PHENOLIC COMPOUNDS OF SORGHUM, THEIR CHEMOPREVENTIVE PROPERTIES AND ABSORPTION

A Dissertation

by

VICTOR MANUEL TALEON ALBAN

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Chair of Committee,	Lloyd W. Rooney
Committee Members,	Joseph Awika
	Susanne Talcott
	William L. Rooney
Head of Department,	Jimmy Keeton

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ABSTRACT

Sorghum contains many phenolic compounds which have potential antioxidant, anti-inflammatory, and chemopreventive properties as well as natural colorants in foods. Phenolic compounds of stalks, sheaths, leaves, glumes and grains from tan (ARTx631/RTx436), red (Tx2911) and purple (Tx3362) sorghum plants were characterized by UPLC-MS/MS. Antiproliferative properties of selected sorghum extracts were evaluated using HT-29 colon cancer cells and absorption of their polyphenolics was determined by a Caco-2 *in vitro* model system.

Phenolic acids, flavones, 3-deoxyanthocyanidins and chalcones were found in all plant components. Phenolic acids were predominant in the stalks, sheaths and grains of all sorghum types. Flavone glycosides were predominant in leaves, sheaths and stalks while flavone aglycones in glumes and grains. 3-Deoxyanthocyanidins and chalcones were mostly found in sheaths, leaves, glumes and grains of Tx2911 (red) and Tx3362 (purple) genotypes. Sorghum leaves showed high levels of flavone glycosides while glumes had high levels of flavones aglycones.

Glume extract of ATx631/RTx436 (tan) and Tx2911 genotypes had the strongest antiproliferation activity (IC_{50} = 85-178 μg/ml), these extracts had also the highest levels of flavone aglycones (19.6-49.8 mg/g). Absorption of flavones (30.4-42.3 %) was higher than 3-deoxyantocyanidins (1.4-11.3%), while absorption of methoxylated 3-deoxyanthocyanidins (11.3%) was higher than non-methoxylated 3-deoxyanthocyanidins (1.4 – 1.6 %). Flavones had high absorption compared to other sorghum phenolics

suggesting that sorghum flavone aglycones are more bioavailable than other sorghum phenolic compounds. Consequently, sorghum glumes could be used as used as a source of phytochemicals to increase value of sorghum crop.

DEDICATION

To my family.

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CHAPTER I

INTRODUCTION

Sorghum is the fifth most cultivated cereal in the world and in many regions it is a staple crop due to its adaptation to drought (FAOSTAT 2012; Rooney and Waniska, 2000; Blum 2005). Its grain is used for human consumption, animal feed and alcohol while its non grain material is used to produce forage, hay and silage (NRC., 1996; Kleih et al 2007). Natural dye is produced from the colored leaf sheaths of sorghum and used as textile or food colorants and natural medicine (Sereme et al 1993; Kouda-Bonafos et al 1994; Kayode et al 2011). There are no reports on utilization of by-products of the sorghum alcohol industry to extract colored compounds or nutraceutical products. Apart from chlorophyll, phenolic compounds are the main source of color in sorghum leaves and sheaths, similar to sorghum glumes and grains (Njongmeta, 2009).

In sorghum grains, three flavonoid subgroups of phenolic compounds have been found in high amounts in different genotypes: 3-deoxyanthocyanidins, flavones and flavanones (Dykes and Rooney 2006; Dykes et al 2009; Dykes et al 2011). 3-Deoxyanthocyanidins, found in high concentration in black sorghum grains, have shown anti-proliferation of cancer cells (Shih et al 2007; Guajardo, 2008; Yang et al 2009), and also potential as natural colorants in food systems at low pH (Awika et al 2004; Bjoroy et al 2009). Flavanones and flavones also have shown chemopreventive activity (Kuntz et al 1999; Seelinger et al 2008) and are found in high concentrations in lemon yellow and red sorghums with tan secondary plant color (Dykes et al, 2009). Furthermore,

extracts of phenolic compounds from sorghum bran have shown antioxidant and anti-inflammatory activity (Burdette et al 2010).

There are three groups of plants based on secondary plant color: tan, red and purple, controlled by the P and Q genes (Rooney and Waniska, 2000). Njongmeta (2009) identified high levels of 3-deoxyanthocyanidins, as well as several unknown flavones in sheaths, leaves and glumes of sorghum with red and purple secondary plant color. 3-deoxyanthocyanidins and flavones were found previously in sorghum sheaths and leaves (Stafford, 1965; Mueller-Harvey and Reed, 1992; Khalil et al 2010; Kayode et al 2011). Characterization of phenolic compounds found in non-grain material is necessary because many compounds detected are still unknown. Phenolic compounds of sorghum leaf sheaths have shown hepatoprotective and hematoprotective functions, but there are no reports about their anticancer properties (Akande and Biobaku, 2010).

HPLC or UPLC systems coupled with mass analyzers are used to determine the mass to charge ratio (m/z) and structural characteristics of phytochemicals. These systems have been used to characterize phenolic compounds in sorghum previously, for example Gonzalez-Montilla et al (2012) identified flavonoids in sorghum grains using a time of flight detector with electro-spray ionization. Tandem mass spectrometry (MS-MS) coupled to other systems also has been used to determine fragmentation patterns of sorghum polyphenols. For example, Geera et al (2012) used a hybrid quadrupole/time of flight system with APcI source to identify 3-deoxyanthocyanins in sorghum leaf sheaths and Svensson et al (2010) identified phenolic acids and flavonoid derivatives from fermented and non-fermented red sorghum using a quadrupole ion trap system. A

tandem quadrupole mass analyzer has not been widely used to identify structures of phytochemicals, but advances in software technology has allowed the use of this flexible and less expensive system to characterize flavonoids in fruit juices as shown by Abad-Garcia et al (2009) and cowpeas (Ojwang et al 2012). Consequently, the use of a tandem quadrupole mass analyzer could facilitate the identification and characterization of phenolic compounds in sorghum plant components.

Absorption of phenolics compounds is variable due to their different properties such as solubility and molecular size. For example, Zumdick et al (2012) demonstrated that absorption of oligomeric procyanidins decreased when the molecular weight increased. Anthocyanins are rapidly absorbed but they have poor bioavailability relative to other flavonoids (McGhie and Walton, 2007). Absorption of polyphenolics can be done using an *in vitro* model with Caco-2 cells (Pacheco-Palencia, 2009; Tian et al 2009). Caco-2 cells are commonly cultured on permeable supports that allow the measurement of polyphenolics transportation from the apical to the basolateral side of the cell monolayers or from the basolateral to the apical side. Transport efficiency is strongly influenced by chemical structure, concentration and the presence of additional matrix components (Deprez et al 2001; Yi et al 2006). Absorption of sorghum polyphenolics has not been studied yet.

In the present study, sorghum stalks, sheaths, leaves, glumes and grains were evaluated to determine their potential use as a source of beneficial phytochemicals which could create sub-products with high economical value. To determine their potential

benefits it was necessary to identify and chemically characterize them and determine their antioxidant and chemopreventive properties as well as their bioavailability.

To accomplish these needs, the following objectives were established:

1. Chemical characterization of phenolic compounds present in sorghum stalks, sheaths, leaves, glumes and grains of plants with tan, red and purple secondary color (ATx631/RTx436, Tx2911 and Tx3362) by UPLC MS/MS.

2. Determination of the anti-carcinogenic potential of grains, leaves and glumes of sorghum plants with tan, red, and purple secondary plant color on human cancer cells HT-29 by measuring mitochondrial activity.

3. Evaluation of the absorption of phenolic extracts of sorghum with the highest anticancer potential, and 3-deoxyanthocyanidin standards, using an *in vitro* intestinal absorption model with Caco-2 cells monolayer.

CHAPTER II

LITERATURE REVIEW

Sorghum Grain Utilization

In Asia and Africa sorghum grain is used mostly for human foods (FAOSTAT 2012). The types of sorghums used include white, yellow or red sorghums. The most common types of foods prepared are flat breads from fermented or unfermented dough, thin or thick fermented or unfermented porridges, cous cous, and fried products (Rooney and Waniska 2000; Leder, 2004; INTSORMIL, 2006). In the United States, South America and Australia, sorghum grain it is used principally for alcohol production and animal feed. In these regions, sorghum for human food is mostly white sorghum, used as a substitute for corn and wheat. The production of specialty sorghums with or without tannins and high in flavonoids is important (Rooney and Waniska 2000), because they have natural colorants (Awika et al 2004) and phytochemicals beneficial for human health (Shih et al 2007; Bralley et al 2008; Yang et al 2009; Burdette et al 2010).

Uses of Sorghum Plant Material

Forage, hay and silage are produced with the non-grain material of sorghum (NRC, 1996). In the United States the plant material is being extensively studied as a potential source of sugars and starch, to produce alcohol (Almodares and Hadi, 2009). In some regions of Nigeria, Sudan, Burkina Faso and Benin a red dye used as a colorant

in textile and foods is extracted from the sheaths and leaves of sorghum (Sereme et al 1993; Kouda-Bonafos et al 1994; Jansen and Cardon 2005; Kayode et al 2011).

Sorghum Color

Genetic factors determine pericarp color, pericarp thickness, presence of a pigmented testa and pigmentation of the glumes (Rooney and Waniska, 2000). Sorghum grains are classified by pericarp color as red, yellow and white. These properties are determined by the *R* and *Y* genes. The pericarp is white when the gene *Y* is homozygous recessive (*rryy* or *R_yy*). It is yellow when genes *R* and *Y* are homozygous recessive and homozygous dominant, respectively (*rrYY*). When both genes *R* and *Y* are dominant (*R_Y_*), the pericarp is red. Some sorghums with (*R_Y_*) genes in the presence of intense light turn black at maturation, such sorghums are known as black sorghums (Dykes et al 2005). The three main groups of flavonoids: anthocyanins, flavones and flavanones, are important compounds that give color or function as co-pigmentation factors (Rein, 2005). Sorghum plants are classified in three groups based on their

secondary plant color: tan, red and purple. The genes controlling these colors are *P* and *Q* (Rooney and Waniska, 2000). Sorghums with homozygous dominant *PQ* genes produce plants with purple or red secondary plant color, while recessive *pq* genes produce tan plants.

Polyphenolic Compounds of Sorghum Grains

Polyphenols are components of most plant seeds and grains. In sorghum, polyphenols are located mainly in the pericarp, testa, and aleurone layer (Awika et al 2005). The polyphenolic groups present in sorghum are classified as: phenolic acids, flavonoids and proanthocyanidins (Dykes and Rooney, 2006).

Phenolic acids are phenylpropanoids with an aromatic ring and a three carbon side chain. Phenolic acids are found in nature in free and bound forms as esters and glycosides; for example, chlorogenic acids are a family of esters formed between trans cinnamic acids and (-)-quinic acid (Plazonic et al 2009). Twenty one phenolic acids have been reported in sorghum grains (Table 1). The predominant phenolic acids reported are ferulic, cinnamic, *p*-coumaric and protocatechuic acids (Dykes and Rooney, 2006).

Table 1. A summary of the phenolic acids reported in sorghum grains.

Phenolic Acid	Reference
Gallic	Hahn et al., (1983); Awadelkareem et al., (2009)
Protocatechuic	Hahn et al., (1983); Awadelkareem et al., (2009)
Protocatechuic aldehyde	Svensson et al., (2010)
p-Hydroxybenzoic	Hahn et al., (1983); Waniska et al., (1989)
Gentisic	Waniska et al., (1989); Awadelkareem et al., (2009)
Salicylic	Waniska et al., (1989)
Vanillic	Hahn et al., (1983)
Syringic	Waniska et al., (1989); Awadelkareem et al., (2009)
Ferulic	Hahn et al., (1983); Awadelkareem et al., (2009)
Diferulic	Chiremba et al., (2012)
Feruloyl-caffeoylglycerol	Yang et al., (2012)
Caffeic	Hahn et al., (1983); Awadelkareem et al., (2009)
Caffeoylglycerol	Svensson et al., (2010)
1,3-Dicaffeoylglycerol	Svensson et al., (2010)
p-Coumaric	Hahn et al., (1983)
Coumaric-caffeic acid hexoside	Yang et al., (2012)
Coumaroylglycerol	Svensson et al., (2010)
1,3-Coumaroyl-caffeoylglycerol	Svensson et al., (2010)
Cinnamic	Hahn et al., (1983); Waniska et al., (1989)
Sinapic	Waniska et al., (1989)
1,3-Coumaroyl-feruloylglycerol	Svensson et al., (2010)

The second group of polyphenolic compounds is the flavonoids. They are secondary metabolites derived from products of aromatic amino acid biosynthesis (phenylalanine) and the Krebs cycle (acetyl Co A). Flavonoids have a C_6-C_3-C_6 configuration and in nature the principal subgroups are anthocyanins, flavones, flavonols, flavanones, and flavanols (Shirley, 1998). All the major subgroups of flavonoids have been identified in sorghum grains (Table 2). The subgroups 3-deoxyanthocyanidins, flavones and flavanones were found in high amounts in a diverse range of sorghums grains (Dykes et al 2009, 2011). The 3-deoxyanthocyanidins are a special kind of anthocyanidins that have been found in only a limited number of species including sorghum and maize (Winkel, 2006). The difference between the 3-deoxyanthocyanidins and anthocyanidins is that the first group does not have a hydroxyl group in position 3 of the C ring (Figure 1), which gives the 3-deoxyanthocyanidins more stability (Awika et al 2004). In sorghum grains, four 3-deoxyanthocyanidins have been found in significant amounts: apigeninidin, luteolinidin, 5-methoxyluteolinidin and 7-methoxyapigeninidin. Luteolin and apigenin are commonly found flavones and eriodictyol and naringenin are the principal flavanones. Most of the flavonoids in sorghum grains are located in the outer layers i.e: pericarp, testa and aleurone layers (Winkel, 1998; Awika et al 2005; Dykes et al 2009).

Table 2. Flavonoids found in sorghum grains.

Flavonoid	Reference
3-Deoxyanthocyanins:	
Apigeninidin	Nip and Burns (1971); Gous (1989); Dykes et al., (2009)
Apigeninidin-5-glucoside	Nip and Burns (1971); Wu and Prior (2005)
Luteolinidin	Nip and Burns (1971); Gous (1989); Dykes et al., (2009)
5-Methoxyluteolinidin	Seitz (2005); Wu and Prior (2005); Dykes et al., (2009)
7-Methoxyluteolinidin	Seitz (2004)
5-Methoxyluteolinidin-7-glucoside	Wu and Prior (2005)
7-Mexhotyapigeninidin	Pale et al., (1997); Wu and Prior (2005); Dykes et al., (2009)
Luteolinidin-5-glucoside	Nip and Burns (1971); Wu and Prior (2005)
5-Methoxyapigeninidin	Seitz (2005)
7-Methoxyapigeninidin-5-glucoside	Wu and Prior (2005)
Flavan-4-ols:	
Luteoforol	Bate-Smith (1969)
Flavonol:	
Quercetin	Afify et al (2012)
Kaempferol	Afify et al (2012)
Flavones:	
Apigenin	Gujer et al., (1986); Seitz (2005); Dykes et al., (2009)
Luteolin	Dykes et al., (2009)
7-O-Methylluteolin	Yang et al., (2012)
7-O-Methylapigenin	Yang et al., (2012)
Flavanones:	
Eriodictyol	Kambal and Bate-Smith (1976); Dykes et al., (2009)
Eriodictyol-5-glucoside	Gujer et al., (1986)
Naringenin	Gujer et al., (1986); Dykes et al., (2009)
Naringenin-7-o-glucoside	Yang et al., (2012)
Flavonols:	
Kaempferol-3-rutinoside-7-glucuronide	Nip and Burns (1969)
Dihydroflavonols:	
Taxifilin	Gujer et al., (1986)
Taxifolin-7-glucoside	Gujer et al., (1986)

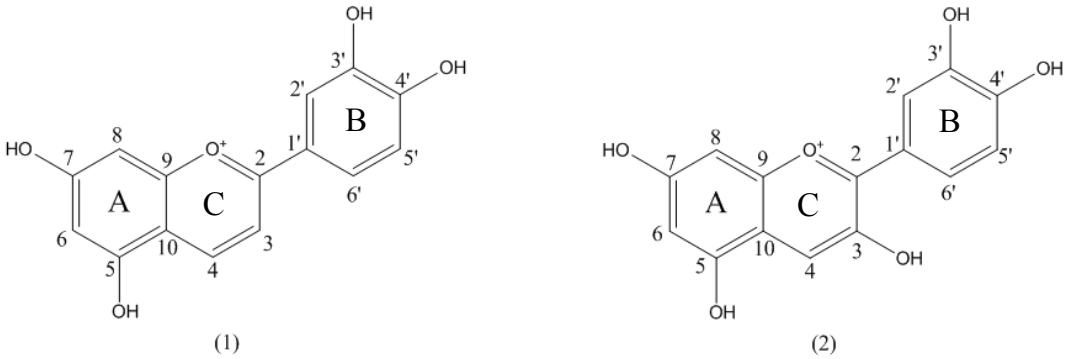

Figure 1. Structure of the 3-deoxyanthocyanidins (1) and anthocyanidins (2).

The third group of polyphenolic compounds present in sorghums is the condensed tannins, also known as proanthocyanidins (Dykes and Rooney, 2006). Sorghum grains with tannins are characterized by having the $B_1\_B_2\_$ genes. The main proanthocyanidins found in sorghum grains are summarized in Table 3.

Table 3. Proanthocyanidins reported in sorghum grains.

Proanthocyanidin	Reference
Catechin	Gupta and Haslam (1978); Gujer et al., (1986)
Procyanidin B-1	Gupta and Haslam (1978); Gujer et al., (1986)
Epicatechin-(epicatechin)n-catechin	Gupta and Haslam (1978); Gujer et al., (1986)
Prodelphinidin	Brandon et al., (1982)
Proapigeninidin	Dykes and Rooney (2006)
Proluteolinidin	Dykes and Rooney (2006)

Polyphenolic Compounds of Sorghum Leaves, Sheaths, Glumes and Stalks

Polyphenolic compounds have been reported in sorghum leaves and sheaths (Table 4). Phenolic acids (*p*-cuomaric, ferulic, salicylic, caffeic, vanillic, gentisic, *p*-hydroxybenzoic, sinapic and syringic) have been identified individually in glumes and stalks (Ring et al 1988) while cinnamic acid has been reported in glumes only. The 3-deoxyanthocyanidins (luteolinidin, apigeninidin, 5-methoxyapigeninidin and 7-methoxyapigeninidin) are found in sorghum glumes and stalks of plants with red secondary plant color (Njongmeta, 2009). The flavones (luteolin and apigenin) have also been reported in glumes (Siame et al 1993; Njongmeta, 2009), but only apigenin has been reported in the stalk (Njongmeta, 2009).

Biosynthesis of Flavonoids

Flavonoids have a C_6-C_3-C_6 configuration. For most of the sorghum flavonoids, the biosynthesis pathway is the same, where phenylalanine and acetyl-CoA are the precursors of the basic flavonoid molecules, the chalcones (Lo and Nicholson, 1998; Winkel, 2006; Vermerris and Nicholson, 2006) (Figure 2).

Table 4. Polyphenolics found in sorghum leaves and sheaths.

Polyphenolic compoud	Reference
p -hydroxycinnamic acid	Stafford (1967)
p -hydroxybenzoic acid	Ring et al., (1988)
p -coumaric acid	Mueller-Harvey and Reed (1992)
Chlorogenic acid	Kojima and Conn (1982)
Ferulic acid	Stafford (1967)
Sinapic acid	Stafford (1967)
Gentisic	Ring et al., (1988)
Caffeic acid	Ring et al., (1988)
Vanillic acid	Ring et al., (1988)
Syringic acid	Ring et al., (1988)
Salicylic acid	Ring et al., (1988)
Cinnamic acid	Ring et al., (1988)
Apigenin	Mueller-Harvey and Reed (1992)
Apigenin-*C* -glycoside	Njongmeta (2009)
Apigenin-7-*O* -glucoside	Mueller-Harvey and Reed (1992)
Luteolin-7-*O* -glucoside	Mueller-Harvey and Reed (1992)
Malvidin	Kayode et al., (2012)
Luteolininidin	Stafford (1965); Nicholson et al., (1987)
Apigeninidin	Stafford (1965); Nicholson et al., (1987)
Luteolinidin-5-glucoside	Stafford (1965)
Apigeninidin-5-glucoside	Stafford (1965)
Caffeic acid ester of arabinosyl-5-*O* -apigeninidin	Hipskind et al., (1990)
5-Methoxyluteolinidin	Lo et al., (1996)
7-Methoxyapigeninidin	Wharton and Nicholson (2000)
Pyrano-apigeninidin 4-vinylphenol	Khalil et al., (2010)
Apigeninidin-flavene dimer	Geera et al., (2012)
Apigeninidin-7-*O* -methylflavene dimer	Geera et al., (2012)
Apiforol	Watterson and Butler (1983)

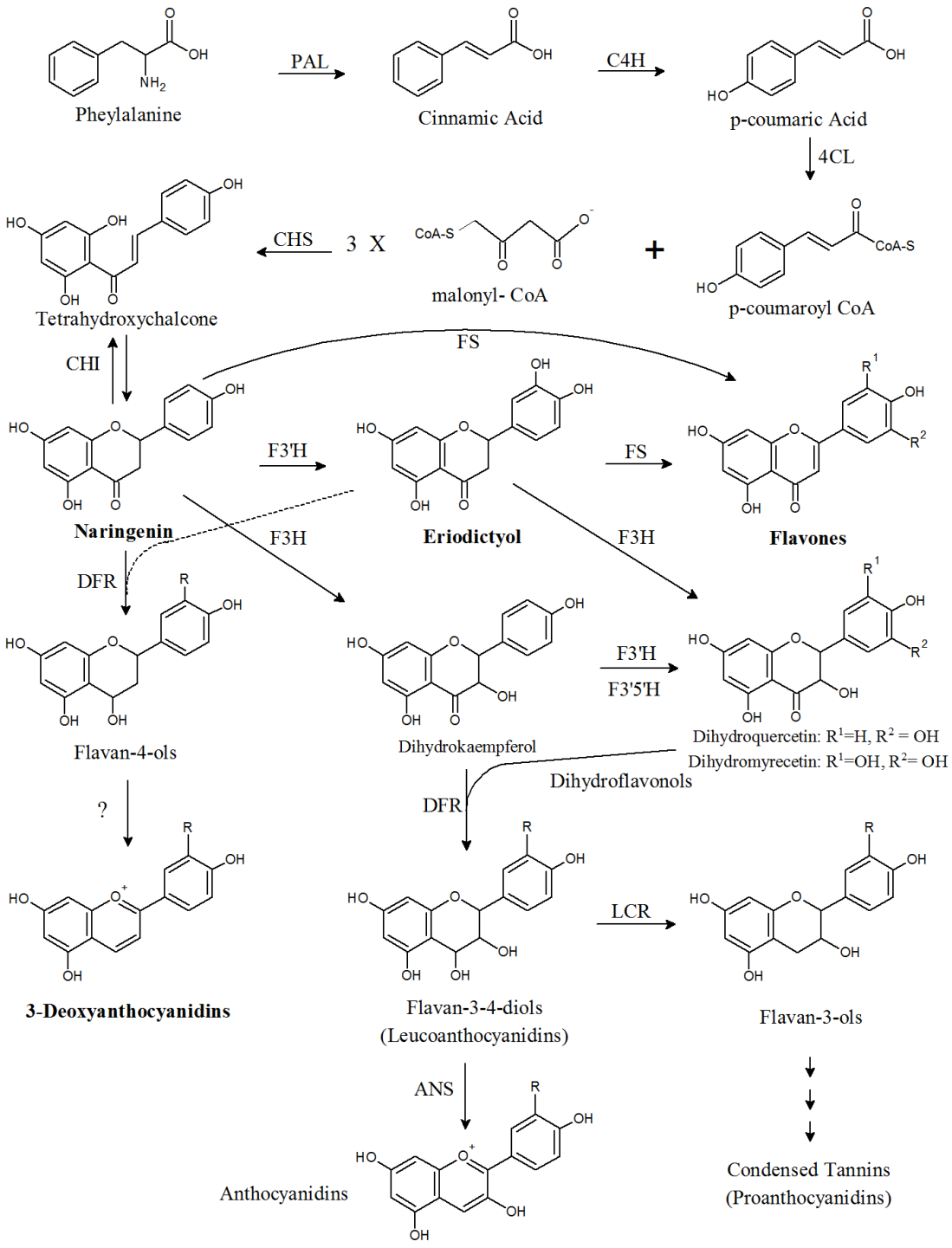

Figure 2. Biosynthesis of sorghum flavonoids. (Adapted from Winkel, 2006 and Davies and Schwinn, 2006).

Importance of Sorghum Polyphenolics

The main class of polyphenolic compounds studied in sorghum is the 3-deoxyanthocyanins because of their special phytoalexin properties which also have shown cancer cell anti-proliferation (Shih et al 2007; Guajardo, 2008; Yang et al 2009; Gonzalez-Montilla et al 2012). These compounds contribute to the black, purple, red and yellow pigmentation in sorghum grains, glumes, sheaths, stems and leaves (Awika et al 2011). 3-Deoxyanthocyanidins have potential as natural colorants in food systems at low pH (Awika et al 2004). They also have fungicidal activity, especially the metholylated forms (Aida et al 1996). They are found in high concentrations in black sorghum grains (Dykes et al 2009). Levels of 3-deoxyanthocyanidins in leaves and sheaths of red sorghums were between 13.7 and 35.5 mg/g while in black sorghum bran the levels were between 4.7 and 11.0 mg/g (Kayode et al 2011). Flavanones and flavones are high in yellow, and red sorghum with a tan secondary plant color (Dykes et al 2009, 2011), respectively. These two groups also have shown anticancer activity (Kuntz et al 1999). Crude phenolic extracts of sorghum bran have shown antioxidant and anti-inflammatory activity (Burdette et al 2010).

Extraction of Phenolics

The extraction of phenolic compounds in sorghum grains is difficult because their location in the cell wall matrix reduces their availability to the extraction solvent. In whole grains, 75 to 85 % of the phenols are present in the bound form (Hahn 1984; Waniska et al 1989) while in fruits and vegetables there are more free than bound

phenolic compounds (Liu, 2007). Different methods are used in the extraction of phenolic compounds from plant materials, depending on the matrix of the material. In sorghum, Hahn (1984) and Gous (1989) found that 1% HCl in methanol was an efficient solvent. Other solvents have been used for the extraction of phenolic compounds from fruits and vegetables and cereals. For example, aqueous acetone has been considered a good solvent for the extraction of procyanidins, anthocyanins and other phenolic compounds in fruits and vegetables (Kallithraka et al 1995; Garcia-Viguera et al 1998). However, Lu and Foo (2001) observed significant anthocyanins interaction when aqueous acetone was used as extraction solvent in fruits and vegetables. Awika et al (2004) also observed modification of the spectral absorbance of 3-deoxyanthocyanins associated with the formation of pyranoluteolinidin and pyranoapigeninidin when aqueous acetone was used as extraction solvent. Combinations of acetic, citric and tartaric acids in aqueous ethanol gave the same profiles of 3-deoxanthocyanins as the commonly used 1% HCl in methanol extractions (Njongmeta et al 2007).

For extraction of flavonoids in leaves, sheaths and stalks 1 % HCl in methanol has been effective (Njongmeta, 2009). Removal of chlorophyll and lipids using petroleum ether is done to obtain cleaner extracts that give better separation during HPLC analysis of flavonoids. 50 % aqueous methanol solution also is a good solvent to extract 3-deoxyanthocyanidins from sorghum leaves (Geera et al 2012). Extraction with water with a neutral pH and magnetic or mechanical stirring at room temperature was an optimum method to obtain polyphenolics from leaves of red sorghum in an environmental friendly semi-industrial scale (Agbangnan et al 2012).

Mass Spectrometry

HPLC or UPLC systems coupled with mass analyzers (detectors) such as quadrupole mass filter, quadrupole ion trap or time of flight are used to determine mass to charge ratio *m/z*, expressed as atomic mass units (*amu*), and structural characteristics of phytochemicals that are previously charged by an ion source (Rijke et al 2003; Pati et al 2009). The common ion sources used to charge molecules detected by mass analyzers are: matrix-assisted laser desorption/ionization (MALDI), fast atom bombardment (FAB), atmospheric-pressure chemical ionization (APcI), thermospray (TSP) and electro-spray ionization (ESI) (Fabre et al 2001; Stobiecki, 2000; Rijke et al 2006). The charge applied to the molecule can be positive or negative (Cuyckens et al 2001; Ablajan et al 2006).

Mass Spectrometry to Identify Phenolic Compounds

Mass spectrometry systems have been used as an important tool for detection, identification and structural characterization of phenolic compounds in plants because of its speed, sensitivity and specificity (Cabrera, 2006; Rijke et al 2006; Prasain and Barnes, 2007). These systems have been used to characterize polyphenolic compounds in sorghum previously, for example Gonzalez-Montilla et al (2012) identified flavonoids in sorghum grains using a time of flight detector with electro-spray ionization. Tandem mass spectrometry (MS-MS) coupled to other systems also has been used to obtain fragmentation patterns of sorghum flavonoids to determine molecular mass, structure of aglycone, acylation of sugar hydroxyl groups, methylation or sulphation of aglycone

hydroxyls and number of sugar rings (Plazonic et al 2009) . For example, Geera et al (2012) used a hybrid quadrupole/time of flight system with APcI source to identify 3-deoxyanthocyanins in sorghum leaf sheaths and Svensson et al (2010) identified phenolic acids and flavonoid derivatives from fermented and non-fermented red sorghum using a quadrupole ion trap system. A triple quadrupole has not been widely used to identify structures of phytochemicals, but advances in software technology has allowed the use of this flexible and less expensive system to characterize flavonoids in fruit juices (Abad-Garcia et al 2009) and cowpeas (Ojwang et al 2012).

The type of charge applied to the compound to identify polyphenols by the mass detector can be positive or negative depending on the type of phenolic compound. For example, anthocyanidins produce higher intensities using positive ion mode because these molecules exist in cationic forms (Lin et al 2011; Sun et al 2012). On the other hand, flavonoids such as flavones, flavonols and flavanones have higher intensities using negative ion mode (Fabre et al 2001) due to their partially negative charge (Han et al 2010). For some polyphenolic compounds, using a combination of negative and positive modes improve the identification of quasi-molecular ions (Es-Safi et al 2005; Abad-Garcia et al 2009; Mazerolles et al 2010).

Fragmentation Nomenclature of Polyphenols

Fragment ions of polyphenols are usually designated using a widely accepted nomenclature system for aglycones developed by Mabry et al (1970), and Ma et al (1997). Flavonol, flavones and flavanone aglycones produce two major types of fragmentation: small molecule losses and retro Diels-Alder cleavages (Davis, 2007). The small molecule losses involve the removal of some atoms from the flavonoid skeleton: H_2O, CO and CO_2. The retro Diels-Alder fragments involve the cleavage of two bonds of the C ring, resulting in complementary fragments containing an intact A or B ring (Cuyckens and Claeys, 2004; March et al 2004). Domon and Costello (1988) developed a notation for glycoconjugates where Y_o indicates the loss of an intact saccharide moiety cleaved at the glycosidic bond, and $^{i,j}X$ indicates the cross-ring cleavage of a sugar ring, with ^{i} and ^{j} indicating the specific bonds of the saccharide ring that were cleaved (Figure 3). Ma et al (1997) proposed a notation for such fragments: $^{i,j}A^{+/-}$ and $^{i,j}B^{+/-}$, where ^{i} and ^{j} denote the numbers of the two broken bonds of the C ring. A or B denote that the fragment ion contains an intact A or B ring, and either a $^{+}$ or $^{-}$ sign indicates the charge of the ion.

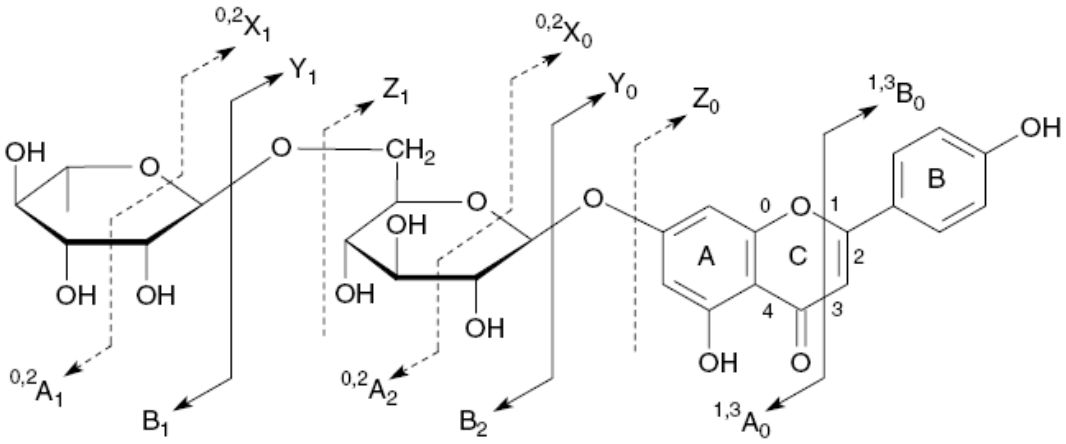

Figure 3. Fragment nomenclature commonly applied for flavonoid glycosides (Costello and Domon, 1988; Ma et al 1997).

Structural Characteristics of Polyphenolics with Glycoside Groups

Flavonoids commonly occur as flavonoid *O*-glycosides, in which one or more hydroxyl groups of the aglycone are bound to a sugar with formation of an acid labile glycosidic O–C bond (Abad-Garcia et al 2009). The 7-hydroxyl group in flavones and flavanones, the 3- and 7-hydroxyls in flavonols and flavan-3-ols and the 3- and 5-hydroxyls in anthocyanidins are the most common glycosylation sites (Abad-Garcia et al 2009; Ojwang, 2011). Glucose is the most commonly attached sugar to flavonoids; galactose, rhamnose, xylose and arabinose are also found (Abad-Garcia et al 2009). Disaccharides are often bound to flavonoids; the most common ones are rutinose (rhamnosyl-(1→6)-glucose) and neohesperidose (rhamnosyl-(1→2)-glucose). Tri- and tetra saccharides are also found with less frequency (Williams, 2006). Acylated glycosides, in which one or more of the sugar hydroxyls are esterified with an acid, also occur.

Glycosylation also can occur by direct linkage of the sugar to the basic nucleus of the flavonoid, via an acid-resistant C–C bond, to form flavonoid C-glycosides. Flavonoid C-glycosides are commonly further divided into mono-C-glycosyl-, di-C-glycosyl-, and O,C-diglycosyl-flavonoids (Abad-Garcia et al 2009). Hydrolyzable sugar is linked either to a phenolic hydroxyl group or a hydroxyl group of the C-glycosyl residue in the case of O,C-diglycosyl-flavonoids. C glycosylation has been found only at the C6 and/or C8 position for the flavonoid nucleus (Abad-Garcia et al 2009).

Fragmentation Properties of Phenolic Compounds by MS/MS

Fragmentation properties of polyphenols by mass spectrometry help determine their basic structural characteristics (Vukics and Guttman 2008). Hydroxycinnamic acids usually have an ion product that lost H_2O and CO molecules from the precursor ions as well as loss of CH_3OH in acids with methoxyl groups (ferulic and sinapic acids) (Clifford et al 2003; Abad-Garcia et al 2009). The products of hydroxybenzoic acids have fragments with the loss of H_2O, CO, CO_2 and CH_3OH in acids with methoxyl groups (Abad-Garcia et al 2009; Jaiswal et al 2011).

Naturally, flavonoids generally occur as O- or C-glycosides. MS-MS spectra of flavonoid glycosides have typical patterns, which depend mainly on the number or nature of the bound saccharides and their C- or O-glycosidic linkages (Gattuso et al 2007). The loss of fragments with well-defined mass from the pseudo-molecular ion provides precise information about the linked saccharide (Gattuso et al 2007). Fragmentation of an O-glycoside starts from the cleavage of the O-sugar bond. During

MS/MS fragmentation of O-glycosides, the glycosidic bond is cleaved and the precursor ion results with loss of 162 amu (hexose: glucose or galactose), 146 amu (deoxyhexose: rhamnose), 132 amu (pentose: xylose or arabinose), and 176 amu (glucuronic acid) (Ojwang, 2011). Fragmentations of flavonoid that require cleavage of two C-C bonds of the C-ring gives informative $^{i,j}A^{+/-}$ and $^{i,j}B^{+/-}$ ions (Figure 3) which are the most important fragment for structural elucidation (Pinheiro and Justino, 2012). These $^{i,j}A^{+/-}$ and $^{i,j}B$ provide information on the number and type of substituents in the A- and B-rings (Cuyckens and Clayes, 2004). For flavonoid C-glycosides, the sugar linked to the flavonoid nucleus with the C-C bond is fragmented creating fragments with *m/z* of $[(M-H)-120]^-$, $[(M-H)-90]^-$ and $[(M-H)-134]^-$for hexoses and $[(M-H)-90]^-$, $[(M-H)-60]^-$ and $[(M-H)-104]^-$ for pentoses, corresponding to the $^{0,2}X$, $^{0,3}X$ and $^{1,5}X$ residues, respectively (Cuyckens and Clayes, 2004).

Importance of Cancer in the United States

Cancer is a chronic disease that caused 25 % of the deaths in the United States in 2011 (Siegel et al 2012). The cancers with the highest incidence are lung, breast and colon cancer (Bertoldi, 2009). Colon cancer represented about 9 % of the deaths by cancer in the United States in 2011 (American Cancer Society, 2012).

Neoplasia and Cancer

Neoplasia is an abnormal and uncontrolled proliferation of cells, which may be benign or malignant, as a result of damage in mechanisms of cell cycle regulation or alteration in genes that regulate the growth and differentiation of cells (Bertoldi, 2009). Cancer is a disease that involves cells that have defects in their regulatory circuits (mutant cells), which governs normal cell proliferation and homeostasis (Hanahan and Weinberg, 2011).

Phases and Causes of Cancer

Carcinogenesis is a complex process that is divided into three stages: initiation, promotion and progression (Stoddart, 1983). Initiation occurs when the DNA or RNA is damaged, producing cell mutation that leads to unregulated cell growth (Klaunig et al 2010). This phase is rapid, reversible and affected by intracellular (inherited genes, oxidative stress, inflammation and hormones) and extracellular (exposure to carcinogens, diet and lifestyle) factors. A single malignant cell reproduces itself to form more malignant cells in the process called promotion which is reversible and may take several years to occur (Klauning et al 2010; Tan et al 2011). In the progression stage, preneoplastic cells undergo neoplastic transformation, which forms a tumor with invasive and metastasis potential (Surh, 2003). Cancer cell attributes can be divided into six common characteristics: sustaining proliferative signaling, evading growth suppressors, resisting programmed cell death (apoptosis), enabling replicative immortality, inducing angiogenesis and activating tissue invasion and metastasis. Due to

the malignant growth, cancer cells form a microenvironment that create their own growth needs and nutrient supply, which impairs the normal metabolism of the specific tissue where the tumor locates (Hanahan and Weinberg, 2011).

Chemopreventive Agents

Chemopreventive agents are dietary or pharmacological compounds with specific natural or synthetic agents designed to prevent, suppress or reverse the carcinogenesis process (Tan and Spivack, 2009; Swanson et al 2010). Two classes of chemopreventive agents have been identified: mono-functional inducers and bi-functional inducers. Mono-functional inducers stimulate Phase II enzymes while bi-functional inducers stimulate Phase I and Phase II enzymes. Since Phase I enzymes can bioactivate or detoxify carcinogens, mono-functional inducers of Phase II enzymes appear to be more effective chemopreventive agents than bi-functional inducers (Pezzuto et al 2005; Yun et al 2010). Knowing the activity of both Phase I and Phase II enzymes caused by chemopreventive agents is important, but the ratio between Phase I and Phase II enzyme activity is more important than the specific activity of either category of enzymes because higher levels of Phase I enzyme activity compared to Phase II enzyme activity can be negative, depending on the specific Phase I enzyme activated (Buck, 2003). A third class of chemopreventive agents, known as dual-acting agents, has been described to stimulate Phase II enzymes but inhibit Phase I enzymes. The dual-acting agents would be the ideal compounds for preventing carcinogen activation (Goossens et al

2007). 4-Methyl catechol, a-tocopherol and red wine decreased phase I enzyme activities while inducing total Glutathione S-transferases activity (Manson et al 1997).

Relevance of Sorghum Polyphenolics in Cancer Research

The main flavonoids found in sorghum: 3-deoxyanthocyanidins, flavones and flavanones (Dykes and Rooney 2006; Dykes et al 2009; Dykes et al 2011) have demonstrated chemopreventive potential. 3-Deoxyanthocyanidins, have shown anti-proliferation of cancer cells *in vitro* (Shih et al 2007; Guajardo, 2008; Yang et al 2009). Bran of black sorghum has shown chemopreventive activity against colon carcinogenesis in rats (Lewis, 2008). Anticancer activity of black sorghum can be attributed to the induction of superoxide dismutase which is involved in blocking the activator protein 1 (AP-1) (Lewis, 2008). AP-1 is a critical modulator of the proliferation and cycle of cancer cells (Suto et al 2004; Ashida, et al 2005). High activity of AP-1 is associated with tumor promotion and progression of cancers (Hou et al 2004). Black sorghum has been demonstrated to induce the phase II enzyme NQO (Awika et al 2009). Flavanones and flavones, found in high concentrations in lemon yellow and red sorghums with tan secondary plant color (Dykes et al 2009; 2011), also have shown anticancer activity (Kuntz et al 1999; Seelinger et al 2008). Flavones of sorghum induced apoptosis in nonmalignant colonocytes by the induction of estrogen receptor ERβ (Yang et al 2012). Also the flavone apigenin and their methoxylated forms have demonstrated to induce cell cycle arrest and apoptosis in prostate cancer cells (Shukla and Gupta, 2006; Walle et al 2007). Furthermore, Njongmeta (2009) identified

high levels of 3-deoxyanthocyanidins, as well as several unknown flavones in sheaths, leaves and glumes of sorghum with red and purple secondary plant color.

Cancer Cell Proliferation Assays

Evaluating the ability of a compound to directly inhibit cancer cell proliferation helps to determine its potential anticancer properties. This ability can be measured by the IC_{50} value, which indicates the amount of compound needed for a 50% inhibition of the net cell proliferation (Baguley et al 2002). Many methods are available to determine cell proliferation, for example: direct counting of viable cells using an hemocytometer or an electronic counter (Macleod and Langdon, 2004), measurement of metabolic activity like the MTT (3-(4,5-Diomethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay that measures mitochondrial metabolic activity, and radioactive and non radioactive methods to determine DNA synthesis (Yu et al 2009; Wang et al 2010).

Proliferation and Mitochondrial Activity

Generally, mitochondrial activity of viable cells is constant; consequently a change in the number of viable cells could be correlated to mitochondrial activity (Meerloo et al 2011). The MTT assay is a commonly used non-radioactive method to indirectly determine proliferation of cells by measuring mitochondrial activity. The MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT to form dark blue formazan crystals which accumulates within healthy cells. The crystals formed are largely

impermeable to cell membranes which help to quantify them. The number of surviving cells is directly proportional to the level of the formazan crystals created (Mosmann 1983; Yang, 2009).

Xenobiotic Transport Mechanisms

Absorption of xenobiotic compounds across the intestinal epithelium can occur through five mechanisms: passive transcellular route, passive paracellular route, carrier-mediated transport, carrier-mediated efflux, and transcytosis (Grassi, 2006; Whocely, 2006). Passive transcellular absorption occurs when the xenobiotic penetrates the membrane surrounding the epithelial cells. When transport occurs via the pores between the epithelium cells is called passive paracellular transport (Engman, 2003). Carrier-mediated transport occurs when a compound is actively transported by a specific transporter. When a compound that enters the cytoplasm of mucosal cells is transported back to the intestinal lumen it is called carrier-mediated efflux (Li, 2001). Trancytosis occurs when compounds are entrapped in vesicles due to invagination of the apical membrane (Whocely, 2006).

Absorption of Flavonoids

Absorption is an important factor to relate *in vitro* antioxidant and chemoprotective properties to in-vivo outcomes in cancer prevention by polyphenolics (Balimane et al 2000; Yi et al 2006). Absorption of flavonoids depends on factors such as chemical structure, molecular size, solubility, methoxylation and glycosylation which

determine the transport mechanisms (Piskula, 2000; McGhie and Walton 2007; Fernandez et al 2012). Active transport of flavonoids could occur through two main carrier superfamilies, the ATP-binding cassettes (ABC) and solute carriers (SLC). ABC transporters such as multidrug resistance protein (MRP) and p-glycoprotein (P-gp) are found to transport flavonoids (Wang et al 2005; De Castro, 2006; Dai et al 2008; Chabane et al 2010). SLC proteins such as monocarboxylic acid transporters (MCT) and organic anion transporters (OAT) also have been found to transport flavonoids (Hu et al 2003; Wang and Morris, 2007; Fale et al 2012).

Anthocyanins have very low bioavailability compared to other flavonoids such as quercetin, a widely described flavonoid (McGhie and Walton 2007; Borska et al 2012). Bioavailability of anthocyanins is known to be low *in vitro*, (McGhie and Walton 2007; Faria et al 2009; Pacheco-Palencia et al 2010), and *in vivo* (Borges et al 2007; Bo et al 2010; Gonzalez-Barrio et al 2010) although it has been reported high in some *in vivo* cases (Steinert et al 2008; Talavera et al 2003). The apparent absorption differs depending upon whether the compound is measured from the gastrointestinal tract or in the blood or urine (Prior and Wu, 2006). Absorption of flavones such as apigenin is high compared to quercetin (Gradolatto, et al 2005). Bioavailability of flavones with methoxylated groups were up to 10 times higher than the non methoxylated flavones (Wen and Walle, 2006; Walle et al 2007). Furthermore, absorption of oligomeric procyanidins decreased when the molecular weight increased (Zumdick et al 2012). Absorption of compounds *in vivo* was found to correlate with permeability coefficients *in vitro* using Caco-2 cells (Artursson and Karlsson, 1991; Artursson et al 2001).

In vitro Models to Measure Phenolic Absorption

The Caco-2 cell line is a recognized model used *in vitro* for determination of intestinal absorption potential of phytochemicals based on significant correlation between absorption in the Caco-2 model and percent of absorption in humans (Elsby et al 2008; Pacheco-Palencia, 2009; Tian et al 2009). The Food and Drug Administration recognized the model system as useful in classifying the absorption characteristics of a compound and classified them according to the Biopharmaceutics Classification System (Hu et al 2004; Chen et al 2012).

Caco-2 cells from human colon adenocarcinoma undergo a process of differentiation during long-term culture when they reach confluence on a porous polymer membrane, leading to the formation of cell monolayers that presents a model for a single layer of epithelial cells that covers the inner intestinal wall which is the rate-limiting barrier for the absorption of drugs (Hu et al 2004; Hubatsch et al 2007). Caco-2 cell monolayer models have been used to assess intestinal transport of several groups of polyphenolics, including phenolic acids (Konishi et al 2003; Pacheco-Palencia et al 2008), flavonoids (Tian et al 2009) and procyanidins (Nielson et al 2010; Ou et al 2012). Using Caco-2 model, active transport of quercetin glucosides has been shown (Williamnson 2003).

Caco-2 cells are commonly cultured on permeable supports that allow free access of nutrients to both sides of the cell monolayer, which resemble the conditions in the intestine in-vivo (Artursson 1990). All polyphenolic groups tested on Caco-2 cells have the ability to be transported from the apical to the basolateral side of cell monolayers.

Transport efficiency is strongly influenced by chemical structure, concentration, and the presence of additional matrix components (Deprez et al 2001; Yi et al 2006). Caco-2 cells are deficient in LPH which is the enzyme responsible for deglycosylation of glycosylated flavonoids, hence the sugar transporter cytosolic β-glucosidase pathway is the predominant pathway in these cells (Williamson, 2005). Transport efficiency is usually higher for low molecular weight compounds, decreasing as the molecular mass increases (Konishi et al 2003). Some procyanidin dimers and trimers are absorbed by Caco-2 cell monolayers, while polymers with a higher degree of polymerization are not (Scalbert et al 2000).

Data Analysis of Caco-2 Transport Model

The rate of transport is obtained from the amount transported and the time curve using linear regression. The following equation is used to make comparisons among permeability of different compounds:

$P = (V/SC)(dC/dt) = (dM/dt)(1/SC)$

Where, V is the volume of the receiver (cm^3), S is the surface are of the cell monolayer (cm^2), C is the initial concentration in μM, and dC/dt is the rate of concentration change on the receiver side in units of nmol/min.

Another important attribute to evaluate is the percent recovery which determines how much compound is metabolized. The percent recovery is calculated using the following equation:

$\% \text{ Recovery} = (M_x + M_d + M_c) / M_L * 100$

Where, M_x, M_d, and M_c are amounts of the drug obtained from the upper chamber, the basolateral chamber and cell monolayer at the end of the experiment, respectively and m_L represents the amount of compound loaded in the upper chamber at the beginning of the experiment (Hu et al 2004; Zuo et al 2006).

CHAPTER III

IDENTIFICATION OF PHENOLICS FROM DIFFERENT SORGHUM MORPHOLOGICAL PARTS

In this study, a tandem mass spectrometer was used to characterize the main phenolic compounds in different morphological parts (stalks, sheaths, leaves, glumes and grains) of sorghum plants with tan, red and purple secondary plant color.

Materials and Methods

Samples

Physiologically mature plants with tan (ATx631/RTx436), red (Tx2911) and purple (Tx3362) (Miller et al 1986; Miller et al 1992; Rooney et al 2000; Rooney et al 2013) secondary plant color were collected from nurseries grown at College Station and Weslaco in 2011. The stalks, sheaths, leaves, glumes and grains were the plant components analyzed.

Standards and Reagents

Gallic acid, and 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) and Folin-Ciocalteu reagent were obtained from Sigma (St. Louis, MO). Trolox was obtained from Aldrich (Milwaukee, WI). Petroleum ether, methanol HPLC grade, acetonitrile LC/MS and methanol LC/MS grade were obtained from Sigma-Aldrich (St. Louis, MO). Formic acid was obtained from Fischer Scientific (Pittsburgh, PA).

Apigenin, luteolin, luteolin-7-O-glucoside, apigenin-7-O-glucoside and naringenin, were obtained from Indofine Chemical Co., Inc. (Hillsborough, NJ). Eriodictyol, luteolinidin chloride, and apigeninidin chloride were obtained from ALSACHIM (Strasbourg, France) and 7-methoxyapigeninidin, 5-methoxyapigeninidin and 5-7-dimethoxyapigeninidin chloride were obtained from ChromaDex (Santa Ana, CA).

Sample Preparation

Plants were collected at maturation. Each of the five plant components was separated. The glumes were removed mechanically and the grains were cleaned. Stalks, leaves and sheaths were freeze dried and stored at -20 °C until analysis.

Extraction for Total Phenols and Antioxidant Activity

All samples were ground through a cyclotec mill (UDY Corp., Fort Collins, CO) (0.5 mm mesh) prior to extraction. For total phenols and antioxidant activity analysis, 0.1-0.5 g was extracted in centrifuge bottles with 25 mL 1% HCl/methanol (v/v) for 2 h while shaking at low speed using an Eberbach shaker (Eberbach Corp., MI). All extracts were centrifuged at 2790g for 10 min in a Sorvall SS-34 centrifuge (DuPont Instruments, Wilmington, DE) and decanted. To avoid oxidation, extracts were stored in the dark at -20°C and analysis was performed within 24 hours.

Total Phenol Analysis of Plant Components

Total phenols of the acidified methanol extracts were measured using the modified Folin-Ciocalteu method of Kaluza et al (1980). A 0.1 mL aliquot of each extract was dissolved in 1.1 mL of water and reacted with 0.4 mL of Folin-Ciocalteu reagent and 0.9 mL of 0.5M ethanolamine. After 20 min of reaction at room temperature, the absorbance at 600 nm was measured and compared to a gallic acid standard to determine total phenols expressed as gallic acid equivalents (GAE)/g of extract. The total phenolic content was determined for all components for each plant type.

Antioxidant Activity of Plant Components

Antioxidant activity of acidified methanol extracts was measured *in vitro* by the ABTS assay. The $ABTS^{\cdot+}$ solution was obtained by reacting 3 mM of $K_2S_2O_8$ with 8 mM ABTS salt in distilled, deionized water for 16 h at room temperature in the dark. The $ABTS^{\cdot+}$ solution was then diluted with a pH 7.4 phosphate buffer (50:42.5:9.5; water: 0.2 M Na_2HPO_4:0.2 M NaH_2PO_4) solution containing 150 mM NaCl (PBS) to obtain an initial absorbance of 1.5 at 734 nm. Fresh $ABTS^{\cdot}$+ solution was prepared each day of analysis. 0.05 g of each crude extract was solubilized in acidified methanol for 2 h while shaking at low speed using an Eberbach shaker (Eberbach Corp., MI). A 0.1mL aliquot of each crude extract solution and standard was reacted separately, with 2.9 mL of ABTS solution for 30 min at room temperature. The absorbance was measured at 734

nm and compared to a trolox standard curve. Results were expressed as micromolar Trolox Equivalent Antioxidant Capacity per g (μmol TE/g) of extract.

Extraction of Phenolic Compounds for Mass Spectrometry Analysis

All plant components from tan, red and purple plants were analyzed. For leaves glumes and sheaths, 0.5 g of ground samples was extracted in 10 mL of methanol, for 2 h in a shaker. For stalks and grains, the same extraction was performed using 1 g of sample. The extracts were centrifuged at 2790g for 10 min and decanted. Samples were washed with petroleum ether to remove chlorophyll using a 250 mL separatory funnel. After equilibration at room temperature all extracts were filtered using a 0.2 μm nylon membrane filter (Whatman Inc., Maidstone, UK) prior to UPLC analyses.

Identification of Phenolic Compounds Using UPLC-DAD and UPLC-TQD-MS/MS

Extracts of sorghum plant components were analyzed on an Acquity® UPLC System (Waters Corp., Milford, MA) with a Waters PDA eλ detector and TQD Detector. Sorghum phenolics were separated using a Kinetex C18 column (150 mm x 2.1 mm i.d., 2.6 μm) from Phenomenex (Torrance, CA). Column temperature was maintained at 40 ºC. The injection volume was 1 μL. The mobile phase consisted of 0.05% formic acid on water (v/v) (Solvent A) and acetonitrile (Solvent B).

The solvent flow rate was 0.4 mL/min. Phenolic compounds were separated using the following gradient: 0-23.5 min., 10-41% B; 23.5-25.5 min., 41-75% B; 25.5-28.5 min., 41-75% B; 28.5-29.5 75-10% B; 29.5-34.5 10% B. Every sample was scanned to determine the λ_{max} with the PDA, and the mass to charge ration (*m/z*) with the TQD using ESI ionization in negative ionization mode for all phenolics except 3-deoxyanthocyanidins which used positive mode.

To determine the *m/z* of the compounds, cone voltages (CV) between 30-45 V for negative mode and 60 V for positive mode were used. The collision energy values used for 3-deoxyanthocyanidins were 35-50 V and for the other polyphenols were 15-35 V. Each MS scan was recorded at 50 – 1000 Da. Fragmentation of each compound was performed applying optimized cone voltage and collision energy voltage for each compound. Fragments were compared with standard's fragments and with information in the literature. Identification of sorghum flavonoids was based on UV-Vis spectra, MS data and retention times of available commercial standard (described in standard and reagents section). Data acquisition and processing was performed using Empower 2 software (Waters Corp., Milford, MA).

Results and Discussion

Phenolic Acids of Sorghum

In the UPLC chromatograms from sorghum plant components obtained at 325 nm, thirteen predominant peaks (compounds 1-7, 9, 32, 39, 41, 44 and 46) (Figure 4 and Figure 5) had UV spectra similar to phenolic acids (Appendix 1). Further identification of these compounds was achieved by comparison of UV spectra and mass spectra of precursor and product ions of each compound with those available in the literature.

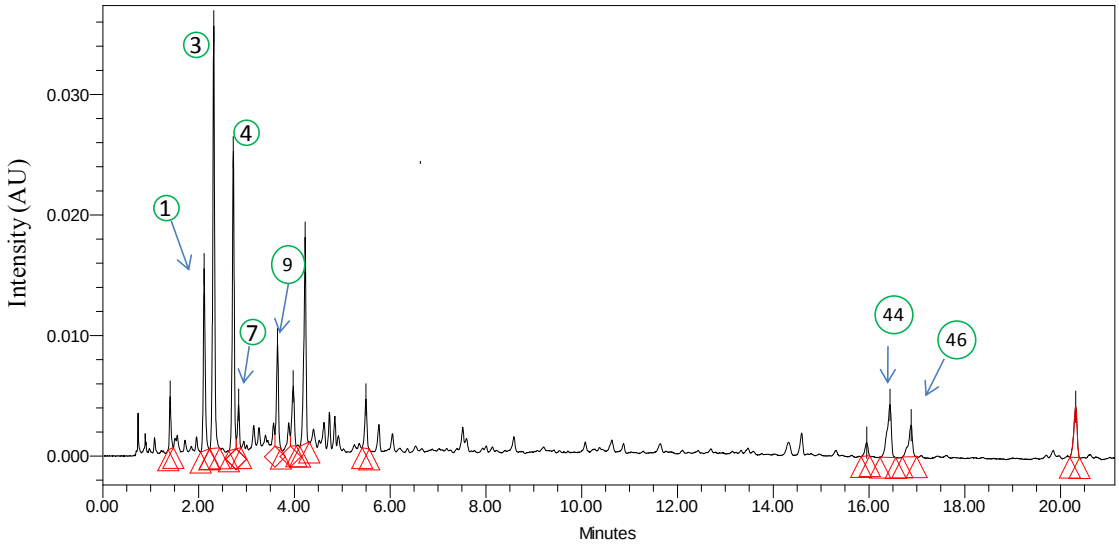

Figure 4. Chromatogram of ATx631/RTx436 sorghum stalk extract, taken at 325 nm, showing the peaks 1, 3, 4, 7, 9, 44, and 46 corresponding to phenolic acids.

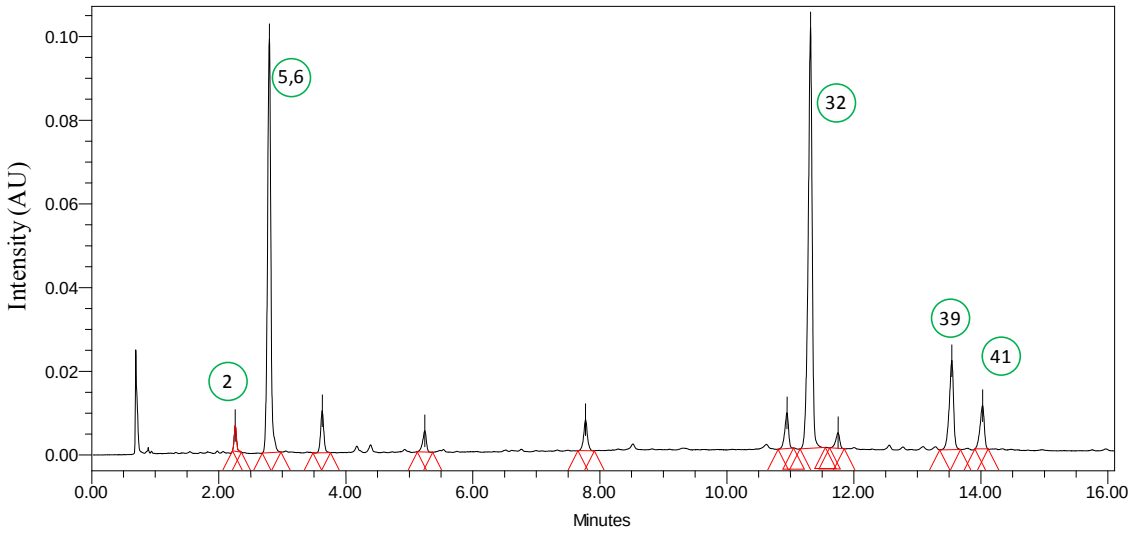

Figure 5. Chromatogram of Tx2911 sorghum grain extracts taken at 325 nm, showing the peaks 2, 5, 6, 32, 39 and 41 corresponding to phenolic acids.

Peak 1 (t_R = 2.12 min) showed a λ_{max} at 326 nm, molecular ion $[M - H]^-$ at *m/z* 353 and MS/MS fragments at *m/z* 191, 179, 173 and 161 (Table 5). The fragment *m/z* 191 represents a [quinic acid – H]$^-$ fragment while the fragment at *m/z* 179 represents a [caffeic acid – H]$^-$ fragment (Lozovaya et al 2006; Ma et al 2007). Using its fragmentation information, UV spectra characteristics and retention time of commercial standard, *peak 1* was identified as 3-caffeoylquinic acid also called chlorogenic acid (Appendix 2). This compound was previously identified in sorghum leaves (Kojima and Conn, 1982). In this study, chlorogenic acid was found in stalks, sheaths and leaves of all sorghum types (Table 6).

Table 5. Fragmentation properties of phenolic acids from sorghum stalks, sheaths, leaves, glumes and grains.

Peak	t_R (min)	λ_{max}	$[M-H]^-$ *(m/z)*	MS/MS (m/z, % Intensity)	Proposed Identity
1	2.12	326	353	191 (100) 179 (5) 173 (6), 161 (5)	Chlorogenic Acid
2	2.25	325	253	161 (100), 135 (17)	Caffeoyl-glycerol
3	2.32	326	367	193 (100), 173 (4), 149 (6), 134 (11)	Feruloylquinic Acid
4	2.73	328	355	193 (19), 175 (100)	Feruloyl hexoside
5	2.79	324	253	179, (16), 161 (100), 135 (32)	Caffeoyl-glycerol
6	2.79	325	179	135	Caffeic Acid
7	2.84	332	385	223 (6), 205 (100), 190 (14)	Sinapoyl hexoside
9	3.66	325	367	193 (20), 173 (100), 149 (5), 134 (7)	Feruloylquinic Acid
32	11.28	325	415	253 (100), 179 (43), 161 (85), 135 (11)	Dicaffeoyl-glycerol
39	13.50	314	399	253 (54), 235 (17), 179 (14), 163 (100), 145 (29)	Coumaroyl-caffeoyl-glycerol
41	13.99	325	429	193 (100), 253 (32), 175, (18), 161 (32), 134 (22)	Feruloyl-caffeoyl-glycerol
44	16.47	315	413	193 (78), 163 (100), 134 (37)	Coumaroyl-feruloyl-glycerol
46	16.80	325	443	193, 175, 160, 134	Diferuloyl-glycerol

Table 6. Phenolic acids found in sorghum stalks, sheaths, leaves, glumes and grains.

Peak	Proposed Identity	Stalks			Sheaths			Leaves			Glumes			Grains		
		T*	R	P	T	R	P	T	R	P	T	R	P	T	R	P
1	Chlorogenic Acid	✓+	✓+	✓+	✓	✓	✓	✓	✓	✓						
2	Caffeoyl-glycerol														✓	
3	Feruloylquinic Acid	✓+	✓+	✓+	✓+	✓+	✓+									
4	Feruloyl hexoside	✓+	✓+	✓+	✓+	✓+	✓+									
5	Caffeoyl-glycerol										✓+			✓	✓	✓
6	Caffeic Acid										✓+			✓	✓	✓
7	Sinapoyl hexoside	✓+	✓+	✓+	✓+	✓+	✓+									
9	Feruloylquinic Acid	✓+	✓+	✓+	✓+	✓+	✓+									
32	Dicaffeoyl-glycerol													✓	✓	✓+
39	Coumaroyl-caffeoyl-glycerol													✓	✓	✓+
41	Feruloyl-caffeoyl-glycerol													✓	✓	✓+
44	Coumaroyl-feruloyl-glycerol	✓+	✓+	✓+				✓+	✓+			✓+			✓	✓+
46	Diferuloyl-glycerol	✓+	✓+	✓+				✓+							✓+	✓+

* T = Tan plant (ATx631/RTx436), R= red plant (Tx2911), p = purple plant (Tx3362)

✓+ = Found for the first time in sorghum

Peak 2 (t_R = 2.25 min) had a λ_{max} at 325, molecular ion $[M - H]^-$ at *m/z* 253 and MS/MS fragments at *m/z* 161 and 135 (Table 5). Fragment ion at *m/z* 135 $[M - H - 64 - 44]^-$ indicated the presence of a decarboxylated caffeic acid residue. *Peak 2* was identified as caffeoyl-glycerol isomer (Appendix 3), based on its UV spectra, and similar fragments as *peak 5*. Caffeoyl-glycerol was previously reported in sorghum grains (Svensson et al 2010; Yang et al 2012). In this study, it was found in all grains, and glumes of the red plant (Table 6).

Peak 3 (t_R = 2.32 min) had a λ_{max} at 326 nm, molecular ion $[M - H]^-$ at *m/z* 367 and MS/MS fragments at *m/z* 193, 173, 149 and 134 (Table 5), which are characteristic fragments of feruloylquinic acids. Main fragments at *m/z* 193 $[(M-H)-174]^-$ were obtained due to the loss of the ferulic acid. The fragment at *m/z* 134 $[M-H-174-44-15]^-$ was obtained due to decarboxylation of the quinic acid and CH_3. These fragments are common in 1-feruloylquinic acid (1-FQA), 3-feruloylquinic acid (3-FQA) and 1-isoferuloylquinic acid (1-iFQA) but only 3-feruloylquinic acid (3-FQA) is known to produced main fragments at *m/z* 173 (Kuhnert et al 2010). Using its fragmentation information, *peak 3* was identified as 3-FQA (Appendix 4). This compound has not been previously reported in sorghum and in this study was found in stalks, sheaths and leaves of all sorghum types (Table 6).

Peak 4 (t_R = 2.73 min) had a λ_{max} at 328, molecular ion $[M - H]^-$ at *m/z* 355 and MS/MS fragments at *m/z* 193 and 175 (Table 5). Fragment ion at *m/z* 193 $[M - H - 162]^-$ represents a feruloyl residue after the loss of a hexosyl radical. Consequently, *peak 4* with the ion at *m/z* 355 was identified as feruloyl hexoside (Appendix 5). Only

ferulic acid has been reported previously in sorghum (Hahn et al 1983; Stafford, 1967) but not its glycoside, which in this study was identified in stalks and sheaths of all sorghum plants evaluated (Table 6).

Peak 5 (t_R = 2.79 min) showed a λ_{max} at 324, molecular ion $[M - H]^-$ at *m/z* 253 and MS/MS fragments at *m/z* 179, 161 and 135 (Table 5). Fragment ion at *m/z* 179 $[M - H - 74]^-$ corresponded to the caffeic acid residue, while fragments at *m/z* 135 $[M - H - 64 - 44]^-$ indicated the presence of a decarboxylated caffeic acid residue. The fragment *m/z* 179 $[M - H - 74]^-$ represents the loss of glycerol residue from the parent ion (Ma et al 2007). Hence, *peak 7* is identified as caffeoyl-glycerol (Appendix 3). Caffeoyl-glycerol was previously reported in sorghum grains (Svensson et al 2010; Yang et al 2012). In this study it was found in the grains of all plant types and glumes of the red plant (Table 6).

Peak 6 (t_R = 2.79 min) showed a λ_{max} at 325, molecular ion $[M - H]^-$ at *m/z* 179 and MS/MS fragments at *m/z* 135 (Table 5). Based on these characteristics peak 6 was identified as caffeic acid (Appendix 6). This compound was found in all grains and in glumes of the red plant (Table 6).

Peak 7 (t_R = 2.84 min) had a λ_{max} at 332, molecular ion $[M - H]^-$ at *m/z* 385 and MS/MS fragments at *m/z* 223, 205 and 190 (Table 5). The fragment at *m/z* 205 $[M - H - 162–18]^-$ corresponding to the loss of the hexosyl radical plus water represents the sinapic acid fragment (Ferreres et al 2006). Thus, *peak 7* was identified as sinapoyl hexoside (Appendix 7). This compound was found in all sorghum stalks and sheaths (Table 6) and is the first report of sinapic acid attached to a hexoside in sorghum.

Previous studies reported the presence of sinapic acid in sorghum glumes, stalks and leaves but without a hexoside group (Ring et al 1988).

Peak 9 (t_R = 3.66 min) showed a λ_{max} at 325, molecular ion $[M - H]^-$ at *m/z* 367 and MS/MS fragments at *m/z* 193, 173,149 and 134 (Table 5). Fragmentation pattern of *peak 11* had the main fragment at *m/z* 173 which is characteristics of 3-isoferuloylquinic acid (3-iFQA), 5-isoferuloylquinic acid (5-iFQA), 4-feruloylquinic acid (4-FQA) and 4-isoferuloylquinic acid (4-iFQA) (Kuhnert et al 2010; Nandutu et al 2007). But these compounds do not produce main fragments at *m/z* 149 and 134 which are major fragments in 3-FQA, 1-FQA and 1-iFQA (Kuhnert et al 2010). Taking in consideration its major fragments and reported information, *peak 9* was identified as an isomer of feruloylquinic acid (Appendix 4). This compound has not been previously reported in sorghum and in this study was found in stalks and sheaths of all sorghum types (Table 6).

Peak 32 (t_R = 11.28 min) showed a λ_{max} at 325, molecular ion $[M - H]^-$ at *m/z* 415 and MS/MS fragments at *m/z* 253, 179, 161 and 135 (Table 5). Fragments with *m/z* 179 and 135 suggest the presence of caffeic acid in the parent ion. Fragments at *m/z* 253 indicate the presence of a caffeoyl glycerol ion, as described in *compound 2*. Consequently, *peak 32* could be identified as dicaffeoyl-glycerol (Appendix 8). This compound was previously reported in red and white sorghum grains (Svensson et al 2010; Yang et al 2012). In the present study, this compound was found in all grains (Table 6).

Compound 39 (t_R = 13.50 min) showed a λ_{max} at 314, molecular ion $[M - H]^-$ at *m/z* 399 and MS/MS major fragments at *m/z* 253, 235, 179, 163 and145 (Table 5). Fragments at *m/z* 235 could be a product of the loss of a *p*-coumaric acid moiety [M-H-164]$^-$. Fragments at *m/z* 163 represent the ion of *p*-coumaric acid. Fragments at *m/z* 145 could be products from the loss of caffeoylglycerol residue by cleavage of the ester bond. Using the λ_{max}, fragmentation patterns and UV spectra, *compound 39* was characterized as coumaroyl-caffeoyl-glycerol (Appendix 9). This compound was identified in all sorghums grains (Table 6). Previously it was found in white and red sorghum grains (Svensson et al 2010; Yang et al 2012).

Compound 41 (t_R = 13.99 min) showed a λ_{max} at 325, molecular ion $[M - H]^-$ at *m/z* 429 and MS/MS major fragments at *m/z* 253, 193, 179, 161, and 134 (Table 5). These properties are similar to feruloyl-caffeoyl-glycerol (Appendix 10) which was previously reported in red and white sorghum grains (Yang et al. 2012). In the present study, *compound 41* was found in all sorghum grains (Table 6).

Compound 44 (t_R = 16.47 min) had a λ_{max} at 315, molecular ion $[M - H]^-$ at *m/z* 413 and MS/MS major fragments at *m/z* 193, 163 and 134 (Table 5). Fragments at *m/z* 193 indicate the presence of a feruloyl ion, while *m/z* 163 represents *p*-coumaric acid, as described above. Using the λ_{max}, fragmentation patterns UV spectra from references, *compound 44* was identified as coumaroyl-feruloyl-glycerol (Appendix 11). This compound has been reported in red grains (Svensson et al 2010). In this study coumaroyl-feruloyl-glycerol was found in all sorghum stalks, red and purple leaves, red grains and purple glumes and grains (Table 6).

Compound 46 (t_R = 16.80 min) had a λ_{max} at 325, molecular ion $[M - H]^-$ at *m/z* 443 and MS/MS major fragments at *m/z* 193, 175, 160 and 134 (Table 5). Fragments at m/z 193 and 175 are characteristic of ferulic acid fragments (Appendix 12). Consequently, compound 46 could be characterized as a diferuloyl-glycerol. This compound was found in all stalks, grains of red and purple plants and leaves of the purple plant (Table 6). This compound has not previously been reported in sorghum.

Most of the phenolic acids identified in sorghum were found in the stalks, sheaths and grains (Table 6). Phenolic acids found in sorghum plant components such as caffeic acid and chlorogenic acid have demonstrated anticancer potential (Belkaid et al 2006; Prasad et al 2011). Health benefits of feruloylquinic acids, and feruloyl and sinapoyl hexosides found in sorghum stalks and sheaths for the first time have not been evaluated. Eight phenolic acids identified in grains and glumes were derivatives with a glycerol group attached (Table 6). Health benefits of phenolic acids with glycerol molecules attached had not been evaluated. Phenolic acids found in sorghum plant components need evaluation to determine their potential antioxidant and anticancer properties.

Flavones of Sorghum

Eleven distinct peaks (compounds 10, 12, 17, 24, 26, 27, 29, 35, 37, 42 and 49) (Figure 6 and Figure 7) with UV-vis spectra matching the flavones group (Appendix 13) were detected in the sorghum plant components based on chromatograms acquired at 340 nm. Further identification was achieved by comparison of UV-vis spectra, retention

time of commercial standards, *m/z* ratio of precursor and product ions of compounds with those available in the literature.

Compound 10 (t_R = 4.24 min) showed a λ_{max} at 334, molecular ion $[M - H]^-$ at *m/z* 563 and MS/MS major fragments at *m/z* 503 $[(M-H)-60]^-$, 473$[(M-H)-90]^-$, 443$[(M-H)-120]^-$, 383$[A + 113]^-$ and 353$[A+ 83]^-$(Table 7). All fragments of *compound 10* are characteristic of *di*-C-hexosyl-pentosyl flavones (Colombo et al 2008). In *C*-glycosyl flavones, sugars at position 6 are easily fragmented relative to the sugar at position 8. Also, it is known that ion at *m/z* 503 results from the fragmentation of the pentose group (Ferreres et al 2003). Consequently relatively high intensities of fragments at *m/z* 503 indicate the presence of the pentosyl group at position 6 in *di-C*-hexosyl-pentosyl flavones. The relatively high intensity of fragment at *m/z* 503 compared to its other fragments and its λ_{max} UV spectra, suggests that *compound 10* could be identified as **6-***C*-pentosyl-8-*C*-hexosyl apigenin (Appendix 14). This compound has not been reported in sorghum before and in this study was found in all sorghum stalks, sheaths and leaves (Table 8).

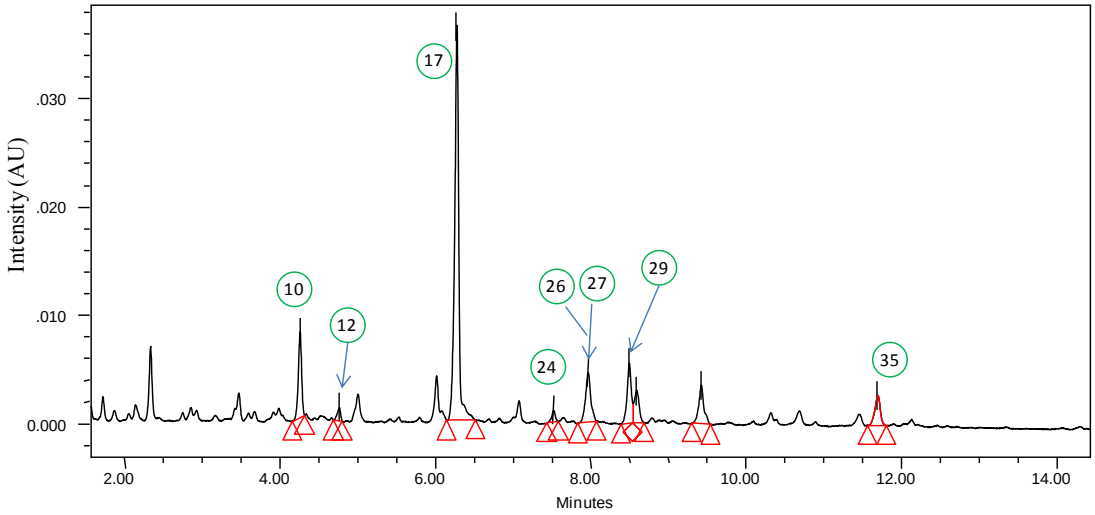

Figure 6. Chromatogram of Tx3362 sorghum leaf extract taken at 340 nm showing the peaks 10, 12, 17, 24, 26, 27, and 29 corresponding to flavones.

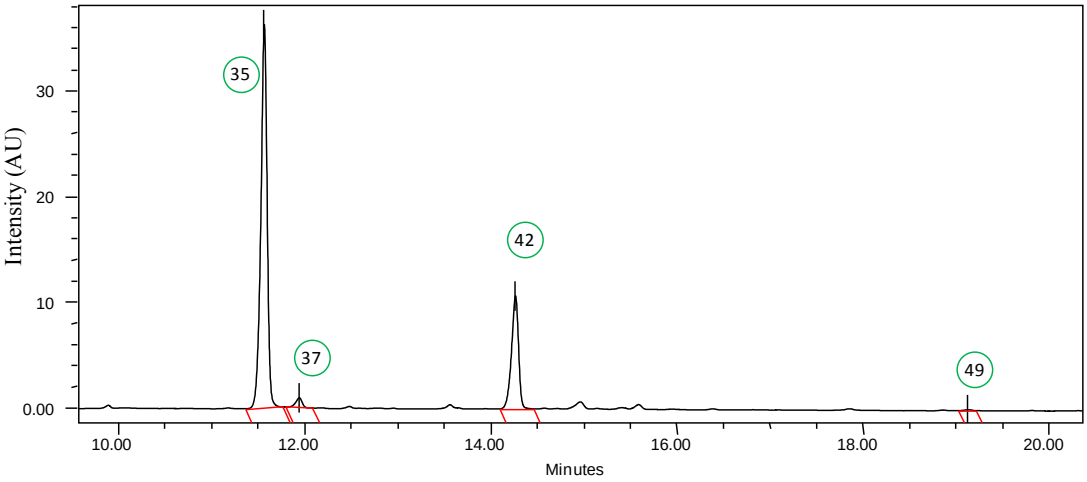

Figure 7. Chromatogram of ATx631/RTx436 plant glumes extract taken at 340 nm showing the peaks 35, 37, 42 and 49 corresponding to flavones.

Table 7. Fragmentation properties of flavones from sorghum plant components.

Peak	t_R (min)	λ_{max}	$[M-H]^-$ *(m/z)*	MS/MS (m/z, % Intensity)	Proposed Identity
10	4.24	334	563	503 (30), 473 (47), 443 (46), 383 (99), 353 (100)	6-*C*-pentosyl-8-*C*-hexosyl Apigenin
12	4.78	338	563	443 (9), 383 (11), 353 (100)	6-*C*-hexosyl-8-*C*-pentosyl Apigenin
17	6.21	349	447	285 (100), 175 (9)	Luteolin-7-*O*-glucoside
24	7.51	337	577	269 (100)	Apigenin-7-*O*-rutinoside
26	7.92	337	431	269 (100)	Apigenin-7-*O*-glucoside
27	7.95	348	447	285 (100)	Luteolin-*O*-glucoside
29	9.41	330	491	328(33),313(100),285(18)	Tricin-*O*-hexoside
35	11.65	348	285	151 (6), 133 (100)	Luteolin
37	11.95	347	315	300 (72), 201 (49), 137 (100)	Tetrahydroxy methoxy flavone
42	14.26	334	269	117 (100)	Apigenin
49	19.13	343	343	313 (64), 298 (100), 285 (11), 270 (55)	Dihydroxy trimethoxy flavone

Table 8. Flavones found in sorghum plant components.

Peak	Proposed Identity	Stalks			Sheaths			Leaves			Glumes			Grains		
		T	R	P	T	R	P	T	R	P	T	R	P	T	R	P
10	6-*C*-pentosyl-8-*C*-hexosyl Apigenin	✓+	✓+	✓+	✓+	✓+	✓+	✓+	✓+	✓+						
12	6-*C*-hexosyl-8-*C*-pentosyl Apigenin			✓+				✓+	✓+	✓+						
17	Luteolin-7-*O*-glucoside				✓	✓	✓	✓	✓	✓						
24	Apigenin-7-*O*-rutinoside								✓+	✓+						
26	Apigenin-7-*O*-glucoside							✓	✓	✓						
27	Luteolin-*O*-glucoside							✓+	✓+	✓+						
29	Tricin-*O*-hexoside							✓+	✓+	✓+						
35	Luteolin	✓			✓			✓	✓	✓	✓	✓	✓	✓		✓
37	Tetrahydroxy methoxy flavone										✓+	✓+	✓+			✓+
42	Apigenin	✓			✓	✓			✓		✓	✓	✓		✓	✓
49	Dihydroxy trimethoxy flavone										✓+		✓+			✓+

* T = Tan plant (ATx631/RTx436), R= red plant (Tx2911), p = purple plant (Tx3362)

✓+ = Found for the first time in sorghum

Compound 12 (t_R = 4.78 min) showed a λ_{max} at 334, molecular ion $[M - H]^-$ at *m/z* 563 and MS/MS major fragments at *m/z* 443[(M-H)-120]$^-$, 383[Aglycone + 113]$^-$ and 353[Aglycone+ 83]$^-$ (Table 7). These characteristics are similar to the 6-*C*-pentosyl-8-*C*-hexosyl apigenin (Ferreres et al 2003), but the absence of fragments at *m/z* 503 suggests that *compound 12* might be identified as 6-*C*-hexoyl-8-*C*-pentosyl apigenin (Appendix 15). This compound has not been reported in sorghum before and in this study was found in all sorghum leaves and stalks of the purple plant (Table 8).

Compound 17 (t_R = 6.21 min) showed a λ_{max} at 349, molecular ion $[M - H]^-$ at *m/z* 447 and MS/MS major fragments [(M-H)-162]$^-$ at *m/z* 285 (Table 7). *Compound 17* showed the characteristic fragmentation of *O*-glycosyl luteolin, which includes the fragment ions at *m/z* 285 produced by the loss of the sugar moiety (Havattum and Ekeberg, 2003). Using the λ_{max}, fragmentation patterns, UV spectra, and retention time of a commercial standard, *compound 17* was characterized as luteolin-7-*O*-glucoside (Appendix 16). Luteolin-7-*O*-glucoside was previously identified in sorghum leaves (Mueller-Harvey and Reed, 1992) and in this study was identified in sheaths and leaves of all plant types (Table 8).

Compound 24 (t_R = 7.51 min) showed a λ_{max} at 337, molecular ion $[M - H]^-$ at *m/z* 577 and MS/MS major fragments at *m/z* 269 [(M-H)-308]$^-$ (Table 7). Compound 24 showed the characteristic fragmentation of a flavone rutinoside which includes fragment ions [(M-H)-162-146]$^-$ at *m/z* 269, representing the loss of a glucose and a rhamnose (Lin et al, 2010). Using the λ_{max}, fragmentation patterns, UV spectra from references, *compound 24* was identified as apigenin-7-*O*-rutinoside (Appendix 17). This compound

was found in leaves of red and purple plants which is the first report of apigenin-7-*O*-rutinoside in sorghum.

Compound 26 (t_R = 7.92 min) showed a λ_{max} at 337, molecular ion $[M - H]^-$ at *m/z* 431 and MS/MS major fragments at *m/z* 269 $[(M-H)-162]^-$. *Compound 26* had the characteristic fragmentation of flavones *O*-glycosides. Using the λ_{max}, fragmentation patterns, UV spectra and retention time of commercial standard, *compound 26* was identified as apigenin-7-*O*-glucoside (Appendix 18). Apigenin-7-*O*-glucoside was previously identified in sorghum sheaths (Mueller-Harvey and Reed, 1992). In this study apigenin-7-*O*-glucoside was found only in sorghum leaves (Table 8).

Compound 27 (t_R = 7.95 min) showed a λ_{max} at 348, molecular ion $[M - H]^-$ at *m/z* 447 and MS/MS major fragments at *m/z* 285 $[(M-H)-162]^-$. *Compound 27* had the characteristic fragmentation of luteolin-7-O-glucoside but with a longer retention time. Luteolin-4-O-glucoside elutes after luteolin-7-O-glucoside in a typical separation using a C-18 column (Abad-Garcia et al 2009; Obied et al 2007). Using the fragmentation patterns, retention time and UV spectra from references, *compound 27* could be identified as luteolin glucoside, which has not been reported in sorghum and in this study was found in all sorghum leaves (Table 8).

Compound 35 (t_R = 11.65 min) showed a λ_{max} at 348, molecular ion $[M - H]^-$ at *m/z* 285 and MS/MS major fragments at *m/z* 151 $[(M-H)-134]^-$, and 133. Using fragmentation patterns, UV spectra and retention time of commercial standard, *compound 35* was identified as luteolin (Appendix 20). Luteolin was found in all plant components of the tan plant, and leaves, glumes and grains of the purple plant (Table 8).

Luteolin was previously reported in leaves, sheaths and grains (Njongmeta, 2009; Dykes et al 2009, 2011) but this is the first time it was identified in glumes and stalks (Table 8).

Compound 37 (t_R = 11.95 min) had a λ_{max} at 347, molecular ion $[M - H]^-$ at *m/z* 315 and MS/MS major fragments at *m/z* 300 $[(M-H)-15]^-$, 201, and 137. Fragments at *m/z* 300 indicated the loss of a methoxy group. Using the λ_{max}, fragmentation patterns UV spectra from references, *compound 37* was identified as tetrahydroxymethoxy flavone (Appendix 21). This compound has not been reported in sorghum; in this study it was present in grains of purple plant and in all sorghum glumes (Table 8).

Compound 42 (t_R = 14.26 min) showed a λ_{max} at 334, molecular ion $[M - H]^-$ at *m/z* 269 and MS/MS major fragments at *m/z* 117. Using the λ_{max}, fragmentation patterns, UV spectra and retention time from commercial standard, *compound 42* was identified as apigenin (Appendix 22). Apigenin was found in all glumes, stalks and sheaths of the tan plant and sheaths, leaves and grains of the red plant (Table 8). Apigenin has been reported in leaves, sheaths and grains (Gujer et al 1986; Mueller-Harvey and Reed, 1992; Dykes et al 2009, 2011), but this is the first time it was identified in glumes (Table 8).

Compound 49 (t_R = 19.13 min) showed a λ_{max} at 343, molecular ion $[M - H]^-$ at *m/z* 343 and MS/MS major fragments at *m/z* 313 $[(M-H)-30]^-$, 298, 285 and 270. These fragments indicated the loss of three methoxy groups. Using the λ_{max}, fragmentation patterns, UV spectra from references, compound 49 was identified as dihydroxytrimethoxy flavone (Appendix 23). This is the first report of

dihydroxytrimethoxyflavone in sorghum, and it was found in glumes and grains of purple plants (Table 8).

Flavones identified in stalks, sheaths and leaves had mostly hexoside or pentoside groups attached to luteolin or apigenin. Leaves contained more flavones that the other plant parts. In glumes and grains, the flavones were found only in the aglycone form (Table 8). Eight flavones were identified for the first time in sorghum plant components (Table 8). The evaluation of sorghum plant material with flavones is necessary to determine their potential health benefits because flavones had demonstrated health benefits (Kuntz et al 1999).

3-Deoxyanthocyanidins of Sorghum

Twelve compounds with λ_{max} characteristic of 3-deoxyanthocyanidins (Appendix 24) were found (compounds 11, 14-16, 21-23, 40, 43, 45, 47 and 48) in the sorghum plant components (Figure 8 and Figure 9). 3-Deoxyanthocyanidins had stronger ionization using positive mode.

Compound 11 (t_R = 4.28 min) showed a λ_{max} at 487, molecular ion $[M + H]^+$ at *m/z* 271. MS/MS major fragments at *m/z* 141 $[M+H-130]^+$ and 115 were obtained (Table 9). Based on retention time of commercial standard, *m/z* ratio, and λ_{max}, compound 11 was identified as luteolinidin. Luteolinidin has been reported previously in leaves, glumes and grains (Stafford, 1995; Nicholson et al 1987, Dykes et al 2009). In this study, luteolinidin was found in sheaths, leaves, glumes and grains of the purple plant and in glumes of the tan plant (Table 10).

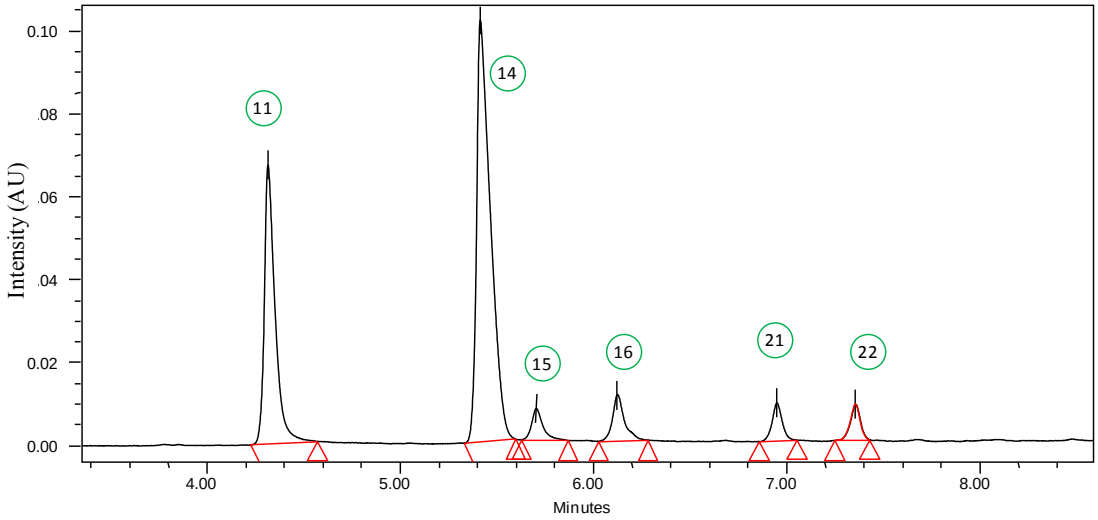

Figure 8. Chromatogram of Tx3362 sorghum glumes extract taken at 485 nm showing the peaks 11, 14-16, 21 and 22 corresponding to 3-deoxyanthocyanidins.

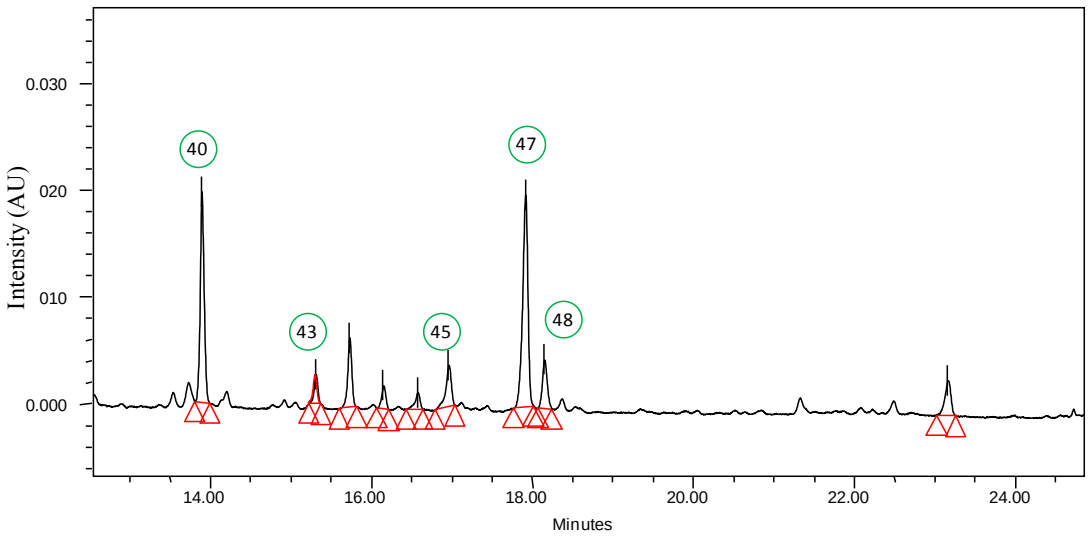

Figure 9. Chromatogram of Tx2911 sorghum glumes extract taken at 485 nm showing the peaks 40, 43, 45, 47 and 48 corresponding to 3-deoxyanthocyanidins.

Table 9. Fragmentation properties of 3-deoxyanthocyanidins from sorghum plant components.

Peak	t_R (min)	λ_{max}	$[M+H]^+$ *(m/z)*	MS/MS (m/z, % Intensity)	Proposed Identity
11	4.28	487	271	141 (52), 115 (100)	Luteolinidin
14	5.38	471	255	171 (100), 157 (79)	Apigeninidin
15	5.58	486	285	270 (10), 242 (100), 213 (16)	7-Methoxyluteolinidin
16	6.12	487	285	270 (10), 242 (100), 213 (53)	5-Methoxyluteolinidin
21	6.79	472	269	254 (13), 226 (100), 197 (21), 169 (22)	7-Methoxyapigeninidin
22	7.35	477	269	254 (100), 226 (96), 197 (26), 169 (36)	5-Methoxyapigeninidin
23	7.49	487	299	284 (32), 256 (100), 241 (11)	Dimethoxyluteolinidin
40	13.64	481	509	395 (10), 383 (15), 371 (100), 343 (17)	Apigeninidin dimer
43	14.98	478	523	507, 371(100), 415, 342, 383 at 60,50	Apigeninidin-methoxyapigeninidin
45	16.59	481	523	523(40), 283(100)	Apigeninidin-methoxyapigeninidin
47	17.73	481	537	521 (39), 505 (22), 385 (100), 370 (99), 356 (74)	Methoxyapigeninidin dimer
48	17.95	493	509	475(19), 373(65), 255(100)	Apigeninidin dimer

Table 10. 3-Deoxyanthocyanidins found in sorghum plant components.

Peak	Proposed Identity	Stalks			Sheaths			Leaves			Glumes			Grains		
		T	R	P	T	R	P	T	R	P	T	R	P	T	R	P
11	Luteolinidin						✓+			✓	✓		✓			✓
14	Apigeninidin			✓+		✓	✓		✓	✓	✓	✓	✓		✓	✓
15	7-Methoxyluteolinidin						✓+			✓			✓+		✓	✓
16	5-Methoxyluteolinidin						✓+			✓	✓		✓		✓	✓
21	7-Methoxyapigeninidin			✓+		✓	✓		✓	✓			✓		✓	✓
22	5-Methoxyapigeninidin					✓			✓		✓	✓+	✓+			
23	Dimethoxyluteolinidin						✓+			✓+						
40	Apigeninidin dimer					✓+			✓+			✓+	✓+			
43	Apigeninidin-methoxyapigeninidin					✓+			✓+			✓+				
45	Apigeninidin-methoxyapigeninidin											✓+	✓+			
47	Methoxyapigeninidin dimer					✓+			✓+	✓+						
48	Apigeninidin dimer											✓+	✓+			

* T = Tan plant (ATx631/RTx436), R= red plant (Tx2911), p = purple plant (Tx3362)

✓+ = Found for the first time in sorghum

Compound 14 (t_R = 5.38 min) showed a λ_{max} at 471, molecular ion $[M + H]^+$ at *m/z* 255. MS/MS major fragments at *m/z* 171 $[M+H-84]^+$ and 157 were obtained. Based on retention time of commercial standard, *m/z* ratio, and λ_{max}, compound 14 was identified as apigeninidin (Appendix 25). Apigeninidin was found in all plant components of the purple plant, also, in sheaths, leaves glumes and grains of the red plant and in glumes of the tan plant (Table 10).

Compound 15 (t_R = 5.58 min) showed a λ_{max} at 486, molecular ion $[M + H]^+$ at *m/z* 285. MS/MS major fragments at *m/z* 270 $[M+H-15]^+$ and 157 were obtained. Fragment $[M + H-15]^+$ at *m/z* 270 indicate the loss of a methoxy group. Based on its retention time, *m/z* ratio, and λ_{max}, compound 15 was identified as **7**-methoxyluteolinidin (Appendix 26). This compound was previously found in grains and leaves of sorghum (Wharton and Nicholson, 2000; Dykes and Rooney, 2006). In this study, 7-methoxyluteolinidin was found in sheaths, leaves, glumes and grains of the purple plant and in grains of the red plant (Table 10).

Compound 16 (t_R = 6.12 min) showed a λ_{max} at 487, molecular ion $[M + H]^+$ at *m/z* 285. MS/MS major fragments at *m/z* 270 $[M+H-15]^+$, 242 and 213 were obtained. Fragment $[M + H-15]^+$ at 270 indicate the loss of a methoxy group. Based on retention, *m/z* ratio, fragmentation pattern and λ_{max}, compound 16 was identified as 5-methoxyluteolinidin (Appendix 27). This compound was previously found in grains, leaves and glumes of sorghum (Lo et al 1996; Dykes et al 2009; Njongmeta, 2009). In this study, 5-methoxyluteolinidin was found in sheaths, leaves, glumes and grains of the purple plant, in glumes of the tan plant, and in grains of the red plant (Table 10).

Compound 21 (t_R = 6.79 min) showed a λ_{max} at 472, molecular ion $[M + H]^+$ at *m/z* 269. MS/MS major fragments at *m/z* 254 $[M+H-15]^+$, 226, 197 and 169 were obtained. Fragment $[M + H-15]^+$ at *m/z* 254 indicate the loss of a methoxy group. Based on retention time of standard, *m/z* ratio, fragmentation pattern and λ_{max}, compound 21 was identified as 7-methoxyapigeninidin (Appendix 28). In this study, 7-methoxyapigeninidin was found in all plant components of the Tx3362 genotype, and in sheaths, leaves, and grains of the Tx2911 genotype (Table 10).

Compound 22 (t_R = 7.35 min) showed a λ_{max} at 477, molecular ion $[M + H]^+$ at *m/z* 269. MS/MS major fragments at *m/z* 254 $[M+H-15]^+$, 226, 197 and 169 were obtained. Fragment $[M + H-15]^+$ at *m/z* 254 indicate the loss of a methoxy group. Based on retention time of standard, *m/z* ratio, fragmentation pattern and λ_{max}, compound 22 was identified as 5-methoxyapigeninidin (Appendix 29).

Compound 23 (t_R = 7.49 min) showed a λ_{max} at 487, molecular ion $[M + H]^+$ at *m/z* 299. MS/MS major fragments at *m/z* 284 $[M+H-15]^+$, and 241 were obtained. Fragment $[M + H-15]^+$ at *m/z* 284 indicate the loss of a methoxy group. Based on its retention time, *m/z* ratio, and λ_{max}, compound 23 could be a dimethoxy-luteolinidin (Appendix 30). This is the first report of dimethoxy luteolinidin in sorghum sheaths (Table 10).

Compound 40 (t_R = 13.64 min) showed a λ_{max} at 481, molecular ion $[M + H]^+$ at *m/z* 509. Fragments $[M + H-126]^+$ at *m/z* 383 and $[M + H-138]^+$ at *m/z* 371 were obtained. Based on its retention time, *m/z* ratio, and λ_{max}, compound 40 could be an apigeninidin dimer. This is the first report of apigeninidin dimer in sorghum. In this

study, it was found in sheaths, leaves and glumes of the red plant and glumes of the purple plant (Table 10).

Compound 43 (t_R = 14.98 min) had a λ_{max} at 478, molecular ion $[M + H]^+$ at *m/z* 523. Fragments $[M + H-16]^+$ at *m/z* 507, $[M + H-108]^+$ at *m/z* 415, and $[M + H-152]^+$ at *m/z* 371 were obtained. Based on its retention time, *m/z* ratio, and λ_{max}, compound 43 could be an apigeninidin-methoxyapigeninidin (Appendix 31). Compound 45 (t_R = 16.59 min) showed a λ_{max} at 481, molecular ion $[M + H]^+$ at *m/z* 523. Fragments $[M + H-240]^+$ at *m/z* 283 were obtained. Based on its retention time, *m/z* ratio, and λ_{max}, compound 45 also can be identified as apigeninidin-methoxyapigeninidin.

Compound 47 (t_R = 17.73 min) showed a λ_{max} at 481, molecular ion $[M + H]^+$ at *m/z* 537. Fragments $[M + H-16]^+$ at *m/z* 521 and $[M + H-32]^+$ at *m/z* 505, 385, 370 and 356 were obtained. Fragment at *m/z* 521, 505 indicate the presence of apigeninidin with a loss of two methoxy groups and an apigeninidin. Based on its retention time, *m/z* ratio, and λ_{max}, compound 47 could be a dimer of methoxyapigeninidin (Appendix 32). This compound has not been reported in sorghum and in this study was found in leaves of the purple plant and in sheaths and leaves of the red plant (Table 10).

Compound 48 (t_R = 17.95 min) showed a λ_{max} at 493, molecular ion $[M + H]^+$ at *m/z* 509. Fragments $[M + H-34]^+$ at *m/z* 475, $[M + H-136]^+$ at *m/z* 373, and $[M + H-254]^+$ at *m/z* 255 were obtained (Appendix 33).

Five dimers of methoxyanthocyanidins were found for the first time in red and purple sorghum plants. 3-Deoxyanthocyanidins were found in the stalks of the purple plant, in sheaths, leaves and grains of the red and purple plants, and in all glumes (Table

10). Due to the antioxidant and anticancer properties attributed to 3-deoxyanthocyanidins (Yang et al 2009), the determination of potential health benefits of these new 3-deoxyanthoycyanidins dimmers found in sheaths, leaves and glumes is necessary.

Chalcones of Sorghum

Five compounds with λ_{max} characteristic of chalcones (Appendix 34) were found (compounds 13, 20, 28, 30 and 34) (Figure 10) in the sorghum plant components (Table 11). Optimum CV values to ionize chalcones were 35-40 V. Optimum CE values to obtain fragment of chalcones was 15-20 V.

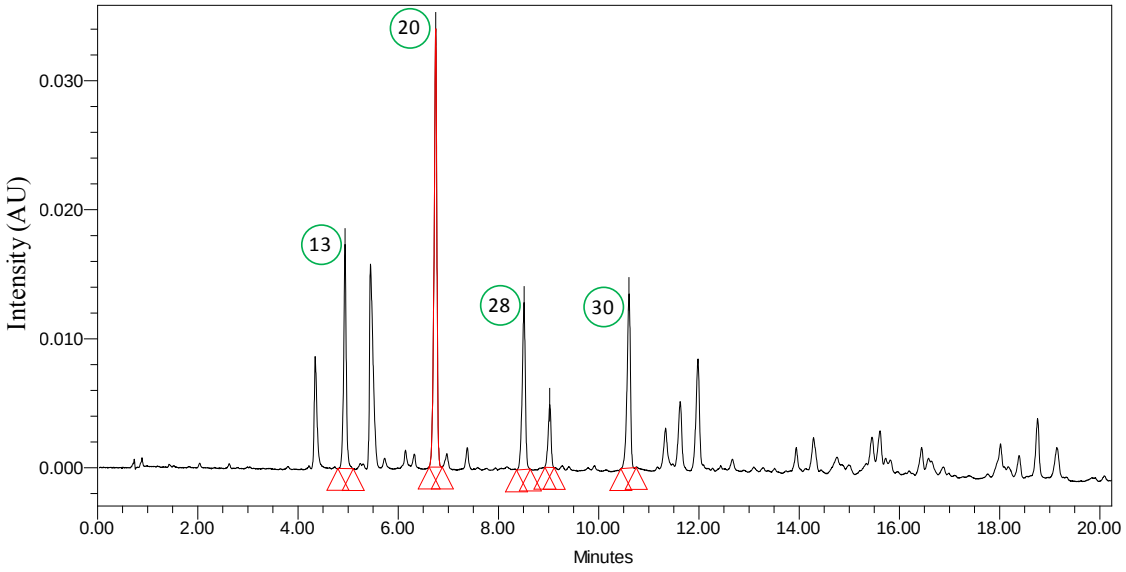

Figure 10. Chromatogram of Tx2911 sorghum glumes extract taken at 370 nm showing the peaks 13, 20, 28 and 30 corresponding to chalcones.

Table 11. Fragmentation properties of chalcones from sorghum plant components.

Peak	t_R (min)	λ_{max}	$[M-H]^-$ *(m/z)*	MS/MS (m/z, % Intensity)	Proposed Identity
13	4.96	379	287	177 (15), 161 (36), 151 (100), 125 (84)	Eriodictyol Chalcone
20	6.68	375	271	177 (41), 161 (4), 151 (100)	Naringenin Chalcone
28	8.54	377	301	191 (9), 176 (3), 165 (76), 161 (100), 150 (11)	Tetrahydroxymethoxychalcone
30	10.62	370	285	191 (25), 176 (24), 165 (100), 150 (25)	Trihydroxymethoxychalcone
34	11.4	377	315	191 (51), 176 (6), 165 (100), 123 (27)	Trihydroxydimethoxychalcone

Compound 13 (t_R = 4.96 min) showed a λ_{max} at 379, molecular ion $[M - H]^-$ at *m/z* 287. Compound 13 had the fragmentation characteristics of eriodictyol (Milbury et al 2006) (Appendix 35) but the λ_{max} corresponded to a chalcone. Tentatively compound 13 could be described as eriodictyol chalcone. This is the first report of eriodictyol chalcone in sorghum (Table 12).

Table 12. Chalcones found in sorghum plant components.

Peak	Proposed Identity	Stalks			Sheaths			Leaves			Glumes			Grains		
		T*	R	P	T	R	P	T	R	P	T	R	P	T	R	P
13	Eriodictyol Chalcone						✓+			✓+			✓+			✓+
20	Naringenin Chalcone					✓+			✓+	✓+		✓+	✓+		✓+	✓+
28	Tetrahydroxymethoxychalcone						✓+			✓+			✓+			✓+
30	Trihydroxymethoxychalcone			✓+		✓+	✓+		✓+	✓+		✓+	✓+		✓+	✓+
34	Trihydroxydimethoxychalcone						✓+			✓+		✓+				

* T = Tan plant (ATx631/RTx436), R= red plant (Tx2911), p = purple plant (Tx3362)

✓+ = Found for the first time in sorghum

Compound 20 (t_R = 6.68 min) showed a λ_{max} at 375, molecular ion $[M - H]^-$ at m/z 271 (Appendix 27). Compound 20 had the fragmentation characteristics of naringenin (Milbury et al 2006) (Appendix 36) but the λ_{max} corresponded to a chalcone. Tentatively compound 20 could be described as naringenin chalcone which was found in sorghum grains (Gujer et al 1986).

Compound 28 (t_R = 10.62 min) showed a λ_{max} at 370, molecular ion $[M - H]^-$ at m/z 301 (Appendix 37). Compound 28 had the fragmentation characteristics of eriodictyol chalcone plus a methoxy group in the A ring with *m/z* 165 and $[M - H–135]^-$. This compound was identified as tetrahydroxy-methoxychalcone.

Compound 30 (t_R = 8.54 min) showed a λ_{max} at 370, molecular ion $[M - H]^-$ at m/z 285 (Appendix 38). Compound 33 had the fragmentation characteristics of naringenin plus a methoxy group in the A ring, with *m/z* 165 and $[M - H–120]^-$. This compound was identified as trihydroxy-methoxychalcone.

Compound 34 (t_R = 11.4 min) showed a λ_{max} at 377, molecular ion $[M - H]^-$ at m/z 315 (Appendix 39). Compound 34 had the fragmentation characteristics of naringenin plus a methoxy group in the A and C rings with *m/z* 165 and $[M - H–150]^-$. This compound was identified as trihydroxy-dimethoxychalcone.

Most of the chalcones were found in the sheaths, leaves glumes and grains of the purple plant. Naringenin chalcone and trihydrxy-methoxychalcone also were found in red sorghum. None of the chalcones found in this study were previously reported in sorghum except for naringenin chalcones in grains.

Flavanones of Sorghum

Four compounds with λ_{max} characteristic of flavanones (Appendix 40) were found (compounds 18, 31, 33 and 36) in the sorghum plant components (Table 13). Optimum CV values to ionize flavanones were 35-40 V. Optimum CE values to obtain fragment of flavanones were 15-25 V.

Compound 18 (t_R = 6.43 min) showed a λ_{max} at 308, molecuar ion $[M - H]^-$ at *m/z* 479. Tentatively compound 18 could be described as trihydroxymethoxyflavanone glucoside (Appendix 41). This is the first report of trihydroxymethoxyflavanone glucoside in sorghum (Table 14).

Compound 31 (t_R = 10.92 min) showed a λ_{max} at 278, molecuar ion $[M - H]^-$ at *m/z* 287. Tentatively compound 31 could be described as tetrahydroxyflavanone (Appendix 42). This is the first report of tetrahydroxyflavanone in sorghum (Table 14).

Compound 33 (t_R = 11.31 min) showed a λ_{max} at 277, molecuar ion $[M - H]^-$ at *m/z* 287. Tentatively compound 33 could be described as tetrahydroxyflavanone. This is the first report of tetrahydroxyflavanone in sorghum (Table 14).

Compound 36 (t_R = 11.84 min) showed a λ_{max} at 306, molecuar ion $[M - H]^-$ at *m/z* 317. Tentatively compound 36 could be described as tetrahydroxymethoxyflavanone (Appendix 43). This is the first report of tetrahydroxymethoxyflavanone in sorghum (Table 14).

Other Compounds on Sorghum

Four compounds detected in sorghum plant components were not fully identified, peaks 8, 19, 25 and 38 (Table 13).

Peak 8 (t_R = 3.61 min) showed a λ_{max} at 318, molecular ion $[M - H]^-$ at *m/z* 468 and MS/MS fragments at *m/z* 332, 306, 161 and135 (Table 13). A compound with these characteristics was found in eggplant pulp and characterized as dicaffeoylspermidine (Singh et al 2009). Thus, compound 8 could be characterized as dicaffeoylspermidine. This compound was detected in all sorghum grains evaluated (Table 14), and has not been reported in sorghum.

Peak 19 (t_R = 6.55 min) showed a λ_{max} at 277, molecular ion $[M - H]^-$ at *m/z* 273 and MS/MS fragments at *m/z* 137, 135 and 125(Table 13). Peak 25 (t_R = 7.75 min) showed a λ_{max} at 283, molecular ion $[M - H]^-$ at *m/z* 851 and MS/MS fragments at *m/z* 689, 563, 401 and 389(Table 13). Peak 38 (t_R = 12.51 min) showed a λ_{max} at 295, molecular ion $[M - H]^-$ at *m/z* 689 and MS/MS fragments at *m/z* 509, 401, 389 and 255(Table 13).

Table 13. Fragmentation properties of flavanones and unknown compounds from sorghum plant components.

Peak	t_R (min)	λ_{max}	$[M-H]^-$ *(m/z)*	MS/MS (*m/z*, % Intensity)	Proposed Identity
8	3.61	318	468	332 (72), 306 (100), 161 (51), 135 (63)	Dicaffeoylspermidine
18	6.43	308	479	317, 193, 177, 151, 139	Trihydroxymethoxyflavanone glucoside
19	6.55	277	273	137 (11), 135 (2), 125 (100)	Unknown
25	*7.75*	*283*	*851*	689 (16), 563 (82),401 (100), 389 (13)	Unknown
31	10.92	278	287	193 (11), 151 (27), 147 (53), 139 (100), 135 (6)	Tetrahydroxyflavanone
33	11.31	277	287	193 (67), 151 (39), 147 (67), 139 (100), 135 (9)	Tetrahydroxyflavanone
36	11.84	306	317	193, 177, 151, 139, 108	Tetrahydroxymethoxyflavanone
38	12.51	295	689	509 (10), 401 (100), 389 (10) 255 (7)	Unknown

* T = Tan plant (ATx631/RTx436), R= red plant (Tx2911), p = purple plant (Tx3362)

✓+ = Found for the first time in sorghum

Table 14. Other polyphenolic compounds present in sorghum plant components.

Peak	Proposed Identity	Stalks			Sheaths			Leaves			Glumes			Grains		
		T	R	P	T	R	P	T	R	P	T	R	P	T	R	P
8	Dicaffeoylspermidine													✓+	✓+	✓+
18	Trihydroxymethoxyflavanone glucoside						✓+			✓+						
19	Unknown											✓+	✓+			
25	Unknown											✓+	✓+		✓+	✓+
31	Tetrahydroxyflavanone											✓+	✓+			
33	Tetrahydroxyflavanone					✓+	✓+		✓+	✓+		✓+	✓+		✓+	✓+
36	Tetrahydroxymethoxyflavanone						✓+			✓+						
38	Unknown											✓+	✓+		✓+	✓+

* T = Tan plant (ATx631/RTx436), R= red plant (Tx2911), p = purple plant (Tx3362)

✓+ = Found for the first time in sorghum

Total Phenols of Sorghum

Total phenols of stalks, sheaths, leaves, glumes and grains with tan, red and purple secondary plant color ranged from 1.9 to 11.8 mg (GAE/g). These values are within the values found by Njongmeta (2009) for sorghum plant components with values of 1.2-28.2 mg GAE/g. There were significant differences among each plant component except for stalks ($p < 0.01$). Leaves from the red plant Tx2911 had the highest total polyphenolic content across all components from its respective plant color, while in the purple plant Tx3362, the leaves and glumes had the highest levels ($p < 0.01$). On the other hand, the lowest levels of polyphenolics were observed in the plant components of the ATx631/RTx436 plant except for their leaves, as well as the stalks and grains of red plant and stalks of purple plant ($p < 0.01$) (Table 15). Grains of tannin sorghum also were measured to compare it with the plant components evaluated.

Antioxidant Activity of Sorghum Plant Components

Antioxidant activity of stalks, sheaths, leaves, glumes and grains of all plant types ranged from 6.8 to 296.1 μmol TE/g. Sorghum plant components evaluated previously had antioxidant activity values from 11.9 to 412.2 μmol TE/g (Njongmeta, 2009). Significant differences were found among each plant component ($p < 0.01$).

Table 15. Total soluble polyphenols and antioxidant activity of sorghum plant components.

Plant Component	Plant Color	Total Polyphenols (mg GAE/g)	Antioxidant Activity (umol TE/g)[b]
Stalks	Tan	1.9^{fg}	38.5^{j}
	Red	2.2^{efg}	45.0^{ij}
	Purple	2.1^{fg}	39.3^{j}
Sheaths	Tan	3.1^{ef}	81.4^{g}
	Red	4.0^{e}	93.6^{g}
	Purple	8.8^{c}	172.2^{de}
Leaves	Tan	6.6^{d}	184.9^{d}
	Red	11.8^{a}	296.1^{a}
	Purple	10.7^{ab}	276.2^{b}
Glumes	Tan	2.8^{efg}	59.7^{h}
	Red	9.9^{bc}	292.8^{a}
	Purple	10.1^{abc}	237.1^{c}
Grains	Tan	1.2$_{g}$	6.8^{k}
	Red	1.8^{fg}	57.6^{hi}
	Purple	8.7^{c}	152.3^{f}
	Red (tannin)	9.0^{bc}	158.1^{ef}

[a] GAE = Gallic acid equivalents

[b] TE = Trolox equivalents

Across all components from the three plant types, leaves and glumes from the red plant Tx2911 had the highest antioxidant activity, while grains from the tan plant ATx631/RTx436 had the lowest ($p < 0.01$) (Table 15). Grains from the ATx631/RTx436 plant had lower antioxidant activity compared to their other plant components while for Tx2911 and Tx3362 genotypes, the lower levels were found in the stalks ($p < 0.01$) (Table 15).

Correlation Between Total Phenols and Antioxidant Activity of Sorghum

The correlation between total phenols and antioxidant activity of sorghum plant components was high ($r^2 = 0.91$, $p<0.01$) (Figure 11). The high correlation of total phenols and antioxidant activity of sorghum plant components was expected because most of the radical quenching capacity of the samples analyzed is due to the hydroxyl groups of the phenolics. Previous studies demonstrated high correlation between antioxidant activity and total polyphenols in sorghum grains (Dykes et al 2005; Njongmeta, 2009), but there were no previous reports of correlations between total phenols and antioxidant activity of sorghum plant components.

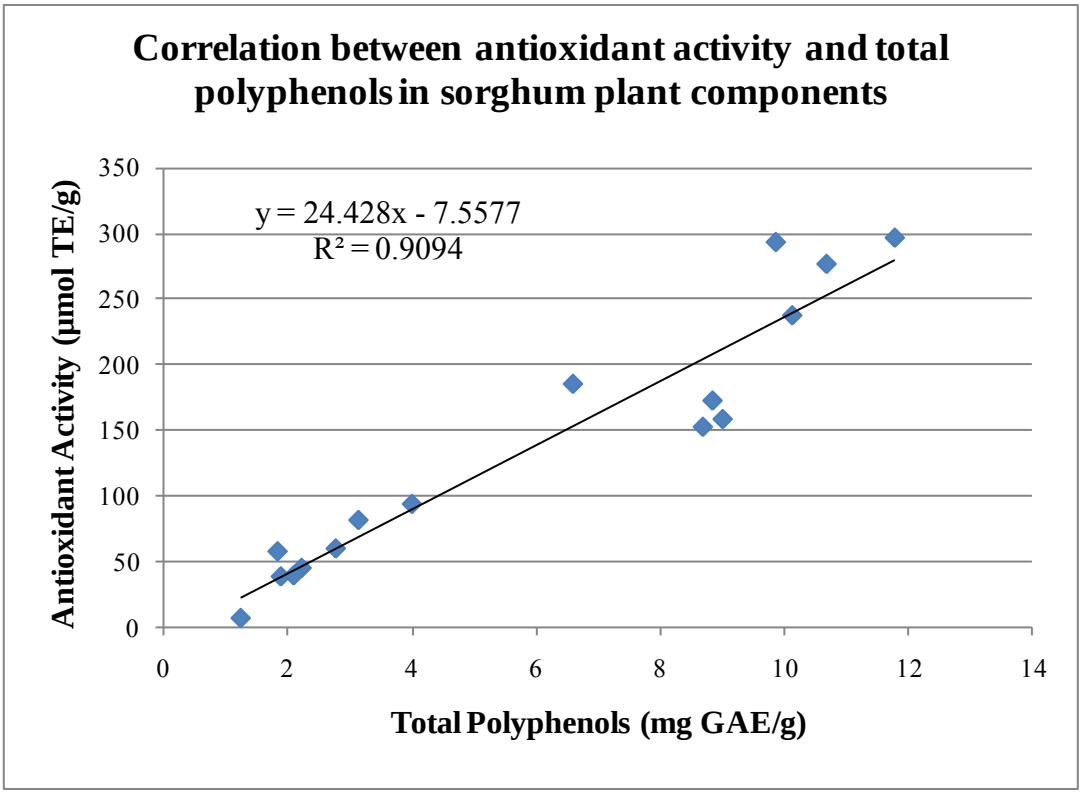

Figure 11. Correlation between total phenols and antioxidant activity of sorghum plant components.

Sorghum Plant Components as Source of Phytochemicals

Polyphenolics from sorghum grains have demonstrated potential use as an antioxidant and chemopreventive agent (Yang et al 2012). Other sorghum plant components have not been studied extensively to determine their potential phytochemical uses. Forty nine compounds were found in the plant tissue, the compounds were mostly phenolic acids, flavones, flavone glycosides, 3-deoxyanthocyanidins, flavanones and chalcones. Phenolic acids such as chlorogenic acid and caffeic acid have shown anticancer potential (Belkaid et al 2006; Prasad et al

2011). Chlorogenic acid was found in stalks, leaves and sheaths while caffeic acid was in glumes and grains. In general, even though sorghum stalks contained phenolic compounds, their levels were significantly lower than leaves, sheaths and glumes of their respective plant types (Table 15), which indicates that stalks are not a good source of phytochemicals compared to other sorghum plant components. Based on their total polyphenolic content and polyphenolic profile, sorghum glumes, leaves and sheaths could be a potential source of phytochemicals. Leaves and sheaths had generally similar polyphenolic profile including flavone glycosides, a unique group of polyphenols compared to other plant components. Furthermore, sheaths had lower levels of polyphenolics than their respective leaves. Based on these results, leaves, grains and glumes of all sorghum types should be tested in future studies to evaluate their chemopreventive properties and determine if value added products could be obtained from sorghum, specifically from alcohol production.

Conclusions

Forty nine phenolic compounds were found in sorghum plant components, the compounds were mostly phenolic acids, flavones, flavone glycosides, 3-deoxyanthocyanidins, flavanones and chalcones. Five dimers of methoxylated 3-deoxyanthocyanidins were found for the first time in sorghum. These findings suggest that not only grains but leaves, sheaths, and glumes are good sources of polyphenolics.

Sorghum leaves and glumes could have similar or higher health benefits than whole sorghum grains because of their high phenolic content compared to grains and because of their phenolic profile. Consequently, extracts from leaves, grains and glumes should be tested to evaluate their chemopreventive properties and bioavailability and determine the potential to produce high value sub-products from sorghum.

CHAPTER IV

CHEMOPREVENTIVE POTENTIAL OF SORGHUM PHENOLIC EXTRACTS

Considering the presence of polyphenolic compounds in leaves and glumes of sorghum, the anti-proliferative properties of these plant components were evaluated using HT-29 cells.

Materials and Methods

Samples

Leaves, glumes and grains, of sorghum plants with tan (ATx631/RTx436), red (Tx2911) and purple (Tx3362) secondary plant color, which had the highest amount of phenolic compounds (1.2-11.8 mg GAE/g) and the most distinct phenolic profile from the previous chapter, were evaluated.

Extraction of Phenolic Compounds

To obtain the crude phenolic extract, leaves, glumes and grains of the three sorghum genotypes were extracted in methanol. 1 g of sample was extracted in 10 mL of methanol for 2 h while shaking at low speed in an Eberbach shaker (Eberbach Corp., MI). All extracts were centrifuged twice at 2790g for 10 min in a Sorvall SS-34 centrifuge (DuPont Instruments, Wilmington, DE) and decanted. Samples were washed with petroleum ether to remove chlorophyll. Washed extracts were concentrated in a rotavap and stored at -20 °C until analysis.

Total Soluble Phenolics of Sorghum Phenolic Extracts

Total phenols of the crude sorghum extracts were measured using a modified Folin-Ciocalteu method (Kaluza et al 1980). 0.05 g of each crude extract was solubilized in acidified methanol for 2 h while shaking at low speed in an Eberbach shaker (Eberbach Corp., MI). A 0.1 mL aliquot was dissolved in 1.1 mL of water and reacted with 0.4 mL of Folin-Ciocalteu reagent and 0.9 mL of 0.5M ethanolamine. After 20 min of reaction at room temperature, the absorbance at 600 nm was measured and compared to a gallic acid standard curve to determine total phenols expressed as GAE/g of crude extract.

Antioxidant Activity of Phenolic Extracts

Antioxidant activity of the crude phenolic extracts was measured *in vitro* by the ABTS assay. The $ABTS^{\cdot+}$ solution was obtained by reacting 3 mM of $K_2S_2O_8$ with 8 mM ABTS salt in distilled, deionized water for 16 h at room temperature in the dark. The $ABTS^{\cdot+}$ solution was then diluted with a pH 7.4 phosphate buffer (50:42.5:9.5; water: 0.2 M Na_2HPO_4:0.2 M NaH_2PO_4) solution containing 150 mM NaCl (PBS) to obtain an initial absorbance of 1.5 at 734 nm. Fresh $ABTS^{\cdot+}$ solution was prepared each day of analysis. 0.05 g of each crude extract was solubilized in acidified methanol for two hours while shaking at low speed using an Eberbach shaker (Eberbach Corp., MI). One aliquot (0.1mL) of each crude extract solution and standard was reacted separately, with 2.9 mL of ABTS solution for 30 min at room temperature. The absorbance was

measured at 734 nm and compared to a trolox standard curve. Results were expressed as micromolar Trolox Equivalent Antioxidant Capacity per g (μmol TE/g) of extract.

Antiproliferative Capacity of Sorghum Extracts

Human colon adenocarcinoma cells HT-29 were obtained from ATCC (Manassas, VA). HT-29 cells were cultured in 75 cm^2 flasks (Sigma-Aldrich, Saint Louis, MO) containing α -MEM medium (Invitrogen, Carlsbad, CA) supplemented with 1% penicillin/streptomycin and 10% of fetal bovine serum (Hyclone, South Logan, UT). Cells were maintained in a humidified incubator (5% CO_2, 37 °C) for 72 h before plating into a clear FALCON 96-well tissue culture plate (FALCON, Franklin Lakes, NJ) at a population density of 5,000 cells/well with 50 μL of growth medium. The linearity of this MTT assay was in the range of 100-5000 plated cells tested after 72 h incubation. After plating, the cells were incubated for 24 h. Ten treatments were evaluated in six concentrations. 50 μL of each treatment were added to each well with cells after 24 h incubation. Each well had a final volume of 100 μL and a final concentration of crude sorghum extracts of 0, 66, 133, 266, 533 or 800 μg/mL. After the addition of the treatment, the cells were incubated for additional 48 h. Each treatment level used 3 wells and each well received 100 μL of growth medium + treatment while control cells (0 μg/mL) received 100 μL of growth medium containing 0.1% DMSO only.

All treatment levels had also 3 wells as a blank, containing 100 µL of growth media with the treatment level without cells. After incubation, viable cells were determined using the MTT assay kit that measures mitochondrial activity (Manassas, VA). The MTT reagent (15 µL) was added to each well and incubated for 4 h at 37 ºC. 100 µL of solubilizing/stop solution were added to dissolve the purple crystals for 2 h. Absorbance was read at 570 nm. Blank reading (15 µL of MTT reagent plus 10 µL of solubilizing/stop solution) was subtracted from each well. Relative Growth (%) was calculated using the following formula: Relative Growth (%) = (Absorbance at 570 of test sample – blank – sample blank)/Absorbance at 570 of control cells × 100 %.

Statistical Analysis

For total phenols and antioxidant capacity, three replications of each treatment were analyzed using a General Linear Model procedure. For the anti-proliferation experiment, three replicates (day) of each treatment level were analyzed using a General Linear Modeling procedure. The analysis was done using SPSS version 16.0 (SPSS Inc. Chicago, Il).

Results and Discussion

Total Polyphenols of Sorghum Glume, Grain and Leaf Extracts

Total soluble phenolics (mg GAE/g) and antioxidant activity (μmol TE/g) of the sorghum extracts were measured to determine their relationship to anti-proliferation activity. Previous studies suggest that total phenolics of extracts of sorghum grains and bran are associated with their chemopreventive potential (Lewis, 2009, Yang 2009). Phenolic extracts of tannin sorghum grains had the highest total phenol content among the grains (238.2 mg GAE/g), while the lowest content was observed in the extracts of the tan plant (ATX631/RTx436) which contained 33.2 mg GAE/g (Table 16). These values are in the range of those found by Yang (2009) in grains with values from 33.2 – 238.2 mg GAE/g. In glumes, the extracts of red plant (Tx2911) had the highest phenolic content (98.2 mg GAE/g), extracts from glumes of the tan and purple plant had lower polyphenolic content than their grains (Table 16). The leaves of the red and purple (Tx3362) plants had the highest phenolic content among leaves at 39.0 and 40.7 mg GAE/g, respectively.

Antioxidant Activity of Sorghum Glume, Grain and Leaf Extracts

The highest antioxidant activity in grain extracts was observed in the tannin grain (4972 μmol TE/g). Values from 403-4420 μmol TE/g were reported previously in sorghum grain extracts. Tannin grain extracts had the highest antioxidant activity among grains (Yang, 2009). The glumes from the red plant had the highest antioxidant activity among glumes (3499 μmol TE/g), while in leaves, highest values were in red and purple plants with 1220 and 1108 μmol TE/g, respectively (Table 16).

Table 16. Total polyphenols, antioxidant activity and anticancer activity values (IC_{50}) of sorghum extracts from grains, glumes and leaves.

Plant Component	Plant Color	Total Polyphenols (mg GAE/g)[a]	Antioxidant Activity (umol TE/g)[b]	IC_{50} (μg/mL)
Grain Extract	Tan	33.2^{g}	681.8^{g}	879^{a}
	Red	60.3^{e}	1560.5^{e}	658^{b}
	Purple	128.0^{b}	3034.6^{c}	511^{c}
	Red (Tannin)	238.2^{a}	4972.1^{a}	419^{cd}
Glume Extract	Tan	56.7^{e}	1653.9^{e}	85^{f}
	Red	98.2^{c}	3499.2^{b}	178^{ef}
	Purple	83.6^{d}	1954.7^{d}	248^{e}
Leaf Extract	Tan	24.6^{h}	710.5^{g}	707^{b}
	Red	39.0^{f}	1220.5^{f}	246^{e}
	Purple	40.7^{f}	1108.0^{f}	408^{d}

[a] GAE = Gallic acid equivalents

[b] TE = Trolox equivalents

Total phenols and antioxidant activity of sorghum extracts were higher than their respective plant components. Total phenol content of extracts was between 3 and 27

times higher than their respective plant component. Antioxidant activity of extracts was between 4 to 100 times higher than their respective plant component.

Correlation between total phenols and antioxidant activity of sorghum plant extracts was high ($r^2 = 0.91$, $p<0.01$) (Figure 12). Similar correlation was observed for the sorghum plant components in the previous chapter. The high correlation between total phenols and antioxidant activity of sorghum extracts was expected because most of the radical quenching capacity of the samples analyzed is due to the hydroxyl groups of the phenolics present (Awika et al 2003).

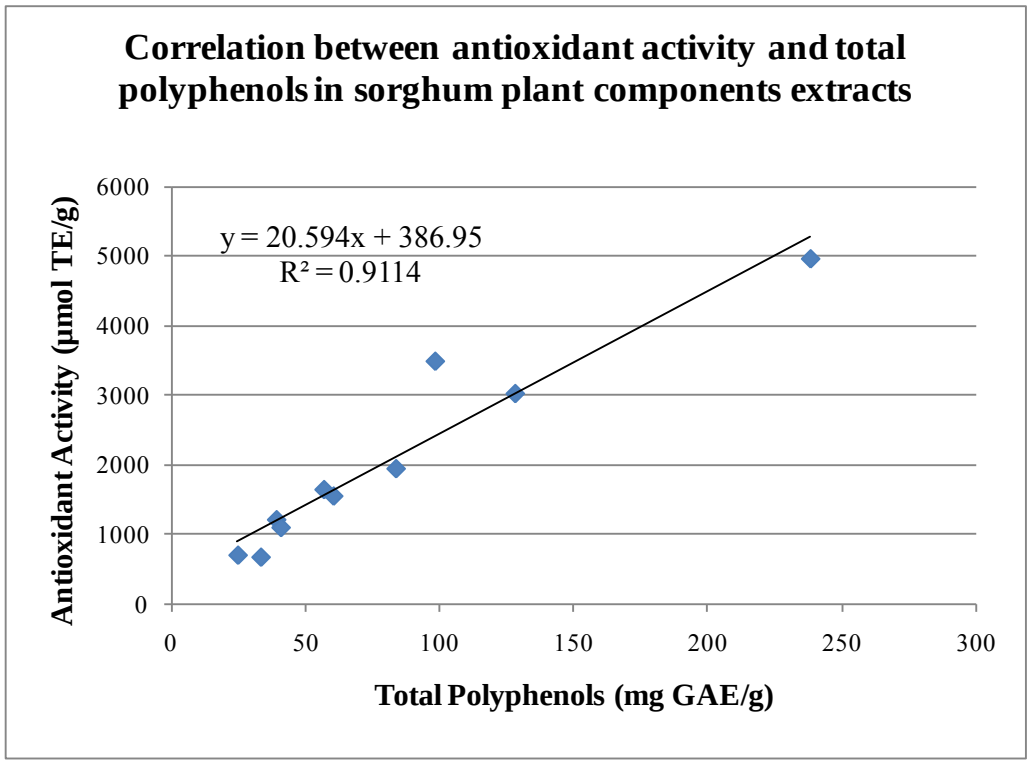

Figure 12. Correlation between total phenols and antioxidant activity of extracts from sorghum leaves, glumes and grains.

Antiproliferation Activity of Sorghum Plant Extracts on Colon Cancer Cells

The antiproliferation activity of sorghum extracts was determined by their IC_{50} values using HT-29 colon carcinoma cells. IC_{50} value indicated the lowest dose of extract needed to inhibit 50 % of cancer cell proliferation. All sorghum extracts showed a dose-response inhibition at different rates. The strongest antiproliferation activity, represented by lower IC_{50} values, was produced by tan plant glume extracts (IC_{50} value = 85 μg/mL) and the lowest by the tan plant grain extracts (IC_{50} value = 879 μg/mL) (Table 16).

Correlation Between Antiproliferation and Antioxidant Activity of Sorghum Extracts

Figure 13 shows a low correlation between antioxidant activity and chemopreventive potential (IC_{50} value) of sorghum plant extracts ($r^2 = 0.13$, $p < 0.01$). Previous data obtained from sorghum grains suggested that antioxidant activity could predict the anticancer potential of sorghum (Lewis, 2008), but the low correlation observed in this study indicates that other factors are also important to determine chemopreventive potential.

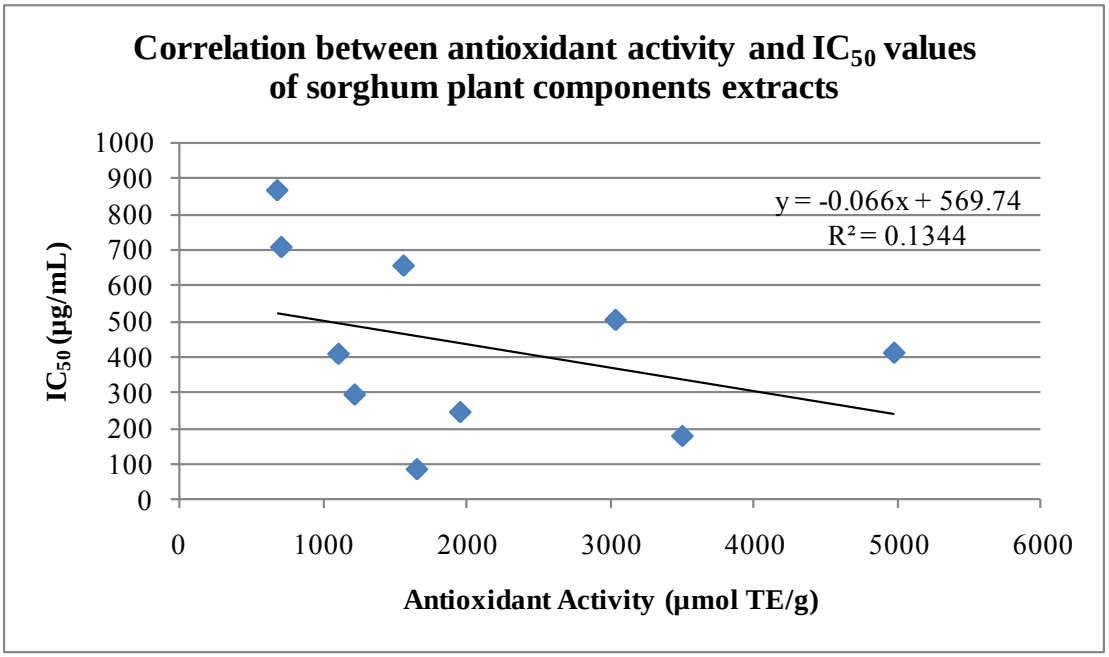

Figure 13. Correlation between antioxidant activity and IC_{50} values from sorghum leaf, glume and grain extracts of tan (ATx631/RTx436), red (Tx2911) and purple (Tx3362) plants.

Table 17 shows the inhibition potential values of the sorghum extracts relative to the grains of the tan plant using a crude extract base (mg of extract) and a total polyphenolic base (mg GAE). The inhibition potential values were calculated by comparing the IC_{50} value of the extracts to the IC_{50} value of the control (tan grains). These values can help to compare the relative antiproliferation properties among the sorghum extracts. On a crude extract base, the highest inhibition was observed in tan glumes, with 10 times more inhibition than the control. Using a corrected value, which considers only the total polyphenols content of the extracts, the potential of tan glumes decreased but still was 6 times stronger than the grains of the tan plant (Table 17). The use of the corrected value helps to analyze the results on a total polyphenolic base assuming that the non-polyphenolic components of the crude extracts does not interfere in the cell growth. The differences obtained between the values of the two methods of comparison indicate an effect of the amount of polyphenolics from extracts on the antiproliferation of cancer cells. Furthermore, differences in inhibition potential among extracts using a total polyphenolic base indicate that not only the amount of total polyphenolics, but also the type of polyphenolics affected their antiproliferation potential.

Table 17. Inhibition of cancer cells (HT-29) proliferation by sorghum leaf, glume and grain extracts.

Plant Component	Plant Color	Inhibition Potential (based on total extract)	Inhibition Potential (based on total polyphenolics)
Grain Extract	Tan	1.0	1.0
	Red	1.3	0.7
	Purple	1.7	0.4
	Red (Tannin)	2.1	0.3
Glume Extract	Tan	10.2	6.0
	Red	4.9	1.6
	Purple	3.5	1.4
Leaf Extract	Tan	1.2	1.7
	Red	2.9	2.5
	Purple	2.1	1.7

[a] GAE = Gallic acid equivalents

[b] TE = Trolox equivalents

Thus, chemopreventive potential of sorghum does not necessarily depend on the total phenolic content or the radical quenching capacity of the extracts. Other factors like type of polyphenolics and their bioavailability may be more important than the amount of phenolics present. Individual analysis of glume, grain and leaf extracts is described next to better understand their effects on cancer cell proliferation.

Chemopreventive Potential of Sorghum Glumes

Sorghum glumes had high levels of polyphenols and antioxidant activity suggesting potential chemopreventive properties (Table 15). Antiproliferative activity of HT-29 cancer cells was measured to evaluate their chemopreventive potential. Polyphenolics from tan glumes had the strongest inhibition of cancer cell proliferation

among glumes, especially at concentrations lower than 400 μg extract/ml (Figure 14). Differences in response could be related to the total phenol content of each extract but also to the type of phenolic present (Table 17). Extracts of tan glumes contained predominantly the flavones luteolin (35080 μg/g) and apigenin (14735 μg/g). The levels of flavones in the other glumes were lower, especially for purple glumes (Table 18). The phenolic compounds have different anticancer activity due to factors such mechanism of action and bioavailability. Bioavailability of the polyphenolic compounds of each sample can be different because they contain different polyphenols. For example, glumes of the tan plant had mostly flavones whereas the purple glumes had mostly 3-deoxyathocyanidins (Table 19). Flavones had demonstrated high absorption on *in vitro* models which suggests that higher chemopreventive potential of the tan glumes is due to presence of flavones. However, Absorption of sorghum 3-deoxyanthocyanidins, found in high levels in purple glumes, has not previously been investigated. Therefore a direct comparison of the effect of 3-deoxyanthocyanidins and flavones is difficult to make.

Chemopreventive Potential of Sorghum Leaves

Polyphenolic extracts from leaves of the red and purple plants had stronger anti-proliferation effects compared to leaves of the tan plant (Figure 15). As observed in glumes, the different chemopreventive potential of the sorghum leaf extracts (IC_{50} 246 – 707 μg/mL) are due to not only their polyphenolic content but also their phenolic profile.

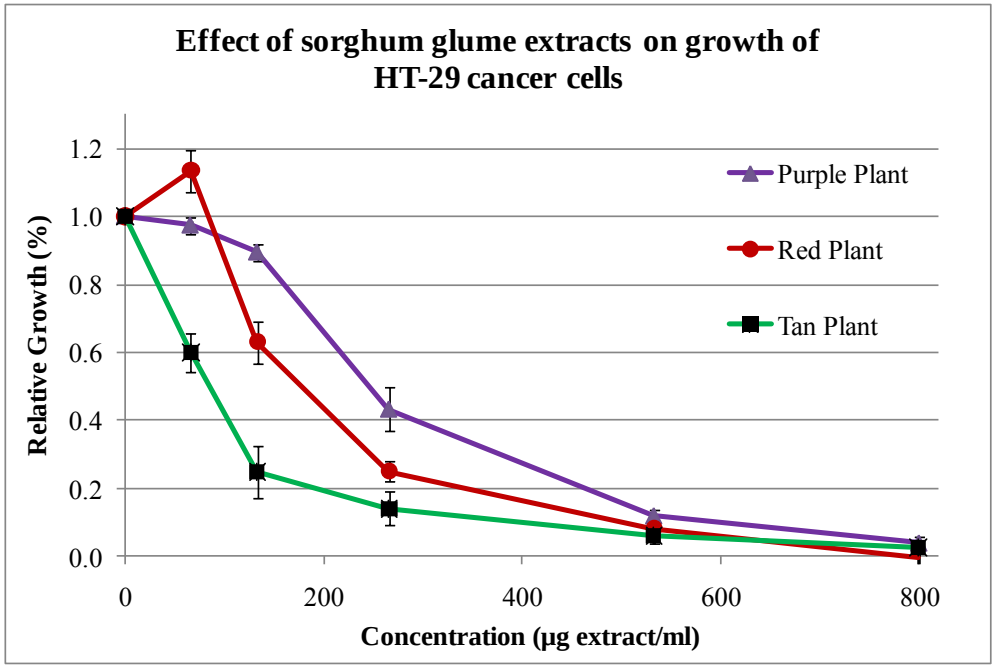

Figure 14. Anticancer potential of sorghum glumes.

For example, when comparing inhibition potential based on total polyphenolic content, only leaf extracts from the red plant had higher chemopreventive potential (Table 17). Levels of flavone glycoside were higher in the red plant (Table 18). Levels of 3-deoxyanthocyanidins in red leaf extracts were also higher than in purple and tan leaf extracts (Table 19). Consequently, higher chemopreventive potential from leaf extracts of red and purple plants can be attributed to their flavone and 3-deoxyanthocyanidin levels and profile.

Table 18. Flavone concentrations of sorghum extracts evaluated for antiproliferation activity.

Plant Component	Flavone concentration (µg/g of extract)			
	Luteolin-7-glucoside	Apigenin-7-glucoside	Luteolin	Apigenin
Tan Grains			4731±167	2953±196
Red Grains				
Black Grains			611±32	240±8
Tan Glume			35080±3007	14735±1021
Red Glume			1677±82	17876±975
Purple Glume			523±45	429±12
Tan Leaves				
Red Leaves	5245±432	10461±378	194±20	154±18
Purple Leaves	8498±155	361±20	592±35	

Table 19. 3-Deoxyanthocyanidins of sorghum extracts evaluated for antiproliferation activity.

	3-Deoxyanthcyanidins concentration (µg/g of extract)			
Plant Component	**Luteolinidin**	**Apigeninidin**	**5-Methoxyluteolinidin**	**7-Methoxyapigeninidin**
Tan Grains				
Red Grains				
Black Grains	143±11	103±5	17±1	51±5
Tan Glume				
Red Glume		145±35		132±8
Purple Glume	465±43	801±66	34±1	168±14
Tan Leaves				
Red Leaves	268±23	257±22		138±9
Purple Leaves	195±21	36±2	35±3	

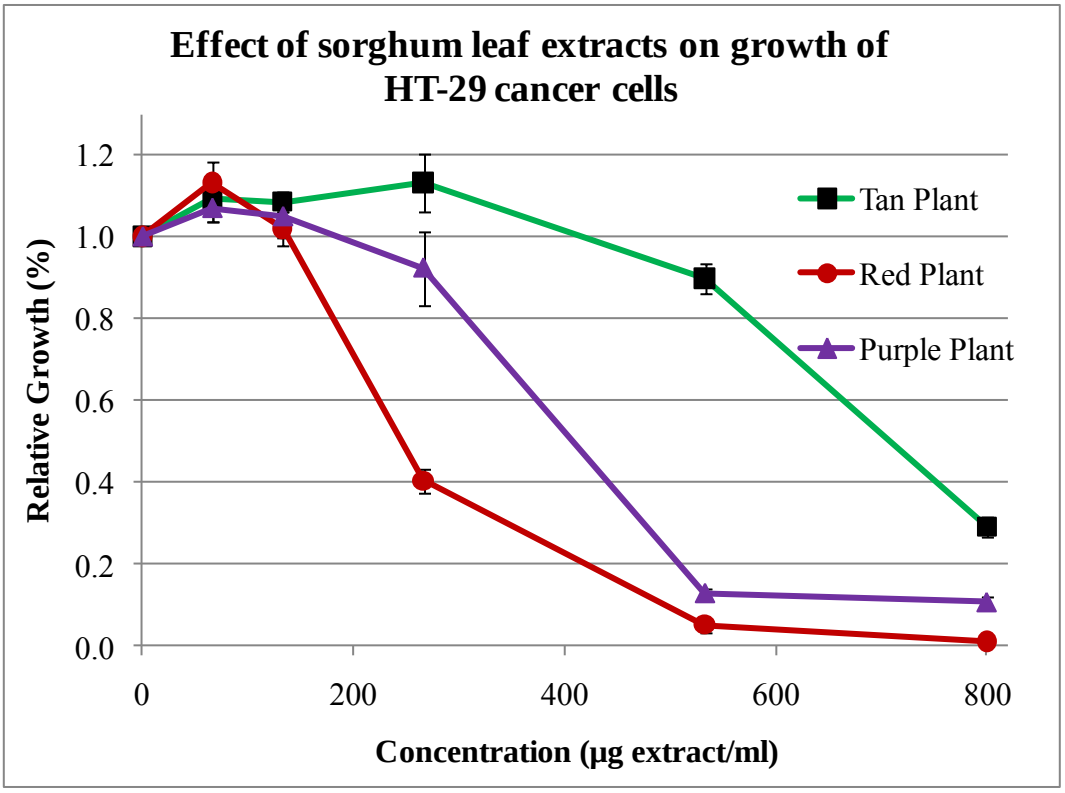

Figure 15. Chemopreventive potential of sorghum leaves.

Chemopreventive Potential of Sorghum Grains

Phenolic extracts from tannin grains caused the strongest inhibition of cancer cells (Figure 16). Previous studies have demonstrated a high chemopreventive potential of tannin sorghums (Guajardo, 2009). When their chemopreventive potential was compared based on total polyphenolic content, the antiproliferation potential of extracts from tannin grains and grains of the red and purple plant were lower than the control (grains of tan plant) (Table 17). This indicates that phenolics present in tan sorghum grains had stronger chemopreventive effect than those present in grains of the red and purple plant and tannin grains. Individual polyphenolic analysis of grain extract from tan plant sorghum revealed that the flavones luteolin and apigenin were the predominant components (Table 18) whereas the predominant compounds in black and red sorghum extracts were 3-deoxyanthocyanidins and phenolic acid derivatives, respectively. These results suggest that flavones are an important factor on the relative high chemopreventive potential of tan plant grain extracts.

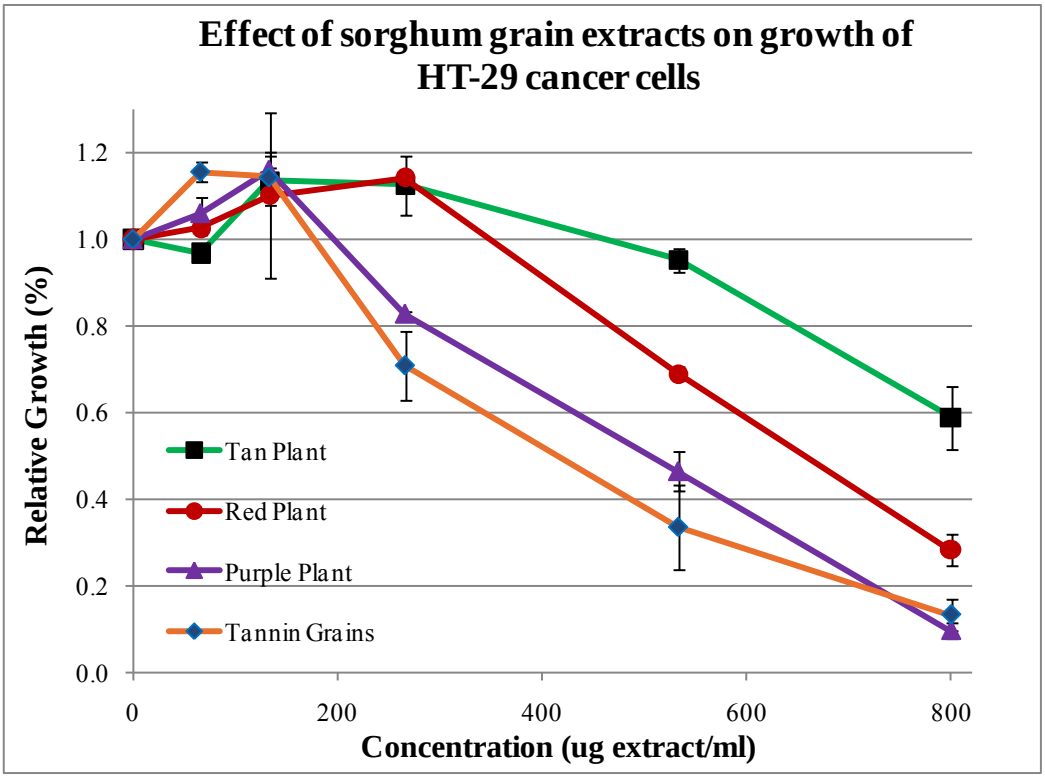

Figure 16. Anticancer potential of sorghum grains.

Sorghum Extracts with the Highest Chemopreventive Potential

The extracts with the highest chemopreventive potential from each plant component were compared against the grain extract of the tan plant ATX631/RTx436, which was considered as the control because it is a widely used sorghum. As observed in Figure 17, sorghum extracts from tan plant glumes and red plant leaves had higher chemopreventive activity *in vitro* than the grain extract with highest chemopreventive activity (tannin grains). IC_{50} values from tan plant glumes (IC_{50} = 85) and the red leaf extracts (IC_{50} = 246) were significantly different than tannin grain extracts (IC_{50} = 419) ($p<0.01$). These results suggest that glumes of tan plant and leaves of red sorghum plant have potentially higher chemopreventive benefits than their corresponding grains when using their crude phenolic extracts. Other cell culture types should be evaluated to determine if glume and leaf sorghum extracts have similar effects across many cancer cell types. Sorghum extracts from grains with tannins have the strongest chemopreventive potential among sorghum grains when using a different cell model (MCF-7) (Guajardo, 2008).

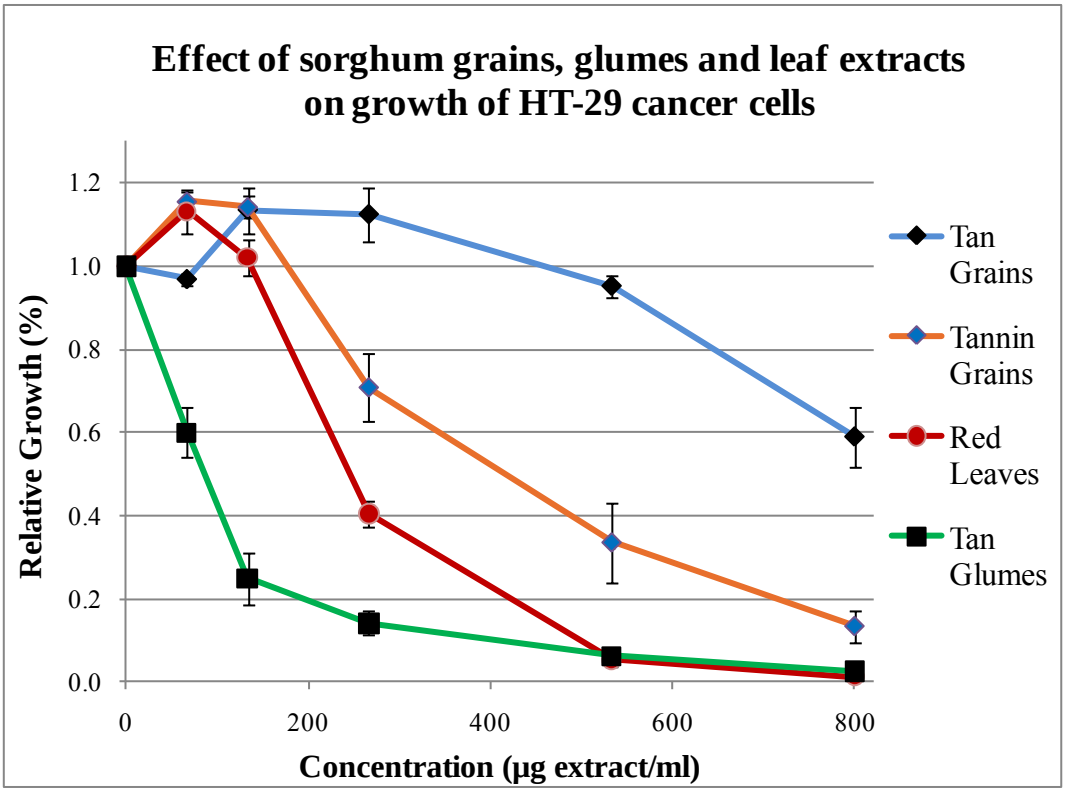

Figure 17. Anticancer potential of sorghum plant components.

The glumes of the tan plant had the highest chemopreventive potential and also had a characteristic phenolic profile with mostly flavones. Previous studies reported chemopreventive activity of the flavones luteolin and apigenin (Seelinger et al 2008; Babcook and Gupta 2012). The presence of flavones was common in the glumes with the highest chemopreventive activity, suggesting that flavones are an important contributor to this activity. Flavones also were present in high levels in the red leaf extracts but they were mostly present with glycoside groups attached and not in the aglycone form as observed in glumes and grains. Flavone glycosides are known to have lower absorption due to their high hydrophilicity which limits their absorption through

the cell membrane (Hollman, 2004). Evaluation of the absorption properties of flavone and flavone glycosides could be useful to understand the chemopreventive properties of leaf and glume sorghum extracts high in these compounds.

Glumes and leaves, represent a small part of the biomass of a sorghum plant, however, compared to its grains, they have higher levels of polyphenolics which can be used as chemopreventive agents. Furthermore, economic benefits resulting from processing of glumes and leaves to extract phytochemicals can be significantly high since these crop residues do not have high economic value. On the other hand, extraction of polyphenolics from grains could be beneficial if these are obtained from bran.

Conclusions

In general, extracts from sorghum glumes had the strongest anti-proliferation activity on HT-29 cancer cells. Their high content of flavones, especially aglycone forms, was associated with the high activity. Polyphenolic concentration or antioxidant activity were not the only factors that affected the chemopreventive potential of sorghum extracts. Other factors such as the type of polyphenolics and their absorption into the cancer cells also could determine their health benefits.

Comparison of the bioavailability of sorghum polyphenols, especially between flavones, flavone glycosides and 3-deoxyanthocyanidins is necessary to understand the high anti-proliferation activity of all sorghum glumes.

Sorghum glumes and leaves are a potential source of natural health compounds which could be extracted and sold as phytochemicals. These results can be used to determine the economic feasibility of producing sorghum plant extracts.

CHAPTER V

BIOAVAILABILITY OF SORGHUM PHENOLIC COMPOUNDS USING A CACO-2 MODEL

Introduction

Phenolic compounds from sorghum demonstrated different chemopreventive potential (previous chapter). The i*n vitro* Caco-2 model can be used to measure % of absorption of the compound through the cell which can be considered as a measure of bioavailability. Consequently, it is important to determine the bioavailability of different flavonoid groups present in sorghum plant components using an *in vitro* Caco-2 absorption model.

Materials and Methods

Samples

Phenolic extracts from leaves, glumes and grains of the red (Tx2911) and purple (Tx3362) plants, and glumes of the tan plant (ATx631/RTx436) used in the anti-proliferation experiment, were tested in the *in vitro* permeability study using a Caco-2 model. These extracts were selected because they had high amounts of phenolic components, a distinct polyphenolic profile and/or higher chemopreventive potential *in vitro* (Table 16). Commercial standards of luteolinidin, apigeninidin, 7-methoxyapigeninidin and 5-7dimethoxyapigeninidin were also evaluated.

Standards and Reagents

Apigenin and luteolin were obtained from Indofine Chemical Co., Inc. (Hillsborough, NJ). Naringenin was obtained from MP Biomedicals (Santa Ana, CA). Luteolin-7-O-glucoside, apigenin-7-O-glucoside and eriodictyol were obtained from Extrasynthese (Genay, France). Luteolinidin chloride, apigeninidin chloride 7-methoxyapigeninidin, 5-methoxyapigeninidin, and 5-7-dimethoxyapigeninidin chloride were obtained from ALSACHIM (Strasbourg, France).

Transepithelial Transport of Sorghum Polyphenolics Using Caco-2 Cells.

Caco-2 colon adenocarcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured into 75 cm^2 flasks (Sigma-Aldrich, Saint Louis, MO) and incubated at 37 °C and 5% CO_2 in growth media. The growth media consisted of DMEM 15 % FBS, 1% non-essential amino acids, 100 units/mL penicillin G, 100 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 10 mM sodium pyruvate (Sigma-Aldrich Co., St. Louis, MO). Cells were split at least three times before seeded into the inserts to stabilize the cell phenotype. Cells were seeded (220,000 cells/insert) into 12 well permeable supports with 0.4 µm pore diameter, 12 mm diameter polyester membrane inserts (Corning Costar Corp., Cambridge, MA), with 0.5 mL and 1.5 mL of growth media in the apical side (donor chamber) and basolateral side (receiver chamber), respectively. Media was replaced after 6 h of incubation to remove non-adherent cells. Growth media was replaced every 2 days in both sides. Seeded cells (passages 38-44) were grown and differentiated to confluent monolayers for 21-23 days.

Confluence and monolayer integrity of cells was monitored by measuring their transepithelial electrical resistance (TEER) value, using a EndOhm Volt ohmmeter equipped with a STX-2 electrode (World Precision Instruments Inc., Sarasota, FL).

Prior to transport experiments, cells were rinsed with Dulbecco's phosphate buffer saline (DPBS) and growth media was replaced by Hank's balanced salt solution (HBSS) (Fischer Scientific, Pittsburgh, PA) containing 10 mM 2-(N-morpholino)ethanesulfonic acid solution (MES, Gibco BRL Life Technology, Grand Island, NY) adjusted to pH 6.5 in the apical side, and HBSS containing N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] buffer solution (1 M) (HEPES, Gibco BRL Life Technology, Grand Island, NY) adjusted to pH 7.4 in the basolateral side, creating a pH gradient similar to the small intestine environment. TEER values were measured after 30 min of incubation with HBSS and at the end of the experiment. Monolayers with TEER values >450 Ω cm^2 at the beginning and >250 Ω cm^2 at the end of the experiment were used for analysis, monolayers with lower TEER values were discarded because lower values of TEER is associated with a non confluent monolayer. After the first measurement of the TEER value, 300 µL of buffer from the basolateral side was taken and HBSS from the apical side was replaced with 400 µL of the treatment. The treatments consisted of HBSS adjusted at pH 6.5 containing different levels of sorghum extracts or standards. Immediately after adding the treatments, 100 µL were removed from the basolateral side of each insert at 0 min and repeated at 30, 60, 90 and 120 min of incubation with the extracts. 100 µL of the buffer at pH 7.4 was added to the basolateral side to compensate for the aliquot taken. The aliquots obtained

were stabilized with 20 µL of 4 N HCl and 100 µL of methanol 1% HCl and filtered thru a 0.2 µm Whatman filter prior to HPLC.

The initial concentration used for these experiments were: 500 µg/mL of crude extract of the tan plant and 30 and 100 µg/mL of 3-deoxyanthocyanidin standards.

Measurement of Phenolic Compounds in Bioavailability Studies

The extracts were analyzed on an Agilent 1200 Series LC system (Agilent Technologies, Santa Clara, CA) with a photodiode array detector (PDA). Phenolic compounds were separated using a Kinetex C18 column (100 mm x 2.1 mm i.d., 2.6 µm) from Phenomenex (Torrance, CA). Column temperature was conditioned at 40 ºC. Injection volume was 20 µL. The mobile phase consisted of 1% formic acid in water (v/v) (Solvent A) and acetonitrile (Solvent B). The solvent flow rate was 0.25 mL/min. The compounds were separated using the following gradient: 0-26 min., 12-40% B; 26-28 min., 40-75% B; 28-31 min., 75% B; 31-32 min., 75-12%B; 32-33min., 12%B, with a run time of 53 min. The 3-deoxyanthocyanins, flavones, and flavanones were measured at 480 nm, 340 nm, and 280 nm respectively. Identification of sorghum flavonoids was based on retention time of commercial standards and UV-Vis spectra. Quantification of each compound was done by comparing peak areas with that of a standard curve of each authentic standard. Data was collected and processed using the ChemStation software (Agilent Technologies, Santa Clara, CA).

Statistical Analysis

Three replicates of each sample collected at 0, 30, 60, 90 and 120 min from the absorption experiment were analyzed using a General Linear Modeling procedure. The analysis was done using SPSS version 16.0 (SPSS Inc. Chicago, Il).

Results and Discussion

Absorption of Flavones

Absorption of phenolic extracts from glumes of the tan plant was investigated to explain its high anti-proliferative activity compared to other extracts from sorghum plant components reported in the previous chapter. Extracts of glumes had mostly flavone aglycones while the leaves had mostly flavone glycosides (Table 18). The highest levels of luteolin (35.1mg/g) and apigenin (14.74 mg/g) were obtained in the tan glumes extracts (Figure 18). These values were converted to gallic acid equivalents by correcting for molecular weight and number of hydroxyl groups. The converted values gave 20.9 mg GAE/g from luteolin and 7.0 mg/g GAE/g from apigenin, resulting in a total of 27.9 mg GAE/g of extract, representing 50 % of the total phenols present in the crude extract of the tan glumes expressed as GAE. Based on this comparison and the chromatograms, most of the polyphenolic compounds present in the tan glumes are luteolin and apigenin. Flavones in extracts of the red plant glumes were significantly lower than in the glumes of the tan plant. The luteolin and apigenin levels in red glumes were 1.7 mg/g and 17.8 mg/g, respectively (Table 18). Based on this information,

extracts of tan glumes were used to evaluate absorption of flavone aglycones from sorghum.

The two major flavones found originally in the tan plant glume extracts were detected in the basolateral chamber of the permeability experiment, which demonstrated that luteolin and apigenin were transported through the cells (Figure 19). Apparent permeability coefficient P_{app} was 1.01×10^{-5} for luteolin and 1.20×10^{-5} for apigenin. Previous studies using Caco-2 models showed permeability coefficients of 6.30×10^{-6} for luteolin and 1.34×10^{-5} and $1.38 \times 10^{-5}$for apigenin (Ng et al 2005; Teng et al 2012). Differences among these studies could be due to differences in cell culture conditions, passage number and concentration of flavone evaluated.

The rate of absorption was linear during the two hours of the experiment with final absorption of 30.4 % for luteolin and 42.3 % for apigenin (Figure 20). Previous experiments have demonstrated a linear absorption when levels of luteolin and apigenin were tested in the range of 10 to 90 μM (Fale et al 2012). In this experiment the initial concentration of luteolin was 102 μM for luteolin and 43 μM for apigenin. This supports other findings that demonstrated that apigenin has higher absorption than luteolin *in vitro* using Caco-2 absorption models (Ng et al 2005) and *in vivo* models using rat intestine (Chen et al 2012).

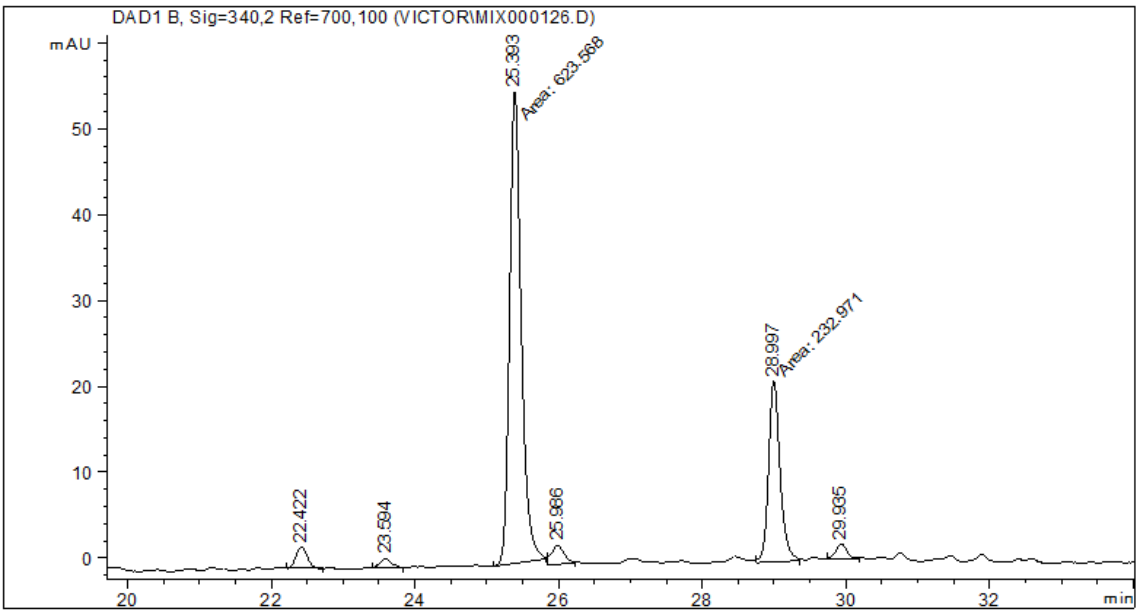

Figure 18. Phenolic compounds, (1) luteolin and (2) apigenin, present in glumes of the tan plant ATx631/RTx436.

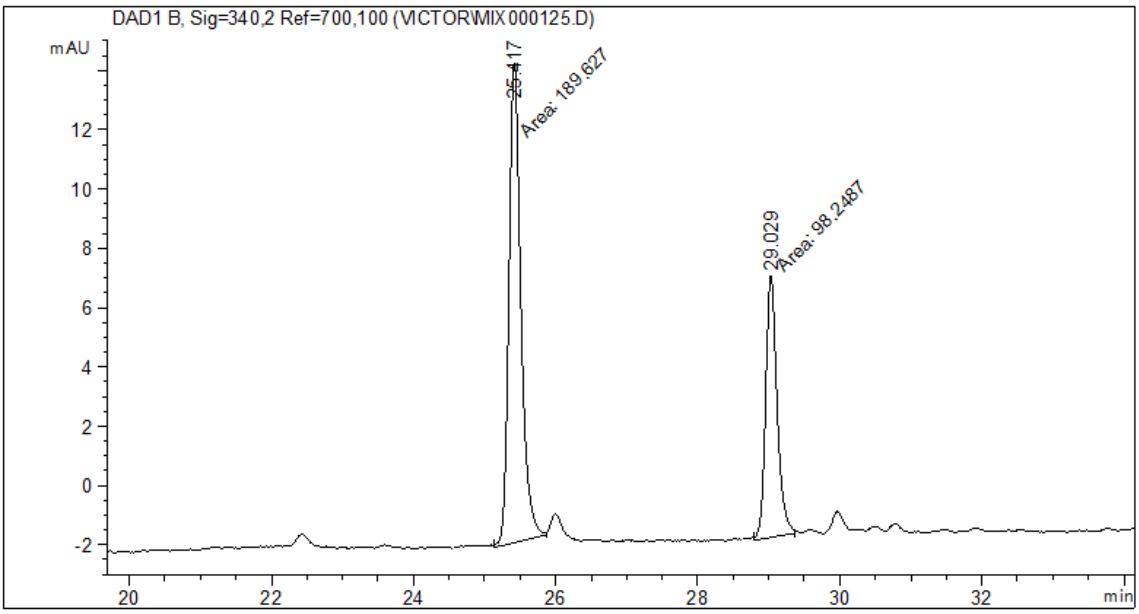

Figure 19. Luteolin and apigenin found in the basolateral chamber of the transepithelial transport experiment with Caco-2 cells.

Recovery from the apical and basolateral side was 63.6 % for apigenin, suggesting that 36.4 % of apigenin was either inside the cells or metabolized. Apigenin quickly metabolizes to form glucuronide and sulfate metabolites in the Caco-2 model and transported by MRPs and OATPs (Hu et al 2003). Recovery of luteolin was 68.4 % which also metabolizes in Caco-2 models (Ng et al 2005).

Chemical structure of apigenin and luteolin are very similar, however absorption of luteolin compared to apigenin could be lower due to the extra hydroxyl group which makes the molecule more hydrophilic reducing its solubility through the cell membrane (Chen et al 2012; Tammela et al 2004) (Figure 21). This could explain the low absorption of luteolin in this experiment.

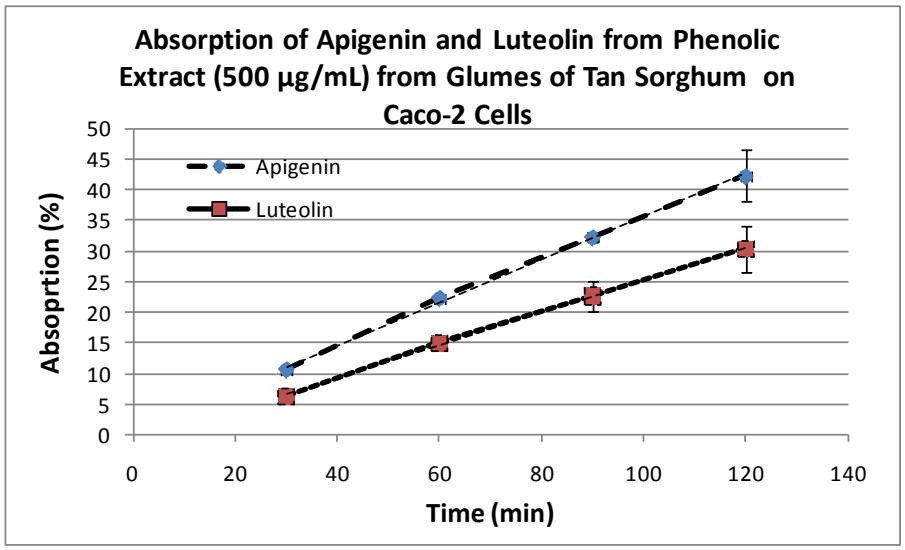

Figure 20. *In vitro* absorption of luteolin and apigenin of tan sorghum glumes at 30, 60, 90 and 120 min of exposure to Caco-2 cells.

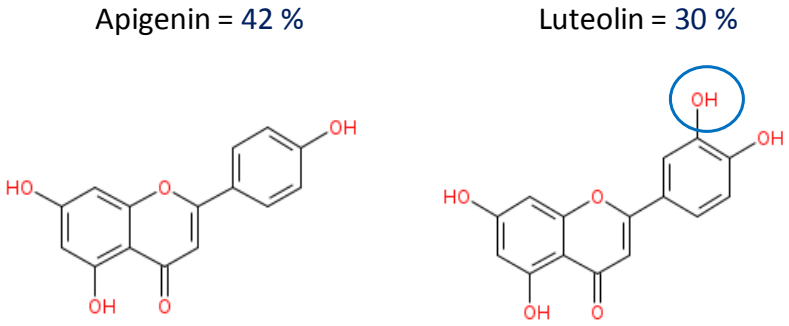

Figure 21. Structure of apigenin and luteolin and % of absorption in a Caco-2 model.

Absorption of Flavones Glycones

Flavone glycones were predominant in sorghum leaves, especially red leaves which demonstrated a high antiproliferation activity of carcinoma cells. To determine if the high antiproliferation activity of leaf extracts was associated with absorption of their flavone glycones, permeability of the flavone glycosides was evaluated using leaves of the red plant which contained the highest levels of flavone glycosides (Table 18). Flavone glycosides were not detected in the basolateral side at the end of the experiment (Figure 22).

Flavones are absorbed less when they are not in the aglycone form because they are less lipophilic which generally causes low permeability through the cell membrane (Akao et al 2000). Consequently, the original levels of flavone glycosides in the extracts of red leaves and their potentially low absorption could be the reason why no flavone glycosides were detected in the receiver chamber.

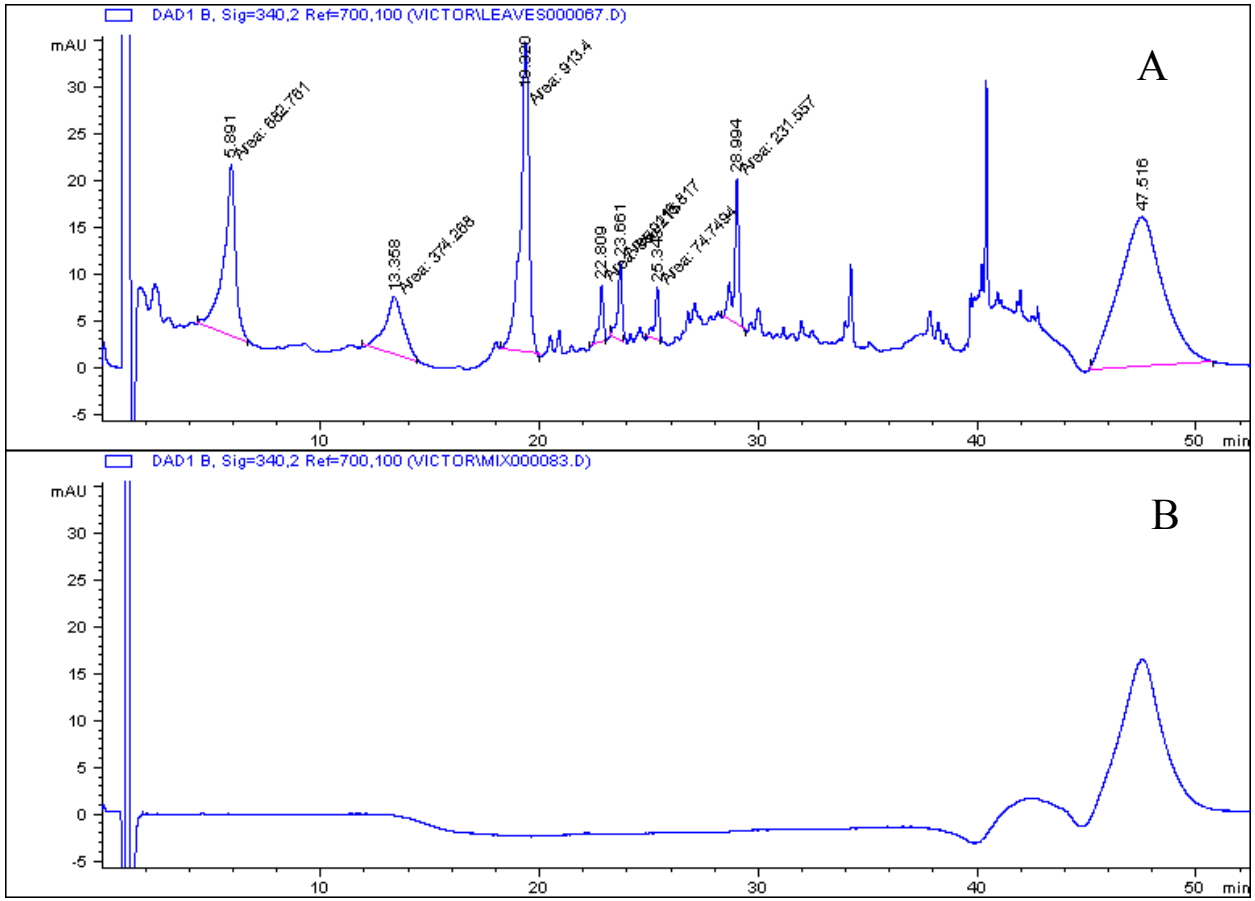

Figure 22. Flavones present in the donor compartment (A) and in the receiver compartment (B) after the transepithelial transport experiment (2 hours) with Caco-2 cells on red sorghum leaf extracts (500 µg/ml).

Absorption of 3-Deoxyanthocyanidins

Extracts from sorghum grains and glumes of the purple plant, which contained the highest levels of 3-deoxyanthocyanidins, were evaluated to measure the absorption of their 3-deoxyanthocyanidins using a concentration of 500 µg extract/mL of buffer at pH 7.4. The initial levels of 3-deoxyanthocyanidins in the apical side were too low to be quantified at the basolateral side in this experiment (Table 19). *In vitro* absorption of 3-deoxyanthocyanidins has not been studied before, but the absence of 3-deoxyanthocyanins in the basolateral side in this study was expected because the *in vitro* absorption of normal anthocyanins is low (Yi et al 2006).

To determine the permeability of 3-deoxyanthocyanidins, standard solutions of luteolinidin and apigeninidin at higher concentrations than found in the samples were evaluated. The highest levels of luteolinidin were observed in the extracts of purple plant glumes (465 µg/g extract) while the highest levels for apigeninidin were also found in the purple plant glumes (801 µg/g extract). For this reason, the level of 3-deoxyanthocyanidin standards used in the experiment was 50 µg/mL.

Permeability coefficient P_{app} were 6.9E-07 for luteolinidin and 4.7E-07 for apigeninidin. Permeability of 3-deoxyanthocyanidins has not been previously evaluated on Caco-2 cells. Absorption after 2 h was 1.6 and 1.4 % for luteolinidin and apigeninidin, respectively (Figure 23). Statistically these two 3-deoxyanthocyanidins had the same absorption when tested at 50 µg/mL. Recovery of luteolinidin was 87.2 % and for apigeninidin was 89.1 %.

These permeability values are low compared to the permeability of flavones found in the tan plant, which suggests that flavones are potentially more bioavailable than 3-deoxyanthocyanidins. The potentially higher bioavailability of flavones compared to 3-deoxyanthocyanidis could explain the high antiproliferation activity of sorghum glumes against cancer cell. Permeability values of 3-deoxhyanthocyanidins (luteolinidin and apigeninidin) were slightly lower than the values reported on anthocyanins from blueberries which were in the range of 3-4 % (Yi et al 2006).

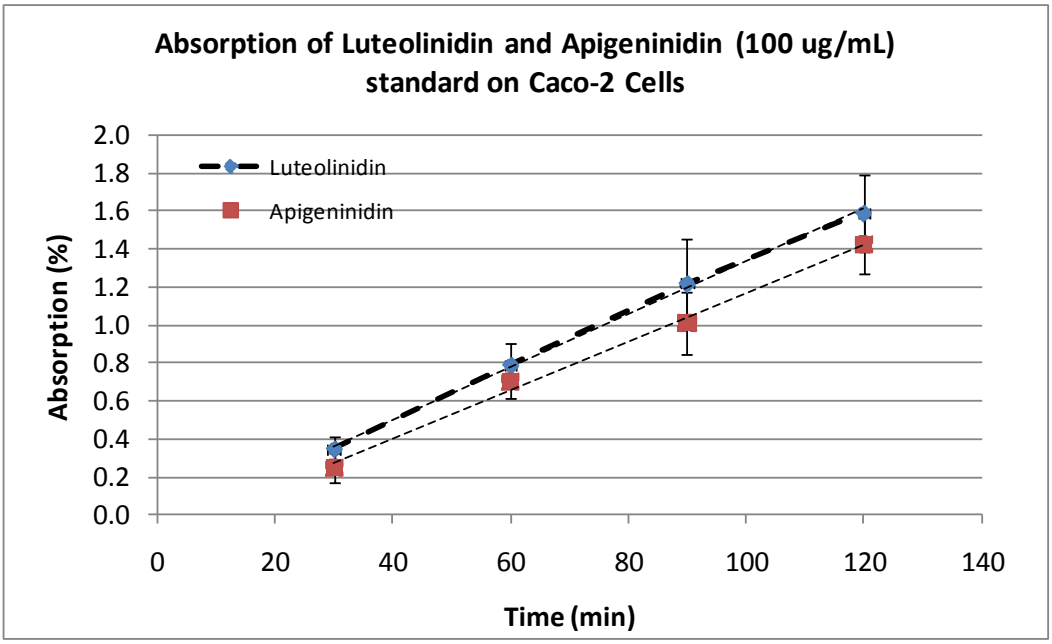

Figure 23. *In vitro* absorption of 3-deoxyanthocyanidins.

Effect of Methoxy Groups in Absorption of 3-Deoxyanthocyanidins

3- Deoxyanthocyanidins with methoxy groups were detected in the samples, which could potentially have higher absorption than the non methoxylated 3-deoxyantocyanidins based on a previous study on anthocyanins which reported higher absorption of anthocyanins with a OCH_3 group (Yi et al 2006). An experiment to evaluate the effect of a methoxy group on absorption of anthocyanidins was performed to determine if the less hydrophilic 3-deoxyanthocyanins could have better absorption than the non methoxylated 3-deoxyanthocyanidins. The compounds evaluated were apigeninidin and 7-methoxyapigeninidin at a concentration of 30 μg/mL.

Permeability of luteolinidin at 30 μg/mL was 8.0E-07 for apigeninidin and 3.1E-06 for 7-methoxyapigeninidin. Recovery was 94.3 % and 70.7 for apigeninidin and 7-methoxyapigeninidin, respectively. A significant increase of absorption was observed in the methoxylated 3-deoxyanthocyanidin compared to the non methoxylated form (Figure 24). Absorption of the methoxylated 3-deoxyanthocyanidin (7-methoxyapigeninidin) was 11.3 % while the non methoxylated form (apigeninidin) was 3.0 %. The higher absorption on 7-methoxyapigeninidin could be attributed to its higher lipophilicity due to the methoxy group (Figure 25). These results indicate that the 3-deoxyanthocyanidins have similar behavior as to the normal anthocyanins which have higher permeability when they have a methoxylated group attached (Yi et al 2006).

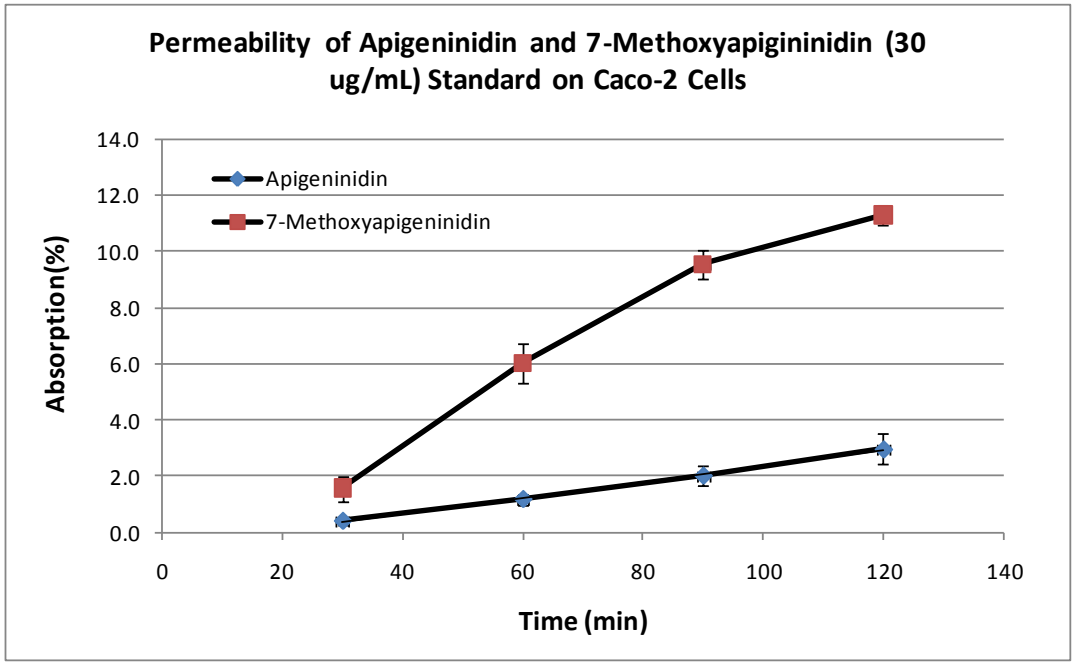

Figure 24. Comparison of absorption between methoxylated and non methoxylated 3-deoxyanthocyanidins.

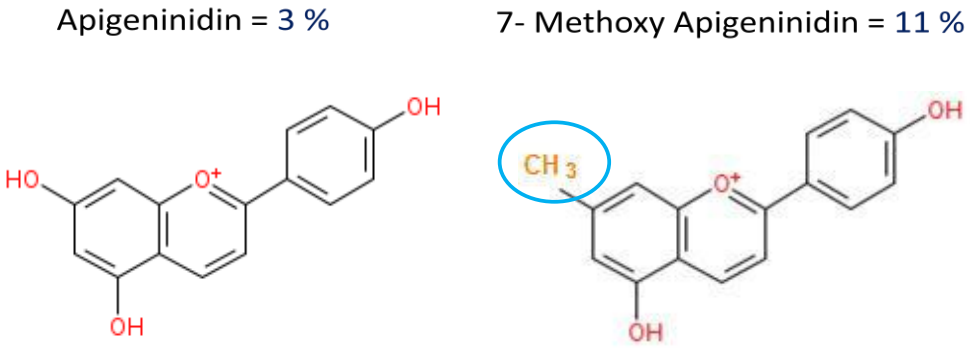

Figure 25. Absorption (%) of methoxylated and non-methoxylated 3-deoxyanthocyanidins.

Relationship Between Cancer Cells Antiproliferation and Absorption of Sorghum Extracts

The absorption of phenolic compounds present in sorghum plant components has a direct effect on their chemopreventive potential. Sorghum extracts with the strongest chemopreventive activity, such as the glume extracts of the tan plant ATx631/RTx436, contained mainly flavone aglycones. Flavone aglycones have higher *in vitro* absorption compared to 3-deoxyanthocyanidins which are present mostly in leaves, grains and glumes of the red (Tx2911) and purple (Tx3362) plants. Flavone aglycones in sorghum could function as an effective chemopreventive agent compared to other sorghum polyphenols due to their higher absorption.

Leaf extracts of the red plant, which contain the highest levels of flavone glycosides show similar antiproliferation activity compared to glume extracts from the purple plant which contained higher levels of 3-deoxyanthocyanidins and flavone aglycones. Furthermore, flavone glycosides were absorbed through the Caco-2 system model suggesting a low absorption *in vivo*. On the other hand, 3-deoxyanthocyanidins had relatively low absorption.

Not only polyphenolic content or absorption, but mechanisms of action of polyphenolics are important factors involved in chemoprevention potential. Also, *in vitro* absorption of flavone glycosides may not be representative of their *in vivo* bioavailability because *in vivo* absorption of flavone glycosides could be affected by hydrolysis of their sugars by intestinal β-glucosidases which could produce highly absorbed flavone aglycones.

Conclusions

Flavones from sorghum glumes had high absorption *in vitro* compared to 3-deoxyanthocyanidins which suggests that sorghum extracts with high levels of flavones could be more available to act as chemoprotective agents than sorghum with similar levels of 3-deoxyanthocyanidins.

Absorption of phenolic compounds affected chemopreventive potential of sorghum plant extracts based on the absorption of 3-deoxyanthocyanidins and flavones and their relationship with chemopreventive potential of sorghum extracts containing these compounds.

Methoxylated 3-deoxyanthocyanidins found in red and purple sorghum extracts had higher absorption than non-methoxylated 3-deoxyanthocyanidins.

Further studies comparing absorption of other phenolics present in sorghum extracts such as phenolic acids and chalcones is necessary to determine their importance on chemopreventive potential of sorghum extracts.

CHAPTER VI

SUMMARY

Forty nine phenolic compounds were identified in stalks, sheaths, leaves, glumes and grains of three sorghum plant types using a UPLC MS/MS system. Five major groups of polyphenolics were identified: phenolic acids, flavones, 3-deoxyanthocyanidins, flavanones and chalcones. Twenty of these compounds have not previously been identified in sorghum. Phenolic content and profile suggested that sorghum plant components, especially glumes and leaves are a potential source of phytochemicals.

Glumes, leaves and grains of sorghum extracts with distinct phenolic profile and high total phenol content were evaluated for chemopreventive properties using HT-29 cancer cells. Extract of tan and red plant glumes had the strongest chemopreventive activity (IC_{50} = 85-178 μg/ml). Extracts of purple plant glumes and red plant leaves also had high chemopreventive activity compared to grains (IC_{50} = 246-248 μg/ml). These results suggest that sorghum plant material could be utilized to produce extracts of phenolic compounds with chemopreventive properties.

Phenolic extracts of glumes which had the strongest chemopreventive activity, had high levels of flavone aglycones. Absorption properties of flavones were evaluated to determine if the flavone aglycones found in sorghum could affect their chemopreventive properties. Absorption of flavone aglycones, found in high levels in glumes of the tan plant, were compared to flavone glycosides, found in high levels in

sorghum leaves. Absorption of these flavones also was compared to 3-deoxyanthocyanidins which were the predominant compounds in the other sorghum extracts and have been associated with chemopreventive properties of sorghum (Yang et al 2009).

Absorption of the flavone luteolin and apigenin aglycones was the highest, 30 and 42 %, respectively. The 3-deoxyanthocyanidins had absorption values between 1.6 and 11.3 %. Methoxylated 3-deoxyanthocyanidins showed higher absorption than non-methoxylated 3-deoxyanthocyanidins. The high absorption of flavone aglycones may be an important factor affecting the high chemopreventive potential of sorghum glumes. Also, the lower absorption of 3-deoxyanthocyanidins but higher chemopreventive properties of extracts containing them, suggests that efficacy of 3-deoxyanthocyanidins as a chemopreventive agent is greater than other sorghum polyphenolics.

Different functionality could be obtained from extracts of different plant parts to add value to the sorghum crop. For example, extracts from red and purple leaves and glumes could be used as natural colorants while extracts of glumes of red and tan plant could be used as a phytochemical due to their potential health benefits *in vitro*. The use of sorghum glumes as a source of phytochemicals could increase the value of sorghum crop. Plant breeders could select and develop sorghum lines with high levels of flavones, 3-deoxyanthocyanins or methoxylated 3-deoxyanthocyanidins to obtain extracts with enhanced phytochemical properties.

It is necessary to compare the chemopreventive potential of sorghum flavones and 3-deoxyanthocyanidins "*in vivo*" using rat models to confirm the results presented in

this study. These results could help to determine the economical feasibility of producing sorghum plant extracts but other factors such as collection and separation of plant components and cost of extraction needs evaluation.

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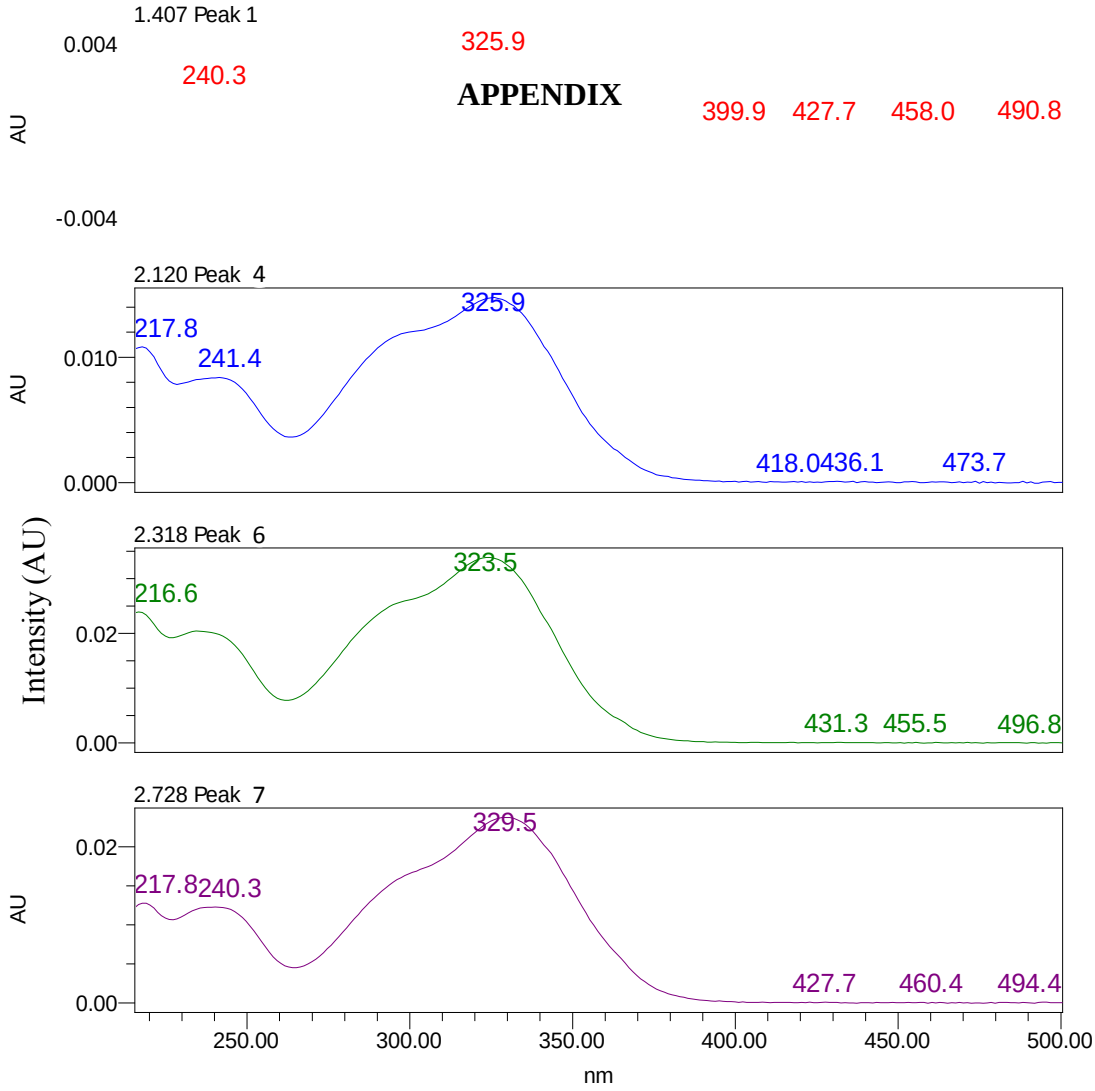
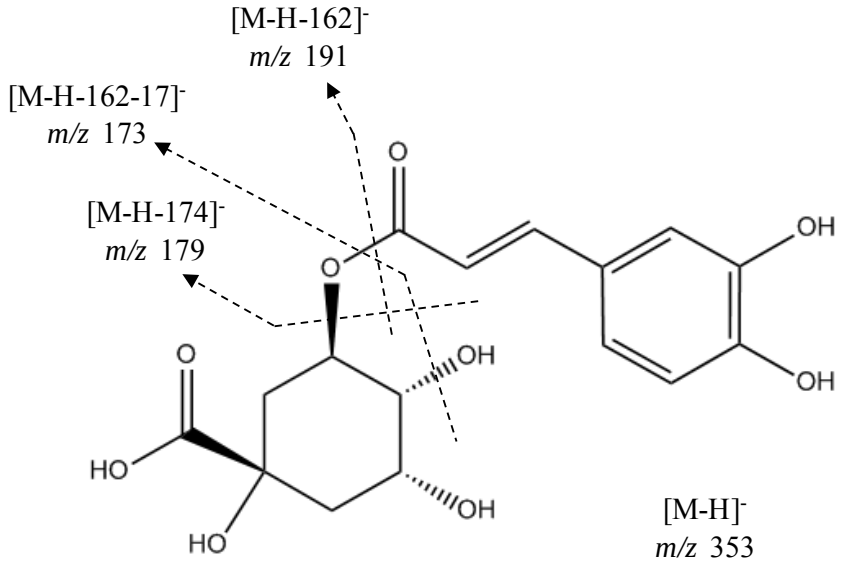
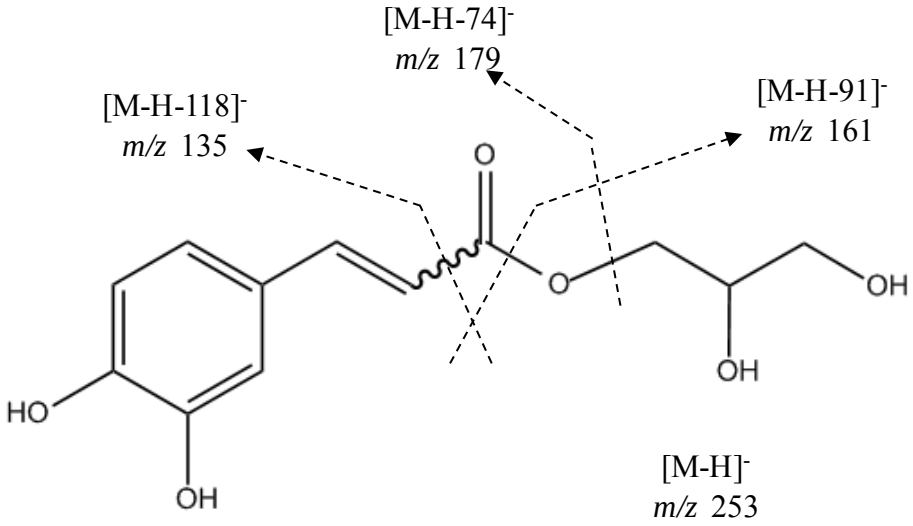


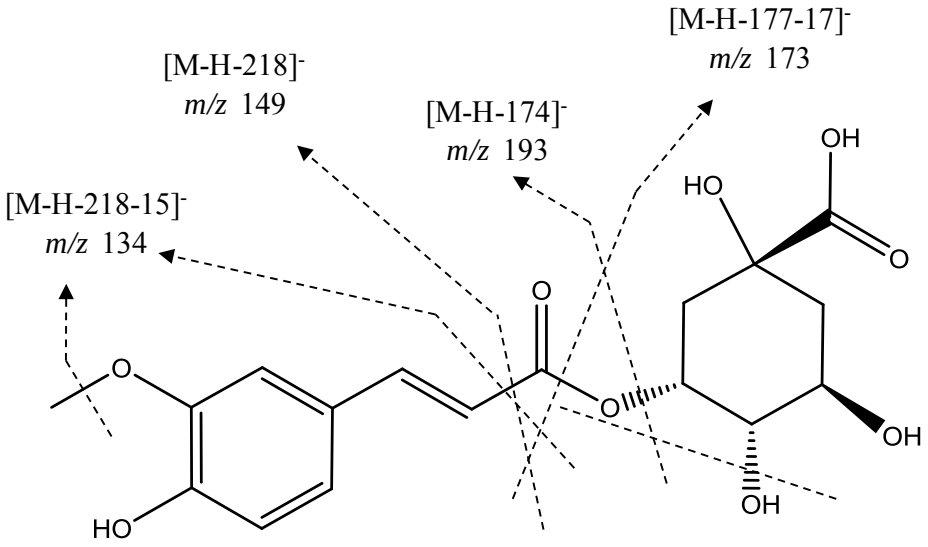
Appendix 1 . UV spectra of phenolic acids identified in sorghum stalks



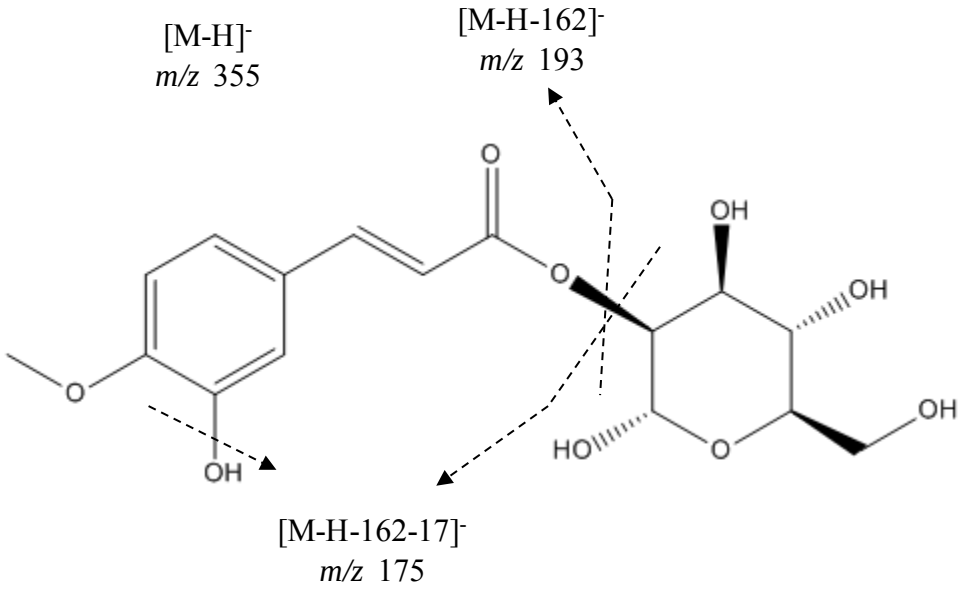
Appendix 2. Fragmentation pattern of 3-caffeoylquinic acid or chlorogenic acid



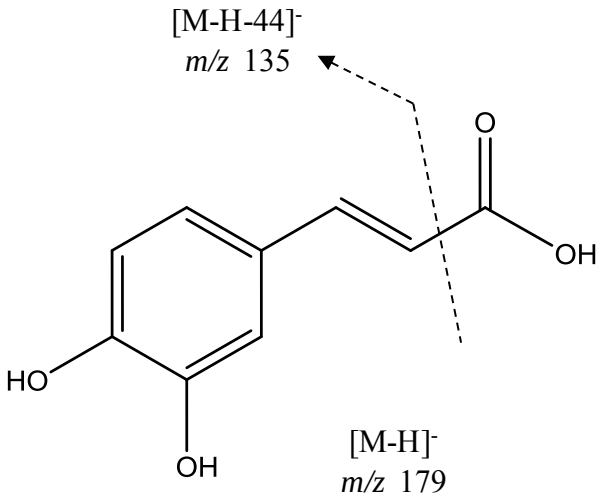
Appendix 3. Fragmentation pattern of caffeoyl glycerol



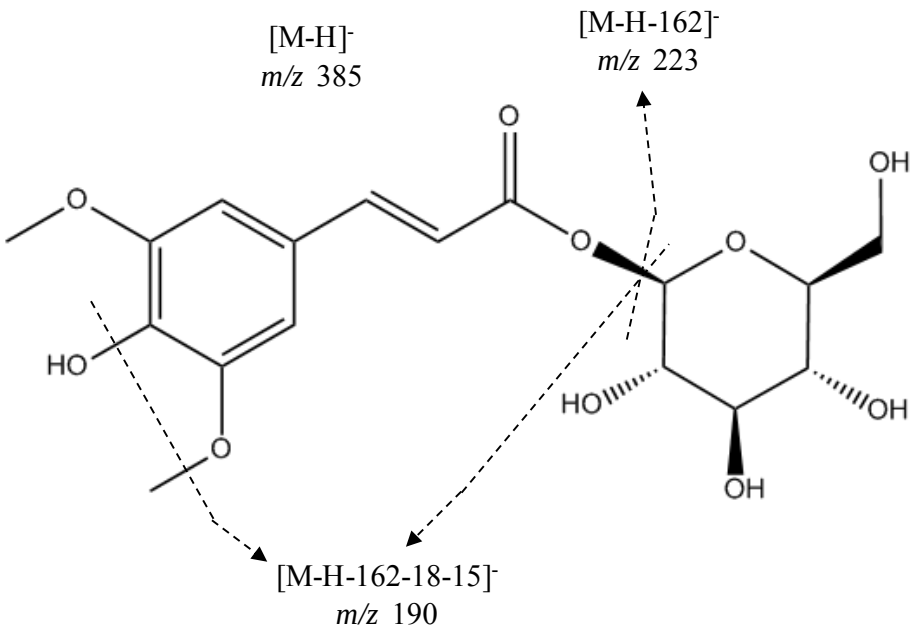
Appendix 4. Fragmentation pattern of 3-feruloylquinic acid



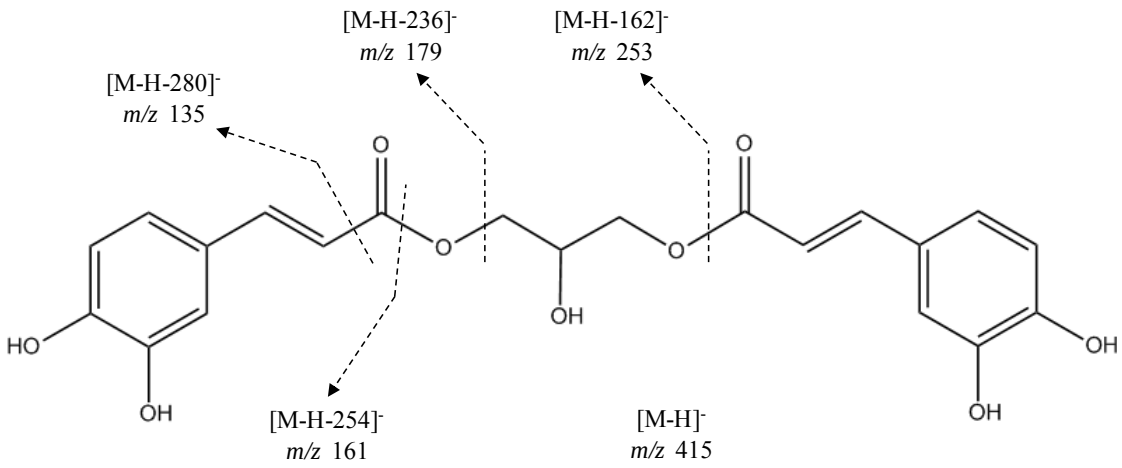
Appendix 5. Fragmentation pattern of feruloyl hexoside



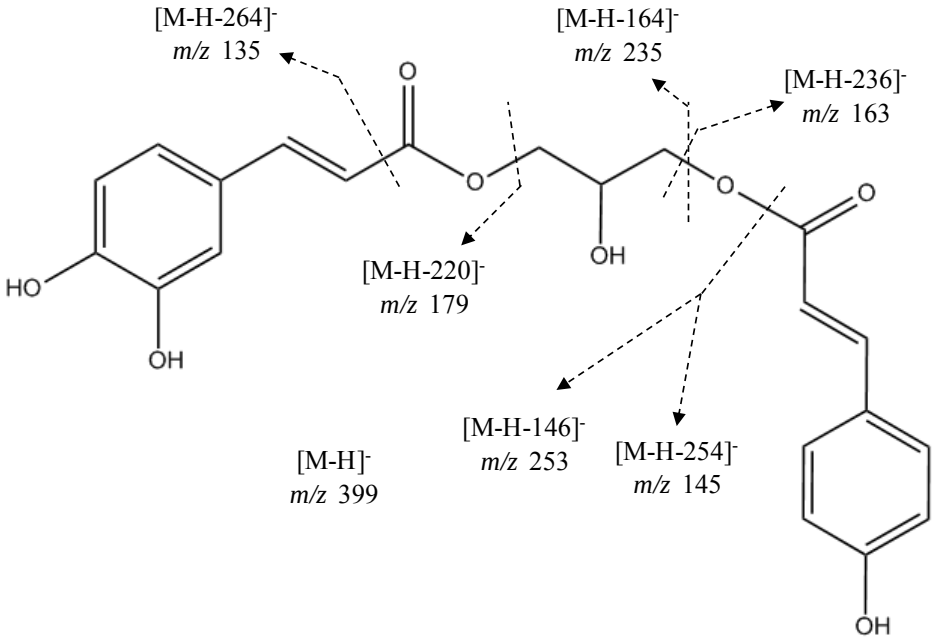
Appendix 6. Fragmentation pattern of caffeic acid



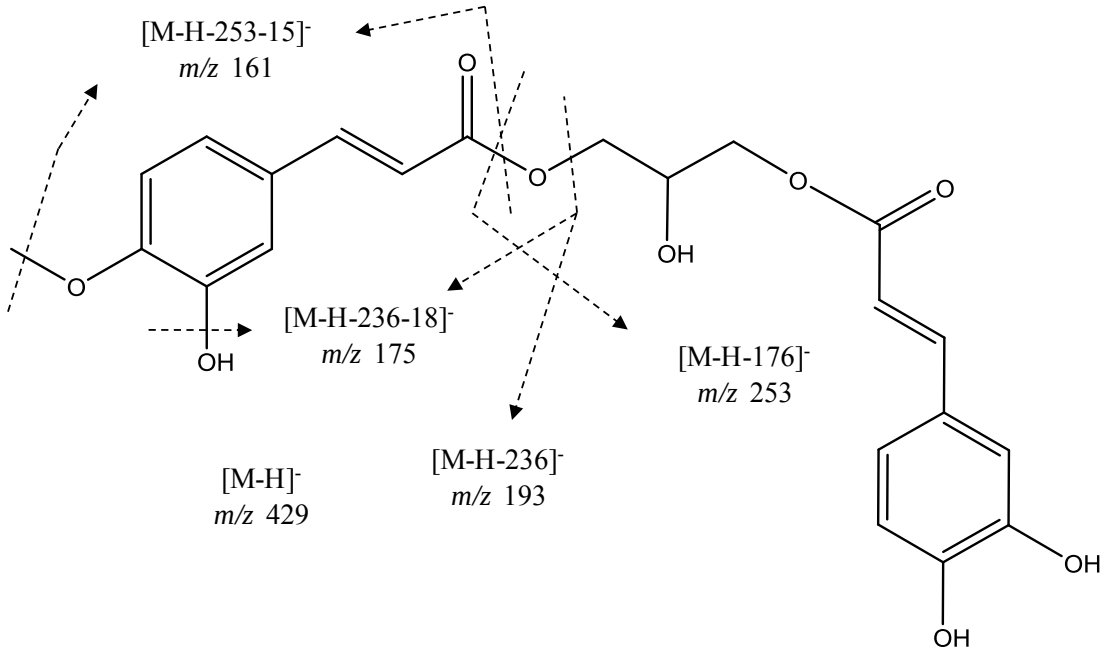
Appendix 7. Fragmentation pattern of sinapoyl hexoside



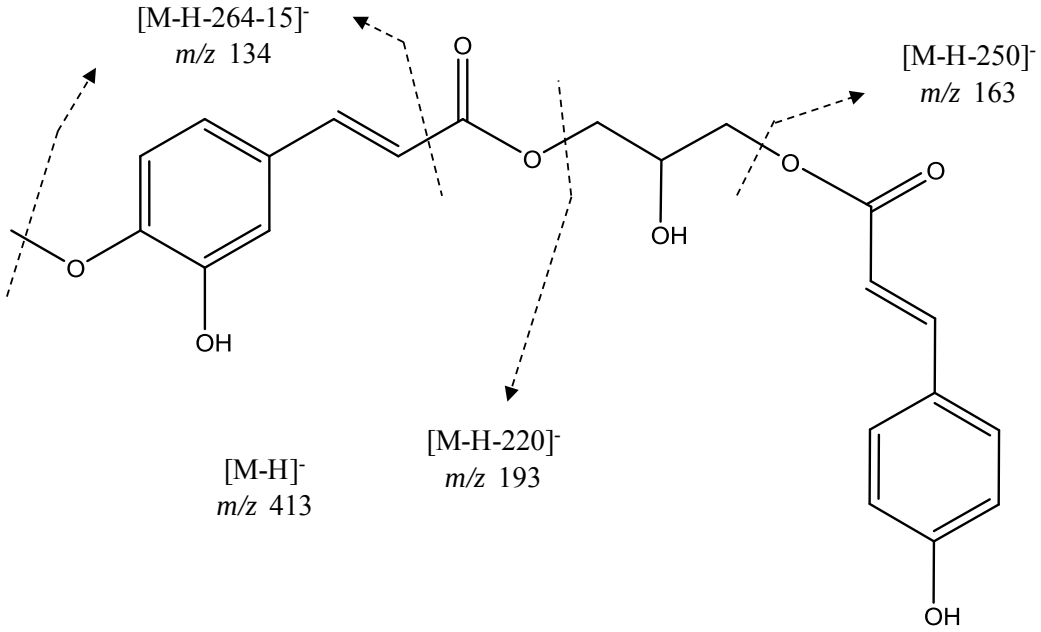
Appendix 8. Fragmentation pattern of dicaffeoyl-glycerol



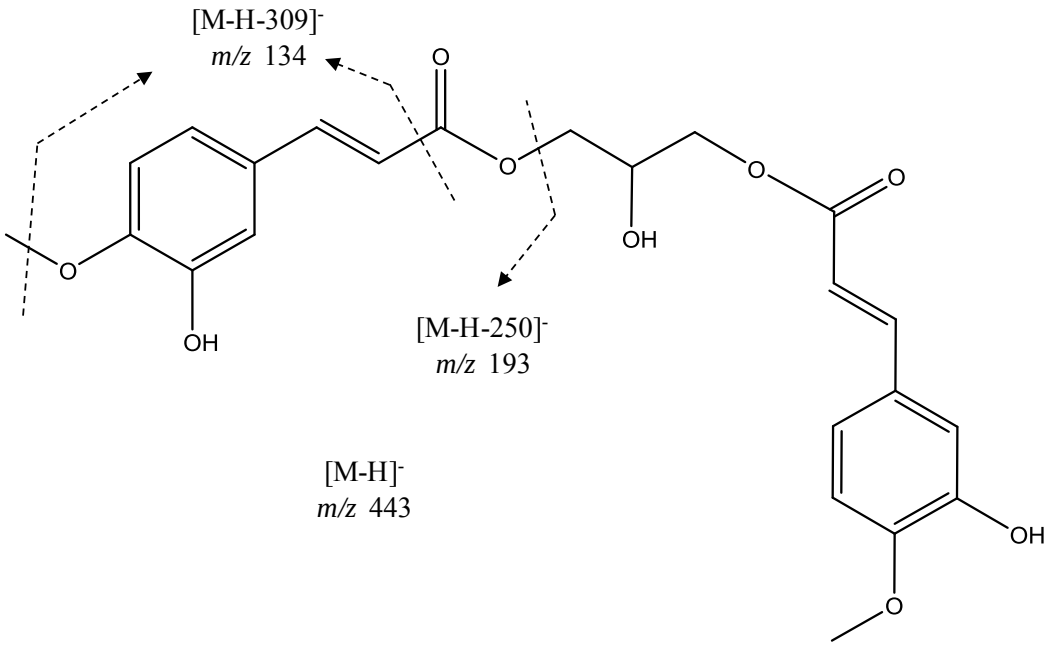
Appendix 9. Fragmentation pattern of coumaroyl caffeoyl glycerol



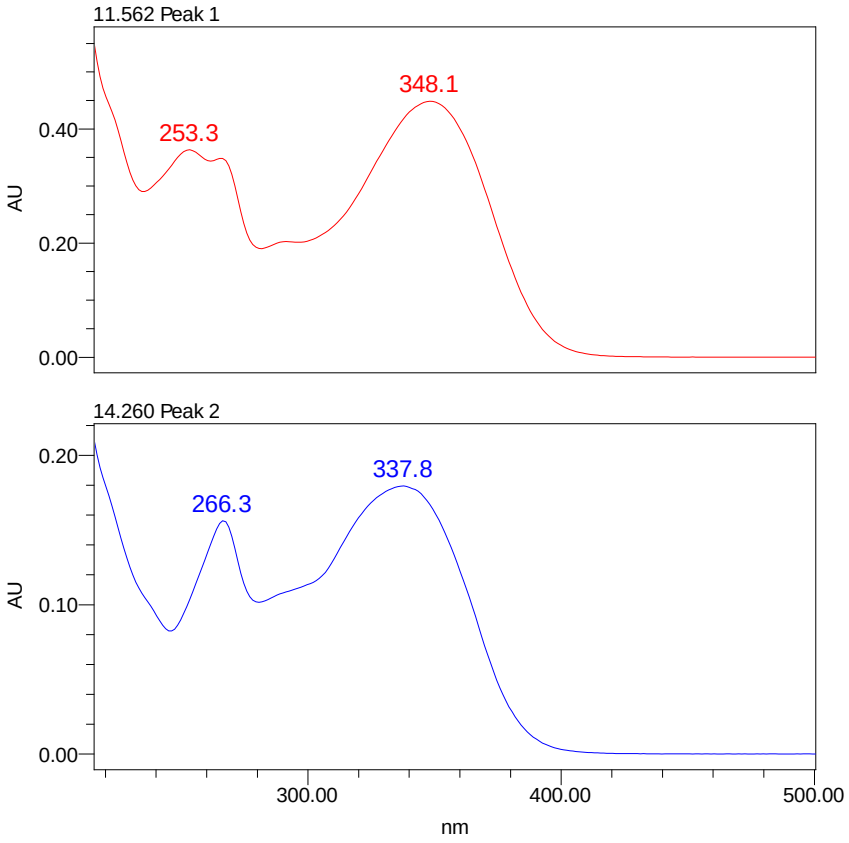
Appendix 10. Fragmentation pattern of feruloyl caffeoyl glycerol



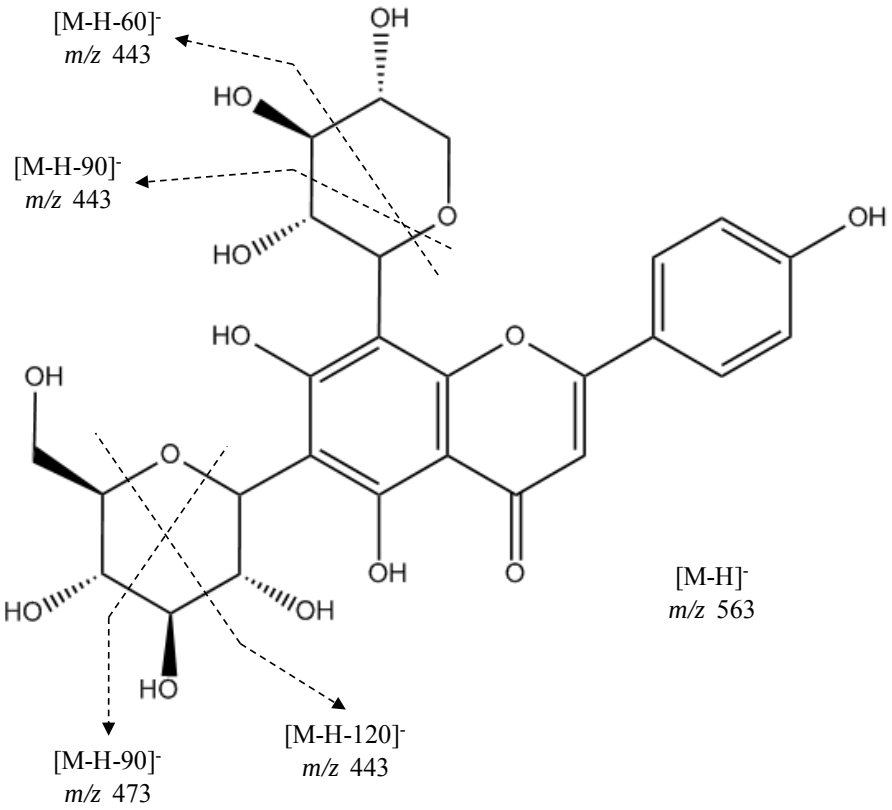
Appendix 11. Fragmentation pattern of coumaroyl feruloyl glycerol



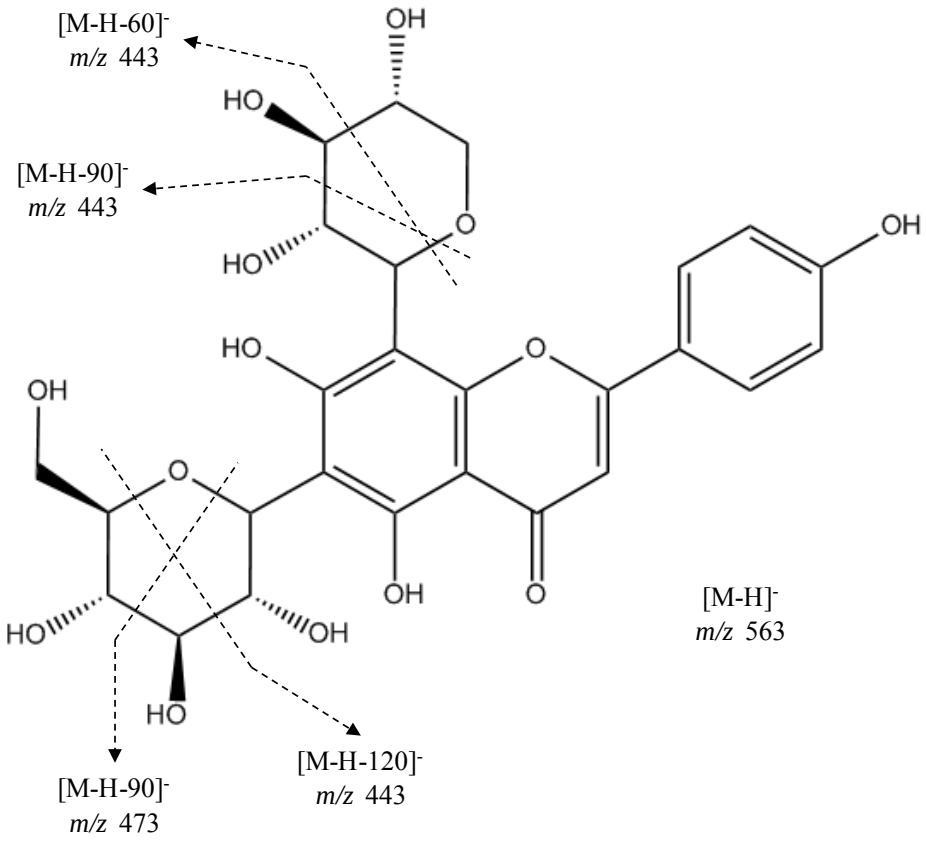
Appendix 12. Fragmentation pattern of diferuloyl glycerol



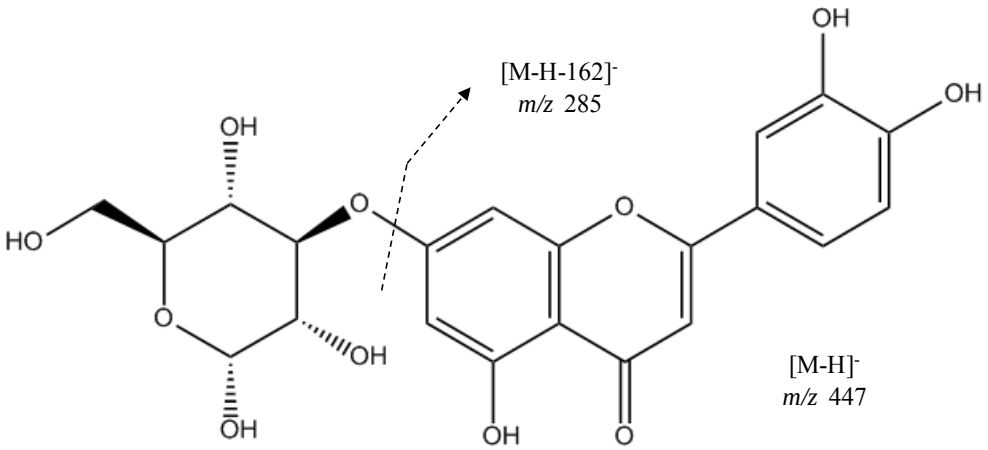
Appendix13. UV spectra of flavones (1) luteolin and (2) apigenin identified in sorghum glumes



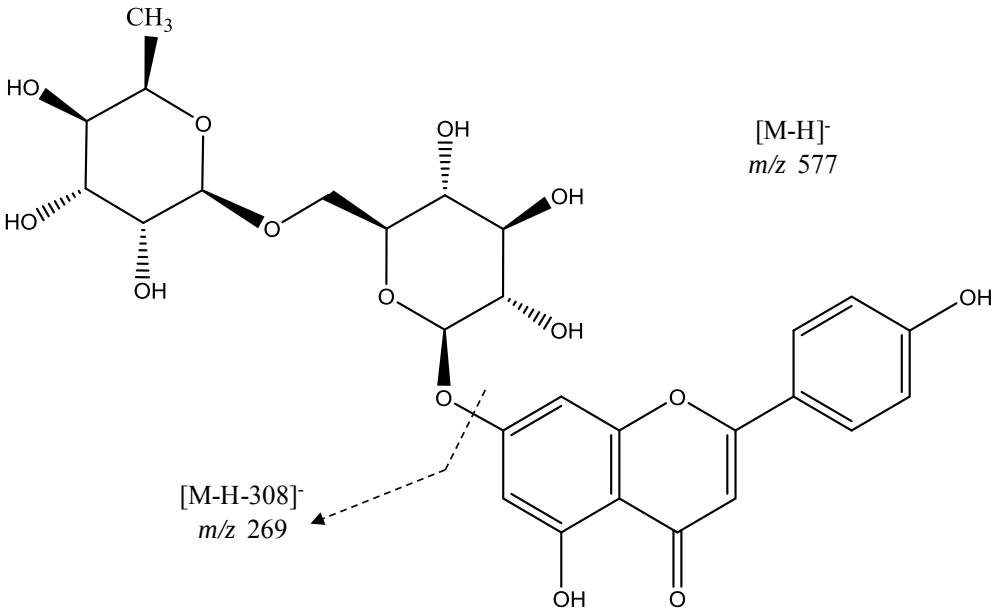
Appendix14. Fragmentation pathway for 6-C-hexosyl-8-C-pentosyl apigenin



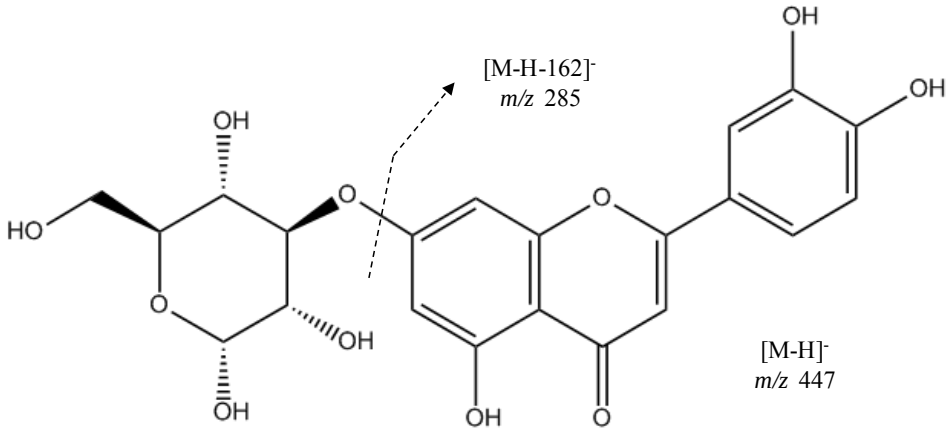
Appendix15. Fragmentation pathway for 6-C-hexosyl-8-C-pentosyl apigenin



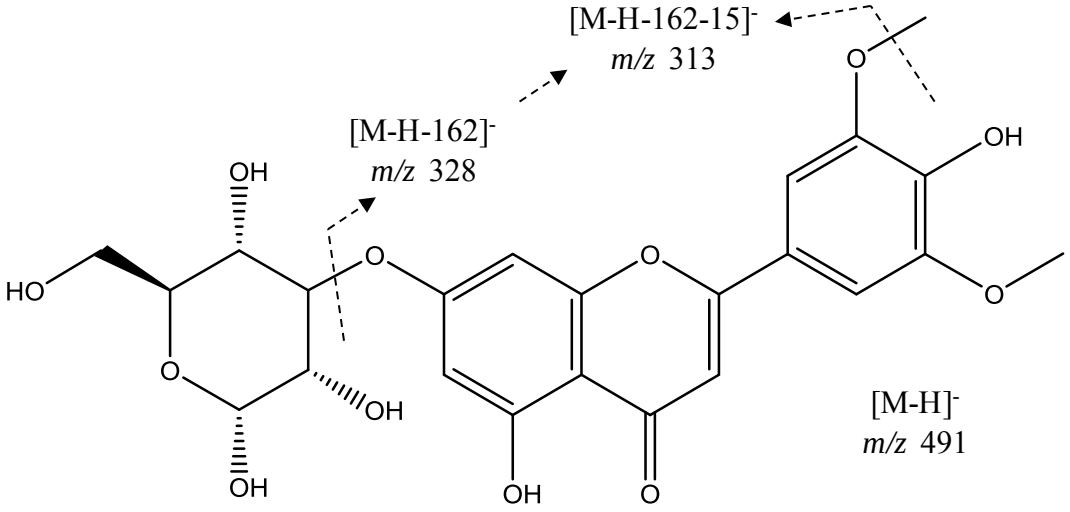
Appendix 16. Fragmentation pathway for luteolin-7-O-glucoside



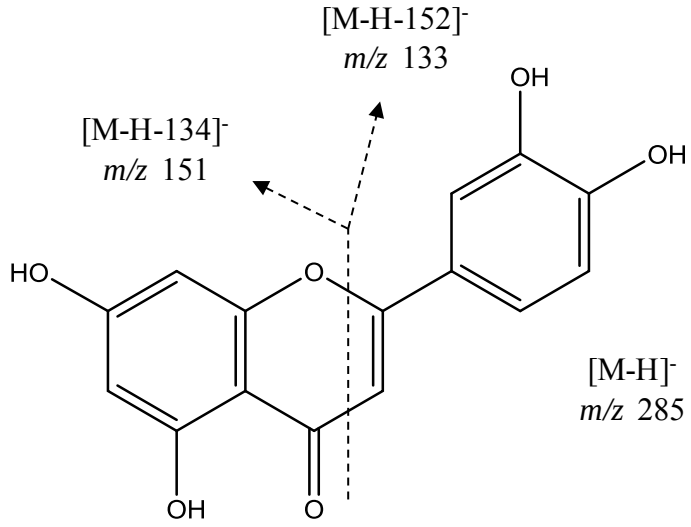
Appendix 17. Fragmentation pathway for apigenin-7-*O*-rutinoside



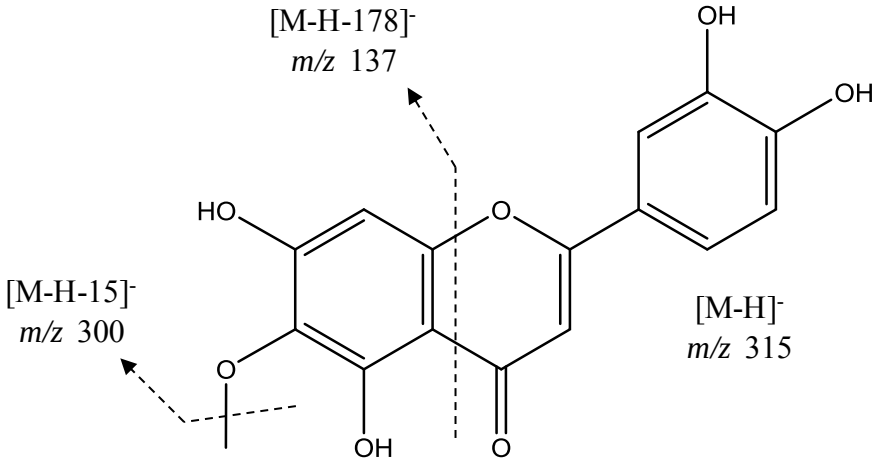
Appendix 18. Fragmentation pathway for apigenin-7-*O*-glucoside



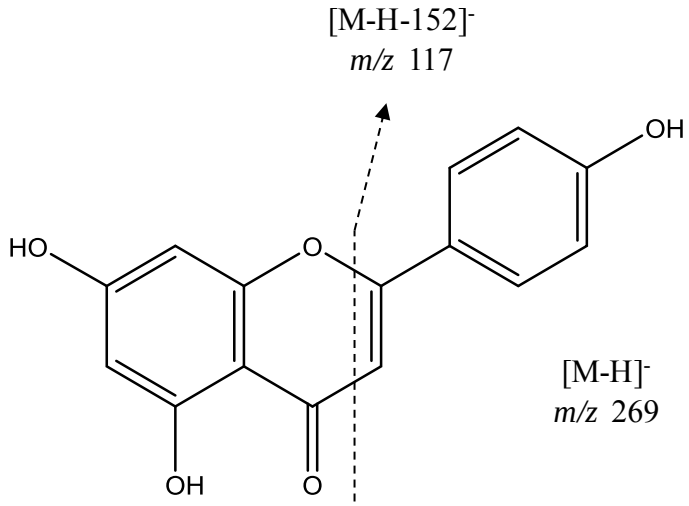
Appendix 19. Fragmentation pathway for tricin-*O*-glucoside



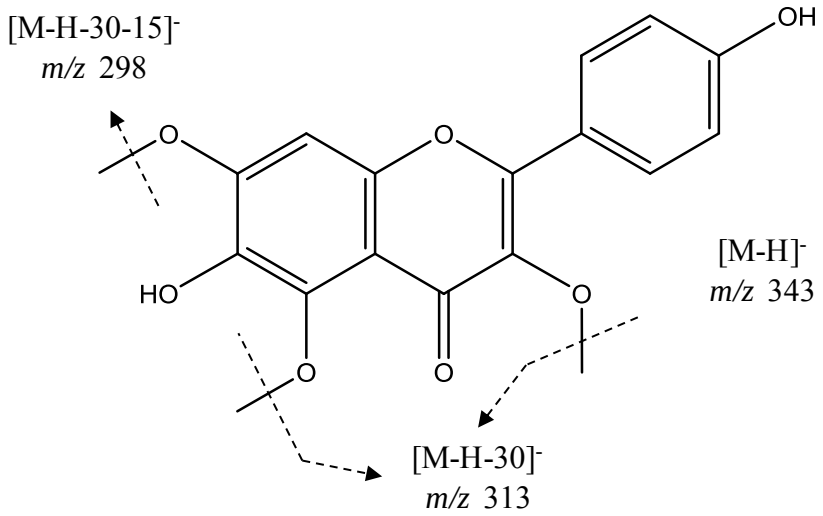
Appendix 20. Fragmentation pathway for luteolin



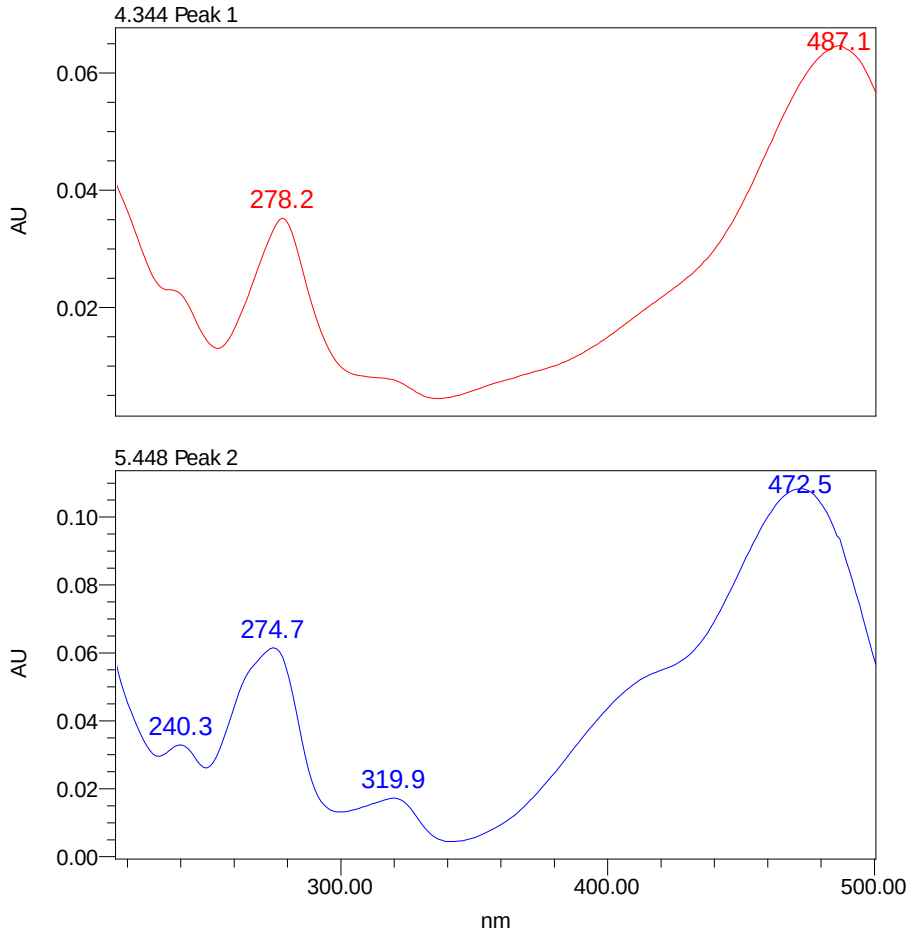
Appendix 21. Fragmentation pathway for tetrahydroxymethoxyflavone



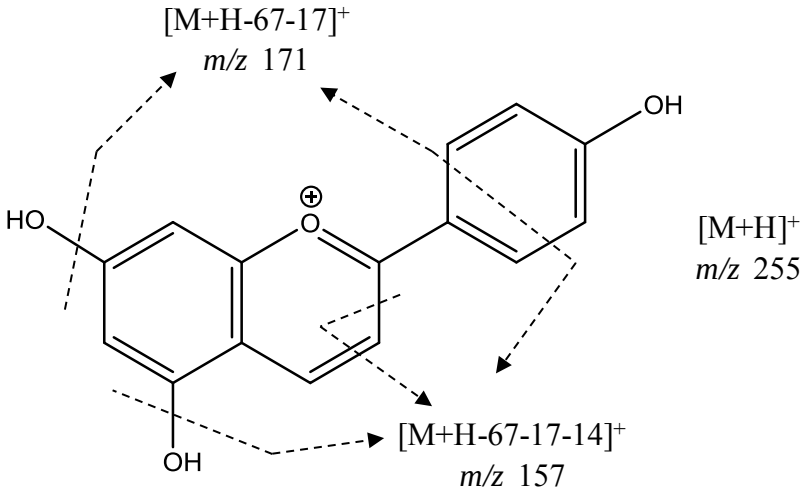
Appendix 22. Fragmentation pathway for apigenin



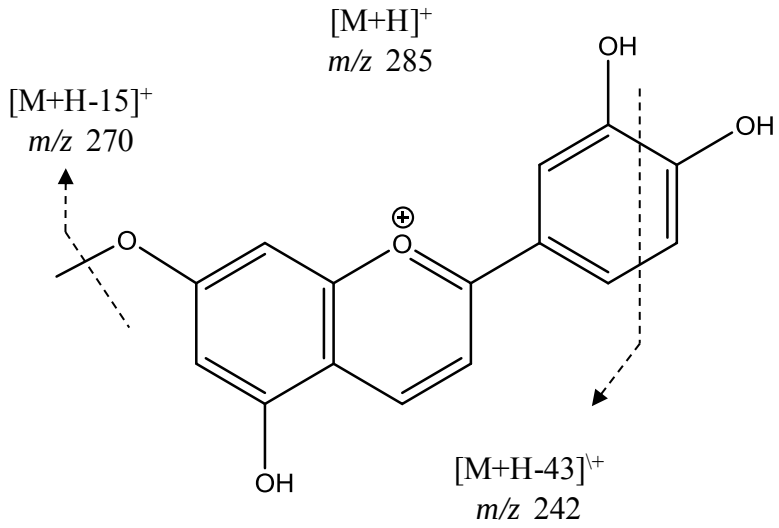
Appendix 23. Fragmentation pathway for dihydroxytrimethoxyflavone



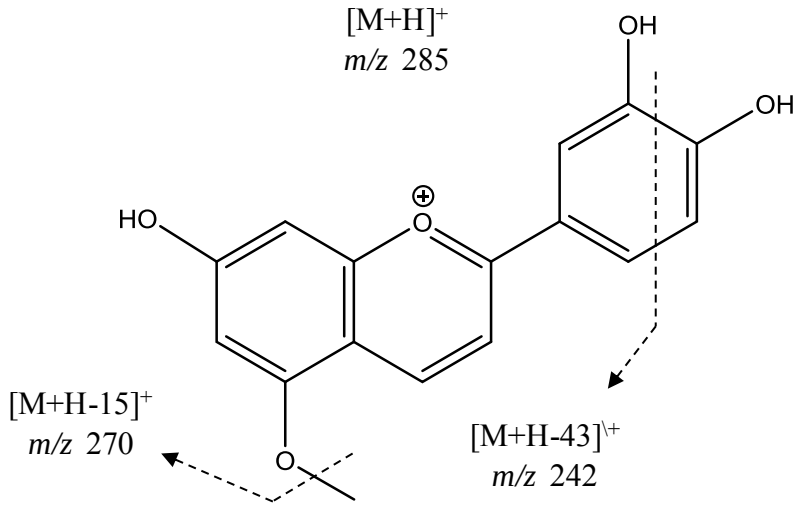
Appendix 24. UV spectra of 3-deoxyanthocyanidins: (1) luteolinidin and (2) apigeninidin, identified in sorghum leaves



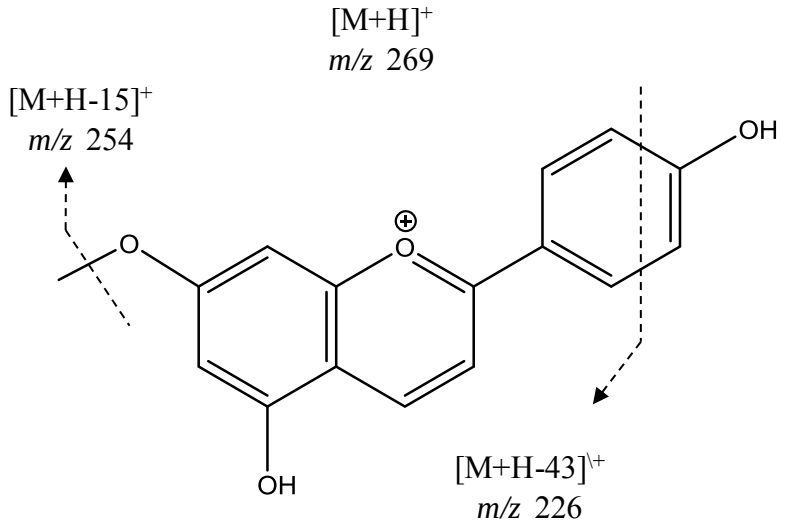
Appendix 25. Fragmentation pathway for apigeninidin



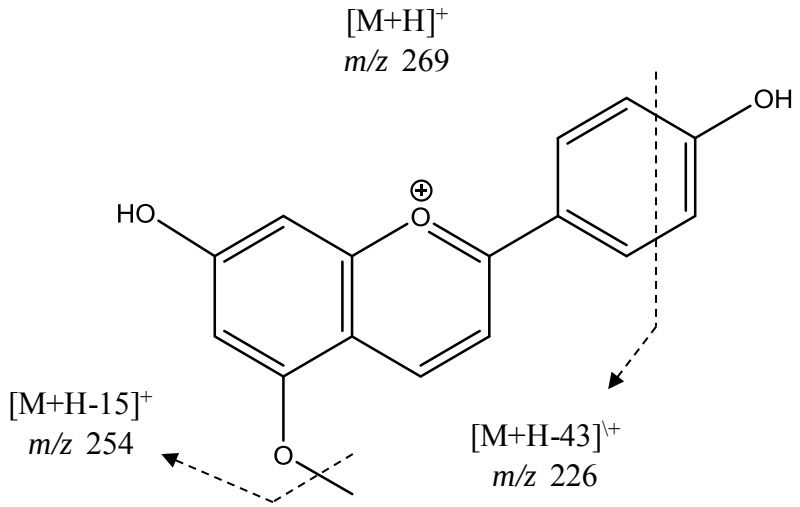
Appendix 26. Fragmentation pathway for 7-methoxyluteolinidin



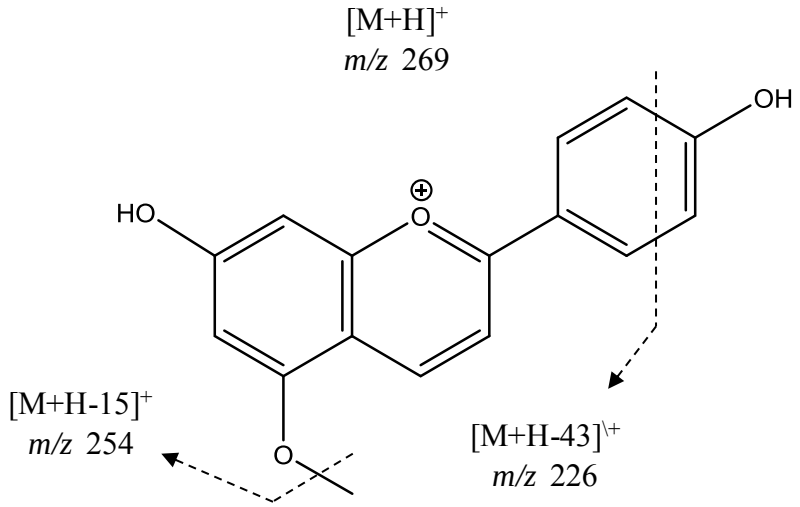
Appendix 27. Fragmentation pathway for 5-methoxyluteolinidin



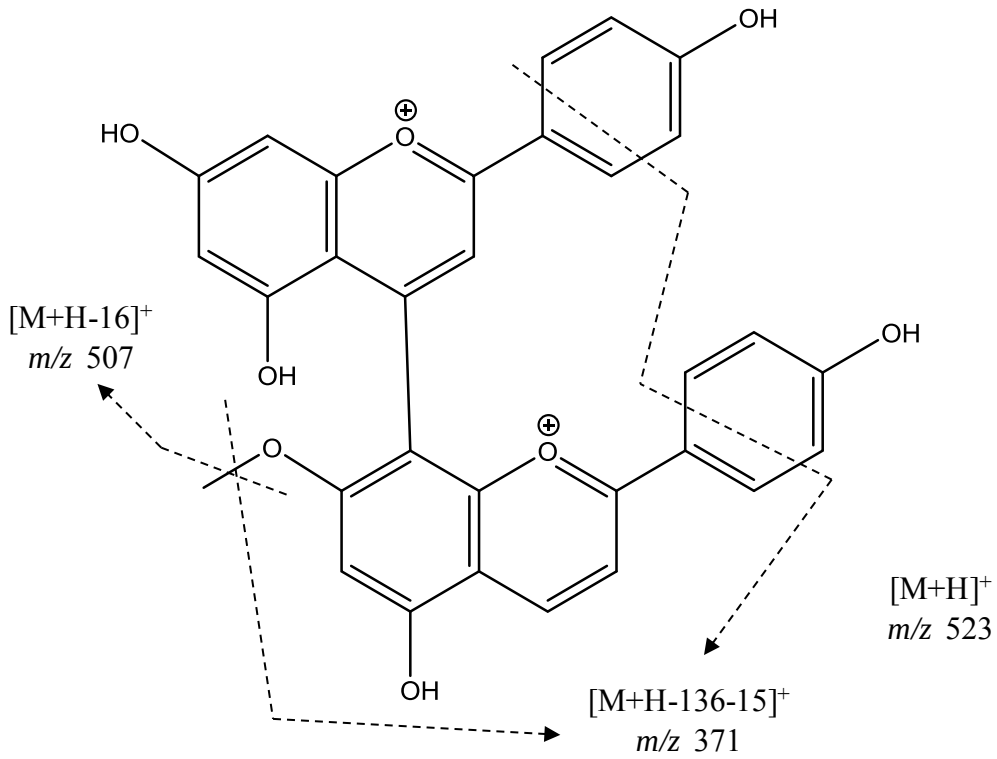
Appendix 28. Fragmentation pathway for 7-methoxyapigeninidin



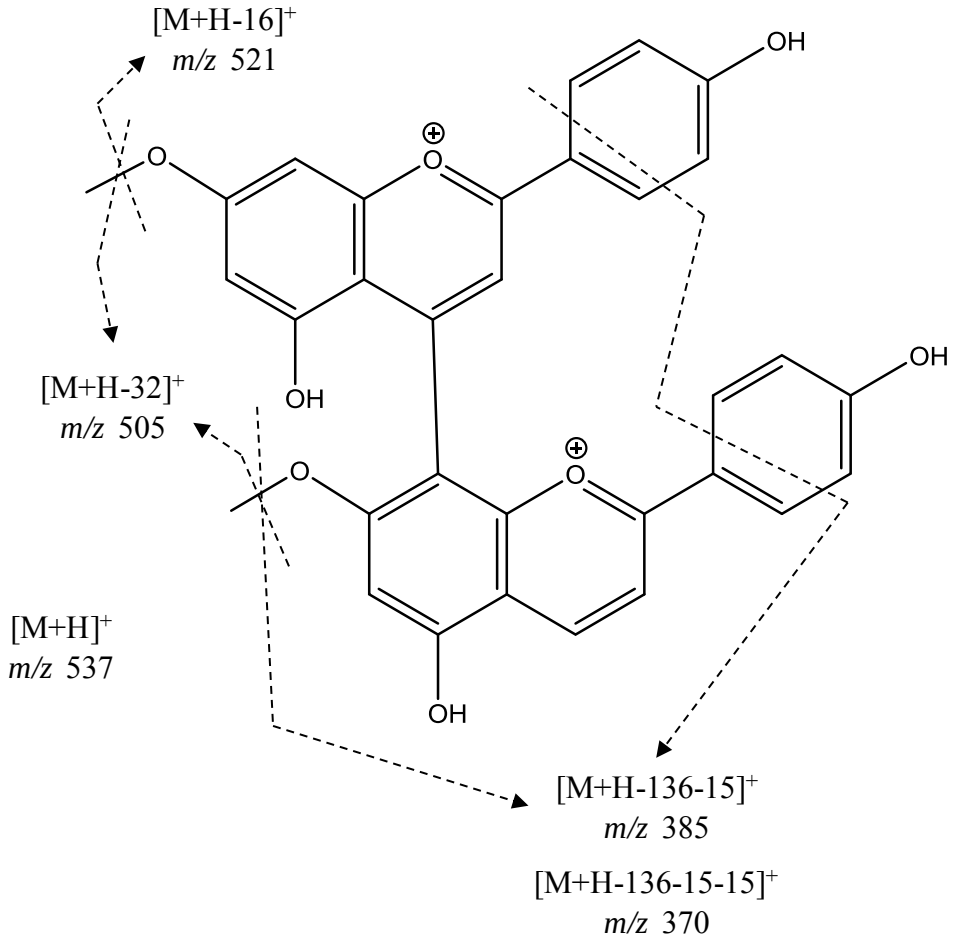
Appendix 29. Fragmentation pathway for 5-methoxyapigeninidin



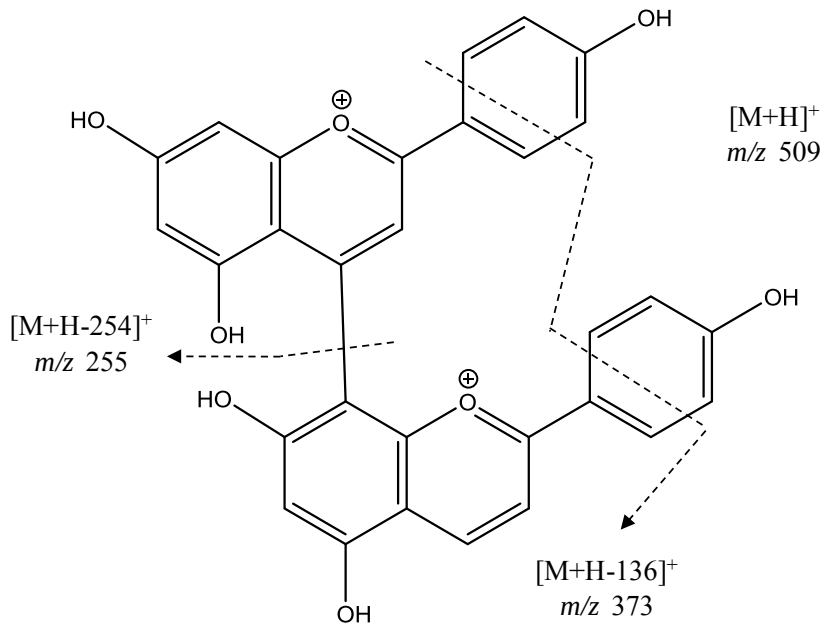
Appendix 30. Fragmentation pathway for dimethoxyluteolinidin



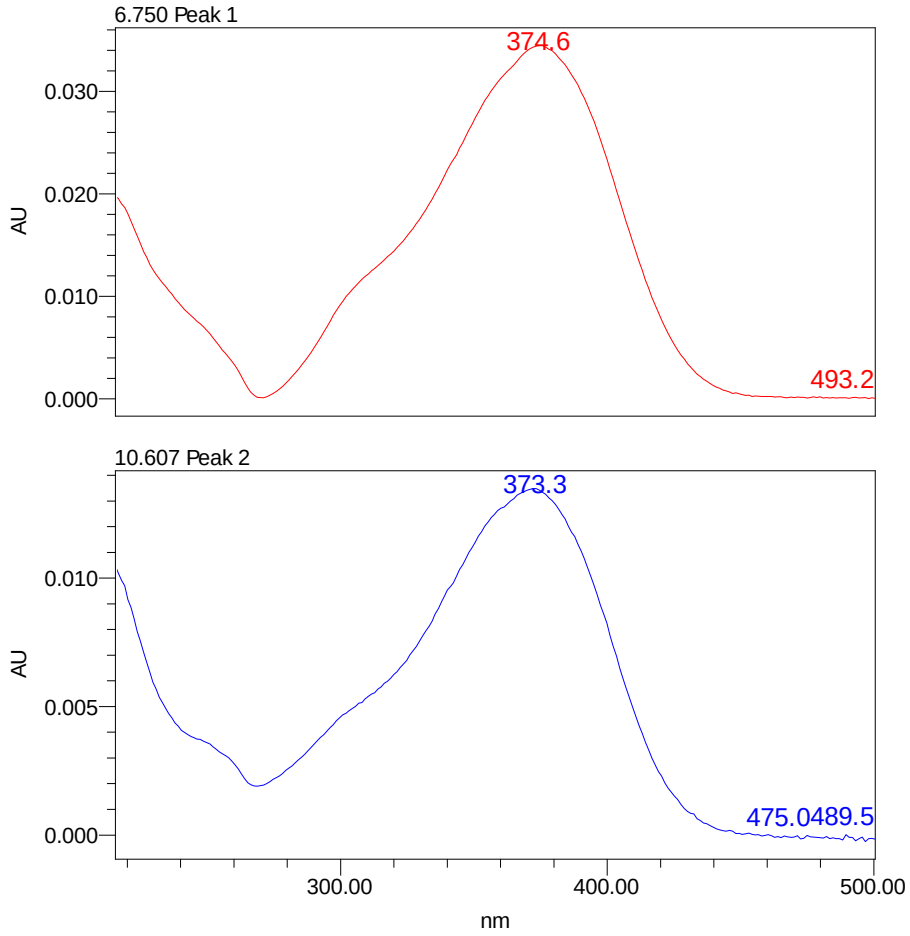
Appendix 31. Fragmentation pathway for apigeninidin-methoxyapigeninidin



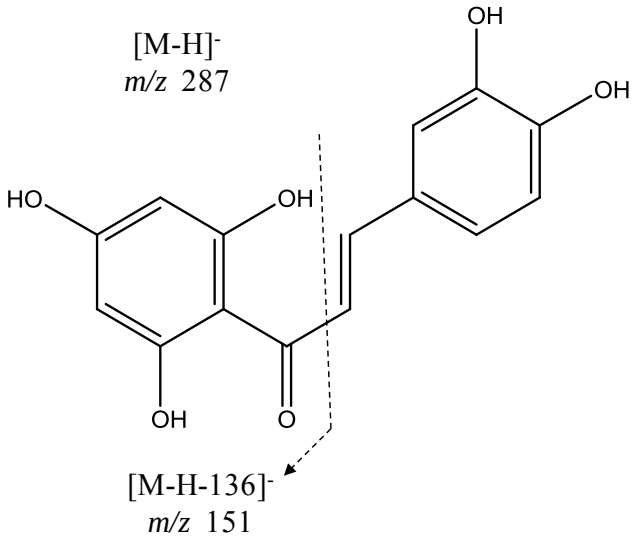
Appendix 32. Fragmentation pathway for methoxyapigeninidin dimer



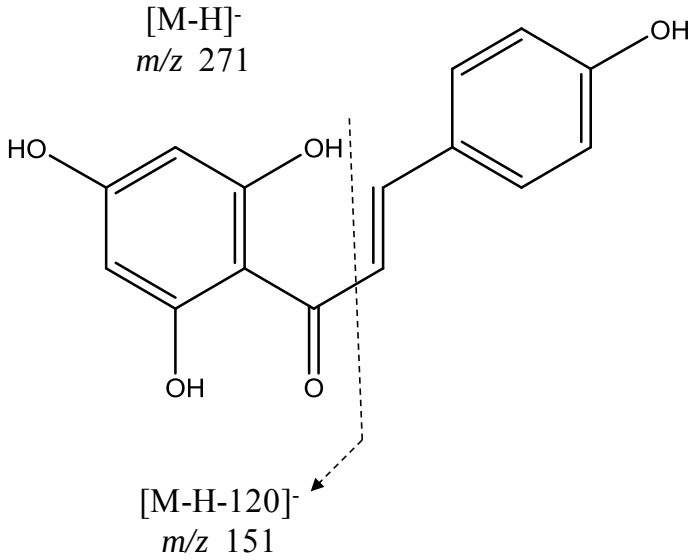
Appendix 33. Fragmentation pathway for apigeninidin dimer



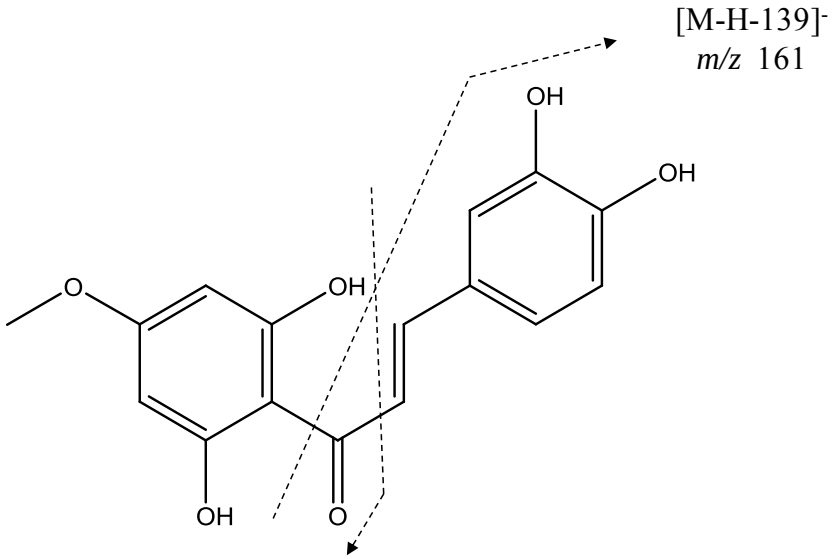
Appendix 34. UV spectra of chalcones: (1) naringenin chalcone and (2) trihydroxymethoxychalcone, identified in sorghum leaves (Tx3362)



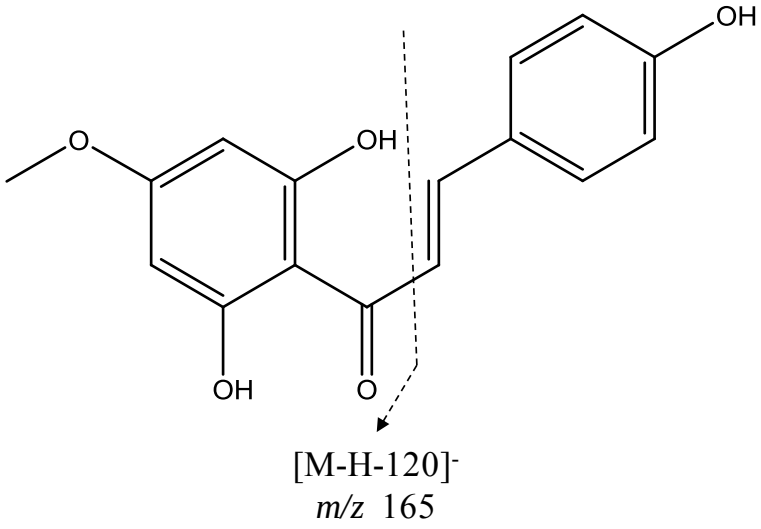
Appendix 35. Fragmentation pathway for eriodictyol chalcone



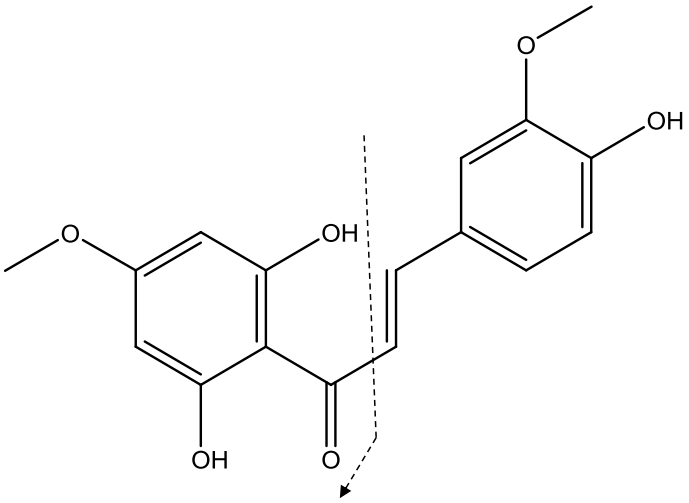
Appendix 36. Fragmentation pathway for naringenin chalcone



Appendix 37. Fragmentation pathway for tetrahydroxymethoxychalcone

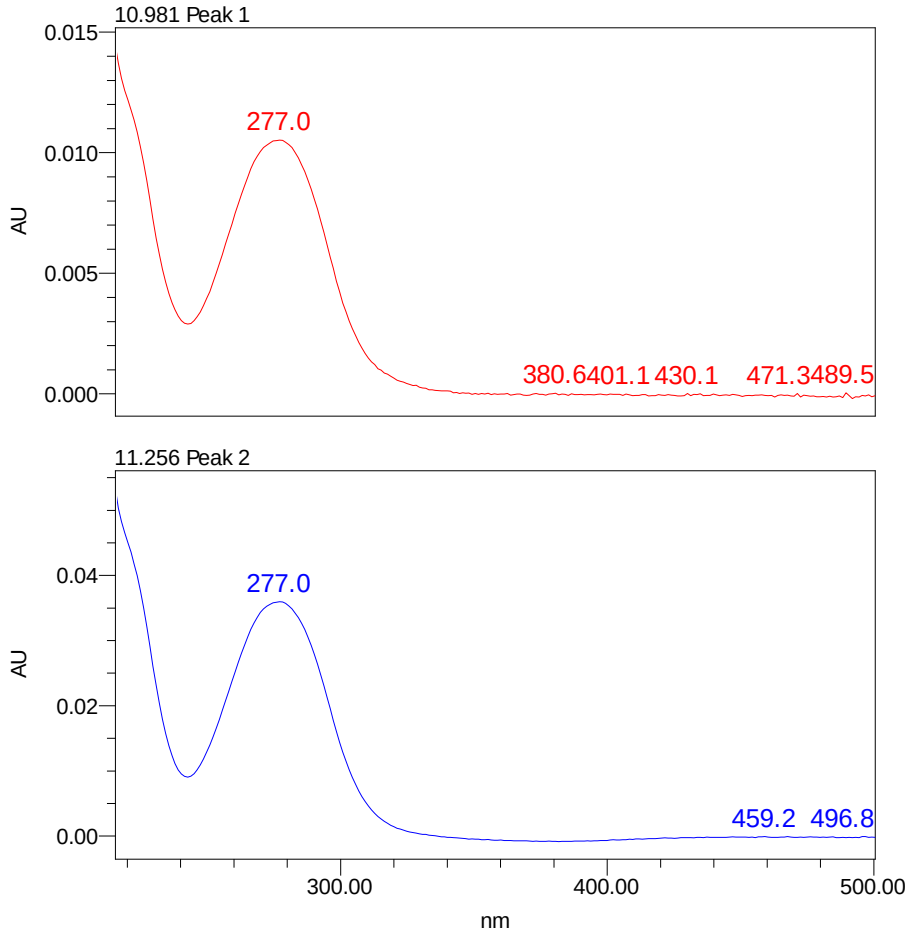


Appendix 38. Fragmentation pathway for trihydroxymethoxychalcone

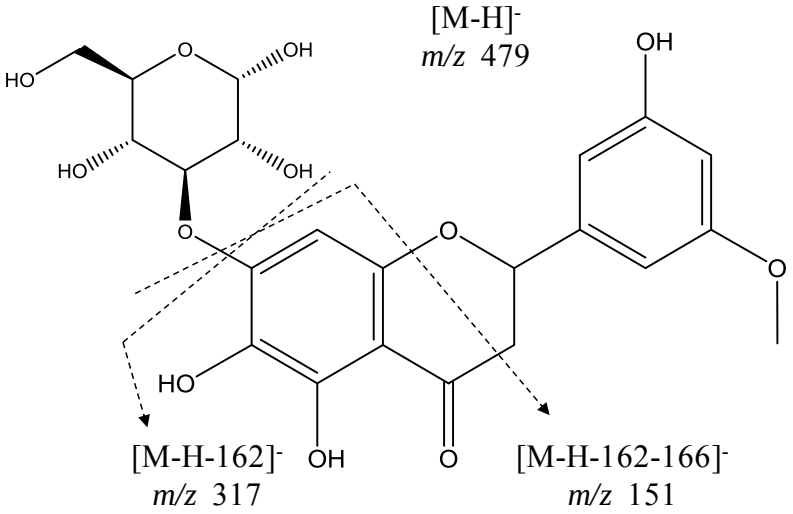


$[M-H-150]^-$
m/z 165

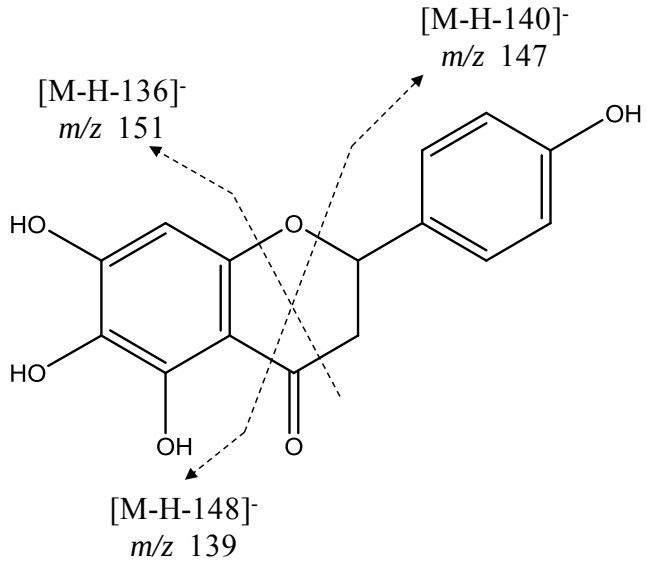
Appendix 39. Fragmentation pathway for trihydroxydimethoxychalcone



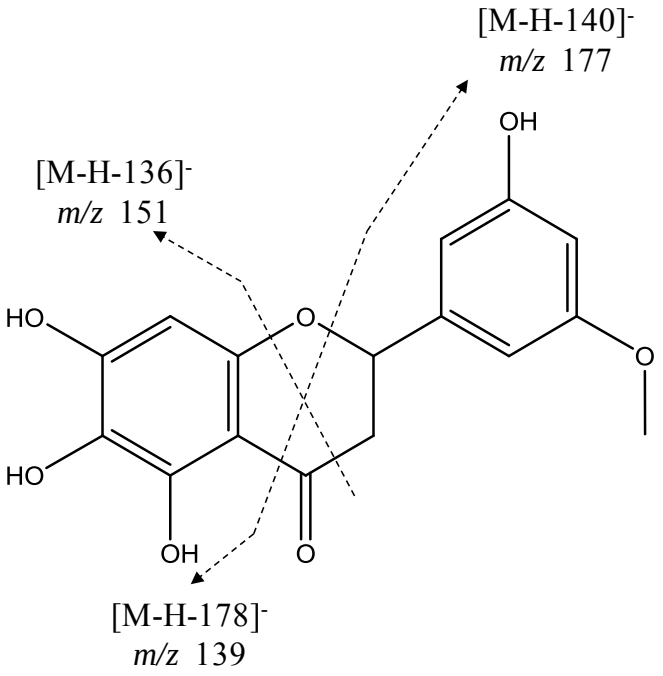
Appendix 40. UV spectra of flavanones (tetrahydroxyflavanones) identified in sorghum leaves



Appendix 41. Fragmentation pathway for trihydroxymethoxyflavanone glucoside



Appendix 42. Fragmentation pathway for tetrahydroxyflavanone



Appendix 43. Fragmentation pathway for tetrahydroxymethoxyflavanone