IDENTIFICATION AND CHARACTERIZATION OF HEALTH-PROMOTING BENEFITS AND BIOACTIVE MOLECULES IN BLACK WALNUTS (*JUGLANS NIGRA* L.) AND WASTE MATERIALS FROM AGROFORESTRY SYSTEMS

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by

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IDENTIFICATION AND CHARACTERIZATION OF HEALTH-PROMOTING BENEFITS AND BIOACTIVE MOLECULES IN BLACK WALNUTS (*JUGLANS NIGRA* L.) AND WASTE MATERIALS FROM AGROFORESTRY SYSTEMS

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This dissertation is dedicated to my husband, Man Huynh, and my sons, Tri Huynh and Huy Huynh for their love, support, and sacrifices throughout my graduate studies. I am truly thankful for having my beloved family in my life.

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ABSTRACT

Black walnut (Fagales: Juglandaceae; *Juglans nigra* L.), known as eastern black walnut or America walnut, is one of the most economically valuable hardwood species and a high value tree for edible nut production in the United States. This native plant species to North America that adapts to grow on a wide range of cultivation conditions is an ideal tree for agroforestry practices. Black walnut agroforestry systems provide landowners the opportunity to develop a solid long-term investment in future generations, both economically and spiritually while allowing establishing a balanced relationship with the environment.

Remarkably, black walnut provides an excellent nutritious food material that contains a rich source of lipids and proteins. Consumption of black walnut has been linked to many health-promoting benefits such as decreased risk of cardiovascular disease, reduced levels of cholesterol, stimulated brain functions and prevention of certain cancers (e.g., prostate and breast cancers). However, the biological activities of black walnuts are remained unknown. We systematically explored multiple biological functions (antibacterial and antioxidant activities, and anti-inflammatory potential) of a numerous of black walnut cultivars that were selected and cultivated for nut production by the University of Missouri - Center for Agroforestry (Columbia, MO, USA). Additionally, it is likely that the bioactive activities of black walnut are correlated with its bioactive constituents including polyphenols. We further identified and characterized bioactive molecules that are driving the health-promoting benefits of black walnuts by modern analytical techniques combined with an array of in vitro bioassays. The exploration of health -promoting benefits in black walnut and its associated bioactive

compounds would promote the development of novel applications of black walnut and its by-products such as new drug discoveries to mitigate human diseases (e.g., diabetes, cancer), cosmetic products (e.g., skin care products, personal care products), and functional foods (e.g., natural dietary supplements), and thereby potentially increase the sustainability of the black walnut agro-industry.

We report the biological properties of black walnuts and their associated bioactive molecules. Black walnuts possessed antibacterial activities against a Gram-positive bacterium (Chapter 2). The antibacterial capacity of black walnuts was variable among different cultivars in which Mystry exhibited the strongest antibacterial capacity. Chapter 2 also describes a discovery of glansreginin A as a novel bacterial compound responsible for the antibacterial capacity of Mystry extract via a metabolomic approach coupled with bioassay-guided fractionation strategy. In Chapter 3, anti-inflammatory potential of black walnuts is described. Our results demonstrated for the first time that black walnut possessed compounds that exerted an inhibitory effect on the secretion of six measured cytokines [tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-8, IL-10, and monocyte chemoattractant protein (MCP)-1] induced by a human promonocytic cell line U937. Black walnut kernels contain a wealth of bioactive metabolites putatively identified through a metabolomics approach.

Chapter 4 describes biological properties of glansreginin A. This compound was presented at moderate levels (6.8 - 47.0 mg/kg) in all 12 black cultivars examined. Glansreginin A possessed moderate antibacterial activities against Gram-positive bacteria including *Staphyloccocus aureus* and *Bacillus anthracis*. However, this polyphenolic compound exhibited no antioxidant activity, did not induced activity of antioxidant

response element signaling pathways, and had no antiproliferative effects on the growth of both the tumorigenic alveolar epithelial cells and non-tumorigenic lung fibroblast cells. Furthermore, we identified and characterized antioxidant and anticancer capacities of 16 phenolic compounds that have been reported in black walnuts using high-throughput screening technologies (Chapter 5). Our findings suggested that penta-*O*-galloyl-*β*-D-glucose, which is one of the most abundant phenolic compounds in Mystry, could be a potential bioactive agent for the cosmetic and pharmaceutical industries. Since several phenolic compounds had anticancer activities against growth of tumorigenic alveolar epithelial cells, revealing that black walnut extracts possibly possess anticancer activities.

In addition to black walnuts, we investigated biological activities and associated bioactive compounds in waste materials [spent coffee grounds and switchgrass (*Panicum virgatum*)] from agroforestry systems. Chapter 6 describes the identification and quantification of bioactive molecules inhibiting pro-inflammatory cytokine production in spent coffee grounds. We found that methanolic extracts of spent coffee grounds obtained from 3 Arabica cultivars possess compounds that exerted inhibitory effects on the secretion of inflammatory mediators (TNF-α, IL-6 and IL-10) induced by a human pro-monocytic cell line U-937. Spent coffee grounds contain a wealth of anti-inflammatory bioactive compounds with caffeine and 5-caffeoylquinic acid as the most abundant compounds identified via robust analytical metabolomic analyses. The health-promoting activities of the extracts from switchgrasses are assessed in Chapter 7. The extractives from switchgrass exhibited anti-inflammatory potentials, whereas no other biological activities (antibacterial and anticancer activities, tyrosinase and elastase inhibition) were observed on the switchgrass extracts. Switchgrass metabolic profile of anti-inflammatory

compounds was putatively identified via untargeted metabolomics profiling and many of them were quantified using targeted metabolomics analyses.

CHAPTER I. INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Black walnut (Fagales: Juglandaceae; *Juglans nigra* L.), known as eastern black walnut or America walnut, is one of the most economically valuable hardwood species (McGranahan and Leslie 1991), distributed throughout most of the eastern half of the United States (Harlow and Harrar 1961). This native plant species to North America adapts to grow on a wide range of soil types and its root system is ideally suited for agroforestry systems (Reid et al. 2009) and has become an ideal plant species for planting in Missouri agroforestry practices (Garrett et al. 1998). Black walnut plantations for nut production are relatively small, but do hold great promising for improving the value of this species (Schmidt 1998). Black walnut is an excellent nutritious food source and its consumption has linked to multiple health-promoting benefits (Câmara and Schlegel 2016). Black walnut agroforestry systems in Missouri provide landowners the opportunity to develop a solid long-term investment in future generations, both economically and spiritually while allowing establishing a balanced relationship with the environment (Clarke 1998).

BLACK WALNUT: ECONOMIC CONSIDERATIONS AND CURRENT STATUS, HARDWOOD AND NUT PRODUCTION, CULTIVARS AND CULTIVATION, AND TREE MANAGEMENT

Economic considerations and current status

Black walnut (*J. nigra*) has been used by humanity for centuries and it has been considered as an important component of forests in North American since the Upper Cretaceous (Elias 1980). Black walnuts are highly value trees for hardwood production and edible nuts production in the United States (Randolph et al. 2013). The value of black walnut growing stock on timberland in the eastern United States is estimated to be over one-half trillion dollars (Table 1) (Newton et al. 2009).

Table 1. Estimated value of black walnut in the eastern United States (Newton et al. 2009)

Region of black walnut	Net volume of growing stock on timberland in cubic feet [1]	Conversion from million cubic feet to thousand board feet [2]	Lumber grade price No. 2 A[3]	Estimated value in dollars for net volume of growing stock in 2002
North	1,125,000,000	205,875,000	$850	$174,993,750,000
South	608,000,000	111,264,000	$850	$94,574,400,000
East	1,733,000,000	317,139,000	$850	$269,568,150,000
Total	3,466,000,000	634,278,000	$850	$539,136,300,000

[1] Source: USDA, Forest Service, Forest Resources of the United States, 2002. General Technical Report NC-241 A.

[2] Calculated as follows: thousand board feet = (Net growing stock cubic feet / 1,000) * 183). Assumes that 1000 board feet (mbf) = approximately 183 cubic feet (range: 160 to 220)

[3] Reported for October 2006 by Hardwood Market Report ($ per thousand board feet) MBF one-inch thick (4/4).

Although walnut ranked second in nut production with 504,000 tons produced in the United States in 2010 (USDA 2012), nut production of black walnut is a secondary consideration, which is a relatively small production with 17,000 metric tons from 16 states in 2001 (Hammons 1998). The Persian walnut (*Juglans regia* L.), known as English walnut, or common walnut, is the most important species in the genus of *Juglans* for nut production while black walnut is the second highest produced walnut nut in the United States with Missouri being the highest ranking producer (USDA 2012).

Over 15.4 million acres of black walnut were seen in the United States in 1990 (Table 2) (Schmidt 1998). Black walnut natural stands were occupied 99% of total inventory area whereas black walnut plantations were very small, only 1% of total black walnut area. Black walnut was seen in natural stands in 30 states and total area of black walnut were estimated to be over 15,400 thousand acres in timberland in 1990 (Schmidt 1998). Four states including Missouri, Kentucky, Ohio, and Virginia were responsible for 50% of the total timberland area in the United States (Figure 1). The Central Hardwood Region (i.e., Missouri, Kentucky, Ohio, West Virginia, Tennessee, Indiana, Illinois, Pennsylvania, Virginia, Iowa, Kansas, and Arkansas) occupied for 90% of total area of black walnut timberland (Schmidt 1998).

Table 2. Area of timberland with black walnut (Schmidt 1998)

States	Most recent inventory	Ownership class (thousand acres)						
		Total	National forest	Misc. federal	Other public	Industry	Private individual	Private corporation
Missouri	1989	2,569.7	101.1	76.0	50.9	8.3	2,124.3	209.1
Kentucky	1988	1,971.2	46.1	-	15.5	10.3	1,770.3	129.0
Ohio	1991	1,759.8	26.8	-	46.6	19.0	1,532.3	135.1
West Virginia	1989	1,357.8	4.6	7.2	22.1	54.9	1,103.7	165.3
Tennessee	1989	1,234.6	7.7	36.2	11.5	59.7	1,088.3	31.2
Indiana	1986	1,064.2	12.9	31.4	27.5	1.9	907.6	82.9
Illinois	1985	941.5	20.1	-	15.8	4.0	853.1	48.5
Pennsylvania	1989	708.7	-	5.3	5.8	5.5	636.0	56.1
Virginia	1991	577.5	15.8	13.5	10.8	15.2	461.4	60.8
Iowa	1990	536.0	-	6.1	17.9	-	484.5	27.5
Kansas	1981	472.7	-	15.3	1.0	3.4	427.3	25.7
Arkansas	1988	451.4	52.0	6.3	6.2	9.7	343.8	33.4
18 other states	-	1,769.7	6.6	38.1	82.2	69.3	1,468.3	105.2
Total all states	-	15,414.8	293.7	235.4	313.8	261.2	13,200.9	1,109.8

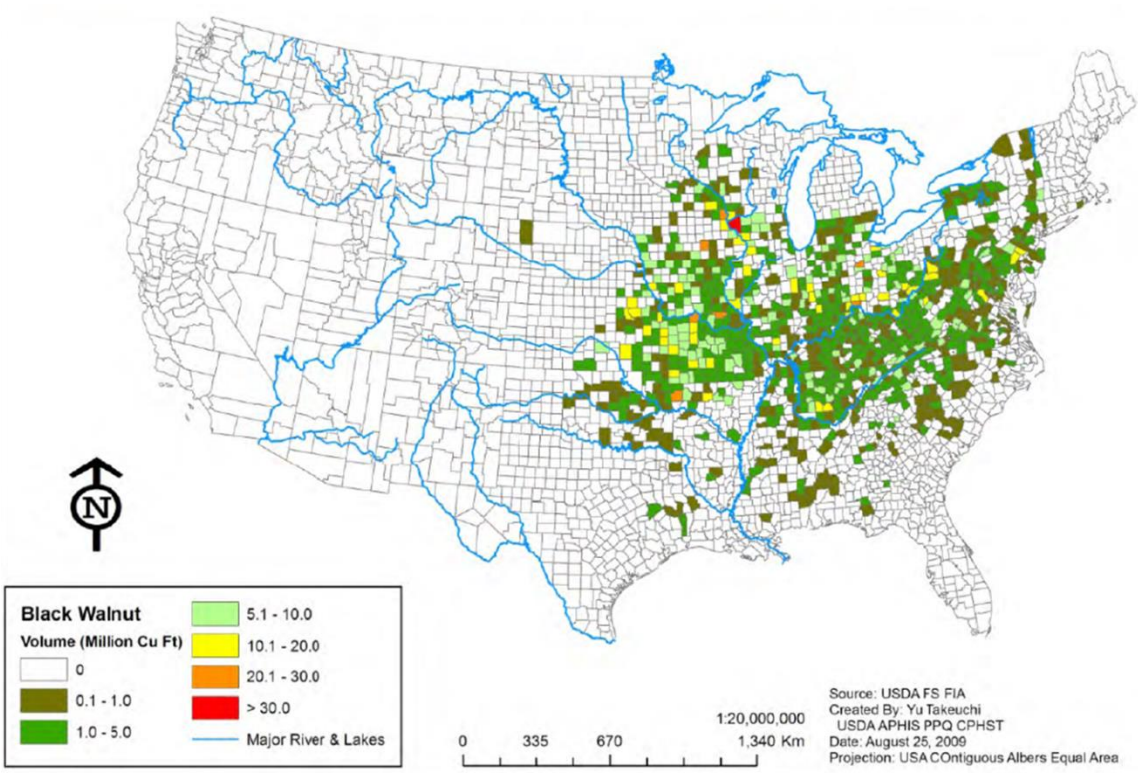

Figure 1. Black walnut (*Juglans nigra*) in the continental United States (source: USDA APHIS)

Hardwood and nut production

The volume of black walnut growing-stock, in the most recent inventories, was more than 1.6 billion cubic including 60% of black walnut sawtimber, 28% poletimber, and 22% sapling-seedling in the United States (Schmidt 1998). The black walnut timberland classified as its productivity at very high to excellent conditions (120+ cubic feet of growth per acre per year) was less than 10% of total area of black walnut timberland while 70% of the black walnut timberland were seen with the productivity level below 84 cubic feet of growth per acre per year and 30% of the black walnut timberland were located at the average productivity class (85 – 119 cubic feet of growth per acre per year) (Schmidt 1998). The quality of walnut lumber in the eastern United

States varies including top grade, middle grade and low grades that are 12%, 50% and 38%, respectively (Dixon and Eves 1991).

Black walnut has been removed annually for wood fiber products. Black walnut (non-growing stock and growing-stock) is currently harvested at an estimation of 12.3 million cubic feet including 82% removals from the site and 18% removals on the ground as logging residue or slash in the North Central Region (Schmidt 1998). Missouri and Indiana were leading states for black walnut annual removals in 1993. An estimated 2.5 million cubic feet of black walnut non-growing stock and 1.8 million cubic feet of black walnut growing stock were harvested in Missouri in 1993 whereas the removals of black walnut growing stock and non-growing stock in Indiana were 1.8 million cubic feet and 0.34 million cubic feet, respectively (Schmidt 1998). Iowa and Wisconsin were also responsible for large percentages of total black walnut harvest, however were smaller in volumes compared to Missouri and Indiana.

Currently, nut production is not a primary consideration of black walnut production in the United States. The black walnut plantations specifically designed for nut production were a relatively small fraction of the total area of black walnut, which were distributed mostly in Ohio, Kentucky and Illinois (Schmidt 1998). The market of nut production is estimated to be about 26 million pounds of nuts in shell by nut companies each year and the demand of consumers is estimated to be approximately 2 million pounds of black walnut kernels in the United States (Hammons 1998).

The total number of black walnut trees is estimated to be over 500 million black walnut growing stock trees and 91 million non-growing stock black walnut trees (rough

or rotten trees) (Schmidt 1998). Currently, black walnut plantations have been consistently increased, but did not have a significant impact on the supply of black walnut nuts or timber. Over 3 million back walnut seedlings have been annually planted in the eastern United States in 1990's and several other black walnut seedlings have been distributed by many private nurseries (Johnson 1994). Black walnut plantations have not met the expectations of landowners and industries (Schmidt and Kingsley 1997) for the hardwood production, but do hold a promising improvement for nut production (Schmidt 1998).

Black walnut nutrients and health-promoting benefits

Consumption of black walnut nuts has been linked to many health benefits that are likely correlated with a wealth source of nutritious food components and health-promoting bioactive compounds present in black walnut (Câmara and Schlegel 2016). With respect to the nutritional components (per 100 g), black walnut provides 59 g of lipids, 24 g of proteins, and 9.9 g of carbohydrate. Lipids are the most abundant nutritive constituents in black walnut, with linoleic acids and monounsaturated oleic acid of essential fatty acids being the highest components. Compared to English walnut, black walnut contains higher levels of monounsaturated fatty acids and phytosterols (USDA 2012). Mineral and vitamin are present in moderate quantities in black walnut (Table 3).

Table 3. Black walnut nutrient composition (value per 100 g of kernels) (USDA 2012, Câmara and Schlegel 2016)

Macronutrients		**Minerals**		**Vitamin**
Energy kcal	618	Calcium	61 mg	Vitamin C 1.7 mg
Protein	24.1 g	Iron	3.1 mg	Vitamin B1 0.05 mg
Carbohydrate	9.9 g	Magnesium	201 mg	Vitamin B2 0.13 mg
Fiber, total dietary	6.8 g	Potassium	523 mg	Vitamin B3 0.47 mg
Sugar, total	1.1 g	Phosphorus	513 mg	Vitamin B6 0.6 mg
Total lipid (fat)	59.0 g	Sodium	2 mg	Vitamin A 2 µg
Fatty acids		Zinc	3.4 mg	Vitamin E 30.1 mg
Total saturated	3.4 g			Vitamin K 2.7 µg
Total monounsaturated	15.0 g			
Total polyunsaturated	35.1 g			
Oleic acid (C18:1)	14.5 g			
Linoleic acid (C18:2)	33.1 g			
α- linoleic acid (C18:3)	2.0 g			

Recent research has demonstrated that black walnut kernels contain several phenolic compounds (e.g., quinic acid, gallic acid, catechin, vanillic acid, syringic acid, rutin, quercetin, ellagic acid, naringin) (Vu et al. 2018). Black walnut also contains high levels of non-cholesterol sterols, such as phytosterols (USDA 2012). Compared to English walnut, black walnuts contain lower levels of polyphenols and higher levels of phytosterols. Many phytochemical compounds found in black walnuts have been correlated with several potential health benefits including decreased risk of cardiovascular disease, reduced levels of cholesterol, stimulated brain functions and prevention of certain cancers (e.g., prostate and breast cancers) (Table 4). However,

systematic identification and characterization of the bioactive activities in black walnuts including kernels, leaves or other parts of black walnut trees, and the correlations between black walnut cultivars and their health-promoting compounds and specific health benefits have not been well established.

Table 4. Potential health-promoting properties of phenolic compounds identified in black walnuts (Vu et al. 2018)

Bioactive compounds	Potential health-promoting properties	Reference
Quinic acid	antiviral, antibacterial	Özçelik et al. (2011)
Gallic acid	antibacterial; antiviral; antifungal	Borges et al. (2013)
1,3,6-Trigalloylglucose	antiviral	Ahn et al. (2002)
p-Hydroxybenzoic acid	antimicrobial	Fernandez et al. (1996)
(+)-Catechin	anti-inflammatory	Andre et al. (2012)
(−)-Epicatechin	anticancer	Saha et al. (2010)
Vanillic acid	antimicrobial	Fernandez et al. (1996)
Syringic acid	antimicrobial	Fernandez et al. (1996)
Rutin	antibacterial	Watt and Pretorius (2001)
	anti-inflammatory	Selloum et al. (2003)
	antiplatelet	Sheu et al. (2004)
	antinociceptive effect	Hernandez-Leon et al. (2016)
Quercetin-3-β-D-glucoside	anti-inflammatory; anti-asthmatic	Rogerio et al. (2007)
	antidiabetic	Zhang et al. (2009)
	anticancer	Chen et al. (2015)
(−)-Epicatechin gallate	anticancer; anti-proliferative; apoptotic antibacterial	Actis-Goretta et al. (2008)
Ellagic acid	anti-atherogenic; osteoblastic	Papoutsi et al. (2008)

	anti-inflammatory; anticancer	Kassim et al. (2010)
	anti-angiogenesis	Wang et al. (2009)
Naringin	antibacterial and antifungal	Tsui et al. (2008)
	anti-inflammatory; neuroprotective	Golechha et al. (2011)
	anti-tumor	Camargo et al. (2012)
	antidiabetic	Mahmoud et al. (2012)
Ferulic acid	anti-apoptosis	Khanduja et al. (2006)
	neuroprotective	Chen et al. (2015)
	anti-tumor	Huang et al. (2014)
	Alzheimer's disease l	Ozaki (1992)
	antibacterial	Borges et al. (2013)
p-Coumaric acid	antiplatelet	Luceri et al. (2007)
Chrysin	vasodilatory effect	Duarte et al. (2001)
	anti-hypertensive	Villar et al. (2002)
	anti-inflammatory; neuroprotective	Gresa-Arribas et al. (2010)
	antidiabetic	Torres-Piedra et al. (2010)
	anti-aging	Anand et al. (2012)

The University of Missouri Center of Agroforestry has been working to improve black walnut nut traits since 1996 (Reid et al. 2009). This breeding program have developed approximately 60 different nut cultivars with top-quality nuts that meet the demand of consumers for a milder flavor and color preferences while providing valuable nutrient sources (e.g. dietary fiber, folate, phytosterols, protein, melatonin). In addition to continuous improving nut quality of black walnut, further efforts might focus on exploring and identifying health benefits of black walnut and its bioactive constituents. The exploration of health-promoting benefits and its associated bioactive compounds in black walnuts would promote the development of novel applications of black walnut and

its by-products such as new drug discoveries to mitigate diseases of humans (e.g., diabetes, cancer), cosmetic products (e.g., skin care products, personal care products), and functional foods (e.g., natural dietary supplements), and thereby potentially increase the sustainability of the black walnut agro-industry.

Cultivars and cultivation

Black walnut has been identified and selected for propagation since late 1800s (Zarger 1969). After the first black walnut cultivar was named as "Thomas" in America in 1881 (Corsa 1896), there were over 700 black walnut cultivars that have been recorded and selected for either nut production or timber during the past century (Williams 1990, Reid 1997). Black walnut plantations are differently for nut production or timber production. Black walnut selected-timber trees often grow over 80 years, which are tall and straight plants in natural stands or man-made plantation while the walnut orchard tree has a short trunk, wide spreading branches and full canopy (Reid et al. 2009). Selection traits for black walnut orchards include several characteristics such as yield, percent kernel, cultivar traits leafing date, flowering dates, growth habit, disease resistance, precocity, productivity, and shelling quality (Reid et al. 2004).

The establishment of black walnut orchards provides high-quality black walnut kernels for family or for a commercially marketable nut crop while planting black walnut trees considered as a hobby (Reid et al. 2009). Several black walnut cultivars are selected and recommended for nut production by University of Missouri Center for Agroforestry (Table 5). Black walnut plantations for nut production require well-drain soils, deep root penetration (more than 3 feet), soils with slightly acidic to slightly base (pH of 6.5 to 7.5),

and high soil moisture during growing season. There are three cultivar groups of ripening periods, namely early, mid-season and late. The orchard design of black walnut is recommended to have plenty of room for the trees that allow maximizing the development of canopy needed for nut production. The tree spacing should be more than 25 x 25 feet. In walnut agroforestry systems, wider plant distances (40 to 60 feet) are recommended to allow growing the intercropping of crops between the rows (Reid et al. 2009).

Table 5. Selective black walnut cultivars for nut production (Reid et al. 2009)

Cultivar	Yield (lbs./tree)	Nut weight (g)	% Kernel	Leafing date[1]	Spur fruiting	Ripening season[2]
Sparrow	15.9	19	32	15	no	early
Sparks 127	4.1	15	33	12	yes	early
Tomboy	-	22	27	7	no	early
Emma K	4.9	19	34	5	yes	mid
Mintle	8.7	16	31	4	no	mid
McGinnis	9.4	17	31	4	yes	mid
Drake	19.8	19	30	17	yes	mid
Kwik Krop	2.6	17	31	15	yes	mid
Sparks 147	3.2	17	36	21	yes	mid
Sauber	-	15	32	13	yes	mid
Football	11.8	22	29	6	yes	late
Hay	-	22	32	23	yes	late
Rupert	22.6	18	26	8	yes	late
Surprise	9.3	20	33	13	yes	late
Thomas	-	22	24	22	no	late

[1]Leafing date is recorded as days after Davidson, the earliest leafing cultivar under trial in central Missouri. Average leafing date for Davidson in central Missouri = April 12.

[2]Average ripening dates in central Missouri: early = Sept. 1-14; mid = Sept. 15-28; late = after Sept. 28.

Tree management

Growing black walnut trees requires monthly management of the trees and the management is differently for non-bearing trees and bearing trees (Figure 2). Commercial walnut orchards require major investments in fertilizers to ensure maximum annual nut production. Nitrogen fertilization applies in March and in May and newly established trees is recommended to be watered one a week, which enhances the developing trees with strong trunks and healthy roots systems (Reid et al. 2009). In late June to early August, a fertilizer application for micronutrients might be needed if the mineral nutrients in walnut foliage are below the normal condition (Reid et al. 2009)

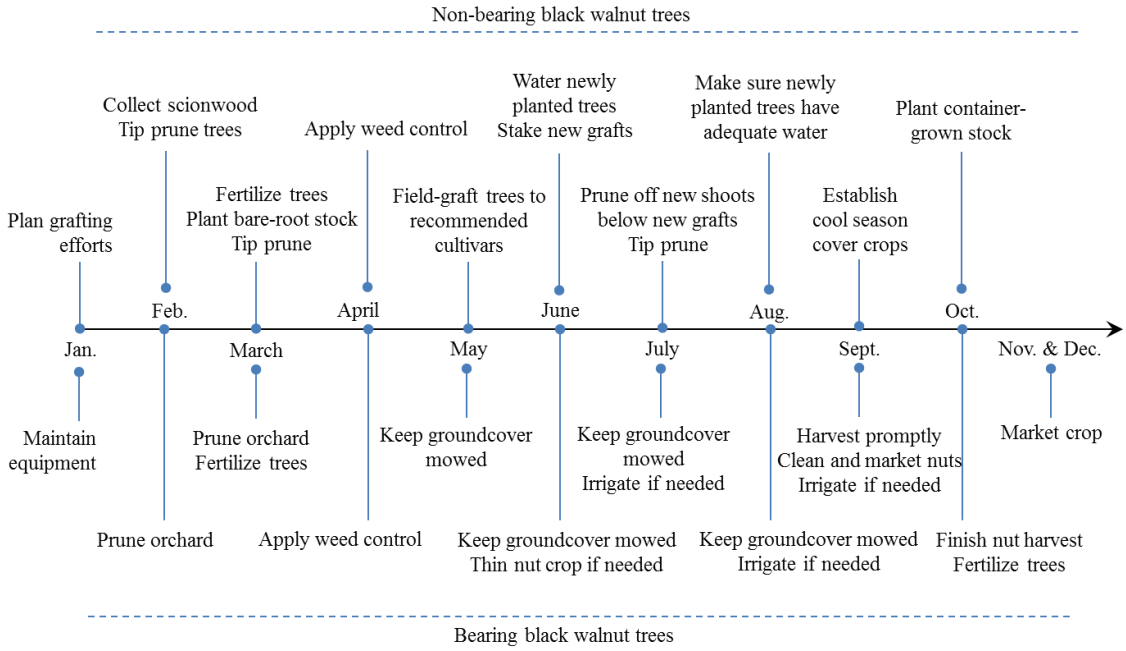

Figure 2. Black walnut grower's calendar for nut production (Reid et al. 2009)

Management of pests and diseases in black walnut orchards is essential. Several key pests (e.g., *Acrobasis* moths, walnut curculio, fall webworm, walnut caterpillar, yellow necked caterpillar, aphids, walnut lace bug, and walnut husk fly) can cause

negative impacts on the production of nuts from black walnut trees. These insect pests appear during growing season and can be controlled through the selected pesticides if occurring at high densities (higher than the economic threshold of each pest). Walnut *anthracnose* is a major disease by an *anthracnose* fungus in black walnut. The disease agent over-winters on decaying leaves and then infects new leaves in the spring. Multiple fungicide applications should be applied to control the walnut *anthracnose*. The timing of the first application is as soon as pistillate flowers are pollinated and a second fungicide should be applied 10 to 14 days after the first spray. Additional fungicide applications could be considered during wet seasons (Reid et al. 2009).

Walnut harvest is a costly part of growing black walnut for nut production due to time consuming and labor requirements (Reid et al. 2009). Once over 50% of the walnut nuts are ripe, a tree shaker can be used to remove the crop form the tree. Larger growers can be used pecan harvesters for picking nuts from the ground while small scale producer might use hand-operated Nut Wizard to collect manually the nuts from the ground. After harvesting, nut hullers can be used to remove nut husks.

BLACK WALNUT IN AGROFORESTRY SYSTEMS IN MISSOURI

Agroforestry is intensive land-use management that combines growing trees and other crops and/or livestock together. Black walnut has been used in alley cropping, one of the five widely recognized agroforestry practices in North America (i.e., alley cropping, silvopastoral management, windbreaks, riparian buffers and forest farming) in Missouri since 1970 (Garrett et al. 1998, Hammons 1998). Black walnut is an ideal tree

for agroforestry practices because black walnut is one of the most commercially valuable timber trees (Newton et al. 2009) and adapts to several growing conditions.

Black walnut in alley cropping practices

Black walnut is an excellent plant species in Missouri alley cropping (Garrett et al. 1998). This species provides high prices for wood and flavorful nut meat. The development of its root system and crow morphology are well adapted to many alley crops. Black walnut roots typical extend deep into the soil with a small amount of feeder roots near the surface of soils, which enables to minimize the competition of water and nutrients of black walnut trees with companion crops. Growing black walnut trees enable to incorporate growth of several row crops, cover crops and specialty crops for both conservation and production benefits (Scott and Sullivan 2007). Growth and shade characteristics of black walnut are compatible with the growth of several companion crops. Roots system of black walnut is deep and adapt to a fairly wide range of soil conditions with neutral pH (Pham et al. 1978, Reid 1997), which reduce the competition for soil nutrients with the companion crops.

The establishment of black walnut trees depends on the tree species of the companion crops planted (Figure 3) and the goals of landowners (short-term and long-term goals, nut production or conservation benefits). Depending on growth rates of black walnut trees, with spacing between rows at 30 x 30 feet, black walnut will begin to crown each other after 20 years (Reid et al. 2009). Alleyway widths should be greater than the width of the primary equipment to be used (Garrett et al. 1998). Alley cropping requires an intensive management of trees that allows maximizing the production of multiple crops. The management of black walnut trees depends on the goals of landowners that grow black walnut for nut production or timber production.

Figure 3. Black walnut in alley cropping practices with corn as a companion crop planted in between rows of black walnut.
Source: centerforagroforestry.org/practices/ac.php

Black walnut has known to produce an allelopathic compound ("juglone") that causes the inhibition of the growth of neighboring plants (Willis 2000). Several parts of black walnut trees (e.g. roots, leaves, and hulls) contain hydrojuglone which is a precursor of juglone (Segura-Aguilar et al. 1992) that can release primarily in a root exudate in the rhizosphere of the black walnut trees (Bertin et al. 2003). Companion crops can be selected based on three phases of juglone accumulation in the black walnut area including short term (<15 years), medium term (15-30 years), and long term (>30 years) (Table 6). The accumulations of allelopathic toxins of black walnut trees in three

timeframes (short term, medium term, and long term) in the growing area are estimated to be none to low, low to high, and very high, respectively (Scott and Sullivan 2007). Many black walnut companion cropping systems are available for growing in the short term and middle term after planting black walnut trees, but only a few for the long term.

Table 6. Suitable companion crops for black walnut trees (Scott and Sullivan 2007)

Species	**Yield**	**Reference**
Short term: <15 years		
Alfalfa (*Medicago sativa*)	Forage	Brooks (1951)
Cereal rye (*Secale cereale*)	Forage	Kallenbach et al. (2006)
Fescue (*Festuca arundinacea*)	Forage	Buergler et al. (2005)
Ryegrass (*Lolium multiflorum*)	Forage	Kallenbach et al. (2006)
Jerusalem artichokes (*Helianthus tuberosum*)	Vegetable	Ross (1996)
Lima bean (*Phaseolus lunatus*)	Vegetable	MacDaniels and Pinnow (1976)
Onion (*Allium cepa*)	Vegetable	MacDaniels and Pinnow (1976)
Parsnips (*Pastinaca sativa*)	Vegetable	MacDaniels and Pinnow (1976)
Sugar beet (*Beta vulgaris*)	Vegetable	Piedrahita (1984)
Wax bean (*Phaseolus vulgaris*)	Vegetable	MacDaniels and Pinnow (1976)
Medium term: 15–30 years		
Fescue (*Festuca arundinacea*)	Forage	Funt and Martin (1993)
Kentucky bluegrass (*Poa pratensis*)	Forage	Funt and Martin (1993)
Red clover (*Trifolium pratense*)	Forage, N-fixer	Boes (1986)

Timothy (*Phleum pratense*)	Forage	Boes (1986)
White clover (*Trifolium repens*)	Forage, N-fixer	Boes (1986)
Black raspberry (*Rubus occidentalis*)	Fruit	Fuchs (1995)
Currant (*Ribes* spp.)	Fruit	Brooks (1951)
Elderberry (*Sambucus canadensis*)	Fruit	Brooks (1951)
Mulberry (*Morus* spp.)	Fruit	Brooks (1951)
Pawpaw (*Asimina triloba*)	Fruit	Brooks (1951)
Persimmon (*Diospyros virginiana*)	Fruit	Gordon (1981)
Autumn olive (*Elaeagnus umbellata*)	N-fixer	Ponder et al. (1980)
Black locust (*Robinia pseudoacacia*)	N-fixer, wood	Ponder et al. (1980)
European alder (*Alnus glutinosa*)	N-fixer, wood	Bohanek and Groninger (2005)
Russian olive (*Elaeagnus angustifolia*)	N-fixer	Burde (1989)
Eastern white pine (*Pinus strobus*)	Wood	Burde (1989)
Red oak (*Quercus rubra*)	Wood	Burde (1989)
Sugar maple (*Acer saccharum*)	Wood	Burde (1989)
White ash (*Fraxinus americana*)	Wood	Burde (1989)
Long term: >30 years		
Bamboo (*Phyllostachys* spp.)	Wood	Scott and Sullivan (2007)
Ginseng (*Panax quinquefolium*)	Medicinal roots	Carroll and Apsley (2004)
Mushroom logs	Mushrooms	Scott and Sullivan (2007)

Black walnut in silvopastoral systems

Missouri ranks second in cow-calf production among all states in the Unites States in 2018 (USDA 2018). Landowners can add a livestock dimension into black walnut agroforestry system (Figure 4) by using alleyways for growing forage crops to graze animals and then an alley-cropping system can be converted to silvopastoral program, another component of the five widely recognized agroforestry practices in North America. Missouri has a lot of well-suited land area to forage production (Garrett et al. 1998). There are about 5.2 million ha of nonfederal pasture and rangeland, in which more than 2.8 million of these hectares are readily in use in alley cropping or silvopastoral management. Additionally, there are approximately 2.4 million ha of cropland in Missouri that have an erodibility index (EI) higher than 8. These areas are well-suited for growing grass and would recommend for silvopastoral systems (Garrett et al. 1998).

Figure 4. Silvopasture practice with black walnut trees in Missouri.
Source: centerforagroforestry.org/practices/sp.php

The management requirements of plant crops and livestock are intensive in the silvopastoral system. Several forage crops (e.g, alfalfa, cereal rye, fescue, ryegrass, Kentucky bluegrass) that are suitable in black walnut agroforestry system can be established in alleyways (Table 7). Cattle (*Bos taurus* L.) and chicken (*Gallus gallus*) can be grazed in black walnut agroforestry system (Ponder et al. 2005, Kallenbach et al.

2006). Black walnut plantations produce adequate amount of forages to maintain cattle utilizing the plating grasses. The forage production and quality, higher calf gains without negative impact on the growth of trees in rotationally grazing are higher compared to that of continuous grazing in black walnut agroforestry practices (Lehmkuhler et al. 1998).

Shade and drought tolerance are key characteristics for selection of tree species for planting in black walnut agroforestry systems. Most forage species are required full sunlight and open fields. Shade is not a major consideration during early years of establishing black walnuts, but light availability of forage crops will be reduced due to the growth of black walnut trees. This results in the establishment of new microenvironments affecting the development of forage crops. Black walnut will begin to crown each other after 20 years if planting with spacing between rows at 30 x 30 feet (Reid et al. 2009). Tall fescue (*Festuca arundinacea*), orchard grass (*Dactylis glomerata*), red clover (*Trifolium pratense*) are known to be shade tolerant species. Tall fescue which plants at an estimated 2.4 million hectares in Missouri can growth normally when grown over 40% shade (Allard et al. 1991) whereas the yield of orchard grass, the second most widely planted grass species in Missouri pastures, is reduced when grown lower 30% incident sunlight (Blake et al. 1966). Based on forage and tree interactions, orchard grass and red clover are two grasses that are recommended in Missouri agroforestry practices (Garrett et al. 1998).

The crop value is also one of important characteristics for the selection of tree species. Since alfalfa (*Medicago sativa*) and timothy (*Phleum pratense* L.) are high value cash crops, alfalfa has been selected for black walnut agroforestry system as an alley crop. Alfalfa performs well under partial shade conditions in Missouri and produces an

extensive root system that might cause water competitions in soils with other crops. The nut yields of black walnut could reduce due to water competition when planting with alfalfa (Garrett et al. 1998) .

Social benefits of black walnut agroforestry systems in Missouri

"Putting land into eastern black walnuts is a good way to invest in future generations, as well as a natural and good thing to do with newly acquired property" (Dilsaver, 1998). Black walnut plantations would provide several social benefits including long-term economic stability for communities and regions, the inherent desire for people to have a balanced relationship with the environment, and infrastructure to support the industry (Clarke 1998). Developing black walnut agroforestry system not only provides short-term economic benefits due to the high value of black walnut trees but also a valuable long-term investment in the future. Black walnut can be cultivated over 100 years (McKenna and Coggeshall 2018) which the benefits can share with next generations.

Growing black walnut is not only economic benefits but also promotes culture development. In southwestern Missouri, black walnut is a native tree species and unique for the location. Black walnuts from wild and plantations provide aesthetic beauty in Missouri and a unique cultural meaning for humans share that landscape (Clarke 1998). Multiple generations of families have been working together for harvesting and processing wild black walnut nuts each fall that enhances the family strengths as well as its relationship with the environment.

Black walnut plantations also promote the infrastructure to support the industry (Clarke 1998). Recently, selected black walnut cultivars with higher yield for nut and timber production have being planted in several regions in the United States (Schmidt 1998). As an increase of interest in black walnut, the industry for nut and wood production will grow. The nut and timber production of black walnut derived from two main sources such as wild crops and commercial crops. Both of them provide the multiple inherent values provided by a "free" natural renewable resource (Clarke 1998).

In general, several benefits would derive from black walnuts. The black walnut agroforestry system provides landowners the opportunity to develop a solid long-term investment in future generations, both economically and spiritually while allowing to establish a balanced relationship with the environment (Clarke 1998).

Economics of black walnut agroforestry systems in Missouri

The black walnut agroforestry systems provide financial advantages by diversifying existing or planned agricultural products. The systems allow landowners the opportunity to develop short-term and long-term investments that optimize the production efficiency while reducing the potential risks through diversification. The profitability of black walnut agroforestry varied for every practitioner depending on their individual financial circumstances and the overall farming operation (Harper and Kurtz 1998). In black walnut agroforestry systems, internal rates of return have been estimated to be from 4% to 11% depending upon the combination of crops studied (Kurtz et al. 1996). The returns are associated with management complexity and site quality and early returns

from intercrops and nut production are key factors of high financial yields (Kurtz et al. 1984)

Nut production of black walnut is not high when growing in wild and unmanaged conditions while nut production of black walnut plantations is difficult to estimate due to the limited data for calculation. Nut yield of the open grown and unmanaged walnut trees in age range from 70 to 100 years was 44 kg per tree per year. Total nut yield per hectare is estimated 3,256 kg if 74 trees are planted per hectare. At a price of $10 per kg, the value of nuts would be $32,560 per ha. Total nut yield of walnut trees in age range from 15 to 20 years is estimated from 900 to 1100 kg per hectare while walnut trees from ages 7 through 15 produce less than 100 nuts per tree per year. Sparrow, Emma K and Kwik Krop are three of the best yielding walnut cultivars in Missouri (Garrett et al. 1998).

Although the sustained profitability of the systems mostly depends on nut production, income from the secondary sources between-the-trees crops (livestock, hay or row crop) provides cash flow especially during the first 10 to 15 years (Harper and Kurtz 1998). Black walnut agroforestry is a long-term commitment. In Missouri Basic System, a net profit of black walnut is present after 10 to 15 years of establishing black walnut trees and the investment will be highly profitable over a long period (over 60 years) (Harper and Kurtz 1998) with the internal rates of return over 11% (Kurtz et al. 1996). The economic decision of black walnut plantations of landowners will challenge with the short-term profitable benefits but could be a decision to benefit the next generations. The long-term goal is under potential risks of natural disasters (e.g., disease, fire and lightning) that might result in the loss of the systems.

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CHAPTER II: IDENTIFYING ANTIBACTERIAL COMPOUNDS IN BLACK WALNUTS (*JUGLANS NIGRA*) USING A METABOLOMICS APPROACH

ABSTRACT

Black walnut (*Juglans nigra* L.) is one of the most economically valuable hardwood species and a high value tree for edible nut production in the United States. Although consumption of black walnut has been linked to multiple health-promoting effects (e.g., antioxidant, antimicrobial, anti-inflammatory), the bioactive compounds have not been systematically characterized. In addition, the associations between different black walnut cultivars and their health-promoting compounds have not been well established. In this study, the kernels of 22 black walnut cultivars selected for nut production by the University of Missouri Center of Agroforestry (Columbia, MO, USA) were evaluated for their antibacterial activities using agar-well diffusion assay. Among the selected cultivars, four black walnut cultivars (i.e., Mystry, Surprise, D.34, and A.36) exhibited antibacterial activity against a Gram-positive bacterium (*Staphylococcus aureus*), whereas other cultivars showed no effect on the inhibition of this bacterium. The antibacterial compounds showing the strongest activity were isolated with bioassay-guided purification and identified using a metabolomics approach. Six antibacterial bioactive compounds responsible for antimicrobial activity were successfully identified. Glansreginin A, azelaic acid, quercetin, and eriodictyol-7-O-glucoside are novel antibacterial compounds identified in the kernels of black walnuts. The metabolomics approach provides a simple and cost-effective tool for bioactive compound identification.

INTRODUCTION

Black walnut (*Juglans nigra* L.), known as eastern black walnut or America walnut, is economically valuable tree for hardwood and nut production (Randolph et al., 2013; McGranahan & Leslie 1991), distributed throughout most of the eastern half of the United States (Harlow & Harrar 1968). The value of black walnut growing stock on timberland is estimated to be over one-half trillion dollars in the eastern United States (Newton et al., 2018). This species is the second highest produced walnut nut in the United States and Missouri is the leading producer of black walnut (Câmara & Schlegel 2016). Black walnut is often preferred in the food industry due to its unique flavor and aroma.

Black walnut has been identified and selected for propagation since late 1800s (Zarger 1969). Currently, over 700 black walnut cultivars have been recorded and selected for either timber or nut production during the past century (Williams 1990; Reid 1997). Selection traits for black walnut orchards include several characteristics such as yield, percent kernel, cultivar traits leafing date, flowering dates, growth habit, disease resistance, precocity, productivity, and shelling quality (Reid et al., 2004). Several black walnut cultivars have been selected for nut production by the University of Missouri (MU) Center for Agroforestry (Columbia, MO, USA) (Reid et al., 2009).

Black walnut is an excellent source for phytochemical compounds including phenolic acids, flavonoids, and catechins (Vu et al., 2018) and monounsaturated fatty acids (Câmara & Schlegel 2016). Our previous studies have revealed that the kernel of black walnuts contain several bioactive compounds such as quinic acid, gallic acid, p-hydroxybenzoic acid, vanillic acid, syringic acid, quercetin-3-d-glucoside, epicatechin

gallate, rutin, naringin, and ferulic acid (Vu et al., 2018). These compounds have been successfully identified from eleven black walnut cultivars using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Vu et al., 2018). In fact, the kernel extraction of black walnuts has been linked to antibacterial properties (Özçelik et al., 2011: Borges et al., 2013; Fernandez et al., 1996; Nakayama et al., 2015; Qin et al., 2013; Watt & Pretorius 2001; Tsui et al., 2008). The stem bark extraction of English walnut (*Juglans regia* L.) has been reported to exhibit the antibacterial activity against methicillin-resistant *Staphylococcus aureus* (Moori et al., 2016). However, the antibacterial activities among the extracts from different black walnut cultivars have never been compared, and predominant bioactive compounds in black walnut have not been isolated or characterized.

Reverse-phase flash chromatography has been widely utilized to fractionate and separate the biomolecules (Blunt et al., 1987). In this technique, the powder resins collated with the sample extraction are loaded on chromatography column packed resin coated with hydrophobic functional groups, such as C18 or C18 Bondesil, and connected to a fraction collector. The phytochemical compounds from the plant extraction are separated based on differences in their physicochemical properties (e.g., hydrophobicity and affinity). The phytochemical compounds migrate through the column at different rates and then are automatically collected at different times. The identification of bioactive compounds can be facilitated via phytochemical screening assays. This technique provides several advantages (e.g., reproducibility) compared to the traditional normal-phase chromatography technique.

For the current paper, we first evaluated and compared the antibacterial properties among the extracts from twenty-two black walnut cultivars selected for nut production. We then isolated and identified bioactive compounds in the kernels of the best cultivar using bioassay-guided purification followed by a metabolomics analysis.

MATERIALS AND METHODS

Black walnut cultivars

The nuts of 22 black walnut cultivars (i.e., Bowser, Daniel, Davidson, Emma, Hay, Hare, Jackson, Kwik Krop, Mystry, Schessler, Sparks, Sparrow, South Fork, Surprise, Thomas, A4, A.36, B.15, B.31, C8, D16, and D.34) were collected at the University of Missouri, Horticulture and Agroforestry Research Center, New Franklin, MO, USA. The black walnuts were hulled mechanically and hang up to dry for 15 days in a dry and darkness place at 24 °C. The hulled nuts were then stored at -20 °C until analysis.

Extraction of bioactive compounds from the kernels of black walnuts

The hulled nuts were manually cracked, and the kernels were shelled and homogenized using a coffee grinder (product # CBG100S, Black + Decker, Beachwood, OH, USA) prior to extraction. The phytochemicals in the kernels of each cultivar (3 g, 20–30 mesh) were extracted with sonication in 15 mL of methanol (HPLC grade, Fisher Scientific, Pittsburg, PA, USA). The extract was sonicated for 60 min followed by centrifugation for 10 min at 4000 rpm and the supernatant was collected. Subsequently, the supernatant was filtered through a 0.2 μm Whatman Anotop syringe membrane filter

(Sigma-Adrich, St. Louis, MO, USA). The aqueous extract was evaporated until dryness under a flow of nitrogen and the final extract was resuspended with dimethyl sulfoxide (DMSO, Sigma-Adrich, St. Louis, MO, USA) at concentration 0.12 g/mL for screening antibacterial activities using an agar-well diffusion assay.

Antibacterial assay

The strain of bacteria used in this study was a gram-positive bacterium (*S. aureus* strain RN 6390) (Novick et al., 1990). *S. aureus* and Methicillin-resistant *S. aureus* (MRSA) are Gram-positive bacteria that are resistant to several antibiotics in the market. Identifying the molecules or scaffolds that could inhibit the *S. aureus* and MRSA could help elucidating the possible new antibacterial modes of action against this pathogen. In addition, many skin infections (acne) or gum disease often results from the infection of the Gram-positive bacteria. From the previous works, many antimicrobial compounds that inhibited the *S. aureus* could also use to treat the skin infections and gum disease (e.g., totarol). Therefore, selecting this specific strain in the early stage screening study will help explore the future commercial application.

The antibacterial activities of black walnut extracts were determined using agar-well diffusion assay as described by Holder and Boyce (1994). The *S. aureus* RN6390 was streaked on Luria-Bertani (LB) agar plates incubated at 37 °C for 12 h. For preparation of 1 L of LB agar, 15 g of agar (Fisher Scientific), 10 g of tryptone (Fisher Scientific), 5 g of yeast extract (Fisher Scientific) and 10 g of NaCl (Fisher Scientific) were suspended in 1 L of distilled water. The mixture was homogenized and autoclaved at 121 °C for 1 h. It was then cooled to approximately 50 °C while mixing with a magnetic stirrer. The liquid LB agar was poured into Petri plates (150 mm diameter) and

allowed to solidify at 25 °C. For preparation of an overnight culture of RN6390, the bacteria were cultured in LB broth at 37 °C for 16 h and then diluted into 5 mL LB broth (to an OD_{600} of 0.02) and incubated in a shaker at 37 °C. Once the culture reached an OD_{600} of 0.1, it was swab inoculated onto LB agar plates. For testing antibacterial activities of the extracts, several wells (4.5 mm in diameter) were cut into the surface of the agar using a cork borer. The extracts (10 µL) were pipetted into the wells and the plates then incubated under aerobic condition at 37 °C for 16 h. Sterilized solvent (DMSO) was used as the negative control. The diameters of inhibition zones were measured by a ruler, with an accuracy of 0.5 mm. Each inhibition zone was measured three times and each extract was replicated at least three times in three different plates.

Identification of bioactive compounds using a metabolic approach

The black walnut cultivar that exhibited the strongest antibacterial activities against the gram-positive bacterium was selected for bioassay-guided purification. The kernel extract of this cultivar was sequentially fractionated using column chromatography (CC) and then the bioactive fraction was further fractionated using high-performance liquid chromatography (HPLC). The agar-well diffusion assay was performed to identify the antibacterial activities of the bioactive fractions. The HPLC sub-fraction that had the strongest antibacterial activities against the Gram-positive bacterium was analyzed by high-resolution mass spectrometry and the mass spectra was analyzed to identify the bioactive compounds responsible for the antibacterial activities.

Column chromatography

Kernels (25 g) were collected and the phytochemicals were extracted twice with methanol (100 mL: 100 mL). The extract was homogenized thoroughly using a blender (Hamilton Beach, Inc., Glen Allen, VA, USA) and was sonicated for 60 min at temperature ≤ 30 °C. The extract was filtered through filter paper (125 mm in diameter, Whatman, GE Healthcare, Chicago, IL, USA) under SPE Vacuum Manifold (Visiprep™ SPE Vacuum Manifold, Sigma-Aldrich, Saint Louis, MO, USA) and then was concentrated by a rotary evaporator (BUCHI Rotary Evaporator R110, Buchi, Flawil, Switzerland) under a vacuum (Buchi), yielding a greenish yellow gum (4 g). The resultant gum (4 g) was dissolved in methanol (5 mL), impregnated with 4 g of sorbents Bondesil C18 (40 µm particle size; Agilent Technologies, Santa Clara, CA, USA) and placed in the hood for 12 h until all the methanol was evaporated, which yielded powder resins coated with the extract. The C18 resins with the extract were stored at 4 °C in darkness and used within a week.

The compounds were separated and fractionated by a Biotage FlashMaster II flash chromatography connected with an ISCO Foxy 200 fraction collector. The powder resins collated with the extract (8 g) were loaded on to the top of chromatography column (3.7 cm in diameter and 13.5 cm long) packed with 34 g of C18 Bondesil resin. The running time was 300 min at a flow rate of 0.5 mL/min. The mobile phase consisted of deionized water (A) and methanol (B) and a linear gradient was optimized as follows 25% B (0–40 min), 25–50% B (40–90 min), 50–75% B (90–120 min), 75% B (120–180 min), 75–100% B (180–240 min) and 100% B (240–300 min), respectively. The elution yielded forty-six fractions collected automatically by a fraction collector. All fractions were

concentrated by nitrogen evaporator and re-dissolved in DMSO at 10× concentration for antibacterial activities testing using an agar-well diffusion assay.

HPLC analysis

The bioactive fraction from column chromatography that exhibited the strongest antibacterial activities against the Gram-positive bacterium was further fractionated by high-performance liquid chromatography (HPLC). The bioactive fraction (50 µL) was injected into a HPLC system consisting of a Shimadzu SCL-10Avp HPLC controller (Shimadzu Co., Columbia, MD), a LC-10ADvp solvent delivery system, SIL-10ADvp auto-injector, a SPD-10Avp photodiode array detector, and a FRC-1500 HPLC micro fractionation collector (Shimadzu). The elution was performed with a Columbus C8 reverse-phase column (250 mm × 4.6 mm, 5 µm particle size; Phenomenex, Torrance, CA). The running time was 30 min at a flow rate of 0.5 mL/min and the signals were monitored at both 254 nm and 220 nm. The mobile phase consisted of deionized water (A) and acetonitrile (B). The elution condition was optimized as follows 10–45% B (0–16 min), 45% B (16–16.2 min), 45–80% B (16.2–17 min), 80–98% B (17–18 min), 98% B (18–19 min), 98–20% B (19–20 min), and 20% B (20–30 min), respectively. The HPLC sub-fractions were collected automatically into fraction collection vials. The fractions having the same retention time window were pooled after 50-time injections of the bioactive fraction from column chromatography. The solvent of each separated fraction was evaporated under the hood and the samples were dissolved in DMSO at 100 × concentration for testing antibacterial activities using an agar-well diffusion assay.

UHPLC-QTOF-MS/MS analysis

The HPLC sub-fraction that exhibited the strongest antibacterial activities was concentrated 100X in 80% methanol containing an internal standard (umbelliferone) and then the sample was analyzed by UHPLC coupled to a maXis impact quadrupole-time-of-flight mass spectrometer (Bruker Daltonics, Gmbh, Bremen, Germany). The separation was achieved on a Waters Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particles size) using a linear gradient of 95%: 5% to 30%: 70% eluents A: B (A: 0.1% formic acid and B: acetonitrile) in 30 min. From 30–40 min, a linear gradient was as follows 70–95% B (30–33 min), 95% B (33–35 min), 95–5% B (35–36 min), and 5% B (37–40 min), respectively. The flow rate was 0.56 mL/min and the column temperature was kept at 60 °C. Mass spectrometry was performed in both negative and positive electrospray ionization modes with the nebulization gas pressure at 43.5 psi, dry gas of 12 L/min, dry temperature of 250 °C and a capillary voltage of 4000V. MS/MS mass spectral data was collected automatically using following parameters including MS full scan from 100 to 1500 *m*/*z*, 3 precursors, threshold with 10 counts, active exclusion with three spectra released after 0.15 min, collision energy depending on mass such as 35 eV at 500 Da, 50 eV at 1000 Da and 70 eV at 2000 Da. The mass spectra were auto-calibrated using sodium formate after data acquisition.

The metabolite annotation in significant peaks in UV chromatogram of the MS/MS mass spectra was identified based on MS/MS fragmentation. The MS/MS fragments were referenced to the Metabolomics library developed by Lei et al. (2015), as well as MetFrag (https://msbi.ipb-halle.de/MetFragBeta/), MassBank of North America (http://mona.fiehnlab.ucdavis.edu/spectra/), and METLIN (http://metlin.scripps.edu).

Statistical analysis

In antibacterial experiments, zones of inhibition of black walnut extracts were analyzed as a randomized complete block design using PROC MIXED in SAS 9.4 (SAS Institute, Cary, NC, USA). If no inhibition was observed in the samples, a value of 4.5 mm, which was the diameter of the wells used in the agar-well diffusion assay, was assigned to these treatments prior to the analysis. The black walnut extract was the fixed effect and replication was the random variable. Differences between extracts were determined using Fisher's LSD.

RESULTS

Antibacterial activity from twenty-two black walnut cultivars

The zones of inhibition caused by kernel extracts from twenty-two black walnut cultivars against the gram-positive bacterium (*S. aureus*) showed significant differences ($p < 0.0001$, $F_{22,75} = 200.19$) (Figure 5). Four black walnut cultivars (i.e., Mystry, Surprise, D.34, and A.36) exhibited the antibacterial activity against the gram-positive bacterium whereas other cultivars (i.e., A4.1010, B.15, B.31, Bowser, C8.04.1003, D16.06.1036, Daniel, Davidson, Emma, Hay, Hare, Jackson, Kwik Krop, Schessler, Sparks, Sparrow, South Fork, and Thomas) had no inhibitory effects on this bacterium. Mystry had the greatest zone of inhibition (11.83 ± 0.75 mm) compared to other cultivars and the zones of inhibition of Surprise, D.34, and A.36 which were 7.42 ± 0.92 mm, 6.75 ± 0.42 mm, and 6.33 ± 0.52 mm, respectively, were significantly different.

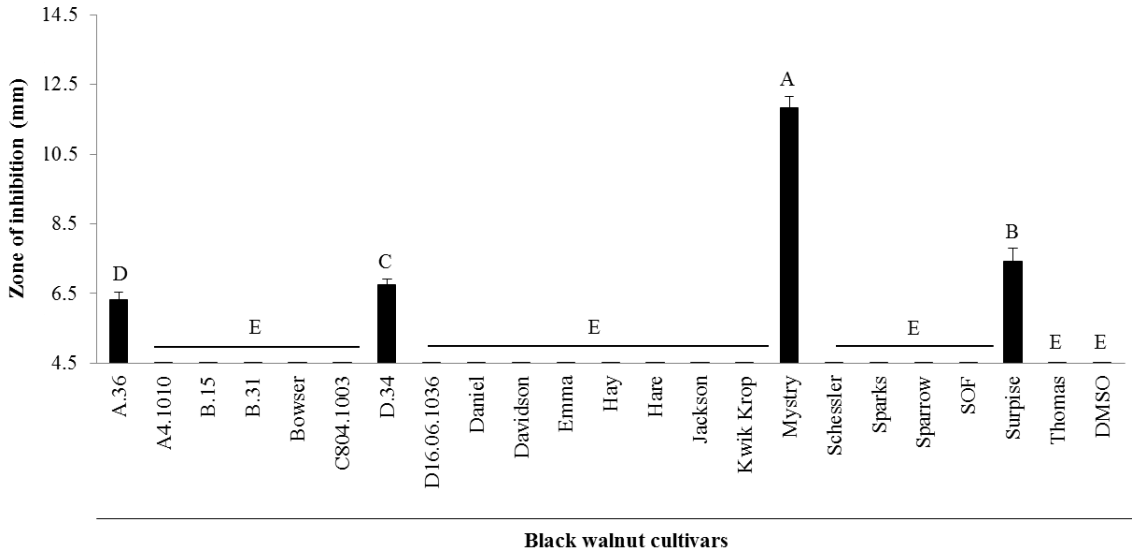


Figure 5. Zones of inhibition of crude kernel extracts of twenty-two black walnut cultivars grown in Missouri for *Staphylococcus aureus*. If no inhibition was observed in samples, a value of 4.5 mm, which was the diameter of the well-used for the agar-well diffusion experiments, was assigned. Means within bars followed by different letters are significantly different ($\alpha = 0.05$, ANOVA). Mean ± SEM.

Identification of antibacterial compounds derived from the kernel extract of Mystry

Column chromatography

The fractionation of kernel extract from Mystry by column chromatography yielded 46 fractions. The antibacterial activities of these fractions against the Gram-positive bacterium (*S. aureus*) were significant different ($p < 0.001$, $F_{47,94} = 37.28$) (Figure 6 and Supplementary Figure 1). The fraction numbers from 4 to 18 had antibacterial activities against *S. aureus* while other fractions had no inhibitory effects on *S. aureus*. The zones of inhibition of fraction 5 and 6 (11.17 ± 0.83 mm and 12.17 ± 0.44 mm, respectively) were significantly higher compared to that of other fractions. No

significant difference was seen between the zones of inhibition of fraction 6 and the crude extract of Mystry whereas the zone of inhibition of fraction 5 was lower than that of the extract of Mystry (Figure 6).

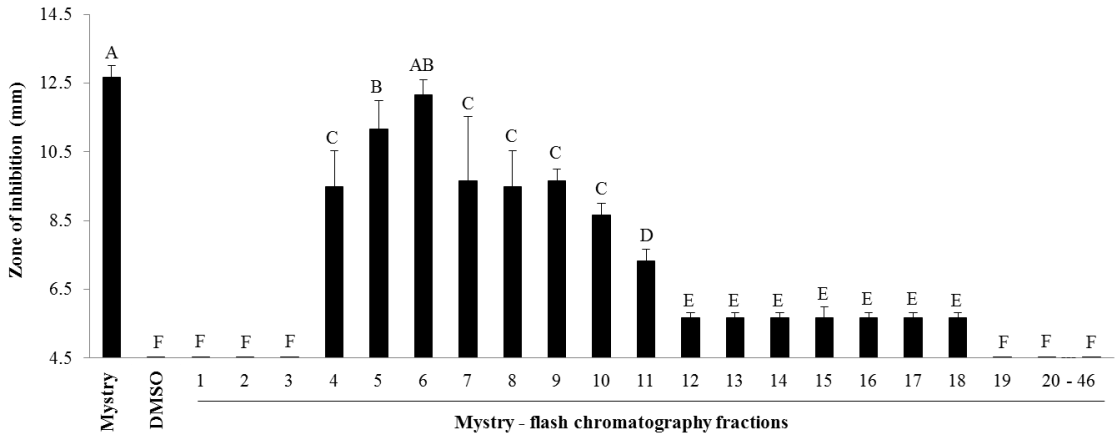


Figure 6. Zones of inhibition of 46 Mystry fractions from column chromatography. 20–46: fraction 20 through fraction 46. If no inhibition was observed in samples, a value of 4.5 mm, which was the diameter of the well-used for the agar-well diffusion experiments, was assigned. Means within bars followed by different letters are significantly different ($\alpha = 0.05$, ANOVA). Mean ± SEM.

HPLC analysis

The further separation of fraction 6 by HPLC resulted in 27 sub-fractions. Only sub-fraction 14 exhibited the antibacterial activity against *S. aureus* while other sub-fractions had no effect on the inhibition of the Gram-positive bacterium (Figure 7). The zone of inhibition of sub-fraction 14 was significantly higher compared to other sub-fractions, but was lower compared to the crude extract of kernel from Mystry ($p < 0.0001$, $F_{28,56} = 466.87$).

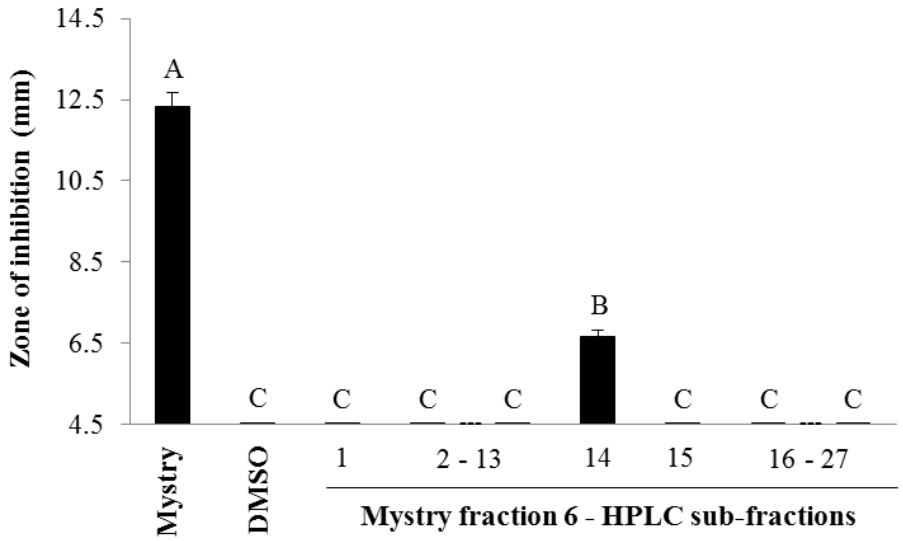


Figure 7. Zones of inhibition of 27 Mystry HPLC sub-fractions of fraction 6 from column chromatography. 2–13, 16–27: fraction 2 through fraction 13 and fraction 16 through fraction 27, respectively. If no inhibition was observed in samples, a value of 4.5 mm, which was the diameter of the well-used for the agar-well diffusion experiments, was assigned. Means within bars followed by different letters are significantly different ($\alpha = 0.05$, ANOVA). Mean ± SEM.

UHPLC-QTOF-MS/MS analysis to identifying the bioactive compounds

Fraction 14 from HPLC fractionation that showed inhibition activity was subject to UHPLC-QTOF-MS/MS analysis in both negative and positive electrospray ionization modes (Supplementary Figure 2). Six major peaks resolved bioactive compounds were identified tentatively by searching their MS/MS spectra with the metabolomics databases (Figures 8-9, Table 7).

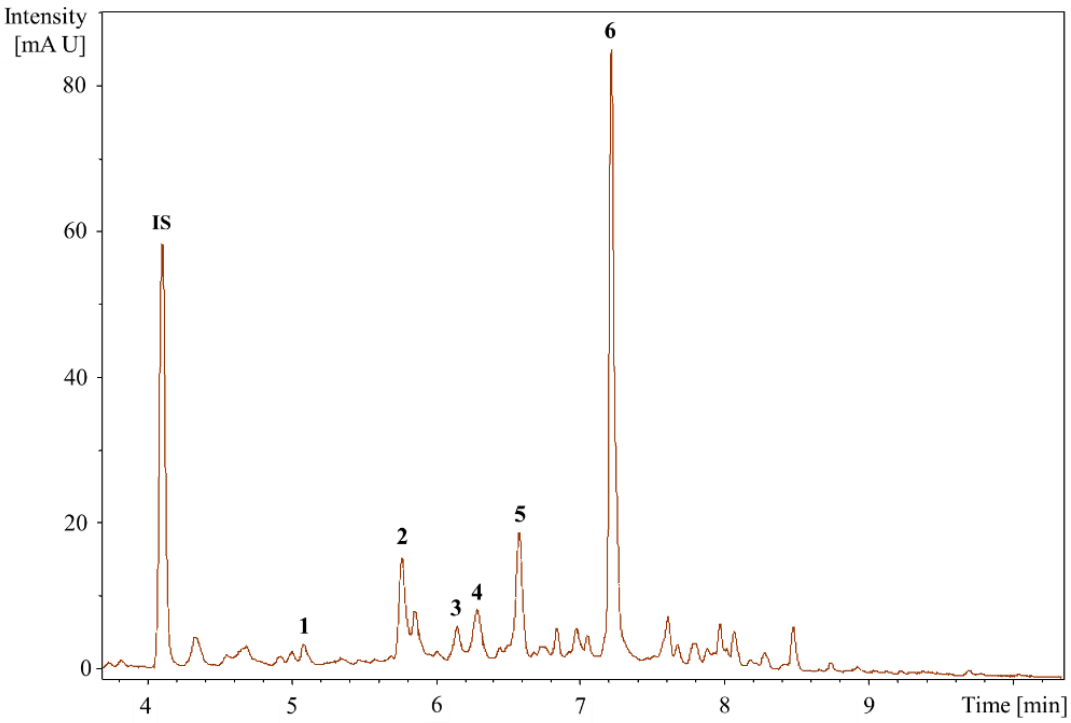


Figure 8. UV chromatogram of Mystry HPLC sub-fraction at 280 nm. 1-6: annotated metabolites, 1: quercetin-3-*O*-glucoside, 2: a catechin derivative, 3: eriodictyol-7-*O*-glucoside, 4: quercitrin, 5: azelaic acid, 6: glansreginin A, and IS: internal standard.

Table 7. Putative bioactive compounds responsible for antibacterial activity in Mystry.

Peak No.	T_R (min)	[M-H]- (*m*/*z*)	Formula	Exact Mass	Δm (ppm)	MS/MS Fragments, *m*/*z*, Intensity (%)	Putative Identification *
1	5.21	463.0888	$C_{21}H_{20}O_{12}$	464.0954	2.6	301.0308 (25.3), 300.0280 (100), 271.0260 (76.8), 255.0316 (34.3), 146.9262 (23.2), 119.9065 (21.1)	Quercetin-3-*O*-glucoside
2	5.77	465.1414	$C_{22}H_{18}O_{11}$	458.0849	3.8	301.0147 (2.7), 300.0280 (3.0), 241.0358 (4.2), 169.0146 (64.2), 125.0253 (100), 107.0132 (17.5)	Agnuside Catechin-4-ol 3'-methyl ether 3-*O*-alpha-L-rhamnopyranoside Catechin 5-*O*-beta-D-glucopyranoside-4'-methyl ether Catechin 7-*O*-beta-D-glucopyranoside-3'-methyl ether
3	6.15	449.1102	$C_{21}H_{22}O_{11}$	450.1162	4.0	299.9964 (12.4), 298.9994 (15.3), 255.0408 (12.4), 200.8817 (11.5), 174.9541 (17.1), 151.0039 (100), 135.0444 (77.5)	Eriodictyol-7-*O*-glucoside
4	6.26	477.0941	$C_{21}H_{20}O_{11}$	448.1006	1.3	301.0373 (43.2), 300.0282 (100), 271.0284 (90.3), 255.0264 (24.8), 178.9994 (29.1), 151.0032 (45.4)	Quercitrin
5	6.58	187.0977	$C_9H_{14}O_4$	186.0892	3.2	144.9013 (46.2), 125.0979 (100), 123.0804 (62.4), 97.0656 (47.8)	Azelaic acid
6	7.18	592.2043	$C_{28}H_{35}NO_{13}$	593.2108	2.1	283.1199 (8.5), 241.1080 (8.2), 223.0979 (19.9), 197.1185 (34.5), 181.0875 (30.8), 144.0456 (100), 137.0972 (12.1)	Glansreginin A

* Tentative identification of compounds on the basis of MS/MS mass spectra and UV spectra reported in the referred databases.

Compound **1** has retention time (rt) and *m*/*z* at 5.21 min and 463.0388, respectively. The MS/MS spectrum displayed fragment ions at *m*/*z* 301 $[M - H - 162]^-$, 300 $[M - H - 162 - H^{\cdot}]^-$, 271 $[M - H - 162 - 30]^-$, 255 $[M - H - 162 - 46]^-$, 146 $[M - H - 162 - 155]^-$, and 119 $[M - H - 162 - 182]^-$. Loss of 162 suggested that it is glycosylated compound and fragment at *m*/*z* of 301 is the aglycone ion. The compound is not methylated as no loss of 15 Da was observed. The MS/MS was matched to quercetin-

3-*O*-glucoside with a score of 834 (out of 1000) in the MassBank library. Thus, the compound is tentatively identified as quercetin-3-*O*-glucoside.

Compound **2** has rt and *m*/*z* at 5.77 min and 465.1414, respectively. The MS/MS spectrum displayed fragment ions at *m*/*z* of 301 $[M - H - 164]^-$, 300 $[M - H - 164 - H\cdot]^-$, 241 $[M - H - 164 - 60]^-$, 169 $[M - H - 164 - 132]^-$, 125 $[M - H - 164 - 176]^-$, and 107 $[M - H - 164 - 194]^-$. This information matches to agnuside, catechin-4-ol 3'-methyl ether 3-*O*-alpha-l-rhamnopyranoside, catechin 5-*O*-beta-d-glucopyranoside-4'-methyl ether, symplocoside (catechin 7-*O*-beta-d-glucopyranoside-3'-methyl ether) in Metlin database with the same mass tolerance (3.8 ppm). MS/MS similarity search showed that major fragments in MS/MS spectrum matched to those of epigallocatechin gallate with the score of 685, but the molecular weight did not match. Thus, it is possibly one of the catechin derivatives.

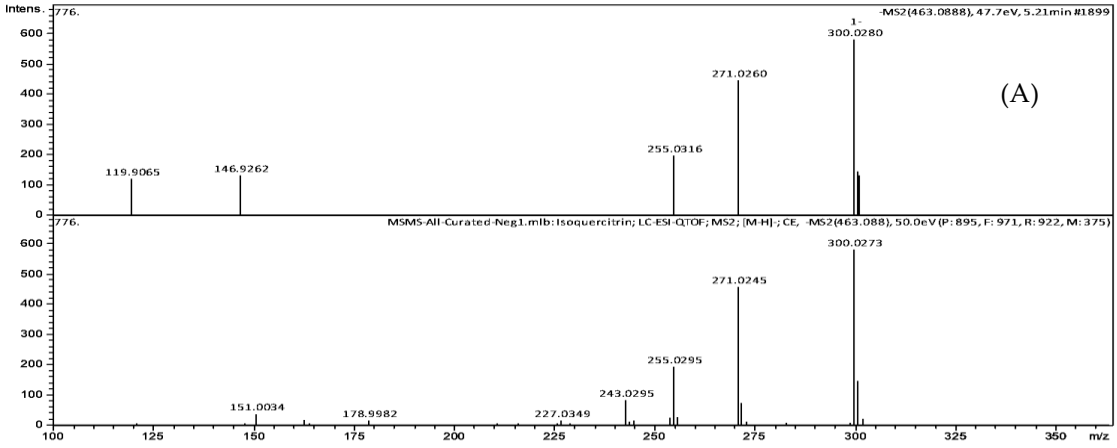
Compound **3** has rt and *m*/*z* at 6.15 min and 449.1102, respectively. The MS/MS spectrum displayed fragment ions at *m*/*z* 300 $[M - H - 149]^-$, 299 $[M - H - 149 - H\cdot]^-$, 255 $[M - H - 149 - 45]^-$, 200 $[M - H - 149 - 100]^-$, 175 $[M - H - 149 - 125]^-$, 151 $[M - H - 149 - 149]^-$, and 135 $[M - H - 149 - 165]^-$. The MS/MS was matched to eriodictyol-7-*O*-glucoside with a score of 872 (out of 1000) in the MassBank library. The ion 449.1102 *m*/*z* gave 151/135 as fragments, corresponding to the fragments previously described by Brito et al. (2014) as key aglycone fragments. Thus, the compound is tentatively identified as eriodictyol-7-*O*-glucoside.

Compound **4** has rt and *m*/*z* at 6.26 min and 477.0941, respectively. The MS/MS spectrum displayed fragment ions at *m*/*z* 301 $[M - H - 176]^-$, 300 $[M - H - 176 - H\cdot]^-$, 271 $[M - H - 176 - 30]^-$, 255 $[M - H - 176 - 46]^-$, 179 $[M - H - 176 - 122]^-$, and 151

[M − H − 176 − 150]⁻. In MS/MS spectrum, the predominant ions at *m*/*z* 301 [M–H–176]⁻ gave the proof of same glucuronyl unit loss and fragment at *m*/*z* of 301 is the aglycone ion. The characteristic product ions at *m*/*z* of 271, 255, 179, and 151 indicate the aglycone of quercetin. On the basis of the mass spectral data, compound 4 is tentatively identified as quercetrin with a score of 823 (out of 1000) in the metabolomics library developed by Lei et al. (2015).

Compound **5** has rt and *m*/*z* at 6.58 min and 187.0977, respectively. The MS/MS spectrum displayed fragment ions at *m*/*z* 144 [M − H − 43]⁻, 125 [M − H − 43 − 19]⁻, 123 [M − H − 43 − 21]⁻, and 97 [M − H − 43 − 47]⁻. The MS/MS product ion spectrum is dominated by *m*/*z* of 125, which corresponds to the combined loss of a molecule water and CO_2 (62 Da). Further fragmentation at *m*/*z* of 125 leads to ions at *m*/*z* of 123 and 97. The MS/MS was matched to azelaic acid in the MassBank library with a score of 813 (out of 1000). Therefore, compound 5 was tentatively identified as azelaic acid.

Compound **6** has rt and *m*/*z* at 7.18 min and 592.2043, respectively. The MS/MS spectrum displayed fragment ions at *m*/*z* of 283 [M − H − 309]⁻, 241 [M − H − 309 − 42]⁻, 223 [M − H − 309 − 60]⁻, 197 [M − H − 309 − 86]⁻, 181 [M − H − 309 − 102]⁻, 144 [M − H − 309 − 139]⁻, and 137 [M − H − 309 − 146]⁻. The ion 592.2043 *m*/*z* gave 241/197 as fragments, corresponding to the fragments previously described by Gómez-Caravaca et al. (2008) to be glansreginins A. Thus, compound 6 is tentatively identified as glansreginin A with score of 1.0 (peaks: 13/21 annotated/matched) via the MetFrag library.


Intens.
776.
-MS2(463.0888), 47.7eV, 5.21min #1899
1-
300.0280
271.0260
255.0316
119.9065
146.9262
(A)
776.
MSMS-All-Curated-Neg1.mlb: Isoquercitrin; LC-ESI-QTOF; MS2; [M-H]-; CE, -MS2(463.088), 50.0eV (P: 895, F: 971, R: 922, M: 375)
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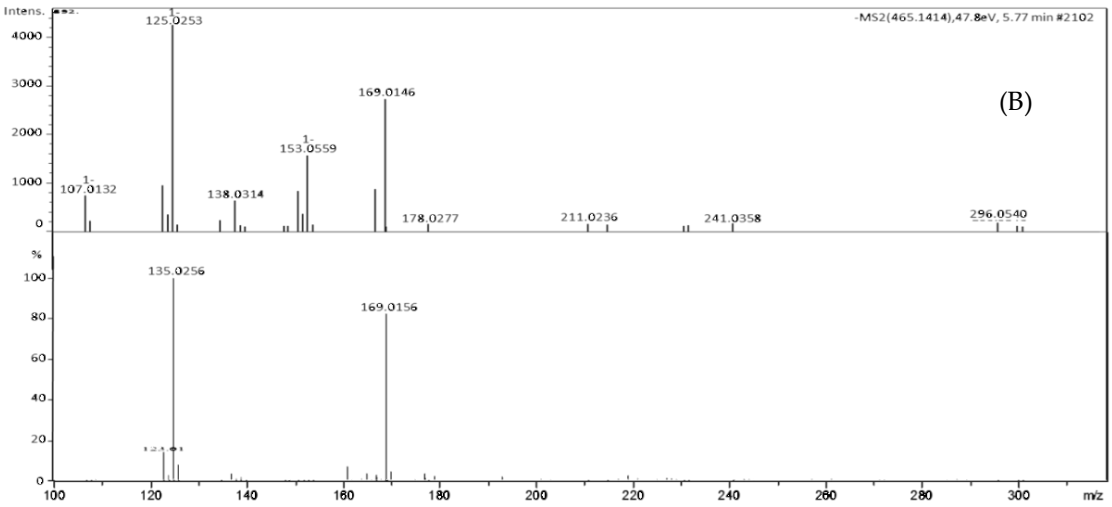
271.0245
255.0295
243.0295
151.0034
178.9982
227.0349
m/z


Intens.
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169.0146
1-
153.0559
1-
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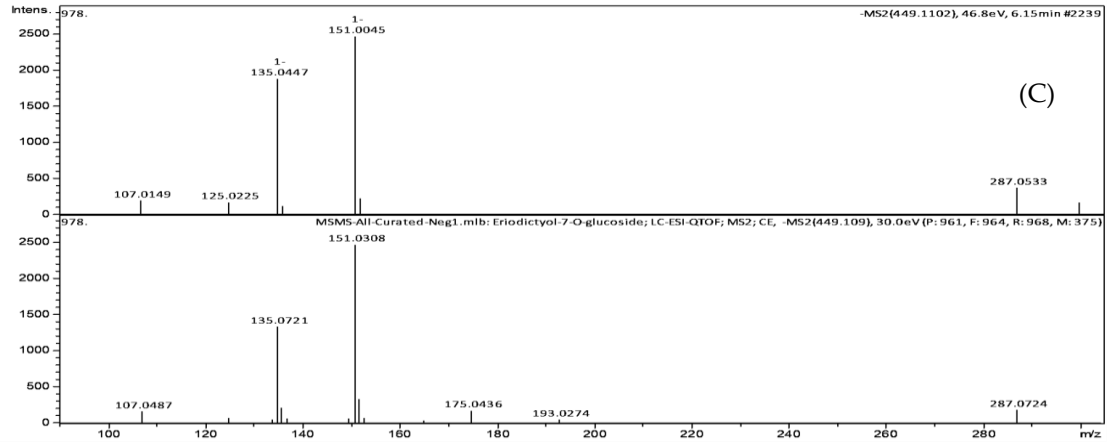
138.0314
178.0277
211.0236
241.0358
296.0540
(B)
%
135.0256
169.0156
m/z


Intens.
978.
-MS2(449.1102), 46.8eV, 6.15min #2239
1-
151.0045
1-
135.0447
107.0149
125.0225
287.0533
(C)
978.
MSMS-All-Curated-Neg1.mlb: Eriodictyol-7-O-glucoside; LC-ESI-QTOF; MS2; CE, -MS2(449.109), 30.0eV (P: 961, F: 964, R: 968, M: 375)
151.0308
135.0721
107.0487
175.0436
193.0274
287.0724
m/z

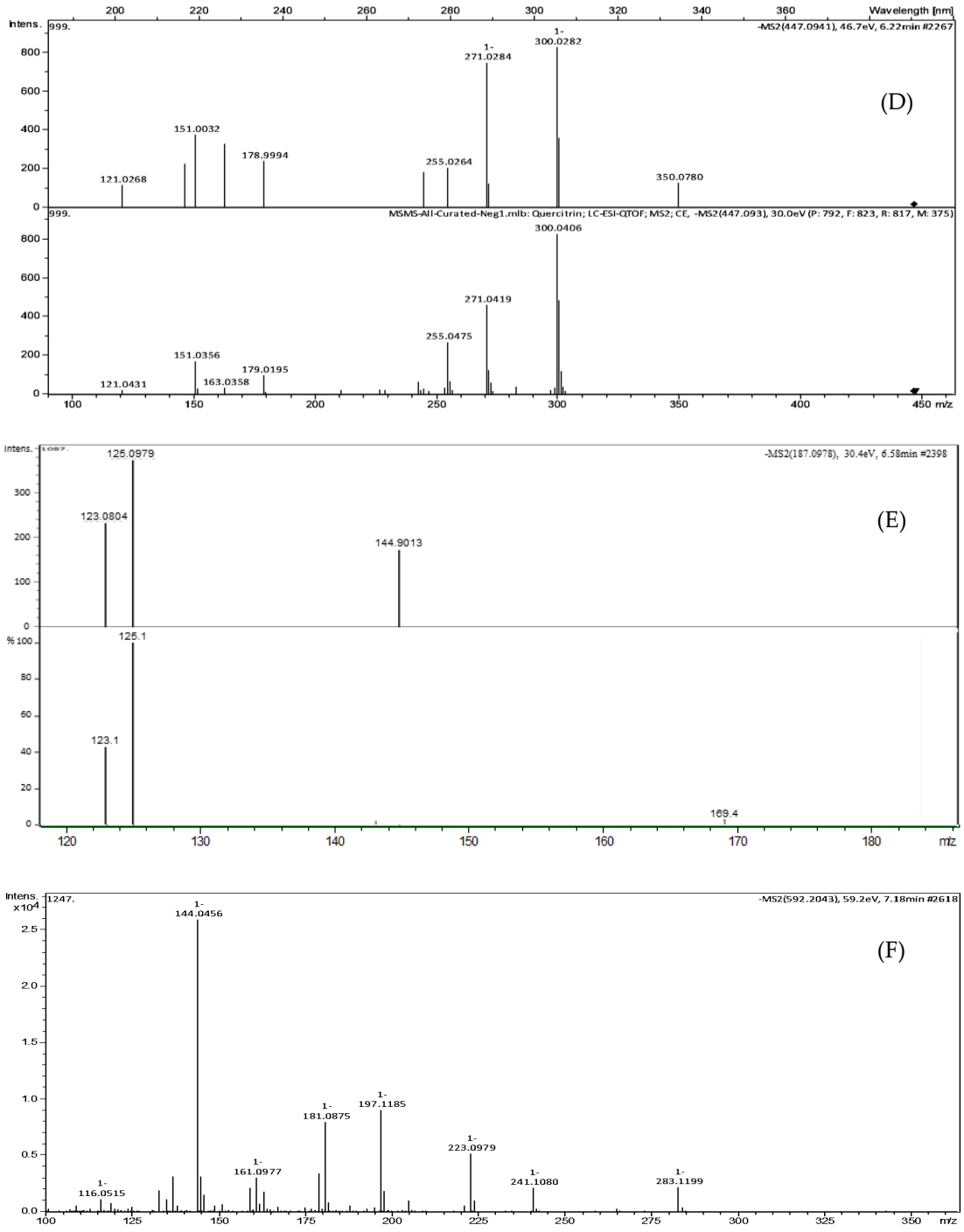
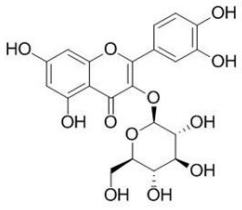


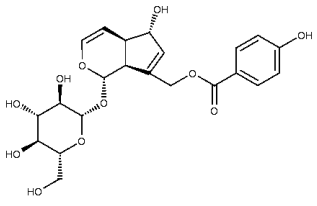
Figure 9. Head-to-tail spectral comparisons between the experimental MS/MS spectra and the referenced library MS/MS spectra. (**A**) Peak 1: Isoquercitrin, (**B**) peak 2: a catechin derivative; (**C**) peak 3: eriodictyol-7-*O*-glucoside, (**D**) peak 4: quercitrin, (**E**) Peak 5: azelaic acid, (**F**) peak 6: glansreginin A. In each comparison, the experimental MS/MS spectra (upper) and the referenced library MS/MS spectra (lower). Since compound 6 was tentatively identified by MetFrag in silico interpretation of the MS/MS spectrum, head-to-tail of this spectral comparison did not show.

DISCUSSION

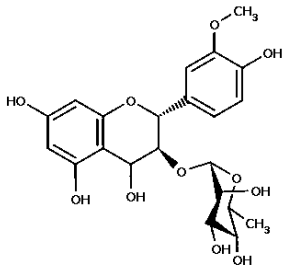
We demonstrated that the antibacterial properties of black walnuts against *S. aureus* were varied among tested cultivars. The four cultivars including Mystry, Surprise, D.34, and A.36 exhibited different antibacterial capacity against the Gram-positive bacterium (*S. aureus RN6390*). Other cultivars tested showed no effects on this bacterium. Several fractions of the kernel of Mystry from the column extraction showed the antibacterial activities (Figure 6), indicating the presence of multiple bioactive compounds and the possibilities of the synergy effects of these compounds that inhibited the bacterial growth. Vu et al. (2018) reported differences in phenolic profiles of 11 different black walnut cultivars (e.g., Daniel, Davidson, Emma, Hay, Jackson, Kwik Krop, Mystry, Sparks, Sparrow, Schessler, Surprise, and Tomboy). Differences in the bioactive activity of black walnut cultivars are highly likely due to the differences in phytochemical profiles of these cultivars. This finding from this study clearly illustrated the differences in antibacterial properties among the black walnut cultivars, which can be used as a selection trait for improving the quality of nuts for nut production.



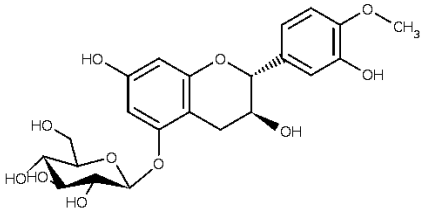
(1) Quercetin-3-*O*-glucoside



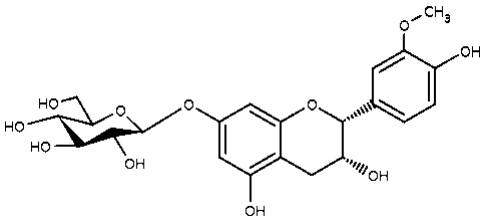
(2a) Agnuside



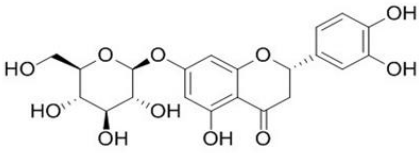
(2b) Catechin-4-ol 3'-methyl ether 3-O-alpha-L-rhamnopyranoside



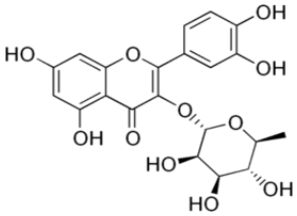
(2c) Catechin 5-*O*-beta-d-glucopyranoside-4'-methyl ether



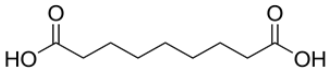
(2d) Catechin 7-*O*-beta-d-glucopyranoside-3’-methyl ether



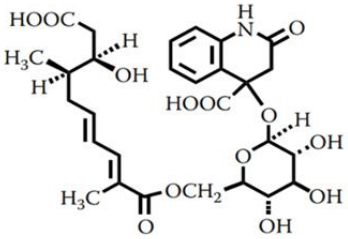
(3) Eriodictyol-7-*O*-glucoside



(4) Quercitrin



(5) Azelaic acid



(6) Glansreginin A

Figure 10. Chemical structures of tentative compounds from black walnut (Mystry).

We found six bioactive compounds (i.e., quercetin-3-*O*-glucoside, a catechin derivative, eriodictyol-7-*O*-glucoside, quercetin, azelaic acid, and glansreginin A) responsible for the antibacterial activity of the kernels of Mystry against the Gram-

positive bacterium (*S. aureus*) via the metabolomics approach combined with bioassay-guided fractionation strategy (Figure 9). The information on the biological activities of these compounds is summarized in Table 8. In addition to other bioactive compounds (i.e., quinic acid, gallic acid, p-hydroxybenzoic acid, vanillic acid, syringic acid, isoquercetin, catechin, epicatechin gallate, rutin, naringin, ferulic acid) in the kernels of black walnuts that have been linked to possess the antibacterial properties (Vu et al., 2018), our results reveal that glansreginin A, azelaic acid, quercetin, and eriodictyol-7-*O*-glucoside are the predominant antibacterial compounds in the kernels of black walnuts according to their hydrophobicity (retention times). Among the six bioactive compounds, glansreginin A was the most abundant in the purified bioactive fraction (Figure 8). However, the analytical standard for glansreginin A is not commercially available (Persic et al., 2018), making it difficult for the validation and qualification of this compound. Future research should focus on validation and characterization of these antibacterial compounds in black walnut cultivars when the authentic standards or purified reference standards are accessible. The antimicrobial agents identified black walnut extracts could be used to prevent growth of spoilage and pathogenic microorganisms in foods. They could also be used as the natural preservatives in the formulation of personal care product.

Table 8. Bioactivities of six bioactive compounds responsible for antibacterial activity in the kernels of black walnut (Mystry).

No.	Compound	Bioactivities	References
1	Quercetin-3-*O*-glucoside	antimicrobial	Wang et al. (2013)
		antioxidant	Chang et al. (2011)
		anti-inflammatory	Li et al. (2016)
		anti-fungal	Yun et al. (2015)
		antidiabetic	Zhang et al. (2011)
		anti-allergic	Rogerio et al. (2007)
		antitumor	Amado et al. (2014), Chen et al. (2016)
		antiviral	dos Santos et al. (2014)
		anti-hypertensive	Junior et al. (2011)
		anti-apoptoti	Zhu et al. (2016)
		diuretic effects	Junior et al. (2012)
2	Catechin derivatives	antimicrobial	Veluri et al. (2004), Hara-Kudo et al. (2005)
		antioxidant	Seeram et al. (2006)
		anti-inflammatory	Mizushina et al. (2005)
		antitumor	Cao et al. (2016)
		antiviral	Song et al. (2007)
3	Eriodictyol-7-*O*-glucoside	antioxidant	Jing et al. (2013)
4	Quercitrin	antimicrobial	Wang et al. (2013)
		antioxidant	Wang et al. (2013)
		anti-inflammatory	Yang et al. (2017)
		anti-allergic	Rogerio et al. (2007)
		antitumor	Liu et al. (2018)

5	Azelaic acid	antimicrobial	Charnock et al. (2004)
		antitumor	Pan et al. (2017), Breathnach (1999)
6	Glansreginin A	antioxidant	Ito et al. (2007), Slatnar et al. (2014)
		anti-inflammatory	Papoutsi et al. (2008)
		antiatherogenic effect	Berryman et al. (2013)
		antinociceptive effects	Raafat (2018)
		reduction of cholesterol absorption	Ren et al. (2018)

Quercetin-3-*O*-glucoside (known as isoquercitin), a flavonoid, is widely present in a variety of plants (e.g., medicinal herbs, fruits and vegetables) such as black walnut (Vu et al., 2018), English walnut (*J. regia*) (Slatnar et al., 2015), and buckwheat (*Fagopyrum esculentum*) (Kalinova & Vichotova 2009). In vitro, isoquercitin has also been documented to possess antibacterial activities against several bacteria such as *S. aureus*, *S. epidermidis*, and *Propionibacterium acnes* in disk diffusion assay (Wang et al., 2013). Many reports revealed that isoquercitin has been linked to versatile biological properties in vitro and in vivo including antioxidant (Chang et al., 2011), anti-inflammatory (Li et al., 2016), antifungal (Yun et al., 2015), antidiabetic (Zhang et al., 2011), anti-allergic (Rogerio et al., 2007), antitumor (Amado et al., 2014; Chen et al., 2016), antiviral (dos Santos et al., 2014), anti-hypertensive (Junior et al., 2011), anti-apoptotic (Zhu et al., 2016), and diuretic effects (Veluri et al., 2004).

Catechin derivatives (e.g., (+)-catechin, (−)-epicatechin, (−)-epigallocatechin) are found as major flavonol components in beverages, vegetables, and fruits (Seeram et al., 2006; Yilmazer et al., 2012). Catechin, epicatechin, and epicatechin gallate have also

detected previously in the kernels of black walnuts (Vu et al., 2018). This phytochemical group has been associated with a variety of biological functions including antibacterial, antioxidant, anti-inflammatory, antiviral, and antitumor effects (Veluri et al., 2004; Mizushina et al., 2005; Cao et al., 2016; Song et al., 2007; Yilmazer et al., 2012).

Eriodictyol-7-*O*-glucoside is found as a major flavonoid component derived from a Chinese herb (*Dracocephalum rupestre*), and from several plants e.g., grapevine (*Vitis vinifera*) (Puhl et al., 2008), pistachios (*Pistacia vera*) (Sonmezdag et al., 2018). The biological property of eriodictyol-7-*O*-glucoside has not been well established. Information on the biological activity of this compound has focused on the neuroprotective effect against oxidative stress in vitro and in vivo via Nrf2/ARE activation (Jing et al., 2013). In contrast to eriodictyol-7-*O*-glucoside, eriodictyol has been associated with a variety of biological properties in vitro and in vivo such as antioxidant (Chatzopoulou et al., 2010), antimicrobial (Chu et al., 2016), anti-inflammatory (Lee 2011), antineoplastic (Liu et al., 2016), and antinociception (Rossato et al., 2011).

Quercetin, a flavonoid, and is commonly found in vegetables (e.g., onion, garlic). The antibacterial properties against several bacteria have been also reported for quercetin (Wang et al., 2013). Quercetin exhibited stronger antibacterial effects against three bacteria (*S. aureus*, *S. epidermidis*, and *P. acnes*) compared to isoquercetin (Wang et al., 2013). This compound has also been linked to multiple biological functions in vitro and in vivo, such as antioxidant (Wang et al., 2013), anti-inflammatory (Yang et al., 2017), anti-allergic (Rogerio et al., 2007), and antitumor (Dajas 2012) capabilities.

Azelaic acid is a naturally occurring saturated dicarboxylic acid derived from a variety of grains such as sorghum (*Sorghum bicolor*) (Mehmood et al., 2008), rye (*Secale cereal*) (Bondia-Pons et al., 2013). This compound has also been reported to exert a variety of biological activities in vitro and in vivo including antimicrobial (Charnock et al., 2004) and antitumor (Breathnach 1999; Ito et al., 2007) capabilities. The U.S. Food and Drug Administration (FDA) approved 15% gel formulation of azelaic acid for the treatment of rosacea in 2002 (Gupta & Gover 2007) and this compound is an excellent antimicrobial agent cosmetic for the treatment of comedonal and inflammatory acnes (Hashim et al., 2018; Fitton & Goa 1991). In vitro, azelaic acid exhibited the antibacterial effect against *S. aureus*, *S. epidermidi*s, and *P. acnes* at pH of 5.6, but no antibacterial effect was seen at pH of 7.3 (Charnock et al., 2004).

Glansreginin A, a dicarboxylic acid derivative, presented dominantly in the bioactive fraction might be mainly responsible for the antibacterial activity of the kernels of Mystry. Glansreginin A has been also found in the kernels of three other nuts including English walnut (Gómez-Caravaca et al., 2008; Ito et al., 2007; Ren et al., 2018; Slatnar et al., 2015), pecans (*Carya illinoinensis*) (Jia et al., 2018), and hazelnut (*Corylus avellana*) (Slatnar et al., 2014). Due to the presence of this compound in the kernels of these nuts, glansreginin A has been linked to multiple biological activities in vitro and in vivo such as antioxidant (Ito et al., 2007; Slatnar et al., 2014; Bati et al., 2015), anti-inflammatory (Papoutsi et al., 2008), antiatherogenic effect (Berryman et al., 2013), and antinociceptive effect (Raafat 2018), reduction of cholesterol absorption (Ren et al., 2018). This is the first time, glansreginin A is reported to be associated with the antibacterial properties.

In recent years, the advancement in mass spectrometry, computation power, metabolomics algorithm and mass spectral libraries in metabolomics allows rapid identification of the bioactive molecules. The metabolomics approach combined with bioassay guided fractionation strategy in this study is a promising tool for putative identification of new bioactive compounds from natural sources. The high-resolution data generated from MS/MS offer the mass accuracy and specific fragmentation fingerprints needed for rapid identification of the antimicrobial molecules. Therefore, it eliminated the time consuming and labor-intensive large-scale purification procedure that is required in the traditional structural elucidation techniques. This approach is cost-effective compared to other approaches for compound identification e.g., nuclear magnetic resonance (NMR), which typically requires at least 1 mg of the purified crystal (Rosengren et al., 2009).

Black walnut is an excellent resource not only for nutrition but also medicinal values. Among twenty-two black walnut cultivars tested, four cultivars exhibited antibacterial activity against the gram-positive bacterium *S. aureus* and six antibacterial compounds in the kernels of Mystry were tentatively identified. With a growing global consumption of organic personal care products and diet supplements, through identifying the novel uses of the black walnut and its byproducts, this study will provide the opportunities to turn abundant, low-value, renewable materials from the black walnut and its byproducts into profitable value-added products for the industry. Future research should focus on exploring of other health-promoting properties (e.g., antioxidant, anti-inflammatory, antitumor) and industrial applications of bioactive compounds of black walnuts through utilizing the same metabolomics strategy.

CONCLUSION

The antibacterial properties of the kernels of black walnuts were successfully characterized. In fact, twenty-two black walnut cultivars (i.e., Bowser, Daniel, Davidson, Emma, Hay, Hare, Jackson, Kwik Krop, Mystry, Schessler, Sparks, Sparrow, South Fork, Surprise, Thomas, A4, A.36, B.15, B.31, C8, D16, and D.34) had shown differences in their antibacterial properties against the Gram-positive bacterium (*S. aureus RN6390*) and Mystry exhibited the strongest antibacterial activity. The antibacterial activities were also seen in Surprise, D.34, and A.36, but no antibacterial effects were seen in the other tested cultivars. Six possible antibacterial compounds in the kernels of Mystry were tentatively identified through the metabolomics approach combined with bioassay-guided purification. This approach is a promising tool for identifying the candidates of bioactive molecules from natural sources.

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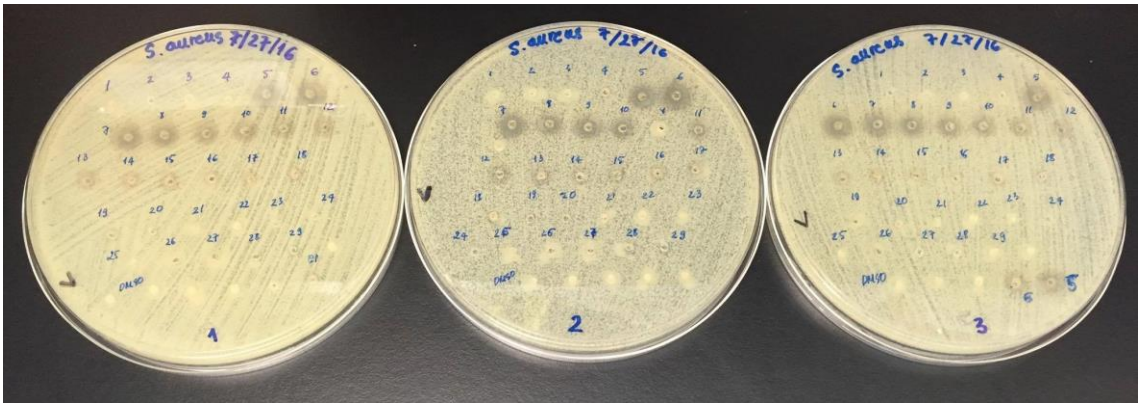
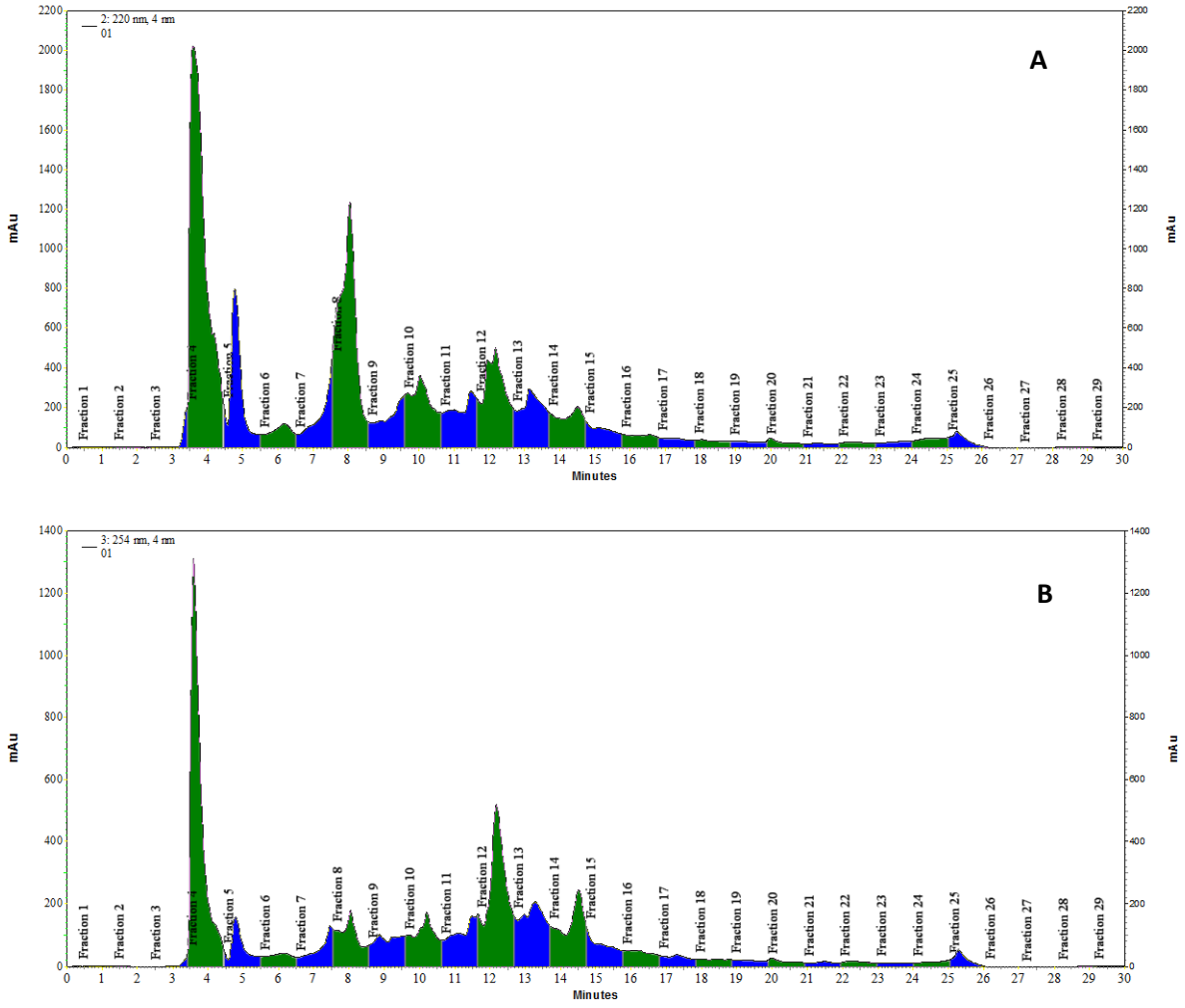
SUPPLEMENTARY INFORMATION



Supplementary Figure 1. Zone of inhibition of 29 Mystry fractions (out of 46) from column chromatography. 1-29: fraction 1 through fraction 29.



Supplementary Figure 2. Shimadzu UV Chromatogram of HPLC sub-fraction F14 at 220 nm (A) and 254 nm (B) generated from SPD-10Avp photodiode array detector.

CHAPTER III: BLACK WALNUT (*JUGLANS NIGRA*) EXTRACTS INHIBIT PRO-INFLAMMATORY CYTOKINE PRODUCTION FROM LPS-STIMULATED HUMAN PRO-MONOCYTIC CELL LINE U-937

ABSTRACT

Black walnut (*Juglans nigra* L.) is an excellent source of health-promoting compounds. Consumption of black walnuts has been linked to many health benefits (e.g., anti-inflammatory) stemming from its phytochemical composition and medicinal properties, but these effects have not been systematically studied or characterized. In this study, potential anti-inflammatory compounds found in kernel extracts of ten black walnut cultivars were putatively identified using a metabolomics profiling analysis, revealing differences in potential anti-inflammatory capacities among examined cultivars. Five cultivars were examined for activities in the human pro-monocytic cell line U-937 by evaluating the effects of the extracts on the expression of 6 human inflammatory cytokines/chemokines using a bead-based, flow cytometric multiplex assay. The methanolic extracts of these cultivars were added at four concentrations (0.1, 0.3, 1, and 10 mg/mL) either before and after the addition of lipopolysaccharide (LPS) to human U-937 cells to examine their effect on cytokine production. Results from cytotoxicity and viability assays revealed that the kernel extracts had no toxic effect on the U-937 cells. Of the 13 cytokines (IL-1β, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, IL-23, IL-33, IFN-α, IFN-γ) measured, only 6 were detected under the culture conditions. The production of the six detected cytokines by PMA-differentiated, LPS-stimulated U-937

was significantly inhibited by the kernel extracts from two cultivars Surprise and Sparrow when the extracts were added prior to the addition of LPS. Other cultivars (Daniel, Mystry, and Sparks) showed weak or no significant effects on cytokine production. In contrast, no inhibitory effect was observed on the production of cytokines by PMA-differentiated, LPS-stimulated U-937 when the kernel extracts were added after the addition of LPS. The findings suggest that the extracts from certain black walnut cultivars, such as Sparrow and Surprise, are promising biologic candidates for potentially decreasing the severity of inflammatory disease.

INTRODUCTION

Inflammation is a complex pathophysiological response of the immune system in response to infections of harmful stimuli or tissue damage (Medzhitov 2010). An uncontrolled inflammatory response might result in development of a variety of chronic inflammatory diseases such as rheumatoid arthritis (RA). Rheumatoid arthritis is the most common inflammatory arthritis affecting approximately 1% of population worldwide (Firestein 2003). This inflammatory disease is associated with articular inflammation and synovial joint damage that can result in disability and increased mortality. In synovial tissues, several cytokines are released and are functionally active in mediating immune responses associated with the pathogenesis of RA (McInnes and Schett 2007). Cytokines are therapeutic targets for the treatment of patients with RA (tumour necrosis factor, TNF) and in RA clinical trials (e.g., interleukin (IL)-1, IL-6, IL-27) (McInnes et al. 2016).

Black walnut (*Juglans nigra* L.) is one of the most economically valuable hardwood species in the United States (Randolph et al. 2013). Native Americans traditionally valued kernels of black walnut as a food source and utilized leaves for medical purposes to treat diarrhea, bilious, and cramp colic (Nolan and Robbins 1999). A recent report indicated that the kernels of black walnut contain a wealth of phytochemical substances (Vu et al. 2018) and many of these phenolic compounds are associated with a variety of biological functions such as antioxidant, antimicrobial, and anti-inflammatory properties. Our previous study demonstrated antibacterial activities of the kernels from black walnut against a Gram-positive bacterium (*Staphylococcus aureus* RN6390), and its antibacterial compounds (e.g., glansreginin A, azelaic acid) were successfully identified (Ho et al. 2018).

Anti-inflammatory capacities of another common *Juglans* species, English walnut (*J. regia* L.) have been well-established in vitro and in vivo. Willis et al. (2010) reported that kernel extracts of English walnut reduced production of TNF-α and nitric oxide synthase induced by BV-2 murine microglial cells stimulated with lipopolysaccharide (LPS) when the cells were pre-treated with the extract prior to stimulation with LPS. Papoutsi et al. (2008) also documented that extracts of English walnut decreased expression of TNF-α-induced endothelial vascular cell adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1 expression in human aortic endothelial cells. In vivo, consumption of English walnut was associated with multiple health benefits with respect to alleviating inflammation, oxidative stress and improving vascular function in both animals and human clinical trials (Ros 2015). In fact, the consumption of English walnut was associated with a lowered risk of type 2 diabetes (Pan et al. 2013) and a

reduction of the incidence of major cardiovascular events (Estruch et al. 2013) in two randomized clinical trials of 137,956 women aged 35-77 over 10 years and of 7447 participants aged 55-80 (47% were men), respectively. Anti-inflammatory functions of English walnut are likely associated with its phytochemical compounds. Remarkably, English walnut and black walnut share a similar phytochemical profile, revealing potential anti-inflammatory capacities of black walnut extracts. In fact, 16 phenolic compounds characterized in English walnut also found in black walnut (Vu et al. 2018). Nonetheless, anti-inflammatory properties of black walnut have never been characterized yet. Exploring the anti-inflammatory properties of black walnut might point to novel uses of black walnut and its byproducts, promoting development of new biological agents for the prevention or even treatment of inflammation as well as increasing the value of black walnuts by identifying new applications and health benefits.

In the present study, we first examined and compared effects of kernel extracts from ten black walnut cultivars via a global metabolomics profiling approach. We then characterized possible anti-inflammatory properties in kernel extracts of five selective cultivars by evaluating the expression of 6 inflammatory mediators (IL-1β, TNF-α, monocyte chemoattractant protein (MCP)-1, IL-6, IL-8, and IL-10) using the human pro-monocytic cell line U-937.

MATERIALS AND METHODS

Black walnut cultivars

The nuts of ten black walnut cultivars including Daniel, Davidson, Hay, Jackson, Kwik Krop, Mystry, Schessler, Sparks, Sparrow, and Surprise were collected at the University of Missouri, Horticulture and Agroforestry Research Center (New Franklin, MO, USA). The black walnuts were hulled mechanically and hung up to dry at 24°C for 15 days. The hulled nuts were then stored at -20°C until analysis.

Sample preparation

The hulled nuts were cracked manually the kernels removed from the shelled and homogenized using a coffee grinder (CBG100S, Black + Decker, Beachwood, OH, USA). For metabolic analysis, the kernels (2.5 g, 20–30 mesh) from ten cultivars were extracted with sonication in 15 mL of methanol. The methanolic extract was sonicated in a water bath (4°C) for 60 min followed by centrifugation for 10 min at 8000 rpm. The supernatant was collected and filtered through a 0.2 µm Whatman Anotop syringe membrane filter (Sigma-Aldrich, St. Louis, MO, USA), and then injected into UHPLC-MS (ultra-high performance liquid chromatography tandem mass-spectrometry). For assays, the kernels (2.5 g) from five selective cultivars (i.e., Daniel, Mystry, Sparks, Sparrow, and Surprise) were extracted with sonication in 10 mL of methanol. The extract was sonicated for 60 min followed by centrifugation for 10 min at 4000 rpm and the supernatant was collected. Subsequently, the supernatant was filtered through the syringe membrane filter (0.2 µm, Whatman). The aqueous extract was evaporated until dryness under a flow of nitrogen and was resuspended with 0.125 mL of DMSO (Sigma-Aldrich,

USA) and then the resulting extract was concentrated 40 times to achieve the concentration of 10,000 mg/mL. Cytokine modulating activities of the extract were identified using multiplex bead-based cytokine assay kits (BioLegendplex™ Human Inflammation panel kits, BioLegend, San Diego, CA, USA).

UHPLC-QTOF-MS analysis to examine metabolite profiles of black walnuts

The kernel extracts from ten black walnut cultivars were analyzed by UHPLC coupled to a maXis impact quadrupole-time-of-flight mass spectrometer (Bruker Co., Billerica, MA, USA). Separations were achieved on a Waters Acquity UHPLC BEH C18 column (2.1x 150 mm, 1.7 µm particles size) using a linear gradient of 95%: 5% to 30%: 70% eluents A: B (A: 0.1% formic acid and B: acetonitrile) in 30 min. Subsequently, the separation was followed by a linear wash gradient as follows 70-95% B (30-33 min), 95% B (33-35 min), 95-5% B (35-36 min), and 5% B (37-40 min), respectively. The column temperature was kept at 60^{o}C and the flow rate was 0.56 mL/min. Mass spectrometry was performed in both negative and positive electrospray ionization modes with the nebulization gas pressure at 43.5 psi, dry gas of 12 L/min, dry temperature of 250°C and a capillary voltage of 4000V. Mass spectral data were collected automatically from 100 to 1,500 m/z, three precursors were selected for auto MS/MS and m/z range auto-calibrated using sodium formate after data acquisition. Each cultivar was analyzed in triplicate along with methanol blank used as a control.

Cell culture and differentiation induction

The human monocyte cell line U-937 was purchased from American Type Culture Collection (ATCC) (CRL-1593.2, ATCC, Manassas, VA, USA). U-937 cells

were cultured in complete Roswell Park Memorial Institute (RPMI) medium (RPMI 1640, ATCC) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 100 µg/mL gentamicin, and then incubated at 37°C in a 5% CO_2 humidified atmosphere. U-937 cells were seeded at 2 x 10^5 cells/well in 96-well plates in a 200 µL volume in the presence of 50 nM of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 48 hours (Rovera et al. 1979) to induce differentiation. After washing the PMA-differentiated cells twice, fresh media was added and the cultures were incubated for an additional 18 hours at 37°C in a 5% CO_2 humidified atmosphere before the addition of extracts. Subsequently, the cultures were pre-treated with extract dilutions from five cultivars (Daniel, Mystry, Sparks, Sparrow, and Surprise) at 4 final concentrations (0.1, 0.3, 1, and 10 mg/mL) for 2 hours prior to stimulation with 1 µg/mL LPS (*Escherichia coli* 0127:B8, Sigma-Aldrich). In some experiments, the PMA-differentiated cells were stimulated with LPS for 2 hours prior to the addition of two cultivar extracts (Sparrow and Surprise). Extracts were prepared in tissue-culture grade DMSO, and the highest concentration of DMSO in any cultivar sample was 0.1%. An immunosuppressant drug, cyclosporin A (CSA, Sigma-Aldrich), was added at a final concentration of 0.002 mg/mL in 0.02% DMSO and served as a positive control for the inhibition of cytokine secretion. Samples without CSA or cultivars were supplemented with 0.1% DMSO and served as vehicle controls. Twenty-four hours after the addition of LPS and extracts, the triplicate culture supernatants from each group were pooled, spun to remove cell debris, transferred to new tubes, and stored at -20°C until analysis. Cytokine secretion levels in LPS-stimulated, cultivar-treated cultures were compared to samples containing DMSO but not

cultivars. Viability of the attached cells was determined using an MTT assay (see methods section below).

A macrophage model system was chosen to investigate the anti-inflammatory potential of black walnut extracts because macrophages are central to the inflammatory response and are active during all phases of inflammation. The U-937 cell line can be induced to differentiate with PMA, and the addition of LPS derived from gram-negative bacterial cell walls results in the release of numerous inflammatory mediators. These cytokines include, but are not limited to, TNF-α, IL-1β, and IL-6, which in turn contribute to the recruitment and activation of other immune cells against bacterial infections (Gordon 2002). Since cytokines such as TNF, IL-6, IL-1β play primary roles in the pathogenesis of rheumatoid arthritis (Smolen and Aletaha 2015), they can be used as key biomarkers of RA.

Quantification of secretion of cytokines/chemokines by macrophages

The LEGENDplex™ human inflammation bead-based immunoassay was used to quantitate the secretion of cytokines in the U-937 culture model system in the absence or presence of black walnut extract, according to the manufacturer's procedure. Initial experiments were performed using a pre-defined panel of 13 inflammatory analytes (IL-1β, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, IL-23, IL-33, interferon (IFN)-α, and IFN-γ) but subsequent experiments focused on a subset of 6 cytokines. Data were collected on a BD LSRFortessa™ X-20 cell analyzer (BD Biosciences, San Jose, CA, USA) using instrument settings recommended by BioLegend. A cytokine standard curve was included in each experiment, and cytokine levels were calculated from a 5-

parameter logistic curve using the software provided in the BioLegend kit. Triplicates were collected at each cultivar concentration in multiple experiments.

Effects of black walnut extracts on cell viability

The MTT assays were performed to evaluate the effect of black walnut extracts on cytotoxicity and/or cell loss at the time of supernatant collection. A colorimetric cell viability assay (CGD1 – 1KT, Sigma-Aldrich) using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) as the substrate (Mosmann 1983) was carried out after collection of the supernatants. The ability of the cells to convert MTT to formazan crystals indicates mitochondrial activity and cell viability. DMEM high glucose phenol red free media (Fisher Scientific, Pittsburgh, PA, USA) containing 1% FBS and MTT was added to each well, and the plates were incubated for 3 hours at 37°C until precipitates were observed. An acidified isopropanol solvent was then added to dissolve the formazan crystals, and the samples were pipetted several times to completely dissolve the crystals. Absorbance was measured within 30 min after solvent addition using a BioTek ELx808 microplate reader (BioTek, Winooski, Vermont, USA). Formazan crystals were detected at a wavelength of 570 nm, and background absorbance was measured at 630 nm.

Statistical analysis

For the metabolic analyses, the original UHPLC-MS data were converted into a format (*.cdf) that is compatible with XCMS Online (www.xcmsonline.scripps.edu) (Tautenhahn et al. 2012) and they were processed using the XCMS Online algorithms. This web-based tool for metabolomics data allows converting data file for peak detection,

peak grouping, retention time correction and alignment. Pairwise analyses between each black walnut cultivar and the control (methanol) were performed to identify single ion (m/z) features that were significant differences at p-value < 0.005 and intensity ≥ 10,000 across the chromatographic time domain. The metabolites of the significant ion features were putatively identified based on the accurate mass of the molecular ions, referenced to METLIN metabolite mass spectral database containing over 1 million molecules (http://metlin.scripps.edu) (Guijas et al. 2018). Metabolites that have been reported to possess anti-inflammatory activities based on a literature search (supplementary Table 1) were selected to initially examine profiles of black walnut cultivars and to identify potential anti-inflammatory compounds in each cultivar. Multivariate analyses such as partial least squares-discriminant analysis (PLS-DA) and heat map were performed using MetaboAnalyst (Chong et al. 2018) to reveal differences in metabolic profiles among black walnut cultivars.

Relative cell viability was measured with the MTT assay according to the manufacturer's protocol. The amount of MTT conversion was determined by subtracting the background absorbance (630 nm) from the absorbance of formazan crystals at 570 nm (A_{570} nm – A_{630} nm = specific MTT absorbance). Cell viability was expressed relatively to the PMA-differentiated, LPS-stimulated sample (control sample) in the absence of black walnut cultivars. The percentage cell viability (%) was calculated by dividing the specific MTT absorbance of the treated sample by the specific MTT absorbance of the control sample and multiplying by 100. The control sample was set at 100%.

Cytokine concentrations were determined in the multiplex bead-based assay by extrapolating the concentration from a 5-parameter logistic standard curve using curve-

fitting software. The absolute concentrations of each cytokine from treated samples were then compared to the control samples, as described above for the MTT assay. The relative percentage (%) was obtained by dividing the cytokine concentration of the treated sample by the cytokine concentration of the control sample and multiplying by 100. The PMA-differentiated, LPS-stimulated sample served as the control sample and was set at 100%.

The data were analyzed as a randomized complete block design using PROC MIXED in SAS 9.4 (SAS Institute, Cary, NC). The black walnut extract was the fixed effect and replication was the random variable. Differences between extracts were determined using Fisher's LSD at p-value < 0.01.

RESULTS

Anti-inflammatory metabolic profiles of black walnut

The metabolic profiles of the kernel extracts from ten black walnut cultivars were characterized using data acquired from liquid chromatography–high resolution MS (LC-HRMS). The UHPLC-HRMS data processed with XCMS Online provided 650 and 420 significant features in positive and negative modes, respectively, which were further annotated using METLIN metabolite database. This resulted in the identification of 26 substances with known anti-inflammatory activities. These metabolites included flavanols, hydroxybenzoic acids, and ellagitannins (supplementary Table 1). The partial least squares-discriminant analysis (PLS-DA) score plot showed significant differences in anti-inflammatory metabolic profiles among ten black walnut cultivars (Figure. 11A). In the score plot, Sparrow and Schessler, Mystry and Sparks, and Daniel and Davidson were

distributed separately on quadrant I, quadrant II, and quadrant IV, respectively, whereas other four cultivars including Kwik Krop, Jackson, Hay and Surprise roughly clustered together in the intersection of quadrant I & IV. Five cultivars (Daniel, Mystry, Sparks, Sparrow, and Surprise) were chosen as representative examples for further examined for their ability to alter cytokine production using human pro-monocytic cell line U-937 because they were selected from different groups distributed in four distinct quadrants (I, II, IV, or the intersection of I and IV). This approach was utilized to select cultivars that capture the most potentially diverse set of anti-inflammatory compounds.

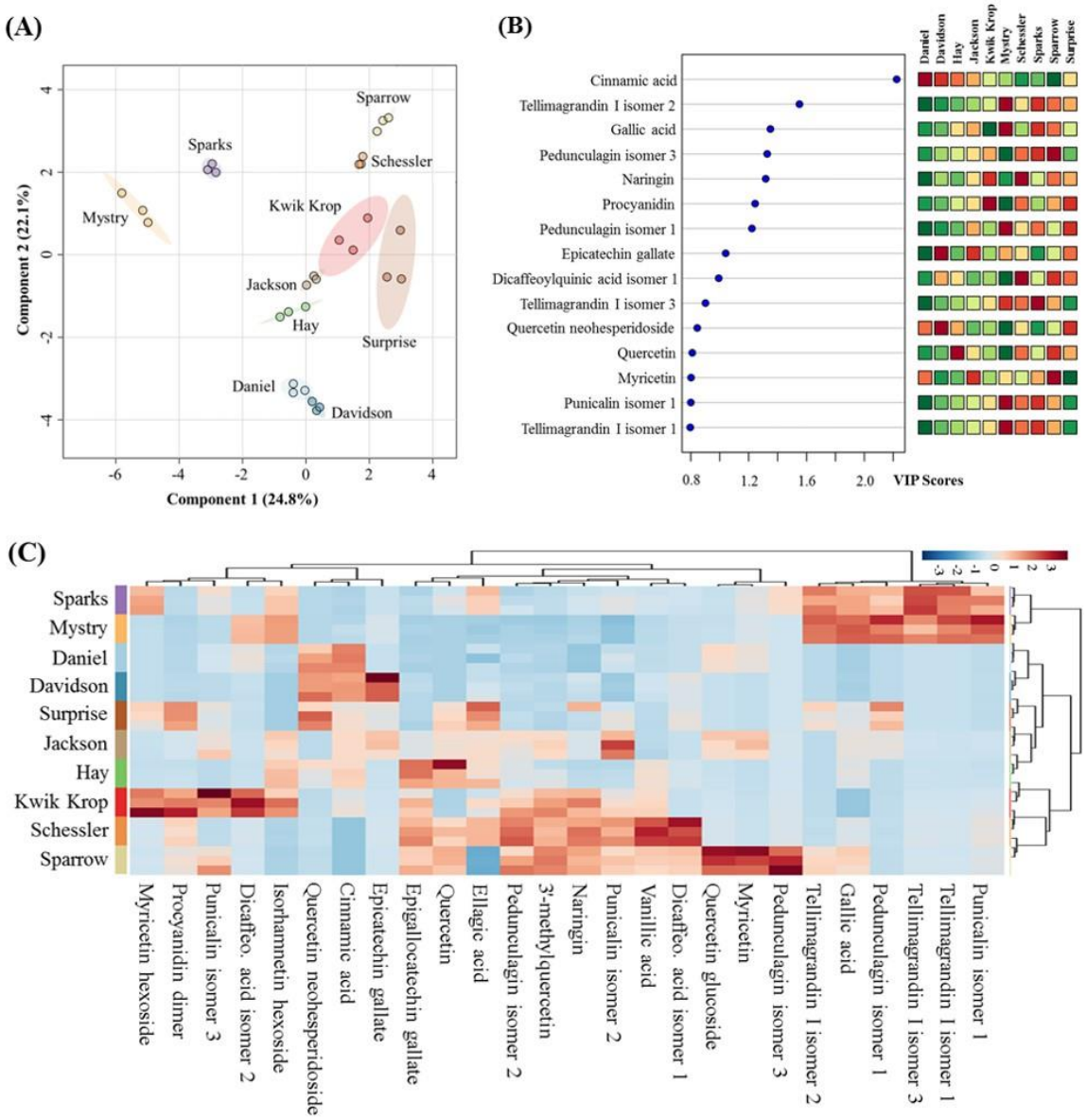


Figure 11. Differences in metabolic profiles of black walnut cultivars. (A) Partial least squares-discriminant analysis (PLS-DA). (B) variable importance in projection (VIP) and (C) heat map. In the PLS-DA plot, circles with same colors represent replicates of metabolic profiles for each cultivar. The colored ellipses indicate 95% confidence regions of metabolic profiles for each cultivar. In the VIP score plot, 15 out of 26 metabolites in total were shown and the colored boxes on the right indicate the relative abundance of the corresponding metabolite in each cultivar. Red represents higher relative abundance, whereas green and blue represent lower relative abundance in the VIP score plot and the heat map, respectively.

Each black walnut cultivar contained several potential anti-inflammatory compounds and the relative concentrations of many compounds were distinct within specific cultivars (Figures 11B & C). Sparks and Mystry contained the highest relative abundance of gallic acid and four ellagitannins including tellimagrandin I isomer 1 & 3, punicalin isomer 1, pedunculagin isomer 1, while cinnamic acid and quercetin neohesperidoside were relatively dominant in Daniel and Davidson. The highest relative abundance of ellagic acid was found in Surprise and this cultivar also contained procyanidin dimer, quercetin neohesperidoside, pedunculagin isomer 1 as major components. Kwik Krop contained the highest relative abundance of myricetin hexoside, procyanidin dimer, punicalin isomer 3, and dicaffeocylquinic acid isomer 2. Schessler and Sparrow also contained a variety of bioactive compounds. Quercetin glucoside, myricetin, and pedunculagin isomer 3 were presented dominantly in Sparrow while vanillic acid and dicaffeoylquinic acid isomer 3 were major compounds in Schessler.

Cell viability assays

The cell viability assays were performed to address possible cytotoxic effects of the black walnut cultivars. A reduction in MTT absorbance could point to a decrease in cell viability, a decrease in mitochondrial activity, and/or cell loss, resulting in a concomitant decrease in cytokine secretion. MTT viability assays were performed at the same time cell supernatants were collected. CSA, a potent immunosuppressive agent capable of inhibiting cytokine production and release, was included in the experiments as a positive control. Black walnut extracts were resuspended in DMSO, and all experimental groups contained up to 0.1% DMSO. Control groups without extracts were supplemented with 0.1% DMSO to account for any adverse effects of the DMSO vehicle.

The results indicated that the viability was not significantly different in any of the groups analyzed (Figure 12). These groups include no treatment (none, no LPS and no black walnut extracts, with DMSO), LPS control cultures (LPS: LPS and DMSO, no extract), treated cells (Daniel, Mystry, Sparks, Sparrow, and Surprise: extracts, DMSO, and LPS), and CSA (CSA: CSA, DMSO, and LPS). Since the extracts of all five cultivars and CSA at all the concentrations tested were non-toxic to the PMA-differentiated U-937 cells, they were selected for examining their effect on cytokine production.

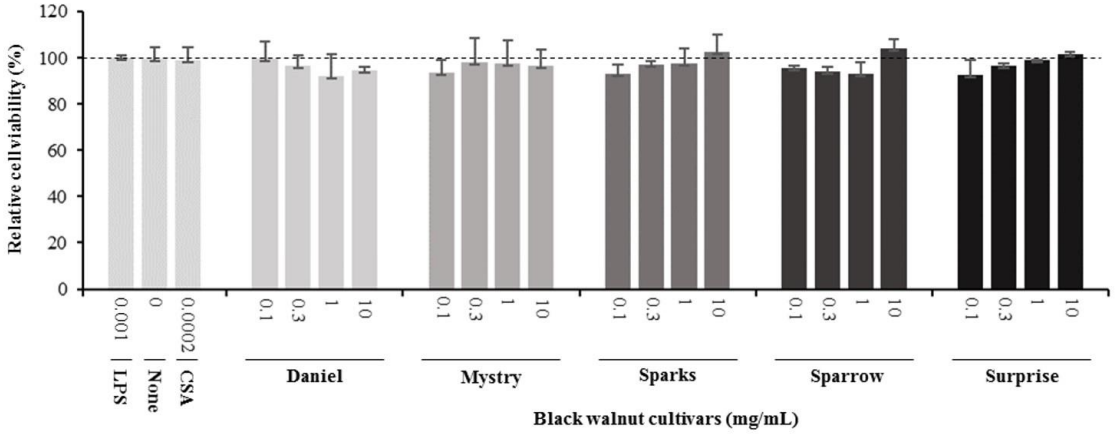


Figure 12. Effect of lipopolysaccharide (LPS), an immune suppressor drug (cyclosporin A, CSA), and black walnut extracts on the viability of PMA-differentiated U-937 cells. None: PMA-differentiated cells in the absence of black walnut extract and LPS. (*) Significant decrease ($p < 0.01$) compared to PMA-differentiated, LPS-stimulated cells in the absence of black walnut extract. Mean ± SEM.

Secretion of cytokines

Secreted cytokine levels were quantified in PMA-differentiated, LPS-stimulated U-937 cells after pre-treatment with 5 representative cultivars at 4 concentrations. A pre-defined human inflammation bead-based multiplex kit was chosen because it could be

used to detect multiple cytokines simultaneously from the same sample with a small amount of the sample. The panel was comprised of 13 cytokines involved in various aspects of the inflammatory process, and many of these were studied previously in the U-937 model system. Of the 6 cytokines initially detected in the supernatants, 5 (TNF-α, IL-1β, IL-6, IL-8, and MCP-15) are pro-inflammatory and 1 (IL-10) is anti-inflammatory. Subsequent experiments focusing on these cytokines showed that secretion by PMA-differentiated, LPS-stimulated U-937 cells was significantly and dose-dependently attenuated by pre-treatment with extracts derived from Sparrow and Surprise compared to control cells (Figures. 13-18). More specifically, cultures pre-treated with Sparrow extracts at the concentrations of 0.1, 0.3, 1, and 10 mg/mL reduced the secretion of TNF-α by 23.9%, 31.7%, 35.5%, and 42.7%, respectively, IL-1β by 17.7%, 16.4%, 23.1%, and 34.1%, respectively, IL-6 by 26.3%, 23.7%, 35.6%, and 43.3%, respectively, IL-8 by 23.4%, 34.1%, 39.8%, and 51.1%, respectively, IL-10 by 22.0%, 30.1%, 25.6%, and 33.9%, respectively, and MCP-1 by 26.5%, 41.9%, 40.8%, and 49.1%, respectively (Figures 13-18). Similarly, at the concentrations of 0.1, 0.3, 1, and 10 mg/mL, cultures pre-treated with Surprise extracts also reduced the secretion of TNF-α by 36.4%, 37.6%, 47.8%, and 55.7%, respectively, IL-1β by 32.1%, 34.8%, 44.0%, and 48.7%, respectively, IL-6 by 24.9%, 34.3%, 58.9%, and 56.3%, respectively, IL-8 by 33.1%, 29.3%, 45.9%, and 59.3%, respectively, IL-10 by 21.2%, 27.8%, 34.9%, and 45.5%, respectively, and MCP-1 by 27.8%, 30.7%, 45.6%, and 46.0%, respectively (Figures 13-18).

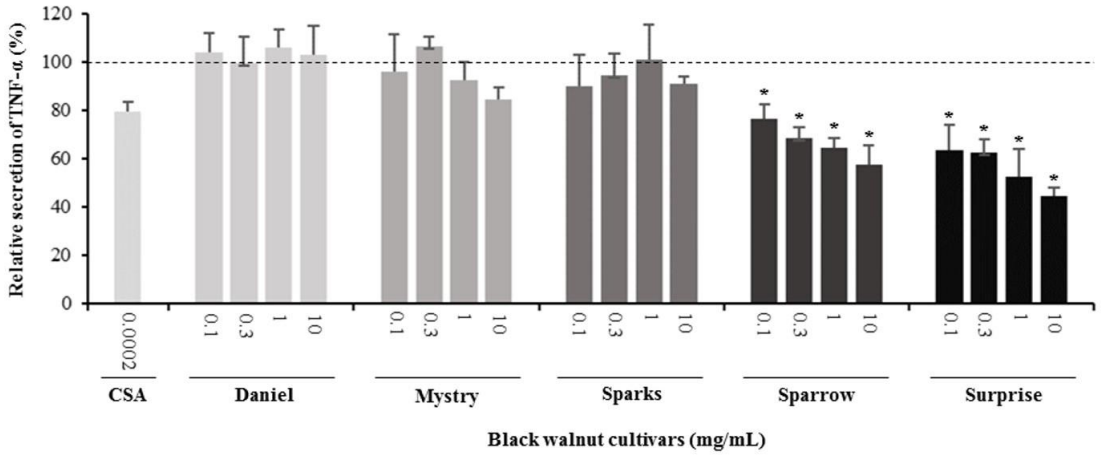


Figure 13. Effect of black walnut extracts on the secretion of TNF-α by PMA-differentiated, LPS-stimulated U937 cells. Cyclosporin A (CSA) was used as a positive control. (*) Significant decrease ($p < 0.01$) compared to PMA-differentiated, LPS-stimulated U937 cells in the absence of extract. Mean ± SEM.

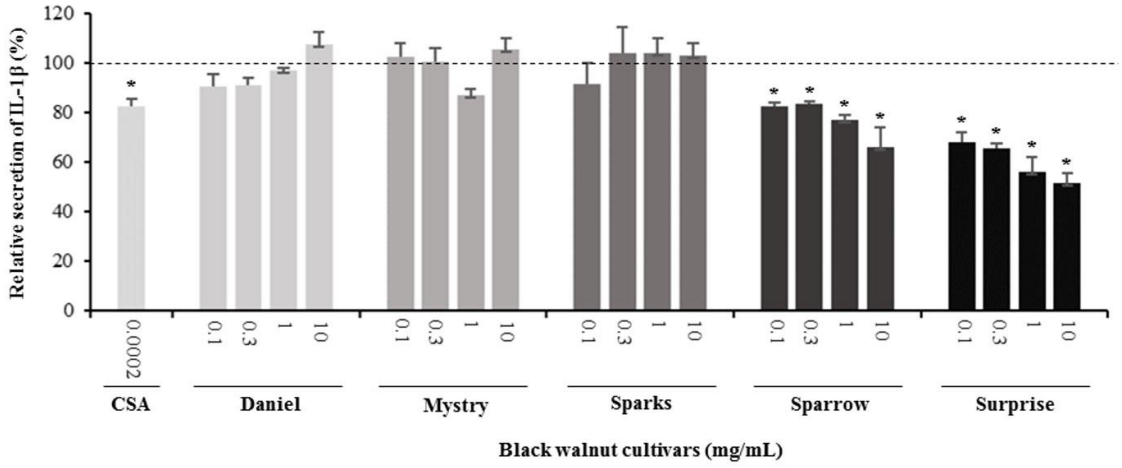


Figure 14. Effect of black walnut extracts on the secretion of IL-1β by PMA-differentiated, LPS-stimulated U937 cells. Cyclosporin A (CSA) was used as a positive control. (*) Significant decrease ($p < 0.01$) compared to PMA-differentiated, LPS-stimulated U937 cells in the absence of extracts. Mean ± SEM.

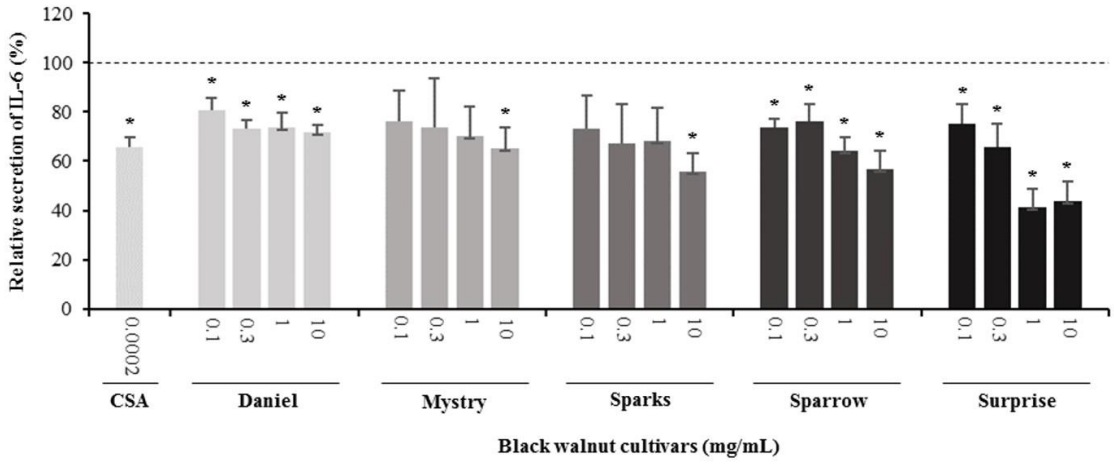


Figure 15. Effect of black walnut extracts on the secretion of IL-6 by PMA-differentiated, LPS-stimulated U937 cells. Cyclosporin A (CSA) was used as a positive control. (*) Significant decrease ($p < 0.01$) compared to PMA-differentiated, LPS-stimulated U937 cells in the absence of extract. Mean ± SEM.

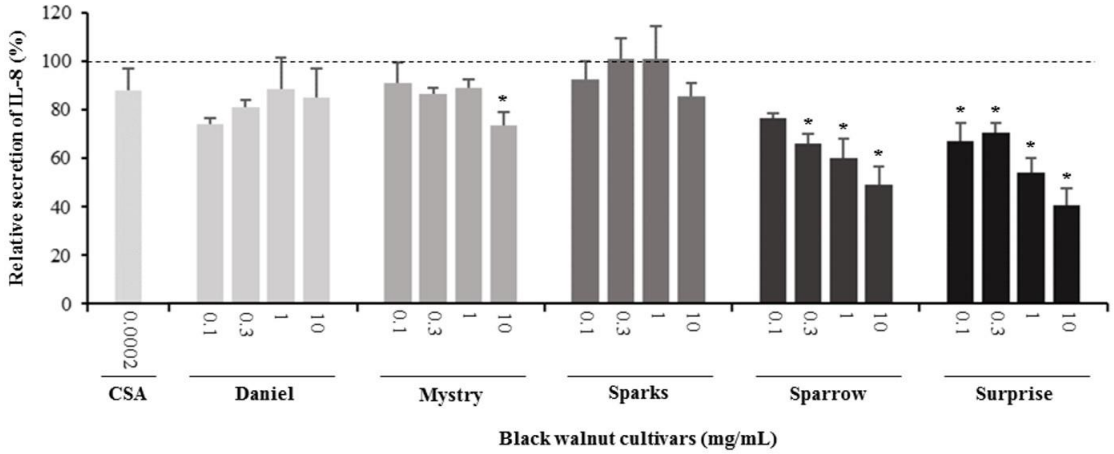


Figure 16. Effect of black walnut extracts on the secretion of IL-8 by PMA-differentiated, LPS-stimulated U937 cells. Cyclosporin A (CSA) was used as a positive control. (*) Significant decrease ($p < 0.01$) compared to PMA-differentiated, LPS-stimulated U937 cells in the absence of extract. Mean ± SEM.

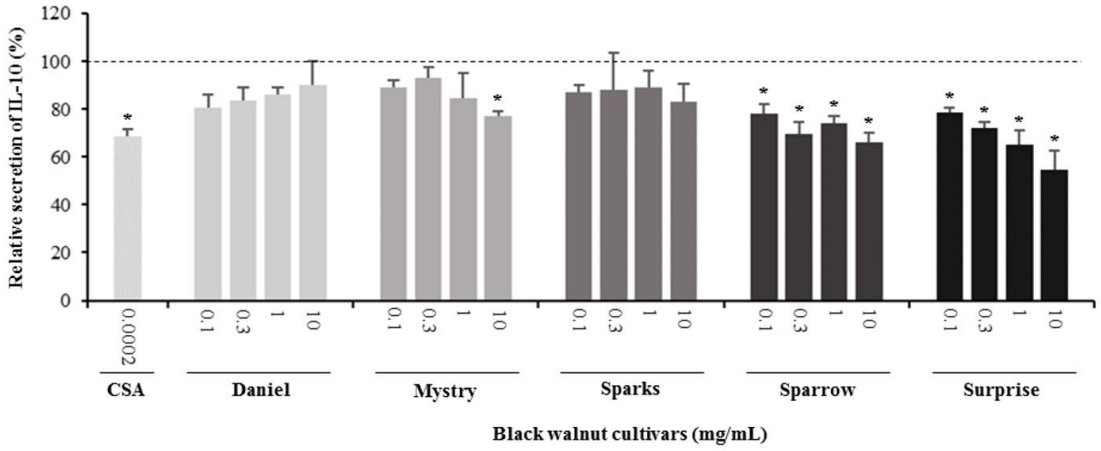


Figure 17. Effect of black walnut extracts on the secretion of IL-10 by PMA-differentiated, LPS-stimulated U937 cells. Cyclosporin A (CSA) was used as a positive control. (*) Significant decrease ($p < 0.01$) compared to PMA-differentiated, LPS-stimulated U937 cells in the absence of extract. Mean ± SEM.

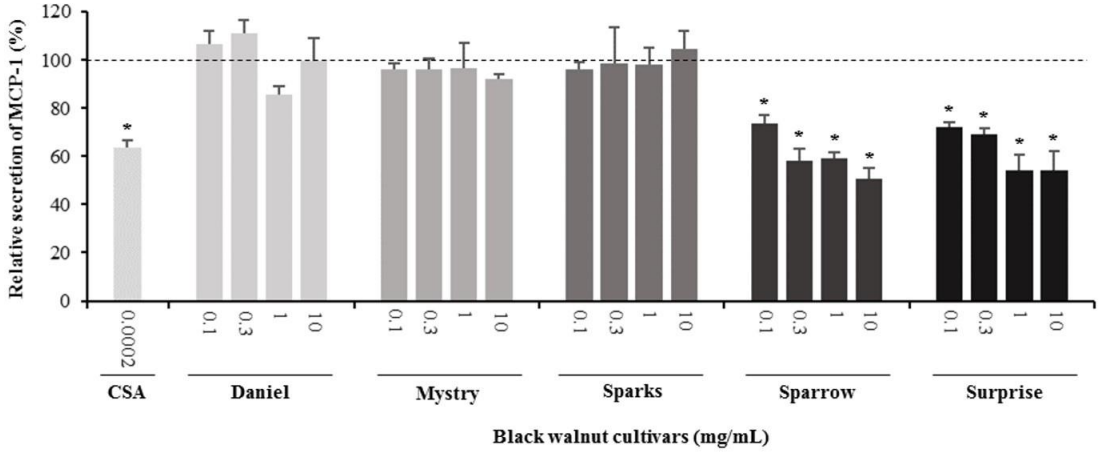


Figure 18. Effect of black walnut extracts on the secretion of MCP-1 by PMA-differentiated, LPS-stimulated U937 cells. Cyclosporin A (CSA) was used as a positive control. (*) Significant decrease ($p < 0.01$) compared to PMA-differentiated, LPS-stimulated U937 cells in the absence of extract. Mean ± SEM.

Minor to no effects were observed in IL-1β, TNF-α, and MCP-1 cytokine levels following pre-treatment with Daniel, Mystry, and Sparks at all 4 concentrations tested. The secretion of IL-6 was significantly inhibited by extracts derived from Daniel (≥ 0.1

mg/ mL), Mystry (10 mg/mL), and Spark (10 mg/mL). More specifically, the extracts of Daniel at the concentrations of 0.1, 1, 0.3, and 10 mg/mL, Mystry and Sparks at a concentration of 10 mg/mL significantly reduced the secretion of IL-6 by 19.5%, 26.7%, 26.5%, 28.5%, 34.9%, and 44.1%, respectively (Figure 15). In addition, the extract of Mystry at a concentration of 10 mg/mL reduced the secretion of IL-8 (26.3%), and IL-10 (22.8%) by PMA-differentiated, LPS-stimulated U-937 cells (Figures 16 & 17).

In order to gain insight into potential mechanism(s) of action, parallel experiments whereby LPS was added 2 hours prior to exposure to extracts were conducted. These experiments focused on Sparrow or Surprise cultivars because of their strong inhibitory effect on cytokine secretion among examined cultivars when the extracts were added prior to the addition of LPS. In these experiments, the cell stimulation and cytokine secretion were initiated before the extracts were added. The results showed that Sparrow and Surprise extracts had no inhibitory effect on the secretion of the 6 cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10, and MCP-1) when LPS was added before the extracts (data not shown).

DISCUSSION

The results of this study demonstrated for the first time that black walnut possesses compounds that exert an inhibitory effect on the secretion of 6 measured cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10, and MCP-1) induced by a human pro-monocytic cell line differentiated with PMA and stimulated with LPS. Cytokines play vital roles in mediating pathological responses in rheumatoid arthritis (RA). TNF, IL-1,

and IL-6 are pivotal cytokines in regulating innate and adaptive immune responses associated with the disease onset and persistence, and TNF and IL-1 are signature innate cytokines in RA. TNF is functionally central to RA pathophysiology, and is involved in leucocyte activation, adhesion and migration, in endothelial-cell adhesion molecule expression, and in stromal-cell, chondrocyte, and osteoclast activation (McInnes et al. 2016); IL-1 plays a role in destruction of bone at inflammatory joint sites, such as in RA, via activation, differentiation, and survival of osteoclasts (Kim et al. 2009) and it also mediates secretion of cytokines from synovial fibroblasts and monocytes, and induces endothelial-cell adhesion molecule expression (McInnes and Schett 2007). IL-6 is a key player in innate and adaptive immune responses, including proliferation, differentiation, cytotoxic T cell activity, antibody production, generation of blood cells and platelets, hepatic acute-phase responses, and neuroendocrine effects (McInnes and Schett 2007). The two proinflammatory cytokines, TNF and IL-6 are therapeutic targets for the treatment of RA (Smolen and Aletaha 2015), whereas inhibition of IL-1 using biologic therapies has not been effective in preventing RA (McInnes et al. 2016). In the present study, we showed that black walnut cultivars Sparrow and Surprise significantly and dose-dependently reduced the secretion of three pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the U-937 model system. Additionally, other cultivars also had an inhibitory effect on IL-6 secretion. Our findings suggest that the extracts from certain black walnut cultivars, such as Sparrow and Surprise, might be promising biologic agents for future investigation on the prevention of inflammatory diseases, such as RA in animal model studies.

Our results also revealed the inhibitory effects of black walnut extracts on three other cytokines/ chemokines (MCP-1, IL-8, and IL-10) in the U-937 model system. MCP-1 and IL-8, members of the CC and CXC chemokine family, respectively, play important roles in acute inflammation. The expression of MCP-1 regulates migration and infiltration of monocytes, memory T cells and dendritic cells (Carr et al. 1994, Deshmane et al. 2009) whereas IL-8 recruits neutrophils and other granulocytes to the site of infection and stimulates phagocytosis (Harada et al. 1994). IL-10 is a potent immune-modulatory cytokine with broad anti-inflammatory properties (O'garra et al. 2008), and is produced by a variety of cells including B cells, Th1, Th2, Th17, T reg, CD8+ T cells, and myeloid cells (Saraiva and O'garra 2010). During inflammation, IL-10 can be produced by regulatory B cells and inhibit the production of TNF (Smallie et al. 2010) as well as inhibit the infiltration and activation of neutrophils in the synovial tissue (Bober et al. 2000). Interestingly, Sparrow and Surprise exhibited inhibitory effects not only on MCP-1 and IL-8 but also on IL-10. Black walnut extracts contain many possible anti-inflammatory metabolites (Figure 11) that might exert the inhibitory effect on the production of IL-8 and MCP-1 (Figure 11), as well as other unidentified metabolites. Some of the unknown metabolites might inhibit the production of IL-10 in the U-937 model system.

Our results indicated a rich source of possible anti-inflammatory bioactive compounds in black walnut. These compounds were tentatively identified through a metabolomics analysis (Figure 11) and many of them including gallic acid, vanillic acid, ellagic acid, quercetin, quercetin 3-*O*-glucoside, naringin, and cinnamic acid, have been confirmed and quantified in black walnut kernels using liquid chromatography-tandem

mass spectrometry (LC-MS/MS) analysis with authentic standards (Vu et al. 2018). The presence of multiple compounds in certain cultivars raises the possibility of synergistic effects on cytokine secretion. We previously identified multiple bioactive compounds (e.g. glansreginin A, azelaic acid, and quercetin) in black walnut that are responsible for antibacterial activities against a Gram-positive bacterium (*S. aureus* RN6390) (Ho et al. 2018). Bioassay-guided fractionation was utilized to isolate and identify the bioactive compounds (based on MS/MS fragmentation data of ions) by a metabolomics approach using high-resolution tandem mass spectrometry (MS/MS) databases (e.g., METLIN, MassBank of North America, MetFrag). Future research will focus on purification, validation, and characterization of compounds responsible for cytokine suppressive activities in Surprise and Sparrow utilizing the same metabolomics strategy. The compounds in black walnut might be useful as natural anti-inflammatory agents to mitigate inflammation.

Interestingly, the inhibitory effect of Surprise and Sparrow cultivars was only observed when U-937 were pre-treated with the extracts of these cultivars before the addition of LPS, whereas the suppressive activity did not occur when U-937 were stimulated with LPS before addition of the extract. These findings suggest the possibility that the extracts are inhibiting cell activation at the level of the receptor rather than affecting downstream signaling pathways. The observed decrease in cytokine levels in the pre-treated experiments could result from the ability of the extract to interfere with the interaction between LPS and its receptor TLR4 and/or co-receptor CD14, or from the direct binding of the extract to the LPS receptor, thereby blocking cell activation. In other words, the extracts might function by preventing LPS from activating U-937 cells.

Importantly, TLR4 receptors are expressed in U-937 cells, and receptor activation leads to a dose-dependent induction of certain inflammatory markers (Vogel et al. 2012). When U-937 were stimulated with LPS before the addition of the extracts, LPS might bind to its receptor and initiate the signaling cascade prior to the addition of the extracts. Additionally, the active compounds may be effective against other receptors and/or affect intracellular signaling pathways. The mechanism of action is not fully understood at this point. Future experiments will be required to elucidate how the active compounds are carrying out their effect.

We also documented that cytokine suppressive activities of black walnut were variable among tested cultivars. Two cultivars (Sparrow and Surprise) exhibited inhibitory effect on the secretion of 6 detected cytokines induced by U-937 differentiated with PMA and stimulated with LPS, while other cultivars (Daniel, Mystry, and Sparks) reduced the secretion of IL-6 alone. Mystry also reduced the secretion of IL-10 and MCP-1, but no inhibitory effect of Mystry on TNF-α, IL-1β, IL-8 was observed. Differences in the suppressive properties of black walnut are likely due to the differences in the composition and proportions of different compounds among these cultivars (Figure 11A). In our previous studies, we also found differences in antibacterial properties among black walnut cultivars (Ho et al. 2018). In fact, Surprise and Mystry exhibited the strongest antibacterial capacities against *S. aureus* among 22 tested cultivars, while Daniel, Davidson, Hay, Jackson, Kwik Krop, Schessler, Sparks, and Sparrow cultivars showed no effect on the inhibition of *S. aureus*. Health-promoting characteristics of black walnut (e.g. anti-inflammatory and antibacterial capacities) could potentially be used to select traits to improve nut quality. Over 700 black walnut cultivars have been recorded

and many of them were selected for nut production in the past century (Williams 1990). Current nut production criteria include yield, percent kernel, leafing date, flowering dates, growth habit, disease resistance, precocity, productivity, and shelling quality (Reid et al. 2004).

CONCLUSION

We demonstrated cytokine suppressive properties of black walnut extracts using the human pro-monocytic cell line U-937. Black walnut kernels contain a wealth of bioactive metabolites putatively identified through a metabolomics approach. The five cultivars (Daniel, Mystry, Sparks, Sparrow, Surprise) tested showed differences in their ability to inhibit the secretion of 6 cytokines/ chemokines (TNF-α, IL-1β, IL-6, IL-8, IL-10, and MCP-1). Sparrow and Surprise showed the strongest inhibitory effects on the secretion of all measured cytokines. Mystry reduced the secretion of IL-6, IL-10 and MCP-1, while Daniel and Sparks only reduced the production of IL-6. Our findings reveal that certain black walnut cultivars may represent promising preventive agents for inflammatory diseases.

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SUPPLEMENTARY INFORMATION

Supplementary Table 1. Phenolic compounds known to produce anti-inflammatory activity and putatively identified in the methanolic extracts of black walnut kernels via a metabolomics approach.

Feature No.	Retention time (min)	Theoretical mass	Exact mass	Δm (ppm)	Adducts	Formula	Putatively identified compound	Anti-inflammatory activity
1	0.59	787.0988	787.0995	0.89	$[M + H]^+$	$C_{34}H_{26}O_{22}$	Tellimagrandin I isomer 1 (di-HHDP glucose isomer)	Kiss and Piwowarski (2018)
2	1.10	785.0832	785.0846	1.78	$[M + H]^+$	$C_{34}H_{24}O_{22}$	Pedunculagin isomer 1 (bis-HHDP-glucose)	Lee et al. (2010)
3	1.23	577.1352	577.1359	1.21	$[M - H]^-$	$C_{30}H_{26}O_{12}$	Procyanidin dimer	Xing et al. (2015)
4	1.87	787.0988	787.0976	-1.52	$[M + H]^+$	$C_{34}H_{26}O_{22}$	Tellimagrandin I isomer 2	Kiss and Piwowarski (2018)
5	2.23	169.0131	169.0129	-1.18	$[M - H]^-$	$C_7H_6O_5$	Gallic acid	Kim et al. (2005)
6	2.35	499.1234	499.1210	-4.81	$[M + H - H_2O]^+$	$C_{25}H_{24}O_{12}$	Dicaffeoylquinic acid isomer 1	Han et al. (2007)
7	2.61	787.0988	787.0995	0.89	$[M + H]^+$	$C_{34}H_{26}O_{22}$	Tellimagrandin I isomer 3	Kiss and Piwowarski (2018)
8	2.74	463.0871	463.0898	5.83	$[M + H - H_2O]^+$	$C_{21}H_{20}O_{13}$	Myricetin hexoside isomer	Wang et al. (2010)
9	3.43	169.0349	169.0490	-2.96	$[M + H]^+$	$C_8H_8O_4$	Vanillic acid	Miles et al. (2005)
10	3.44	785.0832	785.0836	0.51	$[M + H]^+$	$C_{34}H_{24}O_{22}$	Pedunculagin isomer 2	Lee et al. (2010)
11	3.45	783.0675	783.0641	-4.34	$[M + H]^+$	$C_{34}H_{22}O_{22}$	Punicalin isomer 1	Lee et al. (2010)
12	4.37	303.0135	303.0138	0.99	$[M + H]^+$	$C_{14}H_6O_8$	Ellagic acid	Umesalma and Sudhandiran (2010)
13	4.77	319.0448	319.0455	2.19	$[M + H]^+$	$C_{15}H_{10}O_8$	Myricetin	Wang et al. (2010)
14	5.14	463.0882	463.0867	-3.23	$[M - H]^-$	$C_{21}H_{20}O_{12}$	Quercetin 3-*O*-glucoside	Rogerio et al. (2007)
15	5.30	441.0827	441.0790	-8.39	$[M - H]^-$	$C_{22}H_{18}O_{10}$	(-)-Epicatechin 3-*O*-gallate	Kürbitz et al. (2011)
16	6.42	479.1184	479.1191	1.46	$[M + H]^+$	$C_{22}H_{22}O_{12}$	Isorhamnetin hexoside isomer	Abdallah and Esmat (2017)
17	6.59	499.1234	499.1199	-7.21	$[M + H - H_2O]^+$	$C_{25}H_{24}O_{12}$	Dicaffeoylquinic acid isomer 2	Han et al. (2007)
18	8.10	303.0499	303.0498	-0.32	$[M + H]^+$	$C_{15}H_{10}O_7$	Quercetin	Rogerio et al. (2007)
19	10.68	783.0675	783.0678	0.38	$[M + H]^+$	$C_{34}H_{22}O_{22}$	Punicalin isomer 2	Lee et al. (2010)
20	19.21	581.1865	581.1843	-3.78	$[M + H]^+$	$C_{27}H_{32}O_{14}$	Naringin	Golechha et al. (2011)
21	20.84	785.0832	785.0823	-1.15	$[M + H]^+$	$C_{34}H_{24}O_{22}$	Pedunculagin isomer 3	Lee et al. (2010)
22	25.77	149.0597	149.0599	1.34	$[M + H]^+$	$C_9H_8O_2$	Cinnamic acid	Song et al. (2013)
23	28.31	441.0816	441.0817	0.22	$[M + H - H_2O]^+$	$C_{22}H_{18}O_{11}$	(-)-Epigallocatechin gallate	Cavet et al. (2011)
24	32.77	593.1501	593.1532	5.23	$[M + H - H_2O]^+$	$C_{27}H_{30}O_{16}$	Quercetin 3-*O*-neohesperidoside	da Silva et al. (2000)
25	35.59	783.0675	783.0674	-0.13	$[M + H]^+$	$C_{34}H_{22}O_{22}$	Punicalin isomer 3	Lee et al. (2010)
26	39.79	299.0550	299.0557	2.34	$[M + H - H_2O]^+$	$C_{16}H_{12}O_7$	3'-Methylquercetin	Jiang et al. (2006)

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CHAPTER IV: QUANTIFICATION AND CHARACTERIZATION OF BIOLOGICAL ACTIVITIES OF GLANSREGININ A IN BLACK WALNUTS (*JUGLANS NIGRA*)

ABSTRACT

Glansreginin A has been reported as an indicator of the quality of walnuts (*Juglans* spp.). Walnuts are well-known to produce multiple health-promoting benefits that are likely associated with their bioactive constituents, including glansreginin A. However, the bioactive property of glansreginin A has not been adequately explored. In the present study, we quantified concentrations of glansreginin A in black walnuts (*Juglans nigra*) using high resolution liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and performed an array of in vitro bioassays to characterize multiple biological activities (antibacterial, antioxidant, anti-inflammatory capacity) of glansreginin A. Results from HPLC-MS/MS analysis indicated that glansreginin A was presented in all 12 black cultivars examined and its contents were variable among black walnut cultivars, ranged from 6.8 mg/kg (Jackson) to 47.0 mg/kg (Hay). Our results revealed that glansreginin A possessed moderate antibacterial activities against Gram-positive bacteria, with minimum inhibitory concentrations of 100 µg/mL for *Staphyloccocus aureus* and 50 µg/mL for *Bacillus anthracis*. Glansreginin A exhibited no antioxidant activities and did not induced the activity of antioxidant response element (ARE) signaling pathways. Additionally, this compound showed no antiproliferative effects on the growth of both the tumorigenic alveolar epithelial cells and non-

tumorigenic lung fibroblast cells. Our results suggested glansreginin A is one of the major polyphenols in black walnut and has the ability to possess antibacterial activities.

INTRODUCTION

Glansreginin A, a dicarboxylic acid derivative, has been documented as an indicator component of the quality of walnuts (Haramiishi et al. 2020). This compound has been detected as a major constituent in the kernel extracts of English walnut (*Juglans regia*) which is the most important species in the genus of *Juglans* for nut production (USDA 2012, Haramiishi et al. 2020). Glansreginin A has putatively been identified in the kernel extracts of pecan (*Carya illinoinensis*) and hazelnut (*Corylus avellana*) (Slatnar et al. 2014, Jia et al. 2018) and has not been found in other common tree nuts including almond (*Prunus dulcis*), pistachio (*Pistacia vera*), cashew nut (*Anacardium occidentale*), and macadamia nut (*Macadamia* sp.) (Haramiishi et al. 2020). Limited information on the biological functions of glansreginin A is available. Ito et al. (2007) evaluated antioxidant capacities of glansreginin A by SOD like activity and DPPH radical scavenging effects and reported that glansreginin A had no antioxidant activities in both assays. Haramiishi et al. (2020) documented neuroprotective effect of glansreginin A via anti-inflammation in the brain using lipopolysaccharide (LPS)-induced inflammatory model mice. This group reported the LPS-induced abnormal behavior and LPS induced hyper-activation of microglia in the hippocampus were significantly reduced in the LPS-injected mice with oral administration of glansreginin A (50 mg/kg for 8 days) in comparison to those without ingestion of glansreginin A.

Glansreginin A has been putatively identified in another common *Juglans* species, black walnut (*Juglans nigra* L.) that is one of the most economically valuable hardwood species (McGranahan and Leslie 1991). This native plant species is also a highly value tree for edible nut production in North America (Randolph et al. 2013), which is the second highest produced walnut nut in the United States with Missouri being the highest ranking producer (USDA 2012). Our previous study has tentatively identified the presence of glansreginin A in the bioactive fraction in the kernel extract of a black walnut cultivar, Mystry by comparing mass spectral data of this compound in the extract of Mystry with the known spectral data reported in literature (Ho et al. 2018). Since glansreginin A is not commercially available, validation and quantification of concentrations of glansreginin A in black walnut were not investigated in Ho et al. (2018). With respect to the biological function, glansreginin has been found as the major constituent of the bioactive fraction of Mystry that exhibited the antibacterial activity against a Gram-positive bacterium, suggesting that this compound likely possesses antibacterial capacity.

Black walnut has been demonstrated as an excellent food source of health promoting benefits. The extractives from kernels of black walnuts that contain a wealth source of phytochemical compounds have been reported to potentially possess multiple biological functions (antioxidant, antibacterial and anti-inflammatory activities) (Ho et al. 2018, Ho et al. 2019, Vu et al. 2019, Ho et al. 2020a). Glansreginin A has recently documented as the indicator for quality of walnut (Haramiishi et al. 2020), but this compound has not been characterized in black walnuts. In addition to this, the bioactive activities of glansreginin A have not been adequately characterized. In the present study,

we validated the presence of glansreginin A in Mystry and identified the concentrations of glansreginin A in 12 black walnut cultivars that were selected for nut production. We then characterized multiple biological activities (antibacterial, antioxidant, anti-inflammatory capacity) of glansreginin A using in vitro bioassays. The characterization of the biological functions of glansreginin A, a key bioactive compound in walnuts, could promote the development of novel applications of black walnut and its by-products and might point out underexplored bioactive activities of black walnuts as well as other tree nuts in the genus of *Juglans*.

MATERIAL AND METHODS

Black walnut cultivars

The nuts of 12 black walnut cultivars including Bowser, Davidson, Emma, Hay, Hare, Jackson, Mystry, Schessler, Sparks 147, South Fork, Surprise, and Thomas were collected in November 2017 at the University of Missouri, Horticulture and Agroforestry Research Center (New Franklin, MO, USA). The black walnut trees were well cultivated for nut production with typical practices (e.g., lime, pruning, fertilization, weed control, and pest and disease management) (Reid et al. 2009). After the black walnut nuts were collected, these nuts were husked mechanically and dried at 24 °C for 15 days and the hulled nuts were then stored at -20 °C until analysis.

Kernel extraction

The hulled nuts were cracked manually and then the shell were removed to collect the kernels of black walnuts. The kernels were homogenized using a coffee grinder

(CBG100S, Black + Decker, Beachwood, OH, USA). Subsequently, the homogenized samples (100 g, 20–30 mesh) were extracted in 400 ml methanol (HPLC grade, Fisher Scientific, Pittsburg, PA, USA) twice and then sonicated at 10 °C for 60 min, followed by centrifugation for 10 min at 4,000 rpm. The methanolic extracts were filtered through a 0.2 µm Whatman filter paper (GE Healthcare, Chicago, IL, USA) under SPE Vacuum Manifold (Visiprep^{TM} SPE Vacuum Manifold, Sigma-Aldrich, St. Louis, MO, USA). The resulting supernatant was then collected and stored at -20 °C until analysis.

Validation and quantification of glansreginin A in black walnut kernels

Our previous study has putatively identified the presence of glansreginin A in the bioactive fraction in the kernel extract of a black walnut cultivar, Mystry by comparing mass spectral data of this compound in the extract of Mystry with the known spectral data reported in MetFrag metabolite database (msbi.ipb-halle.de/MetFragBeta/) (Ho et al. 2018). Since glansreginin A is not commercially available, concentrations of this compound in black walnut kernels were not determined in Ho et al. (2018). In the present study, a purified glansreginin A that was obtained from a closely related species of black walnut (English walnut) was used for the validation and the determination of the contents of glansreginin A in 12 black walnut cultivars. The purified compound was extracted and isolated from English walnut kernels as described in Ito et al. (2007). The purified compound was formed as a pale yellow amorphous powder.

Validation of glansreginin A

The presence of glansreginin A in the bioactive fraction of Mystry was validated by comparing the mass spectra of this compound in the Mystry fraction with the mass

spectra of the purified compound derived from English walnut. The bioactive fraction of Mystry was obtained from Ho et al. (2018). Mass spectral data of the purified compound spiking to the bioactive fraction and the blank (solvent) were also generated as controls. The mass spectra of all samples (2 µL per injection) were generated by an ultra-high performance liquid chromatography (UHPLC) system coupled to a maXis impact quadrupole-time-of-flight high-resolution mass spectrometer (Q-TOF) (Bruker Co., Billerica, MA, United States) as described previously (Ho et al. 2018). The system was operated in a negative electrospray ionization mode with the nebulization gas pressure at 43.5 psi, dry gas of 12 L/min, dry temperature of 250 °C and a capillary voltage of 4000 V. The mass spectral data were collected at retention time (rt) of 7.18 min and m/z of 592.2043.

Quantification of glansreginin A

The concentrations of glansreginin A in the kernels of 12 different black walnut cultivars was determined using a Waters Alliance 2695 High Performance Liquid Chromatography (HPLC) system coupled with Waters Acquity TQ triple quadrupole mass spectrometer (MS/MS), as described in (Ho et al. 2020b). The system was operated in negative electrospray ionization mode with the nebulization gas pressure at 43.5 psi, dry gas of 12 L/min, dry temperature of 250 °C and a capillary voltage of 1500 V. The kernel extracts of black walnut (20 mL per injection) were separated by a Phenomenex Kinetex C18 reverse-phase column (100 × 4.6 mm; 2.6 µm particle size, Torrance, CA, United States). The MS/MS system was operated in the multi-reaction monitoring (MRM) mode with the optimized collision energy. Waters IntelliStart optimization software was used to optimize collision, ionization energy, MRM transition ions

(molecular and product ions, 592 → 403/343), capillary and cone voltage, desolvation gas flow, and collision energy. Assessment of the sensitivity of the analytical method was determined by calculating limit of detection (LOD) and limit of quantification (LOQ) for glansreginin A that were identified by employing signal-to-noise ratios of three and ten. The concentrations of glansreginin A in the extracts were determined based on a standard curve for this compound generated using the purified glansreginin A at 8 concentrations (0.1, 0.5, 1, 5, 10, 25, 50 and 100 ppm) in six replicates.

Exploration of bioactive activities of glansreginin A in vitro

Antibacterial activity

The purified glansreginin A derived from kernels of English walnut was used to identify antibacterial, antioxidant and anticancer capacities of this compound. The antibacterial capacities of glansreginin A were evaluated on the growth of two Gram-positive bacteria (*Staphyloccocus aureus* strain RN 6390 and *Bacillus anthracis*) using agar-well diffusion assay as described by Holder and Boyce (1994). These bacteria were obtained from Dr. George C. Stewart's lab (University of Missouri, Columbia, Missouri, USA) and were cultured on Luria-Bertani (LB) agar plates as described in Ho et al. (2018). Briefly, the bacteria were cultured in 5 mL LB broth at OD_{600} of 0.02 in a shaker at 37 ^{o}C. Once the culture reached an OD_{600} of 0.1, the bacteria were swab-inoculated onto LB agar plates. Subsequently, a cork borer was used to punch the surface of the agar and the cutting agars were removed to create punched wells (4.5 mm in diameter, 20 wells per plate) in the plates. The compounds tested (10 µL per well) were pipetted into

the punched wells and the plates then incubated under aerobic condition at 37 ℃ for 16 h. Glansreginin A and chlortetracycline (Sigma-Aldrich), a known antibacterial agent, were tested at 9 final concentrations in 2 × serial dilution, ranging from 1.56 - 400 µg/mL. The diluting solvent (DMSO at 100%) was served as vehicle controls. Each treatment was replicated at least 4 times in different LB agar plates. MIC values (minimum inhibitory concentration) of glansreginin A and chlortetracycline were visually identified for each bacterium tested. The diameters of inhibition zones were measured by a ruler, with an accuracy of 0.5 mm. Each inhibition zone was measured three times.

Total antioxidant capacity

The total antioxidant capacity of glansreginin A was determined by a total antioxidant capacity (TAC) colorimetric assay kit (K274-100, BioVision, CA, USA), according to the manufacturer's instructions. Glansreginin A was evaluated its total antioxidant activity at 7 concentrations of 0, 2.5, 15, 30, 80, 120, and 250 µM, whereas DL-sulforaphane and tert-butylhydroquinone (TBHQ), which were used as positive controls, were tested at 7 concentrations of 0, 2.5, 10, 45, 100, 220, and 300 µM. Trolox was included to standardize the antioxidant capacity, as recommended by the manufacturer. For the assays, the tested compounds were added to 384-well plates, followed by an addition of Cu^{2+} working solution (12.5 µL/ well) into the sample wells. The plates were incubated for 1.5 h at room temperature. Subsequently, the absorbance of the samples was then read at 570 nm using a microplate reader (Enspire, Perkin Elmer Inc., Waltham, MA, USA). The total antioxidant capacity of the tested compounds was calculated from a standard curve of Trolox at 7 concentrations (0, 5, 10, 20, 40, 80, and 120 µM) and was expressed as Trolox equivalent (µM).

Antioxidant response element (ARE) activation

The influence of glansreginin A on the activity of ARE signaling pathways that regulates expression of genes encoding over 250 antioxidant and detoxification proteins (Surh et al. 2008) was determined using Steady-Glo® Luciferase assay system (E2510, Promega, Madison, WI, USA). An Nrf2 ARE reporter HepG2 cell line (HepG2-ARE), a stably transfected liver cell line expressing a firefly luciferase gene under the control of the ARE, was purchased from BPS Bioscience (San Diego, CA, USA). The HepG2-ARE cells were grown in modified Eagle's medium (MEM) supplemented with GlutaMAX, 10% fetal calf serum (FBS) and 600 µg/mL Geneticin (Thermo Fisher Scientific, Waltham, MA, USA), and maintained at 37 °C in a humidified incubator with 5% CO_2.

For the assays, the HepG2-ARE cells were seeded at a density of 10^4 cells/well in 384-well plates that contain the complete media (50 µL per well) using a Multidrop Combi dispenser (Thermo Fisher Scientific, Waltham, MA, USA). The plate cultures were incubated at 37 °C in a 5% CO_2 humidified incubator for 20 h. Subsequently, the HepG2-ARE cells were incubated with glansreginin A at 7 concentrations (as described above) for 18 h. The known ARE activator TBHQ at 7 concentrations (as described above) was used as a positive control and the cells treated with 0.35% DMSO and without the tested compounds served as a vehicle control. The cells without additions of DMSO and tested compounds were used for measuring the background luminescence. Steady-Glo® luciferase assay reagent (Promega) was added at 25 µL per well for 30 min to measure the reporter activity using the Multidrop Combi dispenser (Thermo Fisher Scientific). Enspire microplate reader (Perkin Elmer Inc.) was used to read the luminescence intensities of the 384-well plates. The ARE fold induction of the

compounds was calculated by dividing the luminescence absorbance of the treatment by the specific luminescence absorbance of the vehicle control.

Cell proliferation assays

The anticancer activities of glansreginin A were identified by evaluating the effects of this compound on the growth of the tumorigenic alveolar epithelial cells (A549) and non-tumorigenic lung fibroblast cells (MRC-5) using CellTiter-Glo® cell viability assay kit (G7571, BioVision, CA, USA). The A549 and MRC-5 cells were purchased from American Type Culture Collection (ATCC) (CCL-185 and CCL-171, ATCC, Manassas, VA, USA). These cells were grown in RPMI medium supplemented with 10% FBS and maintained at 37 °C in a humidified incubator with 5% CO_2. For the assays, the A549 and MRC-5 cells at densities of 8×10^3 and 3×10^3 cells per well, respectively, were seeded in 384-well plates and the plates were incubated at 37 °C in a 5% CO_2 humidified incubator. Subsequently, the cultures were then treated with glansreginin A and DL-Sulphorane (a known antiproliferative agent as a positive control) at 7 final concentrations (as mentioned above) and a vehicle (0.35% DMSO) and then the treated cultures were incubated for 72 h. Matrix Wellmate dispenser (Thermo Fisher Scientific) was used to dispense CellTiter-Glo Luminescent assay reagent (10 µL per well) (Promega) into the 384-well plates. The plates were incubated for 20 min at room temperature. An Enspire microplate reader (Perkin Elmer Inc.) was used to read the luminescence. Relative cytotoxicity (%) of the compounds was determined by dividing the specific luminescence absorbance of the treated samples by the specific luminescence absorbance of the vehicle control and multiplying by 100.

Data analysis

The concentrations of glansreginin A in different black walnut cultivars obtained from LC-MS/MS analysis were analyzed as a completely randomized design using PROC MIXED in SAS 9.4 (SAS Institute, Cary, NC, United States). The black walnut cultivar (treatment) was the fixed effect and replication was the random variable. Differences among the concentrations of glansreginin A in the black walnut cultivars were determined using Fisher's LSD at $p < 0.05$.

For total antioxidant capacity, GraphPad Prism 8 (San Diego, CA, USA) was used to generate linear regression equation for each compound. Coefficients of the compound equations were compared with that of the Trolox control to determine the relative total antioxidant capacity of each compound. Fold-increase over Trolox was determined by dividing the coefficient of the compound models by that of the Trolox control. For antibacterial and anticancer activities, non-linear regression analysis was performed to identify the dose-response curve for each compound using GraphPad Prism 8. Half maximal inhibitory concentrations (IC_{50} values) of each compound tested were determined from the dose-response curve for the A549 and MRC-5 cell lines.

RESULTS

Validation the presence of glansreginin A in black walnuts

The purified glansreginin A derived from English walnut kernels, the bioactive fraction of Mystry, and the sample with the purified compound spiking to the bioactive fraction had UHPLC chromatograms at a wavelength of 267 with the same major peak

(Figure 19). Glansreginin A in the active fraction of Mystry at retention time at 7.17 was the same as the purified compound at retention time at 7.13 min. The tandem MS and MS/MS spectra of the major peak in the purified compound, the bioactive fraction of Mystry, and the sample including the purified compound and the bioactive fraction were similar (Figure 20).

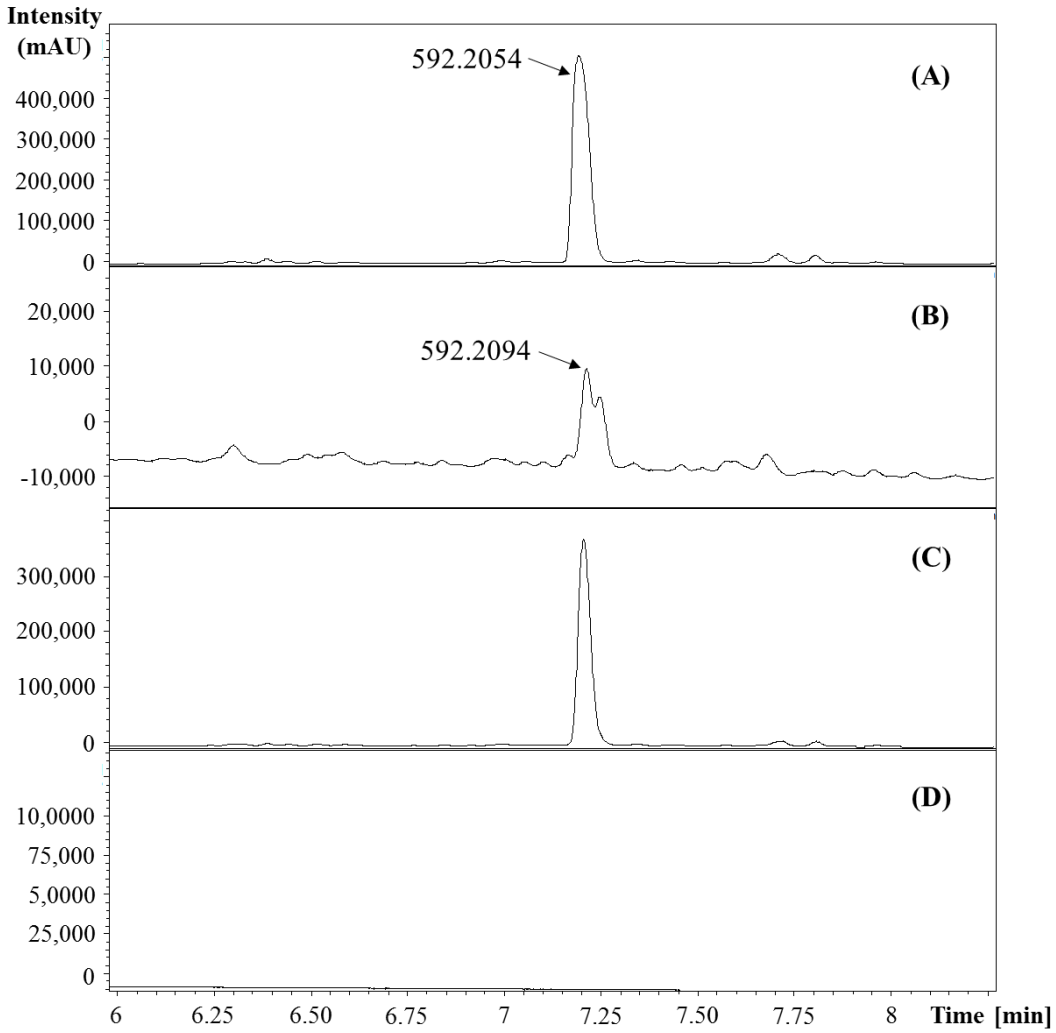

Figure 19. UHPLC chromatograms (detected at 267 nm) of glansreginin A (A), the bioactive fraction in Mystry (B), glansreginin A spiking to the bioactive fraction (C), the solvent (D).

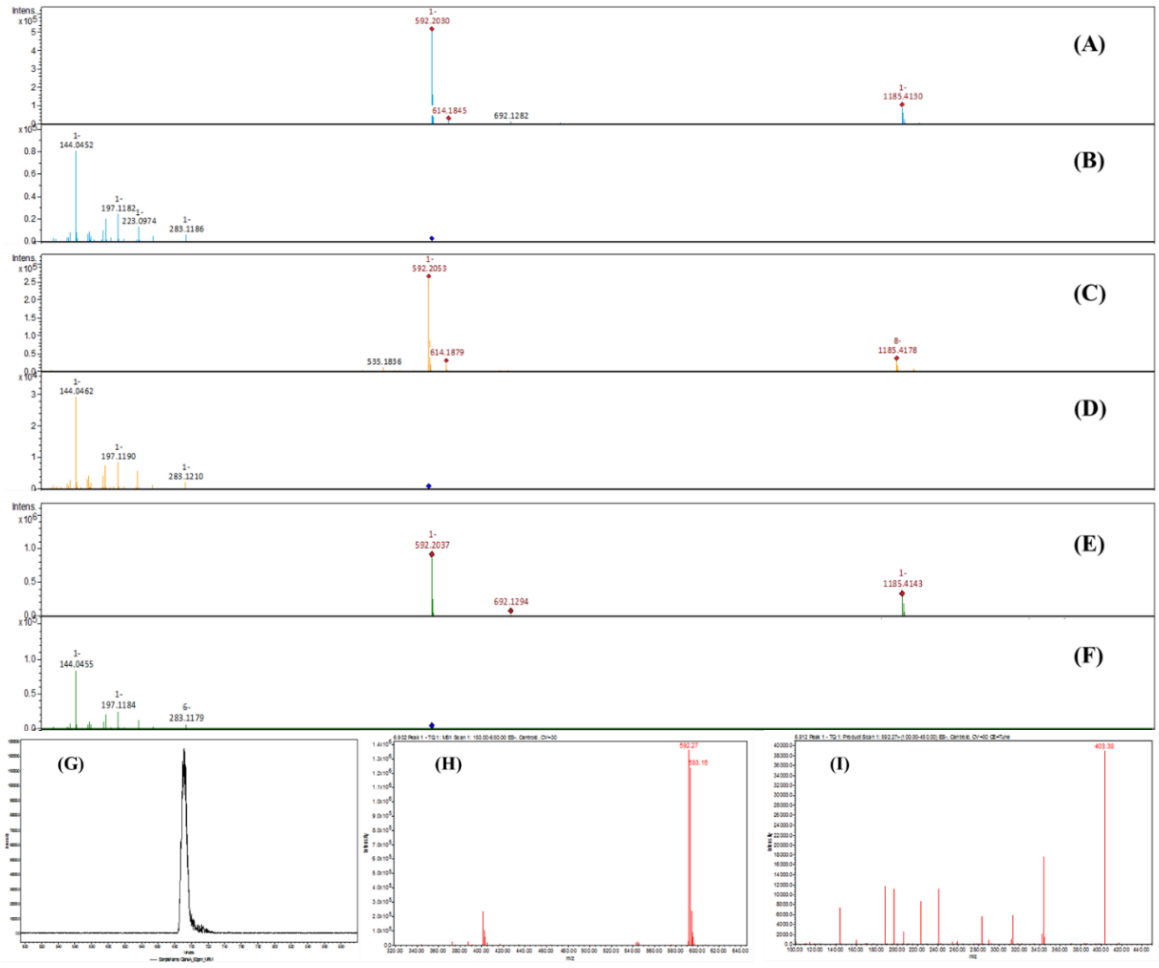

Figure 20. Mass spectra (MS and MS/MS) of purified glansreginin A (A, B and H, I), the bioactive fraction in Mystry (C, D), glansreginin A spiking to the bioactive fraction (E, F). (G): UV chromatogram of the purified glansreginin A.

Quantification of glansreginin A in black walnuts

Glansreginin A was detected in the kernels of all 12 black walnut cultivars. The concentrations of this compound varied among the examined cultivars (Figure 21). This compound was found to be at the highest amount in Hay (47.0 ± 4.0 mg/kg), followed by Emma (38.1 ± 5.3 mg/kg) and Thomas (29.1 ± 4.3 mg/kg), Schessler (25.3 ± 4.3 mg/kg),

Hare (24.2 ± 5.2 mg/kg), and Bowser (20.4 ± 4.0 mg/kg). Compared to these cultivars, the contents of glansreginin A in other tested cultivars were significantly smaller. Specifically, the concentrations of glangreginin A in Surprise, Mystry, Davidson, South Fork and Jackson were 17.3 ± 7.6 mg/kg, 14.5 ± 1.4 mg/kg, 13.1 ±3.3 mg/kg, 10.9 ± 3.0 mg/kg, 6.8 ± 2.3 mg/kg, respectively.

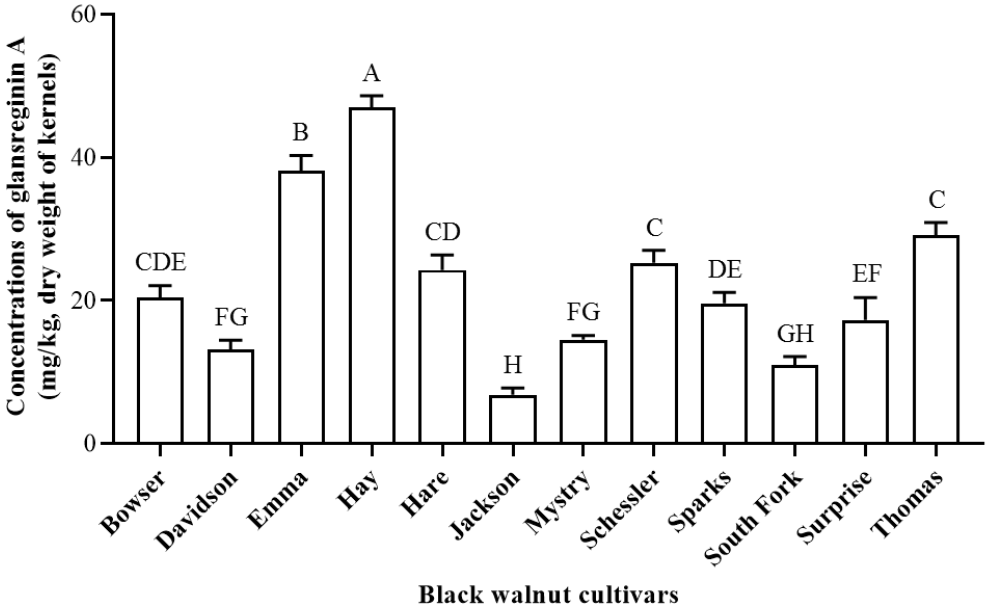

Figure 21. Contents of glansreginin A (mg/kg of dry weight) in kernels of 12 black walnut cultivars. Means with bars followed by different letters are significantly different ($p < 0.05$). Means ± SD.

Bioactive activities of glansreginin A

Antibacterial activities

The MIC values of glansreginin A for *B. anthracis* and *S. aureus* were 50 µg/mL and 100 µg/mL, respectively, whereas the MIC values of chlortetracycline for *B.*

anthracis and *S. aureus* were <1.56 µg/mL and 6.25 µg/mL, respectively. Figure 22 shows the zone of inhibition of glansreginin A and chlortetracycline that were positively correlated with increases in the concentrations of these compounds.

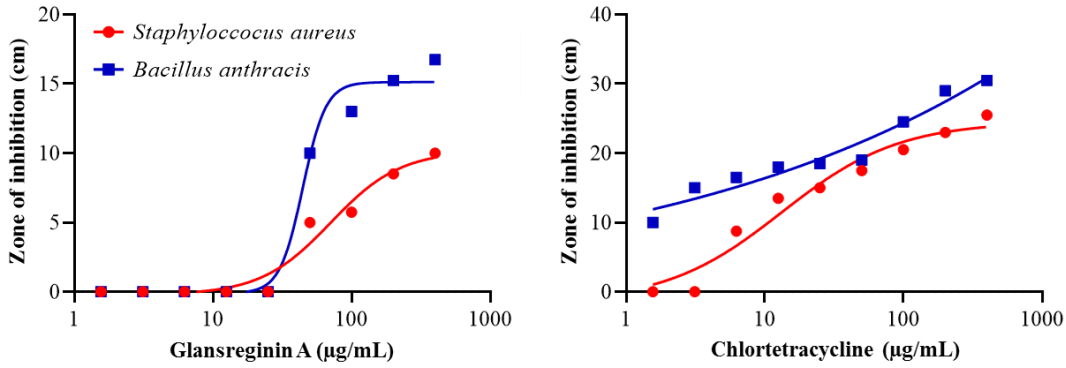

Figure 22. Zones of inhibition of glansreginin A and chlortetracycline for *Bacillus anthracis* and *Staphyloccocus aureus*.

Antioxidant activities and ARE activation

Linear regression models of each compound tested were generated and compared with the Trolox control. The R^2 values of all models were high (>0.98), indicating that these models were reliable (Table 9). Compared with the control (Trolox), the total antioxidant capacity of glansreginin A were significantly lower, whereas TBHQ and DL-sulforaphane exhibited higher total antioxidant capacity (Figure 23). The fold-increase over Trolox of glansreginin A was 0.12 which is 8.3 times lower compared with Trolox. TBHQ and DL-sulforaphane had the fold-increase over Trolox of 10.44 and 1.23, respectively.

Table 9. Antioxidant activities of phenolic compounds in black walnut.

No.	Compound	Slope (in Trolox Equivalents)	R square	Fold-increase over Trolox
1	Trolox	$0.00100 \pm 6.5\times10^{-6}$	0.999	1.0
2	Glansreginin A	$0.00012 \pm 2.3\times10^{-6}$	0.988	0.12
3	text - Butylhydroquinone	$0.01044 \pm 5.9\times10^{-4}$	0.995	10.44
4	DL - Sulforaphane	$0.00123 \pm 7.0\times10^{-5}$	0.999	1.23

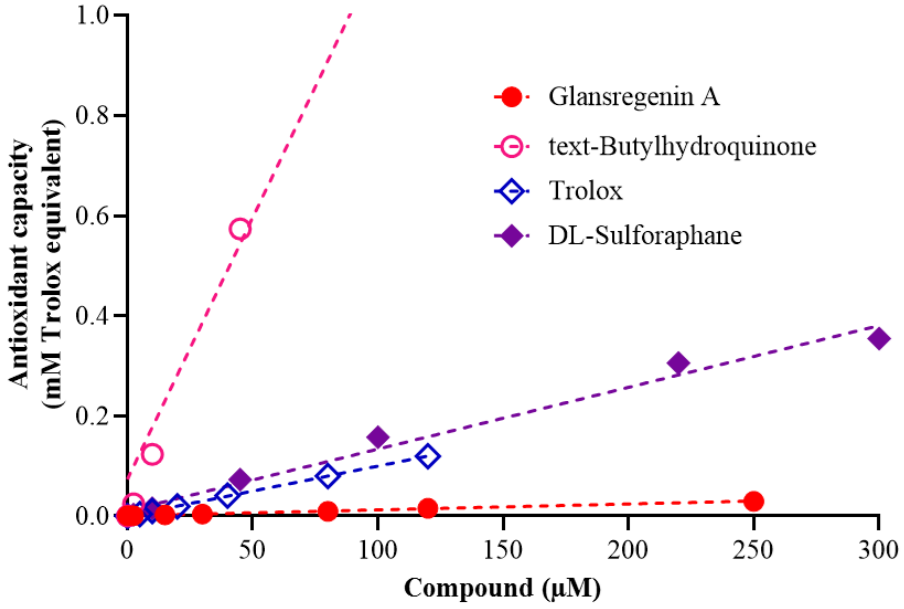

Figure 23. Total antioxidant activity of Glansreginin A, Trolox, text - Butylhydroquinone, DL-Sulforaphane.

Antioxidant Response Element (ARE) activation

Glansreginin A at tested concentrations was not toxic to the HepG2-ARE cells. The ARE fold-increase in HepG2-ARE activation of glansreginin A at tested

concentrations relative to the control was <2 **(**Figure 24**)**. Glansreginin A exhibited a very low total antioxidant capacity and the ARE fold-increase in HepG2-ARE, this compound could not be considered to possess ARE induction activity.

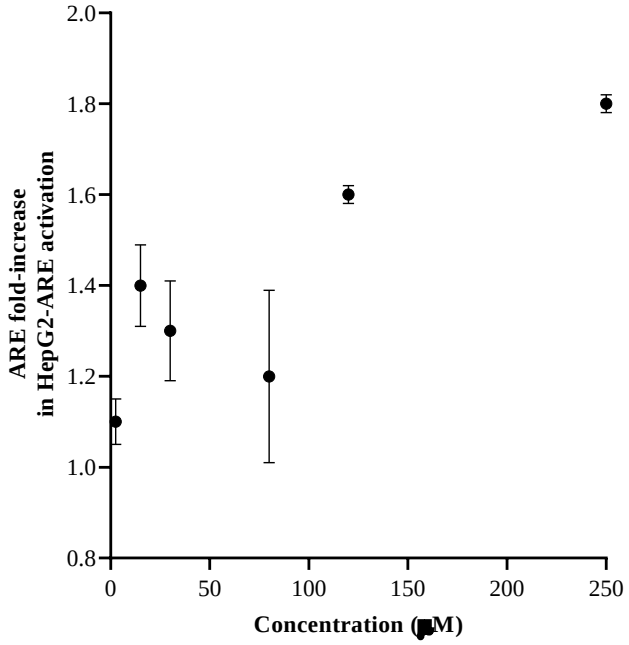

Figure 24. Antioxidant response element (ARE) activation activities of glansreginin A in HepG2-ARE cells.

Anticancer activities

The cytotoxic effects of glansreginin A on the growth of A549 and MRC-5 cells were determined using cell viability assays. A reduction in cell viability or cell number results to a reduction in luminescence absorbance. Results from the assays showed that the vehicle control (DMSO at 0.35%) exhibited no effect on cell number or viability in the A549 and MRC-5 cells, revealing that a reduction in luminescence absorbance in the presence of the tested compounds would indicate a toxic effect of the compounds rather

than the vehicle. The IC_{50} values of glansreginin A were higher than 250 for both A549 and MRC-5 cells (Table 10), while the IC_{50} values of Trolox were higher than 120 for the tested cell lines. The positive control (DL-Sulforaphane) and TBHQ had IC_{50} values for A549 and MRC-5 cells were 16.87 and 9.27, and 139.2 and 108.5, respectively (Figure 25).

Table 10. Half maximal inhibitory concentrations (IC_{50}) of Glansreginin A (μM) in A549 and MRC-5 cell lines.

No.	Compound	A549 cell line	MRC-5 cell line
1	Glansreginin A	>250	>250
2	Trolox	>120	>120
3	text - Butylhydroquinone	139.2	108.5
4	DL-Sulforaphane	16.87	9.27

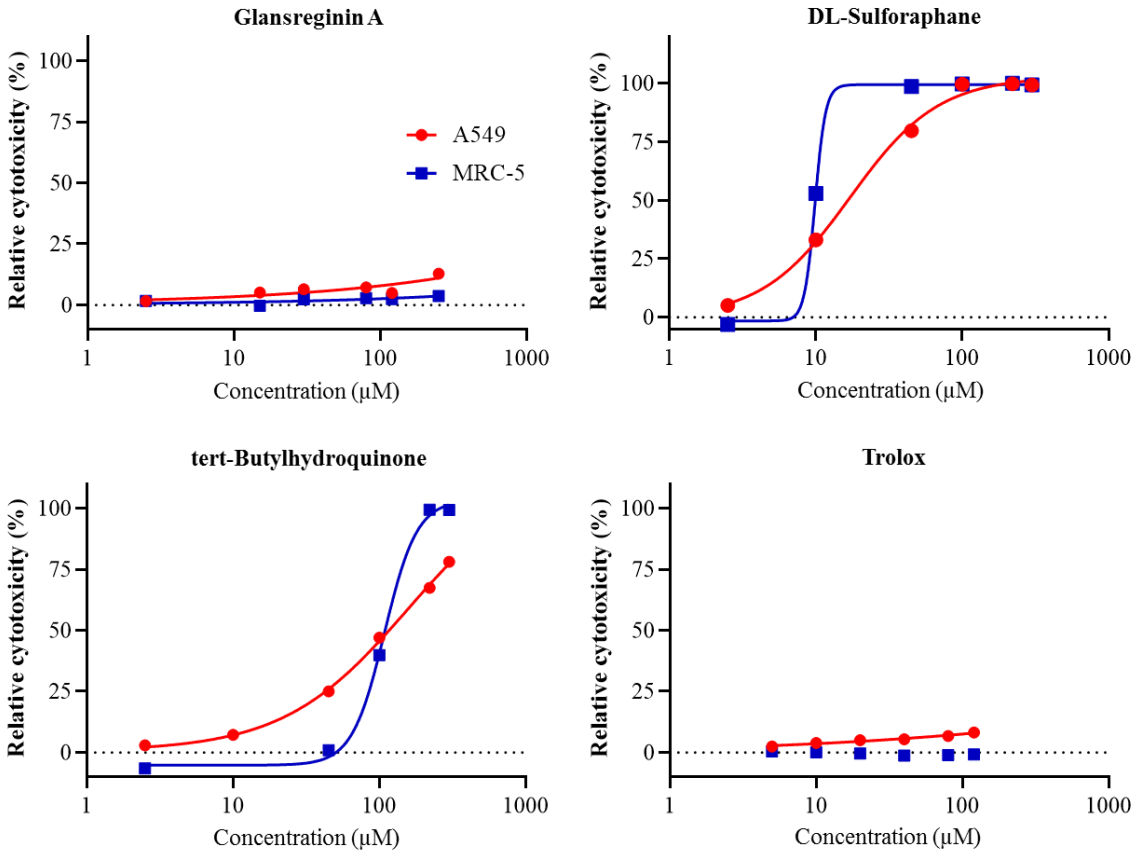

Figure 25. Cytotoxicity (%) of glansreginin A, Trolox, text - Butylhydroquinone, DL-Sulforaphane in A549 and MRC-5 cell lines. Data are expressed as percentages of cytotoxicity in A549 and MRC-5 cells treated with DMSO and the compounds compared with the corresponding vehicle controls that were A549 and MRC-5 cells treated with 0.35% DMSO only.

DISCUSSION

Glansreginin A has recently documented to represent for the quality of walnuts. This compound likely contributes to the biological activities of black walnuts which have been demonstrated as a promising natural source for the medicinal and pharmaceutical industries. In this study, the contents of glansreginin A in the kernels of 12 black walnut cultivars selected for nut production using HPLC-MS/MS analysis were identified and

the biological properties of glansreginin A (antibacterial, antioxidant and anticancer activities) were characterized via an array of in vitro bioassays. The exploration of the biological functions of glansreginin A, a signature compound in walnuts could promote the development of novel applications of black walnut and its by-products in cosmetic and pharmaceutical industries. This could result in profitable value-added products from the abundant, low-value, renewable materials from black walnut, which potentially increases the sustainability of the black walnut agro-industry.

Our previous study has putatively identified glansreginin A in the bioactive fraction in the kernel extract of a black walnut cultivar, Mystry based on the mass spectral information of this compound available in MetFrag metabolite database (Ho et al. 2018). In the current study, glansreginin A was successfully validated in the bioactive fraction from Mystry using a purified glansreginin A derived from English walnut. Our results also that the presence of glansreginin A in all black walnut cultivars examined. Its concentrations varied among the tested cultivars, ranged from 6.8 mg/kg (Jackson) to 47.0 mg/kg (Hay). Among the tested cultivars, Mystry contained a moderate amount of glansreginin A (17.7 mg/kg). Black walnuts have been reported to contain a wealth source of polyphenols such as syringic acid, ellagic acid, and hydrolyzable tannins (Colaric et al. 2005, Slatnar et al. 2015, Figueroa et al. 2016, Vu et al. 2018). The contents of phenolic compounds were found to be highly variable. Ellagic acid has been reported as one of the most abundant polyphenols in black walnuts. Figueroa et al. (2016) reported the concentrations of ellagic acid of 217.3 - 704.7 mg/kg, whereas its concentrations in a range of 11.4 - 97.7 mg/kg in other studies. Vu et al. (2018) characterized phenolic compounds in black walnut and reported a variation in the

concentrations of 16 phenolic compounds detected among and within 11 black walnut cultivars. Ellagic acid was found as the most abundant phenolic compounds in black walnuts, and its concentrations ranged from 9 mg/kg (Tomboy) to 72 mg/kg (Surprise), whereas other phenolic compounds were mostly less than 10 mg/kg in the black walnut cultivars. Compared to the known phenolic compounds in black walnut, glansreginin A presented at a relatively high levels in the kernels of black walnuts.

In order to characterize components of walnuts, Haramiishi et al. (2020) compared the extract derived from English walnut with those of other common tree nuts (almond, pistachio, hazelnut, cashew nut, pecan nut, and macadamia nut) using HPLC analysis. The authors reported that glansreginin A was only present in the walnut extract and was not observed in any other extracts. This group further reported the presence of glansreginin A in every commercial walnut and suggested that glansreginin A as the indicator components in walnuts. Ito et al. (2007) extracted and isolated glansreginin A from a English walnut cultivar, Chandler and obtained 94.2 mg/kg of glansreginin A of dry weight of the kernels. Later, Gómez-Caravaca et al. (2008) quantified the concentrations of glansreginin A in three English walnut cultivars using chlorogenic acid as a referenced standard and estimated its concentrations in a range of 76.3 mg/kg (Chandler) - 335.6 mg/kg (Howard). Compared to English walnuts, black walnuts contain much lower levels of glansreginin A with its concentrations in the kernels (6.8 mg/kg - 47.0 mg/kg). Noticeably, glansreginin A has been putatively identified in hazelnuts and pecan nuts with the reference to the known mass spectral data of this compound (Slatnar et al. 2014, Jia et al. 2018). Using a gallic acid calibration curve, Slatnar et al. (2014)

estimated the concentrations of glansreginin A in the kernels of four hazelnut cultivars in a range of 18 mg/kg (Istrska dolgoplodna leska) - 110.9 mg/kg (Fertile de Coutard).

Our results revealed that glansreginin A exert moderate antibacterial capacities and no antioxidant and anticancer activities. Glansreginin A exhibited antibacterial activities against both Gram-positive bacteria (*B. anthracis* and *S. aureus*) tested. This compound had MIC values of glansreginin A for *B. anthracis* and *S. aureus* were 50 µg/mL and 100 µg/mL, which were lower compared to chlortetracycline, a known antibacterial agent. Additionally, glansreginin A exhibited much lower levels of the total antioxidant capacity compared to Trolox and the ARE fold-increase in HepG2-ARE activation of this compound was <2. This compound did not induce the activity of ARE signaling pathways that functionally regulate the expression of genes encoding over 250 antioxidant and detoxification proteins (Surh et al. 2008) since its ARE fold-increases relative to the control were less than 2 (Roy et al. 2019). Furthermore, glansreginin A showed no antiproliferative activities against both the tumorigenic alveolar epithelial cells and non-tumorigenic lung fibroblast cells. Glansreginin A at the highest concentration tested did not show a significantly inhibitory effect on the growth of the examined cell lines. So far, only a few studies have examined biological functions of glansreginin A. Haramiishi et al. (2020) reported anti-inflammatory activity of glansreginin A via neuroprotective effect on the brain of LPS-injected mice. The LPS-injected mice consumed glansreginin A (50 mg/kg for 8 days) had a significant reduction of LPS-induced abnormal behavior and LPS induced hyper-activation of microglia in the hippocampus, compared to those without the consumption of glansreginin A. Glansreginin A has previously reported to possess no antioxidant activities evaluated by

SOD like activity and DPPH radical scavenging effects (Ito et al. 2007). Haramiishi et al. (2020) documented neuroprotective effect of glansreginin A via anti-inflammation in the brain using lipopolysaccharide (LPS)-induced inflammatory model mice. This group reported the LPS-induced abnormal behavior and LPS induced hyper-activation of microglia in the hippocampus were significantly reduced in the LPS-injected mice with oral administration of glansreginin A (50 mg/kg for 8 days) in comparison to those without ingestion of glansreginin A.

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CHAPTER V: PROFILING ANTICANCER AND ANTIOXIDANT ACTIVITIES OF PHENOLIC COMPOUNDS PRESENT IN BLACK WALNUTS (*JUGLANS NIGRA*) USING A HIGH-THROUGHPUT SCREENING APPROACH

ABSTRACT

Our recent studies have demonstrated multiple health-promoting benefits from black walnut kernels. These biological functions of black walnuts are likely associated with their bioactive constituents. Characterization of phenolic compounds found in black walnut could point out underexplored bioactive activities of black walnut extracts and promote the development of novel applications of black walnut and its by-products. In the present study, we assessed bioactivity profiles of phenolic compounds identified in the kernels of black walnuts using a high-throughput screening (HTS) approach. Black walnut phenolic compounds were evaluated in terms of their total antioxidant capacity, antioxidant response element (ARE) induction, and anticancer activities. The anticancer activities were identified by evaluating the effects of the phenolic compounds on the growth of the tumorigenic alveolar epithelial cells (A549) and non-tumorigenic lung fibroblast cells (MRC-5). Out of 16 phenolic compounds tested, several compounds (penta-*O*-galloyl-*β*-D-glucose, epicatechin gallate, quercetin, (–)-epicatechin, rutin, quercetin 3-*β*-D-glucoside, gallic acid, (+)-catechin, ferulic acid, syringic acid) exerted antioxidant activities that were significantly higher compared to Trolox, which was used as a control. Two phenolic compounds, penta-*O*-galloyl-*β*-D-glucose and quercetin 3-*β*-D-glucoside, exhibited antiproliferative activities against both the tumorigenic alveolar epithelial cells (A549) and non-tumorigenic lung fibroblast cells (MRC-5). The

antioxidant activity of black walnut is likely driven not only by penta-*O*-galloyl-*β*-D-glucose but also by a combination of multiple phenolic compounds. Our findings suggested that black walnut extracts possibly possess anticancer activities and supported that penta-*O*-galloyl-*β*-D-glucose could be a potential bioactive agent for the cosmetic and pharmaceutical industries.

INTRODUCTION

Black walnut (*Juglans nigra* L.) is an economically valuable tree for edible nut production in the United States (Randolph et al. 2013). This native tree nut constitutes a major part of the nut production industry in the U.S. Midwest, with over 15 million pounds processed annually in Missouri (Coggeshall 2011). People traditionally valued black walnut kernels as a health-promoting food source based on its compositional profile and utilized other parts of the trees (e.g., leaves, barks) for multiple medical purposes to treat diarrhea and bilious and cramp colic (Nolan and Robbins 1999). Consumption of black walnut kernels has been linked to potential health-promoting activities, such as lowering cholesterol absorption, anti-inflammatory effects, and prevention of certain cancers (Câmara and Schlegel 2016).

Our recent studies have demonstrated a wide range of biological functions of kernel extracts derived from black walnuts including antibacterial, antioxidant, and anti-inflammatory potential (Ho et al. 2018, Ho et al. 2019, Vu et al. 2019). Ho et al. (2018) reported antibacterial capacities of 22 black walnut cultivars selected for nut production by the University of Missouri Center for Agroforestry (Columbia, Missouri, USA) (Reid

et al. 2009). Several black walnut cultivars (e.g., Mystry, Surprise) exhibited antibacterial activity against a Gram-positive bacterium (*Staphylococcus aureus*) and the antibacterial activities were variable among the tested cultivars. Glansreginin A, azelaic acid, and quercetin were predominant phenolic compounds responsible for the antibacterial activities. These compounds were successfully identified in the bioactive fraction of kernel extracts from Mystry via a bioassay guided purification strategy combined with a metabolomics approach (Ho et al. 2018).

Black walnut kernels have also been reported to possess anti-inflammatory potential. The kernel extracts of black walnuts exhibited inhibitory effects on the production of several anti-inflammatory mediators (e.g., interleukin (IL)-1β, tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein (MCP)-1, IL-6, IL-8) in human promonocytic cell line U-937 model system (Ho et al. 2019). The cytokine suppressive activities were variable among the black walnut cultivars examined. Two cultivars, Surprise and Sparrow, significantly inhibited the cytokine production of all examined cytokines in the U-937 cells. Additionally, our recent findings revealed antioxidant activities of the kernel extracts derived from six black walnut cultivars. Mystry showed the strongest antioxidant capacities compared with other tested cultivars (Vu et al. 2019).

Health-promoting properties of black walnuts are likely associated with a wealth of phytochemicals presented in black walnut kernels. Several polyphenols detected in the kernel extracts of black walnut are known to possess a variety of bioactive functions such as anti-inflammatory, antioxidant, antibacterial, and anticancer activities. Our previous studies have identified 17 phenolic compounds in the kernels of 11 black walnut cultivars

(Ho et al. 2018, Vu et al. 2018) and many of these compounds (e.g., ellagic acid, epicatechin gallate, naringin, penta-*O*-galloyl-*β*-D-glucose, quercetin-3-*β*-D-glucoside) are known to possess antioxidant and anticancer activity (Actis-Goretta et al. 2008, Zhang et al. 2009, Chen et al. 2015).

High-throughput screening (HTS) is a critical tool to expand biomedical knowledge of small molecules that can be used for the drug discovery industry (Macarron et al. 2011). The high-throughput analytical technologies enable us to evaluate the biological functions of large amounts of chemicals or natural materials in the shortest amount of time by integrating chemical analyses, modeling, and machine learning that can result in marketed pharmaceutical products in the lowest cost production (Leavell et al. 2020). In this study, we utilized high-throughput screening assays to identify antioxidant and anticancer potentials of phenolic compounds found in black walnuts. The exploration of biological functions of bioactive compounds in black walnuts could reveal underexplored bioactive activities of black walnut extracts and promote the development of novel applications of black walnut and its by-products.

MATERIALS AND METHODS

Sample preparation

Chemicals including (+)-catechin, (−)-epicatechin gallate, ellagic acid, ferulic acid, gallic acid, naringin, *p*-coumaric acid, *p*-hydroxybenzoic acid, penta-*O*-galloyl-*β*-D-glucose, quinic acid, quercetin, quercetin-3-*β*-D-glucoside, rutin, syringic acid, vanillic acid, Trolox, DL-sulforaphane, and tert-butylhydroquinone (TBHQ) were purchased

from Sigma-Aldrich (purity ≥ 95%, Sigma-Aldrich, St. Louis, MO, USA). The compound, glansreginin A, was not included in this study since this compound was not commercially available. In all assays, all chemicals were solubilized in 100% DMSO (dimethyl sulfoxide, tissue-culture grade, Sigma-Aldrich) to facilitate acoustic transfer and were transferred acoustically to the assay plates using an Echo Liquid Handler (Echo® 555, Beckman Coulter Inc., Brea, CA, USA). All compounds were evaluated for antioxidant and antitumor activities in dose-response assays at 7 concentrations of 0, 2.5, 15, 30, 80, 120, and 250 μM, except for penta-*O*-galloyl-*β*-D-glucose, Trolox, DL-sulforaphane, and TBHQ. The concentrations of penta-*O*-galloyl-*β*-D-glucose tested were 0, 2.5, 15, 30, 80, 125, and 175 μM, and Trolox was screened at concentrations of 0, 5, 10, 20, 40, 80, and 120 μM, whereas DL-sulforaphane and TBHQ were evaluated at concentrations of 0, 2.5, 10, 45, 100, 220, and 300 μM. DMSO was used for backfill and the highest final concentration of DMSO added into the cells was 0.35%. The cells treated with 0.35% DMSO and without DMSO were included as vehicle controls in the assays. In all screening assays, all compounds were tested with one replicate per concentration per assay to generate a linear curve of each activity for each compound. The vehicles and positive controls (DL-sulforaphane, TBHQ) were screened via four replicates to identify variability of data.

Cell lines

An Nrf2 antioxidant response element (ARE) reporter HepG2 cell line, a stably transfected liver cell line expressing a firefly luciferase gene under the control of the ARE, was purchased from BPS Bioscience (San Diego, CA, USA). The human alveolar epithelial cell line A549 and the human lung fibroblast cell line MRC-5 were obtained

from American Type Culture Collection (ATCC) (CCL-185 and CCL-171, ATCC, Manassas, VA, USA). The HepG2-ARE cells were grown in modified Eagle's medium (MEM) supplemented with GlutaMAX, 10% fetal calf serum (FBS) and 600ug/mL Geneticin (Thermo Fisher Scientific, Waltham, MA, USA). The tumorigenic alveolar epithelial cells (A549) and non-tumorigenic lung fibroblast cells (MRC-5) were grown in RPMI medium supplemented with 10% FBS. All cells were grown and maintained at 37 °C in a humidified incubator with 5% CO_2.

Total antioxidant capacity

The antioxidant capacity of the phenolic compounds was evaluated using a total antioxidant capacity (TAC) colorimetric assay kit (K274-100, BioVision, CA, USA), according to the manufacturer's instructions. Briefly, the phenolic compounds tested at 7 concentrations (as described above) were added to 384-well plates. Subsequently, Cu^{2+} working solution (12.5 µL/ well) was added into the sample wells. The 384-well plates were incubated for 1.5 h at room temperature and the absorbance of the samples was then read at 570 nm using a microplate reader (Enspire, Perkin Elmer Inc., Waltham, MA, USA). Trolox was used to standardize the antioxidant capacity, as recommended by the manufacturer. A Trolox standard curve was included, and the total antioxidant capacity of the phenolic compounds was interpolated and expressed as Trolox equivalent (mM) from a seven-parameter logistic curve of the Trolox control using curve-fitting software.

Antioxidant response element (ARE) activation

The impact of the phenolic compounds on ARE activation in the HepG2-ARE cell line was evaluated using Steady-Glo® Luciferase assay system (E2510, Promega,

Madison, WI, USA), following the manufacturer's instructions. In brief, the HepG2 - ARE cells were seeded at a density of 10,000 cells/well in 384-well plates containing 50 µL of the complete media per well using a Multidrop Combi dispenser (Thermo Fisher Scientific, Waltham, MA, USA) and then the plate cultures were incubated at 37 °C in a 5% CO_2 humidified incubator for 20 h. The HepG2-ARE cells were incubated with compounds for 18 h. The known ARE activator TBHQ was used as a positive control and the cells treated with 0.35% DMSO and without compounds tested served as a vehicle control. The cells in the absence of DMSO and the compounds were utilized for measuring the background luminescence. The reporter activity was measured by the addition of 25 µL Steady-Glo® luciferase assay reagent (Promega) for 30 min using the Multidrop Combi dispenser (Thermo Fisher Scientific). The luminescence intensities of the 384-well plates were read on Enspire microplate reader (Perkin Elmer Inc.). Percent cytotoxicity of compounds was normalized to the positive and negative controls on each assay plate.

Cell proliferation assays

Influence of the phenolic compounds on cell growth in the tumorigenic alveolar epithelial cells (A549) and non-tumorigenic lung fibroblast cell (MRC-5) cell lines was investigated using the CellTiter-Glo® cell viability assay kit (G7571, BioVision, CA, USA), according to the manufacturer's instructions. Briefly, the A549 and MRC-5 cells were seeded at densities of 8000 and 3000 cells per well, respectively, in 384-well plates and were then incubated in a 5% CO_2 humidified incubator at 37 °C. The cultures were treated with the phenolic compounds and DL-Sulphorane (a known antiproliferative agent as a positive control) at 7 final concentrations (as mentioned above) and a vehicle

(0.35% DMSO). After 72h of incubation, CellTiter-Glo Luminescent assay reagent (Promega) was dispensed at a volume of 10 µL per well into the 384-well plates for 10 min using the Matrix Wellmate dispenser (Thermo Fisher Scientific). The plates were allowed to incubate at room temperature for 20 min and then the luminescence was read using an Enspire microplate reader (Perkin Elmer Inc.)

Data analysis

For total antioxidant capacity analysis, linear regression analysis was performed to identify the linear regression equation for each compound using GraphPad Prism 8 (San Diego, CA, USA). The coefficient of the compound equation was compared with the coefficient of the Trolox control to determine the relative total antioxidant capacity of each compound. Fold-increase over Trolox was calculated by dividing the coefficient of the compound models by the coefficient of the Trolox control. The compounds that exhibited a fold-increase over Trolox greater than 5 were considered to possess significant total antioxidant capacity (Roy et al. 2019).

The ARE fold induction of the compounds was measured by dividing the luminescence absorbance of the treatment by the specific luminescence absorbance of the control sample and multiplying by 100. The control sample (in the presence of DMSO vehicle and without the compounds) was set at 100%. The compounds that had ARE fold induction to 10-fold over the vehicle controls in one or more concentrations were considered to have significant ARE induction activity (Roy et al. 2019).

The relative cytotoxicity (%) of the phenolic compounds was calculated by dividing the specific luminescence absorbance of the treated sampled by the specific

luminescence absorbance of the control sample and multiplying by 100. The control sample (in the presence of DMSO vehicle and without the compounds) was set at 100%. Non-linear regression analysis of data was performed to identify the dose-response curve for each compound. The IC_{50} values (half maximal inhibitory concentration) of each compound were determined from the dose-response curve for the A549 and MRC-5 cell lines using GraphPad Prism 8. The compounds that exhibited IC_{50} values < 10 µM in the A549 cell line and had no toxic effects on the control cell line MRC-5 were considered to be potent antiproliferative compounds.

RESULTS

Total antioxidant capacity

Our previous studies have documented the presence of 17 phenolic compounds in black walnuts (Ho et al. 2018, Vu et al. 2018). Out of 17 phenolic compounds identified, 16 compounds were evaluated for their antioxidant and anticancer activities, whereas the bioactive activities of glansreginin A were not examined since this compound was not commercially available. Out of 16 phenolic compounds tested, 10 compounds (penta-*O*-galloyl-*β*-D-glucose, epicatechin gallate, quercetin, (–)-epicatechin, rutin, quercetin 3-*β*-D-glucoside, gallic acid, (+)-catechin, ferulic acid, syringic acid) exhibited higher total antioxidant capacity compared with the control (Trolox). Six phenolic compounds including vanillic acid, ellagic acid, naringin, *p*-coumaric acid, *p*-hydroxybenzoic acid, and quinic acid had lower total antioxidant capacity than Trolox (Figure 26). The fold-increase over Trolox of these phenolic compounds ranged from 1.1 to 11.5. Penta-*O*-galloyl-*β*-D-glucose exhibited the highest total antioxidant capacity, followed by

epicatechin gallate, quercetin, (–)-epicatechin, rutin, quercetin 3-*β*-D-glucoside, gallic acid, (+)-catechin, ferulic acid, and syringic acid, respectively (Table 11).

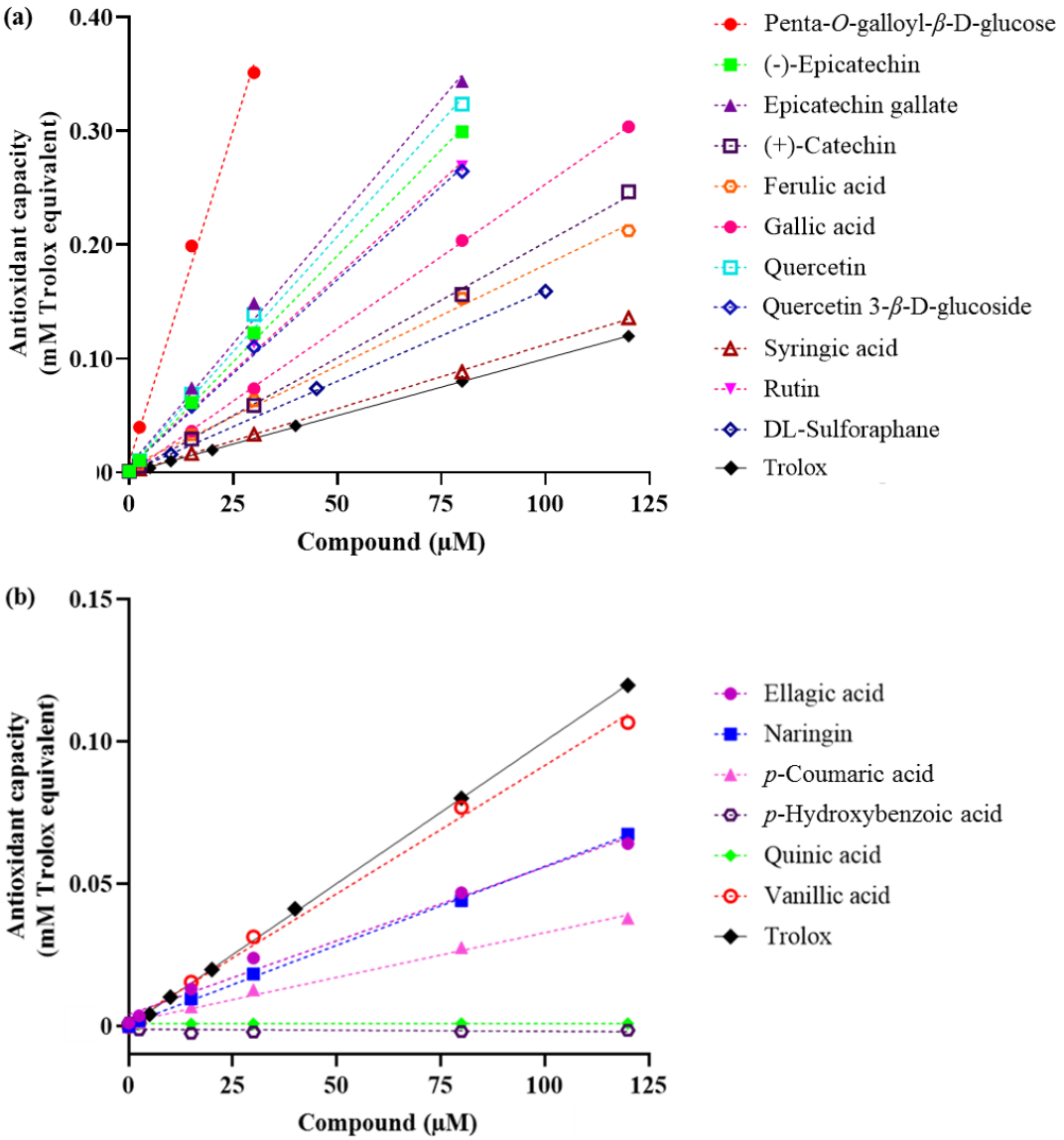

Figure 26. Total antioxidant activity of phenolic compounds in black walnut. (**a**) Compounds with higher antioxidant capacity than Trolox, (**b**) compounds with lower antioxidant capacity than Trolox.

Table 11. Antioxidant activities of phenolic compounds in black walnut.

No.	Compound	Slope (in Trolox Equivalents)	R Square	Fold-Increase Over Trolox
		Control		
1	Trolox	$0.001014 \pm 1.125 \times 10^{-5}$	0.999	1.0
		Antioxidant Capacity Higher than Trolox		
2	Penta-*O*-galloyl-*β*-D-glucose	$0.01167 \pm 5.756 \times 10^{-4}$	0.995	11.5
3	Epicatechin gallate	$0.004294 \pm 1.570 \times 10^{-4}$	0.996	4.2
4	Quercetin	$0.004045 \pm 1.392 \times 10^{-4}$	0.997	4.0
5	(−)-Epicatechin	$0.003729 \pm 7.546 \times 10^{-5}$	0.999	3.7
6	Rutin	$0.002962 \pm 1.551 \times 10^{-4}$	0.989	2.9
7	Quercetin 3-*β*-D-glucoside	$0.002908 \pm 1.522 \times 10^{-4}$	0.989	2.9
8	Gallic acid	$0.002541 \pm 1.392 \times 10^{-5}$	0.999	2.5
9	(+)-Catechin	$0.002029 \pm 3.184 \times 10^{-5}$	0.999	2.0
10	Ferulic acid	$0.001775 \pm 5.026 \times 10^{-5}$	0.997	1.8
11	Syringic acid	$0.001088 \pm 1.057 \times 10^{-5}$	0.999	1.1
		Antioxidant capacity lower than Trolox		
12	Vanillic acid	$0.0007396 \pm 4.245 \times 10^{-5}$	0.984	0.7
13	Ellagic acid	$0.0005183 \pm 2.860 \times 10^{-5}$	0.988	0.5
14	Naringin	$0.0005177 \pm 9.755 \times 10^{-6}$	0.998	0.5
15	*p*-Coumaric acid	$0.0003139 \pm 1.434 \times 10^{-5}$	0.992	0.3
16	*p*-Hydroxybenzoic acid	$-0.000008 \pm 1.260 \times 10^{-5}$	0.086	n/a
17	Quinic acid	$-0.0000004 \pm 1.034 \times 10^{-6}$	0.032	<0.1

Linear regression models of the majority of tested compounds had high R^2 values (>0.98), indicating that these models were reliable (Table 11). Models of two compounds

(*p*-hydroxybenzoic acid and quinic acid) had low values of R^2 since these compounds had minor or no antioxidant capacity under the experimental conditions. Violin plots representing the data distribution of controls (Trolox and tert-butylhydroquinone) showed a relatively small variation of data, indicating that the HTS assay system was reliable (Supplementary Figures 3–4).

Antioxidant response element (ARE) activation

The ARE fold-increase in HepG2-ARE activation of all compounds relative to the control was <2 (Figure 27). Since the ARE fold-increase in HepG2-ARE of examined compounds was <10 (Roy et al. 2019), there were no compounds that could be considered to exert significant ARE induction activity. (−)-Epicatechin showed the strongest ARE activation among all compounds, followed by *p*-coumaric acid, quercetin 3-*β*-D-glucose, and vanillic acid, respectively. Several compounds such as penta-*O*-galloyl-*β*-D-glucose, gallic acid, epicatechin gallate, and quercetin 3-*β*-D-glucose at high concentrations were toxic to cells, which did not induce ARE activation.

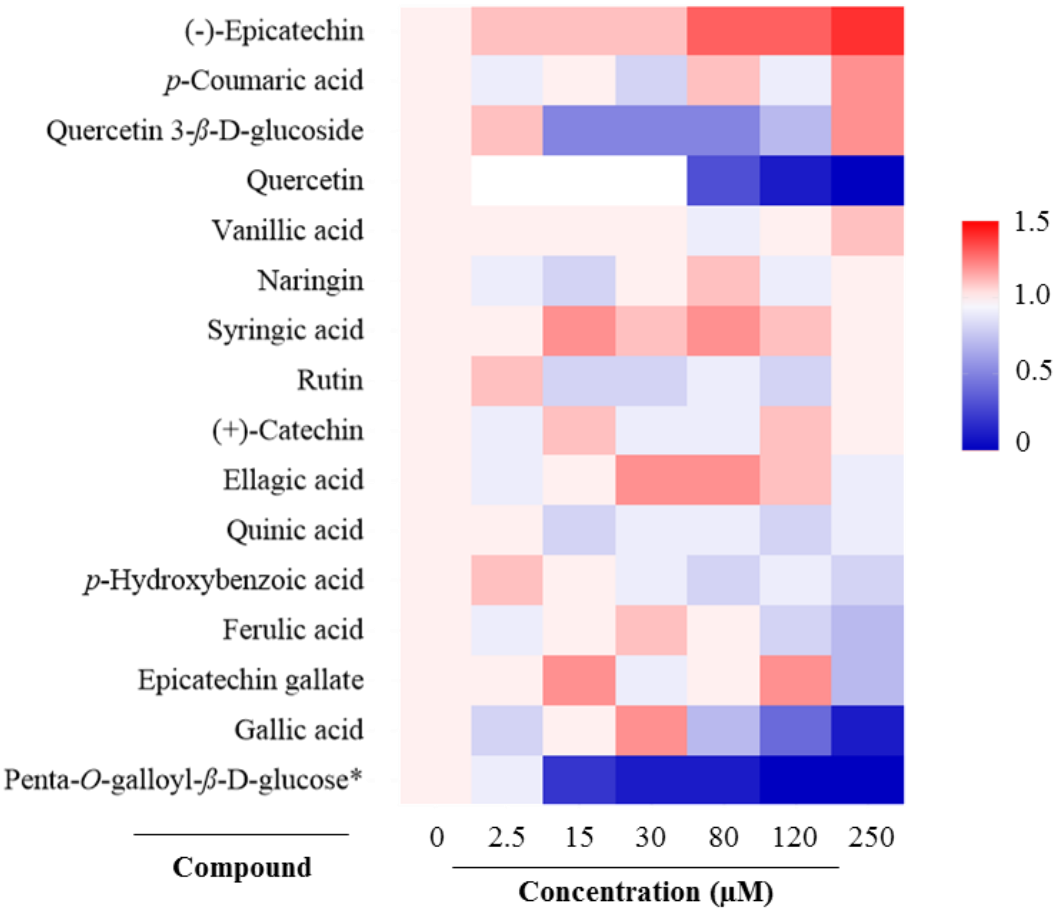

Figure 27. Antioxidant response element (ARE) activation activities of the tested compounds in HepG2-ARE cells. In the heatmap, color represents relative fold-increases in ARE activities in HepG2-ARE cells treated with DMSO and compounds compared with the corresponding vehicle control, the HepG2-ARE cells treated with 0.35% DMSO only. * Penta-*O*-galloyl-*β*-D-glucose was screened at concentrations of 0, 2.5, 15, 30, 80, 125, 175 μM, respectively.

Cell proliferation assays

Cell viability assays were performed to address the cytotoxic effects of the phenolic compounds. A reduction in luminescence absorbance could result from a loss of cell viability and a reduction in cell number. The vehicle DMSO at the highest

concentrations used (0.35%) did not affect cell number or viability in both A549 and MRC-5 cells, indicating that a reduction in luminescence absorbance in the presence of the tested compounds would indicate a toxic effect of these compounds rather than the vehicle. Among 16 tested compounds, penta-*O*-galloyl-*β*-D-glucose and quercetin 3-*β*-D-glucoside had the lowest IC_{50} values in the A549 cells (Table 12). The IC_{50} values of penta-*O*-galloyl-*β*-D-glucose and quercetin 3-*β*-D-glucoside in MRC-5 cells were 6.11 and 6.89 µM, respectively (Figure 28). These compounds were also toxic to the MRC-5 cells. The IC_{50} values of penta-*O*-galloyl-*β*-D-glucose and quercetin 3-*β*-D-glucoside in MRC-5 cells were 10.37 and 12.15 µM, respectively. Epicatechin gallate had IC_{50} values for A549 and MRC-5 cells that were 65.96 and 64.38, respectively, while IC_{50} values of quercetin and gallic acid for A549 and MRC-5 cells were 87.74 and 99.47, and >250 and 48.18, respectively. Other tested compounds had IC_{50} values > 250 µM in both A549 cells and MRC-5 (Table 12).

Table 12. Half maximal inhibitory concentrations (IC_{50}) of phenolic compounds (µM) in black walnuts in A549 and MRC-5 cell lines.

Compound	**A549 cell line**	**MRC-5 cell line**
Penta-*O*-galloyl-*ß*-D-glucose	6.11	10.37
Quercetin 3-*ß*-D-glucoside	6.89	12.15
Epicatechin gallate	65.96	64.38
Quercetin	87.74	99.47
Gallic acid	>250	48.18
Ellagic acid	>250	>250
(-)-Epicatechin	>250	>250
Rutin	>250	>250
(+)-Catechin	>250	>250
Ferulic acid	>250	>250
Syringic acid	>250	>250
Vanillic acid	>250	>250
Naringin	>250	>250
p-Coumaric acid	>250	>250
p-Hydroxybenzoic acid	>250	>250
Quinic acid	>250	>250
DL-Sulforaphane (control)	16.96	9.95

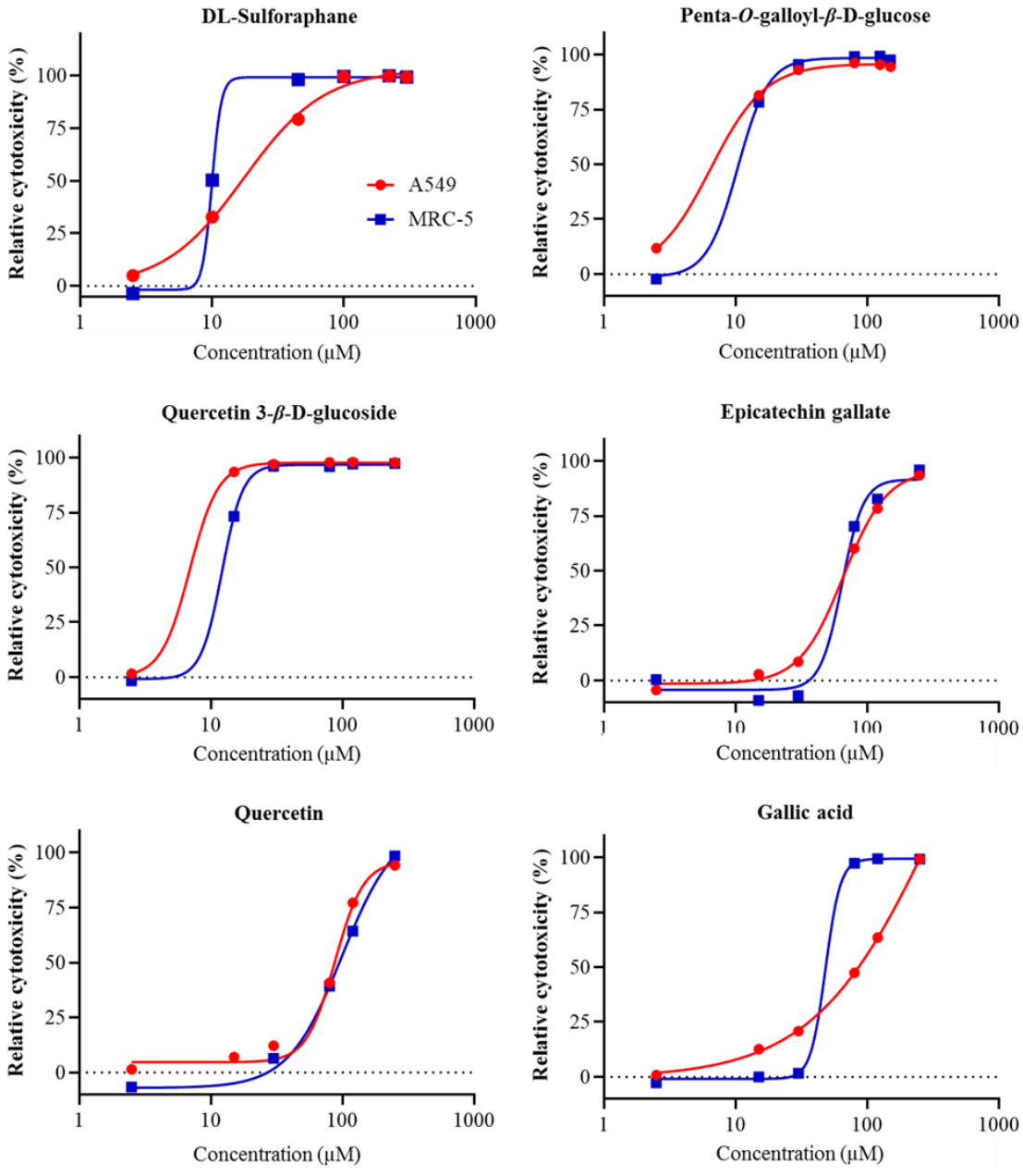

Figure 28. Cytotoxicity (%) of phenolic compounds (penta-*O*-galloyl-*β*-D-glucose, quercetin 3-*β*-D-glucoside, gallic acid, epicatechin gallate, ellagic acid) and the control (DL-sulforaphane) in A549 and MRC-5 cell lines. Data were expressed as percentages of cytotoxicity in A549 and MRC-5 cells treated with DMSO and the compounds compared with the corresponding vehicle controls that are A549 and MRC-5 cells treated with 0.35% DMSO only.

DISCUSSION

Black walnuts have recently been documented as a promising natural source for the medicinal and pharmaceutical industries. The kernels of black walnuts have been reported to possess multiple biological functions which are likely associated with the presence of its bioactive constituents, including polyphenols. In the present study, we utilized HTS technologies to characterize the antioxidant and anticancer activities of 16 phenolic compounds found in black walnut kernels (Ho et al. 2018, Vu et al. 2018). Given the huge availability of black walnuts, the exploration of the biological functions of the bioactive compounds in black walnuts could promote the development of novel applications of black walnut and its by-products, which could provide opportunities to utilize the abundant, low-value, renewable materials from black walnut and its by-products into profitable value-added products and thereby potentially increase the sustainability of the black walnut agro-industry.

Our results indicated that several phenolic compounds found in black walnut kernels exert strong antioxidant activities. Out of 16 phenolic compounds tested, 10 compounds (penta-*O*-galloyl-*β*-D-glucose, epicatechin gallate, quercetin, (−)-epicatechin, rutin, quercetin 3-*β*-D-glucoside, gallic acid, (+)-catechin, ferulic acid, syringic acid) exhibited higher total antioxidant capacity than Trolox. The presence of multiple phenolic compounds also raises the possibility of synergistic activities that are likely responsible for the antioxidant activities observed in black walnuts. Our results indicated that no compound can be considered to exert significant ARE induction activity since all tested compounds had ARE fold-increases relative to the control that were less than 10. Roy et al. (2019) suggested that an ARE fold-increase > 10 was considered to induce the activity

of ARE signaling pathways that functionally regulate the expression of genes encoding over 250 antioxidant and detoxification proteins (Surh et al. 2008). Vu et al. (2019) observed the variation of antioxidant capacities of different black walnut cultivars, in which Mystry exhibited the highest antioxidant activity among the examined cultivars. Future research will focus on purification and characterization of bioactive compounds and its composition in Mystry that mainly drive the antioxidant activity.

Our results also revealed the anticancer potential of the phenolic compounds in black walnuts. Two phenolic compounds, penta-*O*-galloyl-*β*-D-glucose and quercetin 3-*β*-D-glucoside, exhibited antiproliferative activities against both the tumorigenic alveolar epithelial cells (A549) and non-tumorigenic lung fibroblast cells (MRC-5), while no significant inhibitory effects on the growth of these cell lines were observed on other tested compounds. Black walnut and English walnut (*J. regia* L.), another common *Juglans* species, have been reported to share a similar polyphenolic profile in which all 16 phenolic compounds tested were found both in black walnut and English walnut (Table 13). The total contents of these phenolic compounds in black walnut was lower compared with English walnut (Vu et al. 2018). Remarkably, anticancer capacities have been well established both in vivo and in vitro in English walnut. In vivo, English walnut kernels have been shown to inhibit the growth of several types of cancer cells, including colon cancer stem cells, breast cancer cells, and colorectal cancer cells (Lee et al. 2016, Sánchez-González et al. 2017, Koh et al. 2019). Consumption of English walnut kernels by mice was associated with in vitro tumor cell changes in DNA proliferation and apoptosis (Hardman and Ion 2008, Nagel et al. 2012, Reiter et al. 2013). Anticancer functions of English walnuts are possibly associated with its polyphenols (Hardman

2014, Sánchez-González et al. 2017), suggesting possible anticancer capacities of black walnuts and its polyphenols.

Table 13. Concentrations of phenolic compounds (µg/g of dry weight) in kernels of six black walnut cultivars with their known bioactive activities (Figueroa et al. 2016, Ho et al. 2018, Vu et al. 2018, Ho et al. 2019, Vu et al. 2019).

Compound	Black walnut cultivar						English walnut [+]
	Daniel	Hay	Jackson	KK	Mystry	Surprise	
Penta-*O*-galloyl-*β*-D-glucose	n/d	n/d	n/d	n/d	15.2 ± 2.5	n/d	55.9 ± 7.7
Quercetin 3-*β*-D-glucoside	n/d	3.2 ± 0.1	1.6 ± 0.2	n/d	2.1 ± 0.3	1.8 ± 0.3	3.7 ± 0.2
Epicatechin gallate	13.2 ± 0.5	7.0 ± 0.6	3.6 ± 0.7	n/d	2.0 ± 0.2	6.0 ± 0.7	4.9 ± 1.5
Gallic acid	n/d	1.4 ± 0.2	0.7 ± 0.2	0.5 ± 0.03	4.3 ± 0.3	1.0 ± 0.03	8.1 ± 0.7
Ellagic acid	30.4 ± 1.0	40.5 ± 5.9	61.1 ± 3.7	11.4 ± 2.0	65.7 ± 4.8	72.1 ± 8.3	98.4 ± 20.6
Rutin	n/d	n/d	n/d	1.7 ± 0.6	4.2 ± 1.3	n/d	2.7 ± 0.3
(+)-Catechin	n/d	n/d	n/d	n/d	n/d	0.6 ± 0.01	47.9 ± 3.5
Ferulic acid	n/d	n/d	n/d	0.7 ± 0.04	0.6 ± 0.06	4.9 ± 0.08	0.9 ± 0.1
Syringic acid	7.3 ± 1.3	n/d	7.7 ± 1.4	n/d	9.5 ± 2.1	n/d	7.3 ± 2.2
Vanillic acid	n/d	9.9 ± 2.6	8.7 ± 1.7	n/d	6.9 ± 1.2	n/d	7.3 ± 1.8
Naringin	0.5 ± 0.1	n/d	n/d	0.3 ± 0.1	0.5 ± 0.2	4.9 ± 0.1	0.3 ± 0.04
p-Coumaric acid	n/d	0.2 ± 0.03	0.2 ± 0.03	0.2 ± 0.1	0.3 ± 0.02	0.3 ± 0.1	0.5 ± 0.1
p-Hydroxybenzoic acid	n/d	n/d	n/d	n/d	n/d	n/d	1.2 ± 0.5
Quinic acid	4.7 ± 0.2	2.4 ± 0.1	1.4 ± 0.2	4.2 ± 0.2	2.4 ± 0.3	3.9 ± 0.5	6.8 ± 0.4
Quercetin	n/q	n/q	n/q	n/q	n/q	n/q	n/q
(−)-Epicatechin	n/q	n/q	n/q	n/q	n/q	n/q	3.9 ± 0.01
Biological activity of kernel extracts from black walnuts							
Antioxidant	++	+	++	++	+++	+++	n/c
Antibacterial *	.	.	.	.	+++	+++	n/c
Anti-inflammatory potential **	+	n/a	n/a	n/a	+	+++	n/c

+: phenolic contents in an English walnut cultivar (Figueroa et al. 2016, Vu et al. 2018); * antibacterial activities of the extracts against a Gram-positive bacterium (*Staphylococcus aureus*) (Ho et al. 2018); ** : overall cytokine suppressive activities of the extracts on six cytokines/chemokines in (TNF-α, IL-1β, IL-6, IL-8, IL-10, and MCP-1) in human promonocytic cell line U-937 (Ho et al. 2019); n/d: not detected; n/q: identified but not quantified; n/a: not reported; n/c: not comparable since the biological activities of English walnut and black walnut were not reported from the same studies; +: possessing activity; .: no activity. KK: Kwik Krop.

Vu et al. (2018) reported that the contents of phenolic compounds were widely variable among different black walnut cultivars. Among the six black walnut cultivars whose antioxidant capacities have been examined, the kernel extracts from Mystry and Surprise have been documented to possess the strongest antioxidant activities (Vu et al. 2019). Remarkably, penta-*O*-galloyl-*β*-D-glucose only presented in Mystry (15.2 mg/kg) and was not detectable in other cultivars (e.g., Surprise) showing the antioxidant activities (Table 13). The concentration of this compound in black walnut (Mystry) was lower compared with English walnut (58.6 mg/kg). Due to a huge variation in the contents of phenolic compounds in different black walnut cultivars, it is likely that the antioxidant activities of these extracts were possibly driven by not only penta-*O*-galloyl-*β*-D-glucose but also other compounds. Additionally, the presence of multiple phenolic compounds in black walnut raises the possibility of synergistic effects of these compounds that are responsible for the biological activities of black walnuts.

The results from HTS assays indicated penta-*O*-galloyl-*ß*-D-glucose as a potent bioactive compound. This compound exhibited strong antioxidant and anticancer capacities. The fold-increase over trolox of penta-O-galloyl-ß-D-glucose was 11.5, whereas the IC_{50} value of this compound for the tumorigenic alveolar epithelial cell line (A549) was 6.11. Zhang et al. (2009) reported 1, 2, 3, 4, 6-penta-O-galloyl-β-D-glucose, a hydrolysable tannin, as a polyphenolic compound highly enriched in several plants and herbs such as *Acer truncatum*, *Paeonia suffruticosa*, *Rhus chinensis*, *Schinus terebinthifolius*, and *Terminalia chebula*. Previous studies have been documented a variety of biological functions of penta-*O*-galloyl-*ß*-D-glucose including antidiabetic, antibacterial, antioxidant, anticancer, antiangiogenic, antivirus and anti-inflammatory

activities (Zhang et al. 2009, Torres-León et al. 2017). This compound has been showed to possess anticancer effects against multiple cancer cells including lung cancer, prostate cancer, breast cancer (Huh et al. 2005, Hu et al. 2008, Yu et al. 2011). Huh et al. (2005) reported antitumor activities of 1, 2, 3, 4, 6-penta-*O*-galloyl-*ß*-D-glucose that primarily inhibited angiogenesis through cyclooxygenase-2 and phospho-p38 mitogen-activated protein kinase (MAPK)- dependent pathways. In vivo, 1, 2, 3, 4, 6-penta-*O*-galloyl-*ß*-D-glucose exhibited significant inhibitory effects on the proliferation and tube formation in basic fibroblast growth factor-treated human umbilical vein endothelial cells, attenuated the secretion of cyclooxygenase-2, and decreased the expression of vascular endothelial growth factor and prostaglandin E2. In vivo, when mice were intraperitoneally injected 1, 2, 3, 4, 6-penta-*O*-galloyl-*ß*-D-glucose at a daily dose of 20 mg/kg, the growth of tumor angiogenesis induced by Lewis lung carcinoma were significantly inhibited at a rate of 91%. Piao et al. (2009) reported 1, 2, 3, 4, 6-penta-*O*-galloyl-*ß*-D-glucose exerting antioxidant activities that can protect cells against H_2O_2-induced cell damage. Additionally, Bae et al. (2017) suggested 1, 2, 3, 4, 6-penta-*O*-galloyl-*ß*-D-glucose as a potential candidate anti-viral drug to treat varicella-zoster virus (VZV)-associated diseases (e.g., chickenpox) due to its significant suppressive inhibition on VZV-induced c-Jun N-terminal kinase (JNK) activation, expression of viral immediate-early 62 (IE62) protein and VZV replication. Vu et al. (2018) quantified the amount of penta-*O*-galloyl-*β*-D-glucose in kernel extracts from 11 different black walnut cultivars and reported that the highest amount of penta-*O*-galloyl-*β*-D-glucose was found in Mystry (15.2 mg/kg). This cultivar has been documented to possess multiple bioactive activities including

antioxidant, antibacterial, and anti-inflammatory potential. Future research might focus on exploring the anticancer properties of Mystry and its bioactive constituents.

CONCLUSION

We identified the antioxidant and anticancer potentials of phenolic compounds found in black walnuts. Out of 16 tested compounds, several compounds had remarkable antioxidant activities and two compounds had strong anticancer activities. Penta-*O*-galloyl-*β*-D-glucose exhibited the strongest antioxidant and antiproliferative activities against both the tumorigenic alveolar epithelial cells (A549) and non-tumorigenic lung fibroblast cells (MRC-5) among the tested compounds. Antioxidant activities of black walnut cultivars are likely correlated with the synergistic effects of phenolic compounds in black walnut. Black walnut extracts possibly possess anticancer activity. Penta-*O*-galloyl-*β*-D-glucose has been previously documented to exert a wide range of bioactive activities. Our results support the notion that penta-*O*-galloyl-*β*-D-glucose could be a potential bioactive compound for the cosmetic and pharmaceutical industries.

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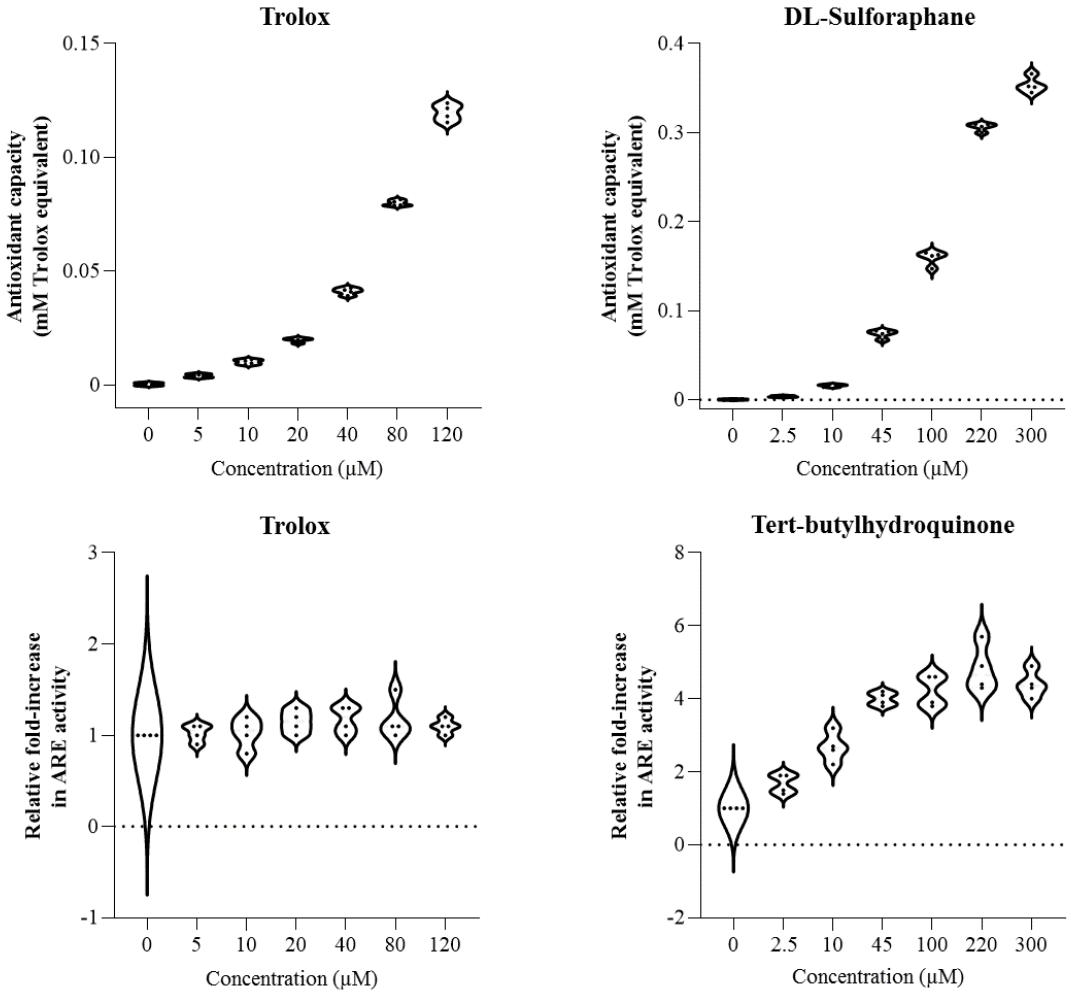
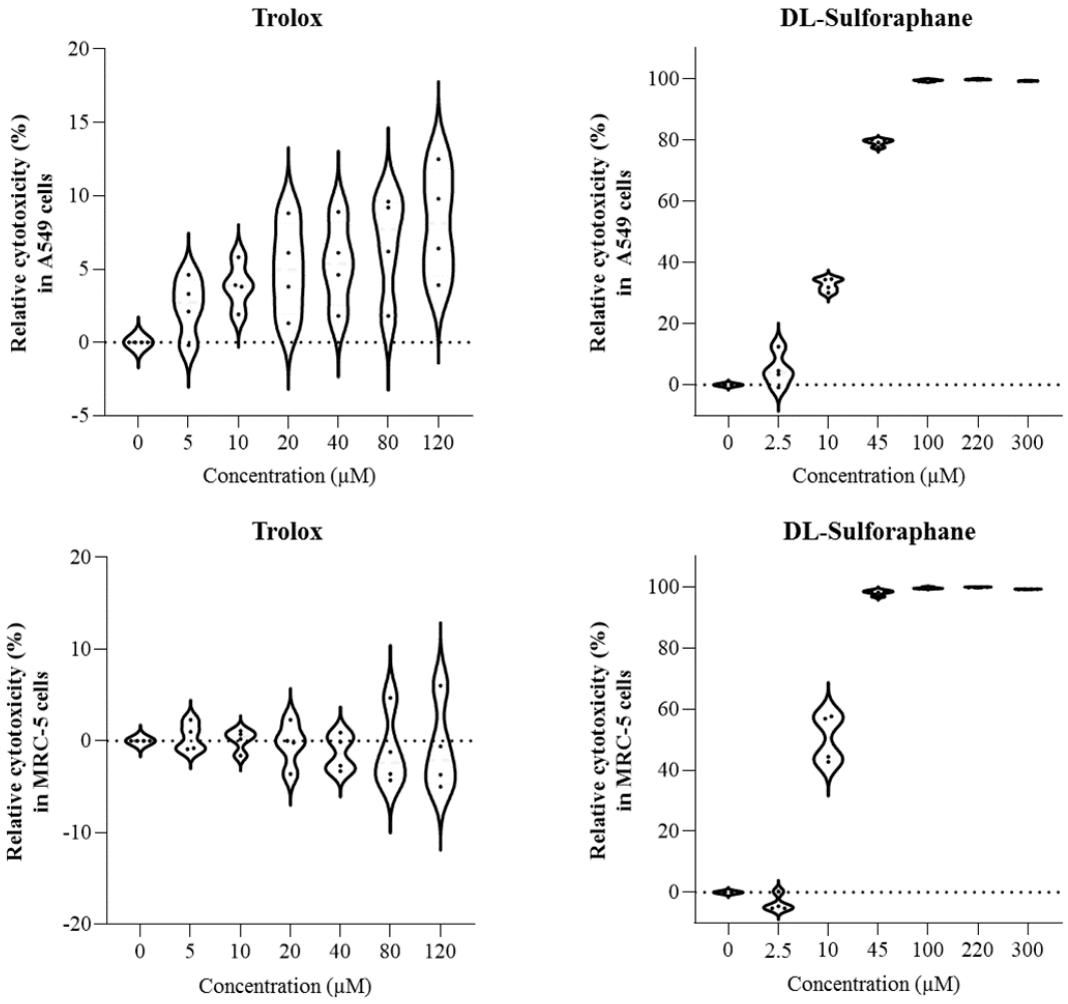
SUPPLEMENTARY INFORMATION



Supplementary Figure 3. Data distribution of controls (Trolox, DL-sulforaphane, tert-butylhydroquinone) in total antioxidant capacity and antioxidant response element (ARE) activation assays. Each violin plot represents the distribution of data for each treatment/concentration. Dot symbols inside violin plots represents data points of each replicate.



Supplementary Figure 4. Data distribution of controls (Trolox, DL-sulforaphane) in cytotoxicity assays. Each violin plot represents the distribution of data for each treatment/concentration. Dot symbols inside violin plots represents data points of each replicate.

CHAPTER VI: IDENTIFICATION AND QUANTIFICATION OF BIOACTIVE MOLECULES INHIBITING PRO-INFLAMMATORY CYTOKINE PRODUCTION IN SPENT COFFEE GROUNDS USING METABOLOMICS ANALYSES

ABSTRACT

In this study, we assessed the anti-inflammatory properties of spent coffee grounds. Methanolic extracts of spent coffee grounds obtained from 3 Arabica cultivars possess compounds that exerted inhibitory effects on the secretion of inflammatory mediators (TNF-α, IL-6 and IL-10) induced by a human pro-monocytic cell line differentiated with PMA and stimulated with lipopolysaccharide (LPS). Our results indicated that the cytokine suppressive activities of the spent coffee ground (SCG) extracts were different among coffee cultivars tested. Hawaiian Kona extracts exhibited inhibitory effects on the expression of 3 examined cytokines, Ethiopian Yirgacheffe extracts reduced the secretion of TNF-α and IL-6, and Costa Rican Tarrazu extracts decreased the secretion of IL-6 only. Untargeted metabolomics analyses of SCG extracts led to the putative identification of 26 metabolites with known anti-inflammatory activities. Multiple metabolites (i.e., chrysin, daidzein, eugenol, naringenin, naringin, oxyresveratrol, pectolinarin, resveratrol, tectochrysin, theaflavin, vanillic acid, and vitexin rhamnoside) identified in the SCGs represent possible novel anti-inflammatory compounds. Of the 26 identified metabolites, the 12 compounds that had high relative intensities in all of the extracts were successfully quantified using liquid chromatography-tandem mass spectrometry analyses. Results from the targeted analyses indicated that caffeine and 5-caffeoylquinic acid (CQA) were the most abundant

compounds in the SCG extracts. The contents of caffeine ranged from 0.38 mg/g (Ethiopian Yirgacheffe) - 0.44 mg/g (Costa Rican Tarrazu), whereas 5-CQA concentrations were in the range of 0.24 mg/g (Costa Rican Tarrazu) - 0.34 mg/g (Ethiopian Yirgacheffe). The presence of multiple anti-inflammatory compounds in SCGs provides a promising natural source for cosmetic and pharmaceutical industries.

INTRODUCTION

Coffee, one of the most frequently consumed beverages, is the second greatest valuable commodity worldwide after petroleum (Murthy & Naidu, 2012). Approximately 6 million tons of spent coffee grounds (SCG), consisting of the solid residues produced during the brewing process, are produced annually on a global level (Getachew & Chun, 2017). The SCGs contain a rich source of amino acids, alkaloids, fatty acids, oils, polyphenols, minerals and polysaccharides (Campos-Vega et al., 2015) and have been investigated for use as value-added products. The SCGs have been proposed to have a wide range of cosmetics application stemming from its phytochemical composition (e.g., phenolic acids, flavonoids, caffeine) (Campos-Vega et al., 2015). Other possible applications include fertilizers, absorbers, fillers and additives for polymer composites, supplements in animal feed, and biofuels (Castro et al., 2011; Givens & Barber, 1986; Moustafa et al., 2017; Park et al., 2016; Ribeiro et al., 2017; Zarrinbakhsh et al., 2016)..

Coffee grounds were utilized as a folk medicine to treat dysentery, external wounds and inflammation (Hänsel et al. 1993). Native people also traditionally valued roasted coffee beans for medical purposes to treat anemia, edema, headaches, hepatitis,

malaria, neuralgia, sleep disorders, and conditions of weakness (Hänsel et al. 1993, Rätsch 2005). The SCGs are thought to exert multiple potential health benefits, but few studies have investigated the anti-inflammatory potential of these coffee residues. Ramalakshmi et al. (2009) reported no inhibitory effects of SCGs on expression of tumor necrosis factor (TNF)-α in the J774A.1 cell model system. In another study, López-Barrera et al. (2016) evaluated the effects of SCG fractions fermented by human gut flora on the cytokine secretion induced by lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. This group reported that these SCG fractions significantly reduced the secretion of 3 cytokines [interleukin (IL)-1β, IL-10, and chemokine ligand (CCL) 17] out of the 40 examined cytokines/ chemokines tested. The cytokine inhibition of the human gut fermented, unabsorbed SCG fractions were reported to be mediated primarily by short-chain fatty acids derived from dietary fiber (López-Barrera et al., 2016). In fact, multiple bioactive compounds in the SCGs (alkaloids and polyphenols) with known anti-inflammatory activities are potential sources of these cytokine suppressive effects. Similarly, bioactive compounds (e.g., caffeine, gallic acid, monocaffeoylquinic acids) identified in SCG have been reported to possess anti-inflammatory activities (Bravo et al., 2012; Hwang et al., 2014; Kim et al., 2005; Köroğlu et al., 2014). Despite these findings, the profiles of anti-inflammatory compounds in SCG from different coffee cultivars have not been adequately compared and characterized.

In this study, we described the inhibitory effects of SCG extracts derived from 3 Arabica cultivars on the expression of 3 inflammatory mediators (TNF-α, IL-6, and IL-10) using the human pro-monocytic cell line U-937. We subsequently identified and quantified the bioactive compounds in SCGs by metabolomics approaches. The

integration of the cellular bioassay, untargeted chemical profiling, and targeted analyses is a powerful way to explore global activity metabolites with known as well as unknown physico-chemical properties (Rinschen et al., 2019).

MATERIALS AND METHODS

Sample preparation

Ground roast coffees from three Arabica cultivars including Costa Rican Tarrazu, Ethiopian Yirgacheffe, and Hawiian Kona were purchased from Lakota Coffee Company (Columbia, Missouri, USA). Spent coffee grounds from these coffee cultivars were obtained from a coffee maker (Bunn VP17-2, Springfield, IL, USA) after brewing of the ground roast coffee for 5 min at 90 °C. The SCG was immediately homogenized using a coffee grinder (CBG100S, Black+Decker, Beachwood, OH, USA). The homogenized samples (25 g wet weight, 78% water) were extracted in 100 mL of methanol (HPLC grade, Fisher Scientific, Pittsburg, PA) twice and then sonicated at 10 °C for 60 min as previously described in Ho et al. (2019). Subsequently, the methanolic extract was filtered through a 125 mm Whatman filter paper (GE Healthcare, Chicago, IL, USA) under SPE Vacuum Manifold (Visiprep™ SPE Vacuum Manifold, Sigma-Aldrich, USA), and then the supernatant was collected and stored at -20 °C until analysis. The extract was allowed to thaw at room temperature, vortexed for 30 s, and then filtered through a 0.2 μm syringe Anotop membrane filter (Whatman) prior to the analysis. For cellular assays, the resulting supernatant was evaporated until dryness under a flow of nitrogen. The dry extract was then resuspended with DMSO (Sigma-Aldrich, USA) at

concentration of 5,000 mg/mL. Cytokine modulating activities of the extract were identified using BD^{TM} cytometric bead array (CBA) kits (BD Biosciences, San Jose, CA, USA). For untargeted chemical profiling and targeted analyses, the extracts from each cultivar were injected in triplicate into ultra-high performance liquid chromatography coupled with high resolution mass spectrometer (UHPLC-HRMS) and HPLC with tandem mass-spectrometer (HPLC-MS/MS), respectively.

Cell culture and differentiation induction

The human monocyte cell line U-937 was obtained from American Type Culture Collection (ATCC) (CRL-1593.2, ATCC, Manassas, VA, USA). Cells were grown in complete Roswell Park Memorial Institute (RPMI) medium (RPMI 1640, ATCC) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 100 µg/mL gentamicin at 37 ^{o}C in a humidified incubator with 5% CO_2. To induce differentiation of U-937 cells into macrophage-like cells, U-937 cells were seeded at 2 x 10^5 cells/well in 96 well plates containing 50 nM of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 48 hours (Rovera et al., 1979). Subsequently, the PMA-differentiated cells were washed twice and cultured in fresh growth media. The cells were incubated for an additional 18 hours before pre-treatment with SCG extracts prepared from three cultivars. The SCG extracts were added in triplicate at 3 final concentrations (0.05, 0.5, and 5 mg/mL) for 2 hours prior to stimulation with 1 µg/mL LPS (*Escherichia coli* 0127:B8, Sigma-Aldrich). Dexamethasone (Sigma-Aldrich), an anti-inflammatory agent known to inhibit cytokine secretion, was used as positive control at a concentration of 0.002 mg/mL. SCG extracts were resuspended in tissue culture-grade DMSO, and the highest concentration of DMSO in any sample was 0.1%. Therefore, a vehicle control of 0.1%

DMSO was included in all experiments and served as a point of reference. After the addition of LPS for 22 hours, the culture supernatants from each triplicate group were pooled, spun to remove cell debris, transferred to new tubes, and stored at -20 °C until analysis.

Cell viability analysis

MTT assays were performed to evaluate possible effects of the SCGs on cytotoxicity and/or cell loss. Briefly, cellular activity of mitochondrial dehydrogenase activity was measured using a colorimetric cell viability assay after removal of the supernatants. MTT substrate (Mosmann, 1983) was added to the cells in DMEM high glucose, phenol-red free media containing 1% FBS for 3 hours at 37 °C until formazan crystals were observed. Crystals were dissolved in acidified isopropanol, and samples were pipetted up and down several times to ensure that the crystals were completely dissolved before readings were taken. Absorbance was measured within 30 min after solvent addition using a BioTek ELx808 microplate reader (BioTek, Winooski, Vermont, USA). Formazan crystals were detected at a wavelength of 570 nm, and background absorbance was measured at 630 nm.

Quantification of cytokines/chemokines expression

A multiplex flow cytometric bead-based assay was used to quantitate the amount of secreted cytokines in the U-937 cells in the absence or presence of the SCG extracts. Multiple experiments were performed, and each cultivar was tested at 3 concentrations. Preparation of samples using the BD Cytometric Bead Array (CBA) human inflammatory cytokines kit was carried out according to the manufacturer's recommendations. For these studies, the analysis focused on a subset of inflammatory cytokines (TNF-α, IL-6,

and IL-10) present in the kit. Triplicate samples were collected on a BD LSR Fortessa X-20 cell analyzer (BD Biosciences, San Jose, CA, USA) using instrument settings suggested by BD and optimized in each experiment. A cytokine standard curve was included in each experiment, and cytokine levels were calculated from a five-parameter logistic curve using a curve-fitting software.

Untargeted metabolomics analyses to putatively identify anti-inflammatory molecules

The extracts were analyzed using an UHPLC system coupled to a maXis impact quadrupole-time-of-flight high-resolution mass spectrometer (Q-TOF) (Bruker Co., Billerica, MA, USA) operated in both negative and positive electrospray ionization modes with the nebulization gas pressure at 43.5 psi, dry gas of 12 L/min, dry temperature of 250 °C and a capillary voltage of 4000V, as described in Ho et al. (2018). The SCG extracts obtained from 3 Arabica cultivars were separated using a Waters Acquity UHPLC BEH C18 column (2.1 × 150 mm, 1.7 µm particles size) at 60 °C. The solvent system was 0.1% formic acid in water (A) and 100% acetonitrile (B). The gradient elution used started with a linear gradient of 95%: 5% to 30%: 70% eluents A: B in 30 min. Subsequently, the separation was followed by a linear wash gradient as follows 70-95% B, 95% B, 95-5% B, and 5% B at 30-33 min, 33-35 min, 35-36 min, and 37-40 min, respectively. The flow rate was 0.56 mL/min. Mass spectral data were collected automatically using a scan range from m/z 100 to 1,500 and auto-calibrated using sodium formate after data acquisition. Each coffee cultivar and methanol blank (served as a control) were analyzed in triplicate.

Targeted metabolomics analysis to determine contents of major anti-inflammatory molecules

Major metabolites that had high relative intensities across the SCG extracts were selected for absolute quantification of their contents using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis with authentic standards. The LC-MS/MS analyses were performed using an HPLC system (Water Alliance 2695, Water Co., Milford, MA, USA) coupled to a Waters Acquity TQ triple quadrupole mass spectrometer operated in negative electrospray ionization mode with the nebulization gas pressure at 43.5 psi, dry gas of 12 L/min, dry temperature of 250 °C and a capillary voltage of 1500 V. The compounds in the SCG extracts (20 µL volume per injection) from three coffee cultivars were separated using a Phenomenex Kinetex C18 reverse-phase column (100 × 4.6 mm; 2.6 µm particle size, Torrance, CA, USA) at 25 ^{o}C. The mobile phases were 0.1% formic acid in water (A) and 100% acetonitrile (B). The gradient elution used were 2% B, 2-80% B, 80-98% B, 2% B at 0-0.5 min, 0.5-7 min, 7.0-9.0 min, 9.0-15.0 min, respectively at a flow rate of 0.5 mL/min. MS detection was performed by MS/MS using the multiple reaction monitoring (MRM) mode. Waters IntelliStart optimization software was used to optimize collision, ionization energy, MRM and SIR (single ion recording) transition ions (molecular and product ions), capillary and cone voltage, desolvation gas flow, and collision energy. Waters Empower 3 software was used to analyze data. The concentrations of major anti-inflammatory compounds found in SCG extracts were determined based on a standard curve for each analyte generated using authentic standards of these compounds (purity > 95%, Sigma-Aldrich) at 8 concentrations (0.01, 0.025, 0.05, 0.1, 0.5, 1, 5, 10 ppm) in triplicate.

Assessment of the sensitivity of the analytical method was performed by calculating the limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ for each compound were calculated by employing signal-to-noise ratios of three and ten. The extraction recover rates of the fortified internal standards colchicine and β-naphthylsulfate were used to determine the efficiencies of the extraction procedure. For the extraction procedure, 100 μg of each internal standard was fortified. The recovery rates were greater than 95%.

Data processing and statistical analysis

Cell viability and cytokine concentrations were determined relative to control samples (in the presence of DSMO vehicle and without cultivars). Relative cell viability (%) was obtained by dividing the MTT absorbance of treated samples by those in control samples, and multiplying by 100. Relative cytokine secretions (%) were calculated by dividing the cytokine concentration of treated samples by those in control samples, and multiplying by 100. Relative cell viability, relative cytokine secretion, concentrations of anti-inflammatory compounds obtained from LC-MS/MS analysis were analyzed as a completely randomized design using PROC MIXED in SAS 9.4 (SAS Institute, Cary, NC). The coffee extract (treatment) was the fixed effect and replication was the random variable. Differences between extracts were determined using Fisher's LSD at p-value < 0.01.

The UHPLC-MS data was analyzed based on the procedure described by Ho et al. (2019). First, the original LC-MS data files were converted to NetCDF format files and were uploaded to XCMS online platform (www.xcmsonline.scripps.edu) (Tautenhahn et

al., 2012). The XCMS data were processed with parameters as follows: pairwise analyses between each coffee cultivar and the control (methanol) were conducted in centWave mode for feature detection (1 m/z = 10 ppm, minimum peak width = 5 s, and maximum peak width = 20 s), an obiwarp method was selected for retention time correction (profStep = 1), chromatogram alignment was set as minfrac = 0.5, bw = 5, mzwid = 0.015, max = 100, minsamp = 1, adducts was optimized for UPLC/ Bruker Q-TOF in both ESI(+) and ESI(-) and plant was selected for sample biosource for identification, and the unpaired parametric t-test (Welch t-test) was used for the statistical analysis. Metabolites of significant features (p-value < 0.01 and intensity ≥ 10,000) were putatively identified based on the accurate mass of the molecular ions, referenced to METLIN metabolite mass spectral database containing over 1 million molecules (http://metlin.scripps.edu) (Guijas et al., 2018). Metabolites with known to possess anti-inflammatory activities were used to predict anti-inflammatory compounds in each cultivar (supplementary Table 2). To further characterize differences in profiles of anti-inflammatory compounds among examined coffee cultivars, partial least squares-discriminant analysis (PLS-DA) was performed via a web-based tool MetaboAnalyst (Chong et al., 2018).

RESULTS

Cell viability analysis

Cell viability assays were performed to address any potential cytotoxic effects of the SCG cultivars. A reduction in MTT absorbance could result from a loss of cell

viability, a reduction in cell number, a decrease in mitochondrial activity, an inhibition of cytokine secretion, or a combination of these factors. The MTT viability assays were carried out immediately after the cell supernatants were collected. Dexamethasone, a known inhibitor of cytokine secretion, was included as a positive control. Preliminary experiments using a wide range of DMSO concentrations indicated that DMSO did not affect U-937 cell number or viability as assessed by trypan blue exclusion (data not shown) at the concentrations used in this study, indicating that DMSO is not toxic under the experimental conditions. Therefore, a reduction in MTT absorbance in the presence of the cultivars would indicate a toxic effect of the cultivar rather than the vehicle.

The results shown in Figure 29 illustrate that cell viability in all treatment groups (PMA-differentiated U-937 cells with the addition of SCGs, dexamethasone, LPS) was not significantly different compared to those in the control cultures (PMA-differentiated U-937 cells with DMSO but without LPS and SCG extracts). These results demonstrate that the SCG extracts and dexamethasone did not show any toxic effects on PMA-differentiated U-937 cells at any of the concentrations tested, and they were further examined for their effects on cytokine secretion.

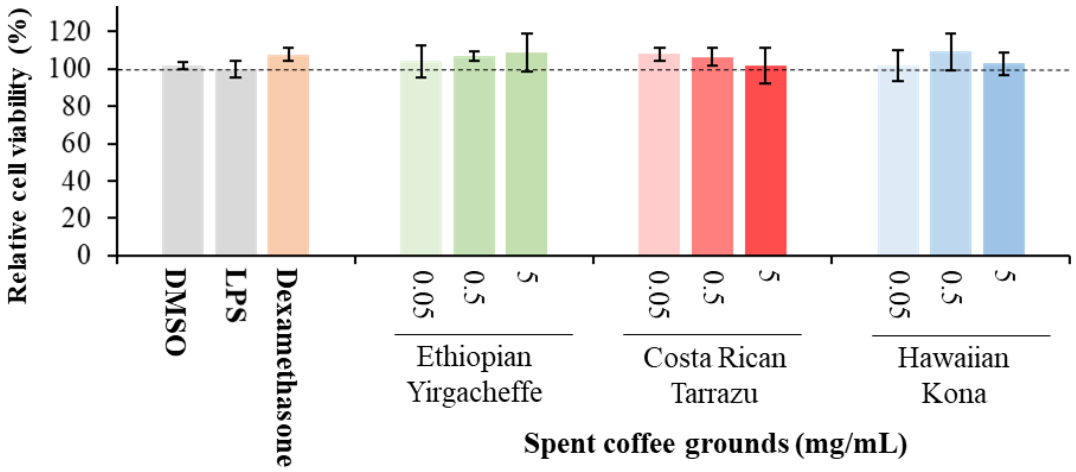


Figure 29. Effect of DMSO, lipopolysaccharide (LPS), Dexamethasone, and spent coffee ground extracts on the viability of PMA-differentiated U-937 cells. DMSO: PMA-differentiated cells treated with 0.1% DMSO, no LPS, no coffee extracts; LPS: LPS and DMSO; coffee extracts: coffee extracts, DMSO and LPS; Dexamethasone: 0.2 µg/mL dexamethasone, DMSO, and LPS. Mean ± SEM.

Impact of spent coffee ground extracts on cytokine secretion

Cytokine secretion was analyzed as a possible indicator of systemic inflammation using the U-937 model system. Secreted cytokine levels were measured in the absence or presence of SGC extracts, with the results expressed relative to control samples cultured with vehicle in the absence of SCG extracts. Three representative cytokines, TNF-α, IL-6, and IL-10, were chosen to reflect both anti-inflammatory as well as pro-inflammatory properties.

As shown in Figures 30-32, inclusion of Hawaiian Kona extracts in the cultures for 2 hours prior to the addition of LPS reduced the levels of all 3 cytokines. This effect was dose-dependent, illustrated by a reduction in TNF-α secretion of 25.8%, 29.9%, and 60.5%, and in IL-6 levels by 32.1%, 49.7%, and 52.5%, at concentrations of 0.05, 0.5,

and 5.0 mg/mL, respectively. TNF-α levels were reduced by 34.4% and 37.8% after pre-incubation of SCG extracts obtained from Ethiopian Yirgacheffe at concentrations of 0.5 and 5.0 mg/mL, respectively, but were not statistically different following pre-incubation with Costa Rican Tarrazu SCGs. A similar reduction in IL-6 levels was observed in cultures containing Ethiopian Yirgacheffe and Costa Rican Tarrazu extracts at the two highest concentrations of 0.5 and 5.0 mg/mL, with a decrease of 40.0%, and 38.9%, and of 47.2% and 36.6%, respectively. No effect on IL-6 levels was observed at the lower concentration of 0.05 mg/mL when U-937 cells were cultured with either extract. The effect on IL-10 secretion was minimal for all three SCG extracts, and the only minor effect noted was a 13.3% reduction after addition of the Hawaiian Kona extract at 5.0 mg/mL. Cytokine levels were reduced to over 90% for all 3 cytokines examined in the presence of the positive control agent dexamethasone, a known inhibitor of cytokine secretion.

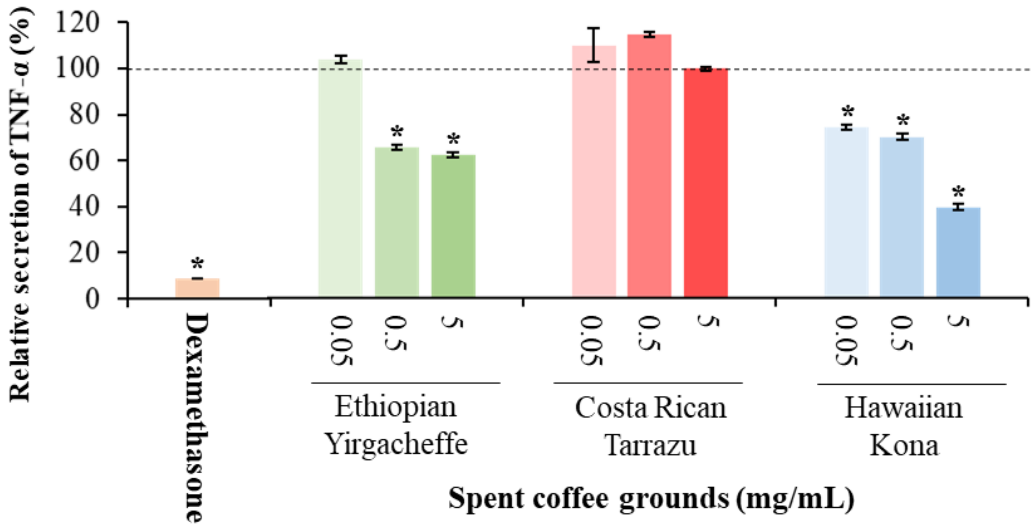


Figure 30. Effect of spent coffee ground extracts on the secretion of TNF-α by PMA-differentiated, LPS-stimulated U937 cells. Dexamethasone was used at 0.2 µg/mL as a

positive control. (*) Significant decrease ($p < 0.001$) compared to PMA-differentiated, LPS-stimulated U937 cells in the absence of extract. Mean ± SEM.

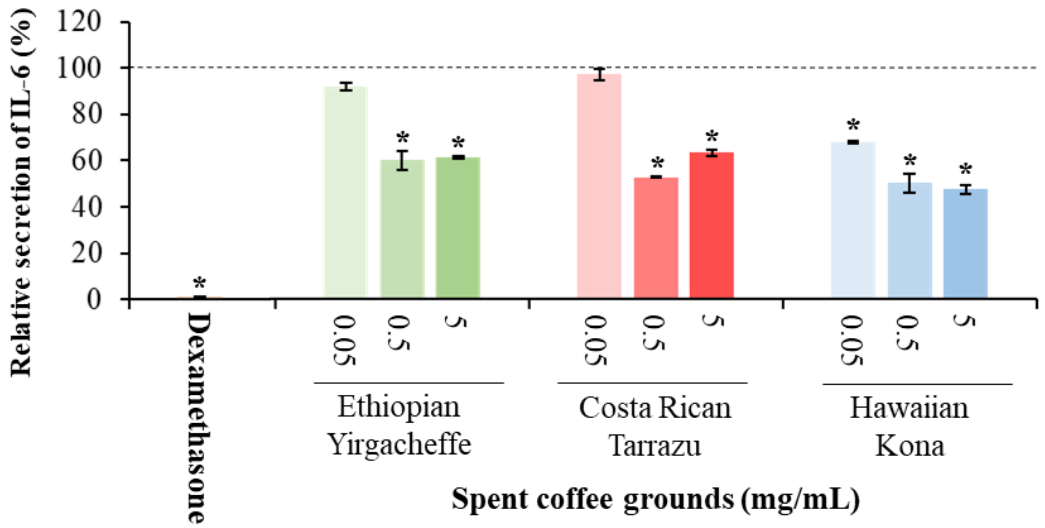


Figure 31. Effect of spent coffee ground extracts on the secretion of IL-6 by PMA-differentiated, LPS-stimulated U937 cells. Dexamethasone was used at 0.2 µg/mL as a positive control. (*) Significant decrease ($p < 0.001$) compared to PMA-differentiated, LPS-stimulated U937 cells in the absence of extract. Mean ± SEM.

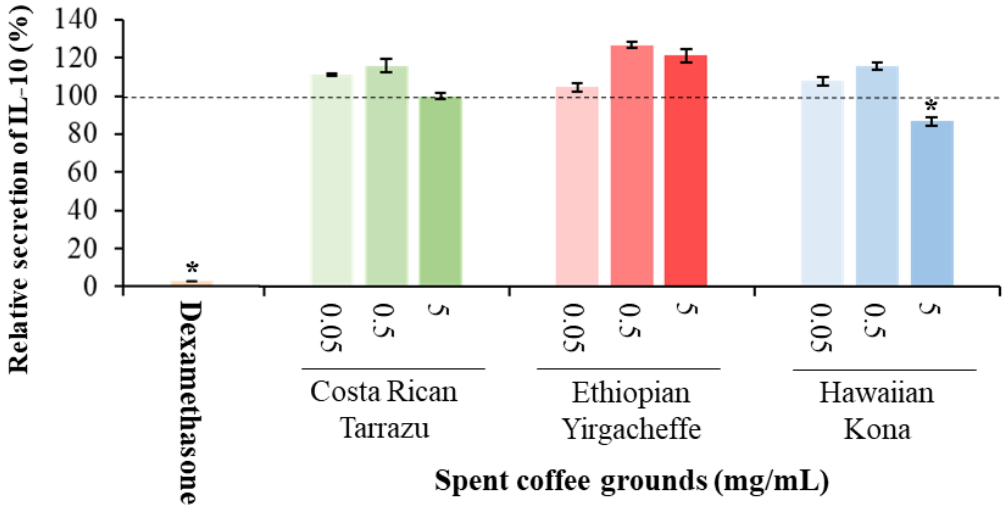


Figure 32. Effect of spent coffee ground extracts on the secretion of IL-10 by PMA-differentiated, LPS-stimulated U937 cells. Dexamethasone was used at 0.2 µg/mL as a positive control. (*) Significant decrease ($p < 0.001$) compared to PMA-differentiated, LPS-stimulated U937 cells in the absence of extract. Mean ± SEM.

UHPLC-HRMS analyses revealed anti-inflammatory metabolite profiles of the spent coffee grounds

LC-MS fingerprints of anti-inflammatory molecules in the extracts were obtained from the liquid chromatography–high resolution MS (LC-HRMS) analysis. The UHPLC-HRMS data processed with XCMS Online resulted in 2,418 significant features in ionization positive mode and 162 features in the negative ionization mode. These features were annotated using METLIN metabolite database, which resulted in the putative identification of 26 metabolites with known anti-inflammatory activities (supplementary Table 2). These metabolites included an alkaloid and polyphenolic compounds (Tables 14 & 15). 12 out of 26 anti-inflammatory metabolites that had high relative intensities across the SCG extracts of all coffee cultivars (Figure 33) were further selected for the absolute quantification of the concentrations in the SCG extracts by LC-MS/MS analyses with authentic standards (Table 16).

Table 14. Metabolites with known anti-inflammatory activities in spent coffee grounds from three Arabica cultivars via untargeted metabolomics analyses. [a]: putative identification by UHPLC-HRMS, [b]: absolute concentrations were determined by LC-MS/MS with authentic standards.

No.	Putatively identified compound	Retention time (min)	Theoretical mass	Exact mass	Δppm	Adducts	Predicted Formula
1	3-Caffeoylquinic acid [a,b]	1.56	353.0866	353.0872	-1.70	[M-H]	$C_{16}H_{18}O_9$
2	5-Caffeoylquinic acid[a,b]	2.24	353.0871	353.0872	-0.28	[M-H]	$C_{16}H_{18}O_9$
3	3,5-Caffeoylquinic acid[a]	6.67	539.1162	539.1165	-0.56	[M+Na]	$C_{25}H_{24}O_{12}$
4	Caffeic acid[a,b]	1.55	179.0348	179.0344	2.23	[M-H]	$C_9H_8O_4$
5	Caffeine[a,b]	0.41	195.0881	195.0882	-0.51	[M+H]	$C_8H_{10}N_4O_2$
6	Catechin[a,b]	3.74	289.0697	289.0712	-5.19	[M-H]	$C_{15}H_{14}O_6$
7	Chrysin[a]	20.28	255.0655	255.0657	-0.78	[M+H]	$C_{15}H_{10}O_5$
8	Daidzein[a]	20.28	255.0655	255.0657	-0.78	[M+H]	$C_{15}H_{10}O_4$
9	Epicatechin[a,b]	2.68	289.0708	289.0712	-1.38	[M-H]	$C_{15}H_{14}O_6$
10	Eugenol[a]	5.55	165.0926	165.0916	6.06	[M+H]	$C_{10}H_{12}O_2$
11	Ferulic acid[a,b]	2.67	193.0504	193.0501	1.55	[M-H]	$C_{10}H_{10}O_4$
12	Gallic acid[a,b]	16.05	208.9846	208.9852	-2.87	[M+K]	$C_7H_6O_5$
13	Naringenin[a]	5.29	271.0610	271.0606	1.48	[M-H]	$C_{15}H_{12}O_5$
14	Naringin[a]	19.21	581.1843	581.1870	-4.65	[M+H]	$C_{27}H_{32}O_{14}$
15	Oxyresveratrol[a]	13.65	407.1341	407.1342	-0.25	[M+H]	$C_{20}H_{22}O_9$
16	p-Coumaric acid[a,b]	2.17	163.0403	163.0395	4.91	[M-H]	$C_9H_8O_3$
17	p-Hydroxybenzoic acid[a,b]	0.76	139.0386	139.0395	-6.47	[M-H]	$C_7H_6O_3$
18	Pectolinarin[a]	31.87	621.1827	621.1819	1.29	[M-H]	$C_{29}H_{34}O_{15}$
19	Quercetin[a]	6.73	489.1359	489.1397	-7.77	[M-H]	$C_{24}H_{26}O_{11}$
20	Quinic acid[a,b]	0.59	191.0562	191.0556	3.14	[M-H]	$C_7H_{12}O_6$
21	Resveratrol[a]	7.71	229.0862	229.0864	-0.87	[M+H]	$C_{14}H_{12}O_3$
22	Rutin[a]	13.65	407.1341	407.1342	-0.25	[M+H]	$C_{20}H_{22}O_9$
23	Tectochrysin[a]	8.42	291.0627	291.0633	-2.06	[M+Na]	$C_{16}H_{12}O_4$
24	Theaflavin[a]	13.01	565.1354	565.1346	1.42	[M+H]	$C_{29}H_{24}O_{12}$
25	Vanillic acid[a,b]	3.70	335.0758	335.0767	-2.69	[M+H]	$C_{16}H_{14}O_8$
26	Vitexin rhamnoside[a]	15.20	601.1539	601.1533	1.00	[M+Na]	$C_{27}H_{30}O_{14}$

Table 15. Molecular and product ions, retention times, linear correlation coefficients, LOD, and LOQ of anti-inflammatory compounds identified in spent coffee ground extracts. Linear equation indicate relationship between peak area of anti-inflammatory compounds and concentration in standard solutions; LOD: limit of detection; LOQ: limit of quantitation

Compound	Molecular ions (m/z)	Product ions (m/z)	Retention time (min)	Polarity	Linear equation[a]	Correlation coefficient (R^2)	LOD[b] (µg/g)	LOQ[c] (µg/g)
5-Caffeoylquinic acid	353	191.04	5.71	ES-	y=96973x	0.9963	0.043	0.142
Quinic acid	190.83	85	2.17	ES-	y=123139x	0.9985	0.023	0.076
Vanillic acid	166.86	-	6.75	ES-	y=21505x	0.9978	0.104	0.346
Caffeic acid	178.88	135	6.65	ES-	y=745425x	0.9994	0.010	0.033
Epicatechin	288.63	109	6.54	ES-	y=22414x	0.9912	0.084	0.279
p-Hydroxybenzoic acid	136.88	93	6.52	ES-	y=269043x	0.9929	0.030	0.100
Catechin	288.65	109.2	6.31	ES-	y=18248x	1.00	0.056	0.186
Ferulic acid	193	134.09	7.43	ES-	y=131247x	0.9993	0.021	0.069
p-Coumaric acid	163.07	119	7.26	ES-	y=403050x	0.9914	0.012	0.04
3-Caffeoylquinic acid	353	191	2.51	ES-	y=541946x	0.9995	0.005	0.017
Gallic acid	169	125	2.48	ES-	y=234571x	0.9862	0.005	0.016
Caffeine	195.01	137.9	6.28	ES+	y=2E-07x	0.9842	0.002	0.007

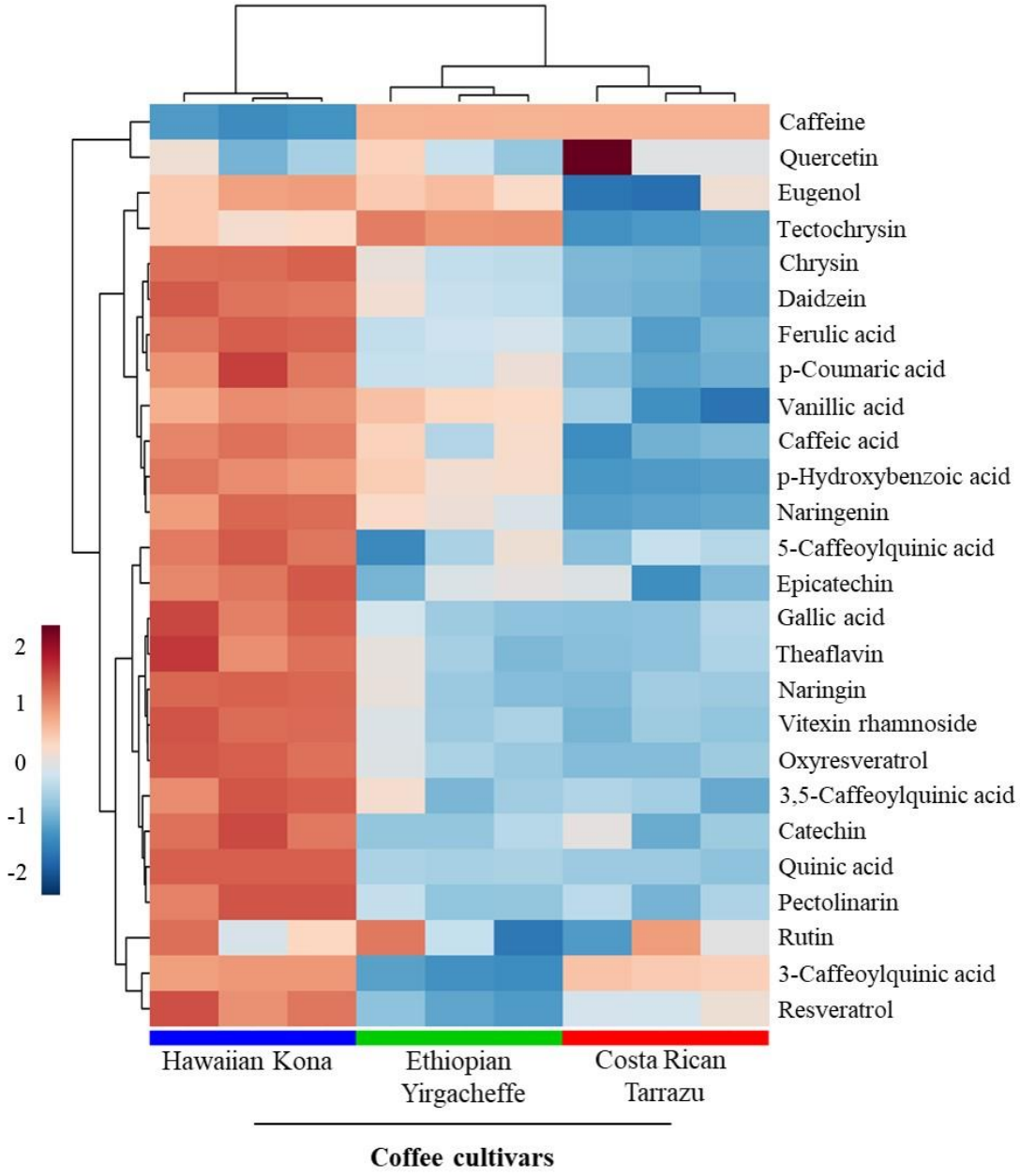


Figure 33. Relative intensities of metabolites with known anti-inflammatory activities in spent coffee grounds. In the heatmap, red represents higher relative abundance, whereas blue represents lower relative abundance.

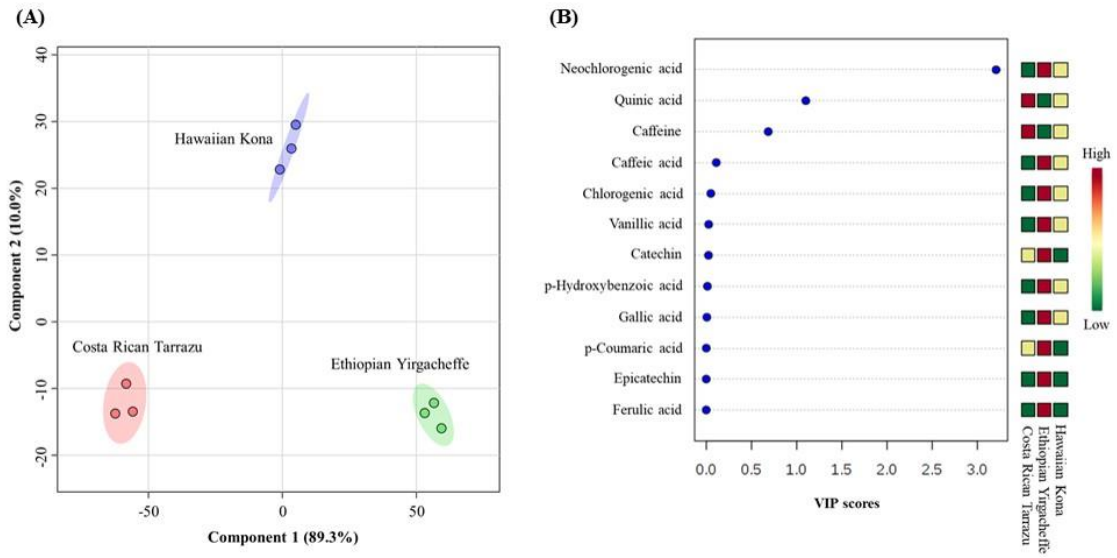


Figure 34. Differences in anti-inflammatory profiles of spent coffee grounds from three coffee cultivars. (A) Partial least squares-discriminant analysis (PLS-DA), (B) Variable importance in projection (VIP). In the PLS-DA plot, circles with same colors represent replicates of metabolic profiles for each cultivar. The colored ellipses indicate 95% confidence regions of anti-inflammatory profiles for each cultivar. In the VIP score plot, the colored boxes on the right indicate the relative abundance of the corresponding anti-inflammatory compounds in each cultivar. Red represents higher relative abundance, whereas green and blue represent lower relative abundance in the VIP score plot.

Table 16. Polyphenols and caffeine (LC-MS/MS) contents (μg/g of dry weight) in spent coffee grounds. *All values are shown as mean ± SE (n=3). In each row, different letter indicates significant differences ($p < 0.01$) among different spent coffee grounds. **LOD: limit of detection.

Polyphenols	Coffee cultivars		
	Ethiopian Yirgacheffe*	Costa Rican Tarrazu	Hawaiian Kona
5-Caffeoylquinic acid	338.1 ± 1.7[a]	236.7 ± 1.5[c]	293.5 ± 1.8[b]
Quinic acid	207.4 ± 0.5[c]	238.3 ± 0.5[a]	218.8 ± 0.3[b]
Vanillic acid	86.2 ± 2.9[a]	53.9 ± 1.3[b]	54.3 ± 1.4[b]
Caffeic acid	54.5 ± 1.5[a]	41.4 ± 0.9[b]	43.4 ± 0.5[b]
Epicatechin	37.2 ± 1.5	<LOD**	<LOD
p-Hydroxybenzoic acid	27.9 ± 0.3[a]	1.9 ± 0.2[b]	2.2 ± 0.1[b]
Catechin	24.0 ± 2.2[a]	1.9 ± 0.3[b]	1.4 ± 0.3[b]
Ferulic acid	21.1 ± 0.6	<LOD	<LOD
p-Coumaric acid	18.3 ± 1.8[a]	0.7 ± 0.1[b]	0.7 ± 0.1[b]
3-Caffeoylquinic acid	3.9 ± 0.5	2.8 ± 0.0	3.7 ± 0.0
Gallic acid	3.1 ± 0.0[a]	1.0 ± 0.0[b]	1.1 ± 0.0[b]
Total polyphenols	821.5 ± 5.7[a]	578.6 ± 1.7[c]	619.1 ± 0.7[b]
Caffeine	384.5 ± 1.1[b]	439.0 ± 4.0[a]	426.9 ± 1.3[a]

LC-MS/MS analyses indicated significant differences in anti-inflammatory profiles of three coffee cultivars

All 12 selected anti-inflammatory metabolites including caffeine, 5-caffeoylquinic acid (5-CQA), quinic acid, vanillic acid, caffeic acid, epicatechin, catechin, ferulic acid, 3-CQA, p-coumaric acid, p-hydroxybenzoic acid, gallic acid were found in Ethiopian Yirgacheffe extracts, while 10 out of 12 metabolites were detected in Costa Rican Tarrazu and Hawaiian Kona extracts (Table 16). The contents of these anti-inflammatory

compounds in SCG vary among tested coffee cultivars. Caffeine, a methylxanthine alkaloid, was the most dominant compound in all examined coffee cultivars. The concentrations of caffeine were found to be the significant higher in Costa Rican Tarrazu (439.0 ± 4.0 µg/g) and Hawaiian Kona (426.9 ± 1.3 µg/g) than that in Ehiopian Yirgacheffe (384.5 ± 1.1 µg/g). The most abundant phenolic compound in all examined coffee cultivars was 5-CQA. Ehiopian Yirgacheffe contained the richest abundance of 5-CQA (338.1 ± 1.7 µg/g), followed by Hawaiian Kona (293.5 ± 1.8 µg/g) and Costa Rican Tarrazu (236.7 ± 1.5 µg/g). The abundance of quinic acid ranked second among the examined phenolic compounds. This compound was found to be at the highest amount in Costa Rican Tarrazu (238.3 ± 0.5 µg/g), followed by and Hawaiian Kona (218.8 ± 0.3 µg/g) and Costa Rican Tarrazu (207.4 ± 0.5 µg/g). The contents of other compounds with an exception of 3-CQA were found to be present at the higher abundances in Ehiopian Yirgacheffe compared to other cultivars, while there were no significant differences in the contents of these compounds in Costa Rican Tarrazu and Hawaiian Kona. The concentrations of vanillic acid, caffeic acid, catechin, p-coumaric acid, p-hydroxybenozic acid in Ehiopian Yirgacheffe were 86.2 ± 2.8 µg/g, 54.5 ± 1.5 µg/g, 24.0 ± 2.2 µg/g, 18.3 ± 1.8 µg/g, 27.9 ± 0.3 µg/g, respectively, while that of Costa Rican Tarrazu and Hawaiian Kona were 53.9 ± 1.3 µg/g and 54.3 ± 1.4 µg/g, 41.5 ± 0.9 µg/g and 43.4 ± 0.5 µg/g, 1.9 ± 0.3 µg/g and 1.4 ± 0.3 µg/g, 0.7 ± 0.1 µg/g and 0.7 ± 0.1 µg/g, 1.9 ± 0.2 µg/g and 2.2 ± 0.1 µg/g, respectively. Epicatechin and ferulic acid were not detectable in Costa Rican Tarrazu and Hawaiian Kona extracts, but were present in Ehiopian Yirgacheffe extracts at concentrations of 37.2 ± 1.5 µg/g and 21.1 ± 0.6 µg/g, respectively. No significant difference in the abundance of 3-CQA was found among all SCG extracts. This

compound was found to be at minor levels in all three coffee extracts with its contents ranging from 2.9 ± 0.2 μg/g (Costa Rican Tarrazu) to 3.9 ± 0.5 μg/g (Hawaiian Kona).

To further characterize differences in anti-inflammatory profiles in SCGs obtained from different coffee cultivars, partial least squares-discriminant analysis (PLS-DA) were performed. A cross-validation method was utilized to evaluate model quality and resulting R2 and Q2 values were 0.99 and 0.97, respectively, indicating that the model was reliable. PLS-DA score plot with two principal components covered 99.3% of total variability of the data (Figure 34A), revealing significant differences in anti-inflammatory profiles in SCG derived from different examined coffee cultivars. The first principal components (PC1) explained 89.3% of the total variability of the data, whereas the second principal components (PC2) accounted for 10.0% of the total variability of the data set. In the PLS-DA score plot, all 3 coffee cultivars were distributed separately along the PC1. Regarding the PC2, Costa Rican Tarrazu and Ethiopian Yirgacheffee relatively shared a similar pattern, and Hawaiian Kona differed from other cultivars. Variable importance in projection (VIP) analyses revealed 5-CQA, quinic acid, and caffeine were the most important compounds (Figure 34B). These compounds also were major compounds found in all tested cultivars (Table 16).

DISCUSSION

Given the huge availability of SCGs, determination of bioactive compounds found within complex mixtures is an important step towards developing novel value-added byproducts that may potentially increase the sustainability of the coffee agro-

industry (Kovalcik et al., 2018). In the present study, we demonstrated that methanolic extracts of SCGs possess compounds that exerted inhibitory effects on the secretion of inflammatory mediators (TNF-α, IL-6 and IL-10) induced in a human pro-monocytic cell line differentiated with PMS and stimulated with LPS. The pro-inflammatory mediators TNF-a and IL-6 are key regulators of innate and adaptive immune responses, and they play a role in disease onset and persistence. As such, they offer potential therapeutic targets for the treatment of acute chronic diseases such as rheumatoid arthritis (Smolen & Aletaha, 2015). IL-10 is a potent immune-modulatory cytokine that has broad anti-inflammatory properties (O'Garra et al., 2008), including the inhibition of TNF production (Smallie et al., 2010). Our results indicated that the cytokine suppressive activities of SCG extracts were different among the coffee cultivars tested. Hawaiian Kona extracts affected the secretion of all 3 examined cytokines in the U-937 model system, whereas Ethiopian Yirgacheffe extracts reduced the secretions of TNF-α and IL-6 only and Costa Rican Tarrazu decreased the secretion of IL-6 only. Cell viability is similar in the absence and presence of all three SCGs extracts, demonstrating that the reduction in cytokine levels is not a result of direct toxic effects. It is possible that the varying levels of cytokine suppressive activities among the Arabica cultivars arise from the differences in the composition and proportions of the anti-inflammatory compounds present in the different SCG extracts (Figure 34). The presence of multiple compounds in the cultivars also raises the possibility that different compounds in the same mixture may modulate each other's activity. For example, the overall cytokine concentration may be determined by a net balance between stimulatory and inhibitory activities. Likewise, there might be synergism between two inhibitory compounds, resulting in a more

profound decrease in cytokine release. The involvement of multiple compounds on cytokine secretion might explain why Hawaiian Kona extracts exhibited inhibitory effects on all three cytokines, whereas the other cultivars had a more limited effect on cytokine secretion. Our findings suggested that SCG extracts from Hawaiian Kona have very distinct profiles of anti-inflammatory molecules and other metabolites as compared to the extracts from other cultivars (Figures 33 & 34). The significant higher levels of the bioactive compounds with known anti-inflammatory activities in the Hawaiian Kona extracts (Figure 33), such as caffeine, 5-caffeoylquinic acid, and quinic acid might be directly or synergistically responsible for the inhibitory activities (Table 16). Furthermore, other materials (e.g., polysaccharides, ash, minerals) found in SCGs (McNutt & Quan, 2018) could be exerting an effect on IL-10 secretion in the U-937 model system. Cytokines are soluble factors that play a role in various steps of acute and chronic inflammation (Brenner et al. 2014). Taken together, our findings suggest that SCGs present a promising source of anti-inflammatory mediators for use in the pharmaceutical and cosmetic industries.

So far, only few studies have examined potential anti-inflammatory activities of SCGs in mouse cell line model systems. Ramalakshmi et al. (2009) evaluated SCG extracts derived from Arabia plantation and Robusta cherry, two varieties of graded coffee beans, on the expression of TNF-α in J774A.1 mouse cell line. Their results showed that TNF-α levels were not suppressed following addition of these two extracts at the 3 concentrations tested (1, 3, and 10 µg/mL). Our results demonstrated inhibitory activity on TNF-α secretion in the human U-937 model system after pre-incubation with Ethiopian Yirgacheffe and Hawaiian Kona extracts. However, the effect was observed in

our study using higher concentrations (500 and 5,000 µg/mL) of SCGs compared to those used in the Ramalakshmi et al. study. López-Barrera et al. (2016) evaluated the effects of SCG fractions fermented by human gut flora on the cytokine secretion in mouse RAW 264.7 macrophages stimulated with LPS. This group reported that out of the 40 cytokines/ chemokines measured, the level of only 3 cytokines (IL-1β, IL-10, and CCL17) was significantly reduced. The cytokine inhibitory activity of the gut fermented, unabsorbed SCG fractions was mainly mediated by short-chain fatty acids derived from dietary fiber (López-Barrera et al., 2016). Our results are consistent with other studies showing an effect of roasted coffee bean extracts on cytokine expression. Jung et al. (2017) investigated the role of different roasting levels (Light, Medium, City and French roast) on the secretion of TNF-α and IL-6 in RAW 264.7 mouse cell line. There findings showed that mRNA expression of TNF-α and IL-6 was reduced in the LPS-stimulated RAW 264.7 cells relative to control cells. In addition, the expression of TNF-α and IL-6 was decreased more as the roasting levels increased. An amount of inhibition comparable to that reported in our study was observed when the light roast coffee extract was tested at the highest concentration, 2 mg/mL, resulting in 42.9% and 36.7% inhibition of TNF-α and IL-6, respectively. The magnitude of this inhibition is comparable to that observed when Hawaiian Kona extracts were tested at 5 mg/mL, whereby a 60.5% and 52.5% reduction was found in TNF-α and IL-6 secreted protein levels, respectively.

Our results demonstrated a diverse range of the anti-inflammatory bioactive compounds in SCGs. In fact, 26 anti-inflammatory metabolites in the SCG extracts were putatively identified via untargeted metabolomics analyses and 12 anti-inflammatory compounds were successfully confirmed and quantified in SCG extracts by LC-MS/MS

analyses with authentic reference standards (Tables 14-16). Among 26 anti-inflammatory metabolites, 12 compounds including chrysin, daidzein, eugenol, naringenin, naringin, oxyresveratrol, pectolinarin, resveratrol, tectochrysin, theaflavin, vanillic acid, and vitexin rhamnoside were the first report possibly present in SCGs, whereas other compounds have been documented as polyphenolic compounds in SCGs (Andrade et al., 2012; López-Barrera et al., 2016). Future research will focus on purification and characterization of compounds mainly driving the cytokine suppressive activities in SCGs.

Among the identified anti-inflammatory compounds, our results revealed that caffeine and 5-caffeoylquinic acid (5-CQA), a monocaffeoylquinic acid (CQA), were the most abundant compounds in the SCG extracts from all examined coffee cultivars. The contents of caffeine ranged from 0.38 mg/g (Ethiopian Yirgacheffe) - 0.44 mg/g (Costa Rican Tarrazu), whereas 5-CQA concentrations were in the range of 0.24 mg/g (Costa Rican Tarrazu) - 0.34 mg/g (Ethiopian Yirgacheffe). Caffeine is well-known as a signature compound in coffee and coffee byproducts. López-Barrera et al. (2016) reported that caffeine contents in Arabica SCG obtained from 2 roasted levels (medium and dark roasted) with Soxhlet extraction were approximately 0.4 mg/g, which roughly shared similar values of caffeine found in this study. However, Bravo et al. (2012) previously reported the contents of caffeine of Arabica SCG with Soxhlet extraction were in the range of 3.6 – 5.2 mg/g depending on the coffeemakers (filter, espresso, and plunger), which were >9 times higher than the values observed in López-Barrera et al. (2016) and our study. Caffeoylquinic acids [monocaffeoylquinic and dicaffeoylquinic acids (diCQA)] have been documented as the most abundant phenolic compounds in

spent coffee grounds (Campos-Vega et al., 2015), in which 5-CQA was the most abundant compound in SCGs obtained from both Arabica and Robusta varieties (Bravo et al., 2012). Bravo et al. (2012) reported that the contents of 5-CQA in Arabica spent coffee grounds obtained from different coffeemakers including filter, espresso, plunger were 3.6, 2.8, 2.5 mg/g respectively, which was 8-15 times higher than the values observed in our study.

Differences in the levels of bioactive compounds in SCG among different studies are likely due to the differences in coffee materials (coffee varieties, cultivars, geographic sources, growth conditions), roasting processes and extraction preparation. Our results indicated that the SCGs obtained from 3 different Arabica cultivars differed on the contents of anti-inflammatory compounds (Table 16). In fact, the total phenolic compounds of SCGs were found to be highest in Ethiopian Yirgacheffe (0.82 mg/g), followed by Hawaiian Kona (0.62 mg/g), and then Costa Rican Tarrazu (0.58 mg/g). The concentrations of bioactive compounds in SCGs have been previously reported to be variable among coffee varieties. Arabica spent coffee grounds contained less caffeine than Robusta SCGs, but the contents of caffeoylquinic acids (i.e., 5-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) in Arabica SCGs were higher than that in Robusta SCGs (Bravo et al., 2012). Furthermore, the amounts of bioactive compounds in SCGs have been previously documented to be highly dependent on extraction techniques and solvents. Many extraction methods (i.e. solid-liquid extraction, supercritical fluid extraction, Soxhlet extraction, and ultrasound, and microwave) with different solvents i.e., polar (methanol and ethanol), medium or non-polar (e.g. dichloromethane, ethyl acetate, hexane, supercritical fluids, subcritical water, deep eutectic and supramolecular

solvents) have been utilized to maximize the recovery of bioactive compounds in SCGs (Andrade et al., 2012; Getachew & Chun, 2017; Mussatto et al., 2011; Pavlović et al., 2013; Torres-Valenzuela et al., 2019; Yoo et al., 2018). Spent coffee grounds defatted with petroleum ether (1:11, w/v) for 3 h at 60°C in a Soxhlet extraction system yielded the highest contents of total phenolic compounds, which ranged 18-22 mg 3-CQA equivalents/g SCG (Andrade et al., 2012). An increase in roasting levels of coffee beans significantly reduced the total phenolic compounds in SCGs. In fact, total phenolic compounds of SCGs derived from medium roasted coffee beans were 9.9 mg/g, while that of SCGs obtained from dark roasted coffee beans were 4.6 mg/g SCG (López-Barrera et al., 2016). Future efforts are ongoing to identify optimum roasting conditions that maximize the cytokine suppresive activties of the SCG extracts.

CONCLUSION

Methanolic extracts of SCGs possessed cytokine inhibition on the human pro-monocytic cell line U-937. The cytokine suppressive effects and the contents of anti-inflammatory compounds in SCGs obtained from different Arabica cultivars were variable. Hawaiian Kona extracts showed the strongest inhibitory effect on the secretion of all 3 cytokines (TNF-α, IL-6, and IL-10), while Ethiopian Yirgacheffe extract reduced the secretions of TNF-α and IL-6 and Costa Rican Tarrazu only decreased the secretion of IL-6. Multiple (26) metabolites in the SCG extracts with known anti-inflammatory activities were identified via an untargeted metabolomics analysis. Targeted (LC-MS/MS) analyses resulted in the quatification of 12 metabolites that had high relative intensities in all of the extracts. Spent coffee grounds contain a wealth of anti-

inflammatory bioactive compounds with caffeine and 5-CQA as the most abundant compounds. Our findings indicate that SCGs could be promising sources of anti-inflammatory bioactive compounds that can be utilized for pharmaceutical and cosmetic industries.

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SUPPLEMENTARY INFORMATION

Supplementary Table 2. Putative metabolites with known to produce anti-inflammatory activities in the methanolic extracts of spent coffee grounds identified via an untargeted metabolomics approach.

No.	Putatively identified compound	Anti-inflammatory activity
1	3-Caffeoylquinic acid	Hwang et al. (2014)
2	5-Caffeoylquinic acid	Kim et al. (2015)
3	3,5-Caffeoylquinic acid	Hong et al. (2015)
4	Caffeic acid	Chao et al. (2009)
5	Caffeine	Köroğlu et al. (2014)
6	Catechin	Nakanishi et al. (2010)
7	Chrysin	Ahad et al. (2014)
8	Daidzein	Liu et al. (2009)
9	Epicatechin	Morrison et al. (2014)
10	Eugenol	Yogalakshmi et al. (2010)
11	Ferulic acid	Zhu et al. (2014)
12	Gallic acid	Kim et al. (2005)
13	Naringenin	Pinho-Ribeiro et al. (2016)
14	Naringin	Sahu et al. (2014)
15	Oxyresveratrol	Wei et al. (2017)
16	p-Coumaric acid	Pragasam et al. (2013)
17	p-Hydroxybenzoic acid	Manuja et al. (2013)
18	Pectolinarin	Lim et al. (2008)
19	Quercetin	Guardia et al. (2001)
20	Quinic acid	Åkesson et al. (2005)
21	Resveratrol	Nunes et al. (2018)
22	Rutin	Guardia et al. (2001)

23	Tectochrysin	Hou et al. (2018)
24	Theaflavin	Zu et al. (2012)
25	Vanillic acid	Kim et al. (2011)
26	Vitexin rhamnoside	Tadić et al. (2008)

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CHAPTER VII: ASSESSING THE HEALTH-PROMOTING ACTIVITIES OF THE EXTRACTS FROM SWITCHGRASSES (*PANICUM VIRGATUM*)

ABSTRACT

Switchgrass is a biomass energy feedstock that potentially possesses multiple health benefits stemming from its phytochemical composition. In this study, we explored potential health-promoting properties (i.e., anti-inflammatory, antibacterial, antimycobacterial, antiproliferative, antityrosinase, anti-elastase activity) of four switchgrass cultivars (Alamo, Kanlow, Liberty, and Show Me) using in vitro bioassays combined with metabolomics approaches. Our results demonstrated that the methanolic extracts of switchgrass possessed compounds that exerted inhibitory effects on the secretion of inflammatory mediators [tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-8, and IL-10] induced by the human pro-monocytic U-937 cell line differentiated with PMA (phorbol 12-myristate 13-acetate) and stimulated with lipopolysaccharide (LPS). Switchgrass extracts from all four cultivars reduced the secretion of all inflammatory mediators examined, except for Liberty that showed no significant effect on IL-10 expression. The degree of cytokine inhibition was variable depending on the particular cultivar, the concentrations tested, and the cytokines examined. Multiple metabolites with known anti-inflammatory activities were putatively identified via untargeted metabolomics profiling and were quantified using LC-MS/MS analyses. Our results also indicated that the switchgrass extracts exhibited no significant inhibition against two bacterial strains (*Cutibacterium acnes* and *Mycobacterium smegmatis*) and two cancer cell lines (HT-29 and UCT-MEL1) and no tyrosinase and

elastase inhibitory effects were observed on the switchgrass extracts. Our findings suggested that switchgrass, particularly Alamo and Kanlow, may represent a promising natural anti-inflammatory source for cosmetic, nutraceutical and pharmaceutical industries. The findings from this study offer an excellent opportunity to increase the overall economic value of switchgrasses in the biorefinery supply chain and promote the development of new sources of biological agents for mitigating inflammation.

INTRODUCTION

Switchgrass (*Panicum virgatum* L.) is a terrestrial, perennial, high biomass, and warm-season C4 grass (Parrish & Fike, 2005). This native herbaceous plant is one of the most common species in North American tallgrass prairies with high productivity, broad geographical adaption, low requirements for agricultural inputs, and positive environmental impacts (Keshwani & Cheng, 2009; Parrish & Fike, 2005). Switchgrass is valued as a multipurpose crop species that is traditionally used for forage production, soil erosion control, wildlife habitat, and ornamentals (Parrish & Fike, 2005). Recently, this grass species has been considered as the critical cellulose source for cellulosic bioethanol production (McLaughlin & Kszos, 2005). Switchgrass can also play a role in phytoremediation as a vegetative filter that can absorb pesticide residues in water systems and can remove soil contaminants (e.g., herbicides, polychlorinated biphenyls, and radionuclides) (Belden & Coats, 2004; Blanche et al., 2003; Dzantor et al., 2000; Entry & Watrud, 1998). Other value-added applications of switchgrass include thermal conversion, pulping and paper making (Keshwani & Cheng, 2009).

Switchgrass has been postulated to possess a wide range of biological functions stemming from its phytochemical composition. Switchgrass extracts contain several classes of bioactive phenolic compounds (e.g., caffeic acid, vanillic acid, p-coumaric acid, ferulic acid, rutin, quercitrin) (Hu et al., 2010; Tao et al., 2019; Uppugundla et al., 2009) that are known to exert health-promoting benefits such as antioxidant, antibacterial, anti-inflammatory, and anticancer capacities. Of these potential health-promoting benefits, only antioxidant activities of switchgrass extracts have been definitively shown (Lau et al., 2004; Tao et al., 2019; Uppugundla et al., 2009). Relative to other potential bioenergy crops, switchgrass possesses higher antioxidant capacities than mimosa seed, spinach, and castor foliage, but lower antioxidant properties than mimosa foliage, sericea, velvet bean foliage, kudzu, and arunzo (Lau et al., 2004). Uppugundla et al. (2009) documented two flavonoids, quercitin and rutin as major compounds responsible for the low-density lipoprotein oxidation inhibition activity observed in aqueous switchgrass extracts. Despite the findings, other potential health-promoting characteristics of switchgrass (e.g., anticancer, anti-elastase, anti-inflammatory, antityrosinase capacity) are not yet established. Identifying health-promoting properties of switchgrass might point to novel uses and applications of switchgrass and its by-products that would increase the economic value of switchgrass in the biorefinery supply chain and promote the development of new biological agents for cosmetic and pharmaceutical industries.

In the present study, we first evaluated multiple medicinal activities (i.e., antibacterial, anti-inflammatory, antimycobacterial, antiproliferative, antityrosinase, anti-elastase activity) of four switchgrass cultivars. We then putatively identified anti-

inflammatory molecules in the switchgrass extracts via a global metabolomics profiling approach and subsequently quantified bioactive compounds in the extracts using targeted analyses.

MATERIALS AND METHODS

Switchgrass collection

Four switchgrass cultivars including Alamo, Kanlow, Liberty, and Show Me were obtained from the University of Missouri, Horticulture and Agroforestry Research Center (New Franklin, MO, United States). All cultivars were established from seed in 2016 and the switchgrass samples were harvested in July 2017. After harvesting, the samples were immediately stored at -20 °C until analysis.

Sample preparation

The plant samples from each switchgrass cultivar (10 g, dry weight basis) were homogenized using a coffee grinder (CBG100S, Black+Decker, Beachwood, OH, United States). The homogenized samples were extracted in 200 mL of methanol (HPLC grade, Fisher Scientific, Pittsburg, PA, United States) twice, and sonicated at 10 °C for 60 min. Subsequently, the methanolic extract was filtered through a 0.2 µm Whatman Anotop filter (GE Healthcare, Germany) to collect the supernatant. For cellular assays, the supernatant was evaporated until dryness under a flow of nitrogen and the resulting dry extract was resuspended with 0.125 mL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, United States) and was concentrated 40 times to achieve the concentration of 10,000 mg/mL.

Identification of anti-inflammatory potential of switchgrass

Cell culture and differentiation induction

The human pro-monocyte cell line U-937 was purchased from American Type Culture Collection (ATCC) (CRL-1593.2, ATCC, Manassas, VA, United States). Cell culture and differentiation induction protocols were performed as previously described (Ho et al., 2019). Briefly, U-937 cells were grown in complete Roswell Park Memorial Institute (RPMI) medium (RPMI 1640, ATCC) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 100 µg/mL gentamicin and cultured at 37^{o}C in a 5% CO_2 humidified atmosphere. To induce differentiation, 2 x 10^5 U-937 cells were seeded in a 96-well plate in 200 µL media containing 50 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 48 hours (Rovera et al., 1979). Subsequently, PMA-differentiated cultures were washed twice and supplemented with fresh media. Following an 18 hour incubation, the media was removed and the cultures were pre-treated with four switchgrass cultivars (Alamo, Kanlow, Liberty, and Show Me) at 3 final concentrations (0.1, 1, and 10 mg/mL) for 2 hours before stimulation with LPS (1 µg/mL, *Escherichia coli* 0127:B8, Sigma-Aldrich). Two immunosuppressant agents, dexamethasone (2 µg/mL, Sigma-Aldrich) and cyclosporin A (2 µg /mL, Sigma-Aldrich) served as positive controls for the inhibition of cytokine secretion, and vehicle controls were included in cultures lacking extracts or inhibitors. Twenty-two hours following LPS stimulation, culture supernatants from each triplicate group were pooled, spun to remove cell debris, transferred to new tubes, and stored at -20^{o}C until analysis.

Cell viability analysis

MTT assays were conducted to determine possible effects of switchgrass extracts on cytotoxicity and/or cell loss that can lead to reduced cytokine levels. A colorimetric cell viability assay (CGD1-1KT, Sigma-Aldrich) was used to measure mitochondrial dehydrogenase activity of the attached cells after removal of the supernatants. The MTT assays were performed as described in Ho et al. (2019). Briefly, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) substrate (Mosmann, 1983) prepared in Dulbecco's modified eagle medium (DMEM) high-glucose, phenol red free media (Gibco, Pittsburgh, PA, United States) containing 1% FBS was added to the cells and incubated for 3 hours at 37°C until formazan crystals were observed. Crystals were completely dissolved in acidified isopropanol by pipetting multiple times before taking readings. A BioTek ELx808 microplate reader (BioTek,Winooski, VT, United States) was used to measure absorbance of the formazan crystals at a wavelength of 570 nm within 30 min after the addition of the solvent. Background absorbance was measured at a wavelength of 630 nm. MTT conversion levels were calculated by subtracting the background absorbance (630 nm) from the absorbance of formazan crystals at 570 nm (A_{570} nm – A_{630} nm = specific MTT absorbance).

Quantification of secretion of cytokines/chemokines by macrophages

Soluble cytokine levels were analyzed in the U-937 model system as an indicator of inflammation. Cytokines released into the supernatant were measured in PMA-differentiated, LPS-stimulated cells in the absence or presence of switchgrass extracts. Four representative cytokines, TNF-α, IL-6, IL-8, and IL-10, were chosen to reflect anti-inflammatory as well as proinflammatory properties. Cytokine levels were quantitated

using a Cytometric Bead Array (CBA) human inflammatory cytokine kit according to the manufacturer's procedure (BD Biosciences, San Jose, CA, United States). Samples from each treatment group were run in triplicate on a BD LSRFortessa^{TM} X-20 cell analyzer (BD Biosciences, San Jose, CA, United States) using instrument settings suggested by BD and optimized in each experiment. Cytokine levels were calculated from a standard curve for each cytokine generated from a 5-parameter logistic curve using curve-fitting software.

Identification and qualification of anti-inflammatory molecules in switchgrass

UHPLC-QTOF-MS analysis

For both metabolomic profiling and quantitative analyses, the same methanolic extracts were analyzed by ultra-high performance liquid chromatography with high-resolution mass-spectrometry (UHPLC-HRMS) and tandem mass spectrometer (HPLC-MS/MS). The untargeted metabolomics analysis was conducted as described previously (Ho et al., 2018). Briefly, the methanolic extracts (2 µL per injection) obtained from four switchgrass cultivars were injected into UHPLC coupled with a maXis impact quadrupole-time-of-flight (QTOF) mass spectrometer (Bruker Co., Billerica, MA, United States) operated in both negative and positive electrospray ionization modes. The nebulization gas pressure was at 43.5 psi and a capillary voltage was used at 4000 V. Dry gas was 12 L/min, whereas dry temperature was at 250°C. The Waters Acquity UHPLC BEH C18 column (2.1x 150 mm, 1.7 µm particles size) was used for the separations with a linear gradient as follows 5%-70% B (0-30 min, A: 0.1% formic acid and B:

acetonitrile), 70-95% B (30-33 min), 95% B (33-35 min), 95-5% B (35-36 min), and 5% B (37-40 min). The column was kept at 60^{o}C with a flow rate of 0.56 mL/ min. Mass spectral data were collected automatically and auto-calibrated with a full scan ranging from m/z 100 to 1,500. Each treatment was analyzed in triplicate and the solvent (methanol) was used as a control.

HPLC-MS/MS analysis

There metabolites (quercetin, quercetin 3-glucoside and rutin hydrate) were quantitated using an HPLC system (Water Alliance 2695, Water Co., Milford, MA, United States) coupled to a Waters Acquity TQ triple quadrupole mass spectrometer, as described in Ho et al. (2020). Mass spectrometry of the extracts (20 µL/ injection) was analyzed in triplicate in negative electrospray ionization mode operated with the nebulization gas pressure at 43.5 psi, dry gas of 12 L/min, dry temperature of 250^{o}C and a capillary voltage of 1500 V. The separations were achieved on a Phenomenex Kinetex C18 reverse-phase column (100 × 4.6 mm; 2.6 mm particle size, Torrance, CA, United States) at 25^{o}C with a flow rate of 0.5 mL/min. The linear gradient was used for the elution as follows: 2% B (0–0.5 min, A: 0.1% formic acid and B: acetonitrile), 2–80% B (0.5–7 min), 80–98% B (7.0–9.0 min), 2% B (9.0–15.0 min). Multiple reaction monitoring (MRM) mode was used for MS/MS detection with parameters [e.g., ionization energy, MRM and SIR (single ion recording) transition ions (molecular and product ions), capillary and cone voltage, desolvation gas flow, and collision energy] optimized by Waters IntelliStart software.

Evaluation of other biological activities of switchgrass

Antibacterial activity against Cutibacterium acnes

Cultures of *C. acnes* (ATCC 6919) were grown on Brain Heart Infusion (BHI) agar plates (Anatech Instruments (Pty) Ltd, Randburg, South Africa) from a Microbiologics KWIK-STIK (Anatech Instruments (Pty) Ltd). Inoculated BHI agar plates were incubated in an Anaerocult jar with Anaerocult A (Merck Chemicals (Pty) Ltd, Germiston, South Africa) at 37°C for 72 hours. Antibacterial activity was determined using the method described by Lall et al (2013). The switchgrass extracts and the positive control, tetracycline (Sigma-Aldrich, Modderfontein, South Africa), were tested at 8 final concentrations in 2 × serial dilution, ranging from 3.91 - 500 µg/mL and 0.39 - 50 µg/mL, respectively. A vehicle control (DMSO at 2.5 %) and a BHI broth media control was included. The PrestoBlue reagent was used to visually determine the minimum inhibitory concentration.

Antimycobacterial activity against Mycobacterium smegmatis

The *Mycobacterium smegmatis* (MC^2 155) strain was donated by the Department of Medical Microbiology (University of Pretoria, Pretoria, South Africa). Middlebrook 7H11 agar plates (Sigma-Aldrich) were used to culture *M. smegmatis* at 37°C for 48 hours. Antimycobacterial activity was determined using the method described by Reid et al (2019). The switchgrass extracts and the positive control, ciprofloxacin (Sigma-Aldrich), were tested at 7 final concentrations in 2 × serial dilution, ranging from 15.63 - 1000 µg/mL and 0.16 - 10 µg/mL respectively. A vehicle control (DMSO at 5 %) and a

7H9 media control was included. The MIC was visually determined using the PrestoBlue viability reagent.

Antiproliferative activity against cancer cell lines

The human colorectal adenocarcinoma (HT-29) cells (Cellonex CHT-FL, Separations Scientific Group, Johannesburg, South Africa) were maintained in McCoy's 5A Medium Modified (Separations), whereas the pigmented human malignant melanoma (UCT-MEL1) cells, which were originally isolated from the metastatic lymph nodes of a patient from Groote Schuur Hospital (Cape Town, South Africa) and donated by Dr. Lester Davids (University of Cape Town, Cape Town, South Africa), were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Separations). Culture media was supplemented with 10% FBS (Separations) and 1% PSF (100 µg/mL penicillin, 100 µg/mL streptomycin and 250 µg/mL fungizone). Cells were cultured in T75 flasks (Lasec SA (Pty) Ltd, Midrand, South Africa) and incubated at 37°C and 5% CO_2 until 80% confluence. Cells were detached using 0.1% Trypsin-EDTA (Biocom Africa (Pty) Ltd, Centurion, South Africa) and seeded in 96-well plates at a concentration of 1×10^5 cells/mL and allowed to attach for 24 hours. The switchgrass extracts and the positive control, actinomycin D (Sigma-Aldrich), were tested at 8 final concentrations in 2 × serial dilution, ranging from 3.13 - 400 µg/mL and 3.9×10^{-4} - 0.05 µg/mL, respectively. A vehicle control (DMSO at 2%) and a media control was included. The cell viability was determined using the PrestoBlue reagent where fluorescence was measured using a Victor Nivo multiwell plate reader (PerkinElmer South Africa (Pty) Ltd, Midrand, South Africa) with excitation/emission set to 535nm/615nm.

Elastase inhibition

The porcine pancreatic elastase (PPE) Type IV (E0248, Sigma USA) inhibitory potential was determined spectrophotometrically according to the method described by Lall et al. (2017) with modifications. The reaction mixture contained 100 mM Tris buffer (pH 8.0), 0.5 M HCl and the extracts or positive control (ursolic acid) at 7 final concentrations in 2 × serial dilution, ranging between 7.81-500 µg/mL. The reaction mixture was completed with the addition of 20 µL PPE (5 mM) following incubation at 37°C for 15 min. The enzyme reaction was initiated with the addition of 30 µL of *N*-succinyl-ala-ala-ala-*p*-nitroanilide (4 mM). Two 0% controls were included, one with no enzyme (replaced with buffer) and one with no substrate (replaced with buffer). A 100% activity control, where the samples were replaced with DMSO, was also included. The change in the absorbance of the reaction was kinetically measured using a BioTek PowerWave XS multiwell plate reader for 15 min at 37°C to obtain the relative rate. One unit of elastase activity is defined as the release of 1 µM of *p*-nitroaniline/min.

Tyrosinase inhibition

The tyrosinase inhibitory potential of the switchgrass extracts was determined using a colorimetric assay as described in Lall et al. (2019). Inhibitory activity was determined on the monophenolase activity of mushroom tyrosinase isolated from *Agaricus bisporus* (Sigma-Aldrich). The switchgrass extracts and the positive control, kojic acid (Sigma-Aldrich), were tested at 8 final concentrations in 2 × serial dilution, ranging from 1.56 - 200 µg/mL. A vehicle control (DMSO at 1%) was included. The enzyme reaction was initiated with the addition of the L-tyrosine (Sigma-Aldrich) substrate (637.50 µM) and the change in absorbance was measured kinetically for 30 min

at 492 nm using a BioTek PowerWave XS multiwell plate reader. The relative rate for the reaction was determined and compared to the 0% inhibition control (DMSO at 1%).

Data processing and statistical analysis

Results from experiments addressing cell viability and secreted cytokine levels in treatment groups were expressed relative to the corresponding control groups. The treatment groups included PMA-differentiated, LPS-stimulated U-937 cells in the presence of switchgrass extracts or the known cytokine inhibitors dexamethasone and cyclosporin A. The respective control groups contained the extract vehicle (DMSO) or inhibitor vehicles (DMSO or ethanol), in the absence of extracts. Data were expressed relative to corresponding controls cultured in the presence of vehicle but in the absence of switchgrass extracts that were set to 100%. Relative cell viability (%) was calculated by dividing the MTT absorbance of the treatment group by the MTT absorbance in the corresponding control group and multiplying by 100. Relative secreted cytokine levels (%) were calculated by dividing the secreted cytokine levels of the treatment group by the secreted cytokine levels in the control group and multiplying by 100. Relative cytokine reductions (%) were calculated by subtracting the relative secreted cytokine levels from 100%. The differences in the relative cell viability and secreted cytokine levels between the treatment and the control groups were compared using a two-tailed paired t-test in SAS 9.4 (SAS Institute, Cary, NC). Statistical differences between treated and control groups were determined using least square means at p-value < 0.01.

For untargeted metabolomics analyses, the UHPLC-MS data was analyzed as described by Ho et al. (2020). Briefly, XCMS Online (Tautenhahn et al., 2012) was used for data processing to identify significant features (p-value < 0.05, fold change ≥ 1.5, and

intensity ≥ 10,000) between each switchgrass cultivar and the control (methanol) across the chromatographic time domain. The parameters used as follows: pairwise analyses between each switchgrass cultivar and the control were performed in a centWave method for feature detection (1 m/z = 10 ppm, minimum and maximum peak width of 5 s and 20 s, respectively), an obiwarp mode for retention time correction (profStep = 1), chromatogram alignment (minfrac = 0.5, bw = 5, mzwid = 0.015, max = 100, minsamp = 1), adducts optimized for UPLC/ Bruker Q-TOF in both ESI(-) and ESI(+), and plant selected for sample biosource, and unpaired parametric t-test (Welch t-test) was used for statistical analysis. The metabolite annotation of significant features was based on the accurate mass of the molecular ions (m/z values), referenced to METLIN metabolite mass spectral database with over 1 million molecules (Guijas et al., 2018). Metabolites with known anti-inflammatory activities were selected to predict anti-inflammatory profiles in each cultivar (supplementary Table 3). Partial least squares-discriminant analysis (PLS-DA) and heat map were performed to characterize differences in profiles of anti-inflammatory compounds among switchgrass cultivars using MetaboAnalyst (Chong et al., 2018), a web-based tool for metabolomics data analysis.

For targeted metabolomics analyses, the contents of metabolites in the extracts were extrapolated by a standard curve for each associated analyte generated using authentic standards (purity > 95%, Sigma-Aldrich) at 7 concentrations (0.01, 0.025, 0.05, 0.1, 0.5, 1, 5, and 10 ppm) in triplicate. Differences in concentrations of anti-inflammatory compounds obtained from the LC-MS/MS analyses among the switchgrass cultivars were analyzed as a randomized complete block design using PROC MIXED in SAS 9.4 (SAS Institute). The switchgrass cultivar was the fixed effect and replication

was the random variable. Differences between cultivars were determined using Fisher's LSD at p-value < 0.01.

For other biological activities, minimum inhibitory concentrations (MIC) of each cultivar plus the positive control were visually determined for antibacterial activity against *C. acnes* and antimycobacterial activity against *M. smegmatis*. Half maximal inhibitory concentrations (IC_{50}) of each cultivar plus the positive control were determined from the dose-response curve for the antiproliferative activity against two cancer cell lines (HT-29 and UCT-MEL1), elastase inhibition, and tyrosinase inhibition. The IC_{50} values were generated using GraphPad Prism 4 (San Diego, CA, United States).

RESULTS

Anti-inflammatory potential of switchgrass

Cell viability analysis

MTT colorimetric assays were performed to address potential cytotoxic effects of the switchgrass cultivars on U-937 cell viability since this could account for reduced cytokine levels. The MTT assays were performed on the attached cells immediately after collecting supernatants for cytokine quantitation. Two known anti-inflammatory agents, dexamethasone and cyclosporin A, were included as positive controls to demonstrate that modulations in secreted cytokine levels could be detected under the experimental conditions. To examine the effect of the vehicle used to prepare switchgrass extracts, a 0.1% DMSO control group was included. This concentration represented the highest

amount of DMSO present in any of the extract dilutions as well as the amount used in our previous studies (Ho et al. 2019). Likewise, ethanol and DMSO served as vehicle controls for dexamethasone and cyclosporin A, respectively. The results in Table 17 indicated that no significant differences in cell viability were observed among any of the PMA-differentiated, LPS-stimulated U-937 groups. Specifically, cell viability was similar regardless of whether the cells were exposed to treatment (switchgrass extracts), immunosuppressant agents (dexamethasone and cyclosporine A), or vehicle (DMSO or ethanol). U-937 PMA-differentiated cells cultured in the presence of DMSO but in the absence of LPS showed similar viability to those containing LPS. Taken together, these findings indicated that the switchgrass extracts, inhibitors, and vehicles did not show toxic effects in the U-937 model system.

Table 17. Effect of DMSO, lipopolysaccharide (LPS), anti-inflammatory agents, and switchgrass extracts on the viability of PMA-differentiated U-937 cells. DMSO: PMA-differentiated cells treated with 0.1% DMSO, no LPS, no switchgrass extracts; LPS: LPS and DMSO; switchgrass extracts: switchgrass extracts, DMSO and LPS; Dexamethasone/ cyclosporin A: dexamethasone/ cyclosporin A, DMSO, and LPS. Mean ± SEM.

Treatment	Cell viability (%)
Vehicles/ controls	
LPS (1 µg/mL)	100.0 ± 4.6
LPS (1 µg/mL) + DMSO (0.1 %)	101.9 ± 2.9
LPS (1 µg/mL) + Ethanol (0.2%)	99.1 ± 6.3
Anti-inflammatory agents	
Dexamethasone (2 µg/mL)	107.7 ± 3.6
Cyclosporin A (2 µg/mL)	99.1 ± 5.5
Switchgrass cultivars	
Alamo (0.1 mg/mL)	109.9 ± 6.7
Alamo (1 mg/mL)	95.5 ± 7.9
Alamo (10 mg/mL)	104.7 ± 3.0
Kanlow (0.1 mg/mL)	106.4 ± 2.7
Kanlow (1 mg/mL)	101.0 ± 4.3
Kanlow (10 mg/mL)	111.1 ± 8.9
Liberty (0.1 mg/mL)	127.2 ± 6.5
Liberty (1 mg/mL)	120.0 ± 14.8
Liberty (10 mg/mL)	107.1 ± 14.8
Show Me (0.1 mg/mL)	124.0 ± 7.3
Show Me (1 mg/mL)	111.0 ± 3.1
Show Me (10 mg/mL)	99.3 ± 2.3

Effects of switchgrass extracts on cytokine secretion

The results summarized in Table 17 demonstrated that the switchgrass extracts did not exhibit toxic effects on the U-937 cells, and they were further analyzed for their

effects on cytokine secretion as a possible indicator of inflammation. Dexamethasone and cyclosporin A, agents known to affect cytokine secretion, were included as positive controls. Switchgrass extracts were added to PMA-differentiated U-937 cells for 2 hours prior to the addition of LPS, and the cultures were incubated for a total of 24 hours before cell supernatant analysis. Cytokine levels of TNF-α, IL-6, IL-8, and IL-10 were determined using a quantitative flow cytometric, multiplex bead-based assay, and the results were expressed relative to samples cultured in the presence of the vehicle but without extracts. The results shown in Figures 35-38 illustrated that the degree of cytokine suppression was influenced by the switchgrass extract, the cultivar concentration, and the cytokine analyzed. Of note, the levels of all 4 cytokines were reduced after incubation with the extract at the highest concentration, 10 mg/mL, for all 4 cultivars. The only exception was the absence of an effect of the Liberty extract on IL-10 secretion. Alamo extracts showed a consistent, statistically significant reduction in TNF-α and IL-6 levels at all 3 concentrations tested. Specifically, culture of the Alamo extracts at 0.1, 1, and 10 mg/mL reduced the secretion of TNF-α to 32.3%, 47.6%, and 53.5%, respectively, and of IL-6 by 21.6%, 27.8%, 51.0%, respectively (Figures 35 & 36) of the control response. Secreted IL-8 and IL-10 levels were reduced to 50.8% and 32.5% of the control levels, respectively, at 10 mg/ml, whereas the levels remained unchanged at the two lower concentrations. Addition of the highest concentration of Kanlow, Liberty, and Show Me cultivars reduced secretions in the range of 40-60% of TNF-α control levels (50.6%, 49.8%, and 62.7%, respectively) and 40-60% of the IL-6 control levels (40.9%, 62.1%, and 66.4%, respectively). The effect on the IL-8 response was more variable and was reduced by 54.0%, 19.3%, and 48.4% of control levels for Kanlow,

Liberty, and Show Me extracts, respectively, at the highest concentration. The decrease in the IL-10 response was less pronounced than for other cytokines, resulting in 30.9%, 19.7%, and 38.8% of control levels (Figures 35-38). These same extracts showed minor to no effects on all 4 cytokines at the two lower concentrations tested. Taken together, these results show that the effect of cultivars on cytokine suppression was dose-dependent and variable among the cultivars. While all 4 cultivars showed an effect at the highest concentration tested, with only one exception, the effect at other concentrations depended on the cultivar and individual cytokine.

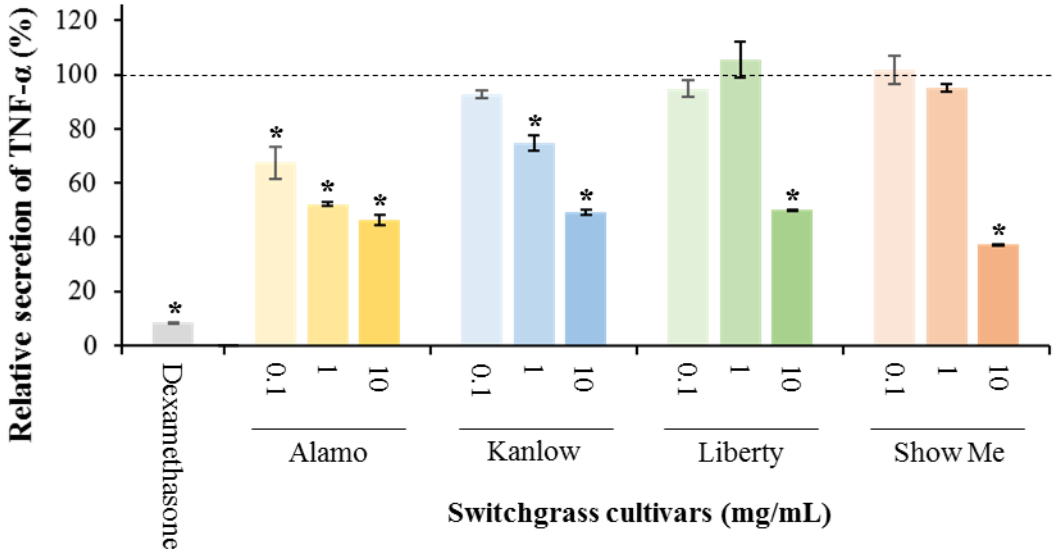

Figure 35. Effect of switchgrass extracts on the secretion of TNF-α by PMA-differentiated, LPS-stimulated U-937 cells. Dexamethasone was used at 2 µg/mL as a positive control. (*) Significant decrease ($p < 0.01$) compared to PMA-differentiated, LPS-stimulated U-937 cells in the absence of extract. Mean ± SEM.

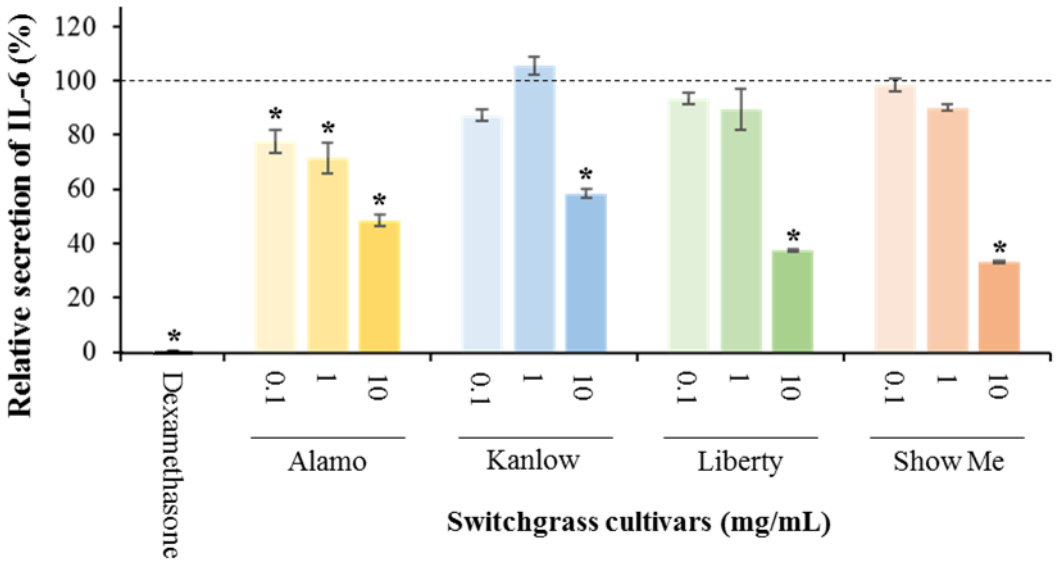

Figure 36. Effect of switchgrass extracts on the secretion of IL-6 by PMA-differentiated, LPS-stimulated U-937 cells. Dexamethasone was used at 2 µg/mL as a positive control. (*) Significant decrease ($p < 0.01$) compared to PMA-differentiated, LPS-stimulated U-937 cells in the absence of extract. Mean ± SEM.

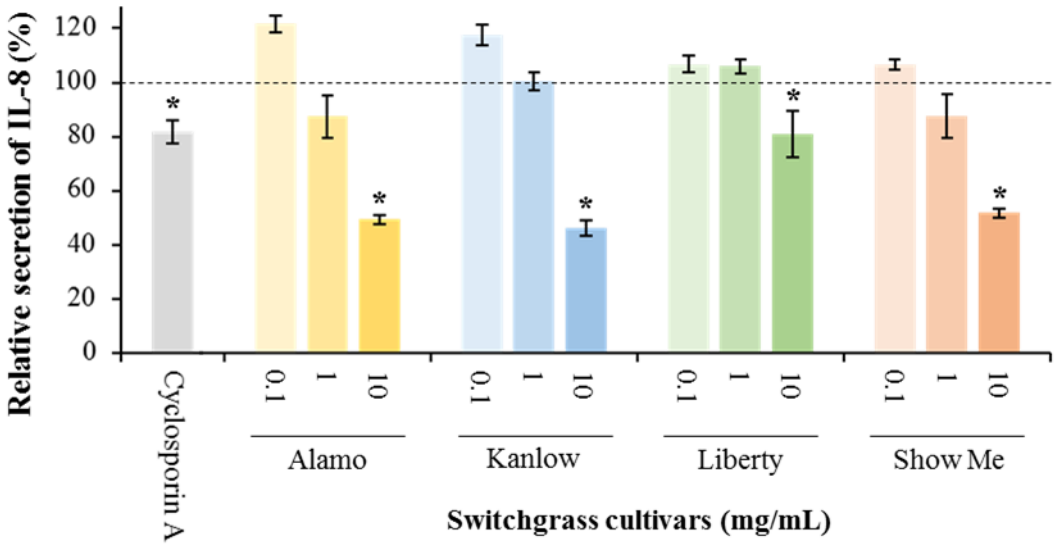

Figure 37. Effect of switchgrass extracts on the secretion of IL-8 by PMA-differentiated, LPS-stimulated U-937 cells. Cyclosporin A was used at 2 µg/mL as a positive control. (*) Significant decrease ($p < 0.01$) compared to PMA-differentiated, LPS-stimulated U-937 cells in the absence of extract. Mean ± SEM.

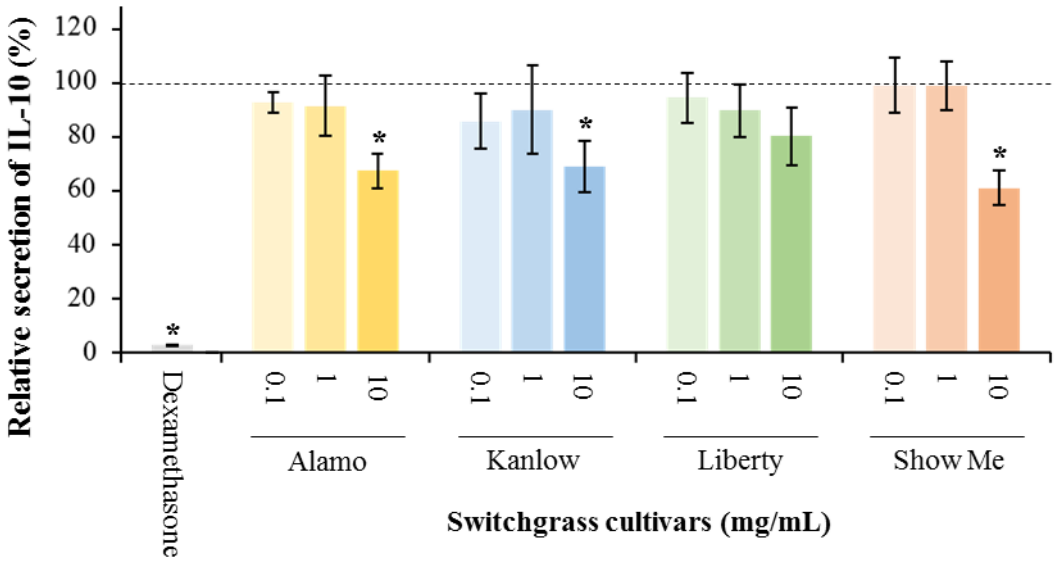

Figure 38. Effect of switchgrass extracts on the secretion of IL-10 by PMA-differentiated, LPS-stimulated U-937 cells. Dexamethasone was used at 2 µg/mL as a positive control. (*) Significant decrease ($p < 0.01$) compared to PMA-differentiated, LPS-stimulated U-937 cells in the absence of extract. Mean ± SEM.

Anti-inflammatory compounds in switchgrass

UHPLC-QTOF-MS analysis

The analyses of UHPLC-HRMS data with XCMS Online combined with METLIN metabolite database resulted in the putative identification of 22 substances with known anti-inflammatory activities (Table 18, supplementary Table 3). Each switchgrass cultivar contained several potential anti-inflammatory compounds and the relative concentrations of many compounds were distinct within specific cultivars (Figure 39). Alamo and Show Me contained the highest relative abundance of okanin 3',4'-diglucoside, quercitrin, and aescin, while formononetin 7-O-rutinoside, tenylidone, quercetin 3-glucoside were relatively dominant in Alamo. Kanlow contained the highest

relative abundance of petunidin 3-glucoside, rutin trihydrate, and baicalin. Altholactone, bergenin, quercetin and osthenol 7-O-β-D-gentiobioside were major metabolites in Show Me, whereas dioscin, 4-hexylresorcinol, bruceine B, auranofin, and gambogic acid were presented dominantly in Liberty. Three (3) metabolites including quercetin, quercetin 3-glucoside and rutin hydrate were further quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis with authentic standards (Table 19).

Table 18. Putative identification of the secondary metabolites with known anti-inflammatory activities in switchgrass through untargeted metabolomics analyses

Compound	Retention time (min)	Formula	Adducts	Theoretical mass	Observed mass	Δm (ppm)
4-Hexylresorcinol	5.75	$C_{12}H_{18}O_2$	$[M + H]^+$	194.1307	194.1302	2.68
Aescin	11.47	$C_{55}H_{86}O_{24}$	$[M-H]^-$	1130.5509	1130.543	6.96
Altholactone	8.86	$C_{13}H_{12}O_4$	$[M + H]^+$	232.0736	232.0731	2.15
Auranofin	5.22	$C_{20}H_{34}AuO_9PS$	$[M + H]^+$	678.1327	678.1335	1.2
Baicalin	6.24	$C_{21}H_{18}O_{11}$	$[M-H]^-$	446.0849	446.0858	2.08
Bergenin	5.2	$C_{14}H_{16}O_9$	$[M+NH4]^+$	328.0794	328.0745	15.03
Bruceine B	4.56	$C_{23}H_{28}O_{11}$	$[M-H]^-$	480.1632	480.1628	0.67
Coumarin	3.17	$C_9H_6O_2$	$[M + H]^+$	146.0368	146.0363	3.56
Dioscin	11.53	$C_{45}H_{72}O_{16}$	$[M + H]^+$	868.482	868.4838	1.98
Formononetin 7-O-rutinoside	8.49	$C_{30}H_{36}O_{17}$	$[M-H]^-$	668.1952	668.1946	0.91
Gambogic acid	9.94	$C_{38}H_{44}O_8$	$[M-H]^-$	628.3036	628.3045	1.47
Kaempferol-7-rhamnoside	7.34	$C_{21}H_{20}O_{10}$	$[M-H]^-$	432.1056	432.1064	1.84
Nevadensin 5-gentibioside	2.29	$C_{30}H_{36}O_{17}$	$[M-H]^-$	668.1952	668.1946	0.91
Okanin 3',4'-diglucoside	6.96	$C_{27}H_{32}O_{16}$	$[M-H]^-$	612.169	612.1704	2.3
Osthenol-7-O-beta-D-gentiobioside	4.64	$C_{26}H_{34}O_{13}$	[M+Na]+	554.1999	554.1997	0.44
Petunidin 3-glucoside	6.47	$C_{22}H_{23}O_{12}$	$[M-H]^-$	478.1111	478.112	1.91
Quercetin	6.22	$C_{15}H_{10}O_7$	$[M + H]^+$	302.0427	302.0423	1.3
Quercetin-3-glucoside	5.17	$C_{21}H_{19}O_{12}$	$[M-H]^-$	464.0955	464.096	1.22
Quercitrin	7.17	$C_{21}H_{20}O_{11}$	$[M-H]^-$	448.1006	448.1012	1.51
Rhoifolin	6.01	$C_{27}H_{30}O_{14}$	[M+Na]+	578.1636	578.1643	1.29
Rutin trihydrate	4.65	$C_{27}H_{36}O_{19}$	$[M-H]^-$	610.1534	610.1543	1.57
Tenylidone	6.09	$C_{16}H_{14}OS_2$	$[M + H]^+$	286.0486	286.0477	3.31

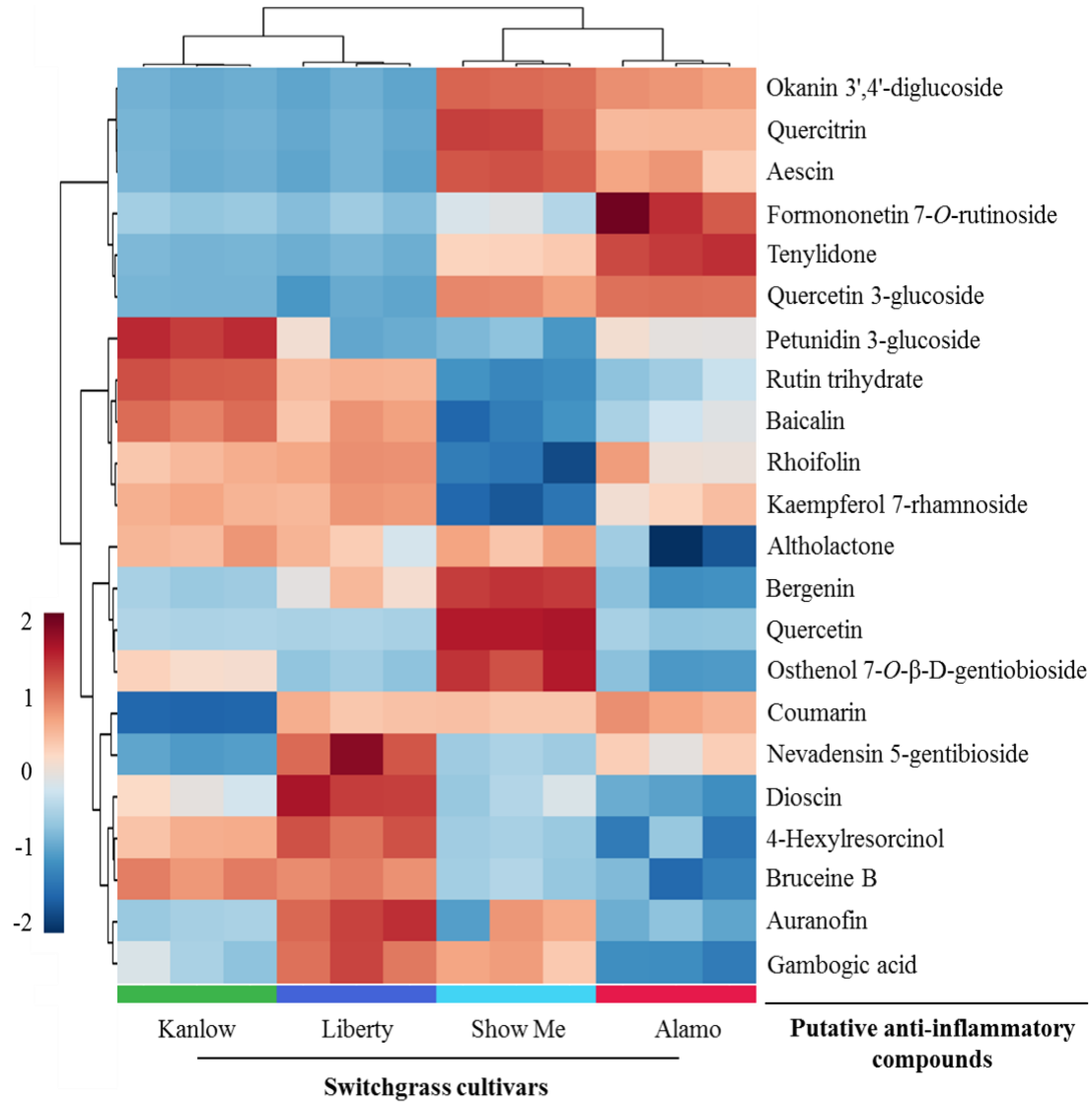

Figure 39. Relative abundance of metabolites with known anti-inflammatory activities in switchgrass. In the heatmap, red represents higher relative abundance, while blue represent lower relative abundance.

The partial least squares-discriminant analysis (PLS-DA) score plot showed significant differences in anti-inflammatory metabolic profiles among four switchgrass cultivars that possibly associated with the variation in anti-inflammatory capacities among the switchgrass cultivars examined (Figures 35-38). Model quality was evaluated using a cross-validation method and resulting R2 and Q2 values were 0.99 and 0.98,

respectively, indicating that the model was reliable. The PLS-DA score plot with three principal components covered 94.8% of total variability of the data (Figure 40). The first principal components (PC1) explained 51.7% of the total variability of the data, whereas the second and third principal components (PC2 and PC3) accounted for 29.3% and 13.8% of the total variability of the data set, respectively. In the PLS-DA score plot, all four switchgrass cultivars were distributed separately. Alamo and Show Me shared a similar pattern according to the PC1 and PC3 but were separated along the PC2. Regarding the PC2, Alamo and Kanlow relatively shared a similar pattern, and differed from Liberty and Show Me.

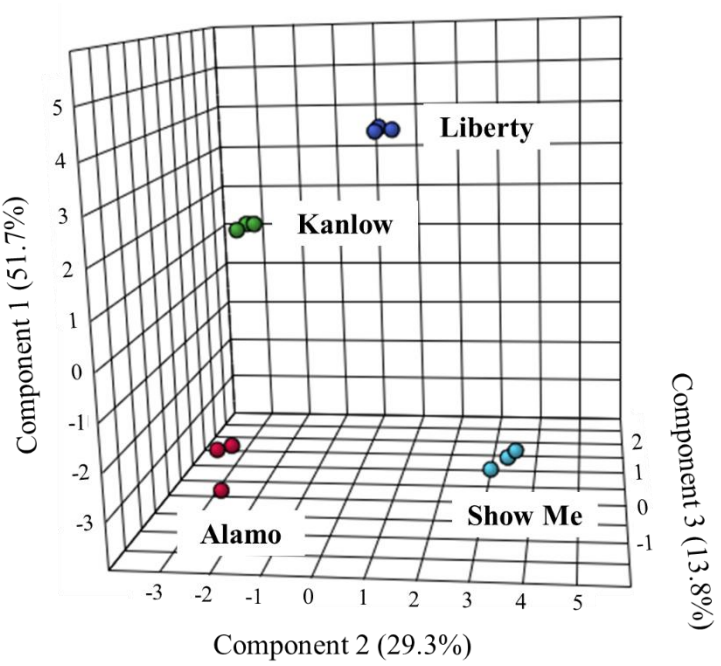

Figure 40. Partial least squares-discriminant analysis (PLS-DA) revealed differences in metabolic profiles of switchgrass cultivars. Circles with the same colors represent replicates of metabolic profiles for each cultivar, whereas the colored ellipses indicate 95% confidence regions of metabolic profiles for each cultivar.

HPLC-MS/MS analysis

Three anti-inflammatory metabolites including quercetin, quercetin 3-glucoside and rutin hydrate were found in the extracts of all cultivars examined (Table 19). The contents of these anti-inflammatory compounds were variable among switchgrass cultivars. In the switchgrass extracts, quercetin 3-glucose and rutin hydrate presented at high concentrations (>150 mg/kg), whereas the contents of quercetin were found at minor levels (< 1 mg/kg). Specifically, Alamo contained the richest abundance of quercetin 3-glucose (346.3 ± 52.1 mg/kg), followed by Kanlow (260.6 ± 18.5 mg/kg), Liberty (215.8 ± 38.7 mg/kg) and Show Me (155.9 ± 7.3 mg/kg), respectively. Similarly, rutin hydrate was found to be present at the highest amount in Alamo (279.4 ± 17.0 mg/kg), followed by Liberty (243.7 ± 13.7 mg/kg), Kanlow (161.5 ± 11.9 mg/kg), and Show Me (152.1 ± 1.1 mg/kg), respectively. The contents of quercetin were found at higher contents in Show Me (0.8 ± 0.04 mg/kg) and Kanlow (0.7 ± 0.05 mg/kg) compared to Alamo (0.2 ± 0.06 mg/kg) and Liberty (0.1 ± 0.01 mg/kg).

Table 19. Contents of anti-inflammatory compounds (mg/kg) in switchgrass determined by UPLC-MS/MS. *All values are shown as mean ± SEM (n=3). In each row, different letter indicates significant differences ($p < 0.01$) among different switchgrass cultivars.

Polyphenols	**Switchgrass cultivars**			
	Alamo*	**Kanlow**	**Liberty**	**Show Me**
Quercetin 3-glucoside	346.3 ± 52.1^{a}	260.6 ± 18.5^{b}	215.8 ± 38.7^{c}	155.9 ± 7.3^{d}
Quercetin	0.2 ± 0.06^{c}	0.7 ± 0.05^{b}	0.1 ± 0.01^{d}	0.8 ± 0.04^{a}
Rutin hydrate	279.4 ± 17.0^{a}	161.5 ± 11.9^{c}	243.7 ± 13.7^{b}	152.1 ± 1.1^{c}

Other biological activities of switchgrass

The four cultivars of switchgrass were tested for potential biological activities against several targets based on the stipulated health benefits. These cultivars at highest concentration tested did not exhibit activity against the selected microbes (*C. acnes* and *M. smegmatis*), antiproliferative activity against the selected cancerous cell lines (HT-29 and UCT-MEL-1) or enzyme inhibition (against elastase and tyrosinase) at the highest concentrations tested (Table 20).

Table 20. *In vitro* biological activities of the extracts derived from the four varieties of switchgrass. Antibacterial and antimycobacterial activities were evaluated against 2 bacterial strains *Cutibacterium acnes* and *Mycobacterium smegmatis*, respectively. Anticancer activity was investigated using human colorectal adenocarcinoma (HT-29) and human malignant melanoma (UCT-MEL-1) cell lines. *Values are expressed as minimum inhibitory concentration (MIC, µg/mL). $^{+}$Values are shown as fifty percent inhibitory concentration (IC_{50}, µg/mL).

Extract	Anti-bacterial*	Anti-mycobacterial*	Anticancer+		Porcine elastase+	Mushroom tyrosinase+
			HT-29	UCT-MEL-1		
Cultivar						
Alamo	>500	>1000	>400	>400	>500	>1000
Kanlow	>500	>1000	>400	>400	>500	>1000
Liberty	>500	>1000	>400	>400	>500	>1000
Show Me	>500	>1000	>400	>400	>500	>1000
Control						
Tetracycline	1.56	-	-	-	-	-
Ciprofloxacin	-	0.31	-	-	-	-
Actinomycin D	-	-	0.040	0.011	-	-
Ursolic acid	-	-	-	-	20.80	-
Kojic acid	-	-	-	-	-	0.45

DISCUSSION

In the present study, we demonstrated that methanolic extracts of switchgrass contain compounds that possessed inhibitory effects on the secretion of inflammatory mediators (TNF-α, IL-6, IL-8, and IL-10) induced in a human pro-monocytic cell line differentiated with PMA and stimulated with LPS. Inflammation is a complex pathophysiological response of the immune system involving a variety of cellular, molecular, and biochemical mediators in response to infections of harmful stimuli or tissue damage (Medzhitov, 2010). Cytokines are inflammatory mediators that play vital roles in various steps of acute and chronic inflammation, which have been widely utilized as possible indicators of systemic inflammation (Brenner et al., 2014). The pro-inflammatory mediators TNF-α and IL-6 are key cytokines regulating innate and adaptive immune responses in disease onset and persistence (McInnes & Schett, 2007) and these cytokines are therapeutic targets and in clinical trials for the treatment of patients with chronic inflammatory diseases such as rheumatoid arthritis (McInnes et al., 2016). IL-8 plays vital roles in acute inflammation that can recruit neutrophils and other granulocytes to the site of infection and stimulates phagocytosis (Harada et al., 1994), whereas IL-10, a potent immune-modulatory cytokine, has broad anti-inflammatory properties (O'Garra et al., 2008), including the inhibition of TNF expression (Smallie et al., 2010). Our results indicated that switchgrass extracts exhibited the suppressive activities on the examined inflammatory mediators. Alamo, Kanlow and Show Me extracts reduced the secretion of all four examined cytokines in the U-937 model system, whereas Liberty extracts decreased the secretions of TNF-α, IL-6 and IL-8 only. Cell viability did not reduce in the presence of all four switchgrass extracts compared to the control cells without extracts,

revealing that the reduction in cytokine secretion is not a result of direct toxic effects. Furthermore, our results demonstrated a diverse range of the anti-inflammatory bioactive compounds potentially present in switchgrass. These compounds are likely responsible for the cytokine suppressive activities observed. Our findings suggest that switchgrass could be considered as a promising source of anti-inflammatory bioactive compounds that can be utilized for pharmaceutical and cosmetic industries. The identification of novel value-added byproducts and applications of switchgrass would potentially increase the sustainability of this important bioenergy crop.

We documented the variation on cytokine suppressive capacities of switchgrass depending upon particular cultivars, concentrations tested, and cytokines examined. In fact, Alamo at all concentrations tested (0.1, 1, and 10 mg/ml) reduced the secretion of TNF-α and IL-6 in the U-937 model system, while other cultivars (Kanlow, Liberty, and Show Me) at lowest concentrations (0.1 and 1 mg/ml) did not exhibit significantly inhibitory effects on the expression of these cytokines. The IL-8 inhibition was observed on all four cultivars at the highest concentration only. Alamo, Kanlow and Show Me also reduced the secretion of IL-10, but no inhibitory effect of Liberty on IL-10 was observed. Tao et al. (2019) reported variation in antioxidant capacities of switchgrass among different cultivars and locations where the samples were collected. Alamo was found to have higher antioxidant activities compared with other cultivars tested including EG1101 (improved ‘Alamo’ cultivar) and EG1102 (improved ‘Kanlow’ cultivar), whereas switchgrass samples collected from different growth locations accounted for up to 20% variation in the antioxidant capacities (Tao et al., 2019). Differences in the bioactive activities of switchgrass are likely associated with differences in anti-inflammatory

metabolic profiles among switchgrass cultivars examined (Figures 39 & 40). The presence of multiple compounds might explain why the switchgrass extracts have broad inhibitory effects that reduce the expression of all four cytokines. Additionally, the involvement of multiple compounds on cytokine secretion also raises the possibility of interaction effects between these compounds on the inhibitory activities that may have synergism among the bioactive compounds. Chemical compositions of switchgrass included carbohydrates, fatty acids, fatty alcohols, glycerol, sterols, organic acids and other constituents (inorganic ions and alkane). Monosaccharides were the major component occupied by 56-60% of total mass balance for the switchgrass extractives (Chen et al., 2010; Yan et al., 2010). The switchgrass chemical constituents may contain materials that interfere the suppressive activities or could have inhibitory effects on IL-10 expression in the U-937 model system. Tao et al. (2019) found that EG1101 and EG1102 had similar levels of total phenolic contents but were different in antioxidant capacities and the contents of free sugar (sucrose, fructose, and glucose). They reported that the high levels of free sugar in EG1101 reduced antioxidant capacities of this cultivar (Tao et al., 2019).

Our results demonstrated a diverse range of the anti-inflammatory bioactive compounds in switchgrass. In fact, 22 anti-inflammatory metabolites in the switchgrass extracts were putatively identified via an untargeted metabolomics analyses and three bioactive compounds (quercetin 3-glucoside, quercetin, rutin hydrate) were successfully quantified in switchgrass extracts by LC-MS/MS analyses with authentic reference standards (Tables 18 & 19). Among 22 anti-inflammatory metabolites, 18 compounds including 4-hexylresorcinol, aescin, auranofin, baicalin, bergenin, bruceine B, dioscin,

formononetin 7-*O*-rutinoside, gambogic acid, kaempferol 7-rhamnoside, nevadensin 5-gentibioside, okanin 3',4'-diglucoside, osthenol-7-*O*-β-D-gentiobioside, petunidin 3-glucoside, quercetin, quercetin-3-glucoside, rhoifolin, tenylidone were the first reported possibly present in switchgrass, whereas other compounds have been documented as polyphenolic compounds in switchgrass. Uppugundla et al. (2009) assessed antioxidant capacities of aqueous extract of switchgrass and found two flavonoids such as quercitrin and rutin as major compounds responsible for the low-density lipoprotein oxidation inhibition activity of switchgrass. In order to further characterize the anti-inflammatory properties of switchgrass, future research will focus on identification and purification of compounds that are driving the cytokine suppressive activities in switchgrass.

Our results also revealed that the contents of anti-inflammatory compounds (quercetin, quercetin 3-glucoside, and rutin hydrate) in switchgrass were variable among the cultivars examined. The contents of quercetin 3-glucoside ranged from 155.9 mg/kg (Show Me) to 346.3 mg/kg (Alamo), whereas the concentrations of rutin hydrate were in range of 152.1 mg/kg (Show Me) to 279.4 mg/kg (Alamo). The presence of quercetin in switchgrass was at minor concentrations, ranging from 0.1 mg/kg (Libery) to 0.8 mg/kg (Show Me). In total, these phenolic compounds of switchgrass were found at the highest abundance in Alamo, followed by Liberty, Kanlow, and Show Me, respectively. The levels of bioactive compounds in switchgrass have also been previously documented to be variable depending on several factors (cultivars, geographic sources, ages, and extraction preparation) (Chen et al., 2010; Hu et al., 2011; Tao et al., 2019). Tao et al. (2019) reported that Alamo contained higher content of hydroxycinnamic acid (6.1 mg gallic acid equivalents (GAE)/g) compared with that in EG1101 (5.6 mg GAE/g) and in

EG1102 (5.3 mg GAE/g), whereas the contents of 6 phenolic compounds (i.e., caffeic acid, vanillic acid, p-coumaric acid, ferulic acid, rutin, and quercitrin) were found to be not significantly different among Alamo, EG1101, and EG1102. Additionally, switchgrass grown in different locations has variation in total phenolics contents. In 4 different growth locations, the total phenolics contents in Alamo, EG1101, EG1102 ranged 6.0-10 mg GAE/g, 7.4-13.8 mg GAE/g, 6.6-11.6 mg GAE/mg, respectively (Tao et al., 2019). Furthermore, the amounts of bioactive compounds in switchgrass have been reported to be highly dependent on extraction solvents and temperatures. Many different solvents (e.g., methanol, ethanol, water) with a few extraction methods (Soxhlet extraction, microwave) have been utilized to maximize the recovery of bioactive compounds in switchgrass (Chen et al., 2010; Uppugundla et al., 2009). Uppugundla et al. (2009) reported that 60% methanol extraction yielded higher recovery rates of rutin and quercetin compared with water extraction. Hu et al. (2011) documented that quinic acid was found in leaves of Alamo when extracted in benzene/ ethanol (2:1, v/v), but this compound was not detectable in those when extracted in hot water. Future efforts will focus on identification of optimum conditions (e.g., extraction methods, solvents) that can maximize the recovery of bioactive compounds in switchgrass.

The antibacterial activity against *C. acnes* was conducted due to the role of this opportunistic microorganism on the stimulation of the inflammatory response in both sebocytes and keratinocytes through the activation of Toll-like receptor 2 (TLR2) in sebocytes and both TLR2 and TLR4 in keratinocytes. This activation leads to the secretion of numerous pro-inflammatory cytokines including IL-1, IL-6, IL-8 and TNF-α (Cong et al., 2019). It is estimated that over 50% of *C. acnes* strains are resistant to

antibiotics, with the highest frequency of resistance reported for macrolide antibiotics (Zhang et al., 2019). The progression of acne is, however, a multifactorial process with the activation of the inflammatory response playing an important role in complete rupture of the pilosebaceous follicles (Coenye et al., 2008). Our results showed that the switchgrass cultivars did not exhibit direct antibacterial activity against *C. acnes*. Since switchgrass extracts exhibited the secretion of IL-8, IL-10 and TNF-α in the LPS-induced U-937 model system, future research may focus on the utilization of the cytokine inhibition of switchgrass extracts as a potential anti-inflammatory treatment for inflammatory acne patients that experience decreased therapeutic activity of antibiotic treatments due to the onset of resistance.

Investigating the antimycobacterial activity was conducted due to the global impact of its related disease as well as the ability of *Mycobacterium tuberculosis* to survive intracellularly and alter the cytokine profiles of macrophages. Li et al. (2014) showed that U-937 cells infected with recombinant cultures of *M. smegmatis* MC^2 155 (expressing the *M. tuberculosis* Rv3402c gene) activate increased protein expression of IL-1β and TNF-α. While there was no direct antimycobacterial activity against the *M. smegmatis* strain (MC^2 155), the switchgrass cultivars may exhibit a therapeutic effect via maintaining normal cytokine levels secreted by macrophages infected with *Mycobacterial* species (Li et al., 2014). The current study showed the effects of switchgrass extracts on TNF-α secretion in U-937 cells. Future experiments could investigate the effects on other cytokines/ chemokines such as IL-1β, which is a pro-inflammatory cytokine found to be activated in many other inflammatory disorders.

The extracts derived from the switchgrass cultivars showed no antiproliferative activity against the human colorectal adenocarcinoma (HT-29) or pigmented human malignant melanoma (UCT-MEL1) cell lines at the highest concentration tested (400 µg/mL). Inflammatory bowel syndrome has been linked with the chronic exposure of intestinal epithelial cells to high levels of TNF-α. While the switchgrass showed no antiproliferative activity against these cancer cells, the inhibition of TNF-α in U-937 cells by these extracts has potentially identified extracts with the ability to treat inflammatory bowel syndrome and ulcerative colitis, since they require agents that can neutralize excessive levels of TNF-α (Bruno & Kaetzel, 2005). This cytokine is also a major inflammatory cytokine present within the tumor microenvironment, contributing towards tumor progression and metastasis (Zhao & Zhang 2018). TNF-α secreted by macrophages has been reported to stimulate the secretion of vascular endothelial growth factor (VEGF) in melanoma cells, furthermore TNF-α has been reported to increase migration and invasion of colon cancer cells (Varney et al., 2005; Zhao & Zhang 2018).

The non-specific protease activity of elastase enzymes enables these enzymes to degrade the elastin and collagen fibers of the connective tissues of the human skin. Elastin and collagen play a structural role in the skin providing the epidermis with its elastic properties. The splitting of elastin, however, contributes to the inflammatory burden, reduced elasticity and skin ageing (Lee et al., 1999). The switchgrass cultivars exhibited no activity against porcine pancreatic elastase (PPE) at the highest tested concentrations of 500 µg/mL. Ortwerth et al. (1994) reported differences in inhibitory activity of α-crystallin on PPE and human neutrophil elastase (HNE) inhibition. Inhibitors of neutrophil-derived elastases may identify potential therapeutics for not only

inflammatory lung, bowel and skin inflammation but also for the ischaemia-reperfusion injury observed in patients that have suffered myocardial infarction, stroke and organ transplants (Hendriksen, 2014). The extracts should be considered for investigating potential HNE inhibition. Lee et al. (1999) also observed many extracts with low inhibitory activity at a concentration of 1000 µg/mL.

Sato & Toriyama (2011) investigated the inhibitory potential of non-steroidal anti-inflammatory drugs (NSAIDs) on mushroom tyrosinase monophenolase and diphenolase activity. The anti-inflammatory compounds diflunisal and indomethacin showed inhibitory effects on monophenolase and diphenolase activity to different degrees. The current study focused largely on the monophenolase inhibitory activity of mushroom tyrosinase [conversion of L-tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine)]. Our results indicated no monophenolase activity of the switchgrass extracts at the highest concentration of 200 µg/mL. Future experiments might explore the effects of switchgrass extracts on the exhibit diphenolase inhibition (L-DOPA to dopaquinone). The investigation of diphenolase inhibitory activity, which is catalysed by the tyrosinase enzyme could identify beneficial switchgrass cultivars with the ability to inhibit melanogenesis, a process that is upregulated during the inflammatory response (Yeom et al., 2014). While the extracts did not show direct tyrosinase inhibition, they can be investigated for other mechanisms of melanin formation i.e. melanin production in melanocytes and the inhibition of melanin transfer from the melanocytes to the keratinocyte cells (Chen et al., 2020).

CONCLUSION

We demonstrated the anti-inflammatory potential of switchgrass extracts from four cultivars (Alamo, Kanlow, Liberty, and Show Me), while no other biological functions of the extracts including antibacterial, antimycobacterial, antiproliferative, antityrosinase, anti-elastase activity were observed under the experimental conditions tested. In fact, methanolic extracts of switchgrass exerted bioactive compounds that suppressed the secretion of inflammatory mediators induced in the human pro-monocytic cell line U-937. The levels of cytokine inhibition were relatively variable depending on particular cultivars, concentrations tested, and cytokines examined. Switchgrass extracts from all cultivars showed inhibitory effects on the secretion of all 4 cytokines (TNF-α, IL-6, IL-8 and IL-10), except for Liberty which did not significantly affect IL-10 expression. Multiple metabolites in the switchgrass extracts with known anti-inflammatory activities were identified via UHPLC-MS analyses and were quantified using LC-MS/MS analyses with authentic standards. The inflammatory properties of the switchgrass extracts suggest that the abundant availability of switchgrass and its by-products could be an excellent source of the raw materials for the nutraceutical and cosmetic industries. The findings from this study offer the opportunities to increase the overall economic value of switchgrasses such as Alamo and Kanlow in the biorefinery supply chain and promote the development of new source of the biological agents for mitigating inflammation.

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SUPPLEMENTARY INFORMATION

Supplementary Table 3. Putative identification of the secondary metabolites with known anti-inflammatory activities in switchgrass through untargeted metabolomics analyses.

Compound	References
4-Hexylresorcinol	Ahn et al. (2016), Frankos et al. (1991)
Aescin	Sirtori (2001)
Altholactone	Al Momani et al. (2011), Jiang et al. (2017), Inayat-Hussain et al. (2002)
Auranofin	Thangamani et al. (2016), Suarez-Alamazor et al. (2000)
Baicalin	Zhao et al. (2016), He et al. (2017), Zhu et al. (2016)
Bergenin	Pushpalata et al. (2015), Arora et al. (2011), Srivastava et al. (2014)
Bruceine B	Bawm et al. (2008)
Coumarin	Kawatra et al. (2015), Venugopala et al. (2013)
Dioscin	Lv et al. (2013), Aumsuwan et al. (2016)
Formononetin 7-O-rutinoside	Singh et al. (2017), Wu et al. (2016)
Gambogic acid	Kashyap et al. (2016), Huang et al. (2015)
Kaempferol-7-rhamnoside	Sim et al. (2017), Abbasi et al. (2017)
Nevadensin 5-gentibioside	Alhusainy et al. (2014)
Okanin 3',4'-diglucoside	Kil et al. (2012)
Osthenol-7-O-beta-D-gentiobioside	Kuo et al. (2017)
Petunidin 3-glucoside	Huang et al. (2018)
Quercetin	Li et al. (2016), Babujanarthanam et al. (2010)
Quercetin-3-glucoside	Olthof et al. (2000), Walton et al. (2006)
Quercitrin	Askari et al. (2012), David et al. (2016)
Rhoifolin	Rao et al. (2011), Qin et al. (2018)
Rutin trihydrate	Ganeshpurkar & Saluja (2016), Al-Dhabi et al. (2015)
Tenylidone	Yadav et al. (2010), Panic et al. (2015)

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CONCLUSION

Black walnut is an excellent source for health-promoting benefits. This nutritious tree nut has been demonstrated to exert a wide range of biological functions including antibacterial, antioxidant, and anti-inflammatory potential and it likely possesses anticancer activities. The bioactive activities of black walnuts vary among different cultivars, and some of the cultivars consistently exhibit strong bioactive capacities. Mystry and Surprise exhibited the strongest antioxidant and antibacterial activities, whereas Sparrow and Surprise represent promising preventive agents for inflammatory diseases.

Black walnut contains a wealth of health-promoting bioactive compounds, including polyphenols that were identified through the robust metabolomics analyses combined with in vitro bioassays. Among the identified compounds, penta-*O*-galloyl-*β*-D-glucose could be a potential bioactive agent for cosmetic and pharmaceutical industries. Glansreginin A is the major antibacterial compound in black walnuts. This is the first report for the antibacterial activity of glansreginin A.

Switchgrass and spent coffee grounds have been found to possess multiple anti-inflammatory compounds inhibiting the expression several inflammatory mediators in vitro. These waste materials represent promising natural anti-inflammatory sources for cosmetic, nutraceutical and pharmaceutical industries. Our findings offer an excellent opportunity to increase the overall economic value of switchgrasses in the biorefinery supply chain and spent coffee grounds, which could promote the development of new sources of biological agents for mitigating inflammation.

LIST OF PUBLICATIONS

To prepare for the dissertation, the author has been published several chapters of the dissertation in peer-reviewed journals and has prepared other ongoing manuscripts to be submitted for publications.

Khanh-Van Ho, Anuradha Roy, Sarah Foote, Phuc H. Vo, Namrita Lall, Chung-Ho Lin. **2020.** Profiling anticancer and antioxidant activities of phenolic compounds present in black walnuts (*Juglans nigra*) using a high-throughput screening approach. Molecules, 25 (19): 4516

Khanh-Van Ho, Kathy L. Schreiber, Jihyun Park, Phuc H. Vo, Zhentian Lei, Lloyd W. Sumner, Charles R. Brown, and Chung-Ho Lin. **2020**. Identification and quantification of bioactive molecules inhibiting pro-inflammatory cytokine production in spent coffee grounds using metabolomics analyses. Frontiers in Pharmacology, 11: 229

Khanh-Van Ho, Kathy L. Schreiber, Danh C. Vu, Susan M. Rottinghaus, Daniel E. Jackson, Charles R. Brown, Zhentian Lei, Lloyd W. Sumner, Mark V. Coggeshall, and Chung-Ho Lin. **2019**. Black walnut (*Juglans nigra*) extracts inhibit pro-inflammatory cytokine production from LPS-stimulated human pro-monocytic cell line U-937. Frontiers in Pharmacology, 10: 1059

Khanh-Van Ho, Zhentian Lei, Lloyd W. Sumner, Mark V. Coggeshall, Hsin-Yeh Hsieh, George C. Stewart, and Chung-Ho Lin. **2018**. Identifying antibacterial compounds in black walnuts (*Juglans nigra*) using a metabolomics approach. Metabolites 2018, 8(4), 58.

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Khanh-Van Ho et al. Assessing the health-promoting activities of the extracts from switchgrasses (*Panicum virgatum*) (tentatively submitted in 10/2020).

Khanh-Van Ho et al. quantification and characterization of biological activities of glansreginin A in black walnuts *(Juglans nigra)* (tentatively submitted in 11/2020)

Ezequiel Rossi, Zahra Salahshoor, **Khanh-Van Ho**, Chung-Ho Lin, Maria Ines Errea, Maria M. Fidalgo. Detection of Chlorantraniliprole residues in tomato using field-deployable MIP photonic sensors (submitted)

Kriuba Krishnaswamy, Salma Akter Antora, **Khanh-Van Ho**, et al. Upcycling: Sustainability in a nutshell with black walnut processing (submitted soon)

APPDENDIX

In order to identify major anti-inflammatory compounds in black walnuts, Thomas, a black walnut cultivar that exhibited the strong inhibitory effects on production of cytokines, was selected for bioassay-guided purification combined with metabolomics analyses, compared with other cultivars. The kernel extract of this cultivar was sequentially fractionated using column chromatography and then the bioactive fraction was further fractionated using high-performance liquid chromatography (HPLC). Immunology assays were performed to evaluate cytokine inhibitory effects of each fraction on the expression of TNF-α in the human monocyte U-937 model system using BioLegend TNF-α ELISA kit (Cat. No. 430206, San Diego, CA). The bioactive fractions that exhibited the strongest cytokine suppressive activities were then selected for the identification of anti-inflammatory compounds. The anti-inflammatory compounds in the bioactive fractions were putatively identified using the global metabolomics profiling analysis. These bioactive compounds will be validated and quantified in different black walnut cultivars via targeted metabolomics analyses and the biological activities of these compounds will be determined using high-throughput screening assays.

Here, we presented preliminary data on the isolation and the identification of anti-inflammatory compounds in Thomas. Figure appendix 1-3 showed UV chromatograms of selective HPLC bioactive fractions of Thomas that exhibited the strong inhibitory effects on the production of TNF-α in the human monocyte U-937 model system. Table appendix 1 revealed possible candidates with their m/z values and retention time from the bioactive fractions identified by UHPLC-MS/MS.

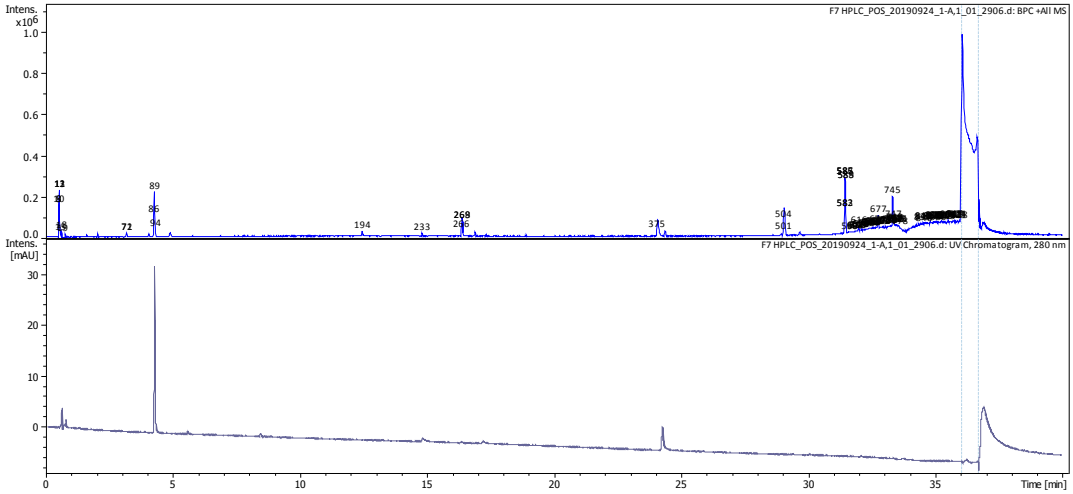

Figure appendix 1. UV chromatogram of active fraction 7

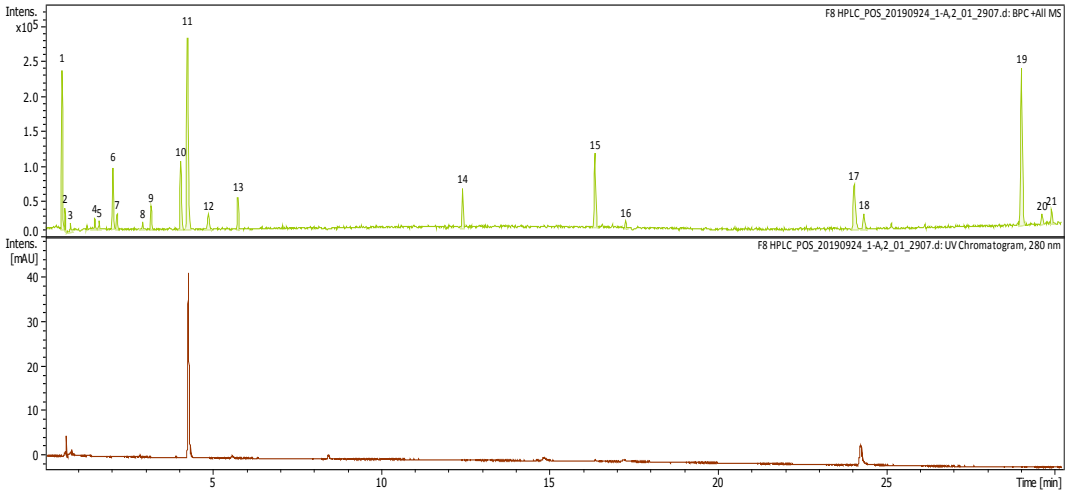

Figure appendix 2. UV chromatogram of active fraction 8

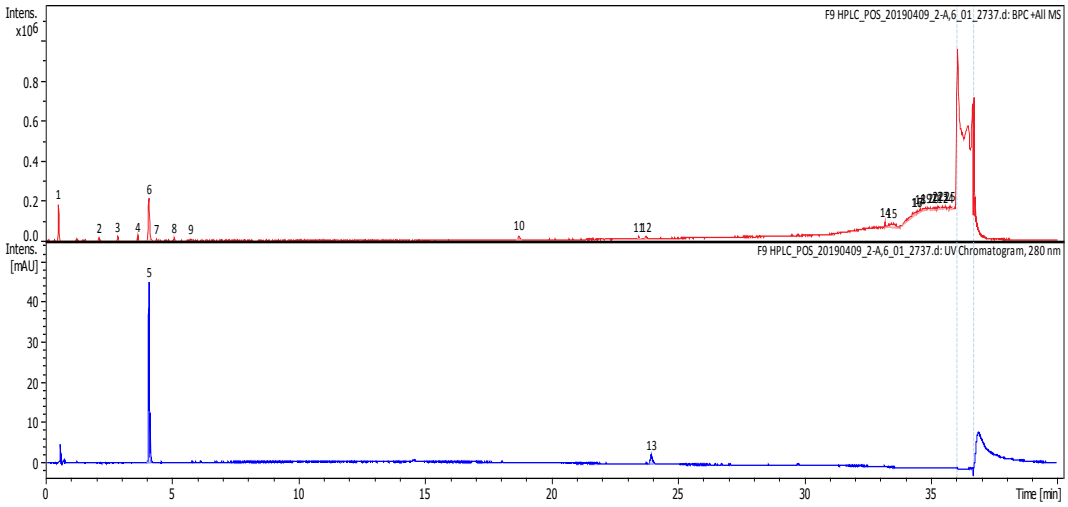

Figure appendix 3. UV chromatogram of active fraction 9

Table appendix 1: List possible candidates with their m/z value from bioactive fractions of black walnut kernel identified by UHPLC-MS/MS.

Active Fraction numbers	Retention time, RT (min)	$[M+H]^+$, (m/z)	Mass
	1.65	302.1973	301.1895
	2.08	227.1757	226.1679
	3.22	316.2132	315.2054
	4.09	340.2605	339.2527
	4.93	261.9792	260.9714
	12.47	226.1807	225.1729
F #7	14.85	283.1193	282.1115
	16.39	387.1809	386.1731
	16.95	420.2014	419.1936
	18.95	387.1942	386.1864
	24.12	421.2335	420.2257
	24.37	361.2235	360.2157
	29.07	438.3795	437.3717
	1.54	332.1349	331.1271
	2.05	227.1761	226.1683
	2.95	283.1766	282.1688
	3.19	316.2128	315.205
	4.08	340.2601	339.2523
	5.77	453.3446	452.3368
F #8	12.44	226.1083	225.1005
	16.38	404.2074	403.1996
	17.27	449.1498	448.142
	24.07	229.1621	228.1543
	24.34	383.205	382.1972
	29.04	256.2639	255.2561
	29.65	270.2798	269.272
	1.52	332.1331	331.1253
	2.16	261.1316	260.1238
	2.9	305.1568	304.149
	3.68	344.2281	343.2203
	4.41	388.2544	387.2466
F #9	5.12	432.2801	431.2723
	5.78	490.2858	489.278
	6.38	520.3321	519.3243
	18.76	164.147	163.1392
	23.5	313.1423	312.1345
	23.77	579.2923	578.2845

VITA

Van Ho was born in Can Tho city, Vietnam. She graduated from Can Tho University in Can Tho city, Vietnam in 2008 with a bachelor's degree in Food Technology. She conducted her undergraduate research in Dr. Thuy Nguyen's lab in the years 2007 - 2008. Her bachelor's thesis was entitled "The effects of post harvesting treatments on quality of fresh orange in various storage conditions". In 2010, she got an award for pursuing studying in the InterUniversity Programme in Food Technology (IUPFOOD), a Master of Science in Food Technology programme, is jointly organised by the faculties of Bioscience Engineering at KU Leuven and Ghent University (UGent). She conducted her master thesis in Dr. Bart Nicolai's lab at the Mebios Postharvest Group, KU Leuven with the master research entitled "Measurement gas exchange properties in tomato fruit". Van Ho received a master's degree in Food Technology at the KU Leuven in 2012. After graduation in 2008, she started to work as a research specialist and teaching assistant in the Department of Food Technology at the Can Tho University and, 5 years later (2013), she became a faculty member at the Can Tho University. As the faculty, her responsibilities are research, teaching and extension. In Fall 2015, Van Ho started her Ph.D. program in School of Natural Resources at the University of Missouri with Dr. Lin Chung-Ho.