycopene may have occur for longer period in cultivars that have a longer ripening cycle. The levels of *trans*-lycopene in the ‘India Green’, ‘India White’ and ‘Large Top’ cultivars were relatively similar, 18.40 ± 4.91, 30.70± 10.19, and 29.60 ± 6.98 µg/g, respectively. Additionally, low levels of 5-*cis*-lycopene were measured in the immature seed membrane material ranging from 1.01-4.10 µg/g. 5-*cis* lycopene was not detected in the ‘Japan Spindle’ cultivar. Furthermore, low levels of 15-*cis* lycopene were measured in the ‘India Green’ and ‘India White’ cultivars (0.93 ± 0.36 µg/g and 0.85 ± 0.55 µg/g, respectively).

In bitter melon, the white mesocarp is continuous with the seed membrane material present in immature fruits. During maturation the mesocarp degrades to create a cavity inside the fruit containing only mature seed enclosed in bright red arils. As in the ripe bitter melon pericarp, low levels of lutein were observed in the ripe aril material ranging from 0.09 to 0.59 µg/g, while only trace levels were observed in the ‘Japan Spindle’ cultivar. The level of β-carotene varied among the mature aril material evaluated. Overall, the highest level of β-carotene was observed in the ‘Large Top’ (42.61 ± 0.77 µg/g) cultivar, while the lowest level was measured in the ‘Hong Kong Green’ cultivar (15.05 ± 0.75 µg/g) (Table 2). The accumulation of β-carotene in mature arils was observed in all cultivar when compared to immature seed membrane material, with the exception of the ‘Hong Kong Green’ cultivar, which remained relatively similar throughout development. A recent study reported a similar accumulation of

β-carotene in mature bitter melon seed arils, but the level in the mature pericarp were considerably lower than the arils [94]. In our study we observed greater levels of β-carotene in mature pericarp material than in the mature aril. Even with lower levels of β- carotene compared to the pericarp, arils could still be considered as a source rich in β-carotene compared to fruits and vegetable known for high levels of β-carotene [259-262].

In the immature stage, the mesocarp of the fruit has a white appearance composed primarily of complex polysaccharides, such as starch, cellulose and pectin [263-265]. During fruit maturation, enzymes such as invertase, amylase, cellulose, polygalactrouronase, and pectin methyl esterase, cause the degradation of the mesocarp and softening of the fruit in general [266]. At physiological maturity, the mesocarp that was previously occupied in its entirety by pulp and immature seed, becomes a cavity containing mature seeds enclosed in bright red seed arils. The drastic change in color and texture of the mesocarp composition may be due to the production of ethylene which induced fruit softening, reduction of cell adhesion, and the alteration of carotenoid metabolizing enzymes, all characteristic of fruit ripening[267-269].

According to previous reports involving bitter melon and other *Momordica* species, the bright red color of the seed aril is principally determined by the level of lycopene within the tissue matrix[94, 246] . The levels of lycopene quantified from various *Momordica* species range from around 200 to over 6000 µg/g [87, 94]. Our results indicate high levels of *trans*-lycopene in the seed arils of all the bitter melon cultivars evaluated. The highest level of *trans*-lycopene was observed in the 'India White' cultivar (993.77 ± 64.66 µg/g) (**Table 6-2**). The level of the *trans*-lycopene was similar among the remaining cultivars, ranging from 562.17 – 866.09 µg/g. The overall levels of *trans*- lycopene we report were similar or in some cases higher than those

previously reported in bitter melon, but definitely within the range reported in other *Momordica* species. The higher level of *trans*-lycopene we report may be due to genotypic differences between previous studies and the cultivars evaluated in this study. Additionally, the level of 5-*cis*-lycopene is similar among the cultivars evaluated ranging from 18.23 to 42.87 μg/g. Furthermore, the levels of 15-, 13-, and 9- *cis* isomers were quantified in bitter melon for the first time in this study. The levels of 15-cis lycopene ranged from 16.83- 88.07 μg/g in aril material, while low levels of 13- and 9-*cis* lycopene were observed, ranging from 0.77 – 3.65 μg/g, respectively.

Our results indicate that bitter melon is a rich source of carotenoids particularly of lycopene. Compare to other crops known to have high levels of lycopene, such as watermelon and tomato, we report substantially higher levels of lycopene in the bitter melon seed arils regardless of the cultivars[259]. In addition to having high levels of lycopene, a study reported that *Momordica spp* aril lycopene has higher bioaccessibility than tomato and carrot food samples[270]. Higher bioaccessibility carotenoid from gac arils was correlated to differences in the localization of carotenoids within cellular structures and possibly the lipid profile of the aril tissues. While it is widely accepted that cis-lycopene isomers are more bioavailable, it has been reported that the presence of these isomers can enhance the bioavailability of trans-lycopene [271]. As such, we evaluated the bioaccessibility of trans-lycopene from aril of five bitter melon cultivars.

Table 6-4. Quantification of *trans*-lycopene (µg/g) from bitter melon mature seed arils, supernatant and digesta with and without the presence of bile acids during *in vitro* digestion.

	Supernatant		Digesta	
	Without Bile Acids	With Bile Acids	Without Bile Acids	With Bile Acids
India Green	15.21 ± 3.33	101.73 ± 19.51	761.46 ± 123.61	1164.43 ± 32.78
Large Top	23.48 ± 5.75	158.16 ± 44.10	817.96 ± 59.14	1042.07 ± 28.68
India White	22.94 ± 7.38	117.40 ± 25.90	828.42 ± 40.72	1097.95 ± 61.55
Hong Kong Green	19.30 ± 2.78	88.39 ± 8.96	713.92 ± 180.07	1051.37 ± 40.20
Japan Spindle	24.95 ± 7.18	105.82 ± 11.45	665.50 ± 22.68	930.53 ± 51.18

Levels of lycopene expressed as the means of three biological replicates ± standard deviation. Differences in lettering of groups within the supernatant and digesta indicate significant differences at $p \leq 0.05$.

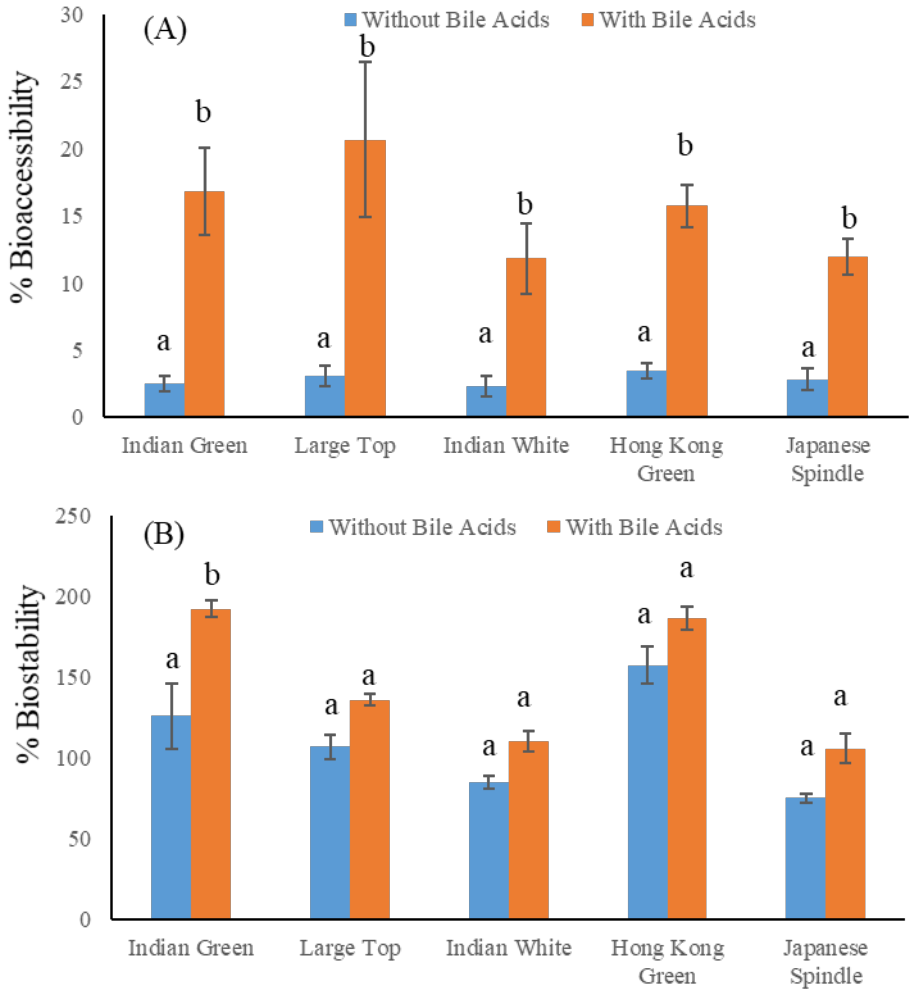

Figure 6-4. The bioaccessibility (A) and biostability (B) of bitter melon aril lycopene after *in vitro* digestion with and without the presence of bile acids. These values were obtained utilizing aril lycopene levels before in vitro digestion and comparing them to lycopene levels after in vitro digestion in supernatant and digesta. The illustration is a result from means of three replicates ± standard deviation. Statistical significance is illustrated by difference in lettering within groups at $p \leq 0.01$.

6.3.4. Bioaccessibility and biostability of trans-lycopene in seed aril

The level of *trans*-lycopene in mature bitter melon arils before undergoing the digestive process ranged from 604.97– 993.77 µg/g (**Table 6-2**), for 'India Green' and 'India White', respectively. After subjecting aril material to *in vitro* digestive processes, the level of *trans*-lycopene was measured in the resulting supernatant and digesta (pellet) to determine lycopene's bioaccessibility and biostability, respectively. Additionally, the effect of bile acids on these two processes was also evaluated. Significantly higher levels of *trans*-lycopene were bioaccessible in samples treated with bile acids regardless of the cultivar. The levels of bioaccessible *trans*-lycopene in samples not treated with bile acids ranged from 15.21 – 24.95 µg/g, while the level of bioaccessible *trans*-lycopene increased significantly with bile acid treatment, 88.39- 158.16 µg/g (**Table 6-4**). Similarly, the levels of biostable *trans*-lycopene were significantly increased with the use of bile acids, but only in the 'India Green' cultivar. The levels of *trans*-lycopene in digesta free of bile acids ranged from 713.46 – 824.42 µg/g, while the levels of *trans*-lycopene in digesta with bile acids ranged from 930.53 – 1164.43 µg/g. The percent bioaccessibility and biostability are presented in **Figure 6-4**.

Our results indicate that bitter melon aril *trans*-lycopene bioaccessibility is similar to levels reported for tomatoes [272]. An increase in bioaccessibility and biostability was observed in samples containing bile acids, possibly due to their role in the formation of mixed micelles. Bile salts are able to encapsulate lipophilic molecules, such as *trans*-lycopene, therefore facilitating absorption. While the bioaccessibility of bitter melon aril *trans*-lycopene is similar to those reported for tomatoes, higher levels of aril *trans*-lycopene may potentially be absorbed

due to higher overall levels in the aril matrix [273]. Additionally, samples treated with bile acids resulted in higher biostability of *trans*-lycopene from aril digesta. The detergent nature of bile salts possibly aided in the release of *trans*-lycopene from the food matrix. The linear structural nature of lycopene and its high lipophilicity may have left high *trans*-lycopene residues in the digesta that was not incorporated into the micelles. As such bile salts may have aided the biostability of lycopene from bitter melon arils and increased the release from the food matrix.

Surprisingly, no studies have evaluated the cultivar difference in *trans*-lycopene levels among bitter melon cultivars, and only three studies have evaluated the level of lycopene in bitter melon seed aril specifically [94, 246, 274]. With increase in demand for carotenoid and specifically lycopene supplements, crops such as bitter melon and other *Momordica* species have the potential to server as a source for lycopene supplements or nutritious food additive. Generally, the bitter melon pericarp is used as food in various cultures, the use of the bitter melon seed arils has not been explored for culinary purposes. In a closely related species, *Momordica cochinchinensis,* commonly known as gac, the seed arils also contain high levels of lycopene and are commonly used in Thailand as a food additive/coloring for its high levels of carotenoids[242]. Bitter melon seed arils have the potential to a value-added product in bitter melon production.

6.4. Conclusion

While studies of carotenoid content in some *Momordica spp* are common, relatively few studies have evaluated the level of carotenoids in *Momordica charantia*, bitter melon. Bitter melon is known to undergo characteristic color changes as it matures, changes that involve the accumulation of various carotenoids, principally β-carotene and lycopene. In the present study

we evaluated the levels of lutein, β-carotene, and lycopene in bitter melon pericarp and seed membranes/ aril during development and their intraspecific variation. Differences in levels of carotenoids were observed between cultivars. Additionally, the level of β-carotene and lycopene in mature seed arils were found to be significantly higher than other foods known for high levels of these carotenoids. Data from *in vitro* digestion of seed arils indicated that while the bioaccessibility of lycopene is similar to reported bioaccessibility of other foods, the bioavailability of lycopene may be higher due to overall higher levels within the aril matrix. Furthermore, the presence of these carotenoids was confirmed by high resolution mass spectrometry. Therefore, we suggest that bitter melon, especially mature seed arils, may be a rich, natural source of carotenoids for the nutraceutical, cosmetic, and supplement industries. In addition, the use of bitter melon seed aril food additive as a value-added product, merits more attention because of its high levels of lycopene.

7. BITTER MELON EXTRACTS AND CUCURBITANE-TYPE TRITERPENOID GLYCOSIDES ANTAGONIZE LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION VIA SUPPRESSION OF NLRP3 INFLAMMASOME

7.1. Introduction

Reactive oxygen species (ROS) are free radicals that are highly reactive due to unpaired valence shell electrons and are endogenously generated by routine cellular activity. ROS generation in humans is a crucial component of the innate immune system, such as responding against pathogen invasions and inflammation. Under homeostatic conditions, endogenously generated free radicals can be quenched, avoiding intracellular accumulation. However, at high concentrations these mechanisms become overwhelmed leading to oxidative stress [275]. Chronic oxidative stress in turn, leads to chronic inflammation. The activation of a chronic inflammatory response has been reported as a key component of various diseases such as heart disease, cancer, hypercholesteremia, obesity and diabetes[276]. Diabetes, type-2 particularly, is characterized by increased expression of pro-inflammatory cytokines, a response activated by the autooxidation of excessive glucose, glycated proteins and glycation of antioxidative enzymes [275].

Recently, studies have correlated ROS with the activation of a multiprotein complex named nucleotide-binding domain and leucine-rich repeat-containing family, pyrin domain-containing-3 (NLRP3) inflammasome[277]. The inflammasome complex is made up of NLRP3, ASC/PYCARD (apoptosis-associated speck-like protein containing a CARD) and pro-caspase-

1 [278]. This complex of proteins are responsible for recognizing exogenous and endogenous danger signals and initiating an inflammatory response, culminating in the production of IL-1β. Increased levels of IL-1β have been reported in patients with type-2 diabetes, resulting in insulin resistance in peripheral tissues, apoptosis and impaired β-cell function [140]. Furthermore, patients with high dietary fatty acid consumption, insulin resistance, obesity and type-2 diabetes have all been reported to show increased expression of *NLRP3*. As such, the use of therapeutic agents inhibiting the activation of the NLRP3 inflammation has been proposed by various studies [279]. NF-κB is one of the major transcriptional factors involved in the control of inflammasome formation. Targeting NF-κB could be of great values in finding therapeutic agents that target inflammasome formation and ultimately inflammation.

Bitter melon (*Momordica charantia* L.) is a fruit that is commonly consumed in many countries for its nutritive and medicinal properties. Various studies have reported bitter melon to have beneficial effects against several chronic diseases such as cancer, cardiovascular diseases, and metabolic disorders [280]. Several animal and cell models have been employed to elucidate the anti-obesity, anti-diabetic and anti-inflammatory effects of bitter melon [281]. Inflammation is a hallmark of several chronic diseases including type-2 diabetes, hence it is important to fully understand the ability of bitter melon to modulate inflammation. Early studies using bitter melon extracts reported anti-inflammatory effects *in vivo* and *in vitro*, ranging from reduction of inflammatory cytokines to influencing the migration and polarization of macrophages. [282-285]. Additionally, compounds isolated from bitter melon have been shown to suppress inflammatory markers (IL-1β, PTP-1B, iNOS) in FL83B hepatocytes [286]. Similarly, bitter melon extracts have been observed to suppress inflammatory markers in

immune cells such as monocytes and macrophages [146, 287-292]. Bitter melon bioactive compounds such as taikuguasin A-D, (23E)-25-methoxycucurbit-23-ene-3β,7β-diol (2), (23E)-5β,19-epoxycucurbita-6,23-diene-3β,25-diol (3), and 3,7-dioxo-23,24,25,26,27-pentanorcucurbit-5-en-22-oic acid and 5β,19-epoxy-25-methoxycucurbita-6,23(E)-dien-3β-ol have been evaluated for their anti-inflammatory effects on RAW 264.7 cells. These compounds inhibited the expression of *iNOS* and *COX-2*, and inhibited the production on many pro-inflammatory cytokines [293-295]. However, systemic profiling of bioactive compounds and characterization of their anti-inflammatory potency are still lacking.

In our previous study, we found that Indian bitter melon acetone and methanol extracts had higher levels of total phenolics, total saponins and potent inhibitor α-amylase, factors that have all been reported to ameliorate diabetic conditions [296]. Additionally, we recently isolated several compounds from the acetone extract of Indian and Chinese bitter melon and screened them for various bioactivities. The isolated compounds from both bitter melon cultivars were found to be potent amylase inhibitors, but were also found to differentially affect the expression of inflammatory markers *IL-6, iNOS, Cox-2 and TNF-α* [297]. Furthermore, our recent studies indicate that compounds from various Chinese bitter melon extracts differentially affected the expression of genes involved in the inflammatory response such as *IL-1β, IL-6, iNOS, NF-κB* and *TNF-α* [298, 299]. Results from our studies and others carried out to date have led us to speculate about the possible influence bitter melon has on the formation of the inflammasome complex. It was recently reported that bitter melon modulates adipose tissue inflammasome gene expression in high-fat diet fed mice [300]. Furthermore, obese and diabetic mice display enhanced intestinal permeability and metabolic endotoxemia, a condition of

increased levels of bacterial toxin lipopolysaccharide (LPS), that participate in the occurrence of metabolic disorders [301, 302]. Hence, we hypothesize that bitter melon extracts and compounds may exert their anti-inflammatory activity by suppressing the formation of inflammasome complex in the macrophages, thus leading to reduced systemic inflammation. We will test this hypothesis *in vitro* using LPS-induced inflammation in a murine macrophage cell line. Macrophages will be treated with bitter melon extracts and five purified compounds to evaluate their possible influence on the formation of the NLRP3 inflammasome complex. Furthermore, in silico molecular docking studies will be undertaken to establish the possibility of a structural-activity relationship between isolated bitter melon compounds and NF-κB. To the best of our knowledge, this is the first report to explore the effects of bitter melon extracts and its purified compounds on the expression of genes relating to the formation of the NLRP3 inflammasome complex in macrophage cells. The results presented in this study suggest bitter melon and bitter melon triterpenoid glycosides can potentially function as suppressor of NLRP3 inflammasome potentially by the inhibition of NF-κB.

7.2. Materials and Methods

7.2.1. Plant material

Indian bitter melon fruit was purchased at a local market (BCS Food Market) in College Station, TX. The fruit pericarp was manually separated from the mesocarp and seeds. The pericarp was then freeze-dried, powdered and processed into a fine powder for the extraction.

7.2.2. Isolation of bioactive compounds

Freeze-dried bitter melon powder was sequentially extracted using a Soxhlet apparatus. Pericarp powder was loaded to Soxhlet type apparatus and extracted with hexane, acetone and

methanol. The extraction process for each solvent was carried out for 16 h. The extracts were concentrated using rotary evaporator (Büchi, New Castle, DE, USA) under vacuum to obtain minimum volume and freeze-dried. The resulting dried extracts were used for purification [297] and certain in vitro bioassays [296]. Results of these studies lead to the selection of methanol and acetone extract for the further investigation of anti-inflammatory properties. Momordicoside A, momordicoside L, karaviloside VI, karaviloside VIII, and charantoside XII were purified and identified from acetone extract of Indian bitter melon according to our recent publications [297, 299].

7.2.3. Cell Culture

RAW 264.7 mouse macrophage cell lines (ATCC, USA) were cultured in RPMI-1640 media containing 10% FBS and 1% penicillin/streptomycin solution. The cells were maintained at 37°C in an atmosphere containing 5% CO_2. After attaining 80% confluency cells were passaged into new flasks. After six passaging events cells were used for *in vitro* assays.

7.2.4. RNA isolation and quantitative reverse transcriptase polymerase chain reaction(qRT-PCR) assay

RAW 264.7 cells were seeded into 6-well plates at a density of 5.0×10^5 cells/well. Cells were incubated at 37 °C in an atmosphere-controlled chamber maintained at 5% CO_2 for 24h. Following incubation, the cells were treated with the desired concentration of extract (50 µg/mL or 100 µg/mL) or compound (50 µM). After 1 h, LPS (lipopolysaccharide) (1 µg/mL) was added to specified wells and allowed to incubate for an additional 18h. Following the incubation period, the total RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA). RNA concentrations were determined using a NanoDrop 2000

spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). From each sample, 500 ng of total RNA was then used for first-strand cDNA synthesis (iScript cDNA Synthesis Kit, Bio-Rad). The qPCR gene expression analysis was carried out using a CFX 384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Aliquots of newly synthesized cDNA were assayed with SsoAdvanced Universal SYBR Green Supermix (Bio-rad) to quantitatively detect the expression of genes in response to bitter melon extract or compound treatments. PrimePCR SYBR green primers were obtained from Bio-Rad Laboratories and had the following unique assay identification numbers: qMmuCID0005641 (*IL-1β),* qMmuCID0005357 *(NF-κB),* qMmuCID0010647 *(NLRP3)*, qMmuCED0047869 (*PYCARD)*, qMmuCID0026983 (*CASP1*), qMmuCED0041193 (*HMGB1*), qMmuCID0040051 (*HO-1*). All reactions and assays were carried out according to manufacturer's specifications. The real-time PCR results were then normalized to *GAPDH* (housekeeping gene) and quantified using comparative C_t method ($2^{-[\Delta][\Delta]Ct}$). Each reaction was performed in triplicate. In this experiment the negative control consisted of RAW 264.7 cells without LPS stimulation, while the positive control consisted of RAW 264.6 cell stimulated with LPS.

7.2.5. *In silico* molecular docking studies

The molecular interactions of purified cucurbitane-type triterpenoids from bitter melon and the pleiotropic transcription factor NF-κB, *in silico* molecular docking, were carried out using Autodock 4.2. Simulated molecular docking studies allow for the prediction of possible ligand formation within the constraints of the target protein receptor binding site. The 3D structure of NF-κB (p50) was obtained from the online Protein Data Bank (PDB code:1NFK) and crystal structure of NF-κB without DNA was used for this study. The active site of protein was carried

out using COACH software. All the water and nucleic acid molecules were removed and only one p50 subunit was used for modeling. The molecular docking was executed with AutoDock Tools (ADT, version: 1.5.6) with the help of AutoDock 4.2 package (Autodock and Autogrid). Additional protein and ligand preparation steps were followed according to our previous published paper [298]. The protein structure was contained cubic grid box (coordinate of the center: x = 15.423, y = 20.846, z = 11.101) set to 80 Å with a spacing of 0.375 Å. The 2D visualization of ligand-protein interactions was analyzed using DS 4.5, and PyMOL molecular graphics system (PyMOL Molecular Graphics System, San Carlos, CA, USA) [298].

7.2.6. Statistics

Statistical analysis on the means of triplicate experiments were performed using the ANOVA procedure of the InStat® software,version 3.0 (GraphPad, San Diego, CA). Tukey's test was used for *post-hoc* analysis. A p-value of $p< 0.05$ was accepted as statistically significant, results are expressed as mean ± SEM.

7.3. Results and discussion

7.3.1. Isolation of compounds

Isolation and purification procedures are reported in our previous publication [303]. Momordicoside A, momordicoside L, karaviloside VI, karaviloside VIII, and charantoside XII were purified from acetone extract from Indian bitter melon. The spectral data presented in our previous publication positively confirmed the identity of these compounds Figure (7-1).

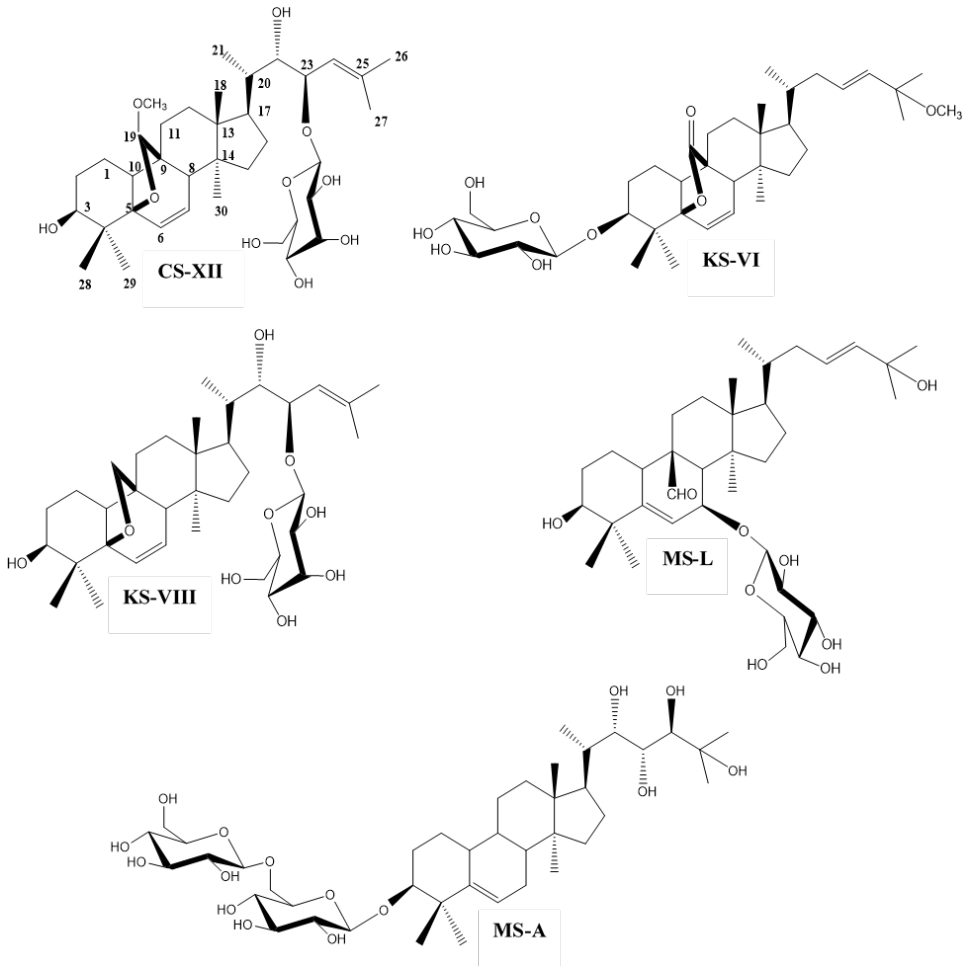

Figure 7-1. Structures of compounds isolated from bitter melon acetone extracts. Abbreviations: CS-XII-charantoside XII; KS-VI-karaviloside VI; KS-VIII- karaviloside VIII; MS-L- momordicoside L; MS-A- momordicoside A.

7.3.2. *In vitro* studies

Macrophages are known as an essential cell population of innate immunity that respond to various stimuli [304, 305]. Activated macrophages control inflammatory responses by secreting inflammatory cytokines, by direct macrophage infiltration into the tissues, and/or by activation of resident macrophages [306, 307]. The unique properties of macrophages directly contribute to

host defense as well as in the maintenance of tissue homeostasis and systemic insulin resistance [308-310]. Insulin resistance is one of the major systemic events that characterizes Type-2 diabetes. The progression of type-2 diabetes is complex, but various studies indicate that inflammation plays a very important role in diabetes pathogenesis [311]. Hyperglycemic conditions lead to the production of pro-inflammatory cytokines such as IL-6 and IL-1β, which in turn exacerbate the inflammatory response in surrounding tissues. ROS production in macrophages leads to the expression of the NLRP3 inflammasome complex [312]. The formation of the NLRP3 inflammasome complex ultimately results in the production of additional pro-inflammatory cytokines such as IL-1β and HMGB-1 (high mobility group box-1) [313]. A viable therapeutic strategy for the mitigation of inflammation is to hinder the formation of the inflammasome complex. In this study we present data in which bitter melon extracts and purified compounds affect the expression of various genes involved in inflammation and the formation of the NLRP3 inflammasome complex.

Initially, acetone and methanolic extracts were screened for their anti-inflammatory effects in RAW 264.7 cells. Our positive control (LPS stimulated cells) showed an increase in expression of the inflammation-related genes compared to the negative control. Two concentrations (50 and 100 µg/mL) of each extract were evaluated for their effects on the expression of various genes involved in the inflammatory response. Our results indicate that acetone extract at 50 µg/mL concentration significantly decrease the expression of *IL-1β* (**Figure 7-2A**). In general, there was a notable decrease in expression of *IL-1β* in the remaining extracts evaluated but

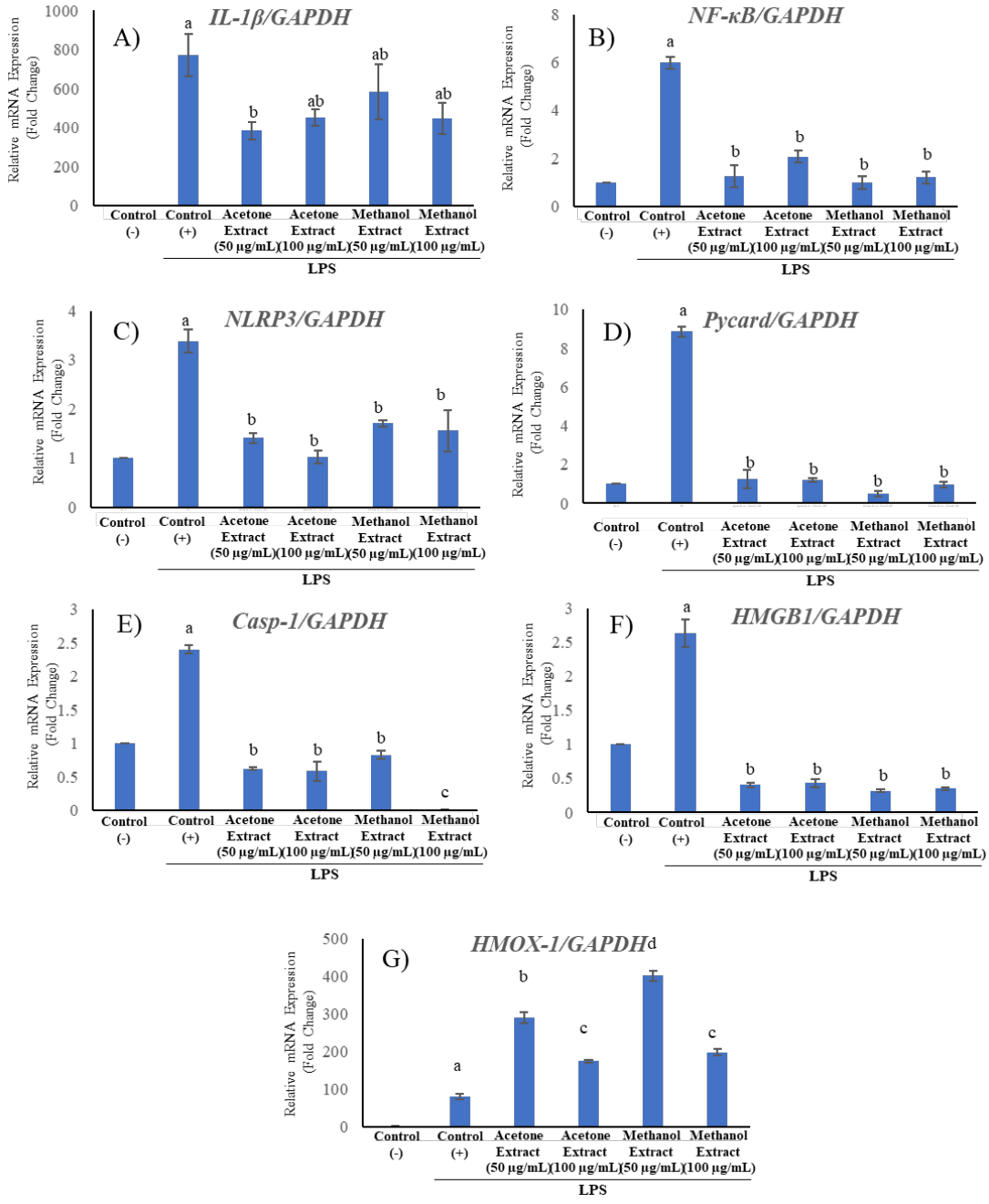

Figure 7-2. Bitter melon extracts have an antagonistic effect on genes related to the NRLP3 Inflammasome formation. Results of RT-qPCR expression analysis illustrated in panels A) *IL-1β*, B) *NF-κB* C) *NLRP3* D) *Pycard* E) *Casp-1* F) *HMGB1* and G) *HMOX-1*. Data is presented as mean ± SEM (n=9). Difference in lettering indicates statistical significance at p≤0.05 compared to the LPS positive control.

the levels were not significantly different when compared to the positive control (LPS only treatment) (**Figure 7-2A**). Pro-inflammatory *IL-1β* binds to membrane receptors such as TLR-4 which in turn activate the NF-κB complex [314]. Our results indicate that all the bitter melon extracts significantly decreased the expression of *NF-κB* mRNA (**Figure 7-2B**). The increase in NF-κB transcriptional factors and ROS are known to result in the production of the NLRP3 inflammasome complex [315]. This inflammasome complex is comprised of three major components an NLRP3 sensor, ASC adaptor (encoded by the *Pycard* gene) and caspase-1 protease[316]. A plausible strategy to manage the formation of the inflammasome complex would be to interrupt the expression of the genes responsible for formation of this multiprotein complex. Our results indicate that both acetone and methanol extract significantly decrease the expression of *NLRP3* (Figure 7-2C). Similarly, there was a notable decrease in expression of *Pycard* and *Casp1* mRNA levels in cells treated with both bitter melon extracts (**Figure 7-2D and 2E**). This data leads us to speculate that some component of the bitter melon extract is involved in the modulation of the inflammasome complex. Additionally, all extracts evaluated significantly decreased the expression of *HMGB1*(**Figure 7-2F**). HMGB1 is a damage-associated molecular pattern (DAMP) protein that has been reported to be released in response to the activation of the NLRP3 inflammasome. Furthermore, HMGB-1 has also been reported to interact with TLR-4 to ultimately induce the expression of the NLRP3 inflammasome[317]. Hence, lower levels of *HMGB1* expression may be due to lower expression of the genes related to the formation of the NLRP3 inflammasome complex, which in turn is down-regulated by lower HMGB-1 levels. Studies have reported higher levels of HMGB-1 in patients with type-2

diabetes and diabetic complications. Additionally, metformin, an anti-diabetic drug, has also been reported to have anti-inflammatory effects along with the decrease levels of HMGB-1 [318]. Again, strengthening the concept of the use of anti-inflammatory agents as a therapeutic strategy against diabetes and diabetic complication.

Lastly, bitter melon extracts significantly increase the expression of *HMOX-1* mRNA (**Figure 7-2G**). Recent studies have reported that the induction of heme oxygenase-1 (HO-1) down-regulates the production of IL-1β and the activation of the NLRP3 inflammasome complex [319]. The anti-inflammatory activities of HO-1 seem to be focused on the reduction of oxidative stress experienced by cells. Patients with type-2 diabetes have been reported to have lower levels of HO-1[320]. As such, the use of therapeutics in the induction of HO-1 may prove to be an additional viable option for the amelioration of a chronic inflammatory response that may lead to the progression of chronic diseases such as diabetes. Overall, the compounds that were observed to antagonistically affect the expression of the genes involved in the formation of the inflammasome complex were momordicoside A and karaviloside VI (**Figure7-3**). Both of these compounds significantly decreased the expression of *IL-1β, NF-κB, NLRP3, Pycard, Casp-1*, and *HMGB1*. Similarly, momordicoside L was observed to significantly decrease the expression of *IL-1β, NLRP3*, and *Casp-1* (**Figure 7-3A, 3C and 3E**). Furthermore, these compounds displayed differential effects on the expression of *HMOX-1* (**Figure 7-3F**). Momordicoside A significantly upregulated the expression of *HMOX-1* when compared to the LPS positive control. Karaviloside VI and momordicoside L either did not affect the expression of *HMOX-1* when compared to the positive control or was downregulated, respectively. Charantoside XII was observed to decrease the expression of *IL-1β, NF-κB*,

Pycard, *Casp-1*, and *HMGB1*, Surprisingly, the levels of *NLRP3* mRNA remained similar to the levels of the LPS control. An increase in the levels of *HMOX-1* mRNA levels was also observed by this treatment. Two possible explanations for this increase may be that the treatment of RAW 264.7 cells with charantoside XII directly induces the expression of cytoprotective heme oxygenase -1 or the cells may be reacting to the expression of *NLRP3* mRNA. The expression of *NLRP3* leads to the possible production of the inflammasome complex resulting in inflammation. In response, the cells produce the cytoprotective heme oxygenase-1 to counteract the inflammatory response. The expression of *IL-β* mRNA significantly decreased in the cells treated with karaviloside VIII compared to the LPS control group (**Figure 7-3A**), while the levels of, *Pycard*, *NLRP3*, *NF-κB*, *Casp-1*, *HMOX-1* and *HMGB1* mRNA remained similar to the LPS control group. Based on our results, a working model is presented in **Figure 7-4.**

7.3.3. *In silico* Molecular docking study of NF-κB

In our previous studies, we have demonstracted various cucurbitane-type triterpenoids to exhibit significant anti-inflammatory activity in RAW 264.7 macrophages [298, 299]. The molecular docking results for charantoside XII, karaviloside VI, karaviloside VIII, momordicoside L, momordicoside A reinforce our current hypothesis concerning the interactions these molecules have with NF-κB. The crystallized structure of NF-κB consists of two p50 subunits and a DNA molecule resulting in a homodimer protein. The separation of both p50 subunits exposes the molecular surface of the DNA binding region (DBR) [321]. The binding site of the NF-κB p50 monomer protein was defined according to previously reported amino acid residues [321]. Residues Arg54, Arg56, Try57, Cys59, Gly306, Lys241, Pro243,

His141, and Asp239 are required for the interaction between DNA and the binding site of the NF-κB p50 monomer. As shown in **Figures 7-5 (A-E)** and **Figure 7-6 (A-E)** all five cucurbitane type triterpenoids bound to the *NF-κB* monomer through the formation of various hydrogen bonds.

The binding energies for the purified compounds ranged from -9.69 to -13.54 kcal/mol. Momordicoside A showed the lowest binding energy (-13.54 kcal/mol), followed by karaviloside VI (-12.81 kcal/mol), charantoside XII (-11.45 kcal/mol), karaviloside VIII (-10.88 kcal/mol) and momordicoside L (-9.69 kcal/mol).

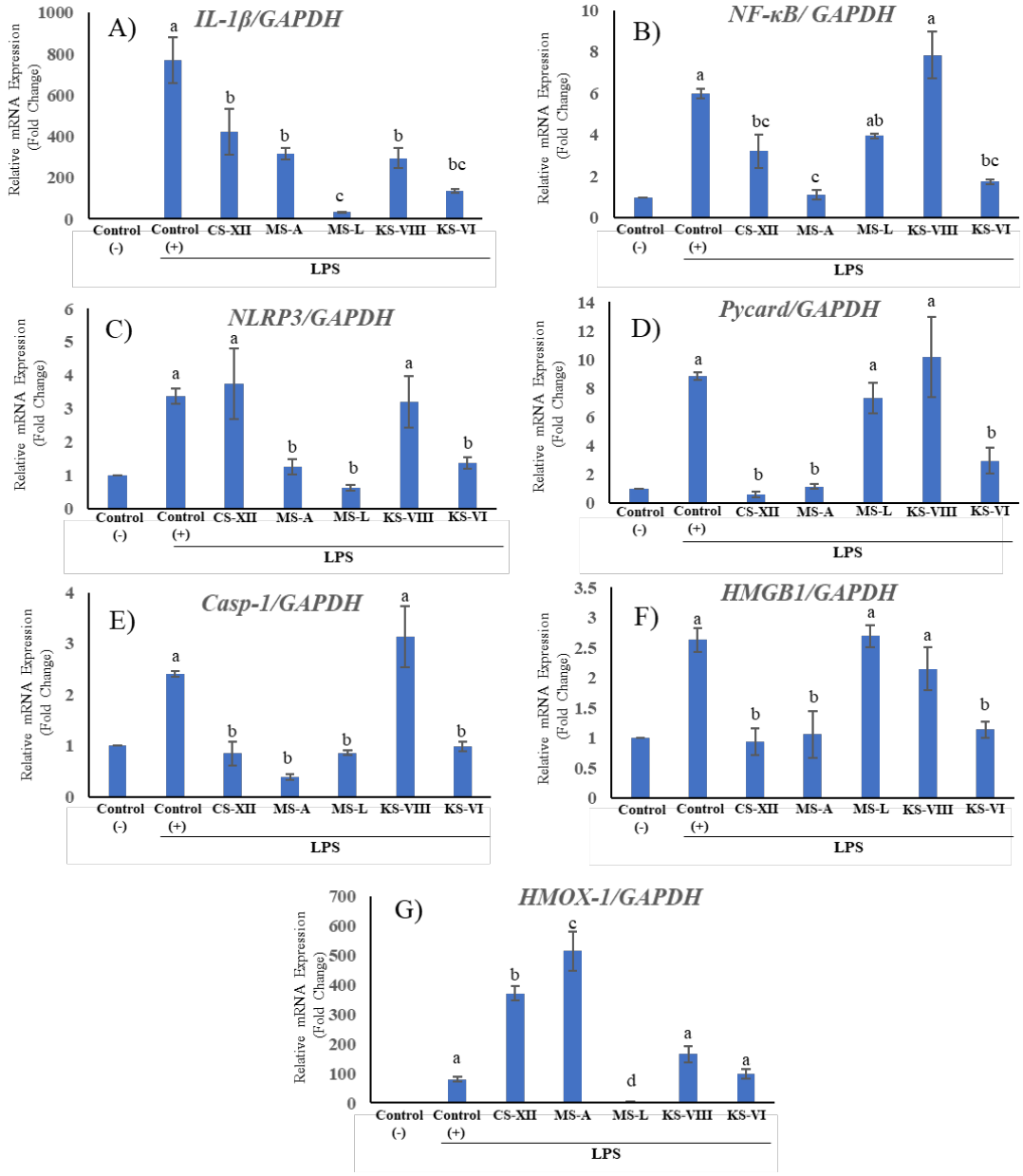

Figure 7-3. Bitter melon purified compounds have a differential effect on the expression of genes related to the NRLP3 Inflammasome formation. Results of RT-qPCR expression analysis illustrated in panels A) *IL-1β*, B) *NF-κB* C) *NLRP3* D) *Pycard* E) *Casp-1* F) *HMGB1* and G) *HMOX-1*. Data is presented as mean ± SEM (n=9). Difference in lettering indicates statistical significance at p≤0.05 compared to the LPS positive control. Abbreviations: CS-XII-charantoside XII; KS-VI-karaviloside VI; KS-VIII- karaviloside VIII; MS-L-momordicoside L; MS-A- momordicoside A.

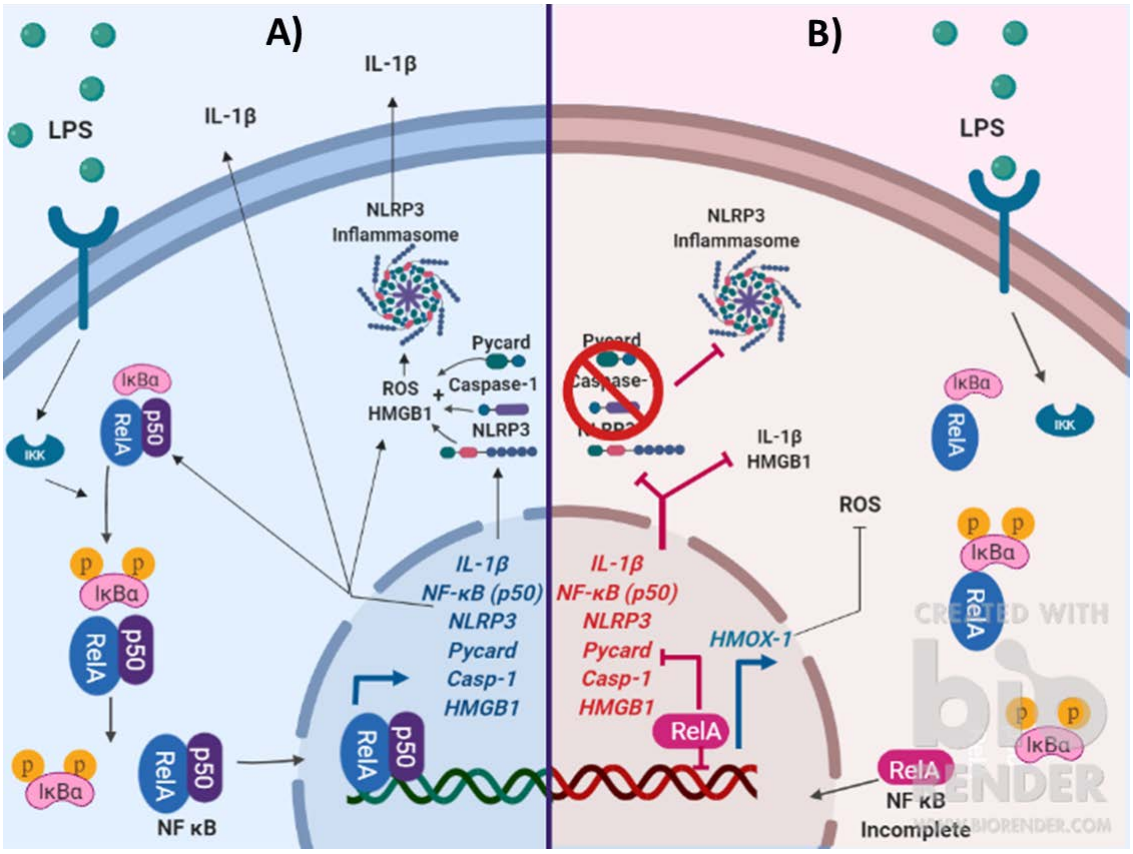

Figure 7-4. Hypothetical model summary of gene expression results. Panel A) depicts the inflammatory response to LPS in RAW 264.7 cells. LPS binding to membrane receptor TLR4 stimulates a signaling cascade that result in the activation and migration of NFκB to the nucleus. Once bound to DNA, NFκB exerts it pleotropic effects by stimulating the expression of various genes related to inflammation and inflammasome formation. Panel B) illustrated the results of RAW 264.7 cells stimulated with LPS and treated with either bitter melon extract or purified compounds. Depending on the treatment, various pro-inflammatory gene were down regulated, while the expression of anti-inflammatory *HMOX-1* was upregulated.

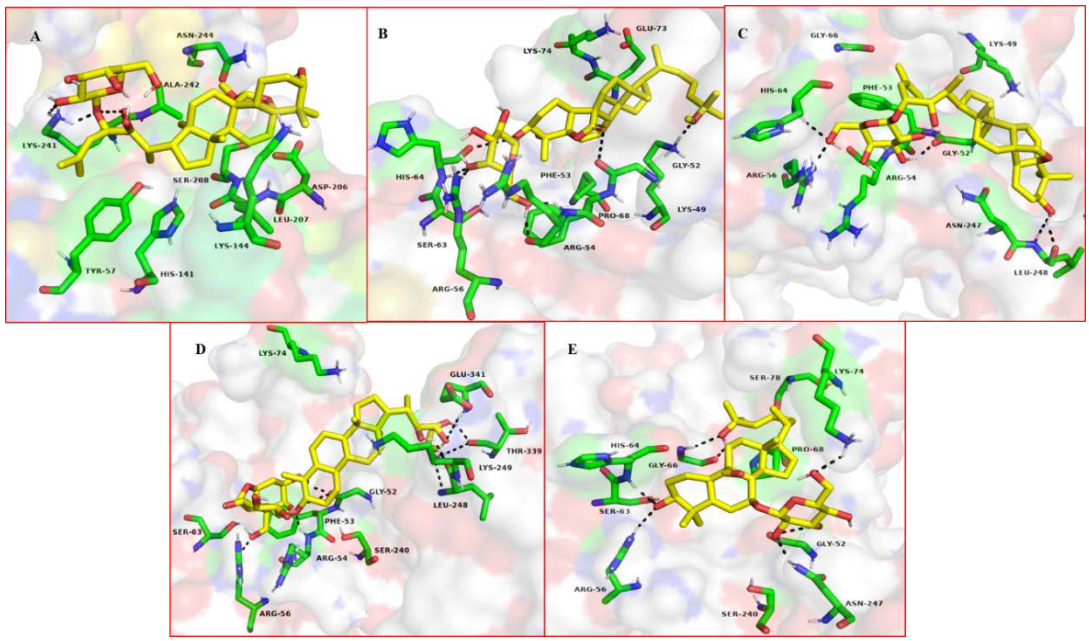

Figure 7-5. **The 3D ligand-protein interactions for (**A**) charantoside XII, (**B**) karaviloside VI, (**C**) karaviloside VIII (**D**) momordicoside L, and (**E**) momordicoside A in the binding pocket of NF-κB. The ligands in the 3D structure are shown in yellow and hydrogen bonds are shown as black dashed lines.**

Lower binding energy corresponds to easier binding of a ligand to the receptor [322]. Hence momordicoside A is most favorable to bind with NF-κB. Furthermore, a detailed analysis of the docked poses regarding ligand-protein binding energy, the number of H-bonds, and the inhibition constant are shown in **Table 7- 1**. In **Figure 7-5 (A-E**), the ligand in the DBR active site of the NF-κB monomer is displayed as a stick model and shown in yellow color. The amino acid residues are depicted as green, blue and red for carbon, hydrogen and oxygen atoms respectively. The hydrogen bonds are shown as black dotted lines depicting the interaction between protein and ligand. As shown in Figure 5 and Figure 6, the purified compounds were

surrounded by crucial amino acid residues including Arg54, Arg56, Try57, Lys241, Pro243, His141, and Asp 239 in the DBR site of NF-κB.

The binding site for purified compounds was inside the binding DBR site of the NF-κB monomer where the compounds formed various hydrogen bonds. Momordicoside A was surrounded by Lys74, Arg54, Gly52, Phe53, Ser240, Ser63, Arg56, His64, Lys249, Asn247, Leu248, Thr339, Ser340, and Glu338. Refined docking of momordicoside A resulted in the best pose with the lowest inhibition constant of 0.11 nM and the lowest binding energy (-13.54 kcal/mol). Incidentally, momordicoside A treatment of RAW 264.7 cells was the most potent at reducing *NF-κB* expression in our cell culture studies, and significantly decreased *IL-1B, NLRP3, Pycard, Casp-1, and HMGB-1* mRNA expression. Momordicoside A formed 10 hydrogen bonds at the NF-κB binding site, notably these hydrogen bonds were formed at Arg 54 and Arg56 Figure 5E and Figure 6E. These bonds have been reported to be essential for the binding of DNA to NF-κB binding site [321]. Similarly, karaviloside VI had a low binding energy of -12.81 kcal/mol and a low inhibition constant (0.40 nM), making it a potential NF-κB inhibitor. Only two hydrogen bonds were made between the ligand and the protein, but the bonds were made at Lys241, a bond considered essencial for NF-κB bonding to DNA. Additionally, Van der Waals interactions were observed at Asp 239 in addition to two Pi-Alkly interactions with Try57 and His141. Interestingly, karaviloside VI also displayed promising anti-inflammatory activity in our cell culture data.

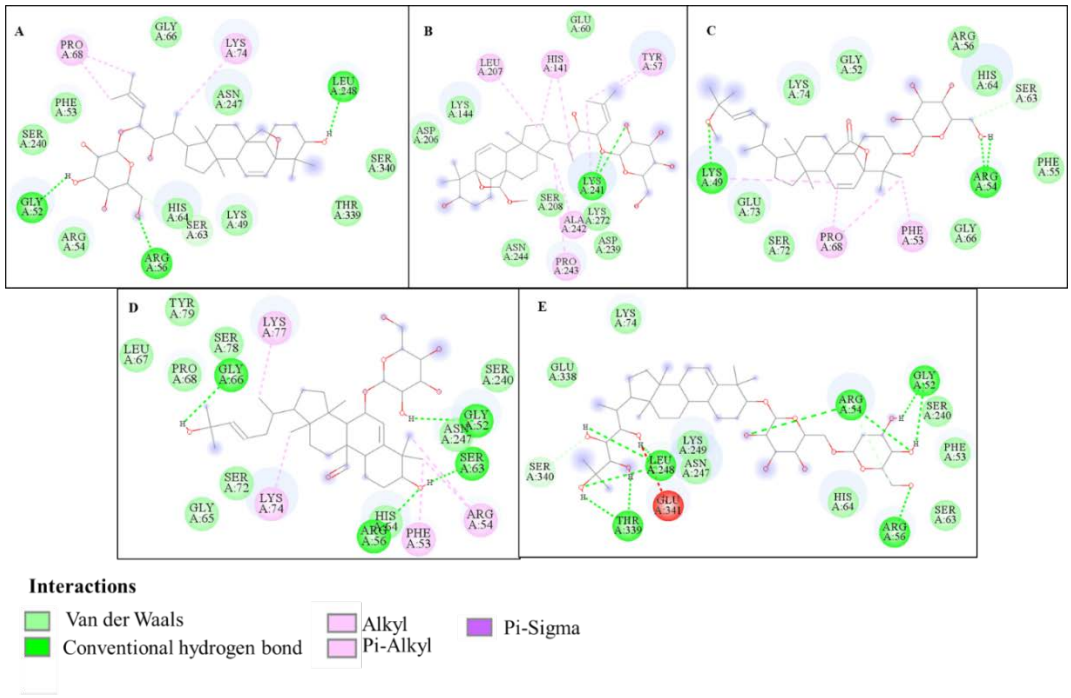

Figure 7-6. The 2D interactions between bitter melon triterpenoids and residues of NF-κB binding site. (A) charantoside XII, (B) karaviloside VI, (C) karaviloside VIII (D) momordicoside L, and (E) momordicoside A in the binding pocket of NF-κB. Legends below the figure explain different protein ligands interactions.

Table 7-1. Binding energy, minimum inhibition constant of the ligand-receptor complex and the number of hydrogen bonds between purified compounds charantoside XII, karaviloside VI, karaviloside VIII, momordicoside L, momordicoside A with NF-κB (PDB code:1NFK).

Receptor	Compound	Binding energies (kcal/mol)	Hydrogen bonds	Interactions	Hydrogen bonds in Å	Inhibition constants (nM)
NF-κB (PDB code:1NFK)	Charantoside XII	-11.45	4	Ser240, Phe53, Pro68, Gly66, Lys74, Asn247, Leu248, Ser340, Thr339, Lys49, Ser63, His64, Arg56, Arg54, Gly52	Gly52 (2.8 Å) , Arg56 (3.3 Å), His64 (3.8Å), Leu248(4.3Å)	4.06
	Karaviloside VI	-12.81	2	Asp206, Lys144, Leu207, His141, Glu60, Tyr57, Lys241, Lys272, Asp239, Ala242, Pro243, Ser208, Asn244	Lys241 (3.1 and 3.2 Å)	0.406
	Karaviloside VIII	-10.88	4	Lys74, Gly52, Arg56, His64, Ser63, Arg54, Gly66, Phe53, Pro68, Ser72, Glu73, Lys49	Lys49 (3.6 Å), Arg 54(2.8 Å), Ser 63 (2.9 Å)	10.56
	Momordicoside L	-9.69	4	Leu67, Tyr79, Pro68, Ser78, Gly66, Lys77, Ser240, Gly52, Asn247, Ser63, Arg54, Phe53, His64, Arg56, Lys74, Ser72, Gly65	Gly66 (3.3 Å), Arg56 (2.6 Å), Ser63 (3.4 Å), Gly52 (3.6 Å)	78.4
	Momordicoside A	-13.54	10	Lys74, Arg54, Gly52, Phe53, Ser240, Ser63, Arg56, His64, Lys249, Asn247, Leu248, Thr339, Ser340, Glu 338	Thr339 (2.8, 3.0 Å), Leu248(3.0, 3.3 and 3.1 Å), Arg54(3.2 and 3.6 Å), Arg56 (3.2 Å), Gly52(3.3 and 3.4 Å)	0.11

Charantoside XII was surrounded by Ser240, Phe53, Pro68, Gly66, Lys74, Asn247, Leu248, Ser340, Thr339, Lys49, Ser63, His64, Arg56, Arg54, and Gly52 and had a binding energy of -11.45 kcal/mol. Four hydrogen bonds were formed between the ligand and active site of NF-κB, but only one was made at an essential residue (Arg56) resulting in and an inhibition constant of 4.06 nM. Charantoside XII also reduced the expression of most genes related to inflammasome formation with the exception of *NLRP3* mRNA. Karaviloside VIII and momordicoside L both had the highest binding energies of the compounds evaluated in this study (**Table 7-1**). The effect of these compounds on the expression of the gene in this study was variable, showing reduction in expression in some genes but not on others (**Figure 7-3**). The results of the molecular docking for these compounds are presented in **Table 7-1**.

In the present molecular docking study, we have observed the formation of hydrogen bonds at various sugar moieties present on the different positions of the purified compounds. Additionally, hydroxyl groups associated with the triterpenoid side chains were also favored for the formation of hydrogen bonds between the ligands and proteins. For example, momordicoside A had the lowest binding energy and inhibition constant as well as the highest number of hydrogen bonds (10) between the ligand and the protein. Momordicoside A is a di-glucoside and has multiple hydroxyl groups on side chain. Half of the hydrogen bonds presented form momordicoside A were present in the glucose moieties while the other half were present of the side chain. Similarly, the rest of the compounds used for the molecular docking in this study had the formation of hydrogen bonds with the sugar moieties and/or side chains of the molecules. The formation of hydrogen bonds was also observed with hydroxyl groups on the A ring of the triterpenoid

nucleus for charantoside XII and momordicoside L, but data may be required to possibly attribute this interaction with any activity.

7.4. Conclusion

The results of this study provide critical insight on into the possible role of bitter melon in suppressing inflammation and inflammatory conditions. The use of anti-inflammatory agents as a therapeutic strategy is currently gaining momentum in various fields of research involving chronic diseases. In this respect, the use of bitter melon for the inhibition of the NLRP3 inflammasome is in line with the hypothesis that bitter melon has anti-inflammatory and anti-diabetic properties. While the anti-inflammatory properties have been previously reported, much more information is still needed to define the exact molecular pathway. Our data demonstrates that suppression of inflammasome complex formation contributes to the anti-inflammatory properties of bitter melon extracts and triterpenoids. Furthermore, our molecular docking results predict that these compounds may be exerting this activity by inhibiting the activity of transcriptional factor NF-κB.

8. SUMMARY AND CONCLUSION

Bitter melon is a fruit that is rich in a wide-array of bioactive compounds and nutrient, all of which have been reported to have several health benefits ranging from anti-cancer, anti-diabetic, anti-oxidant, and anti-inflammatory. As an underutilized horticultural crop, bitter melon has the potential to be a profitable specialty crop in Texas. Relatively little information is available for the production of bitter melon in the US, as such more is formation is need to determine its commercial feasibility. Furthermore, the analysis of its phytochemicals content and health benefit can aid in the promotion of it use by US consumers. In the present study, five bitter melon cultivars were successfully grown in College Station, Texas. The yields varied among the cultivars evaluated but they were comparable to yields observed in area of commercial production. The quality of bitter melon was measured by the levels of ascorbic acid. Bitter melon was observed to have high levels of ascorbic acid. Additionally, the metabolic profiles of two commercially available bitter melon cultivars (Indian and Chinese) were compared. Notable differences were observed between cultivars, extracts and evaluated tissues. Furthermore, non-polar to medium polar metabolites were observe to be potent inhibitors of carbohydrate metabolizing enzymes such as α-amylase and α-glucosidase. It was noted that the acetone extracts of the Indian bitter melon pericarp was the strongest inhibitor of α-amylase activity. These activities have been correlated to the phytochemical content of fruit and the content is known to change in response to postharvest conditions.

We evaluated the change in quality of bitter melon under several storage temperatures, times and in response to processing conditions. Ascorbic acid, amino acids, total phenolics

and antioxidant activities were uses to compare the quality of bitter melon under different storage parameters. Under refrigerated condition, levels of ascorbic acid were observed to decrease in processed fruit, while higher levels were observed in fruits stored under lower temperatures. The levels of amino acids were differentially fluctuated depending on the particular amino acids because of the various physiological roles they play in the metabolism of the plant. Furthermore, the antioxidant activities of bitter melon stored for prolonged periods of time under frozen conditions were observed to increase. The changes of phytonutrients not only change during storage but also during maturation. Bitter melon is a climatic fruit and as such continues to mature after harvest turning from green to orange. The changes are manifested by changes in carotenoid. We measured the levels of various carotenoid in bitter melon and identifies several isomers not previously reported in bitter melon. The isomers were found in mature tissues that are considered a postharvest loss. These “waste” material can be used for the production of carotenoid supplements.

Lastly, evaluated bitter extracts and purified compounds for their anti-inflammatory properties using RAW 264.7 cells. Bitter melon extracts were observed to have a wide range of anti-inflammatory effects such as the down regulation of genes involved in the production of pro-inflammatory cytokines and the NLRP3 inflammasome complex. Furthermore, five compounds purified from bitter melon acetone extracts were screened for their anti-inflammatory activities. Some of these compounds were found to inhibit the formation of the NLRP3 inflammasome complex. We observed through in silico molecular docking studies that this activity may be due to the inhibition of NF-κB transcriptional factor.

The results of this study demonstrate the potential of bitter melon for becoming a profitable specialty crop in Texas. Data presented in this study and information from others on the phytochemical content and health benefits of bitter melon can be a very effective tool in the promotion of its used to manage various inflammatory diseases such as diabetes.

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APPENDIX

PUBLICATIONS AND PRESENTATIONS

Accepted/Submitted:

1) **Jose L. Perez**, GK Jayaprakaska, Bhimanagouda S. Patil. Influence of Postharvest Storage, Temperatures, and Processing on the Levels of Bioactive Compounds Found in Bitter Melon. *Postharvest Biology* (Submitted)
2) B.S. Patil, J. Singh, P. Acharya, R. Metrani, S. Shivanagoudra, **Jose L. Perez,** G.K. Jayaprakasha. Hidden treasures of the Cucurbitaceae: Health benefits of melons, from watermelon to bitter melon. *Acta Horticulturae* (Submitted)
3) Siddanagouda R.S, Wilmer H. Perera, **Jose L Perez**, Giridhar Athrey, Yuxiang Sun, Chia Shan Wu, G. K. Jayaprakasha, Bhimanagouda S. Patil, In vitro and in silico elucidation of antidiabetic and anti-inflammatory activities of bioactive compounds from Momordica charantia *Bioorganic and Medicinal Chemistry. (Accepted)*
4) **Jose Luis Perez**, G.K. Jayaprakasha, Bhimanagouda S. Patil. Metabolite Profiling and in vitro Biological Activities of Two Commercial Bitter Melon (Momordica charantia Linn.) Cultivars. *Food Chemistry* (2019) 178-186.
5) Siddanagouda R.S, Wilmer H. Perera, **Jose L Perez**, Giridhar Athrey, Yuxiang Sun, G. K. Jayaprakasha, Bhimanagouda S. Patil. Cucurbitane-type compounds from Momordica charantia L.: isolation, in vitro antidiabetic and anti-inflammatory activities, and in silico modelling approaches. *Bioorganic Chemistry* (2019) 31-42.
6) **Jose L. Perez,** GK Jayaprakaska, Kevin Crosby, Bhimanagouda S. Patil. Evaluation of Bitter Melon (Momordica charantia) Cultivars Grown in Texas and Levels of Various Phytonutrients. *Journal of the Science of Food and Agriculture* (2019) 379-390.
7) **Jose L Perez**, G. K. Jayaprakasha, Bhimanagouda S. Patil, Separation and Identification of Cucurbitane-Type Triterpenoids from Bitter Melon, *Instrumental Methods for the Analysis and Identification of Bioactive Molecules.* (2014) 51-78 Doi:10.1021/bk-2014-1185.ch003

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1) Jose L. Perez, GK Jayaprakaska, Bhimanagouda S. Patil. Bitter melon (Momordica charantia) mediates inflammation in LPS induced RAW264.7 cells by down regulating inflammasome gene expression. *Free Radical Biology*
2) Wilmer H. Perera , Siddanagouda R. S. , **Jose L. Perez,** Guddadarangavva Jayaprakasha and

Bhimanagouda S. Patil. Anti-inflammatory activity of monoglycoside cucurbitane-type triterpenes from Momordica charantia L. (Cucurbitaceae): in vitro α-amylase and α-glucosidase evaluation and in silico modelling. *Food Chemistry*

3) **Jose L. Perez,** GK Jayaprakaska, Bhimanagouda S. Patil. Intraspecific variation in the levels of carotenoid of *Momordica charantia* cultivars. *Industrial Crops and Products*

Oral Presentations:

1) The Answer Is In The Plants. Blinn Research Appreciation Conference, Bryan, TX November 11, 2018.
2) A Bitter Crop with Sweet Potential for Producers and Human Health, America Society of Horticultural Sciences, New Orleans, LA, August 5,2015.
3) Exploring Bitter Melon's (*Momordica charantia*) Health Benefits and its Feasibility as a Specialty Crop in Texas, Student Research Week, College Station, March 24-28,2014
4) Separation of cucurbitane-type triterpenoids from bitter melon using flash chromatography, American Chemical Society Meeting, Indianapolis, IN, September 9, 2013.

Posters:

1) Bitter Melon (*Momordica charantia*) Extracts have Differential Anti-inflammatory effects. **Jose Luis Perez**, Yuxiang Sun, Chia Shan Wu, G.K. Jayaprakasha, Bhimanagouda S. Patil, Horticulture Graduate Council Poster Competition, College Station, TX November 2017.
2) Bitter Melon(*Momordica charantia*): A Potential Specialty Crop for Texas to Reduce Risk of Diabetes, **Jose Luis Perez** , GK Jayaprakasha, Kevin Crosby, Bhimanagouda S. Patil. Southern Region American Society of Horticultural Sciences, San Antonio, TX February 5-7, 2016
3) Bitter Melon *(Momordica charantia*): A Potential Specialty Crop for Texas with Several Possible Health Benefits. **Jose Luis Perez**, GK Jayaprakasha,Rammohan Uckoo, Bhimanagouda Patil. Student Research Week, College Station, March 24-28,2014.
4) Bitter Melon (*Momordica charantia*): A Potential Specialty Crop for Texas with Several Possible Health Benefits**. Jose Luis Perez**, GK Jayaprakasha, Rammohan Uckoo, Bhimanagouda Patil. Vegetable and Fruit Improvement Center Conference, College Station, February 25-26, 2014.
5) Bitter Melon (*Momordica charantia*) as a potential specialty crop of Texas and the use of botanical extracts as a bio-pest management agent against the

leaffooted bug (*Leptoglossus zonatus*). **Jose Luis Perez**, GK Jayaprakasha, Bhimanagouda Patil Texas Plant Protection Association Annual Conference, Bryan , Texas, December 10-11, 2013.

6) Bitter Melon (*Momoridica charantia*):A Potential New Specialty Vegetable in Texas and its Anti-Diabetic Properties. **Jose Luis Perez**, GK Jayaprakasha, Bhimanagouda Patil. North American Agricultural Biotechnology Council 25th Annual Conference, College Station, Texas, June 4-6, 2013.