ANTI-INFLAMMATORY PROPERTIES OF COWPEA PHENOTYPES WITH DIFFERENT PHENOLIC PROFILES

A Dissertation

by

LEONNARD ODHIAMBO OJWANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Food Science and Technology

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Co-Chairs of Committee,	Joseph M. Awika
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ABSTRACT

Anti-inflammatory Properties of Cowpea Phenotypes with Different Phenolic Profiles.

(May 2012)

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Cowpea (*Vigna unguiculata*) is a highly nutritious, drought tolerant crop with several agronomic advantages over other legumes. This study demonstrated the association of different cowpea phenotypes with specific phenolic profiles, antioxidants activity, anti-inflammatory properties on non-malignant colonic (CCD18co) cells challenged with a lipopolysaccharide (LPS), and the effect of boiling on their individual and total flavonoid content.

Only the black and green phenotypes had detectable anthocyanins; and their levels were highest in the black IT95K-1105-5 variety. The red cowpea phenotypes had the highest level of flavonols (858 – 941 μg/g cowpea flour) and white Early Acre variety had the least. Quercetin derivatives were the major flavonols detected, followed by myricetin derivatives. Monomeric, dimeric and polymeric procyanidins also made up a large proportion of cowpea phenolics. The light brown 09FCV-CC27M cowpea variety had the highest average flavan-3-ol content (13,464 μg/g cowpea flour); whereas white

and green varieties did not contain detectable levels of flavan-3-ols. Thus, seed-coat color was a good indicator of the accumulation of specific flavonoids in cowpeas.

The black, red and light-brown cowpeas had the highest antioxidant activity measured by ORAC and ABTS methods, correlating with their higher total phenol content (TPC) and condensed tannin content (CTC); whereas the white and green varieties had the least. Boiling significantly affected the phenolic profiles, TPC and CTC of all cowpea varieties studied, as well as the antioxidant activity associated with these compounds. The net reduction in antioxidant activity after boiling was less than the net TPC reduction, indicating that the heat-induced phenolic products may still have radical scavenging capacity.

Overall, proinflammatory genes regulation, intracellular ROS inhibition, and modulation of miR-126 and its target gene VCAM-1 by cowpea were found to be dependent on cowpea variety, phenolic composition and concentrations. The underlying mechanism by which cowpea induced miR-126 may be associated with inhibition of ROS and down-regulation of transcription factor NF-κB. These results emphasize the importance of the cancer inhibitory potential of phenolic compounds from cowpea and their possible role in preventing anti-inflammatory disorders. Further *in vivo* studies with cowpea diets are required to validate their clinical relevance to human health.

DEDICATION

To my Mum Claris Martha A. Ojwang and Dad John W. O. Ojwang.

Your constant reminder of nothingness of my yesterday and abundance of today keeps urging me to pursue greater heights. I was fortunate to have been born to the two of you.

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1. INTRODUCTION

1.1 Need for research

Epidemiologic evidence show that regular consumption of products containing grain legumes reduce the risk of cancer (colon, breast, prostrate) (*1*), cardiovascular diseases (*2*) and diabetes (*3*). For example, consumption of pinto beans has been shown to decrease levels of LDL-cholesterol (*4*) by partially interrupting the enterohepatic circulation of bile acids (*5*). Anthocyanidins from black soybean seed coat were found to suppress cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) mRNAs in tissue plasminogen activator (TPA) stimulated HT-29 cells (*6*). Phenolic compounds, like anthocyanins, exert a strong antioxidant activity and are thereby able to protect cells against reactive oxygen species (ROS). ROS act as mitogens to stimulate COX-2 expression via nuclear factor κappa B (NF-κB) activation, thereby inducing inflammatory responses (*7*). NF-κB is the main molecular link between inflammation and inflammatory-linked diseases. Therefore, plant compounds that can effectively down-regulate NF-κB (*8*) and scavenge ROS may reduce inflammatory responses and alleviate the related pathological conditions.

This dissertation follows the style of *Journal of Agriculture and Food Chemistry*.

Cowpea (*Vigna unguiculata* L. (Walp)) is a highly nutritious crop that can be grown in places not suitable for the growth of most food legumes because of its heat and drought tolerance. It is cultivated for its immature pods, mature seeds and fodder, which are rich in lysine and tryptophan – limiting amino acids in cereals.

Cowpea is considered a low glycemic index (GI) food (*9*), a good source of proteins and other nutrients and is especially consumed in developing countries (*10*). Low GI foods have been shown to reduce post-prandial blood glucose and insulin responses in normal and diabetic patients (*11*). Cowpea is also important for the rural farmers who cannot afford fertilizers since it fixes nitrogen into the soil thus improves soil fertility. Cowpea has good quality proteins, carbohydrates, phytochemicals, and calorically compares favorably with soybean, chickpea and kidney-beans. It is cheaper than other protein sources such as meat, milk and fish. This makes production of cowpea more viable for regions pressed by social, cultural, religious and economic issues.

Cowpeas also contain significant quantities of polyphenols such as simple phenols, flavonoids and tannins (*12*), which could act as inflammatory modulators. Inflammatory reaction occurs in response to infection, irritation, trauma, noxious stimuli or other injury that provoke an immune reaction. These conditions may be caused by bacterial, viral, parasitic and chemical agents (*13*). These responses are necessary so that the body can eliminate viral infections, repair tissue injury, and suppress tumorigenesis (*14*). Polyphenols have been proposed to exert their anti-inflammatory property through various mechanisms such as inhibition of ROS and modulation of cytokines/chemokines (*15*). However, no studies have been performed on the potential of cowpea polyphenols

to prevent or delay the onset of inflammatory-linked diseases. Thus, the polyphenolic and physiological properties of different cowpea varieties increases the need for research on the bioactive compounds present in cowpeas that could be exploited for improved human health.

An expanding body of research suggests a connection between consumption of flavonoids, down-regulation of inflammatory mediators and prevention of inflammatory-linked diseases. When NF-kB is activated, it regulates inflammatory responses of many cell types by activating production of pro-inflammatory cytokines, cell adhesion molecules and chemokines (*7*). These inflammatory mediators are important inflammatory markers involved in several chronic inflammatory conditions, which could be alleviated by plant polyphenols. Cowpea flavonoids and their potential to reduce risk of inflammatory-linked chronic diseases should be investigated. The association between the antioxidant activity of cowpea polyphenolics with their anti-inflammatory potential and prevention of chronic diseases is still unknown.

1.2 Significance

Cowpea is an inexpensive source of protein, dietary fiber and minerals (**Table 1**), which are required for good health but are often inadequate in our everyday diets. However, many consumers still view cowpea as a ‘poor man’s food,’ since its production and consumption is mostly associated with the rural farmers in poor and drier regions of developing countries. Thus, increased scientific information on potential health benefits of cowpea consumption is critical in promoting cowpeas to the urban-

middle class, who still do not appreciate the potential value of cowpea in human nutrition. Due to the rising health concerns in the urban demographic, the potential to serve this segment of population with functional foods is no longer a niche market. Thus, by establishing association between seed coat color and bioactive properties of cowpeas we may improve the understanding of healthy varieties that could be bred for improved nutritional properties. Additionally, information about the health properties of cowpeas will equip breeders with more parameters to select for and will contribute to cowpea improvement and greater economic returns to the farmers.

1.3 Research objectives

Three research objectives were established to test this hypothesis

1. Determine the phenolic composition of cowpea as influenced by seed coat color
2. Determine effect of boiling on the phenolic profiles of cowpeas
3. Investigate anti-inflammatory and antioxidant properties of the cowpea varieties, and correlate these properties to phenolic composition

2. LITERATURE REVIEW

2.1 Cowpea production

The *Leguminosae* (or *Fabaceae*) is the second largest family of flowering plants containing some of the most biologically active phytochemicals known to improve human health and prevent various diseases. This family comprises of beans, peas, cowpeas, peanuts, soybeans, lentils, etc. Among the grain legumes, cowpea is one of the most important food legumes of Africa, Asia and South America (*16*). Its origin and domestication is disputed in literature but has been associated with pearl millet and sorghum in Africa (*17*). In the US, cowpea is commonly called crowder pea, black-eyed pea or southern pea; while in some parts of the world, it has several names including *kunde* (Swahili), *coupe* and *niebe* (French), or *frijoles* (Spanish).

The world's largest production and consumption of cowpea is in West Africa (Nigeria and Niger). In 2009, Nigeria produced 2.4 million tons of the total world production of 4.5 million tons (*18*). In Nigeria, two out of every three children feed on cowpeas as a source of protein. Brazil, India, Myanmar, Sri Lanka, Haiti, Senegal, Cameron, Burkina Faso, Australia and the U.S. also produce significant quantities (*18, 19*).

In the U.S., the total production of cowpeas is estimated at 60,000 to 80,000 acres, of which the largest market classes are black-eyed pea and pink-eyed southern pea, which are usually harvested for their leafy greens, green pods, fresh shelled green

seeds, and shelled dried seeds (*20*). In the US, Georgia, Arkansas, California and Texas are the leading producers, accounting for about 60% of the total production (*19*).

Cowpea is a heat and drought tolerant crop that requires little agronomic incentives in terms of fertilizer. Maximum yields are obtained at 27/22°C (day/night) temperatures (*21*). Cowpea is able to survive under arid or semi arid soil conditions, since they are morphologically and physiologically able to reduce transpiration (*22*). Some varieties also grow a deep root system, and may have smaller leaf size with thicker cuticles to reduce rate of water loss, which increases their drought tolerance (*22*).

Cowpea is adapted to a wide range of soils, and can survive in sandy, clay and acid soils which support the presence of nitrogen fixing bacteria and good drainage. Good yields are obtained around pH 6, especially in the Oxisol of central Brazil (*23*). Poor soils produce little forage, but generally produce good yield of seed (*24*). No other legumes has been reported as widely adaptable to various soil and climate conditions as cowpea (*24*).

These cowpea's unique traits and low agronomical input requirement are favorable to the poor and low income farmers. The lower income populations are particularly vulnerable to malnutrition because the usual protein sources (meat and milk) are beyond their purchasing ability. Malnutrition, especially during childhood, has been linked to various chronic diseases in adulthood, including diabetes, obesity and cancer, especially in developing countries (*25*). Childhood malnutrition is also closely associated with food insecurity, particularly in developing countries (*25*). Nutritious, less costly,

easily accessible plant protein sources such as pulses (e.g. cowpea) can be used to improve the nutritional status of these low-income populations.

Unfortunately, most rural poor primarily obtain their food energy from cereals (poor sources of lysine, tryptophan and niacin) and starchy tubers. These foods are generally deficient in good quality proteins as well as health-promoting phytochemicals. The increase in consumption of staple diets such as cereal grains in these rapidly growing populations has led to a steady rise in chronic malnutrition and infant mortality and morbidity (*26*). For example, one in three school age Kenyan children are currently stunted from chronic malnutrition which impairs growth, causes poor performance in schools, and increases morbidity and mortality (*27*). From 2005 – 2007, chronic diseases contributed over half of the top twenty causes of morbidity and mortality in Kenya (*28*). In Zimbabwe, acute child malnutrition has increased by almost 75% since 2007, and more than 50% of the population needs humanitarian assistance (*29*). In eastern Congo, the World Vision estimates the number of children under the age of 5 suffering from malnutrition has increased more than ten-fold since 2008 because of poverty and conflict (*30*). Thus, most developing nations are faced with an impending epidemic of chronic diseases.

On the other hand, chronic diseases such as obesity, diabetes mellitus, CVDs and cancers are becoming increasingly significant causes of premature deaths worldwide, placing additional burdens on already overtaxed national health budgets (*31*). Since cowpea may be a “climate-change crop” due to its wide adaptability in several

environmental conditions and soils, it should be exploited for its potential to contribute to prevention of chronic diseases.

Table 1. Proximate composition, mineral content and amino acid profile of selected grain legumes*

Nutrients (g/100 g)	Cowpea	Chickpea	Lentil	Green pea
Crude protein	24.7 ± 0.10	24.0 ± 0.30	26.1 ± 0.09	24.9 ± 0.03
Crude fat	4.8 ± 0.07	5.2 ± 0.01	3.2 ± 0.06	1.5 ± 0.04
Ash	4.2 ± 0.05	3.6 ± 0.04	2.8 ± 0.06	3.6 ± 0.04
Minerals (mg/100 g)				
Sodium	102 ± 5.29	101 ± 3.51	79 ± 2.65	111 ± 2.65
Potassium	1280 ± 8.62	1155 ± 5.00	874 ± 6.43	1021 ± 12.49
Phosphorus	303 ± 7.94	251 ± 6.11	294 ± 3.61	283 ± 3.00
Calcium	176 ± 4.58	197 ± 3.61	120 ± 6.24	110 ± 3.61
Copper	9.7 ± 0.20	11.6 ± 0.20	9.9 ± 0.10	10.0 ± 0.40
Zinc	5.1 ± 0.20	6.8 ± 0.26	4.4 ± 0.20	3.2 ± 0.56
Magnesium	4.8 ± 0.10	4.6 ± 0.04	4.5 ± 0.04	4.2 ± 0.04
Manganese	1.7 ± 0.04	1.9 ± 0.10	1.6 ± 0.03	2.2 ± 0.02
Iron	2.6 ± 0.20	3.0 ± 0.20	3.1 ± 0.26	2.3 ± 0.56
Amino acids (% of protein)				
Arginine	7.5 ± 0.04	8.3 ± 0.21	7.8 ± 0.03	7.2 ± 0.04
Leucine	7.7 ± 0.08	8.7 ± 0.03	7.8 ± 0.05	7.4 ± 0.05
Lysine	7.5 ± 0.04	7.2 ± 0.03	7.0 ± 0.03	8.1 ± 0.07
Methionine	2.2 ± 0.04	1.1 ± 0.04	0.8 ± 0.02	1.1 ± 0.03
Phenylalanine	7.5 ± 0.06	5.5 ± 0.04	5.0 ± 0.12	5.2 ± 0.04
Tryptophan	0.7 ± 0.02	0.9 ± 0.02	0.7 ± 0.03	0.8 ± 0.02
Valine	5.0 ± 0.06	4.6 ± 0.03	5.0 ± 0.05	5.0 ± 0.09
Histidine	3.1 ± 0.03	3.0 ± 0.03	2.2 ± 0.05	2.4 ± 0.05
Isoleucine	4.5 ± 0.03	4.8 ± 0.03	4.1 ± 0.05	4.5 ± 0.06
Leucine	7.7 ± 0.08	8.7 ± 0.03	7.8 ± 0.05	7.4 ± 0.05
Alanine	4.2 ± 0.03	4.97 ± 0.03	4.2 ± 0.04	5.2 ± 0.04
Aspartic acid	10.8 ± 0.08	11.0 ± 0.04	11.8 ± 0.08	11.0 ± 0.06
Glutamic acid	17.2 ± 0.06	17.3 ± 0.08	21.5 ± 0.07	17.5 ± 0.06
Proline	4.0 ± 0.13	3.8 ± 0.05	3.5 ± 0.03	3.8 ± 0.03

*Adapted from Iqbal *et al.* (*32*). Data are presented as means ± SD.

2.2 Nutritional quality of cowpea

Cowpea has a number of attributes that make it valuable in human nutrition. The chemical composition of cowpea seeds vary depending on the genetic origin, as well as the climate, season, soil type and other agronomic practices. **Table 1** provides the comparison of proximate composition, mineral content and amino acid profiles between cowpea and selected common pulses.

Starch is the most abundant form of carbohydrate found in cowpea. Significant quantities of stachyose (2.0 – 3.6%), verbascose (0.6 – 3.1%) and raffinose (0.4 – 1.2%) exist in cowpea. These oligosaccharides are known to cause flatulence (*33*).

Cowpea also contains anti-nutritional factors like trypsin, chymotrypsin, subtilisin and protease inhibitors (*34, 35*). These classes of inhibitors are thermo-labile, thus are eliminated after proper heat processing. Tannins are particularly high in cowpeas having colored seed coat. Legume tannins complex with dietary proteins, decreasing protein digestibility and efficiency ratio (*36*). The presence of phytic acid (the storage form of phosphorus) in legumes decreases the bioavailability of essential minerals such as calcium and iron by making them insoluble; and may also complex with proteins or protein-mineral complexes. The interaction between phytic acid, proteins and starch also causes the ‘‘hard-to-cook’’ phenomenon of legumes seeds (*37*).

The need to improve human health through diet has motivated researchers to examine not only the levels of essential and non-essential nutrients in crop plants (e.g. grains, oilseeds and legumes) but also the contribution of plant components such as phenolic compounds with potential health attributes (e.g. antioxidant activity). Not only

are the antioxidant compounds able to protect cells and organs, as well as food systems, against oxidative damage caused by superoxide, hydroxyl and peroxyl radicals, but they also provide other health benefits such as lowering risk of several diseases. The challenge, therefore, is for breeders to design approaches for improving the "total nutritional quality" and appealing visual properties of popular grains commonly cultivated and consumed by the rural-poor (e.g. cowpea) to provide acceptable, sustainable and inexpensive complements to medical and social programs that are striving to prevent human diseases.

The diets in most poor communities consist mainly of starchy cereals (sorghum, millet, corn, rice, etc.), tubers (cassava, potatoes, etc.) and plantains. Cereal proteins, as well as cassava proteins, are deficient in lysine, in which legumes are much richer. Cereals provide 70 – 85% of total calories, and are usually prepared in the form of "porridge." The practice, commonly used in many rural areas in Africa, of mixing cassava flour with cereal flour to make a staple food, in the form of stiff porridge, and eating this porridge with legume-based soups provides relatively cheap, nutritious diets which would be beneficial even for the urban dwellers. Therefore, increased production and improvement of familiar legumes such as cowpeas, better storage practices to eradicate weevils, increased scientific information on their health benefits, and new industrial methods of processing, should be given high priority.

2.3 Utilization of cowpea for human food

Cowpea is widely utilized in a variety of food preparations, and is mostly consumed as a boiled, steamed, fermented or fried vegetable (*38*). The traditional foods utilizing recipes of cowpea like *ewa* (boiled whole bean), *akara* (deep-fried dehulled cowpea paste), *seke-sin* (fried cowpea cakes with green onions), *gbegiri* (cowpea soup) and *moin-moin* (steamed cakes) are commonplace in West Africa (*39*). In India, it is consumed as cooked whole seed, or cooked green immature pods in the form of curry in conjunction with certain cereals (*40*). In southern US, it is canned and consumed as boiled beans (*41*). In some parts of the world, the young shoots are often consumed as spinach in fresh form (*42*). Cowpeas thus play a vital role in many traditional diets of several regions throughout the world by providing the major proportion of essential nutrients and energy to these populations. Thus, the use of cowpea in both infant and adult foods is recommended since it improves the nutritional balance of the diet in order to reverse the perennial problems of malnutrition in developing countries.

2.4 Constraints to cowpea production and use

Physical degradation and nutrient depletion of soil is a perennial problem for the rural-poor farmers in developing countries, where much grain-legume production and consumption occurs. Many of these farmers cannot afford to use fertilizers, thus limiting their productivity. Diseases, pathogens and pests also affect legume production, especially in tropical and subtropical regions of Africa (*43*). Crop losses occurring as a result of these factors are huge and several strategies to reduce productivity losses have

been proposed, including biological control of diseases, chemical application and use of molecular markers in disease resistance breeding in bean and cowpea (*43, 44*). Unfortunately, the rural-poor and subsistence farmers cannot afford to utilize many of these expensive measures.

Inclusion of legumes in human diet is also problematic due to lack of compelling benefits to consumers, beany flavor, longer preparation time, and the presence of antinutritional factors which dampens demand and economic value. Protein content in legume seeds range from 20 – 30%, and are rich in lysine, complementing the low levels in starchy cereals and tubers in the diet. However, legumes contain low levels of sulfur amino acids and are usually hard to cook. Consumer preferences for particular grains or seed colors also affect marketability and acceptance. Compelling evidence on health effects of grain-legume consumption may promote acceptability among the urban-rich who are looking for healthy foods.

2.5 Opportunities for increased cowpea utilization

By 2030, the rapidly expanding water-stressed areas of the world is likely to worsen the drought problems for legumes (*45*). Therefore, there is increasing need to increase drought and salinity tolerance in legumes in many areas, especially in the face of climate change. The drought-tolerant legumes, such as cowpea may be a good "climate-change crop" candidate due to its wide adaptability to various environments.

Cowpea contains flavonoids such as anthocyanins; glycosides of flavonols (i.e. kaempferol, quercetin and myricetin), isoflavones (i.e. daidzein and genistein), phenolic acids and tannins (*46, 47*). Reports show that these flavonoids have strong anticancer and anti-inflammatory activities (*48, 49*). Thus, cowpea may provide significant health benefits which would be appealing to the urban-rich who are looking for healthier lifestyles.

Unfortunately, legumes seeds, particularly cowpea are still regarded as "poor man's meat", a stigma that undermines these valuable protein sources. From a nutritional standpoint, the high protein (18 – 35%), carbohydrate (50 – 65%) and amino acid profile of cowpea complement that of cereal grains and starchy tubers. However, in general, the use of cowpea in many foods is limited due to the beany flavor. If appropriate and inexpensive technologies can be developed for converting cowpea into nutritious food ingredients and products, it would be appealing to the urban-rich who presently subsist on nutritionally deficient diets. Presently, food applications utilizing cowpea flour include baby/weaning foods, chips, papad (thin-wafer like tortillas), cinnamon sweet rolls, cookies, doughnuts, muffins, biscuits, Chinese type noodles, *moin-moin* and *akara (50).* The beany aroma is acceptable in certain cowpea products such as *moin-moin*, but not in the noodles, baked and snack food products. Thus, further improvement in cowpea flour research to eliminate the beany aroma would enhance its acceptance and economic value.

Cowpea phenotypes are diverse. Such genetic variation helps in the development of improved cultivars. Each cowpea phenotype has seeds with distinctive appearance

and flavor, which can be used as an alternative to common beans (less drought tolerant legume).

Unfortunately, with increasing population growth in many developing countries, as well as natural calamities such as famine, there is reduced availability of food to match the demand of the growing populations. Cowpea could play an important role in reducing hunger and prevention of such diseases associated with protein-calorie intake.

2.6 Flavonoids in cowpea

Cowpea contains phenolic acids in free and bound forms (*47*), as well as other flavonoids, mainly anthocyanins (*46*), flavonols and flavan-3-ols derivatives (*51*). Cai *et al*. (*47*) reported that in cowpea, protocatechuic acid is the major phenolic acid present in esterified forms, ranging from 9.3 to 92.7 mg/100 g of flour. Trace amounts of six other phenolic acids (at <7 mg/100 g of flour), namely, *p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, ferulic acid, 2,4-dimethoxybenzoic acid, and cinnamic acid, were also identified, mainly in free forms. Total phenolic contents determined using Folin-Ciocalteu's reagent ranged from 34.6 to 376.6 mg/100 g of flour. On the other hand, the phenolic acids content of common bean (*Phaseolus vulgaris* L.) reportedly ranged from 19.1 to 48.3 mg/100g of flour (*52*). Ferulic acid was reportedly the most abundant, whereas *p*-coumaric and sinapic acids were present in moderate amounts in all the samples studied. However, no literature is available correlating cowpea phenolic acids with specific phenotypes and/or variety.

Anthocyanins are responsible for the red, blue, purple colors of many fruits, leaves, stems, roots, flowers and cereal grains. They are odorless and nearly flavorless, and are probably the most important subclass of visible plant pigments in nature. Anthocyanins are not commonly present in most legume seeds. However, anthocyanins have been identified in Beluga Black lentils (*53*) and Hakmeitau beans – a black seed cultivar of cowpea (*54*). In cowpea, seven anthocyanins have been reported, namely, delphinidin-3-*O*-galactoside, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside (*46*). However, their levels are unknown. Since anthocyanins are increasingly becoming important as antioxidants, information on anthocyanin content in cowpea would provide evidence on potential health effects associated with ability to reduce oxidative stress.

In legumes, flavonols (e.g. kaempferol, myricetin and quercetin) and isoflavones (e.g. daidzein and genistein) are usually present in their glycosylated forms, often associated with glucose or rhamnose sugar moieties (*55*). Isoflavones have pseudo-hormonal properties, as such are classified as phytoestrogens.

Compared to other legumes, black seed coat cowpea has higher amounts of quercetin (214 – 280 μg/g), but limited amounts of daidzein, genistein and kaempferol (*56*). Other pulses, for example mung bean contain about 17.6 to 22.0 μg/g of myricetin, and limited amounts of daidzein and kaempferol (*56*); while chickpeas contain approximately 0.4 μg/g daidzein, 0.6 μg/g genistein, 1.4 μg/g formononetin and 0.2 μg/g

biochanin A (*12*). However, how seed coat color influences types and levels of flavonols in cowpea is still unknown.

Procyanidins (i.e. condensed tannins) are the predominant phenolic compounds found in legume seeds, and consist of two main monomers, namely (+)-catechin and (-)-epicatechin. They are located mainly in the seed coats and play an important role in the defense system of seeds against many oxidative insults from the environment. Tannins are known to lower protein digestibility thus reduce nutritional quality of tannin-rich diets. Among legumes, lentils, red bean, adzuki bean, pigeon peas and black gram contain significant levels of tannins (*57, 58*); while chick peas and mung beans contain no detectable levels of tannins (*59*). Tannin concentration in cowpea range from 0 – 0.7%, a level that may be nutritionally important (*59*).

How phenotype/variety affects the types and levels of procyanidin compounds in cowpea is unknown. Other pulses such as lentils reportedly contain procyanidin B2, B3, and procyanidin tetramer ranging from 0.1 to 0.5 mg/100 g dry weight, db (*60*); while adzuki bean contain procyanidin dimers and trimers ranging from 15.9 to 213 mg/g (*61*). Additionally, procyanidin B2, C1, and C2 have been identified in the hulls of red, brown, and black beans (*62*). These compounds play a critical role in the sensory properties and biological quality of foods (*63, 64*).

Seed-coat color influences levels of flavonoids in legume seeds (*56*). Literature reports on the differences in flavonoid compositions among legume genotypes suggest that no single legume is a good source of all flavonoids. However, how cowpea seed coat color affects flavonoid composition is unknown. Association between seed coat

color and individual flavonoid content may be an indicator of accumulation of particular flavonoids and the potential ability of specific cowpea phenotypes to prevent chronic disease.

2.7 Importance of legume flavonoids as antioxidants

Several studies have been conducted to screen for antioxidant activity levels in various legumes. Black and red pigmented cowpeas were reported to have the highest antioxidant activity, followed by the pinkeye and black-eye types. The cream types had the lowest antioxidant capacity (*65*). This suggested that compounds responsible for cowpea seed coat color were involved in antioxidant activity. The antioxidant activity of red and black varieties of common bean was found to be higher than those of their white counterparts, and was attributed to the presence of anthocyanins in the seed coats of the colored types (*66*).

Siddhuraju and Becker (*67*) also screened raw and dry-heated seed extracts from two cowpea varieties for their potential antioxidant activities using DPPH, ABTS, FRAP, linoleic acid emulsion and β-carotene–linoleic acid assays *in vitro* and reported that all extracts exhibited good antioxidant activity against the linoleic acid emulsion system. Thus, cowpea could contribute significantly in prevention of degenerative diseases associated with free radical damage.

Xu *et al*. (*68*) showed that black beans (*Phaseolus vulgaris* L.), lentils (*Lens culinaris*), red kidney beans (*Phaseolus vulgaris* L.) and pinto beans exhibited higher antioxidant capacities (5.7 to 6.4 μmol TE/g), than yellow peas, green peas and chickpea

(2.1 to 2.4 μmol TE/g) in both LDL-conjugated dienes (CD-Trolox) assay and LDL-thiobarbituric acid reactive substances (TBARS) assay. The antioxidant activities of these legumes against human LDL-cholesterol oxidation were found to correlate significantly ($p < 0.01$) with their phenolic content, DPPH radical scavenging activity and ORAC values *in vitro* (*68*). These results indicate that intake of legumes such as black beans, lentils and red kidney beans may have great potential in inhibiting LDL-oxidation thus promoting cardiovascular health.

With vast differences in cowpea genotypes, we believe elucidating the chemical composition and antioxidant properties of cowpea as influenced by seed coat color could increase public recognition of the health benefits (such as antioxidant activity and ability to lower inflammatory-linked conditions) of consuming specific cowpea phenotypes, which could increase demand for food uses of cowpea.

2.8 Effect of thermal processing on phenol content and antioxidant activity of pulses

Most legume seeds must be thermally processed before consumption. Severe heating, for example, pressure cooking (*i.e.* 121°C; 103.421 kPa; 60 min) reportedly reduced the phenolic content in common bean seed coats by approximately 90% (*69*). Cooking (*i.e.* boiling for 5 min, microwave cooking for 1 min or steaming for 7.5 min) has also been shown to significantly ($p < 0.05$) reduce phenolic content in peas (*70*). Among the cool season food legumes, the boiling process has been reported to reduce 40

– 60% of total phenol content in green peas, yellow peas and chickpeas; and 60% in lentils (*71*). Thus, in general, thermal treatment is detrimental to the phenolic composition of legumes. Presently, effect of heat on phenol content of different cowpea phenotypes/varieties is unknown.

Reduction of phenolic content as a result of thermal treatment correlates with reduced antioxidant activity of legumes compared to their raw counterparts. For example, boiling process reportedly decreased ferric reducing antioxidant power (FRAP) values of green pea, yellow pea, chickpea and lentils by 58.5 – 64, 57 – 60, 44 – 64 and 68 – 70%, respectively (*71*). Similar trends were obtained using oxygen radical absorbing capacity (ORAC), DPPH radical scavenging activity (*72*) and Trolox equivalent antioxidant capacity (TEAC) on thermally processed cool-season food legumes (*73*). Thus, thermal processing reduces antioxidant properties of legumes, possibly through leaching of the water-soluble antioxidants (*74*) and degradation of phenolic compounds (*75*). Low antioxidant activity of thermally processed legumes reportedly reduces their antiproliferation activities compared to the raw samples (*71*).

How thermal processing impacts antioxidant capacity, antiproliferation activity and anticancer properties of cowpeas is unknown. These could be critical information because the hypothesized potential modes of actions against cancer proliferation are assumed to involve antioxidant capacity, especially after thermal processing. Thus, in terms of human health, the properties of cowpea phenolics after thermal treatment are important.

2.9 Pulses and disease prevention

2.9.1 Pulses in prevention of cardiovascular disease

Cardiovascular disease (CVD) remains the leading cause of death and disability in North America (*76*). Most CVDs are preventable through life-style measures such as diet and exercise.

Consumption of pulses has been shown to effectively manage hypercholesterolemia, a major risk factor for CVD. The hypocholesterolaemic effects of pulses is related to the presence of dietary components such as soluble dietary fiber, vegetable protein, oligosaccharides, isoflavones, phospholipids and fatty acids, phytosterols, as well as saponins (*77, 78*). Consumption of common beans has been shown to decrease serum LDL-cholesterol by 7% and serum triacylglycerols by more than 10%, without significantly affecting serum HDL–cholesterol values (*79*). Increased bile acid binding and decreased re-absorption of bile acids may also play a significant role in the hypocholesterolaemic effects of pulses (*5*).

Common beans are also relatively good sources of folic acid and thiamine – vitamins known to lower serum homocysteine concentrations (*79*). Pulses supply essential minerals that may lower the risk for hypertension and stroke (*80*). Isoflavones from pulses have also been reported to improve serum lipoproteins, antithrombotic effects and anti-inflammatory activity (*81*).

Epidemiological evidence show that consumption of legumes (more than 4 times per week compared with less than once a week) reduced risk of coronary heart disease (CHD) and CVD by 22 and 11%, respectively (*82*). No epidemiological data exists on

how cowpea consumption may reduce total cholesterol, LDL (important CVD risk factor) and HDL levels. Cowpea's nutritional profile is comparable to that of common beans. Thus, cowpea may have important metabolic and/or physiological effects on reducing CVD risk. Further research is needed to determine how cowpea can reduce CHD and CVD risk factors such as intracellular reactive oxygen species (ROS), which correlates with prevention of inflammatory related diseases.

2.9.2 Pulses in prevention of cancer

Several epidemiologic studies show inverse correlation between intake of plant-based foods and protective effects certain cancers. For example, in a large prospective cohort study a reduced postmenopausal breast cancer risk was associated with higher intake of pulses (e.g. common beans) (*83*). A comparative study of lifestyles in Jiangsu Province, China, found that people living in a low-risk area for gastric cancer consumed kidney beans and soya products more frequently than those in a high-risk area (*84*). In Europe, a case control study showed protection against colorectal cancer by most vegetables, including pulses such as beans (*85*). In the Netherlands, a large cohort study revealed that consumption of pulses such as French beans and broad beans was associated with reduced risk of prostate cancer (*86*). In the United States, a cohort study of non-Hispanic white California Seventh-day Adventists showed that individuals who consumed red meat had significant protection against colon and pancreatic cancers if they also consumed legumes frequently (*87*). Other studies have also shown that

consumption of pulses such as dried beans, split peas, or lentils reduced risk of colorectal adenoma (*88*).

No epidemiological evidence exists on consumption of cowpea and reduced risk of cancers. Since cowpea contains flavonoids that can enhance the body's immune system against inflammatory-linked diseases, potential of cowpea to contribute to cancer prevention should be investigated. How heterogeneity in types and levels of polyphenolics from different cowpea phenotypes could affect ability of cowpea against cancer initiation should be established. This information could form the basis for quantitative measures of intakes for specific cowpea varieties or for pulses in general.

2.10 Association of inflammation with cancer

Inflammation process produces reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS significantly impact etiology of several non-communicable diseases, including cancer (*89*), by causing oxidative damage and DNA mutations (*90*). RNS leads to nitration of DNA bases, which increases the risk of DNA mutation and hence cancer (*91*). ROS include singlet molecular oxygen ($-O_2$), peroxide (O-O-H) and lipid peroxides (LOO), while free radicals include superoxide (-O), hydroxyl (OH), Fe, Cu, nitric acid and ozone. It is thought that inflammation may contribute to tumor initiation through excessive generation of ROS and RNS (*92*).

Characteristics of inflammation include redness, swelling and pain. These conditions may be caused by bacterial, viral, parasitic and chemical agents. Gram-

negative bacteria produce a lethal endotoxin known as lipopolysaccharide (LPS) which functions as an effective stimulus in activation of macrophages to release proinflammatory cytokines such as interleukins (IL-6 and IL-8) and TNF-α, as well as other inflammatory mediators such as prostaglandins, nitric oxide and cyclooxygenase enzymes (COX) (*93*). In general, cytokines are a group of substances produced by resident or migrating cells (e.g. mast cells, macrophages and neutrophils), and function at many steps of the inflammatory response to heal wounds and stimulate epithelial cell proliferation (*94*).

Nuclear factor-κB (NF-κB) is a transcription factor that plays a major role in the immune, stress, proliferative, apoptotic and inflammatory response (*95*). NF-κB is sequestered in the cytoplasm, where it is held inactive in a non-DNA-binding form by the inhibitory proteins (IκBs) (*95*). When cells are stimulated with various NF-κB inducers, IκB proteins become rapidly phosphorylated by IκB kinase (IKK) complex – an inhibitor of NF-κB kinase. Thus, inhibition of NF-κB is regarded as a useful strategy for treatment of inflammatory disorders (*96*).

The cytokine response related to transduction signaling, cellular differentiation and disease can be regulated by microRNAs (miRs; small portent non-coding RNA that post-transcriptionally regulate target genes). miRs can modulate anti-inflammatory mechanisms associated with NF-κB activation by inducing mRNA degradation or blocking translation, as well as regulating vascular inflammation (*97*). For example, endogenous miR-126 has been demonstrated to regulate leukocyte adherence to endothelial cells usually associated with tumor development (*97*). It is likely that the

involvement of plant phenolic compounds in regulation of the cytokine system may also affect expression of mRNAs and activity of miRs during immune response. Therefore, further insight into the effect of phenolic compounds on regulation of cytokine signaling by miRs may help design new strategies to modulate inflammation in a clinical context.

Recent data suggest that some phenolic compounds can exert their anti-inflammatory activity by regulating NF-κB activation, as well as acting on multiple steps of NF-κB activation cascade and consequently on downstream effectors (*98*). For example, quercetin may potentially control intestinal inflammation in celiac disease by preventing the activation of NF-κB and mitogen activated protein kinases (MAPK) pathways (*99*); consequently counteracting the expression of cytokines and inducible nitric oxide synthase (iNOS) (*100*). Anthocyanins have also been shown to inhibit TNF-α-induced endothelial leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression in cultured human umbilical vein endothelial cells (HUVEC) (*101*). Cell adhesion molecules play a key role in monocyte recruitment and thus promote tumor development. Phenolic compounds are therefore important metabolic modulators of cellular pathways and molecules. Therefore, their regulatory action on NF-κB pathways and inhibitory property on NF-κB activation cascade is a critical part in the mechanisms associated with anti-inflammatory effects of dietary plant phenolic compounds.

Majority of studies on legumes and their relationship to prevention of chronic diseases focus on specific nutritional components and not the total dietary intake of legumes. Therefore, legumes have been understudied in their relationship to CHD, CVD

and cancers. The ability of cowpea phenolics to prevent or delay the onset of inflammatory-linked diseases is unknown. Research is needed on possible health promoting effects of cowpea which might help promote its consumption. This study, therefore, aims to demonstrate the anti-inflammatory potential of cowpea polyphenols using nonmalignant colon cells with relevance to colorectal cancer prevention.

2.11 Overall goal of this research

For some dietary polyphenols, certain beneficial effects on cell functions (e.g. ability to prevent cancer and cardiovascular disease risks) are suggested by epidemiological studies, some are supported by *in vivo* studies, and others are extrapolated from *in vitro* systems. However, to our knowledge, no literature exists on how cowpea polyphenols may offer a direct or indirect protection on cell functions such as activation of endogenous defense systems or modulation of cellular signaling processes such as NF-κB activation, which may prevent pathogenesis of inflammatory diseases. We hypothesize that cowpea polyphenols may protect cells against oxidative stress by scavenging ROS or blocking ROS production intracellularly. The ability of cowpea phenolic to inhibition ROS could be pivotal in the cellular defense mechanisms against oxidative and xenobiotic stresses, thus inhibiting NF-κB transactivation critical in the prevention of inflammatory-linked diseases such as cancer. Different cowpea phenotypes would have different phenolic profiles which may affect their anti-inflammatory property. Thus, the overall goal of this research is to identify cowpea phenotypes with best potential to prevent chronic disease.

2.12 Potential impact of this research

The ability of cowpea flavonoids to prevent chronic diseases is unknown. Since significant differences may exist in levels of flavonoids (flavonols, isoflavonols, anthocyanins, proanthocyanidins and phenolic acids) present in different cowpea phenotypes/varieties, information about the health properties of cowpeas will: (a) help identify phenotypes/varieties with best potential to prevent chronic disease, (b) provide compelling evidence on health benefits of cowpea consumption to consumers, (c) equip breeders with more parameters for crop improvement, and (d) increase demand and economic value of cowpea to farmers.

3. PHYTOCHEMICAL PROFILES OF DIFFERENT COWPEA PHENOTYPES AND THEIR LEVELS BEFORE AND AFTER THERMAL PROCESSING

3.1 Introduction

Cowpea (*Vigna unguiculata*) is an important part of human diet in many countries around the world, especially in developing countries (*102*). Apart from its traditional role of supplying good quality proteins (25%) essential in preventing protein malnutrition, cowpea is also a low glycemic index food high in carbohydrates (60%), dietary fiber and minerals (e.g. K, Mg, Ca, P, Na, and Fe) (*103, 104*). Cowpea seed and cowpea protein isolates have been reported to lower plasma total cholesterol and increase high density lipoproteins (HDL) (*105*). Antioxidant activities and phenolic constituents in raw cowpeas have also been reported (*47, 51, 56, 106, 107*).

Phenolic constituents of 17 cowpea varieties were reported to be dominated by protocatechuic acid in esterified forms (from 9.3 to 92.7 mg/100 g of flour) (*47*). Six other phenolic acids were reportedly present in small quantities, including *p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, ferulic acid, 2,4-dimethoxybenzoic acid, and cinnamic acid; which may be free, esterified or bound to other non-phenolic compounds (*47*). The chromatograms of the phenolic constituents in the 17 cowpea flours before acid hydrolysis showed significant differences in the number of peaks and peak distribution. This evidence suggests that chromatograms of intact phenolic compounds better described the distributions of polyphenolic compounds and identities of the individual cowpea phenotypes or varieties.

Anthocyanins, such as 3-*O*-glycosides of delphinidin, cyanidin, petunidin, peonidin and malvidin have also been reported in raw cowpea seed coats (*46*). Anthocyanins are known to have anti-inflammatory property (*108*), selective growth inhibitory activity of cancer cells (*109*), as well as ability to reduce risk of coronary heart disease (*110*). How cowpea seed coat color influence anthocyanin composition is unknown.

Apart from phenolic acids and anthocyanins, cowpeas also contains significant amounts of proanthocyanidins (*i.e.* condensed tannins), flavan-3-ols (*e.g.* catechin, epicatechin), flavonols (*i.e.* quercetin, myricetin and kaempferol), and isoflavones (*i.e.* daidzein and genistein) (*56*). For example, cowpea reportedly contains relatively high amounts of myricetin and quercetin especially in the dark colored varieties, but limited amounts of genistein and kaempferol (*56*). However, since identification and quantification was performed after acid hydrolysis, the study never considered the fact that these flavonoids may be associated with other non-phenolic materials such as glycosides or acyl groups, and thus failed to provide the structural properties of the flavonols and isoflavones in cowpea.

Proanthocyanidins (*i.e.* condensed tannins), consist primarily of catechin and epicatechin units, and are considered antinutritional compounds since they precipitate proteins and complex with iron in the lumen, thus reducing the absorption, digestibility and availability of these nutrients (*111*). Most investigations on condensed tannins in legumes have always focused on herbaceous forage legume species rather than the seeds useful in human nutrition (*112*). Elias *et al*. (*113*) showed that the content of tannins in

legume seeds varied with the color of seed coats, with the white varieties containing lower tannin concentrations than the red, black or bronze seed coats. Tannin-rich extracts from legumes with colored seed coat (*i.e* pea, faba bean, lentil and broad bean) were reported to possess stronger antioxidant activity measured by the oxidation rate of phosphatidyl choline in the liposome system, than legumes with white seed coats (i.e. bean, pea and everlasting pea) (*114, 115*). The presence of higher levels of tannins in the seed coat apparently correlates with the antioxidant activity of legume seeds.

Cowpea polyphenolics are located mainly in the seed coats. Cowpea seed-coat color, hue and intensity vary greatly among varieties, and has been established to strongly correlate with antioxidant activity and total phenol content (*65*). Therefore, it is reasonable to suspect that seed-coat color might influence the flavonoid composition and content in cowpea seeds, and that their antioxidant activities would be phenotype dependent.

Cowpea seeds must be processed before consumption, thus, information on the composition and health promoting properties of boiled samples are more relevant from nutritional point of view. Processing methods include soaking and thermal treatment, as well as fermentation or milling into flour to make other food products (*116, 117*). Thermal processes improve flavor and palatability of cowpeas, and increase the bioavailability of nutrients by eliminating or inactivating antinutritional factors (*e.g.* trypsin inhibitor, saponins, agglutinins, lectins, flatulence causing oligosaccharides, etc) (*118-120*). Thermal processing may also lead to complex changes in the phenolic composition of legumes, as well as degradation of polyphenols. Rocha-Guzmán *et al*.

(*69*) reported that severe heating, for example, pressure cooking (*i.e.* 121°C; 103.421 kPa; 60 min) drastically reduced the phenolic content in common beans seed coats by approximately 90%. However, since polyphenolic compositions of cowpea may be phenotype dependent, effect of thermal treatment on the polyphenolic profiles of cowpea may be phenotype dependent as well. Information on the degree of retention of specific phytochemicals and possible formation of polyphenolic-derivatives following thermal treatment would be essential in providing insight into levels and heat stability of individual cowpea polyphenolic compounds and possible recommendations on strategies to enhance their stability during processing.

At present, no information is available on how seed coat color influences types and levels of flavonoid compounds in cowpea grains. Moreover, no information exists on the effect of thermal treatment on retention and/or transformation of polyphenolic compounds in cowpeas. In order to associate flavonoid profiles of different cowpea varieties with health promoting potential, it is important to first identify and quantify the individual polyphenols present in cowpea, and establish how seed coat color and thermal treatment influence their composition. Thus the objectives of this study were to; (1) elucidate the flavonoid profile of distinct phenotypes of cowpea seeds; (2) demonstrate the association of cowpea phenotype with major flavonoid levels; and (3) characterize the effect of thermal treatment on those flavonoids.

3.2 Materials and methods

3.2.1 Plant materials

Dry cowpea seeds within six phenotypes (black, red, green, white, light brown and golden brown) were collected at maturity in late July 2010 at university experimental station in College Station, Texas, and UC-Riverside, California. The cowpea varieties, seed-coat color, texture and size, and origin or collection sites are listed in **Table 2**. Broken and damaged seeds, as well as foreign materials were removed prior to use. The seeds were ground using a coffee grinder (Cuisinart, Model DCG-20N series) to pass through a 60-mesh sieve. The powders were then stored at 4°C until used. To determine the moisture content, triplicate samples of the ground powders were dried in an air circulated oven at 110°C until a constant weight was obtained (*121*). All calculations for flavanoid content, total phenol content, condensed tannin content and antioxidant capacity were on a dry weight basis.

3.2.2 Chemicals and reagents

All reagents were analytical grade. Kaempferol, quercetin, quercetin-3-*O*-rutinoside (rutin), procyanidin dimer B2, taxifolin, phloretin, phloretin-2′-glucoside (phlorizin) and (+)-catechin were purchased from Sigma-Aldrich Chemicals, St. Louis, MO; protocatechuic acid was obtained from MP Biomedicals, LLC, Solon, OH; myricetin was obtained from ACROS Organics, New Jersey, USA. Delphinidin-3-*O*-glucoside, cyanidin chloride, cyanidin-3-*O*-glucoside, malvidin-3-*O*-glucoside,

eriodictyol, eriodictyol-7-*O*-glucoside and quercetin-3-*O*-glucopyranoside were purchased from Extrasynthese Natural Products, Genay Cedex, France.

Table 2. Description of cowpea cultivars selected for the study. See ***appendix A*** for photos of cowpea phenotypes investigated in this study.

Variety	Seed weight[†] (g/100 seeds)	Seed coat property	Origin[‡]
IT95K-1105-5	23.4 ± 0.37	Black, smooth	Riverside, CA
IT98K-1092-1	11.6 ± 035	Black, smooth	College station, TX
IT82D-889	11.3 ± 0.17	Red, smooth	College station, TX
IT97K-1042-3	13.1 ± 0.20	Red, smooth	College station, TX
TX2028-1-3-1	21.6 ± 0.45	Green, freckled, black-eye	College station, TX
IAR-48	22.6 ± 0.22	Light brown, rough	College station, TX
09FCV-CC-27M	14.9 ± 0.40	Light brown, smooth	Riverside, CA
IFE BROWN	15.8 ± 0.16	Golden brown, rough	College station, TX
IT84S-2246	17.5 ± 0.08	Golden brown, smooth	Riverside, CA
EARLY ACRE	11.6 ± 0.16	White, freckled, brown eye	College station, TX

[†]Seed weight expressed as mean ± SD of triplicate weights of 100 seeds. [‡] Samples were grown in university test plots in CA or TX in 2010.

3.2.3 Thermal processing

Triplicate amounts (100 g each) of the whole cowpea seeds from each variety were separately soaked in 450 g distilled water for 12 hours, and were then boiled in beakers at atmospheric pressure for 15 min [the average time adequate tactile texture (resistance to pressure between the fingers) was achieved] (*122*). Cooking time commenced when the cook-water started boiling. The cooked seeds (including the soups) were immediately transferred into 50 mL falcon tubes, and cooled in a chilling water bath. Subsequently, these samples were frozen to –80°C, freeze-dried and ground to pass through 60-mesh screen. The powders were then stored at 4°C until used.

3.2.4 Extraction of polyphenols for HPLC and mass spectrometry analysis

Approximately 5 grams of raw and cooked ground cowpeas were separately weighed into capped polypropylene centrifuge tubes and soaked in 15 mL of 70% acetone acidified with 1% formic acid for 12 hours at 4°C. Then, the mixtures were shaken intermittently for 4 hours in a shaking water bath set at 37°C (Blue M, Blue Island Electrical Company, IL, USA). The extracts were centrifuged (10,000 *g*-force for 10 min) using a Heraeus Megafuge 11R Centrifuge (Thermo Fisher Scientific, Asheville, NC) at 4°C and the supernatants were transferred into new falcon tubes. The solid residues were washed twice with 10 mL of the extraction solvent and further centrifuged. The supernatants were then combined and concentrated under reduced pressure (Buchmann R110 Rotavapor, Westbury, NY).

3.2.5 Extract purification

The cowpea extracts were fractionated on a Sep-Pak Solid Phase Octadecylsilane (C18) cartridges (Sigma, USA) following methods described by Prior *et al*. (*123*) and Monagas *et al*. (*124*) but with some modifications. Briefly, the C18 cartridges were preconditioned with 25 mL methanol:water (1:1) for 1 hr and washed with 50 mL distilled water. At least 5 mL of the concentrated extracts were deposited into the cartridges and washed with 5 mL distilled water to remove the sugars. Then, catechins, oligomeric proanthocyanins (PA) and other small phenolic molecules were eluted with 15 mL ethyl acetate. The ethyl acetate fraction was taken to dryness under vacuum, and re-dissolved in 3 mL of methanol:water (50:50) containing 0.05% formic acid. The

flavonols, isoflavonols and anthocyanin pigments were then eluted with 15 mL methanol acidified with 0.1% formic acid, followed by elution of polymeric proanthocyanidins with 10 mL 100% methanol. The sequential elution procedure was chosen to reduce the number of compounds in each fraction injected into the mass spectrometer, thus enabling proper characterization. The prepared pigments were stored at 4°C until analyzed.

3.2.6 HPLC analysis

Aliquots of the purified extracts (400 μL) were each separately mixed with 400 μL of methanol acidified with 0.05% formic acid and filtered through a syringe with a 0.2 μm polytetrafluoroethylene (PTFE) membrane filter prior to injection into an HPLC system. The HPLC analysis was conducted using an Agilent 1200 series LC system, equipped with a G1322A vacuum degasser, a G1311A quaternary pump, G1364C analytical auto-sampler, G1316B thermostated column compartment, and a G1315C Diode Array and Multiple Wavelength Detector (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation was performed on a ZORBAX Stable Bond Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm, 100A; Agilent Technologies, MD, USA), with a guard column (4.6 × 12.5 mm, 5µm). The mobile phase consisted of aqueous 2% v/v formic acid solution (A) and acetonitrile:methanol (50:50) acidified with 2% formic acid (B). Using an injection volume of 5 µL in each analysis, the elution gradient was carried out at a flow rate of 1.0 mL/min with the column temperature thermostated at 40°C. The gradient profile was programmed at 0% B from 0 – 2 min, 0 – 10% B from 2 – 8 min, 10 – 20% B from 8 – 15 min, 20 – 50% B from 15 – 30 min, 50 – 80% B from 30 – 35 min,

80 – 100% B from 35 – 40 min, followed by 5 min of isocratic elution at 100% B. A 2-min post time was allowed for a system equilibration before each sequential injection. The spectra were monitored at 280 nm for phenolic acids and flavanols; at 360 nm for flavonols and isoflavonoids; and at 520 nm for anthocyanins.

3.2.7 LC-ESI/MS analysis

A Waters - ACQUITY UPLC/MS system (Waters Corp., Milford, MA) was used. The UPLC was equipped with a binary solvent manager, sample manager, column heater; and Photodiode array eλ detector and interfaced with a Mass Spectrometer equipped with a tandem quadrupole (TQD) electrospray ionization (ESI) detector. Anthocyanins were monitored at 520 nm, while other phenolics were at 280 and 360 nm. The column used was a Kinetex C18 column, 150 × 2.10 mm, 2.6 µm (Phenomenex, Torrance, CA). The column temperature for anthocyanins and other phenolics was thermostated at 50 and 40°C, respectively. Mass spectrometric data of the eluted compounds from the column were acquired in positive mode for anthocyanins and negative mode for flavonol glycosides and catechins. Data acquisition and processing were performed using *Empower 2* software (Waters Corp., Milford, MA). The MS scan were recorded in the range of 100 – 1000 Da for anthocyanins, and 100 – 1500 Da for other phenolics. Nitrogen was used both as a drying gas and as nebulizing gas, while argon was used as the collision gas (AOC, Bryan, TX). The nitrogen gas flow conditions were 800 and 50 L/hr for desolvation and at the cone, respectively. Source block temperature and desolvation temperature were set at 150 and 400°C, respectively.

Optimization of ionization conditions was based on the intensity of the mass signals of protonated/deprotonated molecules and aglycones fragments, and was performed for each individual peak/compound detected. Mass parameters were optimized as follows: capillary voltage, 3.5/3.0 kV; and cone voltage, 40/30 V for positive/negative ionization, respectively. The MS/MS scan was optimized as follows: cone voltage, 40/30-55 V; and collision energy, 20/15-40 V for anthocyanins/other phenolics, respectively. The purified phenolic extracts described above (*see extract purification*) were dissolved in methanol/water (50:50) acidified with 0.05% formic acid. The solution was filtered by a syringe filter with a 0.2 μm PTFE membrane, and 1 μL was injected onto the LC column for UPLC-UV and UPLC-ESI-MS analyses.

The solvents were 2% formic acid in H_2O (solvent A) and acetonitrile (solvent B) for anthocyanin analysis; and 0.05% formic acid in H_2O (solvent A) and acetonitrile (solvent B) for other phenolics. A high level of formic acid was required to modify the pH of the mobile phase for proper analysis of anthocyanins (*125*). The gradient was 5% B from 0 – 2 min, 5 – 75% B from 2 – 27 min, 75 % B isocratic from 27 – 30 min, 75 – 5 % B from 30 – 31 min, followed by 5% B isocratic for 5 min to allow for column equilibration before the next injection; at a flow rate of 0.4 mL/min.

3.2.8 Quantification of anthocyanins, flavonols and flavan-3-ols

3.2.8.1 Extraction

Approximately 5.0 g of raw and cooked cowpea flour were separately extracted in 15 mL of 1% HCl in methanol for 2 hours at room temperature with continuous shaking. The extracts were centrifuged (10000 *g*-force for 10 min) using a Heraeus Megafuge 11R Centrifuge (Thermo Fisher Scientific, Asheville, NC) at 4°C and the supernatants were transferred into 50 mL falcon tubes. The solid residues were washed twice with 5 mL of the extraction solvent, further centrifuged, and the supernatants combined with the previously collected supernatants from corresponding samples. An aliquot from these mixtures were filtered using a syringe filter with a 0.2 μm PTFE membrane, and 5 μL was injected onto the LC column for HPLC analyses.

3.2.8.2 Quantification

The assumption made for quantification of anthocyanins, flavonols and flavan-3-ols derivatives with no standards was that their molar absorptivity was similar to those of their aglycones or mono-glycosides. Thus, concentrations of the identified compounds in cowpea were calculated from the HPLC standard curves of pure standards; and were expressed as micrograms per gram (μg/g) of dry seed on a dry weight basis.

3.3 Results and discussion

3.3.1 Identification of anthocyanins in cowpeas

Naturally, anthocyanins generally occur as O-glycosides. During MS/MS fragmentation, the glycosidic bond is cleaved with concomitant proton rearrangement which leads to elimination of the sugar moiety. Typical losses include 162 *amu* (hexose; glucose or galactose), 146 *amu* (deoxyhexose; rhamnose), 132 *amu* (pentose; xylose or arabinose), and 176 *amu* (glucuronic acid). The most abundant and common glycosyl moiety in most anthocyanins and other pigments is glucose. Glycosylation mostly occurs at position C-3 (*126*). In this study, designation of sugar moieties was mostly achieved based on the MS spectra obtained as well as using literature evidence.

In the UPLC chromatograms acquired at 520 nm**,** eight distinct anthocyanin peaks (compounds **1a – 8a**) were detected in only the black (IT95K-1105-5 and IT98K-1092-1) and green (TX2028-1-3-1) cowpea phenotypes (**Figure 1; Table 3**). Their structures are presented in **Figure 2**. All the other phenotypes did not contain detectable levels of anthocyanins. Identification was achieved by elution profile, comparison to pure standards, UV-vis spectra and comparing the molecular ions and product ions of these anthocyanin compounds with those available in the literature. The molecular ions and product ions of anthocyanins in the three cowpea varieties are summarized in **Table 3.**

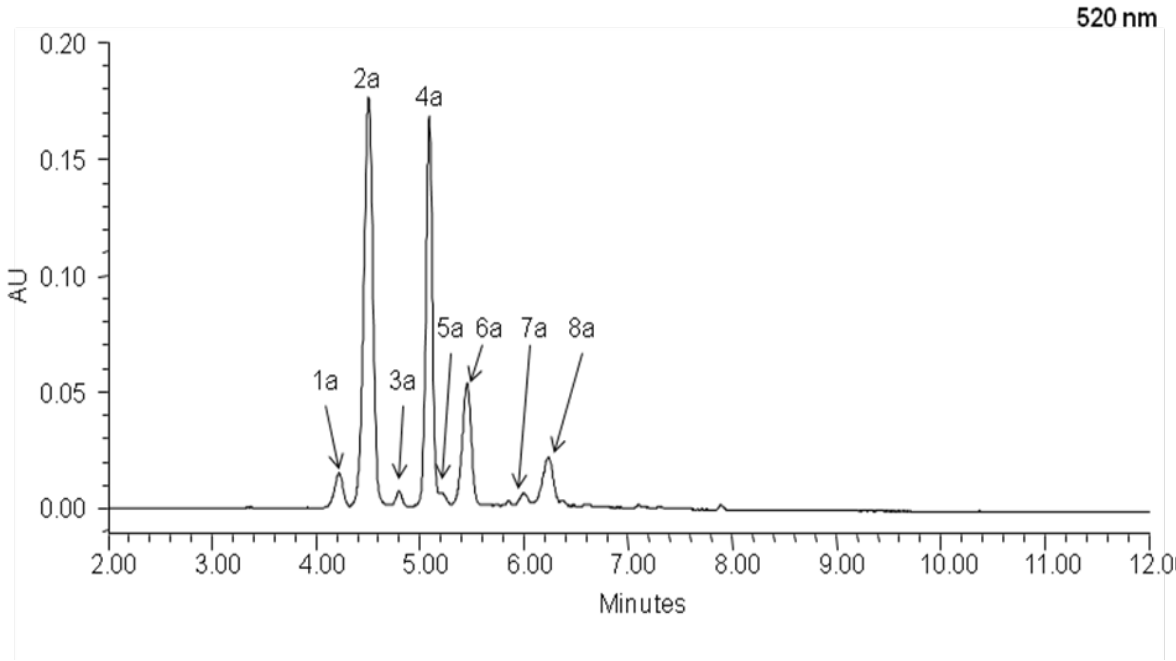


Figure 1. Representative UPLC chromatogram of anthocyanins found in the methanolic fractions from black IT95K-1105-5 cowpea variety monitored at 520 nm. The black IT98K-1092-1 and green TX2028-1-3-1 varieties also had similar profile. See **Table 3** for peak identities and **Figure 2** for structures.

The MS spectrum of *peak 1a* (t_R = 4.22 min) and *2a* (t_R = 4.50 min) showed that the peaks had similar molecular ion $[M + H]^+$ at *m/z* 465, and the MS/MS spectrum showed that the ions at *m/z* 465 had the same fragmentation patterns (*m/z* 303: [M – 162 *amu*]) which corresponded to the loss of a hexose. The MS/MS fragment at *m/z* 303 corresponded to a delphinidin aglycone. Since *peaks 1a* and *2a* differ only by retention time (**Table 3**), *peak 1a* was established as delphinidin-3-*O*-galactoside, on the basis of its shorter retention time; whereas *peak 2a* was elucidated as delphinidin-3-*O*-glucoside, and corroborated by literature data (*46*). The nature of the glycosyl group apparently has

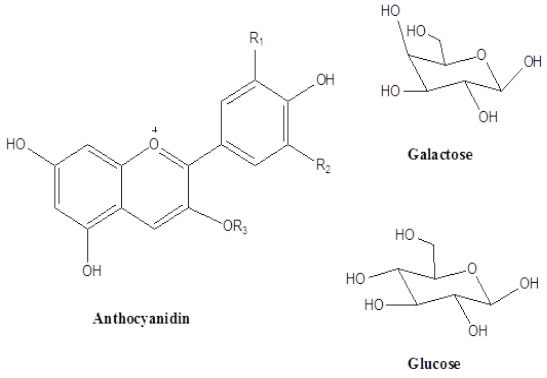
no influence on λ_{max}; however, galactosyl units increases polarity of the pigments through the C18 column than glucosyl units, thus the galactosides elute before glucosides (*46, 127*).

Peaks 3a (t_R = 4.79 min) and *4a* (t_R = 5.08 min) showed a molecular ion $[M + H]^+$ at *m/z* 449 that, when fragmented, gave an ion at *m/z* 287, corresponding to the loss of a hexose molecule (– 162 *amu*). These 2 peaks (*3a* and *4a*) also differed by retention time (**Table 3**). Thus, based on UV-*vis*, MS data, retention time and literature evidence, *peak 3a* was identified as cyanidin-3-*O*-galactoside, while *peak 4a* as cyanidin-3-*O*-glucoside (*46*).

Table 3. Identification of anthocyanins in IT95K-1105-5 (black), IT98K-1092-1 (black) and TX2028-1-3-1 (green) cowpea varieties based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), MS and MS/MS spectroscopic pattern. Peak numbers are referenced to **Figure 1.** The structures of the peaks are reference to **Figure 2.**

Peak no.	t_R (min)	λ_{max} (nm)	MS $[M + H]^+$	MS/MS $[M + H]^+$	Proposed structure
1a	4.22	524	465	303	Delphinidin-3-*O*-galactoside
2a	4.50	524	465	303	Delphinidin-3-*O*-glucoside
3a	4.79	517	449	287	Cyanidin-3-*O*-galactoside
4a	5.08	517	449	287	Cyanidin-3-*O*-glucoside
5a	5.20	526	479	317	Petunidin-3-*O*-galactoside
6a	5.45	526	479	317	Petunidin-3-*O*-glucoside
7a	5.99	516	463	301	Peonidin-3-*O*-glucoside
8a	6.23	526	493	331	Malvidin-3-*O*-glucoside

Similarly, *peaks 5a* (t_R = 5.20 min) and *6a* (t_R = 5.45 min), having a molecular ion at *m/z* 479 from the MS spectrum and one product ion at *m/z* 317 ([M – 162 *amu*]) from the MS/MS spectrum, indicated that the compounds are glucosides of petunidin (**Table 3**). *Peak 6a* was identified as petunidin-3-*O*-glucoside (*46*), while *peak 5a* was identified as petunidin-3-*O*-galactoside based on its shorter retention time relative to its glucosidic analog. This is the first time this pigment is reported in cowpea. In this study, petunidin-3-*O*-galactoside was identified in all three cowpea varieties that contained anthocyanins (black and green). However, since the level of petunidin-3-*O*-galactoside (*peak 5a*) was low (**Figure 1**), a high sensitive LC-MS system is critical in ensuring that the compound is detectable. In this analysis, the UPLC-MS provided exceptional sensitivity due to high LC pressure and greater specificity since the detector could be programmed to select specific ions to fragment with no interferences or ion suppression.



		Substituents		
Peak no.	Anthocyanin structure	R1	R2	R3
1a	Delphinidin-3-O-galactoside	OH	OH	Gal
2a	Delphinidin-3-O-glucoside	OH	OH	Glu
3a	Cyanidin-3-O-galactoside	OH	H	Gal
4a	Cyanidin-3-O-glucoside	OH	H	Glu
5a	Petunidin-3-O-galactoside	OCH3	OH	Gal
6a	Petunidin-3-O-glucoside	OCH3	OH	Glu
7a	Peonidin-3-O-glucoside	OCH3	H	Glu
8a	Malvidin-3-O-glucoside	OCH3	OCH3	Glu

Figure 2. Chemical structures of the eight anthocyanins found in cowpeas. See **Table 3** for molecular masses, maximum absorption wavelengths and peak retention times.

The MS spectrum of *peak 7a* (t_R = 5.99 min) indicated that the peak had *m/z* 463, and the MS/MS spectrum showed it fragmented to *m/z* 301, corresponding to the molecular ion of peonidin aglycone and a loss of a hexose moiety ([M – 162 *amu*]). This compound was identified as peonidin-3-*O*-glucoside. *Peak 8a* (t_R = 6.23 min) was identified as malvidin-3-*O*-glucoside based on MS spectrum molecular ion at *m/z* 493, UV-*vis* spectra and MS/MS fragment at *m/z* 331, corresponding to loss of hexose moiety. Both compounds had previously been identified in cowpea (*46*).

We did not detect anthocyanins in the red, light brown, golden brown and white cowpea varieties studied. Additionally, there are no reports in the literature regarding the anthocyanin profile in green cowpea phenotype. In particular, TX2028-1-3-1 is a variety of cowpea with a green seed coat and a black eye and can be used as a vegetable in many diets such as gravies since it has an appealing look.

The three cowpea (black and green) varieties which contained anthocyanins exhibited similarities in their anthocyanin profiles, suggesting phenotype did not influence the nature of anthocyanins detected. Since the black IT95K-1105-5 variety was grown in UCR while the black IT98K-1092-1 and green TX2028-1-3-1 varieties were grown in College Station, TX, this study provide evidence that genes, light, temperature and agronomic factors do not impact the nature of anthocyanins forms identified in cowpea.

3.3.2 Anthocyanin content in raw cowpeas

On average, the black IT95K-1105-5 and green TX2028-1-3-1 cowpea varieties had the highest seed weight (21.6 – 23.4 g/100 seeds), compared to that of black IT98K-1092-1 variety (11.6 g/100 seeds) (**Table 2**).

Anthocyanin content varied significantly between the three varieties (**Tables 4** and **5**). Among the black phenotypes, the IT95K-1105-5 cowpea variety had the highest average anthocyanin content (2095 ± 35.6 μg/g) despite its larger size, than the smaller seeded black IT98K-1092-1 (1676 ± 33.9 μg/g) suggesting varietal effect (**Table 4**). It is not clear how growth environment influenced anthocyanin content. The IT95K-1105-5 was from UCR while IT98K-1092-1 was from College Station. The green TX2028-1-3-1 cowpea variety had (876 ± 28.4 μg/g) (**Table 5**).

In all the three varieties, the most dominant pigment was delphinidin-3-*O*-glucoside, followed by cyanidin-3-*O*-glucoside and then petunidin-3-*O*-glucoside. Petunidin-3-*O*-galactoside, a new compound first reported in cowpeas in this study, was the least. The average amounts of delphinidin-3-*O*-glucoside in the raw IT95K-1105-5, IT98K-1092-1 and TX2028-1-3-1 cowpeas were 681, 508 and 231 μg/g, respectively; while those of cyanidin-3-*O*-glucoside were 549, 444 and 209 μg/g, respectively (**Tables 4** and **5**).

The literature on anthocyanin content in cowpeas is very limited. Chang and Wong (*54*) reported higher contents of delphinidin-3-*O*-glucoside (1,450 μg/g) and cyanidin-3-*O*-glucoside (1,030 μg/g) in the flour of Hakmeitau beans, a black cowpea cultivar. This is in contrast to investigations by Ha *et al*. (*46*), who showed that area

percent of cyanidin-3-*O*-glucoside (58.1%) in the seed coat was much greater than that of delphinidin-3-*O*-glucoside (22.0%). These differences could be attributed to differences in extractions methods, varieties and environment.

Table 4. Contents of anthocyanins in raw and boiled black (IT95K-1105-5 and IT98K-1092-1) cowpea varieties as determined by HPLC-UV-*vis*[a]

	Black cowpea varieties					
	IT95K-1105-5			**IT98K-1092-1**		
	Anthocyanin content (μg/g cowpea flour)					
Compound	*Raw*	*Boiled*	*% change*	*Raw*	*Boiled*	*% change*
Delphinidin-3-*O*-galactoside	169 ± 5.2[b]	103 ± 1.7[b]	38.9	143 ± 2.4[b]	105 ± 2.5[b]	26.7
Delphinidin-3-*O*-glucoside	681 ± 5.3	115 ± 8.2	83.1	508 ± 6.4	68.2 ± 3.41	86.6
Cyanidin-3-*O*-galactoside	152 ± 4.9[c]	ND[e]	-	142 ± 3.2[c]	ND	-
Cyanidin-3-*O*-glucoside	549 ± 8.5	89.2 ± 3.1	83.8	444 ± 6.0	53.5 ± 2.83	87.9
Petunidin-3-*O*-galactoside	35.2 ± 1.12[d]	ND	-	24.1 ± 3.03[d]	ND	-
Petunidin-3-*O*-glucoside	265 ± 3.6[d]	143 ± 4.0[d]	46.3	202 ± 3.5[d]	90.6 ± 3.81[d]	55.3
Peonidin-3-*O*-glucoside	41.1 ± 2.11[d]	ND	-	38.4 ± 1.09[d]	ND	-
Malvidin-3-*O*-glucoside	202 ± 4.8	60.3 ± 1.0	70.2	174 ± 2.2	41.0 ± 2.01	76.5
Total monomeric anthocyanins	**2094 ± 35.5**	**511 ± 18.0**	**75.6**	**1676 ± 27.8**	**358 ± 14.6**	**78.6**

[a]All values are expressed as mean ± SD of triplicates on a dry weight basis. [b]Expressed as delphinidin-3-*O*-glucoside equivalent. [c]Expressed as cyanidin-3-*O*-glucoside equivalent. [d]Expressed as malvidin-3-*O*-glucoside equivalent. [e] ND = not detectable.

Table 5. Contents of anthocyanins in raw and boiled green (TX2028-1-3-1) cowpea variety as determined by HPLC-UV-*vis* [a]

	TX2028-1-3-1 (green) variety		
	Anthocyanin content (μg/g cowpea flour)		
Compound	*Raw*	*Boiled*	*% change*
Delphinidin-3-*O*-galactoside	98.7 ± 0.80[b]	32.6 ± 1.67[b]	67.0
Delphinidin-3-*O*-glucoside	231 ± 8.3	73.9 ± 2.02	68.0
Cyanidin-3-*O*-galactoside	62.2 ± 3.33[c]	ND [e]	-
Cyanidin-3-*O*-glucoside	209 ± 4.4	43.9 ± 1.02	79.1
Petunidin-3-*O*-galactoside	15.7 ± 2.30[d]	ND	-
Petunidin-3-*O*-glucoside	119 ± 4.4[d]	37.7 ± 1.62[d]	68.4
Peonidin-3-*O*-glucoside	29.3 ± 3.02[d]	ND	-
Malvidin-3-*O*-glucoside	110 ± 1.9	32.7 ± 2.12	70.2
Total monomeric anthocyanins	**875 ± 28.5**	**221 ± 8.5**	**74.8**

[a]All values are expressed as mean ± SD of triplicates on a dry weight basis. [b]Expressed as delphinidin-3-*O*-glucoside equivalent. [c]Expressed as cyanidin-3-*O*-glucoside equivalent. [d]Expressed as malvidin-3-*O*-glucoside equivalent. [e] ND = not detectable.

In this study, the levels of galactosyl-derivatives of anthocyanins were lower than their glucosyl-derivatives. For instance, the average amounts of delphinidin-3-*O*-galactoside in the raw IT95K-1105-5, IT98K-1092-1 and TX2028-1-3-1 cowpeas were 169, 143 and 98.7 μg/g, respectively; while those of cyanidin-3-*O*-galactoside were 152, 142 and 62.2 μg/g, respectively (**Tables 4** and **5**). Similar trends were also reported in levels of anthocyanins in Hakmeitau beans (*54*).

In summary, the levels of the anthocyanins identified in the raw black and green cowpea varieties were in the following descending order: delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, delphinidin-3-*O*-galactoside, cyanidin-3-*O*-galactoside, peonidin-3-*O*-glucoside and finally petunidin-3-*O*-galactoside (**Figure 1; Table 3**). These findings are in agreement with

those of Ha *et al*. (*46*) who reported that cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside were the major anthocyanins in a black seeded cowpea. Other authors (*128, 129*) reported delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside and malvidin-3-*O*-glucoside as the major anthocyanins in black common beans. However, black common beans reportedly contained much higher anthocyanin content compared to the cowpeas used in our study. For example, Xu and Chang (*130*) reported that black beans contained higher levels of delphidin-3-*O*-glucoside (2197 μg/g), followed by petunidin-3-*O*-glucoside (838 μg/g).

3.3.3 Effect of boiling on anthocyanin content in cowpea

Levels of monomeric anthocyanins were significantly reduced by boiling in all three cowpea varieties (**Tables 4** and **5**). In comparison to other anthocyanins, boiling reduced cyanidin-3-*O*-galactoside, petunidin-3-*O*-galactoside and peonidin-3-*O*-glucoside in all the varieties to undetectable levels (**Tables 4** and **5**).

In the case of the black varieties, the most impacted pigments as a result of boiling were delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside (83.1 – 87.9% reduction), and the least was delphinidin-3-*O*-galactoside (26.7 – 38.9% reduction) (**Table 4**). A similar trend was also observed for the green TX2028-1-3-1 variety due to boiling (**Table 5**). Xu and Chang (*130*) reported that on average, thermal processing reduced levels of anthocyanins by >90% in black beans (*Phaseolus vulgaris*). Similar trends have also been reported in thermally processed soybean (*131*).

In general, the black and green cowpea varieties lost on average 75 – 79% of their average monomeric anthocyanin content (measured by HLPC) as a result of boiling (**Tables 4** and **5**). The loss of anthocyanins might be attributed to degradation or decomposition of anthocyanins upon boiling. Thermal degradation of anthocyanins has been reported to follow first-order reaction kinetics, that is, anthocyanins content in foods decrease with increase in temperature (*132*). The decrease in anthocyanin content after boiling may also occur through the formation of less soluble polymerized complexes with other cellular components such as aldehydes, proteins and tannins.

Our results show that monomeric anthocyanins, especially the non-acylated forms found in cowpeas, are highly unstable during processing, and are easily degraded upon heating. Heat-induced degradation of anthocyanins is initiated by opening of the pyrylium ring, leading to formation of their thermolabile chalcone derivatives. This may be followed by degradation of the A-ring to phloroglucinaldehyde; and B-ring to protocatechuic acid or 4-hydroxybenzoic acid as the terminal degradation products (*133*). These colorless phenolics generated upon heating also contribute to the total antioxidant capacity. Phloroglucinaldehyde (*m/z* 155), protocatechuic acid (*m/z* 155) and 4-hydroxybenzoic acid (*m/z* 139) in the boiled samples can be detected by comparing their fragmentation data with typical fragmentation patterns of commercial references using LC/MS.

3.4 Flavonoids in the methanolic fractions

3.4.1 Free phenolic acids and phenolic aldehydes

Peak 1 (t_R = 2.56 min, λ_{max} = 294 nm) had a $[M - H]^-$ at *m/z* 153. The major MS/MS ions produced were at *m/z* 109 (M – 44 *amu*, loss of CO_2), the basis of which the peak was identified as protocatechuic acid. Among the samples investigated, only the red IT97K-1042-3 cowpea variety had detectable levels of protocatechuic acid (**Table 6; Figure 3A-1; Figure 4B**). Protocatechuic acid has been reported in some cowpea varieties (*51*).

Peak 5 (t_R = 3.85 min, λ_{max} = 281 nm) had a $[M - H]^-$ at *m/z* 137, was identified as protocatechuic aldehyde (**Figure 4B**) since its MS and MS/MS ions were similar to those previously reported in literature (*134*)**.** In the MS/MS spectrum, it had strong signals at *m/z* 53, 81, 91, 109 and 119 (**Table 6**). Some leguminous seeds have been reported to contain protocatechuic aldehyde (*135*), however, to our knowledge, this is the first report of protocatechuic aldehyde in cowpeas. Among the varieties studied, protocatechuic aldehyde was only found in the red IT82D-889 variety (**Figure 3B-1**).

Table 6. Identification of phenolic acids and phenolic aldehydes monitored at 280 and 330 nm in methanolic fraction of 10 cowpea varieties based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), and MS–MS/MS spectroscopic pattern. Ionization was performed in the negative mode.

Peak no.	t_R (min)	λ_{max} (nm)	Proposed identifications	$[M-H]^-$ (*m/z*)	MS/MS fragments (*m/z*)
1	2.56	294	Protocatechuic acid	153	109, 107
5	3.85	281	Protocatechuic aldehyde	137	119, 109, 91, 81, 53
9	4.58	322	*trans*-Feruloylaldaric acid	385	209, 191

Peak 9 (t_R = 4.58 min, λ_{max} = 322 nm), which had a $[M - H]^-$ at *m/z* 385 (**Table 6**) was elucidated as *trans*-feruloylaldaric acid based on matching UV-*vis* spectrum and MS data with literature data (*51*). The MS/MS spectrum indicated that the ion at *m/z* 209 (M – 176 *amu*), occurred after cleavage of a glucuronic (aldaric) acid group. The fragment at *m/z* 191 (M – 176 – 18 *amu*) indicated a further loss of water molecule (**Table 6**). In this study, *trans*-feruloylaldaric acid was found in the red IT97K-1092-2 sample (**Figure 3A-1**), the golden brown (IT84S-2246 and Ife Brown) and white cowpea varieties. The presence of *trans*-feruloylaldaric acid has been reported in some cowpea varieties (*51*).

3.4.2 Flavan-3-ols

Peak 3 (t_R = 3.06 min, λ_{max} = 278 nm) had a $[M - H]^-$ at *m/z* 451; its MS/MS showed a major ion at *m/z* 289 (M – 162 *amu*, loss of a hexose unit). Other ions included *m/z* 151 and 179 which matches fragmentation pattern of catechin molecule (**Table 7**). Thus, *peak 3* was identified as catechin-3-*O*-glucoside, a compound which has been identified in lentils (*136*). We found catechin-3-*O*-glucoside in all the varieties studied except in the IT82D-889 (red), TX2028-1-3-1 (green) and Early Acre (white) cowpea phenotypes. This is the first report of glycosyl derivative of flavan-3-ols in cowpea.

Table 7. Identification of flavan-3-ols monitored at 280 nm in methanolic fraction of 10 cowpea varieties based on UPLC retention time (t_r), UV-*vis* spectroscopic characteristics (λ_{max}), and MS – MS/MS spectroscopic pattern. Ionization was performed in the negative mode. Peaks marked (*****) were unidentified.

Peak no.	**t_R (min)**	**λ_{max} (nm)**	**Proposed identifications**	**$[M-H]^-$ (*m/z*)**	**MS/MS fragments (*m/z*)**
2*	2.85	278	Unknown	203	186, 159, 142, 116, 74
3	3.06	278	Catechin-3-*O*-glucoside	451	289, 179, 165, 151
4	3.36	278	Procyanidin dimer-3-*O*-diglucoside-7-*O*-glucoside	901	739, 577, 393, 269
6*	4.19	279	Unknown	885	723, 435, 423
7*	4.45	271	Unknown	508	328, 258, 145, 127, 119
8	4.49	278	Procyanidin dimer B-type	577	425, 407, 289, 125
10	4.82	279	Catechin or epicatechin	289	245, 205, 187, 151, 109
11	5.29	279	Procyanidin trimer T2	865	713, 577, 451, 289, 287
12*	5.36	279	Unknown	885	723, 435, 423, 125
13	5.46	278	Procyanidin tetramer A-type	1154	821, 803, 575, 287
15*	5.85	278	Unknown	387	207, 89, 59
16*	6.03	279	Unknown	721	697, 407, 289, 195
18*	6.16	279	Unknown	885	723, 587, 560, 241, 229
20*	6.36	294	Unknown	317	255, 191, 179, 163, 153, 150
21	6.39	279	Procyanidin trimer C1	865	713, 577, 425, 407, 289, 287
31*	7.48	290	Unknown	349	331, 300, 271, 255, 179, 151
35*	7.73	278	Unknown	723	435, 395, 287, 273, 161, 125
44*	8.40	279	Unknown	809	595, 435, 391, 272, 161

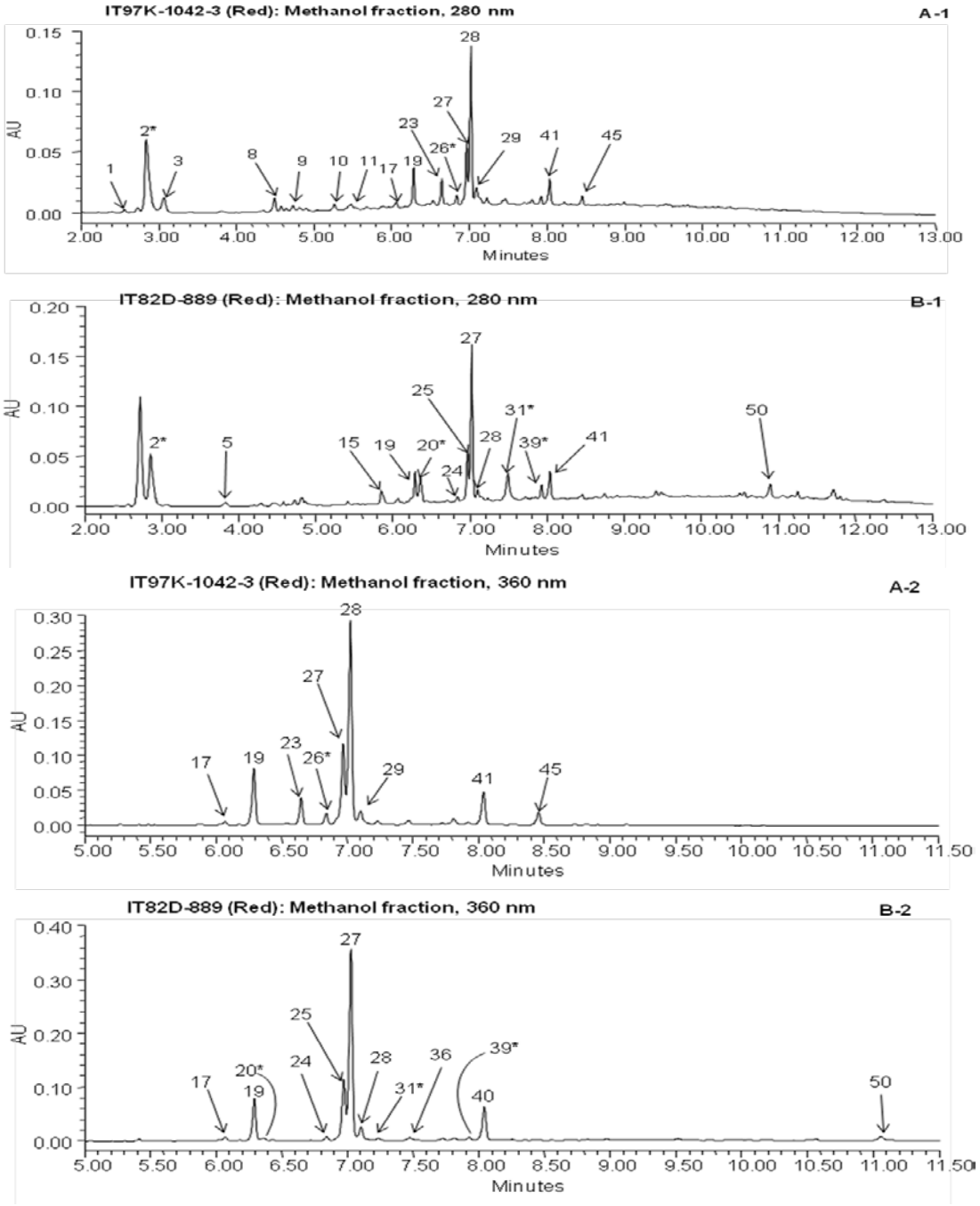


Figure 3. Reverse-phase UPLC chromatogram of phenolic extracts from methanol fractions of red cowpea varieties; IT97K-1042-3 at 280 nm (**A-1**) and 360 nm (**A-2**); and IT82D-889 at 280 nm (**B-1**) and 360 nm (**B-2**). Peaks marked (*****) are unidentified. Peak numbers are referenced to **Tables 6 – 8.**

Peak 4 (t_R = 3.36 min, λ_{max} = 278 nm) had a $[M - H]^-$ at *m/z* 901, and the MS/MS spectrum had fragments at *m/z* 739 (M – 162 *amu*, loss of one hexose unit) and *m/z* 577 (M – 324 *amu*, loss of two hexose units), an indication of 2 glucose moieties attached at 2 different positions on a procyanidin dimer molecule (**Table 6**). Two common positions for the conjugation with sugars are C-3 and C-7 (*137*). Thus, on this basis, *peak 4* is identified as a B-type procyanidin dimer-3-*O*-diglucoside-7-*O*-glucoside, and was only detected in the Ife Brown (golden brown) cowpea variety (**Figure 5B-1**). This is the first report of glycosylated derivatives of proanthocyanidin compounds in cowpeas.

Peak 8 (t_R = 4.49 min, λ_{max} = 278 nm) was identified as procyanidin dimer with a B–type interflavan linkage (**Figure 6**), based on similar fragmentation pattern reported in literature (*124*), and was found in all the cowpea varieties except in the red IT82D-889, IT84S-2246 (golden brown), TX2028-1-3-1 (green) and Early Acre (white) varieties. It had a $[M - H]^-$ at *m/z* 577, with main MS/MS fragments at *m/z* 425, 407 and 289 (**Table 6**). The procyanidins B1-B4, usually characterized by the C4→C8 linkage, are the most common dimers, occasionally accompanied by corresponding **C4→C6** linked isomers (B5-B8) (**Figure 6**) (*138*). The ion at *m/z* 425 resulted from Retro-Diels-Alder (RDA) fission of the heterocyclic ring (**Figure 7**), a characteristic pattern for dimeric procyanidins (*139, 140*). The product of the subsequent water elimination (*m/z* 407) was also detected in significant amounts. The ion at *m/z* 289 was produced from interflavanoid bond cleavage of dimeric procyanidins through the quinone-methine (QM) mechanism, particularly when the base unit is a flavan-3-ol (*139*); whereas the ion at *m/z* 125 is the heterocyclic ring fission (HRF) product (**Figure 7**).

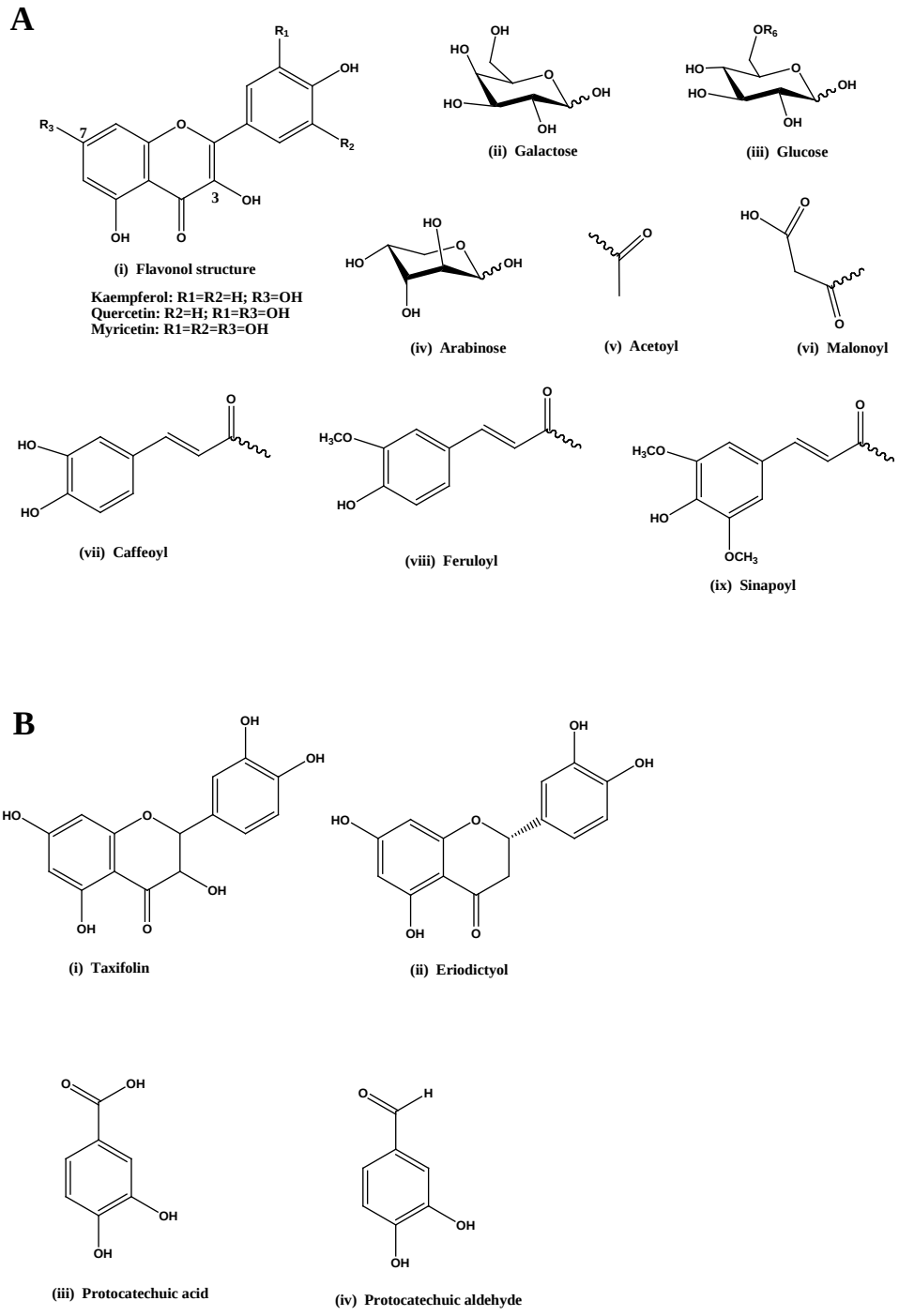


Figure 4. Chemical structures of flavonols and their glycosyl and acyl derivatives, (**A**); and flavanonol (taxifolin), flavanone (eriodictyol), protocatechuic acid and protocatechuic aldehyde identified in cowpea seeds (**B**).

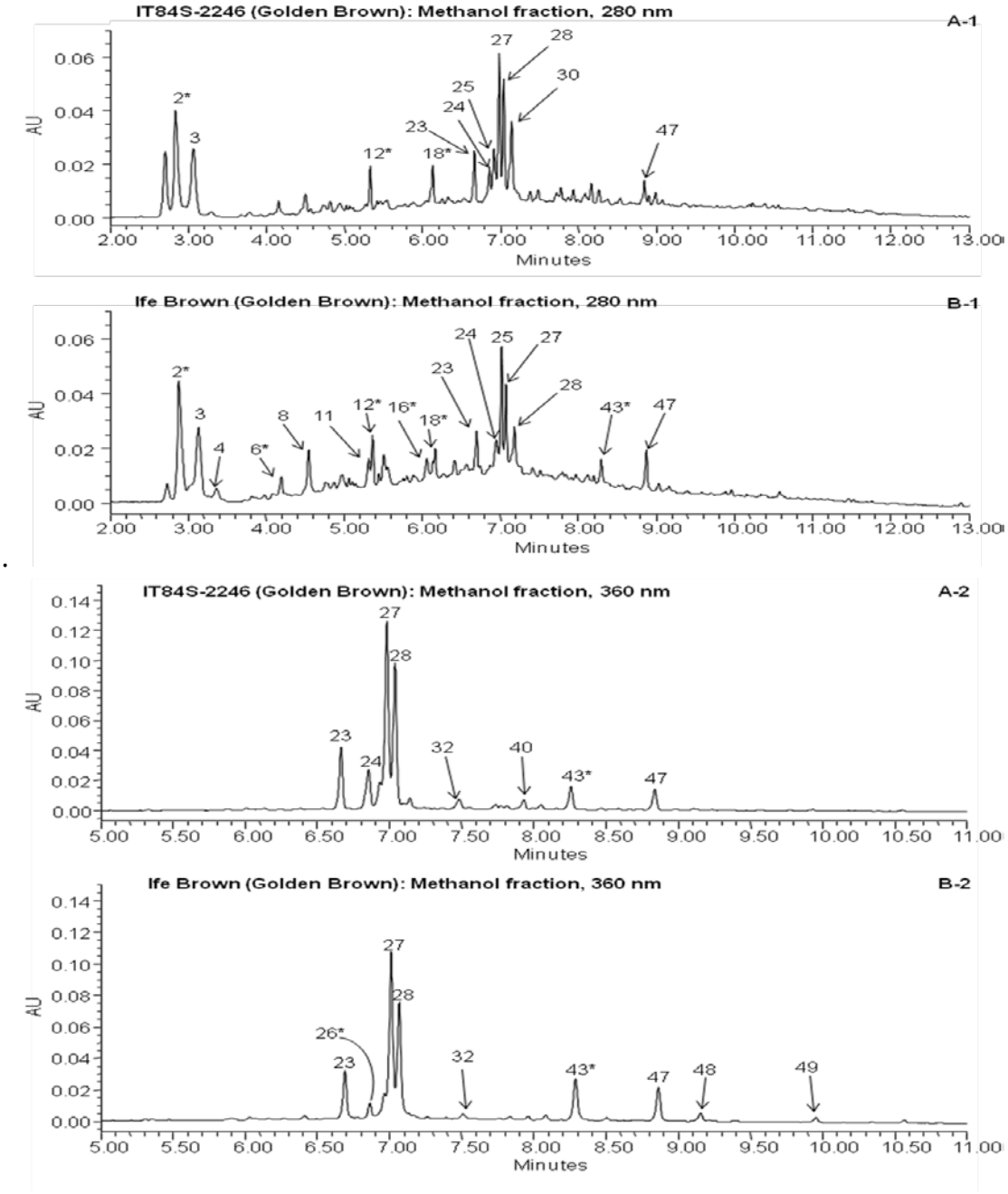


Figure 5. Reverse-phase UPLC chromatogram of phenolic extracts from methanol fractions of golden brown cowpea varieties; IT84S-2246 at 280 nm (**A-1**) and 360 nm (**A-2**); and Ife Brown at 280 nm (**B-1**) and 360 nm (**B-2**). Peaks marked (*****) are unidentified. Peak numbers are referenced to **Tables 7** and **8.**

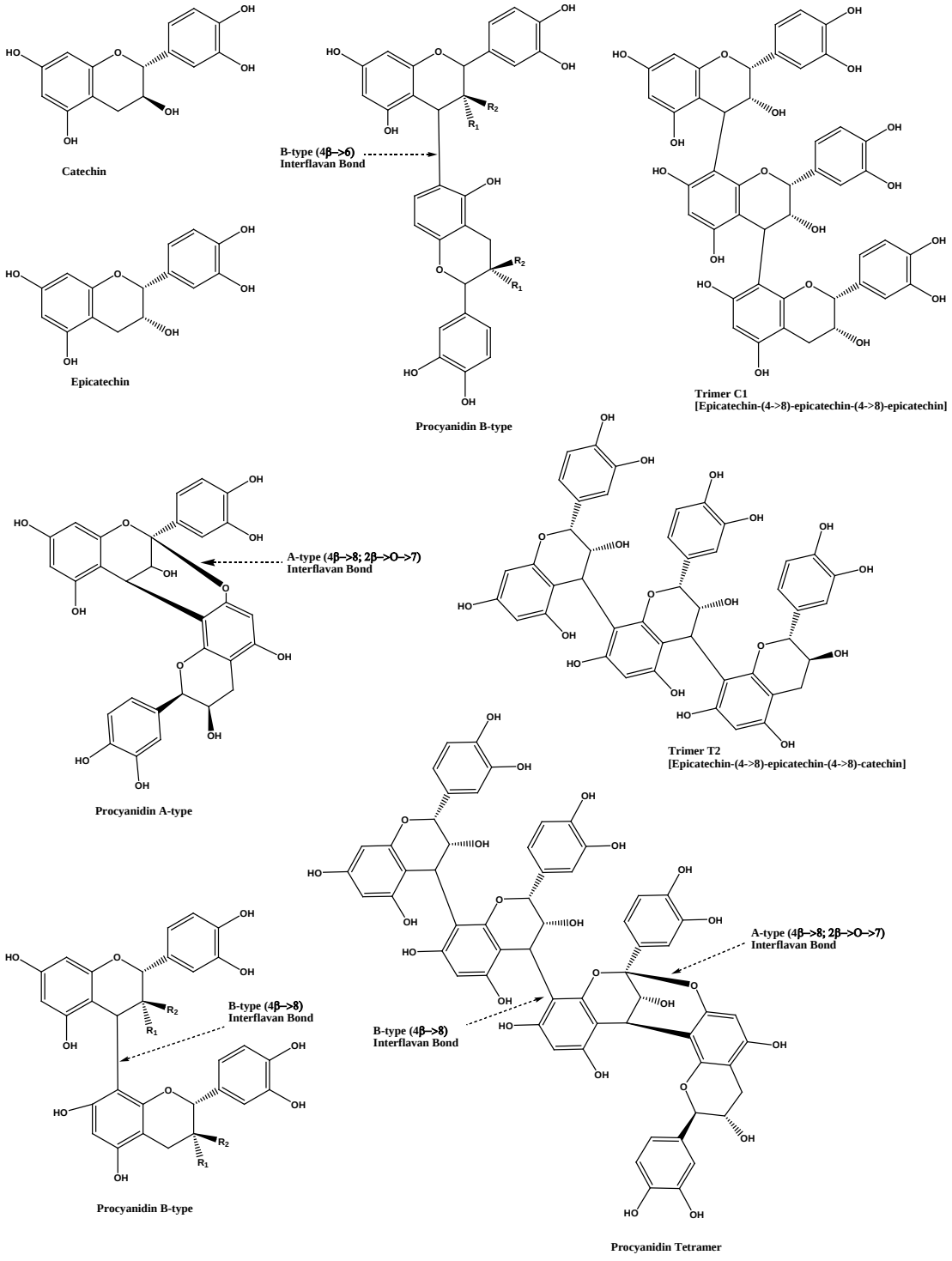


Figure 6. Chemical structures of the most common flavanol monomers, procyanidin dimers and trimers identified in cowpea. **R_1 = H; R_2 = OH.**

Peak 10 (t_R = 4.82 min, λ_{max} = 279 nm), which had a $[M - H]^-$ at *m/z* 289 was elucidated as a procyanidin monomer (either a catechin or epicatechin unit) (**Table 7**) based on standards. It was only found in the red IT97K-1042-3, black (IT95K-1105-5 and IT98K-1092-1) and light brown (IAR-48 and 09FCV-CC-27M) cowpea varieties. These samples also contained the B-type procyanidin dimer (*peak 8*).

Both *peak 11* and *21* had similar $[M - H]^-$ at *m/z* 865 and absorption maxima at λ_{max}=279 nm, and were both established as trimers from literature data (*124*). However, based on differences in elution times of these trimeric procyanidins, *peak 11* (t_R = 5.29 min) was established as procyanidin trimer T2 while *peak 21* (t_R = 6.39 min) was procyanidin trimer C1 (**Figure 6**). The interflavan fragmentation resulted in signals at *m/z* 577 corresponding to $[M_{middle\text{-}base} - H]^-$ and *m/z* 289 (catechin or epicatechin units) (**Table 7**) (*124*), one of which must be B-type unit, regardless of its position in the sequence (*141*). Procyanidin trimer T2 was only found in the IT97K-1042-3 (red) (**Figure 3A-1**), Ife Brown (golden brown) (**Figure 5A-2**), IAR-48 (light brown) and 09FCV-CC27M (light brown) varieties (**Figure 8**); while procyanidin trimer C1 was only found in the 09FCV-CC27M variety (**Figure 8A-1**).

Peak 13 (t_R = 5.46 min, λ_{max} = 278 nm), which had a $[M - H]^-$ at *m/z* 1154 was elucidated as a procyanidin tetramer (four catechin or epicatechin units). This is supported by the MS/MS major products at *m/z* 575 (two catechin or epicatechin units) and at *m/z* 287 (a catechin or epicatechin unit), as well as absorption maxima identical to that of catechin (**Table 7**). The ion at *m/z* 575 suggests the presence of an A-type interflavanoid linkage (4β-8, 2β-*O*-7) (*142*); while the fragment at *m/z* 287 corresponds

to interflavanoid bond cleavage of dimeric procyanidins via the quinone-methine (QM) mechanism, particularly when the methylene-quinone is the top unit (**Figure 7**) (*124*). IAR-48, a light brown phenotype, was the only cowpea variety in which the tetramer was detected (**Figure 8B-1**). This is the first report of A-type procyanidin tetramers in cowpeas.

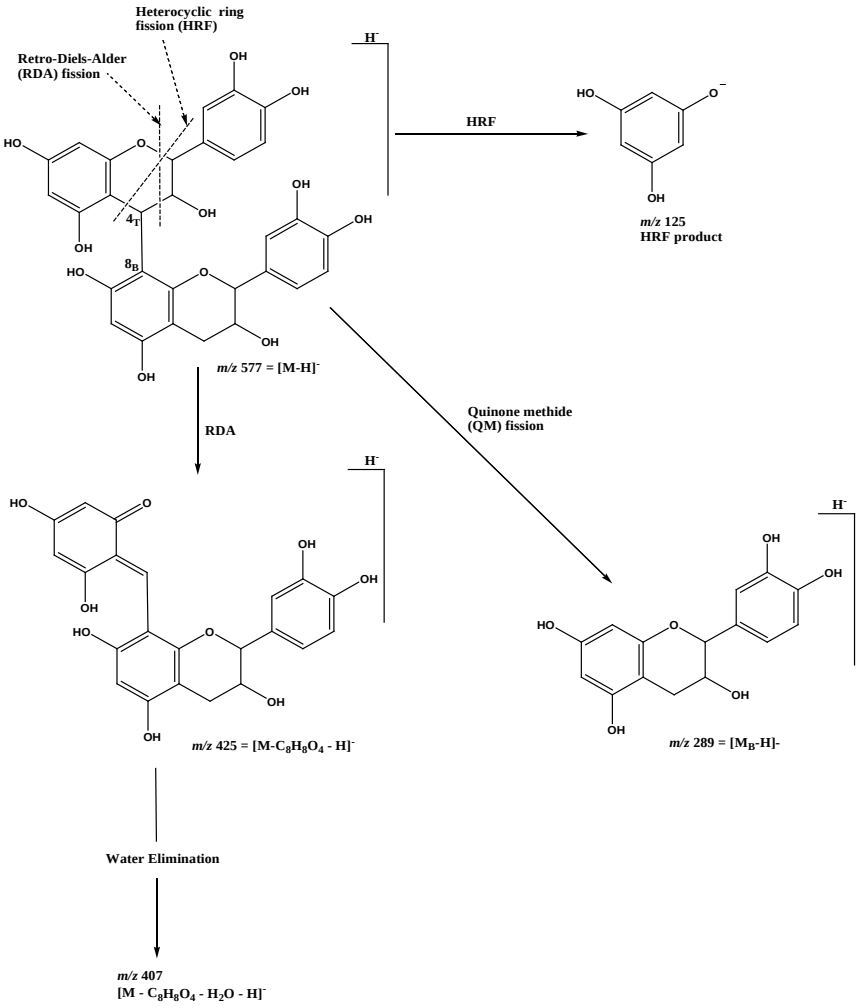


Figure 7. General schematic diagram showing fragmentation of dimeric procyanidins in negative mode following fragmentation mechanism postulated by Friedrich *et al.* (*140*).

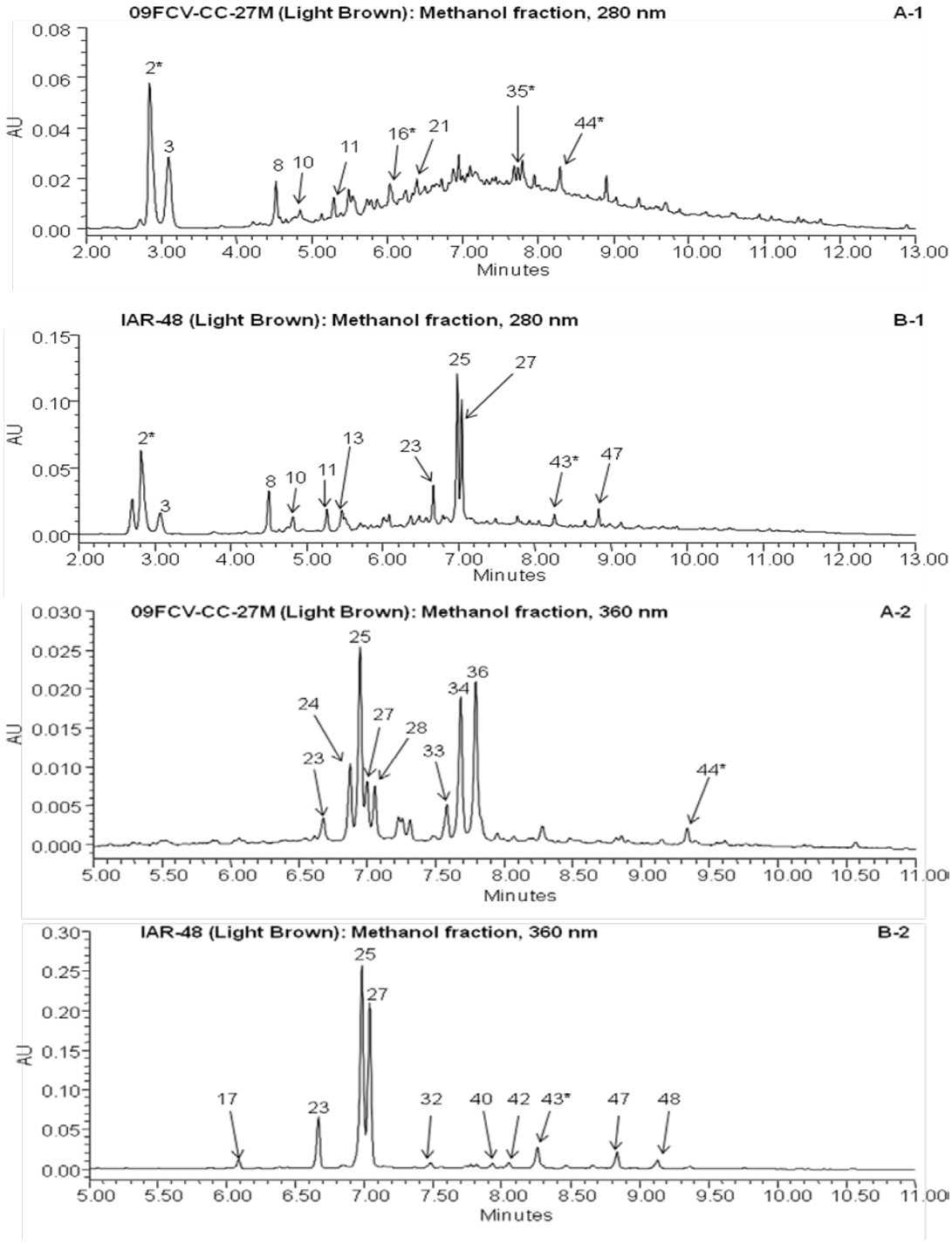


Figure 8. Reverse-phase UPLC chromatogram of phenolic extracts from methanol fractions of light brown cowpea varieties; 09FCV-CC27M at 280 nm (**A-1**) and 360 nm (**A-2**); and IAR-48 at 280 nm (**B-1**) and 360 nm (**B-2**). Peaks marked (*****) are unidentified. Peak numbers are referenced to **Tables 7** and **8.**

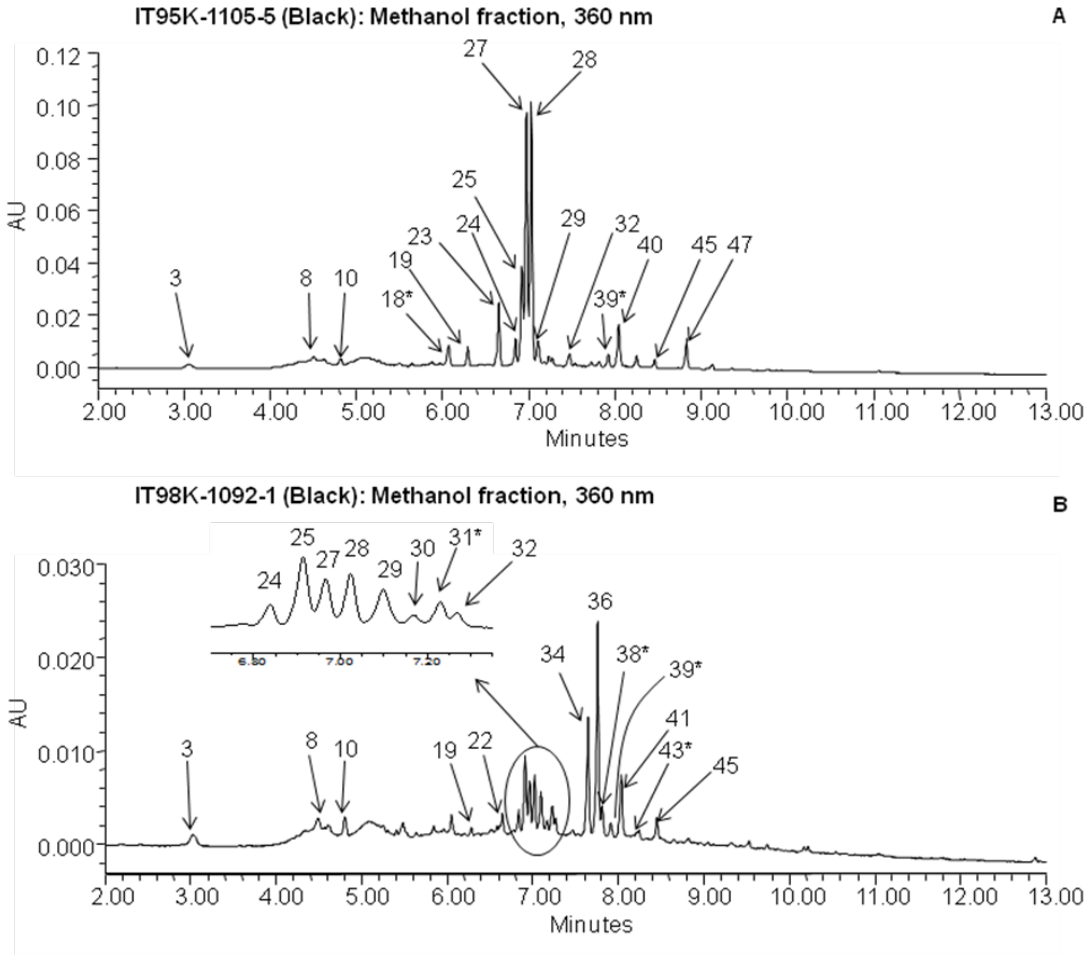


Figure 9. Reverse-phase UPLC chromatogram of phenolic extracts from methanol fractions of black cowpea varieties at 360 nm; IT95K-1105-5 (**A**); and IT98K-1092-1 (**B**). Peaks marked (*****) are unidentified. Peak numbers are referenced to **Tables 7** and **8.**

3.4.3 Flavonols

Peak 14 (t_R = 5.80 min, λ_{max} = 354 nm) was only present in the white Early Acre variety (**Figure 10**) variety and had a $[M - H]^-$ at *m/z* 787. The MS/MS products included *m/z* 301 (M – 486), suggesting loss of 3 glycosyl units (3 × 162 *amu*) that were attached at the same position (**Table 8**). The absorption λ_{max} and other MS/MS products

at *m/z* 179 and 151 matches fragmentation pattern for quercetin (*143*). Because MS/MS cleaves glycosyl units at the glucosidic bonds between the flavylium ring and the sugars directly attached to it (*144*), we propose the identity of this compound as quercetin-3-*O*-triglucoside, since the most common glycosylation position is usually C-3 (*126*).

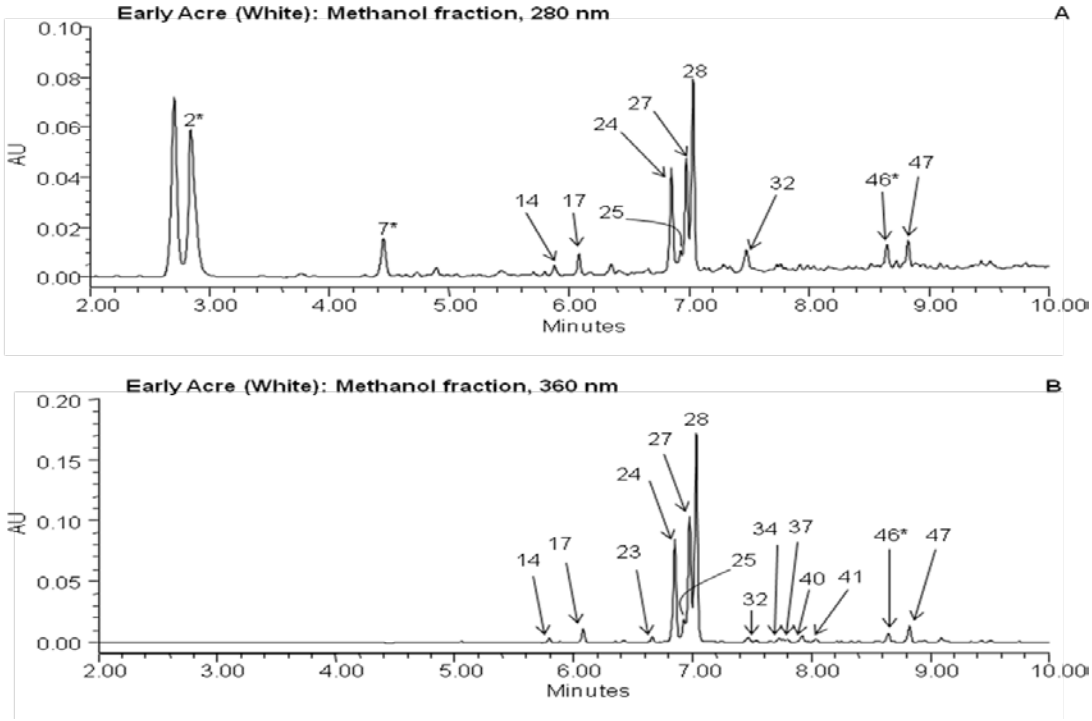


Figure 10. Reverse-phase UPLC chromatogram of phenolic extracts from methanol fraction of white cowpea variety, Early Acre at 280 nm (**A**) and 360 nm (**B**). Peaks marked with (*****) are unidentified. Peak numbers are referenced to **Tables 7** and **8.**

Table 8. Identification of dihydrochalcones (at 320 nm) and flavonols (at 360 nm) in methanolic fraction of 10 cowpea varieties based on UPLC retention time (t_r), UV-vis spectroscopic characteristics (λ_{max}), and MS–MS/MS spectroscopic pattern. Ionization was performed in the negative mode. Peaks marked (*) were unidentified.

Peak no.	t_R (min)	λ_{max} (nm)	Proposed identifications	$[M-H]^-$ (*m/z*)	MS/MS fragments (*m/z*)
14	5.80	354	Quercetin-3-*O*-triglucoside	787	301, 179, 151
17	6.08	345	Quercetin-3-*O*-glucoside-4′-*O*-diglucoside	787	625, 301, 300
19	6.29	357	Myricetin-3-*O*-diglucoside	641	317, 316, 271
22	6.60	351	Quercetin-3-*O*-arabinosyl-diglucoside	757	301
23	6.65	346	Quercetin-3-*O*-arabinoside-7-*O*-diglucoside	757	625, 301
24	6.80	353	Quercetin-3-*O*-digalactoside	625	301, 271, 179, 151
25	6.84	352	Quercetin-3,7-diglucoside	625	463, 301, 271
26*	6.86	376	Unknown	899	737, 585, 556, 539, 407
27	6.90	354	Quercetin-3-*O*-galactosylglucoside	625	300, 151
28†	6.97	355	Quercetin-3-*O*-diglucoside	625	301, 179, 151
29†	7.10	357	Myricetin-3-*O*-glucoside	479	317, 287, 271, 179, 151
30	7.14	325	Phlorizin (Phloretin-2′-glucoside)	435	273, 137, 135, 125
32	7.48	352	Kaempferol-3-*O*-diglucoside	609	285, 255, 169
33	7.58	353	Quercetin-3-*O*-arabinosylglucoside	595	301
34	7.65	355	Quercetin-3-*O*-galactosylrhamnoside	609	301
36	7.76	354	Quercetin-3-*O*-glucosylrhamnoside	609	301
37*	7.81	359	Unknown	467	323, 305, 203, 189, 161
38*	7.85	325	Unknown	467	323, 305, 203, 189, 161
39*	7.90	350	Unknown	597	455, 387, 357, 273, 229, 221, 209, 167
40†	7.93	353	Quercetin-3-*O*-galactoside	463	301, 271, 179, 151
41	8.04	354	Quercetin-7-*O*-glucoside	463	301, 271, 179, 151
42	8.10	357	Quercetin-3-*O*-glucoside	463	301, 271, 179, 151
43*	8.29	376	Unknown	737	407, 394, 287, 271, 229, 189, 165, 161, 125
45	8.48	353	Quercetin-3-(6″-malonoyl)-glucoside	549	301, 271, 179, 151
46*	8.65	364	Unknown	867	517, 349, 331, 317
47	8.82	334	Quercetin-3-(6″-feruloyl)-diglucoside	801	625, 301
48	9.16	363	Quercetin-3-(6″-diacetoyl)-diglucoside	709	625, 301
49	9.96	332	Quercetin-3-(6″-sinapoyl)-rutinoside	815	301
50†	11.05	370	Quercetin	301	301, 179, 151

***Unidentified peak; †Previously identified in cowpea (*51*, *54*).

Peak 17 (λ_{max} = 345 nm) had $[M - H]^-$ at *m/z* 787, and was only present in five varieties including IT97K-1042-3 (red) and IT82D-889 (red) (**Figure 3**), IAR-48 (light brown) (**Figure 8B-2**), Early Acre (white) (**Figure 10**) and TX2028-1-3-1 (green) (**Figure 11**). Since its pseudo-molecular ion is similar to that of *peak 14*, these two compounds are likely related. However, its fragmentation pattern was different from that of *peak 14*, in that the first MS/MS ion at *m/z* 625 (M – 162 *amu*) corresponded to loss of one glucosyl moiety possibly conjugated at C-3 (the most common position for glycosylation) (*54, 126*), while the subsequent ion at *m/z* 301 (M – 162 – 324 *amu*) corresponded to a further loss of two glucosyl moieties possibly from C-4′ position based on UV-*vis* (*145*, *146*). The fact that the glucosyl units were attached at different positions is evident from sequential loss of the glucosyl fragments, as well as the large hypsochromic shift in λ_{max} (9 nm) relative to the quercetin-3-*O*-triglucoside (*peak 14*) (**Table 8**). Based on the MS/MS data and λ_{max}, we propose *peak 17* to correspond to quercetin-3-*O*-glucoside-4′-*O*-diglucoside, a new compound first identified in this study.

Peak 19 (t_R = 6.29 min, λ_{max} = 357 nm) had $[M - H]^-$ at *m/z* 641, which gave a fragment at *m/z* 317 (M – 324 *amu*) corresponding to loss of 2 glycosyl units (**Table 8**). Based on its UV-*vis* profile, elution time and MS data, *peak 19* was identified as myricetin-3-*O*-diglucoside. 3-*O*-Diglycosylated flavonols have been reported in cowpea (*54, 147*). In this study, this peak was found in the black (*i.e.* IT98K-1092-1 and IT95K-1105-5) and red (*i.e.* IT1042-3 and IT82D-889) cowpea phenotypes only.

Peaks 22 and *23* had the same MS profile ($[M - H]^-$ at *m/z* 757) but slightly different retention times (t_R=6.60 and 6.65 min, respectively). The MS/MS data

indicated that both peaks contained quercetin (*m/z* 301) (**Table 8**). However, *peaks 22* MS/MS data showed only one fragment at *m/z* 301 (M – 456 *amu*), corresponding to loss of 2 hexoses (2 × 162 *amu*) and a pentose (132 *amu*) linked together and bonded to the aglycone at one position. The general term pentose is used due to the difficulty in detection of stereochemical structures of glycosyl residues based on MS data alone. It could be either arabinose or xylose, with the former usually preferred (*148*). Thus, based on retention time and MS data, *peak 22* was identified as quercetin-3-*O*-arabinosyl-diglucoside (**Table 8**). This compound was only present in the black IT98K-1092-1 (**Figure 9B**) and green TX2028-1-3-1 varieties (**Figure 11**). On the other hand, *peak 23* MS/MS showed a fragment at *m/z* 625 (M – 132 *amu*), as well as another fragment at *m/z* 301 (M – 132 – 324 *amu*) suggesting a further loss of two glycosyl units (**Table 8**). Thus, based on sequential loss of a pentose group followed by a diglucosyl moiety, *peak 23* was designated as quercetin-3-*O*-arabinoside-7-*O*-diglucoside. It was present in all the cowpea varieties except in the black IT98K-1092-1, green TX2028-1-3-1 and red IT82D-889 varieties. This is the first time that pentose-substituted flavonols has been found in cowpea.

It was also observed that *peaks 24, 25, 27* and *28* had similar MS profiles ([M – H]$^-$ at *m/z* 625). The appearance of an MS/MS fragment corresponding to the loss of 324 *amu* is observed for both peaks (**Table 8**). This corresponds to a loss of a dihexose group to give a fragment at *m/z* 301 which corresponds to quercetin. Based on MS data, *peak 24* (t_R=6.80 min, λ_{max}=353 nm) and *28* (t_R = 6.97 min, λ_{max} = 355 nm) were both assigned quercetin-3-*O*-dihexoside. Based on retention times, it is logical to assume that

one of the sugar moieties for *peak 24* is a galactose unit (which causes slightly shorter retention times and absorption at a lower wavelength), and that both sugars for *peak 28* are glucose units (*149*). Among all the cowpea samples studied, only the light brown IAR-48 and red IT97K-1042-3 varieties did not contain quercetin-3-*O*-digalactoside; while only IAR-48 variety did not contain quercetin-3-*O*-diglucoside. In fact, quercetin 3-*O*-glucoside and quercetin 3-*O*-diglucoside are the two common flavonol glycosides identified in cowpeas (*51, 54*). This compound was found in all the varieties studied except in the red IT97K-1042-3 variety. On the other hand, *peak 27* (t_R = 6.90 min, λ_{max} = 354 nm) was designated as quercetin-3-*O*-galactosylglucoside based on retention time relative to *peaks 24* and *28*, and was found in all the cowpea phenotypes studied. In the case of *peak 25* (t_R = 6.84 min, λ_{max} = 352 nm), the cleavage of the glycosyl units appear to follow a different pattern. Two sequential losses of 162 *amu*, indicated the hexoses are linked at different positions (**Table 8**), we assigned *peak 25* as quercetin-3,7-diglucoside.

Peak 29 (t_R = 7.10 min, λ_{max} = 357 nm) produced a $[M - H]^-$ at *m/z* 479 and an MS/MS spectrum with the predominant ion at *m/z* 317 (M – 162 *amu*, loss of a glucosyl unit) (**Table 8**). Based on this MS spectrum and literature data, we established *peak 29* as myricetin-3-*O*-glucoside (*54*). This compound was identified in only four varieties, that is, red (IT97K-1042-3) (**Figure 3A-1**), black (IT95K-1105-1 and IT98K-1092-1) (**Figure 9**), and green (IT2028-1-3-1) (**Figure 11**). This compound has previously been reported in *Vigna* species (*51, 54*).

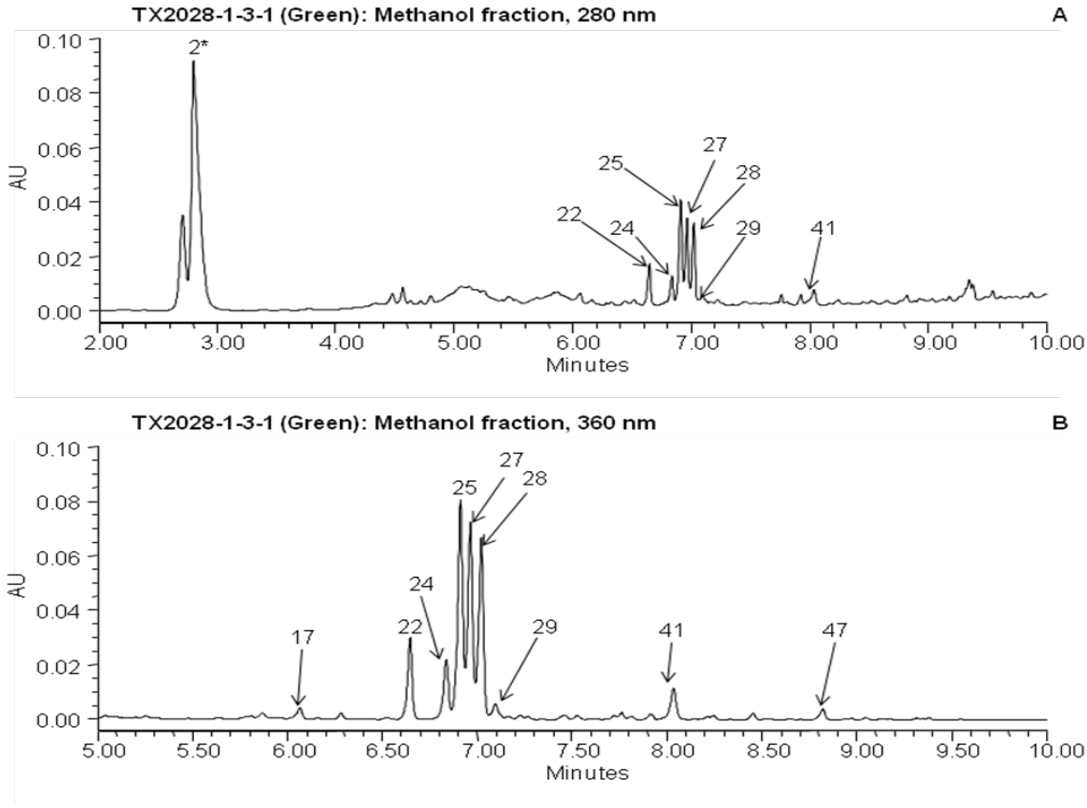


Figure 11. Reverse-phase UPLC chromatogram of phenolic extracts from methanol fraction of green cowpea variety, TX2028-1-3-1 at 280 nm (**A**) and 360 nm (**B**). Peaks marked (*****) are unidentified. Peak numbers are referenced to **Tables 7** and **8.**

Peaks 32 (t_R = 7.48 min, λ_{max}=352 nm), *34* (t_R = 7.65 min, λ_{max} = 355 nm) and *36* (t_R = 7.76 min, λ_{max} = 354 nm) had same MS profiles $[M - H]^-$ at *m/z* 609 (**Table 8**). Their UV-*vis* spectra were also consistent with derivatives of flavonols. However, upon MS/MS fragmentation, *peak 32* had signal at *m/z* 285 (loss of 324 *amu*, corresponding to the loss 2 glycosyl units attached at the same position), which corresponds to kaempferol molecule. Based on this evidence, this peak can be identified as kaempferol-3-*O*-diglucoside. This was found in all the varieties studied except in the red (IT82D-889 and

IT97K-1042-3), green (TX2028-1-3-1) and light brown (09FCV-CC27M) varieties. Kaempferol 3-*O*-glucoside has been reported in some pulses (*150*). As far as we know, this is the first report of diglycosylated kaempferol in pulses.

On the other hand, *peak 34* had a major loss of 308 *amu*, corresponding to loss of a hexose (162 *amu*) + rhamnose (146 *amu*) attached at the same position, indicated by product ion at *m/z* 301 corresponding to that of quercetin molecule. *Peak 36* had similar fragmentation pattern as *peak 34*, and thus are assumed to be structurally related. The nature of the sugar moiety was proposed based on their relative retention times. Since *peak 34* eluted earlier than *peak 36*, *peak 34* was elucidated as quercetin-3-*O*-galactosylrhamnoside, while *peak 36* was quercetin-3-*O*-glucosylrhamnoside. In this study, we found peaks corresponding to quercetin-3-*O*-galactosylrhamnoside only in IT97K-1042-3 (red) (**Figure 3B-2**), 09FCV-CC27M (light brown) (**Figure 8A-2**) and Early Acre (white) (**Figure 10**) varieties; while peaks corresponding to quercetin-3-*O*-glucosylrhamnoside were found only in the red (IT82D-889 and IT97K-1042-3) (**Figure 3**) and light brown (09FCV-CC27M) (**Figure 8A-2**) varieties.

Peak 33 (t_R = 7.58 min, λ_{max} = 353 nm) was only present in the light brown 09FCV-CC27M variety (**Figure 8A-2**) and had $[M - H]^-$ at *m/z* 595. The major signal in MS/MS was *m/z* 301 (M – 294), corresponding to loss of pentose (132 *amu*) + hexose (162 *amu*) attached at the same position on the aglycone. Since arabinose is the most common pentose (*148*), *peak 33* was designated as quercetin-3-*O*-arabinosylglucoside (**Table 8**). This is the first time that this compound has been reported in cowpea.

Peaks 40 (t_R = 7.93 min), *41* (t_R = 8.04 min) and *42* (t_R = 8.10 min) showed [M – H]$^-$ at *m/z* 463. The fragment ion at *m/z* 301 was derived by losing a hexose unit from the parent ion (i.e. quercetin), joined by an *O*-glycosidic linkage (**Table 8**). Thus, all these peaks are proposed to be *O*-glycosyl derivatives of quercetin. Since type and position of glycosylation has been shown to affect the elution characteristics of flavonoids, we used retention time to elucidate the identities of these three peaks. The order of elution of the glycosides on C18 column is galactoside before glucoside, followed by arabinoside (*123*). Therefore, we proposed *peak 40* as quercetin-3-*O*-galactoside based on its shorter retention time and lower UV absorption maximum (λ_{max} = 353 nm), compared to *peaks 41* and *42*. This compound has previously been reported in cowpea (*51*). In this study, quercetin-3-*O*-galactoside was found in five varieties only, that is, IT95K-1105-5 (black) (**Figure 9A**), IT82D-889 (red) (**Figure 3**), IT84S-2246 (golden brown) (**Figure 5A-2**), IAR-48 (light brown) (**Figure 8**) and Early Acre (white) (**Figure 10**). Conjugation of the sugar at 7-position has been shown to increase polarity of quercetin through the C18 column, thus 7-*O*-glucosides are known to elute before 3-*O*-glucosides (*54*). Thus, based on the elution order, *peak 41* was designated as quercetin-7-*O*-glucoside, and *peak 42* as quercetin-3-*O*-glucoside. Both quercetin-7-*O*-glucoside and quercetin-3-*O*-glucoside have been reported in a black seed cultivar of cowpea (*54*). In this study, quercetin-7-*O*-glucoside was found in five cowpea varieties including red (IT97K-1042-3 and IT82D-889) (**Figure 3**), black IT98K-1092-1 (**Figure 9B**), white Early Acre (**Figure 10**) and green TX2028-1-3-1 (**Figure 11**); while

quercetin-3-*O*-glucoside was only present in the light brown IAR-48 variety (**Figure 8B-2**).

Peak 45 (t_R = 8.48 min, λ_{max} = 357 nm) had $[M - H]^-$ at *m/z* 549, and was only present in two phenotypes, that is red IT97K-1042-3 (**Figure 3**) and black (TX95K-1105-5 and TX98K-10921-1) (**Figure 9**). It showed MS/MS fragment at *m/z* 301, corresponding to loss of 248 *amu.* This indicated loss of hexose (162 *amu*) + malonic acid (86 *amu*). The most common positions for acylation are C-3″ and C-6″; however, C-6″ is most preferred position for acylation (*129*). The ions at *m/z* 301, 179 and 151, coupled with the maximum UV absorption for this peak is typical of flavonol quercetin and its derivatives (**Table 8**). Moreover, acyl groups such as acetyl or malonyl usually occur as 6 -*O*-acetylglucoside or 6 -*O*-malonylglucoside (*151*). Thus, *peak 45* was tentatively identified as quercetin-3-(6″-malonoyl)-glucoside (**Figure 4A**). Flavonoids containing 6′ -malonoyl-glucoside groups have been identified in eggplants and red onions (*129*). To our knowledge, this is the first time that this quercetin derivative is identified in cowpea, and thus far, in pulses.

Peak 47 (t_R = 8.82 min, λ_{max} = 334 nm) had precursor ion $[M - H]^-$ at *m/z* 801. The MS/MS spectrum showed product ions at *m/z* 625 and at 301, an indication of sequential cleavage of two groups (**Table 8**). The ion at *m/z* 625 corresponded to a diglucosyl residue (loss of 324 *amu*) while the ion at *m/z* 301 corresponded to a terminal feruloyl derivative (loss of 176 *amu*) in the 3-position of the quercetin aglycone (*152*). Therefore, this compound was proposed as quercetin-3-(6″-feruloyl)-diglucoside (**Figure 4A**). In this study, we found peaks corresponding to quercetin-3-feruloyl-

diglucoside in the black IT95K-1105-5, golden brown (IT84S-2246 and Ife Brown), light brown IAR-48 and green TX2028-1-3-1 varieties. This compound has previously been identified in cowpea (*51*).

Peak 48 (t_R = 9.16 min) showed $[M - H]^-$ at *m/z* 709 and UV-spectrum with maximum at 363 nm. Fragmentation of this compound produced ions at *m/z* 625 (M – 84 *amu*) and 301 (M – 84 – 324 *amu*). This indicates the presence of a terminal di-acetoyl group (2 × 42 *amu*) and di-glucosyl moiety (2 × 162 *amu*). The MS/MS spectral pattern (**Table 8**) showed that the di-acetoyl and the glycosyl groups were sequentially cleaved off the quercetin backbone (*m/z* 301) (**Table 8**). Therefore, this compound was designated as quercetin-3-(6″-diacetoyl)-diglucoside (**Figure 4A**) and also showed much longer retention time compared to its non acylated counterparts. Among all the samples studied, peaks corresponding to quercetin-3-di-acetoyl-diglucoside were only found in the Ife Brown (golden brown) (**Figure 5B-2**) and IAR-48 (light brown) varieties (**Figure 8B-2**).

Peak 49 (t_R = 9.96 min, λ_{max} = 332 nm) had $[M - H]^-$ at *m/z* 815. The fragment at *m/z* 301 (M – 514 *amu*) was attributed to the loss of rutinose (6-*O*-α-L-rhamnosyl-*D*-glucose; 308 *amu*) with the rutinose acylated with sinapic acid (206 *amu*) and finally joined to the aglycone by an *O*-glycosidic bond. The aglycone was revealed by the ions at *m/z* 301, 179 and 151 to be quercetin (**Table 8**). *Peak 49*, which was only found in Ife Brown variety (**Figure 5B-2**), was tentatively designated as quercetin-3-(6″-sinapoyl)-rutinoside (**Figure 4A**) (*129*).

Peak 50 (t_R = 11.05 min) had $[M - H]^-$ at *m/z* 301 corresponding to quasi molecular ion of quercetin in negative ionization mode (**Table 8**). This compound also showed characteristics both in the UV-*vis* spectra (λ_{max} = 370 nm) and in the MS/MS spectrum (ions at *m/z* 179 and 151), coupled with a much longer retention time which are consistent with literature information on properties of quercetin (*54*). In this study, we observed derivatives of quercetin flavonol in these cowpea extracts; thus, the occurrence of quercetin was also expected. However, this peak was only present in the IT82D-889 (red) variety (**Figure 3B-2**). Based on our observations throughout this study, it means that flavonols basically occur in cowpea as glycosides and acylglycosides. We found that glycosides of quercetin are predominant in cowpea. Previous studies reported high levels of quercetin (412.5 μg/g) and myricetin (51.3 μg/g) in black cowpea hydrosylates (*56*).

3.4.4 Dihydrochalcones

Peak 30 (t_R = 7.14 min, λ_{max} = 325 nm), which was only present in IT98K-1092-1 (black) (**Figure 9B**) and golden brown (IT84S-2246 and Ife Brown) varieties (**Figure 5A-1**) produced a $[M - H]^-$ at *m/z* 435. In the MS/MS spectrum, the majority ion at *m/z* 273 (M – 162 *amu*) corresponds to loss of a glycosyl unit. The fragment at *m/z* 273 and λ_{max} at 325 nm (**Table 7**) matches that of dihydrochalcone phloretin (**Figure 13**) known to contribute to the flavor, color and health benefits of apple fruit (*153, 154*). Based on the MS spectrum and literature evidence (*154*), *peak 30* can be identified as phlorizin (phloretin 2′-glucoside), also known to competitively inhibit renal glucose transport

(*155*) and facilitate memory storage in mice (*156*). Until recently, phlorizin and its derivatives were believed to exist only in *Malus* species, primarily in the young shoots, roots, leaves and bark of apple trees (*154*). The presence of phlorizin in legumes has not been reported before.

3.4.5 Phenolic acids

Peaks 55, 56 and *59* all had $[M - H]^-$ at *m/z* 399. The fragment at *m/z* 193 corresponded to cleavage of the aldaric acid moiety. Based on literature data and fragmentation pattern (**Table 9**), we propose the peaks as *trans*-feruloylmethylaldaric acids (**Figure 12**) (*51*). Based on the t_R of *peak 59* (t_R = 7.23 min) > *56* (t_R = 6.66 min) > *55* (t_R = 6.53 min) we suspect that there is a single substituent on the structure of this compound that only differs in the position (*ortho-*, *para-* and *meta-*) at which it is substituted, which may affect their polarity. In this study, we found peaks corresponding to *trans*-feruloylmethylaldaric acid in the red IT82D-889 (**Figure 14**), golden brown (Ife Brown and IT84S 2246) (**Figure 15**), light brown (IAR-48 and 09FCV-CC27M) (**Figure 17**), and white (Early Acre) (**Figure 18**) cowpea varieties. Previous studies have also reported *trans*-feruloylaldaric acid and its methyl derivatives in cowpea (*51*).

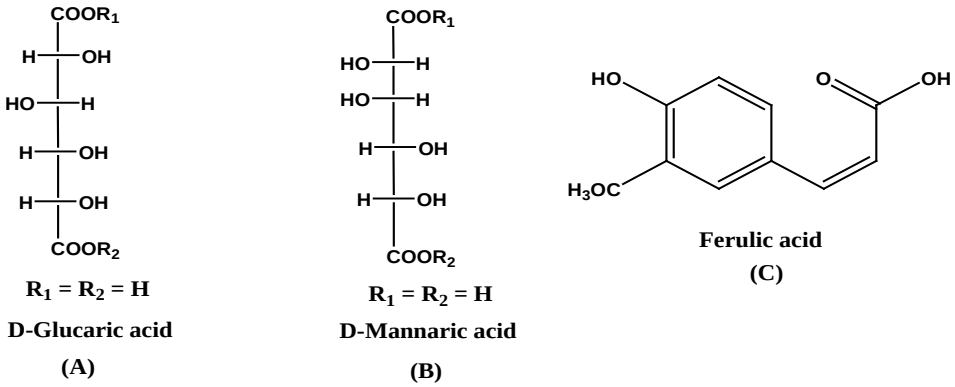


Figure 12. Representative aldaric acid derivatives. Aldaric acid, **$R_1 = R_2 = H$. CH_3** and **ferulic acid** may bond at **R_1** and **R_2** to **A** and **B.**

3.5 Flavonoids in the ethyl-acetate fractions

The elution with ethyl acetate on C18 Sep-Pak cartridges as described under *materials and methods* isolates catechins and oligomeric proanthocyanidin from anthocyanins and flavonols. However, some oligomers and polymeric forms of proanthocyanidins are more strongly associated with the stationary phase, thus, are eluted with stronger solvents, such as methanol. Thus, it is not surprising to find some of these compounds in both methanolic and ethyl-acetate fractions**.** Moreover, non-polar compounds are highly soluble in ethyl acetate, thus would be found in the ethyl acetate extract fractions. The detected peaks (**Figures 14 – 20**), and a summary of their MS and MS/MS data, as well as λ_{max} and retention times (t_R), are presented in **Table 8.** Identification and peak assignments were primarily based on comparison of their retention time and mass spectrometric data with literature guidelines.

Table 9. Identification of flavonoids in ethyl-acetate fraction of 10 cowpea varieties based on UPLC retention time (t_R), UV-*vis* spectroscopic characteristics (λ_{max}), and MS–MS/MS spectroscopic pattern. Ionization was performed in the negative mode. Peaks marked (*) were unidentified.

Peak no.	t_R (min)	λ_{max} (nm)	Proposed identifications	$[M - H]^-$ (*m/z*)	MS/MS fragments (*m/z*)
51	4.73	284	Taxifolin-7-*O*-β-glucoside	465	303, 285, 275, 125
52*	5.28	325	Unknown	865	713, 577, 561, 425, 407, 289
53	6.06	299	Eriodictyol-7-*O*-β-galactoside	449	287, 151, 135
54*	6.11	266	Unknown	281	281, 237, 201, 189, 171
55	6.53	325	*trans*-Feruloylmethylaldaric acid	399	193, 147, 85
56	6.66	326	*trans*-Feruloylmethylaldaric acid	399	193, 147, 85
57	6.79	279	Procyanidin tetramer	1154 (577)	451, 425, 407, 289
58	7.14	278	Di-phlorizin	871 (435)	299, 273, 137, 135
59	7.23	327	*trans*-Feruloylmethylaldaric acid	399	193, 147, 85
60*	7.26	492	Unknown	341	295, 267, 241, 160, 153
61*	7.43	283	Unknown	521	359, 344, 119, 101
62	7.71	283	Eriodictyol-7-*O*-β-glucoside	449	287, 151, 135
63*	8.05	353	Unknown	985	521, 477, 463, 341, 161
64*	8.08	283	Unknown	1043 (521)	359, 344, 119, 101
65*	8.27	381	Unknown	561	409, 273, 125
66	8.74	354	Catechin-kaempferol dimer	575	449, 289, 285, 125
67*	8.75	269	Unknown	849	561, 409, 367, 299, 287, 273, 243
68	9.06	287	Eriodictyol-5-*O*-β-glucoside	449	287, 151, 135
69*	9.38	352	Unknown	493	181, 167, 123
70	9.51	355	Quercetin-3-(6″-succinoyl)-glucoside	563	463, 301, 300, 151
71*	9.85	260	Unknown	553	385, 241, 167
72*	10.38	274	Unknown	579	385, 325, 283, 241, 197

3.5.1 Flavanonols

Peak 51 (t_R = 4.73 min, λ_{max} = 284 nm) $[M - H]^-$ at *m/z* 465 was identified as taxifolin-7-*O*-β-glucoside (*157*). The fragment corresponding to the loss of glucose at *m/z* 303 (M – 162 *amu*) gave the molecular weight of taxifolin aglycone (**Table 9**), and

the UV-*vis* matched that of its standard. Taxifolin (a dihydroquercetin) is categorized as a flavanonol (**Figure 4B**). In this study, we found peaks corresponding to taxifolin-7-*O*-β-glucoside only in the red cowpea varieties, *i.e.* red IT82D-889 and IT97K-1042-3 varieties (**Figure 14**). Taxifolin and its glycosides have not been reported in cowpeas or other pulses before.

3.5.2 Flavanones

The golden brown varieties had *peaks 53, 62* and *68* (**Figure 15**) with $[M - H]^-$ at *m/z* 449. These peaks were identified as eriodictyol derivatives by comparison of their fragmentation pattern with literature data (*157*) and UV spectra with external standard. The loss of 162 *amu* ([M – 287]) indicated presence of a hexose moiety. The most commonly reported hexose conjugated with eriodictyol is glucose (*134, 157*). Moreover, the position and type of the glucose moiety can be used to discriminate between the three peaks based on their retention times (**Table 9**). Flavanone glycosylation mainly occurs at the C-7 position (*158*); however, both 7-*O*-β-glucosylated and 5-*O*-β-glucosylated derivatives of eriodictyol have been reported in nature, especially in sorghum grains (*134, 157*). Glycosylation at C-7 increases polarity of compounds, thus, 7-*O*-glycosides would elute early (*54*). Based on this evidence, *peaks 53* (t_R = 6.06 min), *62* (t_R = 7.71 min) and *68* (t_R = 9.06 min) were identified as eriodictyol-7-*O*-β-galactoside, eriodictyol-7-*O*-β-glucoside and eriodictyol-5-*O*-β-glucoside, respectively. In this study, a peak corresponding to eriodictyol-7-*O*-β-galactoside was only found in the Ife Brown (golden brown) variety; while two peaks corresponding to eriodictyol-7-*O*-β-glucoside

and eriodictyol-5-*O*-β-glucoside were found in the golden brown (*i.e.* IT84S-2246 and Ife Brown) varieties (**Figure 15**). As far as we know, these compounds are reported for the first time in cowpea seeds.

3.5.3 Dihydrochalcones

Peak 58 (t_R = 7.14 min, λ_{max} = 278 nm) is tentatively identified as phlorizin dimer (**Figure 13**), and was only present in the golden brown IT84S-2246 (**Figure 15A-1**) and light brown 09FCV-CC27M cowpea varieties (**Figure 17A-1**). It has $[M - H]^-$ at *m/z* 871, suggesting presence of two units of 435 *amu*. On fragmentation, the ion at *m/z* 435 produced similar products as those observed for *peak 30* (*i.e.* phlorizin aglycone) (**Table 9**). As previously discussed, the monomeric form of phlorizin (*peak 30*) was only found in the golden brown IT84S-2246 (**Figure 5A-1)** and black IT98K-1092-1 varieties (**Figure 9B**). From our observations, it seems therefore, that the black IT98K-1092-1 variety contained the phlorizin only in its monomeric form; while the light brown 09FCV-CC27M only contained the dimeric but not the monomeric form. However, the golden brown IT84S-2246 variety contained both phlorizin and its dimeric form (**Figures 5A-1** and **15A-1**).

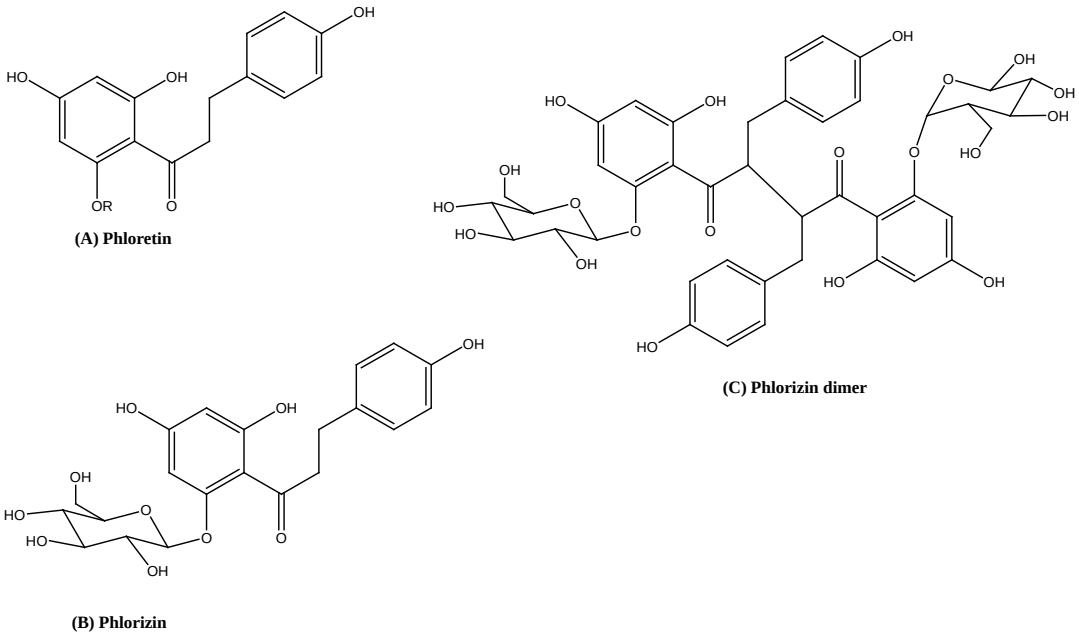


Figure 13. Hydroxychalcones identified in cowpea. Phloretin, **(A)**; phlorizin, **(B)**, and phlorizin dimer, **(C)**. The structure of the dimer is proposed following postulated mechanism for the production of dimeric flavonones from chalcones substituted at C-4 (*159*).

3.5.4 Flavan-3-ols

Among the varieties studied, only 09FCV-CC27M (light brown) (**Figure 16A-1**) and Ife Brown (light brown) varieties (**Figures 16B-1** and **16B-2**) had *peak 57* (t_R = 6.79 min, λ_{max} = 279 nm) which is identified as a procyanidin tetramer. This peak has $[M - H]^-$ at *m/z* 1154, suggesting presence of four catechin units, with major MS/MS fragments at *m/z* 577, suggesting loss of a dimer, and 289, confirming presence of catechin units (*136*). It also showed UV-*vis* absorption maximum of 279 nm, which is typical of proanthocyanidins (**Table 9**). This is the first report indicating that cowpea contains procyanidin tetramers.

Peak 66 (t_R = 8.74 min, λ_{max} = 354 nm) which had $[M - H]^-$ at *m/z* 575 was designated as catechin-kaempferol dimer (**Figure 16**). Its fragmentation pattern included major ions at *m/z* 449, 289, 285 and 125 (**Table 9**). The ion at *m/z* 449 corresponds to HRF of the top unit, causing a loss of 126 *amu.* This fragmentation path leads to the loss of B-ring in the top unit. The ion at *m/z* 289 characterizes catechin as the base unit, following the cleavage of the interflavanoid linkage (*140*), while the ion at *m/z* 285, as well as λ_{max} = 354 nm gives the identity of the flavonol, that is, kaempferol, which forms the terminal (bottom) unit. The strong signal at *m/z* 125 is phloroglucinol (product of HRF) in negative mode (*140, 160*). The proposed fragmentation pathway of this flavonol-flavan-3-ol dimer is shown in **Figure 16.** In this study, we found a peak corresponding to catechin-kaempferol dimer only in the red IT97K-1042-3 variety (**Figures 14A-1** and **2).** This is the first report of the presence of flavanol-flavonol condensed pigment in cowpea, and thus far, in pulses. As far as we know, only the presence of flavanol-anthocyanin condensed pigments in different species of beans (*Phaseolus vulgaris*) has been described by other authors (*161*).

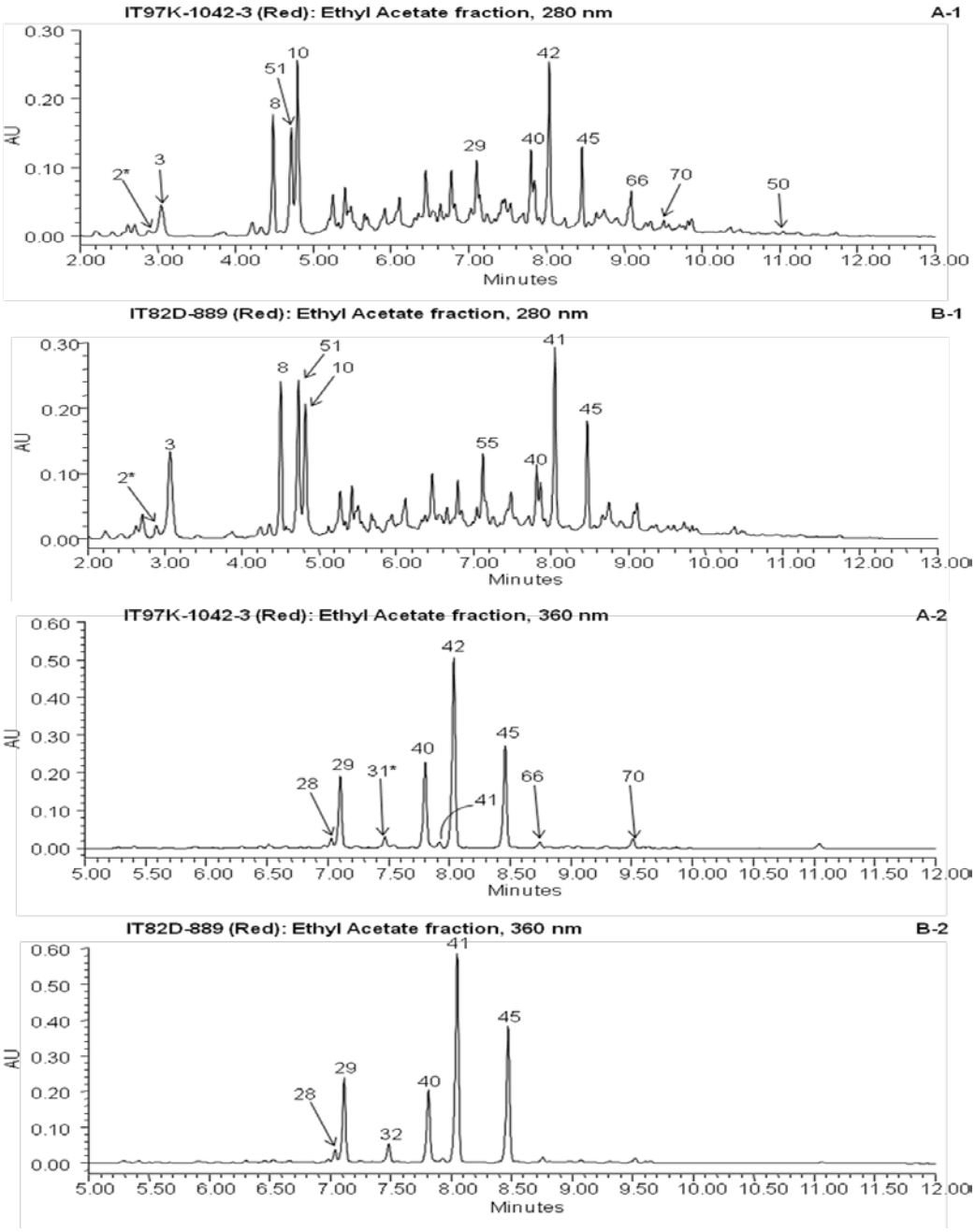


Figure 14. Reverse-phase UPLC chromatogram of phenolic extracts from ethyl-acetate fractions of red cowpea varieties; IT97K-1042-3 at 280 nm (**A-1**) and 360 nm (**A-2**); and IT82D-889 at 280 nm (**B-1**) and 360 nm (**B-2**). Peaks marked (*****) are unidentified. Peak numbers are referenced to **Tables 6 – 9.**

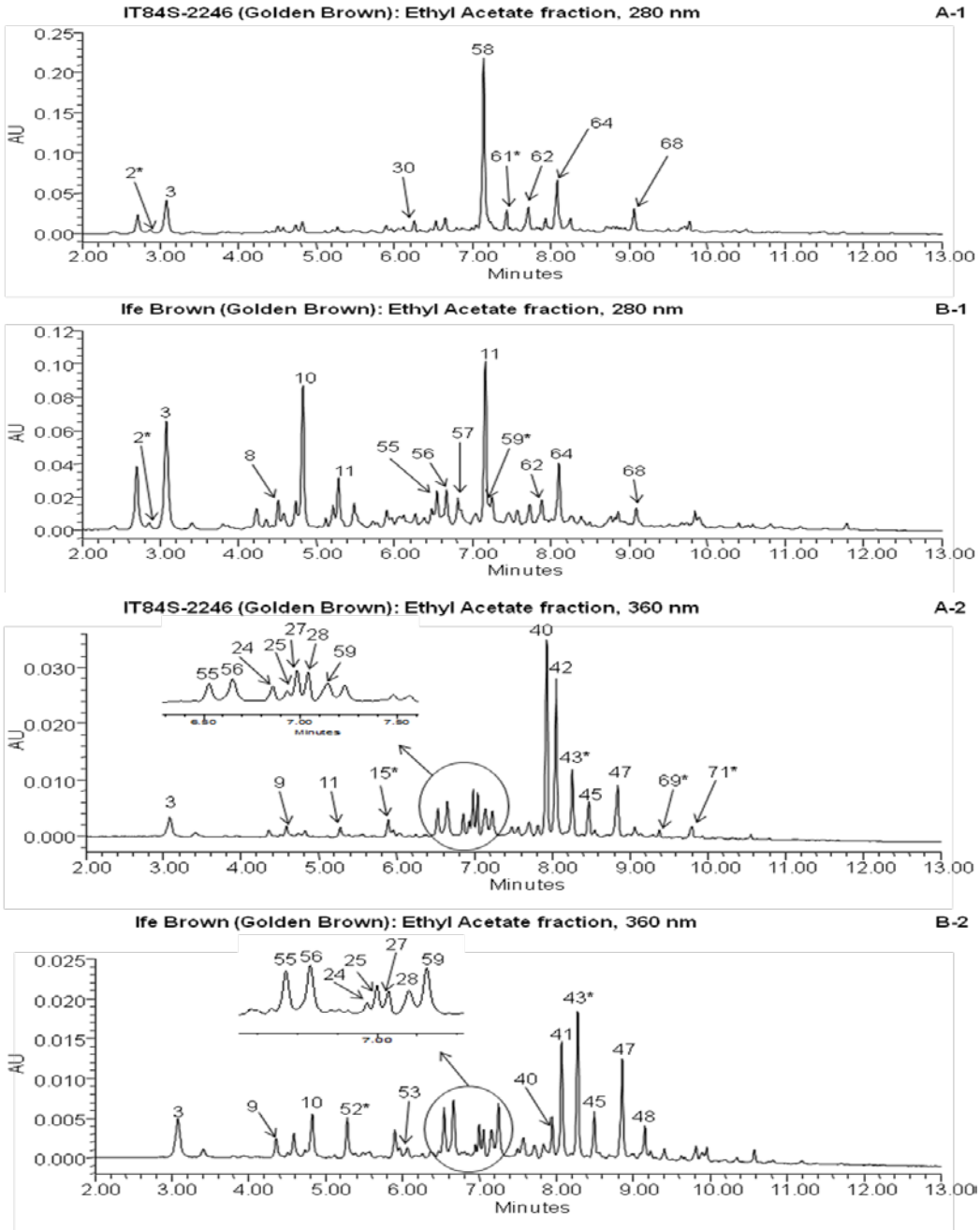


Figure 15. Reverse-phase UPLC chromatogram of phenolic extracts from ethyl-acetate fractions of golden brown cowpea varieties; IT84S-2246 at 280 nm (**A-1**) and 360 nm (**A-2**); and Ife Brown at 280 nm (**B-1**) and 360 nm (**B-2**). Peaks marked (*****) are unidentified. Peak numbers are referenced to **Tables 6 – 9.**

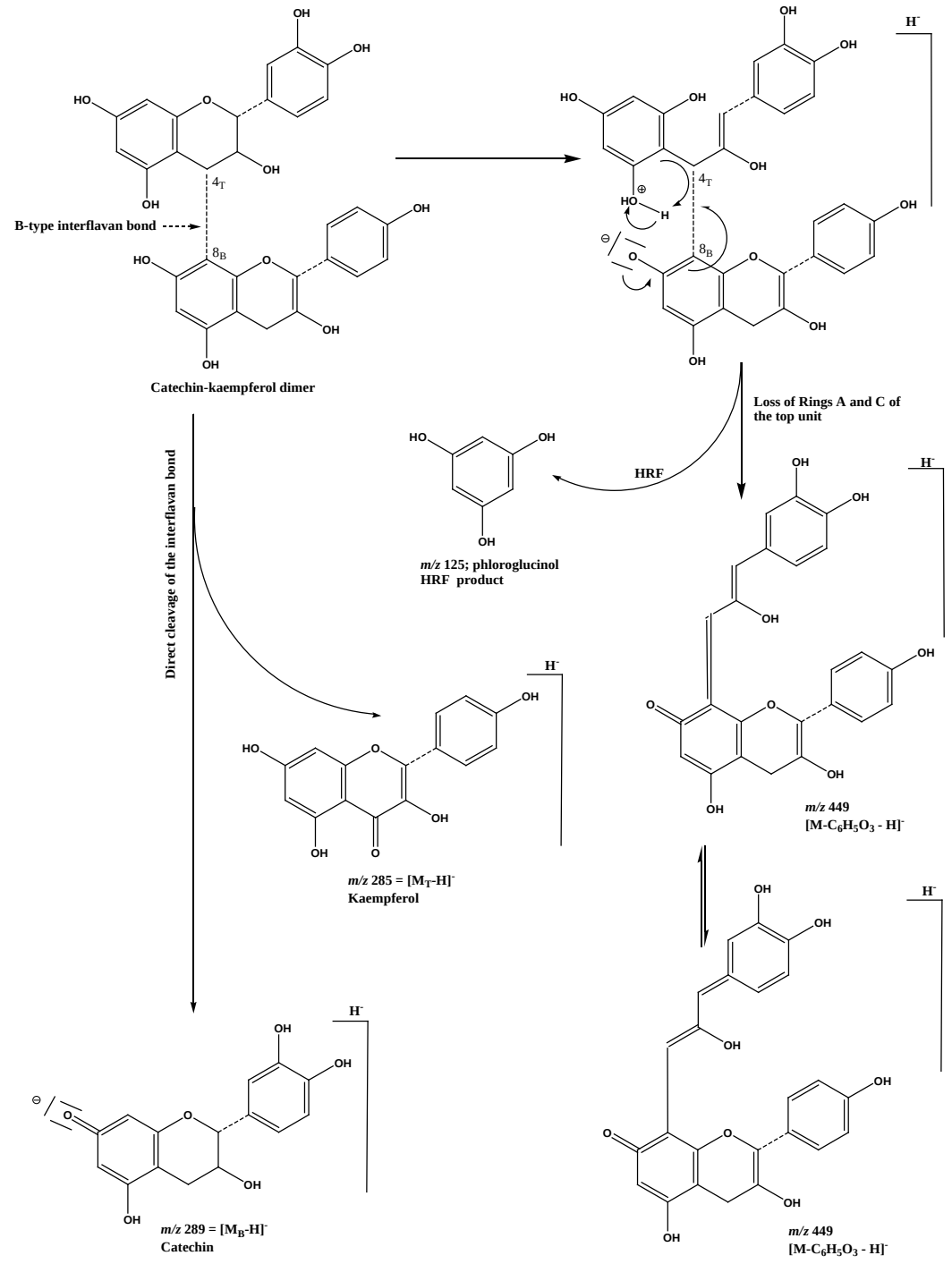


Figure 16. Fragmentation mechanism of flavanol-flavan-3-ol dimer found in cowpea (in negative mode) following general scheme proposed by Friedrich *et al*. (*140*).

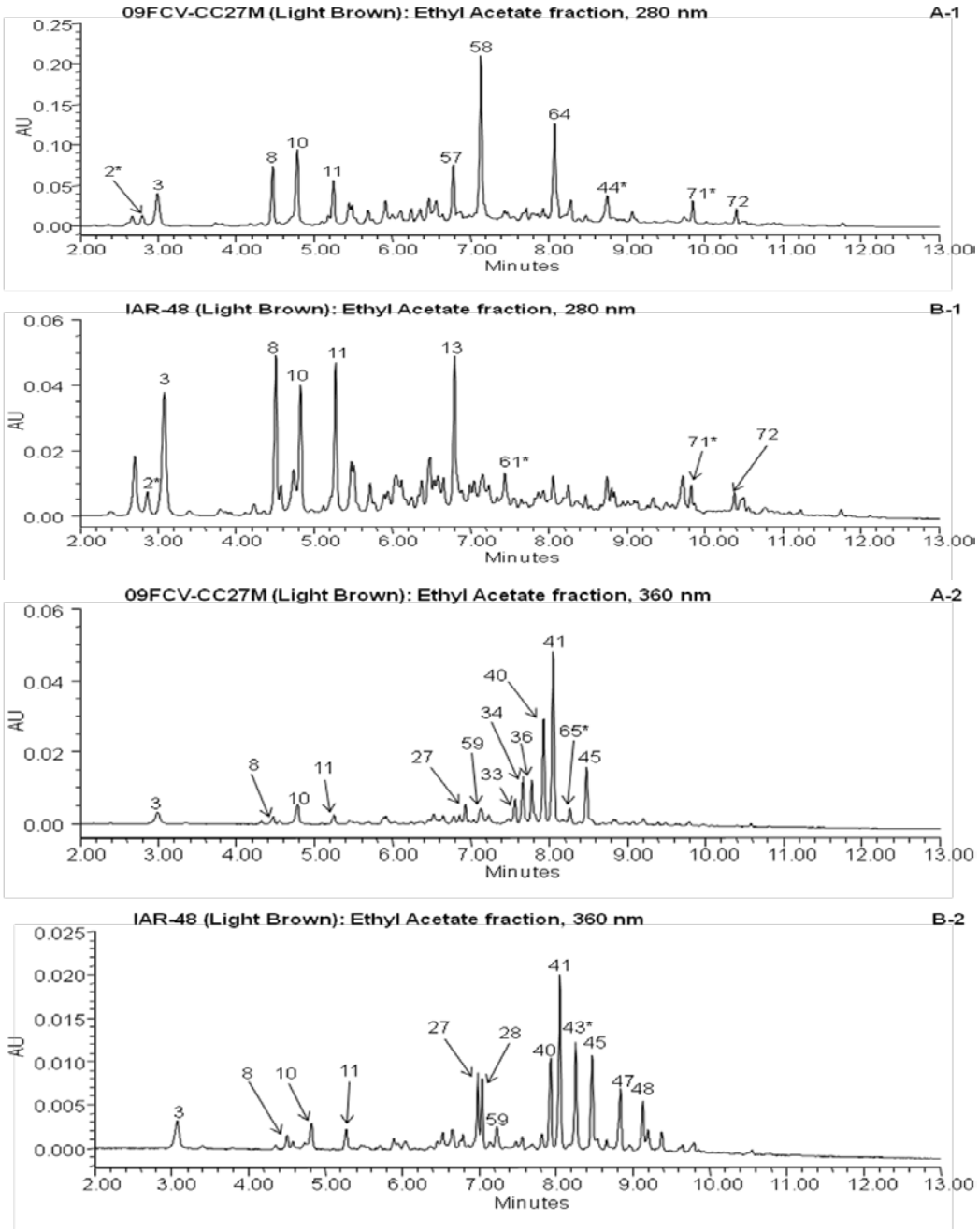


Figure 17. Reverse-phase UPLC chromatogram of phenolic extracts from ethyl-acetate fractions of light brown cowpea varieties; 09FCV-CC27M at 280 nm (**A-1**) and 360 nm (**A-2**); and IAR-48 at 280 nm (**B-1**) and 360 nm (**B-2**). Peaks marked (*****) are unidentified. Peak numbers are referenced to **Tables 6 – 9.**

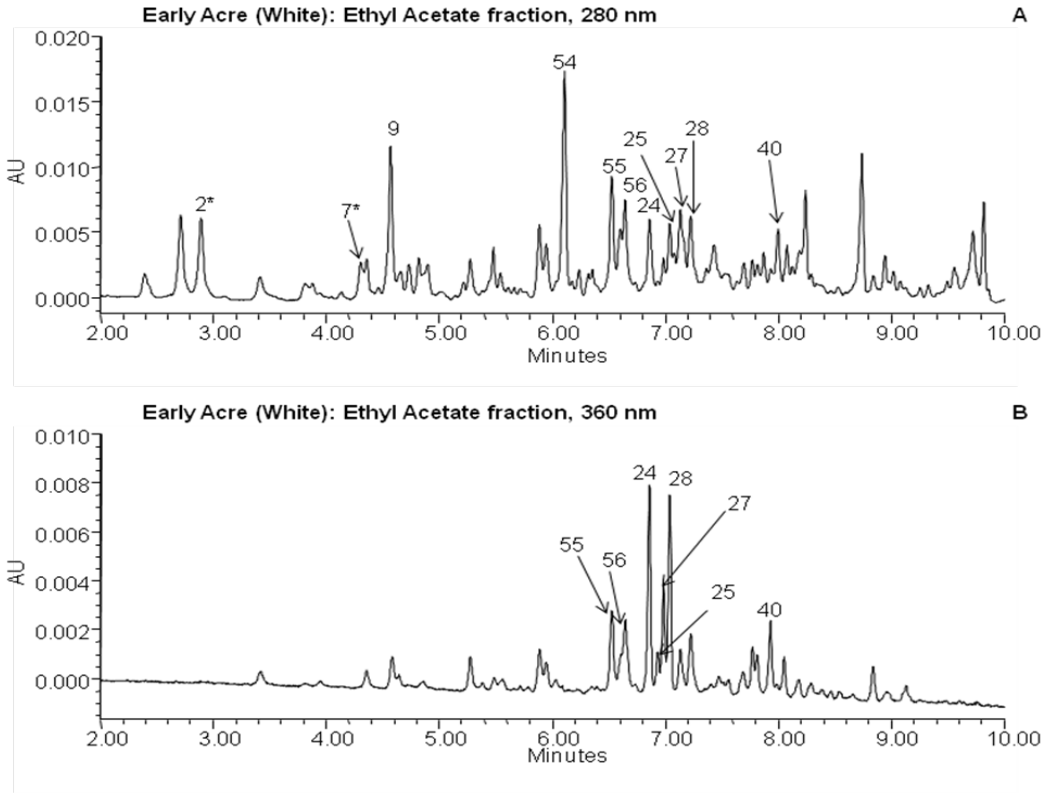


Figure 18. Reverse-phase UPLC chromatogram of phenolic extracts from ethyl-acetate fractions of white cowpea variety, Early Acre at 280 nm (**A**) and 360 nm (**B**). Peaks marked (*) are unidentified. Peak numbers are referenced to **Tables 6 – 9.**

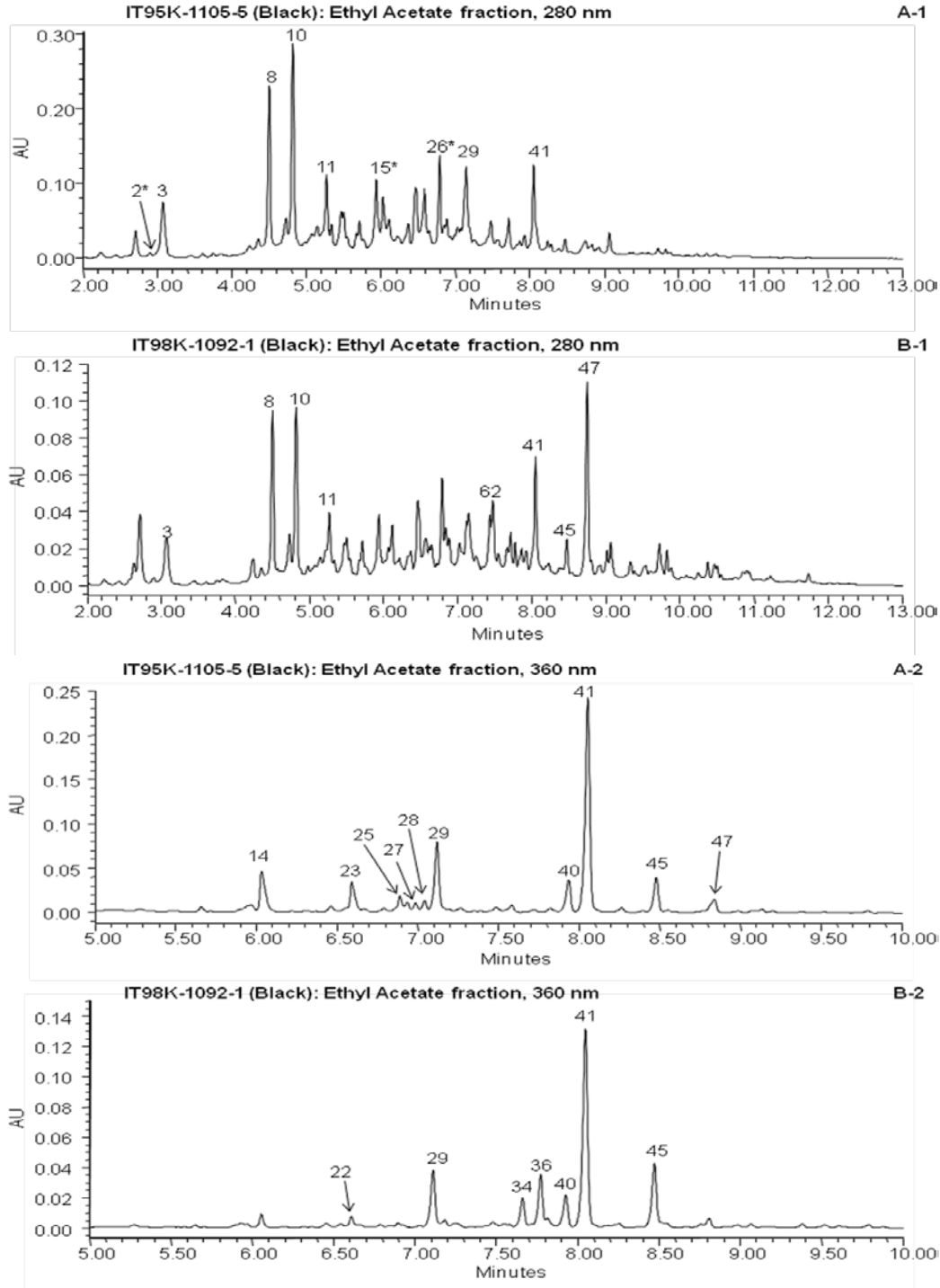


Figure 19. Reverse-phase UPLC chromatogram of phenolic extracts from ethyl-acetate fractions of black cowpea varieties; IT95K-1105-5 at 280 nm (**A-1**) and 360 nm (**A-2**); and IT98K-1092-1 at 280 nm (**B-1**) and 360 nm (**B-2**). Peaks marked (*****) are unidentified. Peak numbers are referenced to **Tables 6 – 9.**

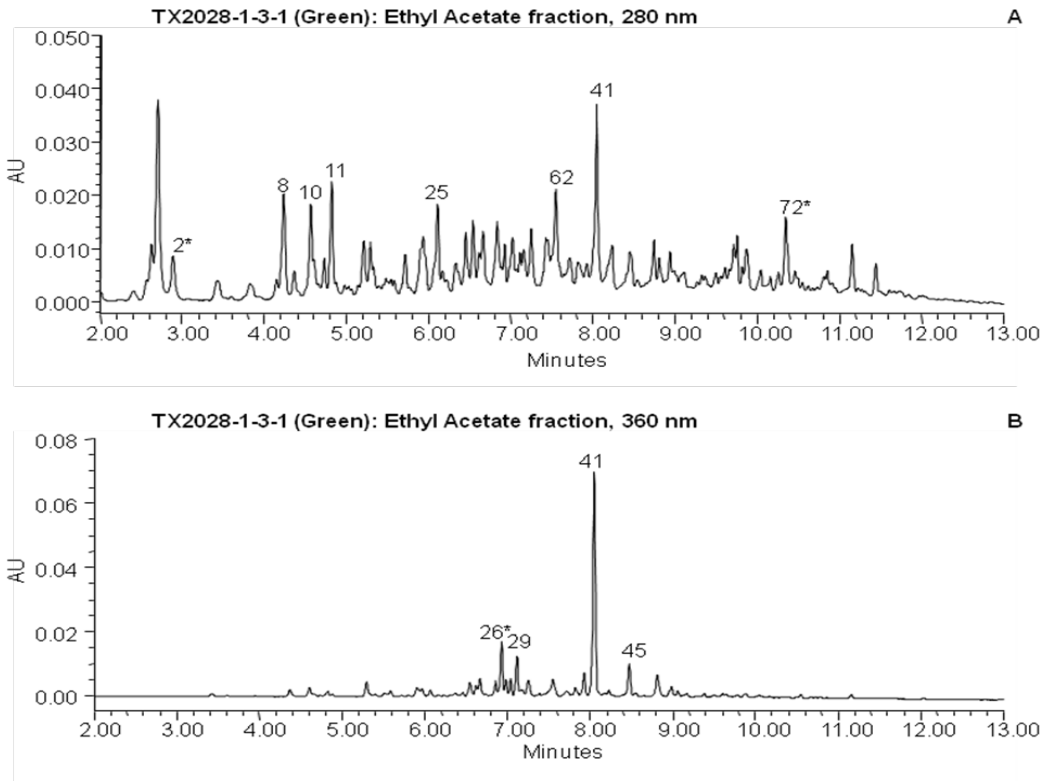


Figure 20. Reverse-phase UPLC chromatogram of phenolic extracts from ethyl-acetate fractions of green cowpea variety, TX2028-1-3-1 at 280 nm (**A**) and 360 nm (**B**). Peaks marked (*****) are unidentified. Peak numbers are referenced to **Tables 6 – 9.**

3.5.5 Flavonols

Peak 70 (t_R = 9.51 min, λ_{max} = 355 nm) had $[M - H]^-$ at *m/z* 563. The MS/MS fragment at *m/z* 301 corresponds to quercetin. Upon fragmentation, the ion at *m/z* 463 (loss of 100 *amu*) occurred, suggesting the compound is acylated with succinic acid; while the ion at *m/z* 301 (M – 100 – 162 *amu*), indicated there was a subsequent fragmentation of a glucose moiety. This peak is thus designated as quercetin-3-(6″-

succinoyl)-glucoside, and was only found in the red IT97K-1042-3 variety (**Figures 14A-1** and **2; Table 8**).

3.6 Flavonol content in cowpeas

With regard to general flavonol content in pulses, most studies focus on common bean, and very limited information is available on other pulses (*47, 54*). The individual (quercetin, myricetin and kaempferol derivatives) and total flavonol contents of the raw and boiled cowpeas are presented in **Tables 10 – 14**.

Overall, twenty nine (29) flavonol compounds were isolated in the cowpea varieties, six of which were unidentified (**Tables 8** and **9**). From this study, a total of twenty three (23) flavonols were newly identified in cowpea. The majority (20 compounds) were quercetin derivatives. Myricetin derivatives were only found in black (IT95K-1105-1 and IT98K-1092-1), red (IT82D-889 and IT97K-1042-3) and green TX2028-1-3-1 varieties. Only one kaempferol derivative (kaempferol-3-*O*-diglucoside) was detected and was only found in the black (IT95K-1105-1 and IT98K-1092-1), golden brown (Ife Brown and IT84S-2246), light brown IAR-48 and white Early Acre varieties. Average flavonol content was significantly higher in the red phenotype (1799 ± 64.9 μg/g), than the other phenotypes (**Tables 10 – 14**).

Table 10. Compositions (μg/g of cowpea flour) and effect of boiling on flavonols and flavan-3-ols in black cowpea varieties [a]

	Black cowpea varieties					
	IT95K-1105-5			IT98K-1092-1		
	Flavonol content (μg/g cowpea flour)					
Compound	*Raw*	*Boiled*	*% change*	*Raw*	*Boiled*	*% change*
Quercetin-3-*O*-glucoside-4′-*O*-diglucoside	34.1 ± 1.14	ND[b]	-	30.5 ± 3.14	ND	-
Quercetin-3-*O*-arabinosyldiglucoside	44.2 ± 0.73	38.8 ± 1.30	12.2	13.9 ± 0.23	ND	-
Quercetin-3-*O*-digalactoside	17.8 ± 0.29	15.6 ± 0.52	12.4	12.6 ± 0.28	ND	-
Quercetin-3,7-diglucoside	12.3 ± 0.55	ND	-	8.54 ± 1.28	ND	-
Quercetin-3-*O*-galactosylglucoside	55.4 ± 1.41	ND	-	12.5 ± 2.81	ND	-
Quercetin-3-*O*-diglucoside	59.0 ± 2.97	ND	-	6.13 ± 1.04	ND	-
Quercetin-7-*O*-glucoside	76.9 ± 2.97	19.8 ± 1.46	74.3	23.3 ± 7.75	12.4 ± 1.21	46.8
Quercetin-3-(6″-malonyl)-glucoside	20.8 ± 0.24	8.36 ± 1.12	59.8	15.4 ± 0.70	7.11 ± 2.65	53.8
Quercetin-3-(6″-feruloyl)-diglucoside	18.8 ± 0.21	8.75 ± 0.15	53.5	ND	ND	-
Quercetin-3-*O*-galactosylrhamnoside	ND	ND	-	53.8 ± 3.37	28.3 ± 1.53	47.4
Quercetin-3-*O*-glucosylrhamnoside	ND	ND	-	67.1 ± 3.57	25.7 ± 0.97	61.7
Subtotal quercetin	**339 ± 7.5**	**91.3 ± 4.55**	**73.1**	**244 ± 24.2**	**73.5 ± 6.36**	**69.9**
Myricetin-3-*O*-diglucoside	23.8 ± 1.39	ND	-	19.3 ± 0.88	ND	-
Myricetin-3-*O*-glucoside	23.2 ± 1.00	ND	-	26.1 ± 1.77	ND	-
Subtotal myricetin	**47.0 ± 2.39**	-	-	**45.4 ± 2.65**	-	-
Kaempferol-3-*O*-diglucoside	13.6 ± 2.44	ND	-	187 ± 9.4	177 ± 5.4	5.28
Subtotal kaempferol	**13.6 ± 2.44**	**ND**	-	**187 ± 9.4**	**177 ± 5.4**	**5.28**
Total flavonols	**399 ± 12.3**	**91.3 ± 4.55**	**77.3**	**476 ± 36.3**	**251 ± 11.8**	**47.3**
	Flavan-3-ols content (μg/g cowpea flour)					
Catechin-3-*O*-glucoside	2288 ± 40.9	571 ± 9.6	75.0	1811 ± 8.3	896 ± 8.6	50.5
Procyanidin dimer B-type	3862 ± 102.8	1770 ± 52.5	54.1	2084 ± 62.7	ND	-
Catechin/epicatechin	272 ± 5.9	157 ± 6.0	42.4	64.1 ± 3.84	28.9 ± 1.6	55.0
Total flavan-3-ols	**6422 ± 149.6**	**2498 ± 68.1**	**61.1**	**3959 ± 74.8**	**924 ± 10.2**	**76.7**

[a]Data are expressed as mean ± SD (n = 3) on a dry weight basis (μg/g of cowpea flour). Concentrations of glycosides or acyl-glycosides of flavonols were calculated from the standard curve of kaempferol, myricetin, quercetin, quercetin-3-*O*-rutinoside and quercetin 3-*O*-glucopyranoside; while those for flavan-3-ols were calculated from standard curve of catechin and procyanidin B2; but were all adjusted on the basis of molecular-weight differences. [b]ND = not detectable.

Table 11. Compositions (μg/g of cowpea flour) and effect of boiling on flavonols and flavan-3-ols in red cowpea varieties [a]

	Red cowpea varieties					
	IT82D-889			**IT97K-1042-3**		
	Flavonol content (μg/g cowpea flour)					
Compound	*Raw*	*Boiled*	*% change*	*Raw*	*Boiled*	*% change*
Quercetin-3-*O*-glucoside-4′-*O*-diglucoside	106 ± 7.9	13.9 ± 0.78	86.9	24.0 ± 3.12	11.9 ± 1.45	50.4
Quercetin-3-*O*-digalactoside	12.5 ± 1.19	ND[b]	-	ND	ND	-
Quercetin-3-*O*-arabinosyldiglucoside	ND	ND	-	93.0 ± 3.89	ND	-
Quercetin-3,7-diglucoside	ND	ND	-	ND	ND	-
Quercetin-3-*O*-galactosylglucoside	102 ± 7.0	35.2 ± 0.71	65.5	70.9 ± 2.25	30.5 ± 0.68	40.4
Quercetin-7-*O*-glucoside	144 ± 3.1	56.7 ± 0.31	60.6	90.0 ± 2.48	82.4 ± 2.18	8.4
Quercetin-3-*O*-diglucoside	313 ± 6.2	118 ± 2.2	62.3	334 ± 5.8	123 ± 6.16	63.2
Quercetin	5.71 ± 0.56	ND	-	ND	ND	-
Quercetin-3-(6″-malonoyl)-glucoside	46.5 ± 3.10	22.2 ± 0.68	52.3	120 ± 4.1	ND	-
Subtotal quercetin	**729 ± 29.1**	**246 ± 4.7**	**66.3**	**732 ± 21.6**	**248 ± 10.5**	**64.2**
Myricetin-3-*O*-diglucoside	129 ± 6.2	88.6 ± 3.22	31.3	138 ± 5.6	93.5 ± 6.13	32.3
Myricetin-3-*O*-glucoside	ND	ND	-	71.4 ± 2.39	68.7 ± 4.87	3.8
Subtotal myricetin	**129 ± 6.2**	**88.6 ± 3.22**	**31.3**	**209 ± 7.99**	**162 ± 11.0**	**22.6**
Total flavonols	**858 ± 35.3**	**335 ± 7.9**	**61.1**	**941 ± 29.6**	**410 ± 21.5**	**56.4**
	Flavan-3-ols content (μg/g cowpea flour)					
Catechin-3-*O*-glucoside	2199 ± 8.3	1118 ± 7.8	49.2	2264 ± 10.3	ND	-
Procyanidin dimer B-type	ND	ND	-	3388 ± 69.9	ND	-
Catechin/epicatechin	ND	ND	-	86.1 ± 4.27	ND	-
Procyanidin trimer T2	ND	ND	-	3833 ± 61.9	ND	-
Total flavan-3-ols	**2199 ± 8.3**	**1118 ± 7.8**	**49.2**	**9571 ± 146.4**	**-**	**-**

[a]Data are expressed as mean ± SD (n = 3) on a dry weight basis (μg/g of cowpea flour). Concentrations of glycosides or acyl-glycosides of flavonols were calculated from the standard curve of myricetin, quercetin, quercetin-3-*O*-rutinoside and quercetin 3-*O*-glucopyranoside; while those for flavan-3-ols were calculated from standard curve of catechin and procyanidin B2; but were all adjusted on the basis of molecular-weight differences. [b]ND = not detectable.

The three most dominant quercetin derivatives identified in the raw cowpea samples were quercetin-3,7-diglucoside, quercetin-3-*O*-galactosylglucoside and quercetin-3-*O*-diglucoside (**Tables 10 – 14**). The average amount of quercetin derivatives were highest in the red varieties (**Table 11**) and lowest in the light brown 09FCV-CC27M variety (**Table 12**). However, the average amounts of the acylated quercetin derivatives were generally low, ranging from 9.1 μg/g of quercetin-3-(6″-diacetoyl)-diglucoside found in golden brown IT84S 2246 variety to 120 μg/g of quercetin-3-(6″-malonoyl)-glucoside in the red IT97K-1042-3 cowpea variety. How growth environment affects synthesis and accumulation of these flavonols is unknown.

Myricetin derivatives (mainly myricetin-3-*O*-glucoside and myricetin-3-*O*-diglucoside) were only detected in the black, red and green cowpea varieties (**Tables 10 – 14**). The average myricetin content was highest in the red IT97K-1042-3 (210 μg/g) variety (**Table 11**), and lowest in the green TX2028-1-3-1 variety (24.8 μg/g) (**Table 14**). From a quantitative point of view, the average myricetin content in raw cowpea varieties studied were higher than those reported in raw cool season food legumes such as green pea (36.2 μg/g), yellow pea (36.7 μg/g), chick pea (32.1 μg/g) and lentils (33.3 μg/g) (*71*).

In conclusion, flavonol compounds of the 10 cowpea varieties studied were found to be dominated by quercetin derivatives, followed by myricetin derivatives. The red phenotype seems to synthesize higher levels of flavonols compared to the other varieties. Five acyl derivatives of quercetin were also identified and were present in small quantities. From this study, we found that the different concentrations of the

identified flavonols and the diversity in peak distribution on chromatograms of the 10 cowpea samples suggest that the identities of the varieties can be described by the flavonol profiles. Total flavonol contents for all the cowpea varieties ranged from 270 to 941 μg/g of flour, suggesting cowpea is a significant source of flavonols in the diet.

3.7 Effect of boiling on flavonol content in cowpea

Boiling significantly reduced total flavonol content in all the cowpea varieties. The greatest loss in total flavonol content was observed in the black IT95K-1105-5 (77.3% reduction) (**Table 10**). Our results are different from earlier report on green beans, which was not much affected by thermal processing (*162*). The differences may be attributed to the difference between dry cowpea seeds in our study and fresh beans used in the literature. Boiling was found to significantly reduce the concentrations of individual flavonols in cowpeas, and in some cases reduced their concentrations to undetectable levels (**Tables 10 – 14**). The changes, however, depended upon the variety of cowpea, as well as the levels of individual flavonols in the raw samples. Thermal processing has also been shown to reduce levels of myricetin in green peas, yellow pea and chick pea (*71*); and kaempferol-3-glucoside and kaempferol-3-acetylglucoside in pinto beans (*130*).

In general, the flavonols were more stable than anthocyanins after boiling. This suggests that the flavonol aglycones are not readily transformed into their intermediate

thermolabile chalcones which permit cleavage into phloroglucinaldehyde, protocatechuic acid and 4-hydroxybenzoic acid.

3.8 Flavan-3-ol content in cowpeas

On average, the light brown phenotype had the highest total flavan-3-ol content, followed by the red, black and then golden brown phenotypes (**Tables 10 – 13**). The green TX2028-1-3-1 and white Early Acre did not contain detectable levels of flavan-3-ols (**Table 14**). In terms of varieties, the light brown 09FCV-CC27M had the highest flavan-3-ols content (13,664 ± 184.5 μg/g), followed by golden brown Ife Brown (9,877 ± 152.8 μg/g) and red IT97K-1092-1 (9,571 ± 146.4 μg/g). Moderate levels of flavan-3-ols were found in the black (IT95K-1105-5 and IT98K-1092-1) (**Table 10**) and light brown IAR-48 cowpea varieties (**Table 12**).

Catechin-3-*O*-glucoside was the most dominant flavan-3-ol compound detected, and was particularly higher in the golden brown IT84S-2246 (**Table 13**), red (IT82D-889 and IT97K-1042-3) (**Table 11**) and black IT95K-1105-5 cowpea varieties (**Table 10**). However, proanthocyanidins polymers with a mean degree of polymerization (mDP) of 7 – 9 were reportedly predominant in lentil seed coat followed by oligomers (mDP of 4 – 5) (*136*). We also observed that polymerization (mDP of 3) was a common feature in most of the flavan-3-ol compounds detected in this study, particularly procyanidin trimers T2 and C1 (**Figure 6**). Our method did not allow for detection of DP > 4.0. The highest amounts of trimer T2 was found in the light brown 09FCV-CC27M

cowpea variety (3,950 ± 69.2 μg/g) (**Table 12**), followed by golden brown Ife Brown, red IT97K-1092-1 and light brown IAR-48 varieties. On the other hand, procyanidin trimer C1 content in the light brown 09FCV-CC27M and IAR-48 varieties were 1,187 and 2,613 μg/g, respectively (**Table 12**). These are the only two cowpea varieties in which trimer C1 was found. Black beans have also been reported to contain procyanidin trimers C1 and C2, as well as B2 dimer (*62*). Proanthocyanidin oligomers containing (epi)gallocatechin have also been reported in black beans (*163*). Procyanidin dimers and trimers in adzuki beans reportedly ranges from 15.9 to 213 mg/g dry weight basis (*135*). Seed coats of lentils have also been shown to contain highly polymerized flavan-3-ols (65 – 75%) and very little in monomeric forms (< 2%) (*136*). The levels of procyanidin B2, B3 and procyanidin tetramer in lentils have been reported to range from 0.1 to 0.5 mg/100 g dry weight basis (*60*). In this study, we also found B-type procyanidin dimer, which was particularly higher in the light brown 09FCV-CC27M sample compared to the other cowpea varieties (**Table 12**).

Catechin was also reported as a major flavan-3-ol in the raw cool season food legumes (*71*). The levels of catechin in green pea, yellow pea, chickpea and lentils were found as 205, 281, 1,509 and 696 μg/g, respectively; whereas epicatechin was only found in chickpea (146 μg/g) and lentils (3,819 μg/g). Chickpea and lentils also contained high levels of epigallocatechin and epigallocatechin-gallate (10.1 – 3,819 μg/g). In our study, the highest concentration of catechin/epicatechin was found in the light brown 09FCV-CC27M variety (1090 μg/g) (**Table 12**) and the least in the black IT98K-1092-1 variety (64.1 μg/g) (**Table 10**).

Table 12. Compositions (μg/g of cowpea flour) and effect of boiling on flavonols and flavan-3-ols in light brown cowpea varieties[a]

	Light Brown cowpea varieties					
	IAR-48			**09FCV-CC-27M**		
	Flavonol content (μg/g cowpea flour)					
Compound	*Raw*	*Boiled*	*% change*	*Raw*	*Boiled*	*% change*
Quercetin-3-*O*-glucoside-4′-*O*-diglucoside	27.5 ± 1.74	ND[b]	-	ND	ND	-
Quercetin-3-*O*-arabinosyldiglucoside	75.4 ± 4.88	28.1 ± 0.12	62.7	23.7 ± 2.90	18.5 ± 1.31	21.9
Quercetin-3,7-diglucoside	314 ± 3.2	180 ± 3.9	42.6	90.7 ± 2.83	36.1 ± 0.75	60.2
Quercetin-3-*O*-galactosylglucoside	ND	ND	-	83.0 ± 8.70	64.8 ± 4.59	21.9
Quercetin-3-*O*-galactoside	17.7 ± 1.91	ND	-	ND	ND	-
Quercetin-3-*O*-glucoside	6.8 ± 0.32	ND	-	ND	ND	-
Quercetin-3-*O*-diglucoside	212 ± 5.0	102 ± 7.0	51.8	16.1 ± 2.77	11.4 ± 0.50	29.2
Quercetin-3-*O*-arabinosylglucoside	ND	ND	-	24.0 ± 3.06	10.7 ± 1.48	55.4
Quercetin-3-(6″-feruloyl)-diglucoside	21.5 ± 1.57	ND	-	ND	ND	-
Quercetin-3-(6″-diacetoyl)-diglucoside	20.0 ± 0.32	11.9 ± 1.07	40.6	ND	ND	-
Quercetin-3-*O*-glucosylrhamnoside	ND	ND	-	88.0 ± 3.38	48.2 ± 1.24	45.2
Quercetin-3-*O*-galactosylrhamnoside	ND	ND	-	96.0 ± 4.73	42.8 ± 1.64	55.4
Subtotal quercetin	**695 ± 18.9**	**322 ± 12.1**	**53.7**	**422 ± 28.4**	**233 ± 11.5**	**44.8**
Kaempferol-3-*O*-diglucoside	13.75 ± 0.87	ND	-	ND	ND	-
Subtotal kaempferol	**13.75 ± 0.87**	**ND**	**-**	**ND**	**ND**	**-**
Total flavonols	**709 ± 19.8**	**322 ± 12.09**	**54.5**	**422 ± 28.4**	**233 ± 11.5**	**44.8**
	Flavan-3-ols content (μg/g cowpea flour)					
Catechin-3-*O*-glucoside	1977 ± 10.6	1188 ± 6.3	39.9	2023 ± 2.7	497 ± 4.5	75.4
Procyanidin dimer B-type	919 ± 45.2	425 ± 59.0	53.8	3788 ± 69.2	1727 ± 69.9	54.4
Catechin/epicatechin	703 ± 1.67	521 ± 9.2	25.8	1090 ± 9.9	917 ± 7.4	15.9
Procyanidin trimer T2	1443 ± 43.7	1080 ± 16.0	25.2	3950 ± 69.2	2801 ± 32.8	16.4
Procyanidin trimer C1	1187 ± 53.2	ND	-	2613 ± 33.5	2008 ± 37.9	44.4
Procyanidin tetramer A-type	601 ± 48.9	ND	-	ND	ND	-
Total flavan-3-ols	**6830 ± 203.3**	**2214 ± 90.5**	**71.7**	**13464 ± 184.5**	**8950 ± 152.5**	**35.4**

[a]Data are expressed as mean ± SD (n = 3) on a dry weight basis (μg/g of cowpea flour). Concentrations of glycosides or acyl-glycosides of flavonols were calculated from the standard curve of kaempferol, myricetin, quercetin, quercetin-3-*O*-rutinoside and quercetin 3-*O*-glucopyranoside; while those for flavan-3-ols were calculated from standard curve of catechin and procyanidin B2; but were all adjusted on the basis of molecular-weight differences. [b]ND = not detectable.

Table 13. Compositions (μg/g of cowpea flour) and effect of boiling on flavonols and flavan-3-ols in golden brown cowpea varieties [a]

	Golden Brown cowpea varieties					
	IFE BROWN			**IT84S 2246**		
	Flavonol content (μg/g cowpea flour)					
Compound	*Raw*	*Boiled*	*% change*	*Raw*	*Boiled*	*% change*
Quercetin-3-O-glucoside-4′-diglucoside	ND	ND[b]	-	14.9 ± 2.24	14.3 ± 0.17	4.03
Quercetin-3-*O*-arabinosyldiglucoside	40.9 ± 8.24	ND	-	91.9 ± 4.67	46.6 ± 2.40	49.2
Quercetin-3-*O*-digalactoside	32.4 ± 6.25	ND	-	16.1 ± 1.27	ND	-
Quercetin-3,7-diglucoside	ND	ND	-	21.6 ± 2.86	11.3 ± 0.86	47.7
Quercetin-3-*O*-galactosylglucoside	177 ± 4.5	31.2 ± 1.04	82.4	188 ± 4.12	88.3 ± 3.87	53.0
Quercetin-3-*O*-diglucoside	148 ± 2.1	102 ± 0.20	31.1	157 ± 5.65	10.3 ± 0.05	93.4
Quercetin-3-(6″-feruloyl)-diglucoside	30.4 ± 4.66	14.4 ± 4.43	52.7	ND	ND	-
Quercetin-3-(6″-diacetoyl)-diglucoside	20.2 ± 2.76	11.4 ± 2.01	43.3	9.1 ± 1.31	ND	-
Quercetin-3-(6″-sinapoyl)-diglucoside	10.1 ± 1.86	ND	-	ND	ND	-
Subtotal quercetin	**459 ± 30.4**	**159 ± 7.68**	**65.4**	**499 ± 22.1**	**171 ± 7.4**	**67.6**
Kaempferol-3-*O*-diglucoside	12.2 ± 1.86	5.76 ± 1.77	52.8	12.9 ± 2.50	ND	-
Subtotal kaempferol	**12.2 ± 1.86**	**5.76 ± 1.77**	**52.8**	**12.9 ± 2.50**	**ND**	**-**
Total flavonols	**471 ± 32.3**	**165 ± 9.5**	**64.9**	**512 ± 24.6**	**171 ± 7.4**	**66.6**
	Flavan-3-ols content (μg/g cowpea flour)					
Catechin-3-*O*-glucoside	877 ± 7.0	550 ± 7.3	37.3	1610 ± 13.9	80.5 ± 5.70	95.0
Procyanidin dimer B-type	1855 ± 59.0	1550 ± 48.1	16.4	ND	ND	-
Procyanidin trimer T2	2948 ± 37.2	1665 ± 52.5	43.5	ND	ND	-
Procyanidin dimer-3-diglucoside-7-glucoside	4197 ± 49.6	2783 ± 72.2	33.7	ND	ND	-
Total flavan-3-ols	**9877 ± 152.8**	**6548 ± 180.1**	**33.7**	**1610 ± 13.9**	**80.5 ± 5.70**	**95.0**

[a]Data are expressed as mean ± SD (n = 3) on a dry weight basis (μg/g of cowpea flour). Concentrations of glycosides or acyl-glycosides of flavonols were calculated from the standard curve of kaempferol, myricetin, quercetin, quercetin-3-*O*-rutinoside and quercetin 3-*O*-glucopyranoside; while those for flavan-3-ols were calculated from standard curve of catechin and procyanidin B2; but were all adjusted on the basis of molecular-weight differences. [b]ND = not detectable.

The identification and quantification of detected flavan-3-ol derivatives in cowpeas, such as catechin-3-*O*-glucoside and procyanidin dimer-3,7-diglucoside, in addition to procyanidin trimer C1, procyanidin trimer T2 and procyanidin tetramer A-type provide new insight on the qualitative flavonoid composition in cowpea that may have health promoting effects in humans. Further characterization of flavan-3-ols in cowpea is still needed. Although the UPLC reversed phase C18 column used in this study had the ability to separate flavan-3-ol monomers and oligomers of equivalent MW into their isomers, the isolation and quantification of heterogeneous flavan-3-ol oligomers (e.g. hexamers; mDP≥ 5) was not feasible. Because of possible overlapping of the isomers with different mean degrees of polymerization or stereochemistry, it is likely that the oligomeric proanthocyanidins co-eluted in some of the large unresolved peak (**Figures 14A-1** and **14B-1; 19A-1** and **19B-1**).

3.9 Effect of boiling on flavan-3-ol content in cowpea

Boiling was found to significantly reduce flavan-3-ol compounds in cowpeas (**Tables 10 – 13**). The greatest impact of processing on total flavan-3-ols was found in the golden brown IT84S-2246 (**Table 13**) and red IT97K-1042-3 (**Table 11**) cowpea varieties, in which boiling reduced most of their flavan-3-ol compounds to undetectable levels. A likely reason for lower flavan-3-ols contents in the boiled cowpea samples is that proanthocyanidins bind with peptides during cooking, forming insoluble complexes held together by hydrogen and/or hydrophobic bonding, thus decreasing their

extractability. Similar interactions between proanthocyanidin and protein have been associated with haze formation in beer and apple juice (*164*).

Table 14. Compositions (μg/g of cowpea flour) and effect of boiling on flavonols in green (TX2028-1-3-1) and white (Early Acre) cowpea varieties [a]

	Green and White cowpea varieties					
	TX2028-1-3-1 (GREEN)			**EARLY ACRE (WHITE)**		
	Flavonol content (μg/g cowpea flour)					
Compound	*Raw*	*Boiled*	*% change*	*Raw*	*Boiled*	*% change*
Quercetin-3-*O*-triglucoside	ND[b]	ND	-	14.9 ± 2.24	14.3 ± 0.17	4.02
Quercetin-3-*O*-glucoside-4′-*O*-diglucoside	35.2 ± 2.82	ND	-	17.4 ± 1.57	11.7 ± 0.53	32.8
Quercetin-3-*O*-arabinosyldiglucoside	60.4 ± 4.50	52.4 ± 2.19	13.2	11.4 ± 0.91	ND	-
Quercetin-3-*O*-digalactoside	22.9 ± 1.56	11.9 ± 0.23	48.0	48.5 ± 6.25	21.1 ± 0.23	56.6
Quercetin-3,7-diglucoside	91.8 ± 7.82	59.4 ± 1.13	35.3	31.4 ± 9.22	20.3 ± 0.33	35.4
Quercetin-3-*O*-galactosylglucoside	34.6 ± 3.14	ND	-	33.7 ± 1.12	ND	-
Quercetin-3-*O*-galactoside	ND	ND	-	5.0 ± 0.70	ND	-
Quercetin-7-*O*-glucoside	28.7 ± 3.37	9.5 ± 0.74	66.8	ND	ND	-
Quercetin-3-*O*-diglucoside	42.1 ± 1.39	21.0 ± 1.06	50.1	77.2 ± 2.80	37.1 ± 0.42	51.9
Quercetin-3-(6″-feruloyl)-diglucoside	11.5 ± 1.27	ND	-	14.5 ± 1.64	ND	-
Subtotal quercetin	**327 ± 25.9**	**154 ± 5.4**	**53.2**	**254 ± 26.5**	**105 ± 1.7**	**52.8**
Myricetin-3-*O*-glucoside	24.8 ± 0.92	ND	-	ND	ND	-
Subtotal myricetin	**24.8 ± 0.92**	**ND**	**-**	**ND**	**ND**	**-**
Kaempferol-3-*O*-diglucoside	ND	ND	-	15.8 ± 2.3	13.5 ± 0.7	14.6
Subtotal kaempferol	**ND**	**ND**	**-**	**15.8 ± 2.3**	**13.5 ± 0.7**	**14.6**
Total flavonols	**352 ± 26.8**	**154 ± 5.4**	**56.3**	**270 ± 28.8**	**119 ± 2.4**	**55.9**

[a]Data are expressed as mean ± SD (n = 3) on a dry weight basis (μg/g of cowpea flour). Concentrations of glycosides or acyl-glycosides of flavonols were calculated from the standard curve of kaempferol, myricetin, quercetin, quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucopyranoside; while those for flavan-3-ols were calculated from standard curve of catechin and procyanidin B2; but were all adjusted on the basis of molecular-weight differences. [b]ND = not detectable.

In general, changes in flavan-3-ols content in cowpea after boiling depended on cowpea variety. Since cowpea must be cooked before consumption, these observations suggest that proper cooking methods should be chosen to preserve these flavonoids and improve health-promoting potential of processed cowpea foods. The deleterious effect of heat on flavan-3-ols was also observed by Xu and Chang (*130*) who reported that regular boiling significantly ($p < 0.05$) decreased flavan-3-ols (catechin, epicatechin and epicatechin-gallate) contents in black beans as compared to the raw black beans.

3.10 Flavonoid content in cowpeas

Among all the samples, the light brown phenotype had the highest average flavonoid content, followed by the black, red, golden brown, green and then the white varieties (**Table 15**). The phenotypic differences in total flavonoid content indicate that seed coat color affect synthesis and/or accumulation of certain groups of flavonoids (**Tables 10 – 14**). For example, the predominant group in the light brown 09FCV-CC27M variety was the flavan-3-ols, contributing 97% of the total flavonoid content (**Tables 12** and **15**); anthocyanins contributed 23.5% of the total flavonoid content in the black IT95K-1105-5 variety (**Tables 4** and **15**); and flavonols contributed 30.8% of the total flavonoid content in the red IT97K-1042-3 variety (**Tables 11** and **15**). However, there were also significant differences in the total flavonoid content between two varieties having the same seed coat color, especially in the light brown IAR-48 and 09FCV-CC-27M (**Table 15**). In general, the light brown 09FCV-CC-27M contained the

highest flavonoid content; followed by the black IT95K-1105-5 and red IT97K-1042-3 variety (**Table 15**).

Table 15. Flavonoid content of raw cowpea samples. See ***appendix A*** for photos of the cowpea phenotypes studied.

	Seed weight[†] **(g/100g seeds)**	**Phenotype**	**Flavonoid content (*μ*g/g)**[‡]
IT95K-1105-5[a]	23.4 ± 0.37	Black	8915 ± 197.4
IT98K-1092-1[a]	11.6 ± 035	Black	6111 ± 138.9
IT82D-889[b]	11.3 ± 0.17	Red	1227 ± 55.3
IT97K-1042-3[b]	13.1 ± 0.20	Red	3057 ± 43.6
TX2028-1-3-1[a]	21.6 ± 0.45	Green	10512 ± 176.0
IAR-48[b]	22.6 ± 0.22	Light brown	8539 ± 223.1
09FCV-CC27M[b]	14.9 ± 0.40	Light brown	13886 ± 232.9
IFE BROWN[b]	15.8 ± 0.16	Golden brown	10348 ± 185.1
IT84S-2246[b]	17.5 ± 0.08	Golden brown	2122± 38.5
EARLY ACRE[c]	11.6 ± 0.16	White	270 ± 28.8

[†]Seed weight expressed as mean ± SD of triplicate weights of 100 seeds. [‡] Sum of HPLC quantified anthocyanins, flavonols and flavan-3-ols (up to tetramers). [a]Contain anthocyanins, flavonols and flavan-3-ols; [b]Contain flavonols and flavan-3-ols; [c]Contain flavonols only.

3.11 Conclusions

From this study, seed-coat color may be a good indicator of the accumulation of flavonoids in cowpeas. Cowpea with dark-colored seed coats, especially the black varieties contain high levels of anthocyanins, whereas the red seed-coated varieties contain high levels of flavonols. The red, black, light brown and golden brown varieties were also found to contain high concentration of flavan-3-ols. Levels of individual phenolic compounds were variety-dependent rather than phenotype-dependent.

Eight anthocyanins and 72 other phenolic compounds in cowpea seeds were identified and characterized using LC coupled with Tandem Quadrupole eλ Detector (TQD). We found, for the first time, petunidin-3-*O*-galactoside in black and green cowpea phenotypes. The concentrations of the anthocyanins identified in the black and green cowpea varieties were in the following order: delphinidin-3-*O*-glucoside > cyanidin-3-*O*-glucoside > petunidin-3-*O*-glucoside > malvidin-3-*O*-glucoside > delphinidin-3-*O*-galactoside > cyanidin-3-*O*-galactoside > peonidin-3-*O*-glucoside > petunidin-3-*O*-galactoside. Relatively higher amounts of these compounds were found in the black IT95K-1105-1, followed by black IT98K-1092-1 and then green TX2028-1-3-1. There were no detectable levels of pelargonidin and its derivatives in all cowpea samples analyzed.

In the case of flavonols, quercetin and its derivatives were the major constituents, followed by myricetin derivatives. The highest amount of quercetin derivatives was found in the red varieties. The three most dominant quercetin derivatives identified in the raw cowpea samples were quercetin-3,7-diglucoside, quercetin-3-*O*-galactosylglucoside and quercetin-3-*O*-diglucoside. The light brown 09FCV-CC27M variety contained the least amount of quercetin compounds. On the other hand, myricetin was only detected in the black, red and green cowpea varieties. Myricetin in cowpeas was mainly in the form of myricetin-3-*O*-glucoside and myricetin-3-*O*-diglucoside; and were highest in red IT97K-1042-3 sample.

In the case of flavan-3-ols, the highest amounts were detected in the light brown 09FCV-CC27M cowpea variety compared to the other samples. Within the light brown

phenotype, the 09FCV-CC27M sample contained twice as much flavan-3-ols as IAR-48 variety, whereas the later had four times as much flavonols than the former; suggesting one synthetic pathway is sacrificed for the other in light-brown phenotypes. The green TX2028-1-3-1 and white Early Acre cowpea varieties did not contain detectable levels of flavan-3-ols. Catechin-3-*O*-glucoside was found as the major flavan-3-ol monomer in cowpea. Polymeric procyanidins made up a large proportion of the cowpea phenolics.

We found, for the first time, acylated derivatives of flavonols in cowpea, specifically malonoyl-, sinapoyl-, feruloyl-, acetoyl- and succinoyl- derivatives of quercetin glycosides, as well as flavononols (taxifolin derivatives), flavanones (eriodictyol derivatives), dihydrochalcones (phloretin derivatives), phenolic acids and phenolic aldehyde. The presence of these compounds in the seeds may contribute to the high antioxidant activity observed for cowpea seeds. The unidentified peaks and information on the position, type and stereochemistry of their substituent groups would require the use of NMR technology. However, this would require extensive sample purification as well as several orders of magnitude more analyte than the low concentrations used in HPLC–MS analysis.

Boiling significantly reduced the levels of individual and total flavonoid contents in cowpea. The decrease in flavonoid content after boiling is related to the losses of individual phenolic compounds, which could be resulting in the formation of other phenolics such as protocatechuic acid, 4-hydroxybenzoic acid and phloroglucinaldehyde. Since these heat-induced degradation products are structurally different from their precursor aglycones, they cannot compensate for the bioactivity of their precursors,

which may lead to lower antioxidant activity in the boiled samples even though these compounds would still be biologically potent. Thus, proper cooking methods should be chosen to preserve flavonoids in cowpea in order to improve health-promoting potential of processed cowpea foods.

In summary, cowpea is a good source of flavonoids; however, great variability in flavonoid content exists within and among cowpea phenotypes. In this study, we show that when specific flavonoids are required in cowpea diets, the black seed-coated cowpea is a good source of anthocyanins; red seed-coated cowpea is a good source of quercetin; and, light brown seed-coated 09FCV-CC27M variety is a good source of flavan-3-ols. Therefore, we recommend that the use of cowpea as a source of flavonoids in diets should consider the differences in flavonoid levels among different cowpea varieties. Since variety has an impact on important traits such as types of flavonoids synthesized, flavonoid content should be incorporated into cowpea breeding programs as a new trait for selection. Further studies are needed to address the effect of growth conditions on synthesis of individual flavonoids identified in this study and to validate the observed relationship between cowpea seed-coat color and their reported flavonoid profiles.

4. EFFECT OF BOILING ON THE ANTIOXIDANT PROPERTIES OF DIFFERENT COWPEA PHENOTYPES

4.1 Introduction

Accumulation of flavonoids in legume seeds is restricted to the seed coat, and affects their antioxidant capacity (*65*). The phenolic compositions of beans comprise condensed tannins, anthocyanins, flavonols, as well as phenolic acids. Of these, condensed tannins are the predominant phenolic compounds found in legume seeds (*113, 165*). The importance of legume seed coat on antioxidant activity of legumes has been reported by several authors, and has been associated with high levels of tannins in dark colored species (*114*). Tannins are considered anti-nutritional since they negatively affect protein digestibility and Fe availability. Tannins are also superior at quenching free radicals than other flavonoids (*166*), suggesting that dehulling or decorticating of cowpeas (legumes) during processing for human consumption may lead to lower tannin content and improved protein digestibility but lower antioxidant activity. In addition, dark colored species of pulses such as beans (*Phaseolus vulgaris* L.) has also been associated with higher antioxidant activity than the white beans due to the presence of anthocyanins in the seed coat of the colored varieties (*167*).

The importance of polyphenolic antioxidants in the maintenance of health and protection from cardiovascular diseases, coronary heart disease and cancer is also raising interest among scientists, food manufacturers and consumers (*168*).

Various processing methods have been shown to significantly reduce the health promoting phenolic compounds and antioxidant activities of legumes (*131*). Soaking and boiling of cowpeas are some of the traditional methods for preparing cowpeas for human consumption in the developing countries, whereas pressure cooking and steaming methods are mostly utilized in the developed countries. Such thermal processes improve flavor and palatability of cowpeas, and increase the bioavailability of nutrients by eliminating or inactivating antinutritional factors (e.g. trypsin inhibitor, saponins, agglutinins, lectins, flatulence causing oligosaccharides, etc) (*119*). However, thermal processing is detrimental to the phenolic composition of legumes. For example, the boiling process destroyed 40 – 60% of total phenol content in green peas, yellow peas and chickpeas; and 60% in lentils (*71*). The overall negative effect of thermal processing on antioxidant properties of polyphenols could be due to leaching of the water-soluble antioxidants, degradation of anthocyanins and other antioxidant components (*75*), interactions between natural and heat-induced antioxidants, and the combined effect of other oxidative reactions during cooking (*169*). The reduction in antioxidant activity by processing caused lower antiproliferation capacities of cool season food legumes against cancer cells compared to the raw samples (*71*).

Cowpea has many nutritional qualities that make it attractive as a functional food. However, even though processed cowpea is useful as human food, the effect of processing on their health-promoting properties such as total phenol content and antioxidant activities remain unexplored. Since antioxidant activity is a fundamental property associated with anti-inflammatory and anti-carcinogenic property of plant

polyphenols, retention of antioxidant activity after commonly used processing methods for cowpea preparation such as boiling becomes important in ensuring that processed cowpea can still prevent inflammatory-related diseases. Therefore, to continue our investigation on thermal processing effects on cowpea phytochemicals, the present study was undertaken to further elucidate how boiling affects bioactive properties such as total phenol content, condensed tannin content and antioxidant activities of cowpea and correlate of these properties to cowpea variety and/or phenotype.

4.2 Materials and methods

4.2.1 Cowpea samples

The cowpeas presented in **Table 2** were used in this investigation. Broken and damaged seeds, as well as foreign materials were removed from the samples. Cooking was conducted by soaking 100 g raw seeds in 450 mL distilled water for 12 hours at room temperature, and then boiling for 15 min [the average time adequate tactile texture (resistance to pressure between the fingers) was achieved] (*122*). Cooking time commenced when the cook-water started boiling. Both the boiled seeds and the soup were then frozen to –80°C and then freeze-dried. Both raw and cooked freeze-dried cowpea seeds were ground using a coffee grinder (Cuisinart, Model DCG-20N series) to pass through a 60-mesh sieve. To determine the moisture content, triplicate samples were dried in an air circulated oven at 110°C until a constant weight was obtained.

4.2.2 Chemicals and reagents

Folin-Ciocalteu reagent, ethanolamine, gallic acid monohydrate, vanillin reagent, (+)-catechin hydrate, fluorescein disodium, ABTS ′(2,2 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), potassium persulfate, sodium fluorescein and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). AAPH (2′,2 -Azobis(2-amidinopropane) dihydrochloride) were purchased from Wako Chemicals USA (Richmond, VA). All other chemicals were analytical grade.

4.2.3 Determination of total phenols content (TPC)

The total phenols content (TPC) was determined by a Folin-Ciocalteu assay described by Kaluza *et al*. (*170*) using gallic acid (GA) as the standard. Briefly, 0.1g of the raw and cooked ground cowpea samples were separately extracted with 10 mL of aqueous 70% acetone by continuously shaking on a Standard Analog Shaker (VWR, USA) at ambient temperature for 8 hours, centrifuged (10000 *g*-force for 10 min) and the supernatants collected. The extractions were performed in triplicates for each individual cowpea variety.

The extracted aqueous samples (0.1 mL) were added into 1.1 mL distilled water then reacted with 0.4 mL Folin reagent and 0.9 mL 0.5 M ethanolamine for 20 min at room temperature. The absorbances were measured using a UV-visible spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan) at 600 nm against a reagent

blank. The TPC values were expressed as micrograms of gallic acid equivalent per gram of cowpeas (μg GAE/g) on dry weight basis through the calibration curve of gallic acid.

4.2.4 Determination of condensed tannins content (CTC)

The condensed tannin content analysis were performed according to the method of Broadhurst and Jones (*171*) with slight modification. Approximately 200 mg of the raw and cooked ground cowpea materials were separately weighed into capped centrifuge tubes and extracted with 8 mL of aqueous 70% acetone in 30°C water bath for 20 minutes. Triplicate extractions were perfomed for each cowpea variety. The tubes were vortexed at 5 min intervals during the incubation period, and were then centrifuged at 10000 *g*-force for 10 min. Two 1-mL aliquots of the respective supernatants were transferred into clean test tubes and placed into the water bath. The absorption was measured using a UV-visible spectrophotometer (UV-2450, Shimadzu, Tokyo, Japan) at 500 nm against a methanol blank. The tannin content was expressed as milligrams of catechin equivalent per gram of cowpeas (mg CAE/g) on dry weight basis using the calibration curve of (+)-catechin.

4.2.5 Determination of scavenging activity on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^{\bullet+}$)

Three replicates of 0.1g of the raw and cooked ground cowpea samples were separately extracted with 10 mL of aqueous 70% acetone by continuously shaking on a

Standard Analog Shaker (VWR, USA) at room temperature for 4 hours and centrifuged (10000 *g*-force for 10 min). The supernatants were used to determine the scavenging activity of $ABTS^{\bullet+}$ (*172*). A mixture of ABTS (8 mM) and potassium persulfate (3 mM) in distilled water was prepared and reacted at room temperature for 12 hours in the dark.

The ABTS working solution was prepared prior to the assay by diluting 10 mL ABTS stock solution with 290 mL of pH 7.4 PBS buffer to an absorbance of 1.5 at 734 nm. The aqueous sample solutions (0.1 mL) was added to the diluted ABTS working solution (2.9 mL) and reacted at ambient temperature for 30 min. These were conducted in triplicates. The standards consisted of a series dilution of Trolox (0, 100, 200, 300, 400, 500, 600, 700, 800, 1000 μM in methanol; r = 0.99). The radical scavenging activity was expressed as μmol Trolox equivalent/g sample on dry weight basis [μmolTE/g (db)].

4.2.6 Oxygen Radical Absorbing Capacity (ORAC) Assay

The ORAC assay was performed using a Biotek Synergy HT plate reader with automatic dispenser (Biotek, Winooski, VT) according to the method described by Talcott and Lee (*173*) with slight modifications. Approximately 100 mg of raw and cooked ground cowpea samples were separately soaked in 10 mL of 70% aqueous acetone in triplicates for 3 hours at 4°C and then shaken for 3 hours at ambient temperature. After centrifuging, 4 mL aliquots of the supernatants were transferred into clean test tubes and stored at 4°C until analyzed. Prior to the analysis, the extracts were properly diluted with distilled water to fit within the linearity range of the Trolox standards. The diluted aqueous cowpea extracts (25 μL) and Trolox standards (25 μL)

were analyzed in triplicates on a solid black 96-well plate. The Trolox standards (0, 6.25, 12.5, 25, 50 and 100 μM) and sodium fluorescein working solution (4×10^{-3} μM) and 2,2'-azobis-2-methyl-propanimidamide dihydrochloride AAPH (153 mM) were prepared using PBS buffer (pH 7.4). Prior to incubation, 150 μL of sodium fluorescein working solution was added to each well and the microplate (sealed with film) was incubated for 30 min in the plate reader set at 37°C. After the incubation period, 25 μL of the peroxyl generator (153 mM AAPH) was automatically dispensed into appointed wells according to the layout to initiate the oxidation reaction.

After shaking the microplate for 15 seconds, the plate reader begun taking the kinetic readings of the fluorescence changes at 1 min intervals for 90 min. The area under the fluorescence decay curve (AUC) (excitation 485 nm, emission 528 nm) was used to calculate the antioxidant capacity; AUC = 0.5 + (R2/R1) + (R3/R1) + (R4/R1) + . . . + (R*n*/R1), where R1 corresponded to the fluorescence reading at the initiation of the reaction and R*n* was the fluorescence reading at the *n*th minute. The net AUC was determined by subtracting the AUC of the blank from that of a sample or standard. A standard curve for the Trolox was then obtained by plotting the net AUC of the series of Trolox standards versus their concentrations (r = 0.99). Trolox equivalent (TE) of each sample was calculated by interpolating the net AUC of sample against the Trolox standard curve. The ORAC values of the cowpea extracts, analyzed in triplicates, were averaged and expressed as μmol TE/g db.

4.3 Statistical Analysis

Data are reported as means ± SD of 3 replicates; and analyzed using 2005 SAS (Version 9.1, SAS Inst. Inc., Cary, N.C., U.S.A.) with one-way Analysis of Variance (ANOVA). Post Hoc test (Fisher's LSD and Tukey-Kramer HSD) after ANOVA was used to compare treatments means. Significant levels were defined using $p < 0.05$. Correlations between variables were performed using Pearson correlation tests.

4.4 Results and discussion

4.4.1 Effect of seed coat color on total phenolic composition of cowpea

The total phenolic content (TPC) and condensed tannin content (CTC) of the extracts from different varieties of cowpeas (raw and cooked) are presented in **Table 16**. Significant differences ($p < 0.05$) in TPC and CTC were found among the raw cowpea phenotypes as well as among the varieties.

Among all the samples, the black, red and light brown phenotypes had the highest TPC values (**Table 16**). This could be due to high amounts of anthocyanins, flavonols and flavan-3-ols in the black (**Table 4**), red (**Table 11**) and light brown varieties (**Table 12**), respectively. Specifically, the light brown 09FCV-CC27M variety had the highest TPC (14.9 mg GAE/g), followed by black and red varieties. The high TPC also correlated with high total flavonoid content reported for these varieties (**Table 15**). The Early Acre (white) and green TX2028-1-3-1 varieties had the lowest TPC (2.6 – 2.9 mg GAE/g) (**Table 16**); this could be due to the much lower levels of flavonol and

no detectable flavan-3-ols in the white and green varieties compared to the other samples (**Table 14**). In general, the CTC values also followed the same trend (**Table 16**).

Table 16. Levels of Total Phenolic Content (TPC) and Condensed Tannin Content (CTC) of raw and boiled cowpeas.* See ***appendix A*** for the photos of the cowpea phenotypes studied.

Cowpea variety	Phenotype	TPC (raw) (mg GAE/g)	TPC (boiled) (mg GAE/g)	TPC % change	CTC (raw) (mg CAE/g)	CTC (boiled) (mg CAE/g)	CTC % change
IT 98K-1092-1	Black	10.6b	6.7b	36.9	8.9b	3.1c	65.5
IT 95K-1105-5	Black	14.4a	8.2b	43.5	11.8a	4.2b	64.1
IT 82D-889	Red	9.8b	6.7b	31.7	7.2b,c	3.1c	56.3
IT 97K -1042-3	Red	10.3b	7.2b	30.4	6.5c	3.0c	54.2
TX 2028-1-3-1	Green	2.9d	2.5d	13.7	0.9d	0.7e,d	27.0
EARLY ACRE	White	2.6d	2.7d	+5.0	0.3d	0.5e	+60.9†
IAR-48	Light brown	4.8c,d	4.0c,d	16.4	1.7d	0.9e,d	44.6
09FCV-CC-27M	Light brown	14.9a	10.2a	31.6	12.6a	5.0a	60.4
IFE BROWN	Golden brown	6.3c	4.8c	23.9	1.9d	1.0d	45.4
IT 84S-2246	Golden brown	6.2c	4.2c,d	32.4	1.7d	0.9e,d	45.9

*Data are expressed as means of triplicate experiments on dry weight basis. †Values determined from measurements falling within margin of error. Values marked by the same letter in each column are not significantly different ($p < 0.05$).

The difference in TPC and CTC values within phenotypes were generally small; except light brown varieties (**Table 16**). This could be due to the significantly high levels of flavan-3-ol compounds in the light brown 09FCV-CC27M variety than in IAR-48 sample (**Table 12**), suggesting that in cowpea, the association of dark-colors with higher phenolic content may be misleading since the 09FCV-CC27M variety is not dark-

colored yet it contained the highest concentrations of total phenolics compared to the black and red seed coated varieties.

The average TPC and CTC values for the cowpea varieties analyzed appear to have the following trend: light brown > black > red > golden brown > green and white (**Table 16**), suggesting cowpea phenolic content and composition may be highly influenced by seed coat color. These observations appear to indicate that in cowpea, dark colored seed coat does not necessarily translate to higher total phenolic compositions.

4.4.2 Effects of boiling on total phenolic composition of cowpeas

Previous literature (*174*) reported that about 26 to 52% of TPC could be lost with the soak water if discarded – a common practice in preparation of legumes for human consumption. Bressani and Elias (*175*) also observed that cooking and discarding the cooking water removes about 30 to 40% of phenolics from common beans. In this study, the soak water was eventually used to cook the seeds, and the remaining water after cooking were freeze-dried to avoid losing water soluble polyphenolics which may have leached into the cook water. As presented in **Table 16**, the TPC and CTC of cooked cowpea samples were significantly reduced ($p < 0.05$) compared to the respective original uncooked seeds, except for the Early Acre (white) variety.

Among all the phenotypes studied, the black cowpea seeds showed the greatest decrease in TPC and CTC as a result of boiling. In fact, about 36.9 – 43.5% of measurable TPC and 65.5 – 64.1% of CTC for the black cowpea varieties (IT 98K-1092-1 and IT 95K-1105-5) were lost due to boiling (**Table 16**). Since these varieties also

contained greater concentrations of anthocyanins (**Table 4**), it is possible that the reduction in polyphenolic compositions may be due to significant degradation of the heat labile anthocyanin pigments.

In general, the observed losses in TPC and CTC of cowpea polyphenolic composition following boiling (**Table 16**) could be due to instability of phenolic compounds to heat treatment causing extensive structural breakdown and transformations of the polyphenolics. Our results showed trends similar to those reported by Xu and Chang (*72, 174*) who demonstrated that thermal treatment decreased TPC in peas and black common beans (*P. vulgaris*) by 50 – 70% and 70 – 80%, respectively; as well as by Xu and Chang (*131*) who reported that about 43 – 62% of TPC and 28 – 36% of CTC were decreased in cooked black soybean.

However, processing can also induce freeing of phenolics from their bound forms, cause formation of new compounds (*176, 177*), as well as alter extractability of phenolic compounds which may affect TPC. Turkmen *et al*. (*70*) reported that conventional cooking methods (boiling and steaming) significantly ($p < 0.05$) increased the TPC for pepper, broccoli and green beans. Steam cooking was also reported to increase the TPC for sweet potatoes by 2 – 13 times (*178*). In our study, we observed approximately 5% increase in the TPC for boiled white cowpea (**Table 16**). Though this increase was not significant ($p < 0.05$), it confirms the ability of the thermal energy to enhance extractability of phenolics by releasing them from their bound forms. Earlier reports (*131*) also showed that thermal processing of yellow soybean, such as pressure steaming increased TPC by about 35%; and that regular boiling, regular steaming and

pressure boiling increased the total flavonoid content of soybeans by 60 – 90% and their CTC by 20 – 60%. The increase in TPC could be due to the freeing of bound phenolic or release of phenolic-like substances from polymeric substances present in the cell wall upon thermal processing; which may also react with Folin-Ciocalteu reagent.

In summary, boiling significantly affected the TPC (14 – 44% reduction) and CTC (27 – 66% reduction) in cowpeas. This effect was variety-dependent. The final TPC and CTC after boiling depended on the initial distributions and compositions of individual compounds in the cowpea samples studied (**Tables 4; 10 – 14; 16**).

4.4.3 Antioxidant capacity of cowpeas with different seed coat colors

Antioxidant activity assays are reaction-mechanism-dependent. The ORAC method uses the hydrogen atom transfer (HAT) mechanism based on how competitive an antioxidant is to trap free radicals; while ABTS uses the electron transfer (ET) mechanism involving non-competitive redox reaction between the antioxidant and oxidant (*179, 180*). These methods have different sensitivity and specificity properties, thus one method may not completely account for all the antioxidants in the extracts. Thus, the use of both HAT and ET assays provides a more reliable determination of the antioxidant activity profiles of the cowpea samples. The antioxidant activities of the raw and cooked cowpeas, based on ABTS and ORAC assays are presented in **Figure 21.**

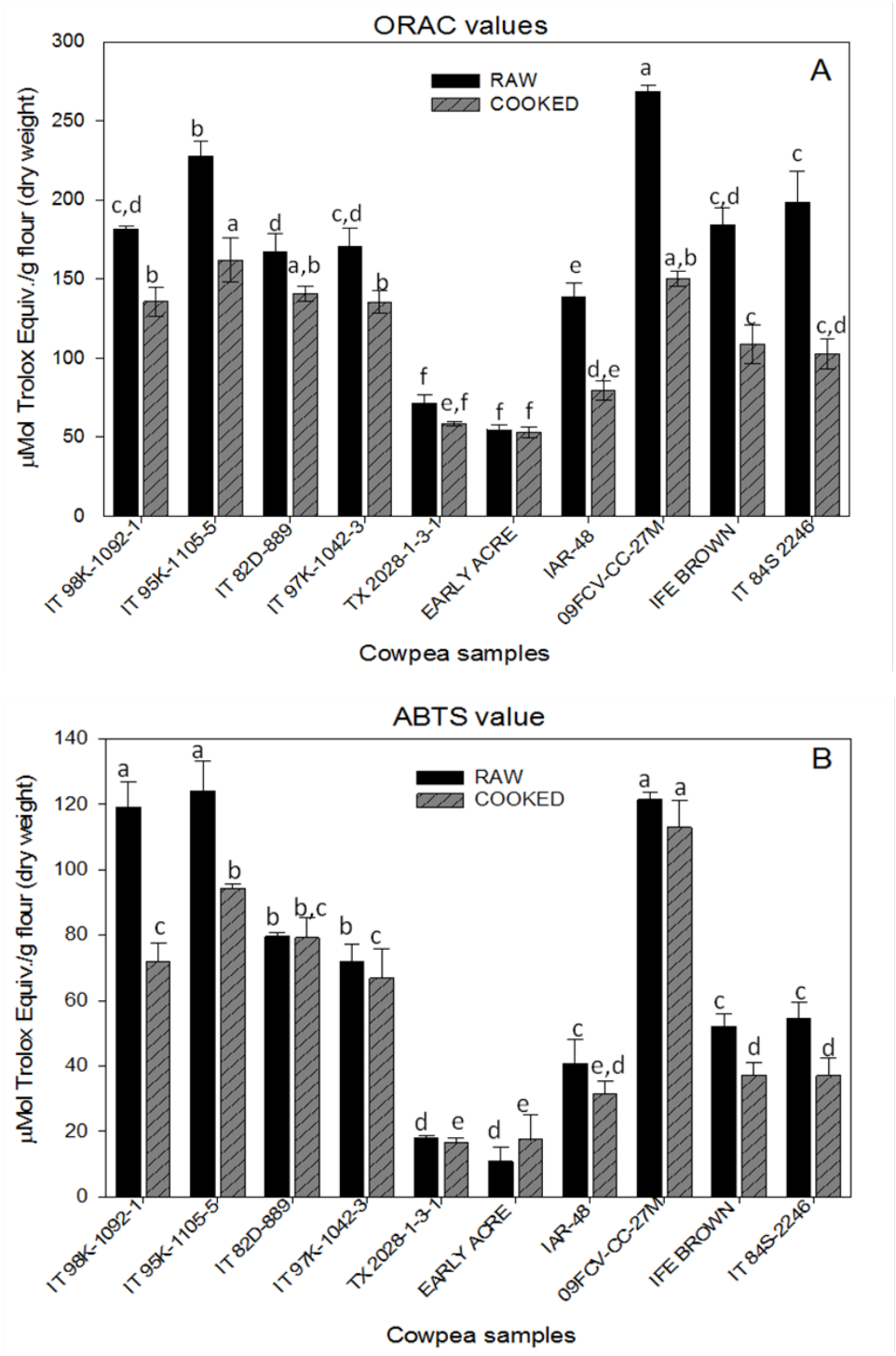


Figure 21. Antioxidant activities of various raw and cooked cowpeas varieties: ORAC values, **A**; and ABTS values, **B**. Bar data are expressed as mean ± standard deviation (n = 3) on dry weight basis. Values marked above the same color bars with the same letter are not significantly different ($p < 0.05$).

In general, the black, red and light brown cowpeas had the highest antioxidant activity determined by both ORAC and ABTS methods, whereas the white and green varieties had the lowest antioxidant activity (**Figure 21**). The presence of high levels of anthocyanins in the black (**Table 4**), flavonols in the red (**Table 11**) and tannin-compounds in the light brown phenotypes (**Table 12**) contributed to their strong ability to quench free radicals. This indicated a positive correlation existed between levels of phenolic compounds and antioxidant activity of cowpea. Xu and Chang (*57*) also found high antioxidant activity of red and black varieties of *Phaseolus vulgaris* than those of their white counterparts. Warrington *et al*. (*181*) also reported significant differences in radical scavenging activity among *Vigna* spp; those with pigmented seed coats, especially black and red varieties had the highest antioxidant capacity values, followed by pinkeye and black-eye types, and then the cream and white types were the lowest.

Our studies show that the average antioxidant activity of the six raw cowpea phenotypes investigated is in decreasing order: black > light brown > red > golden brown > green > white. The relatively higher antioxidant activities of the black, light brown and red phenotypes could be due to their higher TPC and CTC (**Table 16**), as well as their flavonoid compositions. Thus, in addition to their traditional role of preventing protein malnutrition, intake of black, light brown and red cowpea varieties may prevent degenerative diseases associated with oxidative damage.

4.4.4 Effect of boiling on antioxidant activities of cowpeas

Figure 21 shows the effect of boiling on antioxidant activity of different cowpea phenotypes. All phenotypes showed reduction in antioxidant activity after boiling. On average, the greatest losses occurred in the black phenotypes (IT98K-1092-1 and IT95K-1105-5), which showed 25.4 – 28.8% and 24.1 – 39.7% reduction in ORAC and ABTS values, respectively (**Figure 21**); followed by the golden brown, light brown and red phenotypes assayed by the ORAC method (**Figure 21A**). This effect could be due to significant losses in TPC and CTC observed in these phenotypes after boiling (**Table 16**). The overall reduction in antioxidant activity of boiled cowpeas can be attributed to the synergistic effects of heat induced oxidative reactions, breakdown of heat-labile antioxidant compounds and possibly polymerization reactions which reduce extractability of the phenolic components resulting in lower TPC and CTC. Boiling significantly reduced antioxidant activity of other pulses such as pinto beans and black beans (*130*); green pea, yellow pea, chickpea and lentils losses were also attributed to thermal degradation of the antioxidants (*71*).

The reduction in overall antioxidant properties of boiled cowpeas is attributed to the degradation of the phenolic compounds, and heat-induced interactions between the antioxidants and other plant components like starch.

A significant (61.4%) increase in antioxidant activity by boiling was found in the white Early Acre variety (**Figure 21B**). This increase in antioxidant activity of the Early Acre extract was likely due, at least in part, to the release of bound phenolic acids such as feruloylaldaric acid derivatives that were detected in this variety (**Figures 12** and **18**).

Heat treatment has been shown to improve extractability and liberation of low molecular weight phenolic acids (*182*). The presence of protocatechuic acid and protocatechuic aldehyde in the red phenotype (**Figure 3**) may also explain why red cowpea varieties had the least losses of ORAC and ABTS values despite containing higher levels of heat-labile flavonols. Thus, the presence of lower molecular weight phenolic compounds in cowpea may indicate of greater retention of antioxidant activity after heat treatment.

4.4.5 Conclusions

Boiling significantly affected the TPC and CTC of all cowpea varieties analyzed, as well as antioxidant activity associated with these compounds. The net reduction in antioxidant activity after boiling was less than the net TPC reduction, which suggested that phenolic degradation products may contribute to antioxidant activity. It is believed that beneficial plant components are significantly lost through food preparation processes such as sterilization and home-cooking. Exploring processing methods that improve retention of the heat labile antioxidants and phenolic components is recommended.

5. ANTI-INFLAMMATORY PROPERTIES OF COWPEA PHENOTYPES WITH DIFFERENT PHENOLIC PROFILES IN INTESTINAL MYOFIBROBLAST CCD18Co CELLS

5.1 Introduction

Inflammation is generally a protective response induced by microbial infection or tissue injury. The main purpose of inflammation is elimination of disease causing pathogens, healing injured tissues at both cellular and molecular levels and maintenance of homeostasis equilibrium (*14*). Maintenance of this equilibrium is important since the inflammatory response in certain chronic inflammatory diseases such as CVDs and cancers have been shown to cause more damage to the host than the microbe (*183*). Since chronic inflammation can lead to extensive destruction of other neighboring tissues, an ideal inflammatory response should be rapid, destructive and specific, yet self-limiting (*183*). For example, malignant conversion of normal colonic cells following sustained chronic inflammation causes colorectal cancer, the second leading cause of cancer-related deaths worldwide (*184, 185*).

Malnutrition also causes inflammation, and has been one of the main leading causes of mortality and morbidity among the poor and children in developing countries who have limited access to adequate medical care and nutritious foods (*25*). Over-reliance on maize (poor source of tryptophan and lysine) and other cereal grains, especially in sub-Saharan Africa results in malnutrition. These regions are characterized by inadequate rainfall and sandy soils which limit types of crops that may be cultivated.

Cowpea is a heat and drought tolerant legume. It is a nutritious crop that is also highly adaptable to different soil conditions, thus, may be suitable in mitigating malnutrition in such regions. Moreover, the phenolic compounds such as anthocyanins (*46*), flavonols and isoflavonols (*51*) and phenolic acids (*47, 51*) previously identified in cowpeas have been shown to have both anti-inflammatory effects and chemopreventive properties in colon cancer (*186, 187*) as well as ability to reduce risk of coronary heart disease (*110*). Thus, cowpea has great potential for controlling chronic inflammatory states.

Polyphenolic compounds in raw common bean seed coats have antitumor (*188*) antimutagenic (*189*) anti-inflammatory (*190*) and hepatoprotective effects (*191*). Previous studies showed that these positive health effects were probably due to the antioxidative effects exerted by the flavonoids present in the bean seed coat (*191*), suggesting bean antioxidants may be responsible for their physiological effects by counteracting the damages caused by reactive oxygen species (ROS) (*192*), increasing activity of non-enzymatic and enzymatic antioxidant defense systems (*193*), modulating transcription and activity of carcinogen defense and antioxidant enzymes (*194, 195*). ROS is generated in cells during respiration, but can also be derived from external sources. ROS significantly impact etiology of several chronic diseases, including cancer, by causing oxidative damage and DNA mutations (*90*). The presence of diverse groups of phenolic compounds in cowpea suggests it may also have strong ability to inhibit intracellular ROS and prevent chronic inflammatory conditions.

During inflammation, several cytokines and target genes interact to reverse or promote the inflammatory process. For example, pro-inflammatory cytokines such as interleukins and tumor necrosis factor (TNF-α) are associated with proliferative signaling. TNF-α (a signal that induces apoptosis) can induce transcription factors e.g. NF-κB that eventually regulate expression of several target genes (*196*). Activation of NF-κB may lead to development of many different chronic diseases (*197*). Thus flavonoids which inhibit dysregulated activation of NF-κB are capable of suppressing inflammation, tumor cell transformation, proliferation and other chronic disorders (*90, 198*).

Cytokines also activate leukocytes and endothelial cells to express intercellular adhesion molecules (e.g. vascular cell adhesion molecule-1; VCAM-1) as well as their integrin ligands. This promotes leukocyte adherence to endothelial cells and their migration through tissue to the site of injury (*199*). VCAM-1 expression is regulated primarily at the level of transcription, especially by transcription factors such as NF-κB (*200*). Endothelial cells also express microRNAs (miRs), such as miR-126, which regulates VCAM-1 expression and controls vascular inflammation upon bacterial infection. Thus, miRNAs serve as a potential link between inflammation and cancer for their role as potent post-transcriptional regulators of target genes and critical biological processes, including cell differentiation and disease (*201*).

Chronic inflammation of the intestinal tract is a risk factor for colorectal cancer. Previous epidemiological studies have suggested that regular legume intake reduce the risk of colorectal cancer (*202*). *In vitro* and *in vivo* assays have also demonstrated that

legumes such as black soybean can prevent colonic inflammation and cell proliferation, and was associated with antioxidant properties of anthocyanins, proanthocyanidins and isoflavones present in this cultivar (*203*). A recent report, however, show that the evidence associating legumes with reduced risk of inflammatory disorders such as cancer are still inconclusive (*204*), suggesting that more studies are required. Therefore, the aim of this study was to investigate how polyphenolic compounds in different cowpea varieties affect ROS quenching and anti-inflammatory properties on non-malignant intestinal myofribroblast CCD-18Co cells.

5.2 Materials and methods

5.2.1 Chemicals and reagents

The Folin-Ciocalteu reagent, dichlorofluorescein diacetate (DCFH-DA), and lipopolysaccharide (LPS) were purchased from Fisher Scientific (Pittsburgh, PA). Dimethyl sulfoxide (DMSO) was obtained from Sigma (St. Louis, MO). Bradford reagent was obtained from Bio-Rad (Hercules, CA) and VCAM-1 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TaqMan® MicroRNA Assay kit for miR-126 was obtained from Applied Biosystems (Carlsbad, CA). All other chemicals were analytical grade from VWR International (Bristol, CT).

5.2.2 Plant materials and extraction

Five cowpea varieties, namely, black IT95K-1105-5, red IT97K-1042-3, green TX2028-1-3-1, light brown 09FCV-CC-27M and white Early Acre were used in this

study (**Table 2**). These varieties, representing five major phenotypes, were chosen based on their distinct differences in phenolic profiles and total phenol content. These samples were separately soaked in water (1:4.5 w/v) in triplicates for 12 hours, and boiled for 15 minutes. After boiling, the seeds (including the soup) were chilled and frozen to –80°C, freeze-dried and ground to pass through 60-mesh screen. The powders were then extracted with aqueous 70% acetone, roto-evaporated and the extracts freeze-dried. The freeze-dried extracts were stored at –20°C until used.

5.2.3 Total soluble phenolics

Total soluble phenolic (TSP) levels (measure of total metal ion reducing capacity) of the freeze-dried boiled cowpea extracts was determined by a slightly modified Folin-Ciocalteu assay using gallic acid (GA) as the standard (*205*).

5.2.4 Cell culture assays

5.2.4.1 Cell line

Non-malignant colon CCD18Co cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured using high glucose Dulbecco's Modified Eagle Medium, supplemented with 1% penicillin/streptomycin solution, 1% non-essential amino acids (10 mM), 1% sodium pyruvate (100 mM) and 20% Fetal Bovine Serum (Invitrogen, Carlsbad, CA). The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

5.2.4.2 Cell proliferation assay

Cells were seeded (1.5 x 10^4 onto a 24-well plate) and incubated for 24 hr (at 37°C/humidified 5% CO_2 atmosphere) to allow cells to stabilize and attach onto the bottom of the wells. The freeze-dried cowpea extracts were re-dissolved in DMSO, and then diluted with media to known concentrations of total soluble polyphenolics ranging from 0 – 80 mg GAE/L (*205*). The cells were then exposed to the various concentrations of each cowpea phenolic extract for 48 hrs and then quantified with an electronic cell counter (Z1™ Series, Beckman Coulter, Inc).

5.2.4.3 Generation of reactive oxygen species (ROS) assay

Intracellular ROS was assessed using′,7′ -dichlorofluorescin diacetate (DCF-DA) (Molecular Probes, Eugene, OR) as a probe as described by Meng *et al.* (*206*) but with slight modifications. Cell were seeded in a black 96-well plate (3,000 cells/well) for 24 hrs to allow cell attachment, followed by incubation with cowpea phenolic extracts (2 – 20 mg GAE/L) for 24 hrs. The cells were then stimulated with 2 μg/mL LPS (in 100 μL media) to generate ROS for 2 hrs, followed by washing out the spent media using PBS buffer. The cells were then stained *in situ* with 100 μL of 10 μM DCFH, incubated at 37°C and the fluorescence signal was monitored after 15 min at 520 nm emission and 480 nm excitation with a FLUOstar Omega plate reader (BMG Labtech Inc, Durhan, NC). Relative fluorescence units (RFU) were analyzed using Omega Microplate Data Analyse Software and normalized to control cells not treated with cowpea extracts.

5.2.4.4 LPS-induced inflammation assay

Cells were seeded in a 12-well plate (80,000 cells/well) for 24 hrs (37°C/5% CO_2) to allow cell attachment. Cowpea extracts pre-dissolved in DMSO were then diluted to known concentrations of total polyphenolics (2, 5, 10 and 20 mg GAE/L) and normalized to < 0.2% DMSO in the culture medium. The cells were then pre-treated with the extracts for 3 hrs, and then stimulated with 2 μg/mL LPS for 6 hrs, after which messenger RNA (mRNA) and micro-RNA (miRs) were extracted from the lysated cells and analyzed.

5.2.4.5 RNA extraction and real-time PCR analysis of mRNAs and miRNAs

5.2.4.5.1 Total RNA extraction

Total RNA was extracted according to the manufacturer's protocol using the Qiagen extraction kit (Qiagen Inc. Valencia, CA) for mRNA analysis; and using *mir*Vana™ miRNA isolation kit (Applied Biosystems, Foster City, CA) for micro–RNA analysis. The quality and quantity of the isolated RNA were assessed using the NanoDrop® ND–1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

5.2.4.5.2 mRNA analysis

Complementary DNA (cDNA) was synthesized from the isolated RNA using a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY) according to the manufacturer's protocol. Real Time PCR (qRT-PCR) was carried out with the SYBR

Green PCR Master Mix (Applied Biosystems Inc, Foster City, CA) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems Inc, Foster City, CA). The pairs of forward and reverse primers were obtained from Integrated DNA Technologies, Inc., San Diego, CA.

5.2.4.5.3 Primer sequences used for mRNA analysis

The sequences of the primers used were:

IL-8:	F:	5′–CACCGGAAGGAACCATCTCA–3′
IL-8:	R:	5′–AGAGCCACGGCCAGCTT–3′
TNF-α:	F:	5′–TGTGTGGCTGCAGGAAGAAC–3′
TNF-α:	R:	5′–GCAATTGAAGCACTGGAAAAGG–3′
VCAM-1:	F:	5′–ACAGAAGAAGTGGCCCTCCAT–3′
VCAM-1:	R:	5′–TGGCATCCGTCAGGAAGTG–3′
NF-κB:	F:	5′–TGGGAATGGTGAGGTCACTCT–3′
NF-κB:	R:	5′–TCCTGAACTCCAGCACTCTCTTC–3′

5.2.4.5.4 miRNA analysis

The RNA Reverse Transcription (RT) reactions and quantitative real time PCR (qRT-PCR) amplification were performed following TaqMan® MicroRNA Reverse Transcription Kit protocol (Applied Biosystems, Foster City, CA). Briefly, for RT analysis of miR-NU6B and miR-126, 8 μL of the master mix and 5 μL of isolated mRNA containing 7 ng/μL mRNA was used to make the cDNA. The primers for miR-

126 and miR-NU6B were obtained from Life Technologies Corp., Applied Biosystems, Carlsbad, CA. For qRT-PCR analysis of miR-126, the RT product was diluted 1:15 and amplified using TapMan® 2 × Universal PCR Master Mix (No AmpErase® UNG) (Applied Biosystems, Foster City, CA) on a 384-well plate following the manufacturer's recommendations. The miR-NU6B small nuclear RNA was used as an endogenous control.

5.2.4.6 Protein expression

5.2.4.6.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Cells seeded in 10 cm plates (1×10^6) were allowed to attach and stabilize for 24 hrs before subjecting them to treatment with cowpea extracts (0, 2, 5, 10 and 20 mg GAE/L) for 3 hrs followed by LPS stimulation (2 μg/mL) for 6 hrs. Cell culture supernatants were collected and analyzed by ELISA assay for VCAM-1 (Human sVCAM-1 Immunoassay Kit, Invitrogen Corp., Camarillo, CA) according to the manufacturer's protocol. The protein concentration was quantified using Bradford reagent (Bio-Rad) from which the final VCAM-1 (μg/g protein) was calculated according to the manufacturer's protocol and normalized to untreated control cells.

5.2.4.6.2 Transfection assay

Cells seeded in 6-well (35 mm diameter) cluster plates (3×10^5 cells/well) were allowed to attach for 24 hrs to 80% confluency and then transfected with 100 pmol/mL

of anti-sense oligonucleotide (miR-126 inhibitor) (Dharmacon Inc., Lafayette, CO) using Neon™ Transfection System and Lipofectamine® 2000 Reagent kit (Invitrogen, Carlsbad, CA) following the recommendations provided by the manufacturer. For targeted knockdown of miR-126, cells were transfected with a mock siRNA (negative control, NC) in full media according to manufacturer's recommendations. After transfection, the cells were treated with 10 mg GAE/L cowpea extract for 24 hrs followed by LPS (2 μg/mL) stimulation for 24 hrs. Total RNA was then extracted using *mir*Vana™ miRNA isolation kit (Applied Biosystems, Foster City, CA) following manufacturer's protocol and analyzed for miR-126 and VCAM-1 gene expression using qRT-PCR on the Applied Biosystems 7900HT. The cell culture supernatants were also analyzed for VCAM-1 protein expression using ELISA assay kit (Invitrogen, Camarillo, CA).

5.3 Statistical analysis

Data are reported as means ± SD for ROS and protein quantification; and means ± SE for gene expression, of 3 replicates; and analyzed using 2005 SAS (Version 9.1, SAS Inst. Inc., Cary, N.C., U.S.A.) with one-way Analysis of Variance (ANOVA). Post Hoc test (Fisher's LSD and Tukey-Kramer HSD) after ANOVA was used to compare treatments means. Significant levels were defined using $p < 0.05$.

5.4 Results and discussion

5.4.1 Cell proliferation assay

It was important to first investigate cell proliferation property of the cowpea extracts since a chemopreventive agent should have protective effects against cancer without damaging non-cancer cells (*207*). Overall, the maximum concentration of cowpea extracts that supported CCD18Co cell growth after 48 hrs of incubation was 20 mg GAE/L (> 85% cells surviving) (**Figure 22**). Therefore, extract concentrations within a dose range of 2 – 20 mg GAE/L were used in the subsequent assays to assess the anti-inflammatory properties of cowpea.

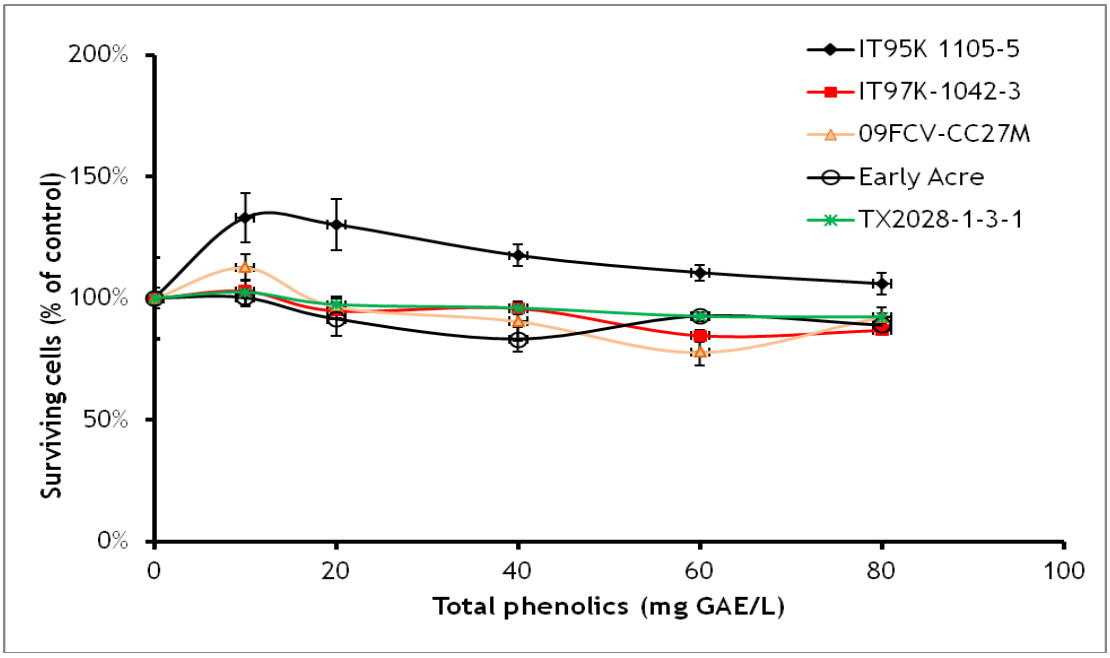


Figure 22. Cell proliferation assay on CCD18Co cells treated with various concentrations of extracts from different cowpea varieties; and assessed after 48 hrs incubation. Values are means ± SD (n = 3).

5.4.2 Cell protection against production of reactive oxygen species

The intracellular reactive oxygen species (ROS) assay was performed to screen for cowpea varieties with greater potency in protecting the non-malignant CCD18Co cells from LPS-induced ROS generation. Results showed that the protection of CCD18Co cells against ROS by the black IT95K-1105-5, red IT97K-1042-3 and light brown 09FCV-CC27M cowpea varieties was achieved in a dose-dependent manner (within 2 to 20 mg GAE/L) (**Figure 23**). At the lowest concentration (2 mg GAE/L), the flavonol-rich red IT97K-1042-3 and anthocyanin-rich black IT95K-1105-5 had the highest inhibitory effect on ROS (37.2 – 39.8% reduction), followed by the light brown 09FCV-CC27M (28.4% reduction) compared to the positive control (**Figure 23**). At highest concentration tested (20 mg GAE/L), the black IT95K-1105-5 had the highest effect on ROS inhibition (55.7%), followed by red IT97K-1042-3 (46.6%) and light brown 09FCV-CC27M (35.0%) compared to the positive control (**Figure 23**). On the other hand, the white Early Acre variety had no significant ($p < 0.05$) effect on ROS inhibition at all concentrations tested (**Figure 23**).

Generation of ROS in cells causes oxidative stress which may lead to cardiovascular disease or promotion of tumor progression which eventually causes cancer (*208*). Therefore, the ability of cowpea extracts to inhibit ROS generation may contribute to delaying or blocking tumor progression. The differences in major types of flavonoid present in these cowpea varieties have a significant effect on their capacity to mitigate generation of ROS in non-cancer cells.

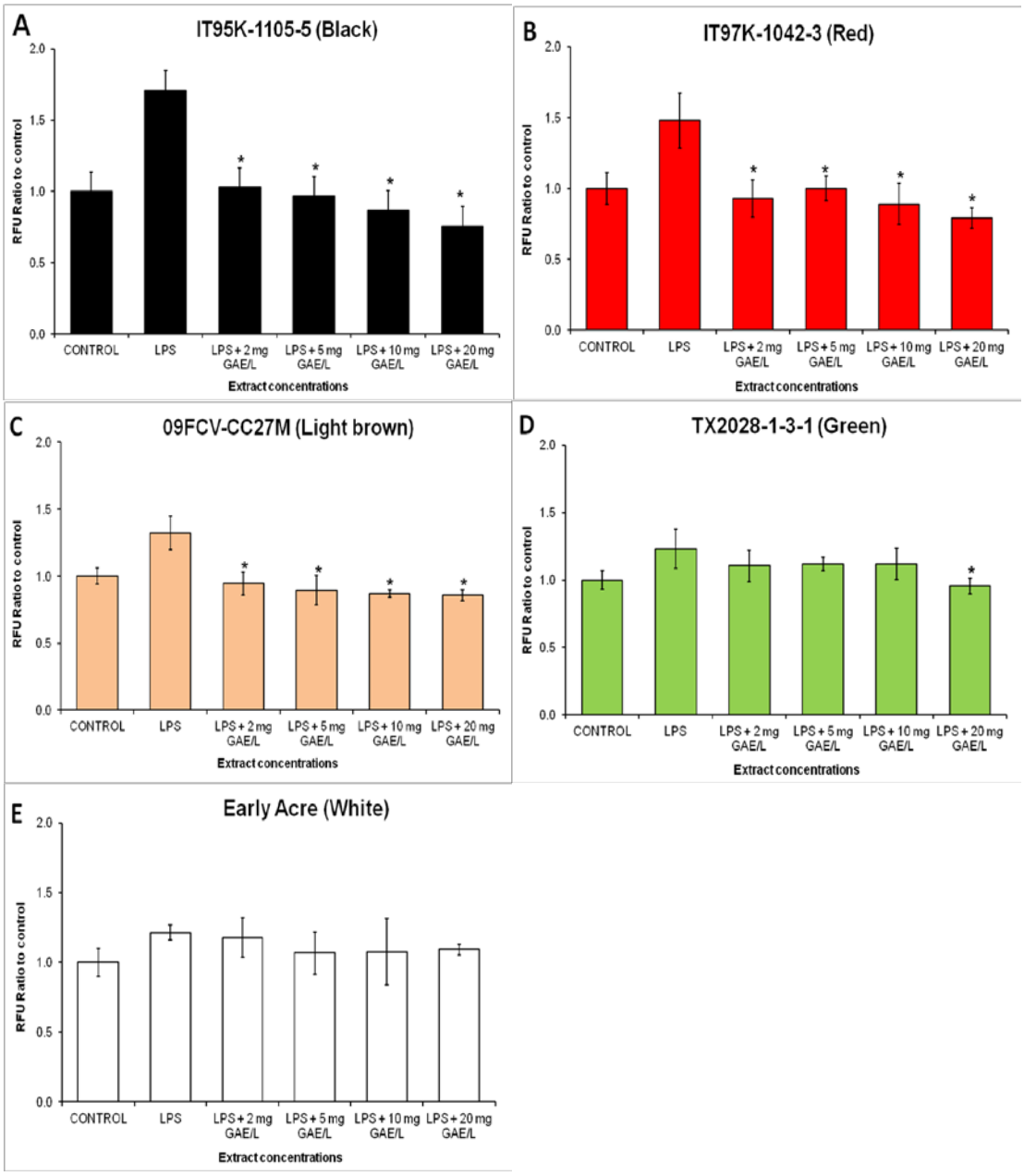


Figure 23. Effect of cowpea extracts on generation of ROS after LPS-induced oxidative damage in non-cancer CCD-18Co cells. Cells were pretreated with extracts (2 – 20 mg GAE/L) from black IT95K-1105-5 (**A**), red IT97K-1042-3 (**B**), light brown 09FCV-CC27M (**C**), green TX2028-1-3-1 (**D**), and white Early Acre (**E**) cowpea varieties for 24 hrs. Values are normalized to control cells not treated with cowpea extracts and presented as means ± SD, $n = 3$; (*****) indicate significance at $p < 0.05$.

Results from this study suggest that cowpea polyphenolics may protect cellular components (e.g. DNA, etc) from oxidative damage or release of proinflammatory cytokines due to their ROS scavenging property, thus, may have a role in treatment of pathological conditions in which ROS production plays an important role.

No previous studies have reported the protection of endothelial cells by cowpea polyphenolics. Other studies showed that flavonols, especially quercetin and kaempferol, had favorable inhibition of TNF-α-induced ROS generation in non-cancer human embryonic kidney HEK-293 cells (*209*). Another study demonstrated differential protective effect of quercetin and kaempferol against oxidative stress induced by proinflammatory stimuli in human hepatocyte-derived cell line (Chang Liver cells) possibly through modulation of some antioxidant enzymes (*210*), suggesting protective effects by flavonols against liver diseases in which prevention of oxidative stress is critical. Cowpea polyphenols may have exerted their cardioprotective property through interference with ROS generation on CCD18Co cells.

5.4.3 Effects of cowpea extracts on proinflammatory cytokines

The mRNA expressions of chemoattractant cytokine IL-8 was significantly up-regulated upon LPS stimulation (**Figure 24**). LPS induced IL-8 gene expression after 6 hrs of stimulation by 2.22-fold compared to untreated control cells (no LPS stimulation). However, all cowpea extracts dose-dependently decreased IL-8 (**Figure 24**). The effect of phenotype and phenolic composition on IL-8 inhibition was also observed even at low extract concentration (2 mg GAE/L). For example, the black IT95K-1105-5 and red

IT97K-1042-3 cowpea varieties significantly ($p < 0.01$) reversed the IL-8 mRNA by 1.95- and 2.22-fold, respectively, compared to untreated control cells; whereas the white Early Acre only decreased IL-8 mRNA by 1.47-fold at 2 mg GAE/L (**Figure 24**). The red variety contained flavonols and flavan-3-ols (**Table 11**) while the black contained anthocyanins in addition to flavonols and flavan-3-ols (**Tables 4** and **10**). The white variety had no detectable levels of anthocyanins or flavan-3-ols (**Table 14**), suggesting the phenolic compounds in the red, black and light brown varieties synergistically contributed to greater IL-8 inhibition. Flavonols (kaempferol and quercetin) have been shown to significantly inhibit IL-8 gene expression (*209*); IL-8 promotes phagocytosis of neutrophils (*211*), an event that causes secretion of ROS which has been implicated in the etiology of several chronic disorders such as colon cancer and IBD (*212*). Since IL-8 are chemokines mostly secreted by endothelial cells under oxidative stress, the inhibition of IL-8 by cowpea extracts containing ROS scavengers (anthocyanins, flavonols, flavan-3-ols, etc) suggest ROS inhibition could be one mechanism by which cowpea may protect endothelial cells from inflammation.

LPS also up-regulated TNF-α mRNA expression by 3.08-fold relative to untreated control cells (**Figure 25**). In this study, all phenotypes showed significant inhibition of TNF-α gene expression even at the lowest concentration tested (2 mg GAE/L), an important step in cancer prevention; except the white Early Acre.

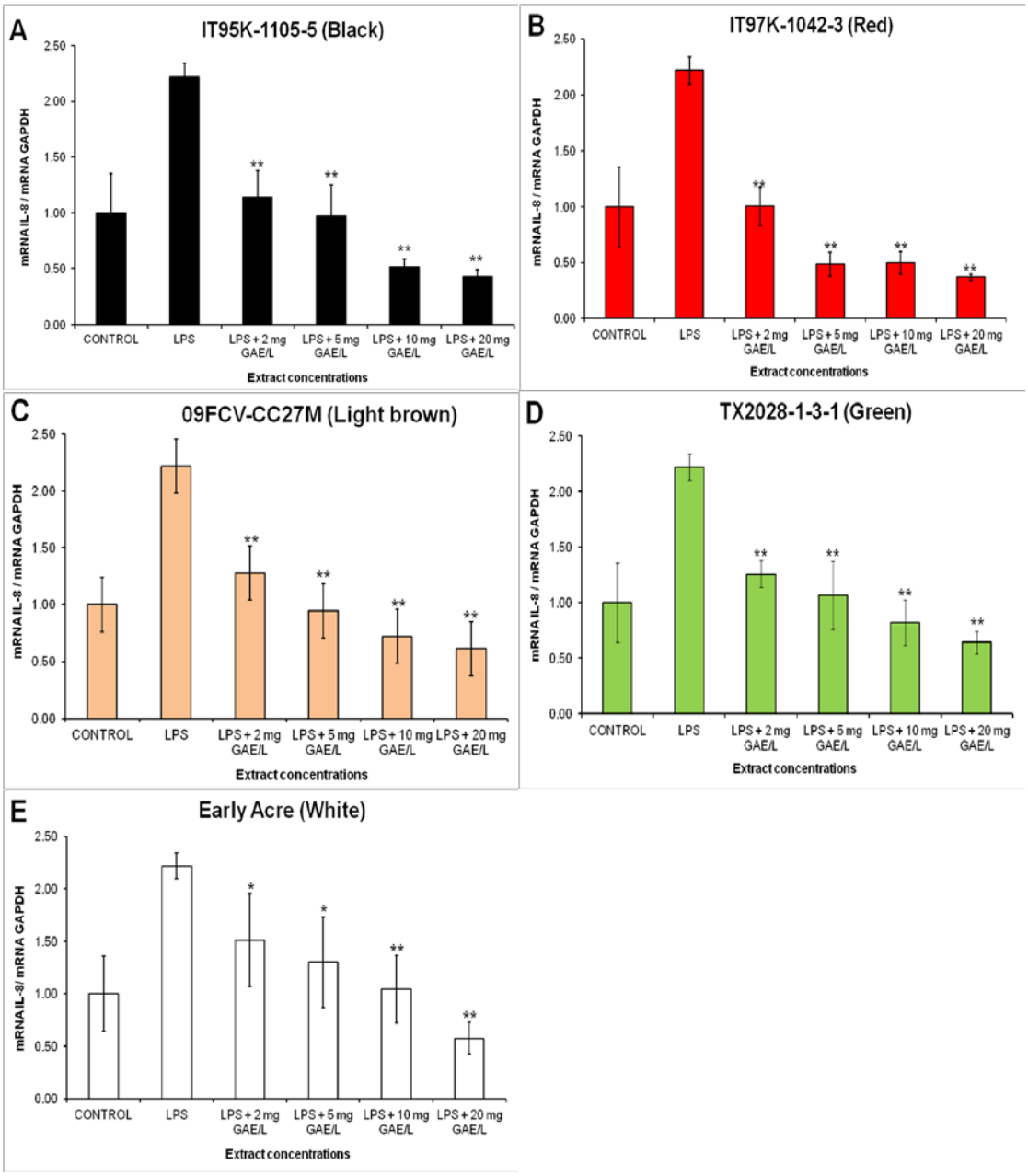


Figure 24. CCD-18Co gene expression of IL-8. Cells were pre-treated for 3 hrs with extracts (2 – 20 mg GAE/L) from black IT95K-1105-5 (**A**), red IT97K-1042-3 (**B**), light brown 09FCV-CC27M (**C**), green TX2028-1-3-1 (**D**), and white Early Acre (**E**) cowpea varieties, then stimulated with LPS for 6 hrs; and analyzed by real time qRT-PCR as ratio to GAPDH mRNA. Values are means ± SE (n = 3); (*****) $p < 0.05$, (**) $p < 0.01$.

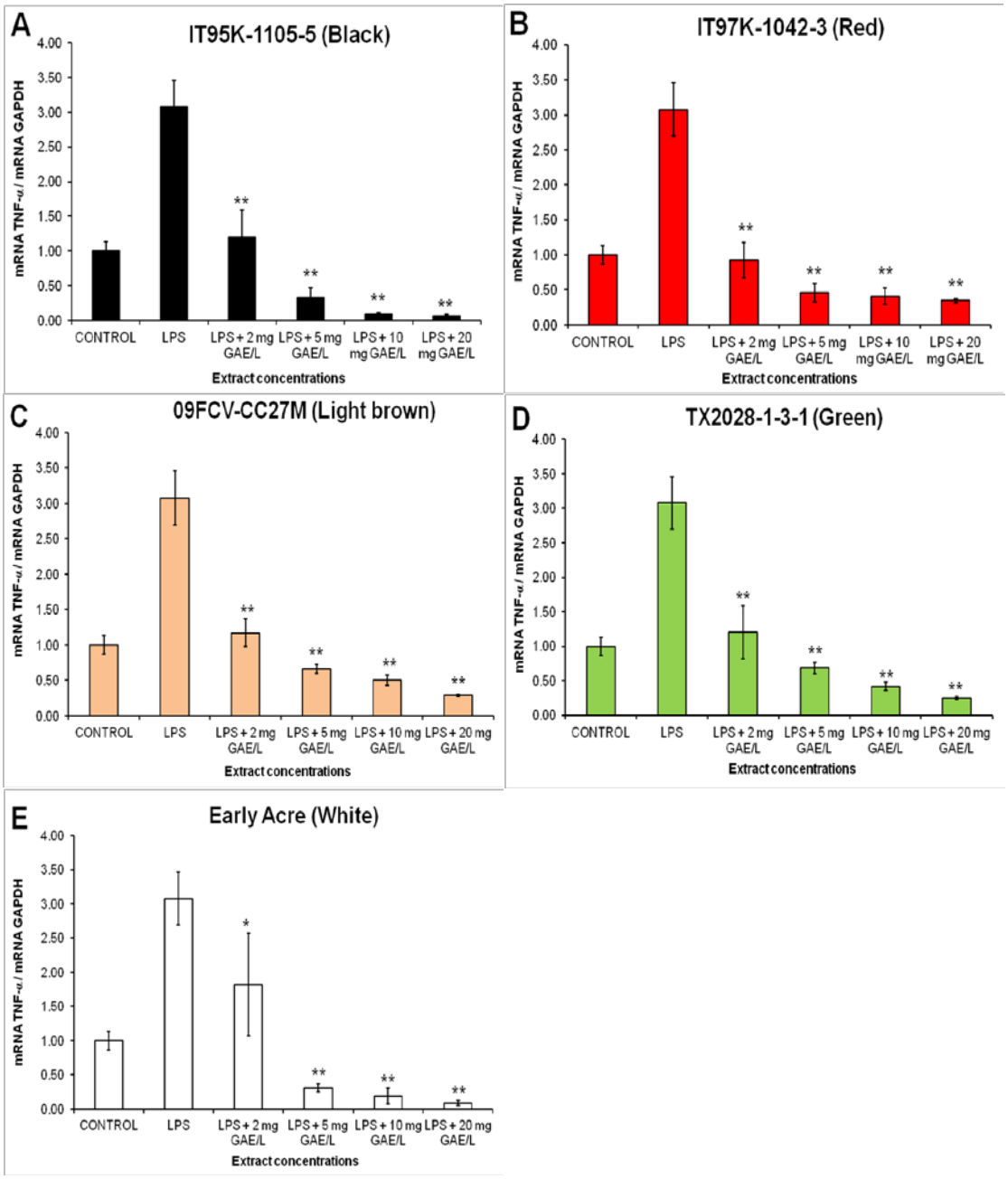


Figure 25. CCD-18Co gene expression of TNF-α. Cells were pre-treated for 3 hrs with extracts (2 – 20 mg GAE/L) from black IT95K-1105-5 (**A**), red IT97K-1042-3 (**B**), light brown 09FCV-CC27M (**C**), green TX2028-1-3-1 (**D**), and white Early Acre (**E**) cowpea varieties, then challenged with LPS for 6 hrs; and analyzed by real time qRT-PCR as ratio to GAPDH mRNA. Values are means ± SE (n = 3); (*****) $p < 0.05$, (******) $p < 0.01$.

At 2 mg GAE/L, the flavonol-rich red IT97K-1042-3 variety had the highest inhibition of TNF-α (by 3.35-fold relative to untreated control cells), followed by the anthocyanin-rich black IT95K-1105-5 variety compared to the white Early Acre variety which only reduced TNF-α by 1.68-fold relative to untreated control cells at this concentration (**Figure 25**). This suggests that cowpea flavonoid profiles strongly influenced inhibition of TNF-α. However, all cowpea extracts down-regulated TNF-α secretion dose-dependently (**Figure 25**). The red cowpea phenotype contained significant levels of quercetin, kaempferol and myricetin derivatives (**Tables 10 – 14**), which may explain why the red IT97K-1042-3 cowpea variety had higher inhibitory effect against IL-8 and TNF-α even at low phenolic concentrations, followed by the anthocyanin-rich black IT95K-1105-5 variety (**Figures 24** and **25**). Down-regulation of proinflammatory cytokines such as TNF-α by flavonoids is generally linked to prevention of chronic diseases such as ulcerative colitis (*213*) and inhibiting tumor growth and angiogenesis, therefore such strategies of down-regulating them has been suggested as therapeutic for colorectal carcinoma (*198, 214*).

This study also investigated the effects of cowpea extracts on expression of transcription factor NF-κB (**Figure 26**). NF-κB induces several pro-inflammatory cytokines including IL-8 and TNF-α in addition to other activities and is activated through phosphorylation. mRNA expression of NF-κB increased by 2.11-fold relative to untreated control cells after LPS challenge (**Figure 26**). This effect was dose-dependently reduced by polyphenolics from all cowpea phenotypes tested. However, our result showed that flavonoid profiles amongst the varieties may influence cowpea's

ability to down-regulate NF-κB genes. For example, whereas the black, red, green and white varieties significantly ($p < 0.01$) inhibited NF-κB at 2 mg GAE/L, the light brown variety only had similar inhibitory effect at 10 mg GAE/L (**Figure 26**). The light brown 09FCV-CC27M contains high levels of polymeric flavan-3-ols which may affect their interactions with certain specific genetic materials such as NF-κB.

In general, higher inhibition of NF-κB was exhibited by the flavonol-rich red IT97K-1042-3 variety at 20 mg GAE/L (**Figure 26**). Flavonols (quercetin and kaempferol) have been reported to inhibit the activation of NF-κB induced by cytokines in parenchymal liver cells, probably via protecting cells against oxidative species, inhibition of anti-inflammatory enzymes and down-regulation of NF-κB pathway (*210, 215*). Our results strongly suggest cowpea may play a critical role on regulating inflammation linked to dysregulated NF-κB gene expression, in the initiation and progression of chronic disorders (*216*).

Since vascular cell adhesion molecule VCAM-1 is a target gene for NF-κB, we also investigated whether cowpea polyphenolics might regulate VCAM-1 as an additional model to assess cowpea's potential protective effects in vascular inflammation. LPS up-regulated VCAM-1 mRNA by 3.55-fold relative to untreated control cells (**Figure 27**). Cell adhesion molecules (CAMs) are known to regulate leukocyte transendothelial migration and cause inflammation (*217*).

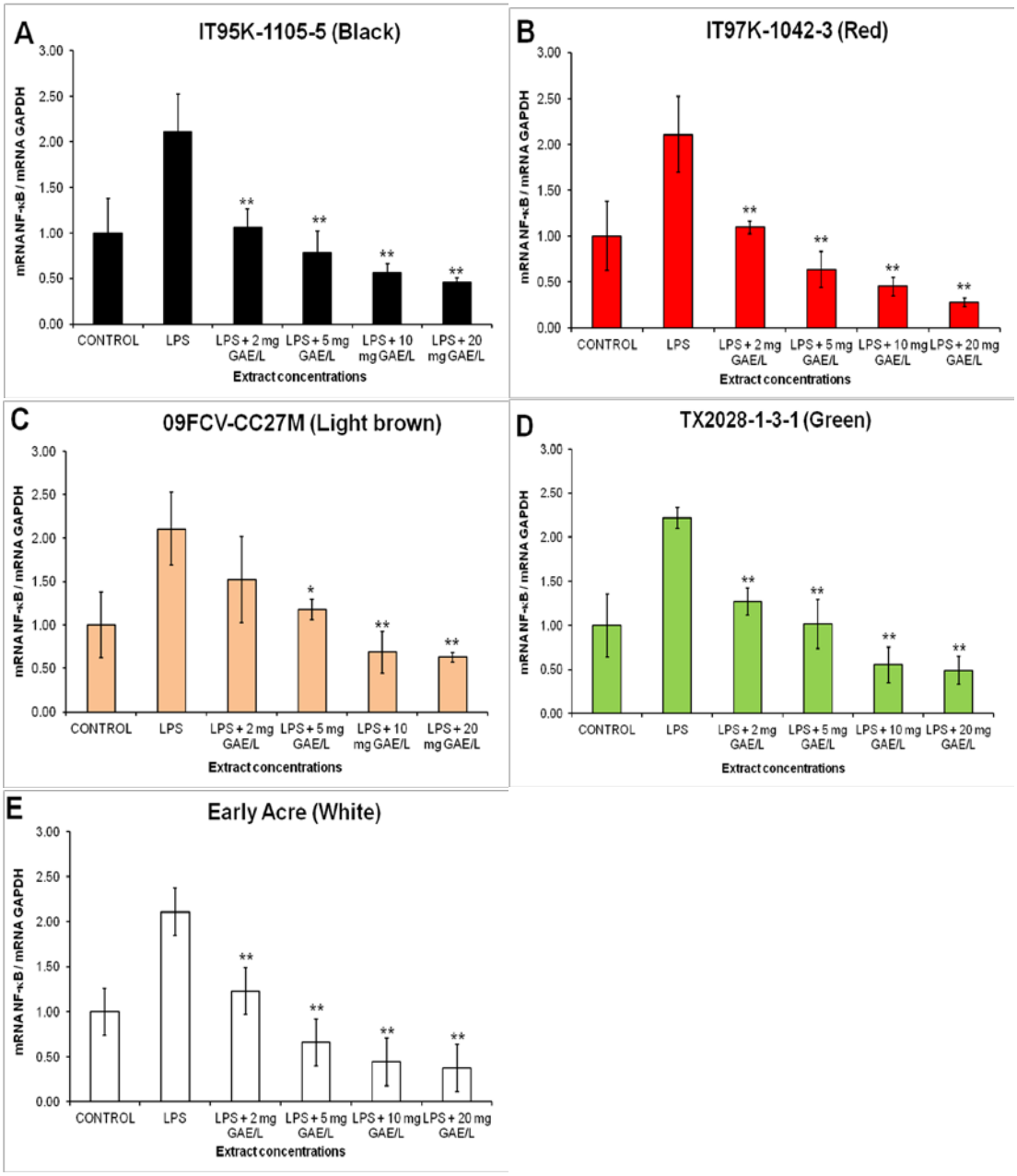


Figure 26. CCD-18Co gene expression of NF-κB. Cells were pre-treated for 3 hrs with extracts (2 – 20 mg GAE/L) from black IT95K-1105-5 (**A**), red IT97K-1042-3 (**B**), light brown 09FCV-CC27M (**C**), green TX2028-1-3-1 (**D**), and white Early Acre (**E**) cowpea varieties, then challenged with LPS for 6 hrs; and analyzed by real time qRT-PCR as ratio to GAPDH mRNA. Values are means ± SE (n = 3); (*****) $p < 0.05$, (******) $p < 0.01$.

All cowpea extracts had significant dose-dependent reduction of VCAM-1 gene expression on CCD18Co cells (**Figure 27**), demonstrating that cowpea polyphenolics have protective effects in endothelial cells stimulated by LPS. Similar results were also reported regarding acaí and red muscadine grape polyphenolics on NF-κB target genes VCAM-1, ICAM-1, and E-selectin (*218*).

The effect of different cowpea phenotypes on VCAM-1 mRNA was also observed (**Figure 27**). Polyphenolics from the anthocyanin-rich IT95K-1105-5 (black), procyanidin-rich 09FCV-CC27M (light brown) and Early Acre (white) varieties seemed to be more effective at down-regulating VCAM-1 gene expression compared to those from red and green at a low concentration of 2 mg GAE/L. This suggests that specific flavonoid profiles modulate different molecular pathways, thus, different cowpea phenotypes may prevent endothelial dysfunction and cardiovascular complications through different mechanisms. Already, proanthocyanidins have been shown to exert inhibitory effect on the expression of CAMs via several mechanisms, including reduction of ROS generation (*219*), inhibition of the AngII-induced MAPK (*220*), down-regulation of NF-κB inflammatory cascade and regulation of TRAF6 – IRAK1 axis (*218*), thus might help prevent development of atherosclerosis.

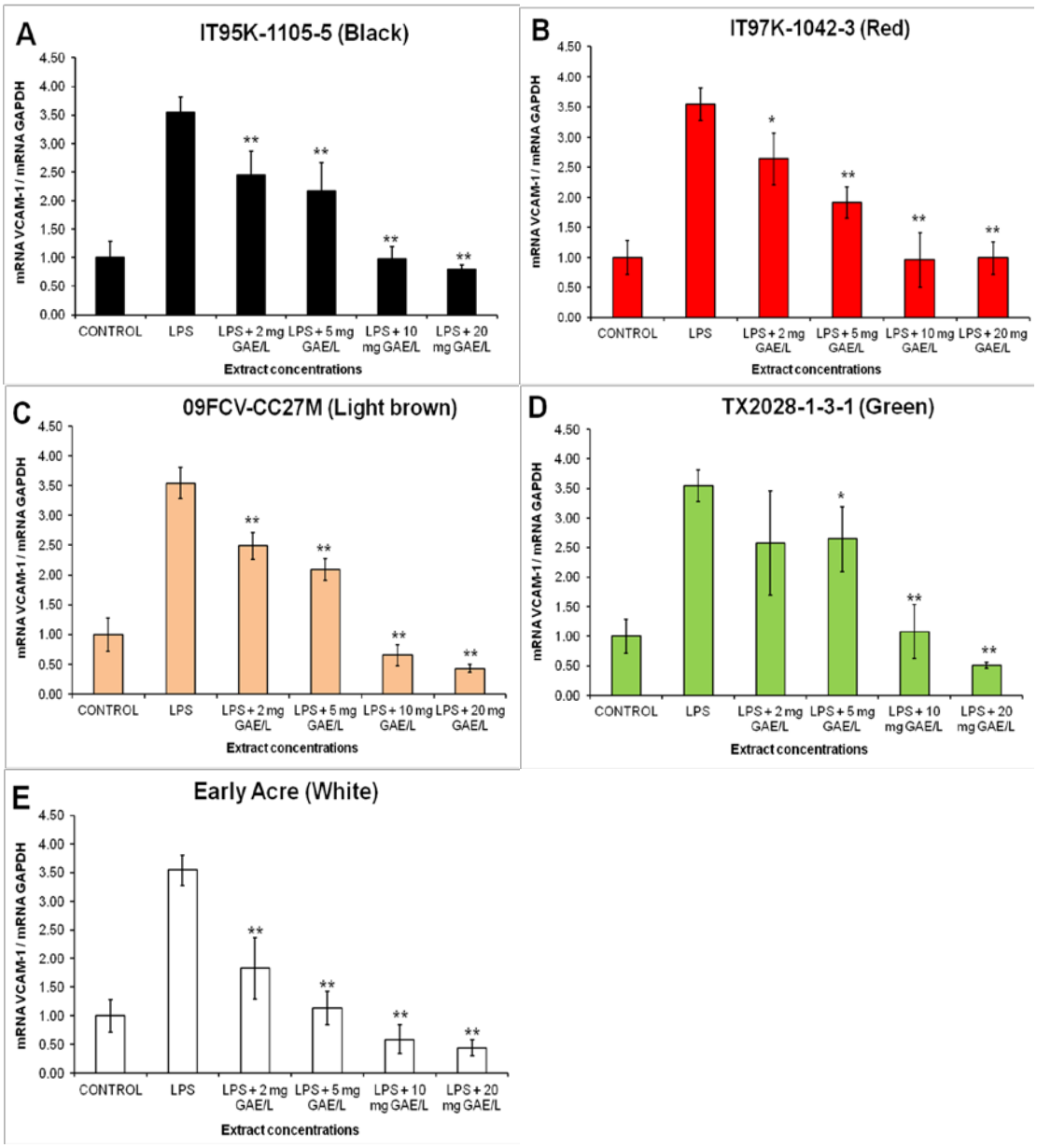


Figure 27. CCD-18Co gene expression of VCAM-1. Cells were pre-treated for 3 hrs with extracts (2 – 20 mg GAE/L) from black IT95K-1105-5 (**A**), red IT97K-1042-3 (**B**), light brown 09FCV-CC27M (**C**), green TX2028-1-3-1 (**D**), and white Early Acre (**E**) cowpea varieties, then challenged with LPS for 6 hrs; and analyzed by qRT-PCR as ratio to GAPDH mRNA. Values are means ± SE (n = 3); (*****) $p < 0.05$, (******) $p < 0.01$.

Our study reveals important differences among cowpea varieties in their capacity to protect the colonic myofibroblast against NF-κB activation and transcription of pro-inflammatory genes in LPS-induced inflammation at the mRNA level. The flavonol-rich red IT97K-1042-3 cowpea variety had superior capacity to down-regulate expression of cytokines/chemokines and NF-κB, followed by the anthocyanin-rich black IT95K-1105-5; suggesting that the modulation of proinflammatory genes by cowpeas is dependent on their phenolic profiles. The light brown phenotype had the least flavonol content. These results provide evidence on how different cowpea phenotypes and their phenolic compositions could affect the management of inflammatory conditions and potential for colon cancer prevention.

Overall, based on the positive effects of cowpea polyphenolics on endothelial function, follow-up studies in animals and humans are required to support these beneficial effects and to determine clinical relevance. Lower concentrations should also be tested to determine the minimum effective doses. Specific flavonoid fractions should also be tested to determine the class of cowpea flavonoids that has the highest potency.

5.4.4 Effect of cowpea extracts on lipopolysaccharide-induced vascular inflammation

LPS induces innate immune responses via cell membrane ligands that initiate the NF-κB cell signaling cascade involving expression of cellular adhesion molecules and interleukins that regulate leukocyte recruitment (*210*). For example, VCAM-1 play a key role in leukocyte adhesion, migration and maintenance of vascular integrity.

Dysregulated expression of VCAM-1 may cause vascular lesions (*221*) via NF-κB activation which may result in atherosclerosis (*222*). Thus, the protective effect of extracts from different cowpea varieties in vascular inflammation was assessed in CCD18Co cells to determine the best variety with greater potential to down-regulate VCAM-1 at the protein level. The amount of VCAM-1 protein excreted to culture media after 6 hrs of LPS stimulation was increased by 6.38-fold compared to untreated control cells (**Figure 28**).

In general, all cowpea extracts dose-dependently reversed LPS-induced up-regulation of VCAM-1 protein expression within 2 – 20 mg GAE/L concentrations tested (**Figure 28**). At 2 mg GAE/L, the greatest decrease in VCAM-1 protein expression was observed with the black and red cowpea phenotypes (**Figure 28**). The black and red cowpea varieties had higher amounts of anthocyanins and flavonols, respectively. At 20 mg GAE/L, all cowpea extracts restored VCAM-1 basal protein excreted into the culture media, indicating that their anti-inflammatory effect on this NF-κB target gene was a response modulated by their polyphenolic profiles. However, as the extract concentration increased, the light brown 09FCV-CC27M cowpea variety seemed to be more efficacious compared to the other varieties; it showed a marked decrease in VCAM-1 protein secretion at 10 mg GAE/L (**Figure 28C**), compared to the other samples at this concentration (**Figure 28**). This suggests that the high levels of procyanidins in 09FCV-CC27M variety may be providing superior protection against adhesion molecules expression than the flavonols and anthocyanins in IT97K-1042-3 and IT95K-1105-5 varieties, respectively. This could be because procyanidins mainly

exert their anti-inflammatory effects through interference with ROS generation and their anti-apoptotic effects (*219*); whereas flavonols mainly provide their anti-inflammatory effects by acting on signaling cascades and their downstream substrates, as well as by inhibiting critical enzymes and membrane proteins often required for cellular specific functions (*223*). Polyphenolics from açai and red Muscadine grape seeds, mainly composed of proanthocyanidins, had a reducing effect on intercellular cell adhesion molecules, namely ICAM-1, VCAM-1 and E-selectin, as well as intracellular platelet endothelial cell adhesion molecule (PECAM-1) by decreasing generation of ROS (*218*).

Overall, in this study, cowpea extracts were effective at decreasing VCAM-1 expression both at mRNA (**Figure 27**) and protein levels (**Figure 28**), suggesting cowpea polyphenolics may prevent dysregulated expression of adhesion molecules on the cell surface of endothelial cells and circulating polymorphonuclear cells and thus may reduce risk of tumors and cardiovascular diseases (*224*). Follow-up studies using *in vivo* models such as rats are therefore required to support these findings.

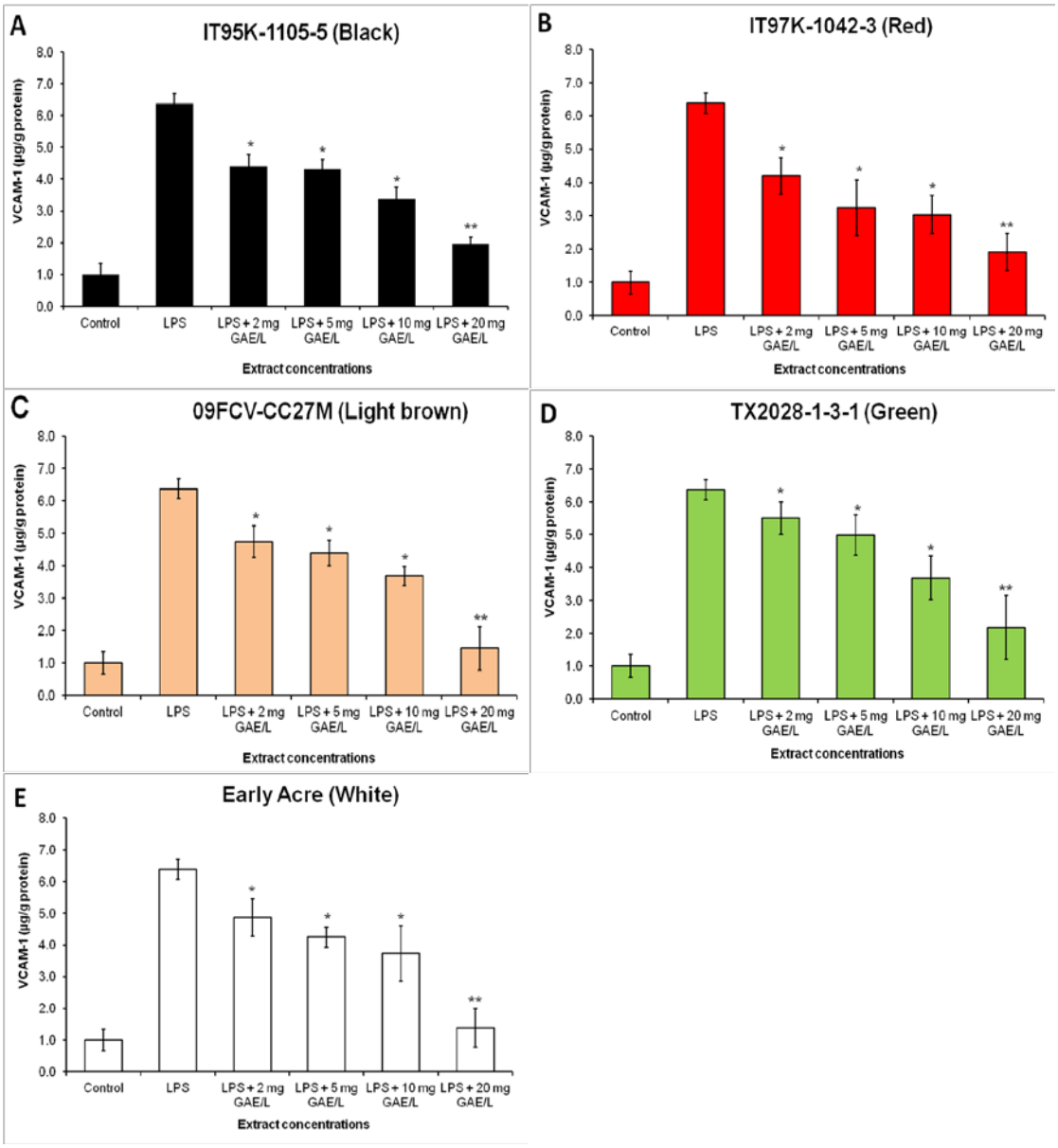


Figure 28. Relative amount of VCAM-1 protein excreted to culture media. Cell culture supernatants from cells pretreated for 3 hrs with solvent (DMSO) or different extract concentrations (2 – 20 mg GAE/L) from black IT95K-1105-5 (**A**), red IT97K-1042-3 (**B**), light brown 09FCV-CC27M (**C**), green TX2028-1-3-1 (**D**), and white Early Acre (**E**) cowpea varieties, and challenged with LPS (2 μg/mL) for 6 hrs were analyzed by ELISA. Values were normalized to protein concentrations relative to untreated control cells. Values are means ± SD (n = 3); (*****) $p < 0.05$, (******) $p < 0.01$.

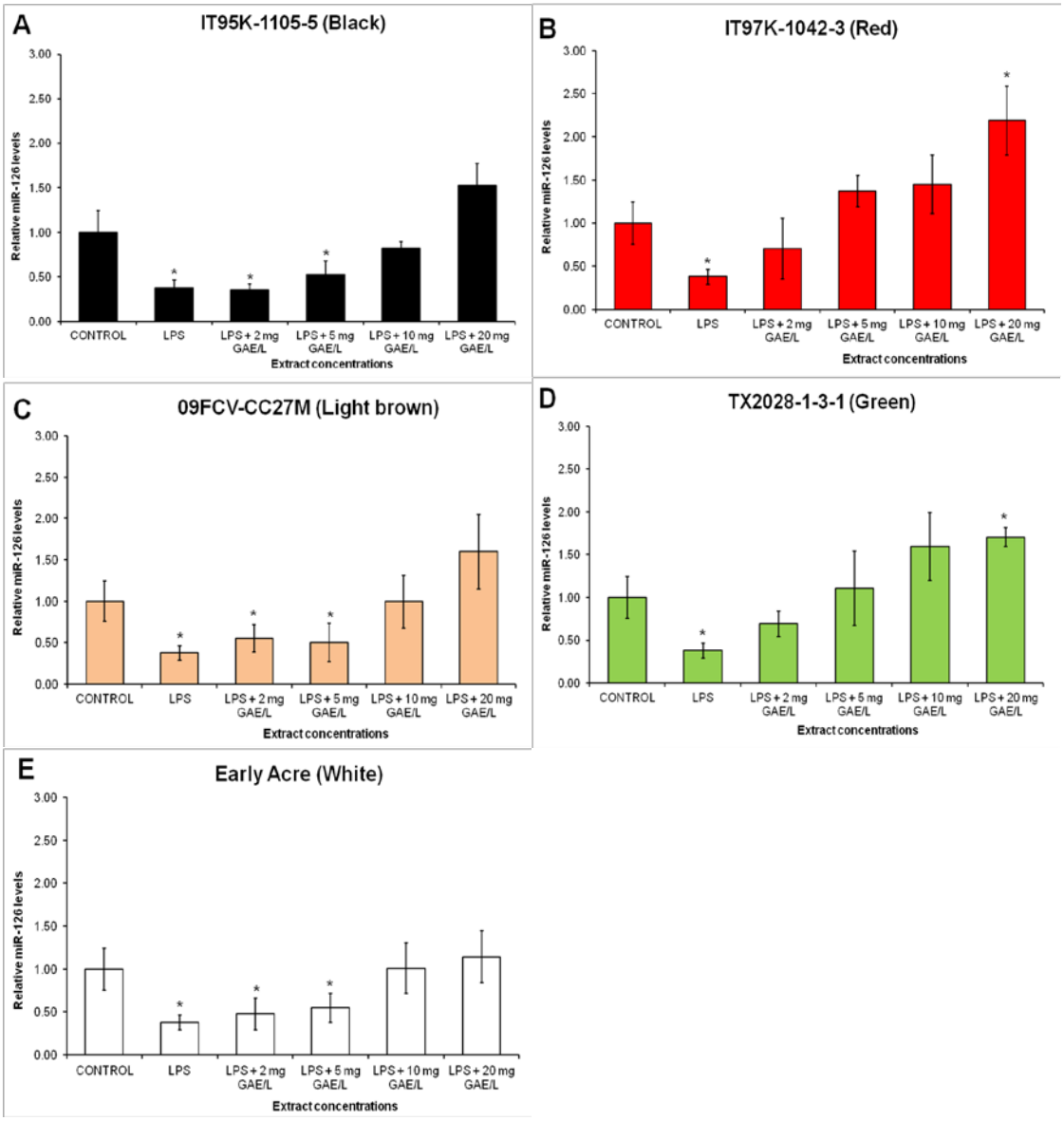


Figure 29. Modulation of miR-126 by cowpea extracts on LPS-challenged CCD18Co cells. Cells were pretreated for 3 hrs with solvent (DMSO) or different extract concentrations (2 – 20 mg GAE/L) from black IT95K-1105-5 (**A**), red IT97K-1042-3 (**B**), light brown 09FCV-CC27M (**C**), green TX2028-1-3-1 (**D**), and white Early Acre (**E**) cowpea varieties, and then stimulated with LPS (2 μg/mL) for 6 hrs. Values are means ± SE (n = 3). (*) within each sample indicate significant difference at $p < 0.05$ compared to control.

5.4.5 Effect of cowpea extracts on miR-126

Resting endothelial cells usually do not express adhesion molecules; however, upon activation by cytokines such as IL-8 and TNF-α, cell adhesion molecules such as VCAM-1 and ICAM-1 may be expressed which eventually mediate leukocyte adherence to endothelial cells (*97*). Such crucial biological processes have been shown to be highly regulated by miRNAs during immune response. For example, miR-126 has been shown to play a key role in regulation of VCAM-1 in vascular endothelial cells (*97*).

In this study, the expression of miR-126 in CCD18Co cells was found to be inversely correlated to VCAM-1 expression at both gene and protein levels, suggesting its participation in the control of vascular inflammation (**Figures 27 – 29**). Results showed that LPS significantly decreased miR-126 expression by 64% relative to untreated control cells (**Figure 29**), and this response was inversely correlated to the expression of VCAM-1 at gene (**Figure 27**) and protein levels (**Figure 28**). However, cowpea extracts (2 – 20 mg GAE/L) exerted a dose-response increase of miR-126 expression (**Figure 29**), which showed corresponding down-regulation of VCAM-1 at both gene and protein levels (**Figures 27** and **28**). Polyphenolics from açai and red Muscadine grape seeds (5 – 20 mg GAE/L) were also reported to induce a dose-response increase of miR-126 expression in LPS-challenged HUVEC cells, and this response was inversely correlated to the expression of adhesion molecules (VCAM-1, ICAM-1, E-selectin and PECAM-1) at both gene and protein expression levels (*218*). This indicates miR-126 is normally involved in inhibiting VCAM-1 expression, confirming VCAM-1 regulation is mediated through miR-126.

To understand the mechanisms by which phenolic compounds in cowpea and miR-126 regulate endothelial biology, we quantified mRNA expression of VCAM-1 (a potential miR-126 target) by qRT-PCR in CCD18Co cells that had been transfected with specific miR-126 inhibitor in the presence of black IT95K-1105-5 cowpea extract at 10 mg GAE/L (**Figure 30**). This sample was chosen since it contained significant amounts of the major flavonoid compounds (anthocyanins, flavonols and flavan-3-ols) identified in cowpea (**Tables 4** and **10**). Upon miR-126 inhibition, LPS increased VCAM-1 mRNA levels by 2.45-fold relative to the negative control (NC)-treated cells, and the black IT95K-1105-5 cowpea extract reversed this effect by 37% (**Figure 30A**). This suggested that cowpea extract were involved in regulation of VCAM-1 genes.

We also assessed the expression of VCAM-1 protein in the cell culture supernatants collected from the control or miR-126-transfected CCD18Co cells using ELISA, but we did not detect significant levels of VCAM-1 protein in these samples. This does not mean that the antibody was ineffective; since VCAM-1 protein was readily detectible in LPS-treated endothelial cells (**Figure 28**); rather it showed that miRNA may regulate stability or translation of target mRNAs as had been demonstrated by other authors (*225*).

Transfection with miR-126 inhibitor significantly ($p < 0.05$) decreased miR-126 levels by 62% of negative control (NC)-treated cells (**Figure 30B**). However, when transfected cells were treated with 10 mg GAE/L black IT95K-1105-5 cowpea extract, the miR-126 levels increased by 1.92-fold of NC-treated (**Figure 30B**).

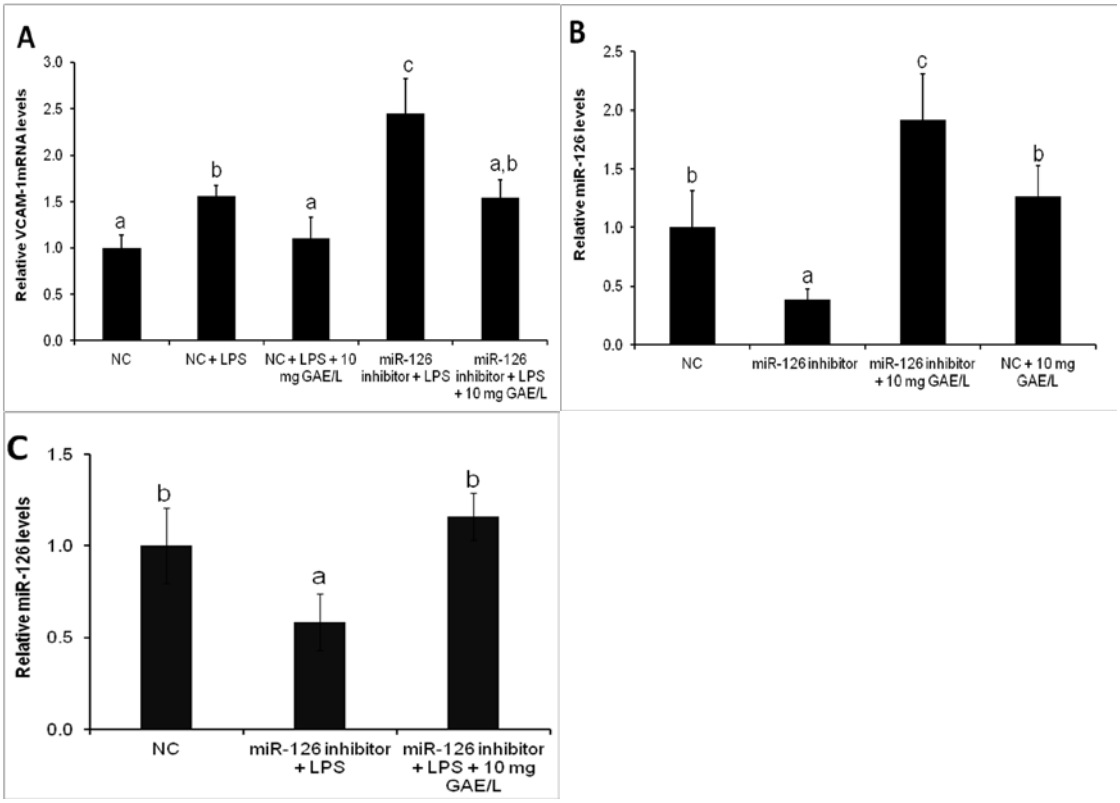


Figure 30. Effect of 10 mg GAE/L polyphenolic extracts from black IT95K-1105-5 cowpea variety on VCAM-1 mRNA levels on LPS-treated transfected cells (**A**); miR-126 levels on transfected cells (**B**); and miR-126 levels on LPS-treated transfected cells (**C**). Cells were pretreated with solvent (DMSO) or the extract for 24 hrs followed by stimulation with LPS (2 μg/mL) for 24 hrs. Using qRT-PCR, relative VCAM-1 and miR-126 levels were analyzed as a ratio to the GAPDH and miR-NU6B endogenous controls, respectively. NC, negative control; LPS, lipopolysaccharide. Data are expressed as mean ± SE (n = 3). Different letters within each assay indicate significant difference at $p < 0.05$.

Upon LPS stimulation, the miR-126 levels on cells transfected with miR-126 inhibitor decreased by 41% relative to NC-treated cells, and the cowpea extract reversed this effect (**Figure 30C**). These results suggest that phenolic compounds in cowpea modulate the expression of endothelial cell-specific miR-126 as one of the underlying mechanisms that protect endothelial cells from inflammation. miR-126 is known to

regulate endothelial expression of VCAM-1 (*97*), thus induction of miR-126 by cowpea polyphenolics following LPS challenge (**Figure 30B**) demonstrates cowpea may protect endothelial cells against atherosclerotic risk factors by decreasing VCAM-1 expression through miR-126 induction as one of the underlying mechanisms. This is supported by the fact that miR-126 expression was inversely correlated to the decreased VCAM-1 gene (**Figure 27**) and protein expression (**Figure 28**). These positive health effects of cowpea associated with regulation of miR-126 is critical since miR-126 expression has been correlated with lung (*226*) and breast (*227*) cancer survival as well as tumor suppression (*228*).

In previous studies, miR-126 was reported to target VCAM-1 in TNF-α treated (*97*) and LPS-treated (*218*) endothelial cells. Additionally, other authors (*97*, *229*) also showed that the 3′-UTR transcript for human VCAM-1 is perfectly complimentary to the nucleotides 604 – 625 of miR-126, which is responsible for decreasing cell adhesion and inflammation in endothelial cells, indicating involvement of miR-126 in modulating VCAM-1 expression during immune response.

5.4.6 Conclusions

Cowpeas contain phenolic compounds which may provide protective effects against NF-κB-linked risk factors associated with cancer and cardiovascular diseases by decreasing levels of VCAM-1 both at the gene and protein expression levels through induction of miR-126 as the underlying mechanism (**Figure 30**). This was further demonstrated when the overall effect of miR-126 inhibitor on VCAM-1 gene and protein

expressions in transfected cells were reversed by polyphenolics from the black IT95K-1105-5 cowpea extract (**Figure 30**).

The molecular pathways targeted by cowpea polyphenolics in the maintenance of cellular homeostasis may be complex, but these results show that their anti-inflammatory effects, ROS inhibition and induction of miR-126 may contribute to their overall prevention of endothelial dysfunction and cardiovascular complications. From these results, cowpea has great potential in prevention of CVDs and cancers. Considering the importance of miR-126 in regulating endothelial biology, further *in vivo* investigations on mechanistic and translational aspects of these positive health effects shown by cowpea extracts would be essential in validating their relevance to human health.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From this study, seed-coat color may be a good indicator of the accumulation of flavonoids in cowpeas. Overall, levels of individual phenolic compounds were variety-dependent rather than phenotype-dependent. Boiling significantly decreased the levels of individual and total flavonoid contents in cowpea. The initial antioxidant capacity of the cowpea varieties was influenced by their phenolic profiles; but was significantly impacted by boiling.

The ability of cowpea phenolic compounds to regulate proinflammatory genes and modulate miR-126 and its target gene VCAM-1 was variety-dependent. This indicated that cowpea's ability to protect the colonic myofibroblast cells from chronic inflammation was strongly influenced by the phenolic profiles. This effect was via inhibition of ROS and down-regulation of transcription factors NF-κB. These studies emphasize the importance of cancer inhibitory potential of phenolic compounds from cowpea and their role in preventing anti-inflammatory disorders. Specifically, the black and red cowpea phenotypes were more efficient than green or white phenotypes in antioxidant cellular protection and in *in vitro* inhibition of inflammatory genes. Taken together, these studies demonstrate that the preventive effect of phenolic compounds from cowpea against inflammatory conditions is variety dependent and, for the first time, demonstrate the potential role of cowpea in prevention of chronic diseases.

6.2 Recommendations for further research

Based upon findings of the study, the following recommendations are relevant:

1. Focus should be placed on isolating, identifying and quantifying heterogeneous flavan-3-ol oligomers (e.g. hexamers; mDP 5) which could be present in cowpea.
2. Determine the type of flavonoid adducts produced by boiling from cowpea polyphenols, since these compounds may contribute to the overall antioxidant activity and anti-inflammatory properties of boiled cowpea samples.
3. Determine the ability of cooked cowpea extracts to regulate and modulate other microRNAs, cell adhesion molecules such as PECAM-1 and E-selectin in non-cancer cell lines, in addition to using various cancerous cell lines would be essential in validating their relevance to human health.
4. Follow-up studies using in vivo animal models and human intervention studies using cowpea diets is required to support these findings.
5. Exploration of processing technologies that permit greater retention of phenolics should be considered.

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APPENDIX A

Cowpea phenotypes investigated in this study

Black phenotypes
(Varieties IT95K-1105-5 and IT98K-1092-1)

Green phenotype
(Variety TX2028-1-3-1)

Red phenotypes
(Varieties IT97K-1042-3 and IT82D-889)

Golden Brown phenotypes
(Varieties Ife Brown and IT84S-2246)

Light Brown phenotypes
(Varieties IAR-48 and 09FCV-CC27M)

White phenotype
(Variety Early Acre)

APPENDIX B

Partly identified and non-identified compounds

The following peaks were characterized as far as possible using the MS/MS-MS and UV-*vis* spectra obtained. Most of the non-identified compounds were found in the ethyl acetate fraction, indicating they are less hydrophobic. They also did not have any shared MS characteristics, suggesting each peak may be structurally different.

Peak 2 (t_R = 2.85 min, λ_{max} = 278 nm) had a $[M - H]^-$ at *m/z* 203 that fragmented on MS/MS to produce ions at *m/z* 159 (M–44, loss of CO_2), 142 (M–44–17 *amu*), 116 (M–44–17–26 *amu*) and 74 (M–44–17–26–42 *amu*), the fragmentation pattern suggest that it could be a simple compound with a carboxylic terminal end, non-glycosylated and may only be consisting of one benzene ring (**Table 7**). This peak was present in all the varieties studied, suggesting it is a major component of cowpea.

Also, the true identities of *peaks 6, 12* and *18* could not be established based on the MS data alone (**Table 7**). All these three peaks had similar $[M - H]^-$ at *m/z* 885 and absorption maxima λ_{max} = 279 nm. Moreover, their MS/MS fragmentation patterns were also similar. However, these peaks had significantly different retention times. The t_R for peaks *6, 12* and *18* were 4.19, 5.36 and 6.16 min, respectively, suggesting that the compounds were structurally related but had varying polarity which affected their elution properties. The MS/MS ion at *m/z* 723 suggested a loss of 162 *amu*, corresponding to a loss of one hexose unit. It therefore means that all these compounds are mono-glycosylated. The position of this glucose unit and the nature of the sugar (glucose or galactose) could also be responsible for the differences in elution times.

Other major products included ions at *m/z* 435 (M – 162 – 288 *amu*) suggesting the presence of flavanol, which must be (epi)-catechin (*161*). The loss of 288 *amu* occurs when the (epi)catechin is situated in the upper unit; otherwise, we would expect a loss of 290 *amu* if the (epi)catechin is found in the terminal unit (*230*). In all likelihood, these compounds could be isomers of the same compound within the catechin (tannin) family.

Another unidentified compound is *Peak 7* (t_R = 4.45 min, λ_{max} = 271 nm) which had an even numbered $[M - H]^-$ ion at *m/z* 508. Further, some of its main MS/MS fragments also gave even numbered ions: at *m/z* 328 (M – 180 *amu*); *m/z* 258 (M – 180 – 70 *amu*); and a major ion at *m/z* 145 (M – 180 – 70 – 113 *amu*) (**Table 7**). Since the experimental conditions were optimized to provide for the best fragmentation conditions for individual compounds, this distinct fragmentation characteristic of this compound in the negative mode indicates the presence of a unique group in the its structure in addition to the usual phenolic skeletons.

Peak 15 (t_R = 5.85 min, λ_{max} = 278 nm) (**Figures 15A-2** and **19A-1)** had a $[M - H]^-$ at *m/z* 387, with MS/MS fragments at *m/z* 207 (M – 180 *amu*), suggesting a loss of caffeic acid (aromatic acid), and *m/z* 89 (M – 180 – 118 *amu*), suggesting a further loss of succinic acid (aliphatic acid) without concomitant loss of water molecule (**Table 7**). This could indicate the presence of these acylated groups bound to the compound. These groups may also be responsible for low λ_{max} = 278 nm exhibited by this compound. However, the two groups may be held together by a simple phenolic compound, which could be esters or other phenolic plant constituents.

Peak 16 (t_R = 6.03 min, λ_{max} = 279 nm) (**Figure 8A-1**) had a $[M - H]^-$ at *m/z* 721, with MS/MS fragments at *m/z* 407 (M – 314 *amu*), and at *m/z* 289 (M – 314 – 117 *amu*). The presence of *m/z* 289 as one of the major MS/MS product ions may suggest it is from the catechin family of compounds. The ion with 314 *amu* is suspected to consist of two units; a galloyl (152 *amu*) and glycosyl (162 *amu*) moieties. The ion with 117 *amu* may be a compound(s) whose constituent(s) are still unknown (**Table 7**).

Peak 20 (t_R = 6.36 min, λ_{max} = 294 nm) showed $[M - H]^-$ at *m/z* 317, which is similar to that of myricetin. Since flavonols have UV absorption maxima ~ 360 nm, this compound cannot be a myricetin (**Table 7**). Because it has similar fragmentation pattern as (+)-catechin, we suspect this compound could be related to the catechin family of molecules. The difference between catechin and this compound is 28 *amu*. Thus, it is possible that this compound consisted of a catechin unit with one aldehyde group linked to the position 6 or 8 of the aromatic A ring (*231*).

Peak 26 (t_R = 6.86 min, λ_{max} = 376 nm) (**Figure 20B**) showed a $[M - H]^-$ at *m/z* 899. In the MS/MS spectrum different fragments are observed, the majority being the ion at *m/z* 737 (M – 162 *amu*), corresponding to loss of a glycosyl unit. The other important fragments are ions at *m/z* 585 (loss of 152 *amu*), *m/z* 557 (loss of 180 *amu*), *m/z* 539 (loss of 198 *amu*) and *m/z* 407 (loss of 330 *amu*), which arise from the cleavage of the ion at *m/z* 73 (**Table 8**). However, on the basis of the MS data, we could not establish the identity of this peak.

Peak 31 (t_R = 7.48 min, λ_{max} = 290 nm) had $[M - H]^-$ at *m/z* 349, and MS/MS spectrum showed fragments at *m/z* 331 (M – 18 *amu*), corresponding to loss of water

molecule; followed by *m/z* 299 (M – 18 – 32 *amu*), corresponding to subsequent loss of a methoxyl group; then *m/z* 271 (M – 18 – 32 – 28 *amu*), corresponding to subsequent loss of an oxo group (C=O). *Peaks 31*, however, had no absorbance in the visible region of the UV-*vis* spectra (**Table 7**), meaning the flavylium structure with conjugated double bonds was interrupted, thus the correct identity of this peak could not be elucidated. *Peak 35* (t_R = 7.73 min, λ_{max}= 278 nm) had $[M - H]^-$ at *m/z* 723. During fragmentation, the major ions were formed at *m/z* 434 (loss of 289 *amu*, possibly catechin), *m/z* 395 (loss of 328 *amu*), *m/z* 287 (loss of 436 *amu*) and m/z 125 (loss of 598 *amu*). The ions with mass 328, 436 and 598 probably consist of multiple parts, the constituents of which are unknown (**Table 7**). This is an unusual structural pattern that, to our knowledge, has never been observed before in phenolic compounds.

According to mass spectral data, *peaks 37* and *38* may have structural features similar to each other. They both consisted of $[M - H]^-$ at *m/z* 467, and MS/MS products at 323 (loss of 144 *amu*), 305 (loss of 162 *amu*), 203 (loss of 264 *amu*), 189 (loss of 278 *amu*) and 161 (loss of 306 *amu*). However, *peak 37* (t_R = 7.809 min) eluted shortly before *peak 38* (t_R = 7.814 min), suggesting it is less polar than *peak 38*; and its λ_{max} (359 nm) was 34 nm higher than that of *peak 38* (**Table 8**).

On the other hand, *peak 39* (t_R = 7.90 min) exhibited a maximum UV absorbance at 350 nm. It gave rise to a precursor ion at *m/z* 597. The fragmentations included ions at *m/z* 454 (M – 143 *amu*), 387 (M – 210 *amu*), 380 (M – 217 *amu*), 357 (M – 240 *amu*), 273 (M – 324 *amu*), 229 (M – 368 *amu*) and 167 (M – 430 *amu*) (**Table 8**). It is evident

that *peak 39* shows an unusual structural pattern that, to our knowledge, has not been observed in flavonols before.

Peak 43 (t_R = 8.29 min) gave rise to $[M - H]^-$ at *m/z* 737. Based on its retention time, it appears that this compound is very polar; while its maximum UV absorbance at 376 nm suggests it may be a chalcone. The major MS/MS ions were at *m/z* 407 (M – 330 *amu*) and 394 (M – 343 *amu*). The intensity of the latter ion (*m/z* 394) was much higher than that of the former (*m/z* 407) in the MS/MS spectrum (**Table 8**). This fragmentation pattern is also unique in the sense that ions with 330 and 343 *amu* may consist of multiple compounds, the nature of which have not been previously observed in chalcone derivatives.

Peak 44 (t_R = 8.40 min, λ_{max} = 279 nm) shows $[M - H]^-$ at *m/z* 809, with MS/MS fragments at *m/z* 595 (M – 214 *amu*), 435 (M – 374 *amu*), 391 (M – 418 *amu*), 272 (M – 537 *amu*) and 161 (M – 648 *amu*) (**Table 7**). This high molecular weight compound is typical of proanthocyanidins (condensed tannins), which are oligomeric and polymeric catechins. However, catechins and catechin-related compounds usually produce specific molecular ions at *m/z* = 289 and 577. Our MS/MS analysis did not detect any of these signals, as well as any glycosyl ions, indicating that this peak is not related to catechins and proanthocyanidins or its glycosidated derivatives. From this evidence, there is a high likelihood that this peak may be a unique compound that has never been identified in cowpea before, thus, application of NMR techniques is suggested.

Peak 46 (t_R = 8.65 min, λ_{max} = 364 nm) gave rise to $[M - H]^-$ at *m/z* 867, which on fragmentation resulted in ions at *m/z* 517 (M – 530 *amu*), 349 (M – 530 – 12 *amu*),

331 (M – 530 – 12 – 6 *amu*) and 317 (M – 530 – 12 – 6 – 2 *amu*) (**Table 8**). The ion with mass 530 can be attributed to a rutinose (6-*O*-α-L-rhamnosyl-*D*-glucose) attached to acetoyl group (42 *amu*). Based on the maximum UV absorbance we assume this peak may be related to a flavonol-like compound.

Peak 52 (t_R = 5.28 min, λ_{max} = 325 nm) had a precursor ion at *m/z* 865. The MS/MS spectra showed ions at *m/z* 577 and 289 (**Table 9**), typical of high molecular weight catechin/epicatechin derivatives. We observed a major fragment at *m/z* 713 (loss of 152 *amu*), corresponding to loss of galloyl unit. Salminen *et al*. (*232*) reported that loss of a galloyl unit indicated that the galloyl unit most probably was bonded via a *m*-depside bond. Thus, this peak could be an *m*-galloyl-linked derivative. Structural properties of the compound with mass 136 (713 – 577 *amu*) is unknown. The ion at *m/z* 425 corresponds to direct Retro Diels-Alder (RDA) cleavage of the pseudomolecular ion in the top unit, while the ion at *m/z* 125 corresponds to the RDA fragment in negative mode (*140*). The ion at *m/z* 407 corresponds to water elimination (425 – 18 *amu*), while the λ_{max} at 325 nm suggest that one of its units may be a flavonol. In this study, we already reported the presence of a flavonol-flavan-3-ol derivative (*peak 66*), thus *peak 52* could also have been formed through a similar mechanism. Nevertheless our analysis did not allow us to establish the position and stereochemistry of the interflavanoid linkage.

Peak 54 (t_R = 6.11 min, λ_{max} = 266 nm) had $[M - H]^-$ at *m/z* 281, and from the MS/MS fragments, it appears that this could be a simple phenolic compound. One of the major signals in MS/MS was caused by the ion at *m/z* 237 (loss of 44 *amu*). A loss of 44

amu is characteristic of a free carboxyl group (*233*). Additionally, only small molecules (80, 92 and 110 *amu*) were lost during fragmentation, with one exception; ion at *m/z* 123 (loss of 158 *amu*). The UV spectrum of *peak 55* is consistent with a derivative of phenolic acids (**Table 9**).

Peak 60 (t_R = 7.26 min) had a molecular ion *m/z* 341, with a UV-*vis* absorption maximum of 492 nm – a property usually observed in some anthocyanins. However, anthocyanidins with a 3-hydroxyl group do not exist naturally in their aglycone form. Anthocyanidins are also soluble in methanol. If this pigment was an anthocyanin, we would expect to find it in greater quantities in the methanolic fraction than in the ethyl-acetate fraction. We did not detect it in the methanol fraction. The MS/MS spectra showed ions at *m/z* 295 (M – 46 *amu*), 267 (M – 74 *amu*), 241 (M – 100 *amu*) and 153 (M – 188 *amu*), clearly indicating that this compound is non-glycosylated and therefore has no relationship with known anthocyanins (**Table 9**). The use of NMR technology may help elucidate the structural features of this compound, which to our knowledge, is identified in pulses for the first time.

Peak 61 (t_R = 7.43 min) appear to be a monomer of *peak 64* (t_R = 8.08 min). *Peak 61* has $[M - H]^-$ at *m/z* 521, with a major MS/MS product ion at *m/z* 359 (M – 162 *amu*) suggesting loss of glucose moiety. *Peak 64* MS/MS spectra showed ions at *m/z* 521 and 1043, indicating that the compound had an aglycone with *m/z* 359 bonded to a glucose unit, and then the two compounds are linked together. For this reason, an ion with high intensity at *m/z* 359 occurs, possibly from breaking of the interflavanoid linkage. Other major fragments occur at *m/z* 344 (M – 162 – 15 *amu*) and 119 (M – 162 – 15 – 225

amu) (**Table 9**). This is an unusual structural pattern that, to our knowledge, has never been described before in phenolic compounds.

Peak 63 (t_R = 8.05 min, λ_{max} = 353 nm) is a high molecular weight compound with $[M - H]^-$ at *m/z* 985. The major MS/MS ions upon fragmentation were at *m/z* 521 (M – 464 *amu*), suggesting loss of glucose + HHDP (302 *amu*). The fragment at *m/z* 477 indicated a further loss of 44 *amu*, in addition to losing the HHDP-glucose group (*i.e.* M – 464 – 44 *amu*). A loss of 44 *amu* corresponds to loss of a free carbonyl. The other ions produced were at *m/z* 463 (M – 464 – 44 – 12 *amu*) and 341 (M – 644 *amu*). Thus far, we suspect that the ion with mass 644 had constituents with mass of 136 in addition to HHDP-glucose group (**Table 9**).

Peak 65 (t_R = 8.27 min, λ_{max} = 381 nm) showed a precursor ion at *m/z* 561. The MS/MS spectrum gave a fragment at *m/z* 409 (M – 152 *amu*), corresponding to a loss of galloyl unit (*234*). Further, another major ion at *m/z* 273 (M – 152 – 136 *amu*) suggesting possible structural relationship with *peak 64* in that the ion with mass 288 (152 + 136 *amu*) could be consisting of a galloyl unit as well as a compound with mass of 136. In addition, they both have UV-*vis* absorption maxima between 350 – 400 nm (**Table 9**).

In *peak 67* (t_R = 8.75 min, λ_{max} = 269 nm), the appearance of a fragment at *m/z* 561, corresponding to the loss of 288 *amu* is observed, and that, we believe, is due to the cleavage of flavanol (epi)-catechin (**Table 9**). A loss of 288 *amu* indicates the (epi)-catechin most probably is situated in the upper unit (*161*). This is followed by a fragment at *m/z* 409 (M – 288 – 152 *amu*), corresponding to subsequent loss of a galloyl unit (152

amu); and at *m/z* 367 (M – 288 – 152 – 42 *amu*), corresponding to another loss of acetoyl unit (42 *amu*). The presence of a strong signal at *m/z* 287 makes us believe that compounds with masses 288 and 287 actually came from an A-type procyanidin (575 *amu*), with additional groups such as galloyl, acetoyl and another compound with a total mass of 80 *amu*.

Peak 69 (t_R = 9.38 min, λ_{max} = 352 nm) had $[M - H]^-$ at *m/z* 493. The fragmentation, however, was different, with main fragments at *m/z* 181 (loss of 312 *amu*), 167 (loss of 326 *amu*) and 123 (loss of 370 *amu*) (**Table 9**). Based on this, the identity of this minor peak cannot be elucidated since the fragmentation sequence does not follow any known pattern.

Finally, *peak 71* (t_R = 9.85 min, λ_{max} = 260 nm) had [M – H]– at *m/z* 553. Its fragmentation pattern of *m/z* 385→ 241→ 167 provided little information to help in its identification. Similarly, we could not correctly elucidate the structure of *peak 72* (t_R = 10.38 min, λ_{max} = 274 nm) which had pseudo-molecular ion at *m/z* 579, and whose fragmentation followed this pattern: *m/z* 385 → 325 → 283 → 241 → 193 (**Table 9**). Small amounts of catechin might be present in the compounds not yet identified, and which have similar spectral characteristics or fragmentation ions as (epi)-catechin. We also believe some of these unidentified peaks might be new compounds never reported before in pulses. To adequately characterize these unidentified peaks, extensive fractionation and purification of these peaks and application of NMR technology is suggested.

VITA

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