Biochemical, Molecular and Pharmacological Studies of the Wheat (*Triticum aestivum* L) Flavone, Tricin

Amira Moheb

A Thesis

in the Department

of

Chemistry and Biochemistry

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

at

Concordia University
Montréal, Québec, Canada

June 2012

CONCORDIA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

This is to certify that the thesis prepared

By: **Amira Moheb**
Entitled: **Biochemical, Molecular and Pharmacological Studies of the Wheat *(Triticum aestivum L)* Flavone, Tricin**

and submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY (Chemistry)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

----------Chair
Dr. S. Shaw

----------External Examiner
Dr. Mario Jolicoeur

----------External to Program
Dr. L. Kalman

----------Examiner
Dr. F. Sarhan

----------Examiner
Dr. L. Cucia

----------Thesis Supervisor
Dr. R. Roy

----------Thesis Co-Supervisor
Dr. C. Wilds

Approved by----------
Dr. H. Muchall, Graduate Program Director

June 08, 2012----------
Dr. B. Lewis, Dean
Faculty of Arts and Science

Abstract

Biochemical, Molecular and Pharmacological Studies of the Wheat (*Triticum aestivum L)* Flavone, Tricin

Amira Moheb, Ph.D.
Concordia University, 2012

Tricin (5,7,4'-trihyroxy-3',5'-dimethoxyflavone), a naturally occurring compound, is a characteristic constituent of the grass family, including cereal grain plants, and has been isolated from rice, oat, maize, and wheat. Apart from being a powerful antioxidant, antimutagenic, and anti-inflammatory agent, tricin has been considered as an efficient chemopreventive agent in growth inhibition of human malignant breast tumour cells and colon cancer cells. However, its high commercial price as a pure compound may hinder further experimentation. Wheat is considered one of the main staple foods in Canada and worldwide, and is the most widely adapted crop to abiotic stresses. The main aim of this study is to investigate the effects of abiotic stress factors, such as cold, drought, and salt treatments, among others, on the biosynthesis and accumulation of tricin in different parts of wheat *(Triticum aestivum L)*, with aim of defining an optimum source for tricin production in this important crop. This thesis consists of four research chapters.

The first chapter focuses on an investigation of the phenolic profile of two varieties of wheat (*Triticum aestivum* L*)* leaves grown under normal and cold stress conditions. The leaf 'phenolomes' were established for two varieties: the winter wheat (*Triticum aestivum L.* var Claire) and spring wheat (*Triticum aestivum L.* var, Bounty) using a combination of HPLC-ESI-MS techniques. Phenolic compounds accumulated at a higher level in the Claire than in the Bounty variety, and detected in significant amounts in the apoplast compartment. The accumulation of a mixture of beneficial flavonoids in

cold-acclimated wheat leaves attests to its potential use as an inexpensive supplement of a health-promoting component to the human diet.

The second chapter describes the distribution of tricin in different parts of wheat with the aim to designate a rich source for its utilization. Winter wheat husk was identified as the most valuable part. Its tricin content is considered the highest in any plant materials suggesting the use of winter wheat husk as a good source of tricin. Moreover, the potential anticancer effect of tricin on two cancer cell lines was evaluated where it was revealed to have a selective anticancer effect.

In the third chapter, the selective anticancer effect of several methylated phenolic and flavonoids compounds were tested *in vitro* on cell cultures, using a LDH-spectrophotometer method to assess the viability of the cell lines. Several candidates were found to possess a remarkable antitumor activity on these malignant cell lines, such as trimethyltricetin, a tricin derivative that exhibited a superior selective activity against human adenocarcinomic alveolar basal epithelial cells (A-549).

In the last chapter, the biosynthesis of tricin is discussed. The expression and the enzyme activity of TaOMT2, the enzyme that catalyzes the methylation of tricetin to tricin, were measured at different wheat developmental stages and in response to different abiotic stresses such as cold, salt and drought. The significant accumulation of tricin in the inflorescences suggests that tricin may play a role in protecting the seeds against biotic and abiotic stresses.

Acknowledgements

There are several people I would like to acknowledge for helping and supporting me during this work.

I would like to express my deep appreciation to my supervisor Pr. René Roy for his supervision, advices, and guidance throughout this work; he is not only a great scientist but also a wonderful person that I feel really lucky to know him.

I gratefully acknowledge Pr. Fathey Sarhan for his continuous support, his encouragement and his contributions that have strengthened this study significantly.

I thankfully acknowledge in particular Pr. Ragai Ibrahim, for his assistance and contribution to the phytochemical aspects of this work.

I would like to thank my co-supervisor Pr. Christopher J. Wilds for his effort and his help throughout this work. Many thanks go to my committee member Pr. Louis Cuccia for his time to serve on the committee and his valuable guidance.

Pr. Heidi Muchall for her time and valuable advices throughout my time as a graduate student in the Chemistry and Biochemistry department

Collective and individual acknowledgments are also owed to all my colleagues at the lab of Pr. René Roy, the lab of Pr. Fathey Sarhan, and the lab of Pr. Ragai Ibrahim, where I really enjoyed working with them.

From my deep heart I would like to thank my mother, my husband and my daughter for everything they've done for me, their encouragement, love and support.

Dedication

This thesis is dedicated to my father

MOHEB ELSHAZLY (1945-2011),

Who encouraged me all the way long to have high expectations and to fight hard for what I believe. He always provided me with love, care, and guidance. His confidence in me and proud were unlimited. Gone now but never forgotten, I will miss him always and love him forever. Thanks for all you did.

Contributions of Other Authors to This Work

Dr. René. Roy supervised the thesis methodology and the chemistry part

Dr. Fathey Sarhan supervised the biological aspect of the work

Dr. Ragai Ibrahim reviewed the phytochemical aspects of the work

Dr. Melanie Grondin for her help in designing the viability tests experiments

Francesca Kanapathy was responsible for the enzyme assays and immunoblot experiments in chapter 5

Table of Contents

List of Figures

List of Tables

List of Abbreviations

COX	Cyclo oxygenase enzyme
DAD	Diode array detector
DFR	Dihydroflavonol reductase
ESI	Electrospray ionization
F3H	Flavanone 3-hydroxylase
FACScan	Fluorescence Activator Cells Sorter
FNS	Flavone synthase
FLS	Flavonol synthase
HPLC-MS	High performance liquid chromatography-mass spectrometry
LC	Liquid chromatography
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
OMT	*O*-Methyltransferase
QQQ	Triple-quadruple mass analyzer
RMS	Root mean square
SAM	*S*-Adenosyl-L-methionine
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TIC	Total ion chromatogram
TOF	Time-of-flight mass analyzer
VIS	Visible light

Chapter 1 GENERAL INTRODUCTION

1. Flavonoids

Flavonoids are naturally occurring phenolic compounds that constitute one of the major classes of the plant's natural products. They are low molecular weight secondary metabolites that are widely distributed in plants performing many functions.

Flavonoids are known for their colour as the anthocyanin pigments responsible for the different shades of orange, red and blue, and the yellow pigments (chalcones, flavones and flavonols) in flowers and food (Brouillard and Cheminat, 1988; Timberlake and Henry, 1986). They account for more than 8000 different compounds described in the literature (Passamonti et al., 2009; Ververidis et al., 2007). They existed in nature for over one billion years and they were interacting with evolving organisms for a very long time (Swain, 1975). The chemical diversity, physical, and biochemical properties of flavonoids allow them to interact with different cellular target locations, thus resulting in different biological effects in plants, and other living organisms (Peer and Murphy, 2007; Taylor and Grotewold, 2005).

1.1 **Occurrence, Distribution of Flavonoids**

Flavonoids are ubiquitous in nature. They are found in all plant organs, including leaves, seeds, flowers, fruits, wood, stems, and barks; being more distributed in higher plants and, to a lesser extent, in lower plants (Harborne, 1988). For humans, flavonoids are important diet constituents as they are consumed regularly within the daily food as olive oil, citrus fruits, and greens. Their average intake was estimated to be *ca.* 23 mg/day of mixed flavonoids, mostly as aglycones of the flavone and flavonol classes

(Hertog et al., 1993). Quercetin, a pentahydroxyflavone, was reported as the most consumed flavonoid, for which tea, onions and apples constitute the richest source.

A database of selected dietary flavonoids was recently established by the United States Department of Agriculture (USDA) with the aim of generating a data source that compiled twenty-six of the most commonly occurring flavonoids compounds in foods (Holden et al., 2005).

1.2 **Flavonoid Structures**

Flavonoids consist of a C15 skeleton, spread over two aromatic ring systems, A and B that are connected by a heterocyclic pyran ring C (Fig. 1-1). Based on the oxidation state and substitution pattern of ring C, flavonoid derivatives are classified into several groups. The main classes are the chalcones, flavanones, flavones, flavonols, isoflavones, and anthocyanidins (Fig. 1-2). In flavonoids, the phenyl group is attached at the 2-position of the pyran ring, whereas in isoflavonoids the attachment is at the 3-position.

Flavonoids could be found in nature in the free forms (as aglycones), or as conjugates. They may undergo further substitution through enzymatic reactions , such as the hydroxylation of rings A and B and their *O*-methylation, glycosylation, sulfonation, acylation, and/or prenylation (Ibrahim, 2001). Due to that diversity of transformations, a vast array-of flavonoid structures are found in nature and their number could reach more than 8000 identified derivatives from vascular plants and bryophytes (Andersen, 2006).

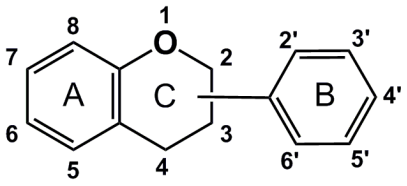


Figure 1-1: Basic structure of flavonoids

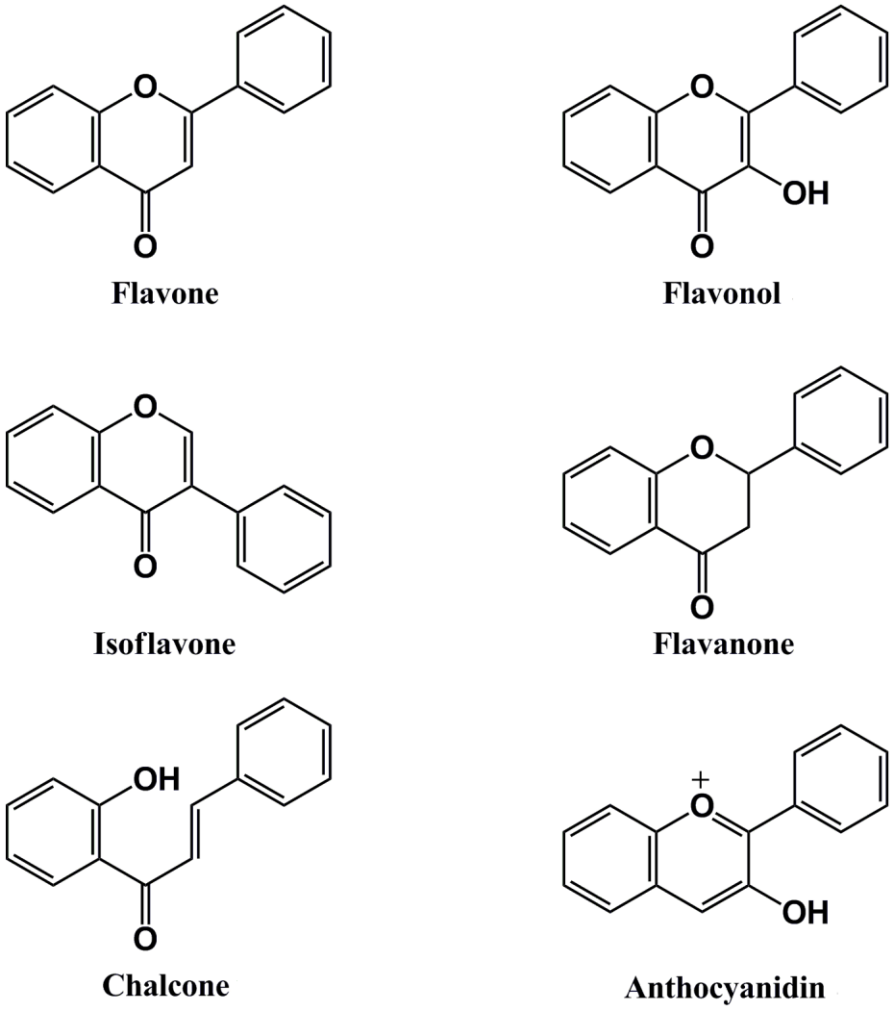


Figure 1-2: Major classes of flavonoids

1.3 **Biosynthesis of Flavonoids**

The use of biochemical, chemical and genetic approaches, resulted in the identification of many pathways involved in the biosynthesis of different classes of flavonoids.

Enzymatic and chemical substitution reactions contribute to the structural and functional diversity of flavonoids. These include: glycosylation, acylation, hydroxylation, methylation and prenylation that take place mostly on the phenolic rings (Ibrahim and Anzellotti, 2003).

The two precursors involved in the flavonoid biosynthesis are 4-coumaroyl CoA and malonyl CoA. The former is derived from the shikimate pathway whereas the latter is formed from acetyl CoA via the acetate pathway. Condensation of both precursors is catalyzed by the enzyme, chalcone synthase (CHS), the first and key enzyme in flavonoid biosynthesis (Winkel-Shirley, 2001); Fig.1-3.

The other enzymes implicated in flavonoid biosynthesis are categorized into several groups: the pre-flavonoid enzymes, such as acetyl-CoA carboxyligase; enzymes involved in the formation of the flavonoid skeleton, such as chalcone synthase (CHS) and stilbene synthase (STS); enzymes of C-ring modification, such as flavone synthase (FS) and flavanone 3-hydroxylase (F3H) and the enzymes catalyzing flavonoid substitution, such as the *O*-methyltransferases (OMTs), glycosyltransferases, among others (Ibrahim, 2001). An excellent review of the biosynthesis and enzymology of flavonoids appeared in the 'Comprehensive Natural Products Chemistry Series'(Forkmann and Heller, 1999).

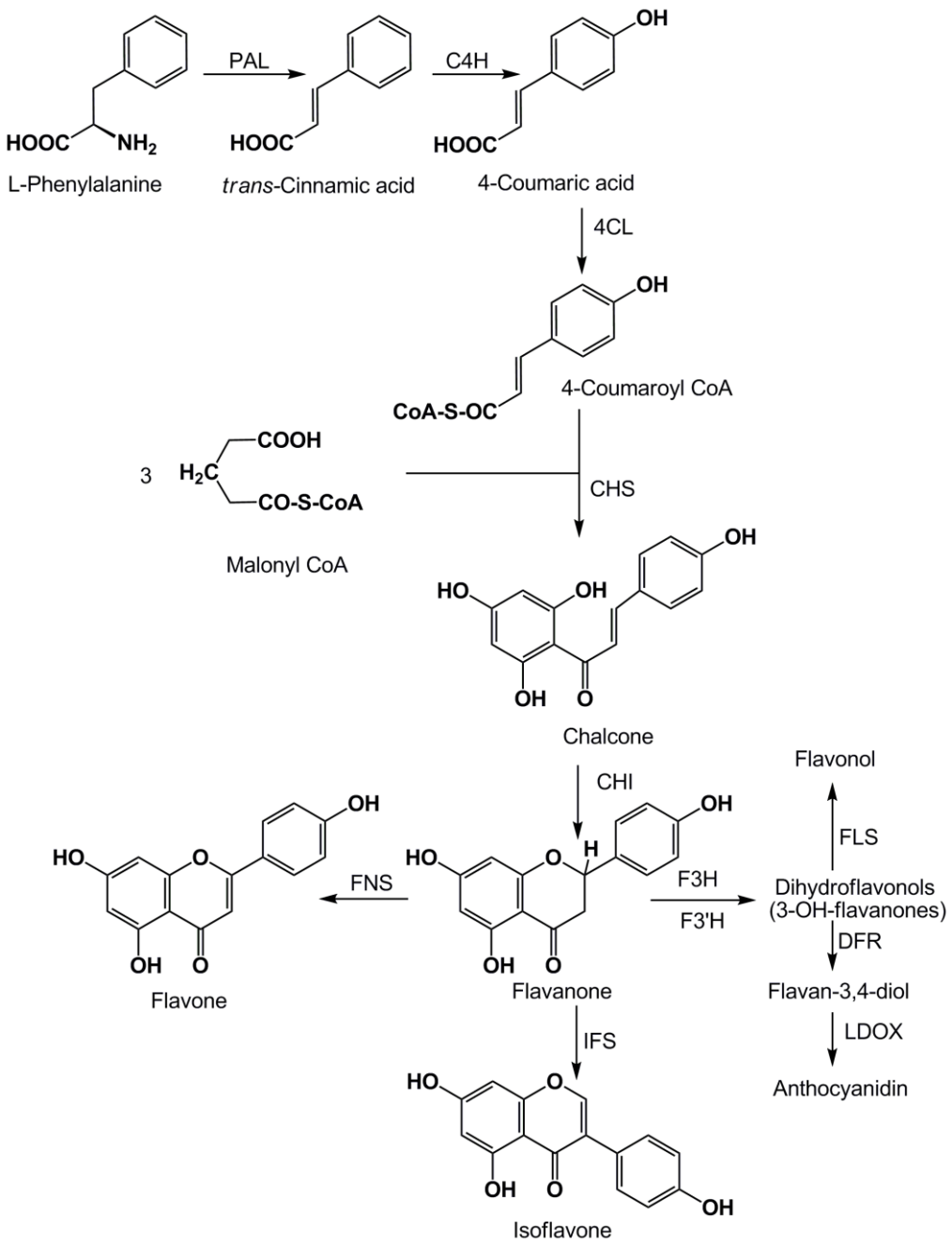


Figure 1-3: Major pathways of flavonoid biosynthesis

*Legends: Phenylalanine ammonia-lyase (**PAL**), cinnamate-4-hydroxylase (**C4H**), 4-coumaroyl:CoA-ligase (**4CL**), chalcone synthase (**CHS**), chalcone isomerase (**CHI**), flavone synthase (**FNS**), flavanone 3-hydroxylase (**F3H**), flavonoid 3' hydroxylase (**F3'H**), flavonol synthase (**FLS**), dihydroflavonol 4-reductase (**DFR**), leucoanthocyanidin dioxygenase (**LDOX**), isoflavone synthase (**IFS**).*

1.4 *O*-Methyltransferases (OMTs)

Enzymatic *O*-methylation, which is catalyzed by a large family of *O*-methyltransferases (OMTs) plays an important role in reducing the toxicity of flavonoids by decreasing the chemical reactivity of their phenolic hydroxyl groups and increasing their lipophilicity; and hence modulates their antimicrobial activity (Middleton and Kandaswami, 1994 and refs therein). Moreover, *O*-methylation of flavonoids plays an important role as signalling molecules in rhizobium–legume symbiotic interactions (Long, 2001), as antiviral agents (Cushnie and Lamb, 2005), and in the reduction of flavonoid mutagenicity in animals (Zhu et al., 1994).

OMTs are both substrate- and regio-specific enzymes (Ibrahim et al., 2000), and are *S*-Adenosyl-L-methionine (SAM)-dependant. They catalyze the transfer of the methyl group of SAM to an appropriate methyl acceptor molecule with the concomitant formation of the corresponding methyl ether derivative and S-Adenosyl-L-homocysteine (SAH), as products.

1.5 Importance of Flavonoids

Functions of flavonoids in plants

The flavonoids play several important roles in the plant's growth and development, and its interaction with the environment. It serves as the flower and fruit's main pigments, protecting the plants against UV radiation and attracting pollinators (Bohm, 1998 and refs therein). They could also inhibit organisms that cause plant diseases eg. *Fusarium oxysporum* (Galeotti et al., 2008), and with interaction with microflora of the rhizosphere such as mycorrhizal fungi.

Flavonoids were reported to accumulate in the progenitor cells for different legume organs (Mathesius et al., 1998; Morris and Djordjevic, 2006) and to have a marked effect on the development of *in vitro* root formation (Imin et al., 2007). This is in addition to their important role in root nodule organogenesis (Wasson et al., 2006; Zhang et al., 2009), and their vital function in the legume-bacterium symbiosis, as they were identified to be responsible for activating nodulation genes in nitrogen fixing bacteria (Rhizobia) (Peters et al., 1986; Redmond et al., 1986). This bacterium was able to sense the flavonoids secreted by legumes such as peas, beans, clover, and soy which triggers the secretion of Nod factors.

They also affect the transport of the plant hormone, auxin (Peer and Murphy, 2007) and can alter the levels of reactive oxygen species (ROS) within the plant (Taylor and Grotewold, 2005).

Potential effects of flavonoids on human health

For humans, apart from being an important diet constituent, flavonoids are found as active ingredients in several medicinal plants where many therapeutic benefits are identified. Flavonoids are present in many natural products preparations, and constitute a major component in such products as seen with the drug profile of *Ginkgo biloba* (Kleijnen and Knipschild, 1992). They possess potential anti-oxidant, anti-inflammatory and pro-apoptotic activities (García-Mediavilla et al., 2007; Taylor and Grotewold, 2005; Williams et al., 2004).

Their functional roles were established as antioxidants, radical scavengers, antiviral and anti-inflammatory agents (Middleton et al., 2000 and references there in).

Recently, a current view of how phytonutrients impact cell signaling as antioxidant was proposed (Fig. 1-4). They were reported to interact through mechanisms independent of their antioxidant properties, by directly affecting the activities of a wide spectrum of cellular targets, including key enzymes and membrane and nuclear receptors (Martin et al., 2011; Virgili and Marino, 2008).

Flavonoids are considered good candidates for combating many forms of cancer as they offer chemopreventive shielding effects. Several studies confirmed their *in vitro* ability to inhibit cell growth and kill some cancer cell lines (Jacquemin et al., 2010; Li-Weber, 2009; Neto, 2007) and refs there in). It was suggested that the health-promoting action of flavonoids may be attributed to their interactions with key enzymes, and signaling cascades that involve cytokines and transcription factors, and/or antioxidant systems (Polya, 2003).

Methoxylated flavones represent a superior anti-cancer flavonoid subclass due to their lipophilicity, resulting in easy access to the target cells and high bioavailability (Deng et al., 2006; Walle, 2007; Walle et al., 2007).

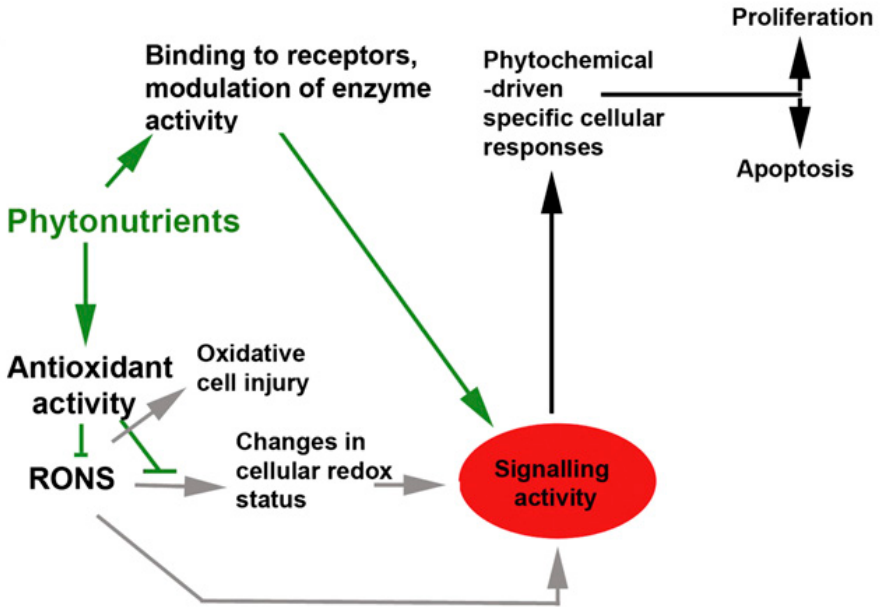


Figure 1-4: The current view of how phytonutrients impact cell signaling

(Figure from (Virgili and Marino, 2008)). Reprinted with permission from Elsevier Ltd. (RONS): reactive oxygen and nitrogen species

1.6 **Bioavailability of Flavonoids**

Bioavailability refers to the proportion of a substance that could reach, in its native form, the blood and the systemic circulation (Ververidis et al., 2007).

The control of the intestinal membrane transport of flavonoids occurs mainly at the cell plasma membrane level. Transporters that promote nutrient absorption are unable to transport flavonoids. The latter are mainly absorbed via the same specific efflux transporters responsible to pump drugs and xenobiotics out of cells (Passamonti et al., 2009).

2. Tricin

5,7,4'-Trihydroxy-3',5'-dimethoxyflavone, tricin (Fig. 1-5), is a naturally occurring flavone of relatively rare and sporadic occurrence. It is mainly found in cereal grain plants, such as rice, oat, maize, barley and wheat. Several studies have revealed the potential importance of this lipophilic flavone in cancer treatment and prevention. It is also considered safe enough for clinical studies (Verschoyle et al., 2006).

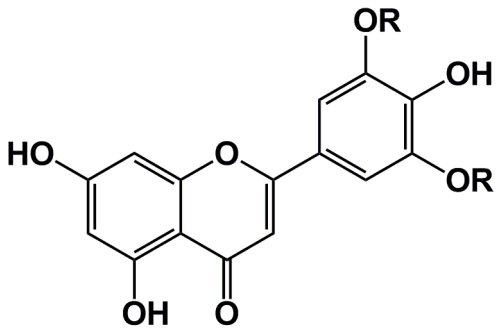


Figure 1-5: Tricin and tricetin structure [R=H, tricetin; R=Me, tricin]

2.1 **Natural Occurrence**

Tricin was first isolated as a free aglycone from a rust-resistant variety of wheat (*Triticum dicoccum L*. var. Khapli) leaves (Anderson and Perkin, 1931). It was later identified as the first flavone detected in butterfly wings feeding on grasses (Harborne, 1967). It was also reported as a typical flavone in grasses and cereals (Harborne and Hall, 1964; Harborne and Williams, 1976). The distribution of tricin and its non-methylated analogue, tricetin (Fig. 1-5) in plants was recently reviewed (Wollenweber and Dorr, 2008). They reported the presence of these compounds in several plant species belonging to unrelated families. For instance the Japanese barnyard millet (*Echinochloa utilis*)

(Watanabe, 1999), Fenugreek (*Trigonella foenumgraecum L.)* (Shang et al., 1998), Alfalfa (*Medicago sativa L*.) (Stochmal et al., 2001), the stem of *Sorghum bicolor* (Kwon and Kim, 2003), *Sasa borealis* (Poaceae) (Park et al., 2007), sugar cane (*Saccharum officinarum L.,* Gramineae) (Colombo et al., 2006), bamboo leaves (*Phyllostachys nigra*) (Jiao et al., 2007), the herb of *Lygodium japonicum* (Zhang et al., 2008), and *(Phoenix hanceana*, Palmae) (Lin et al., 2009), among others.

2.2 **Biological Significance of Tricin**

In general, flavones were reported to possess higher fungicidal activity than flavanones against 34 different fungal species known for their damaging effect to stored seeds (Weidenbörner and Jha, 1997), as well as their superior inhibitory activity against mycelial growth of the plant pathogen *Verticillium albo-atrum* at 1 ppm minimum inhibitory concentration (MIC) (Picman et al., 1995).

Tricin is classified as an example of the flavones subgroup. Moreover, being a methylated molecule enhances its antibacterial activity among this group of flavones (Laks and Pruner, 1989).

Furthermore, tricin was reported to inhibit the growth of both weeds and fungal pathogens in rice, without affecting rice growth. Its large amount found in the soil is probably due to its release from rice root exudation, leaves leaching or seed husk germination. Its accumulation in the soil suggests its possible function as an allelochemical, a natural plant biopesticide, or in protecting rice against pathogens and weeds (Kong et al., 2004). The potential herbicidal activity of tricin, found in the hulls of rice (*Oriza Sativa*) was previously reported (Chung et al., 2005), as well as its an anti-

feedant activity against the boll weevil, *Anthonomus grandis* (Miles et al., 1993) and its feeding deterrent activity in wheat against two aphid species, *Schizaphis graminum* and *Myzus persicae* (Dreyer and Jones, 1981).

Tricin was reported to be implicated in plant-insect interactions in rice; it exhibits antifeedant effect on the nymphae of brown planthopper (BPH), *Nilaparvata* lugens, and antifeedant and oviposition deterrent effects for BPH adults after being allowed to feed on diets containing tricin for 15 days (Bing et al., 2007).

The latter report defined tricin, from ecology point of view, as "one stone killing many birds", due to its value in different steps of rice cultivation such as breeding, cultivation of novel rice varieties and biological engineering technology.

Recently, in rice hulls, tricin and its synthetic aurone isomer, 5,7,4'-trihydroxy-3',5'-dimethoxyaurone, were found to possess a significant fungicidal activity against rice seedling rot disease. Disease incidence was significantly reduced by soil amended with rice hulls. However, aurone itself was more effective than tricin, thus making it an ideal fungicidal compound (Kong et al., 2010).

2.3 **Isolation and Determination of Tricin**

Isolation and separation from different plants

In most cases the EtOAc fraction of the MeOH extract was employed for tricin isolation from different plants, followed by different methods of fractionation and purification. Tricin was isolated from the EtOH extract of the Japanese barnyard millet (*Echinochloa utilis*) grains by Sephadex LH-20 and preparative high-performance liquid

chromatography (Watanabe, 1999), and from the EtOAc and n-BuOH soluble fractions of the aerial parts of *Setaria viridis* (Kwon et al., 2002).

Another method utilized the EtOAc fraction and silica gel column chromatography to produce tricin from freeze-dried foliar parts of rice *(Oriza Sativa*) plants (Kong et al., 2004), while Chung et al., 2005 used the dried hulls of *Oriza Sativa* after soaking it in MeOH for one week, then followed by fractionation of the EtOAc fraction to yield tricin.

Sugar cane (*Saccharum officinarum L*.) juice yielded tricin via successive chromatographical techniques starting by Amberlite XAD-2 resin column chromatography (CC) followed by a Polyamide CC then preparative paper chromatography (Maurício Duarte-Almeida et al., 2006). Tricin was also isolated from the EtOAc soluble fraction of the whole plants of *Sasa borealis* (Gramineae) (Jeong et al., 2007), and of the aerial plant parts from "ear pregnant" stage of resistant rice plant (IR36) (Bing et al., 2007).

Two other derivatives, tricin-4'-*O*-β-D-glucopyranoside and tricin-5-*O*-β-D glucopyranoside , were isolated from hot-water extracts of the leaves of *Sasa kurilensis* after being separated on a Sephadex LH-20 column followed by ODS, HPLC (Hasegawa et al., 2008). Another derivative, tricin 7-O-*β*-D-glucoside was isolated from the leaves of *Ginkgo biloba* (Jun-Ping and Ling-Li, 2008).

Recently, a group of tricin-type flavonolignans and tricin were isolated and characterized from the EtOAc extract of *Calamus quiquesetinervius* (Chang et al., 2010) after using diverse chromatographic techniques.

Methods used for determination of tricin:

Methods for the determination of tricin and estimation of its quantity *in vivo* were mainly carried out by the group of Cai, where a specific and simple high-performance liquid chromatographic (HPLC) method was developed for the determination of tricin with UV–visible detection in human plasma (Cai et al., 2003), and in the plasma and tissues of mice (Cai et al., 2005b).

2.4 **Pharmacological Activities of Tricin**

Chemoprevention

The term 'chemoprevention' entails protection of humans against cancer by various chemical, biological, or nutritional interventions, *via* disrupting oncogenesis in order to avoid or delay the incidence of cancer (Kapetanovic, 2009). In general, three important prerequisites are required to consider flavonoids for clinical evaluation as cancer chemopreventive agents: good efficacy, lack of toxicity and reasonable bioavailability; all of which qualify tricin as a prime candidate in chemoprevention.

In Asia, where rice is the main staple food, the incidence of breast and colon cancer is markedly below that in the western world; this was attributed to the presence of the chemopreventive agent tricin in the rice bran (Hudson et al., 2000). Tricin is considered a potential candidate in chemoprevention of colon or breast cancer. Its effect was studied on cell viability and colony-forming ability (clonogenicity) of human-derived tumour breast cell lines (MDA MB 468) and human-derived colon carcinoma cell lines (SW 480). Tricin-containing extracts from brown rice inhibited the proliferation of human colon and breast cancer cells in vitro (Hudson et al., 2000). Moreover, when the

IC_{50} (inhibition of clonogenicity) of tricin was compared with that of the two anticlonogenic compounds, caffeic and protocatechuic acids, it appeared that tricin was the most potent anticlonogenic agent with cells of either breast or colon tissue origin (Hudson et al., 2000).

Further studies revealed that tricin was capable of arresting nude-mouse MDA-MB-468 tumour cells in the G2/M phase of the cell cycle, without inducing apoptosis (Cai et al., 2004). The latter study also demonstrated that tricin-supplemented diet (0.2%, $w.w^{-1}$) administered one-week prior to MDA-MB-468 cell implantation, resulted in failure to impede tumour development (Cai et al., 2004). The fact that dietary tricin was found in the mouse intestine in concentration greater than that in the plasma or liver, suggested that tricin may have a vital role in intestine by affecting and possibly inhibiting colon carcinogenesis (Cai et al., 2007).

In addition, tricin was reported to inhibit the cyclooxygenase enzymes and interfere with intestinal carcinogenesis in mice. These findings resulted in a recommendation for further preclinical trials aimed at exploring its suitability for trials in humans with intestinal polyps (Cai et al., 2005a)

Additionally, it was found that tricin, isolated from the whole plants of *Sasa borealis* (Gramineae), inhibits P-glycoprotein activity in adriamycin- resistant human breast cancer cells, delays spontaneous mammary tumorigenesis and suppressing apoptosis induced by oxidative stress (Jeong et al., 2007).

Dietary tricin effectively suppressed azoxymethane (AOM)/dextran sodium sulphate (DSS) - induced colon carcinogenesis in mouse model (Oyama et al., 2009), where the

development of colonic adenomas and adenocarcinomas was significantly reduced by feeding with 50 and 250 ppm tricin, respectively (Fig 1-6).

Recently, the *in vitro* effect of tricin on hepatic stellate cells (HSCs) suggested that tricin might be beneficial in therapeutic or chemopreventive applications for hepatic fibrosis. It works by blocking tyrosine phosphorylation of platelet-derived growth factor (PDGF) receptor and its signalling pathways (Seki et al., 2012).

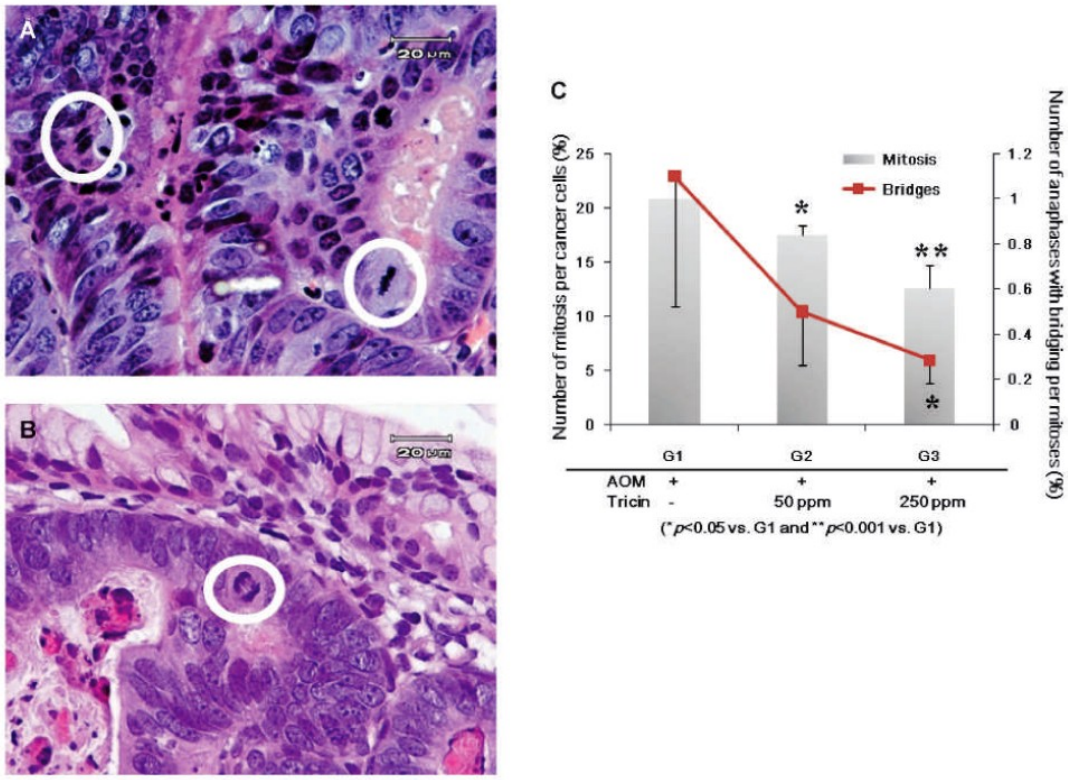


Figure 1-6: The effects of dietary tricin on the MI and ABI.

(Figure from (Oyama et al., 2009) "Annual Copyright License from Copyright Clearance Center"

A, representative mitotic figures (left circle, anaphase; right circle, metaphase) in an adenocarcinoma, (B) representative anaphase bridging (circle) in an adenocarcinoma, and (C) MI (columns) and ABI (lines). Dietary administration of tricin significantly reduced the MI (50 ppm tricin, $P < 0.05$; and 250 ppm tricin, $P < 0.001$) and ABI (250 ppm tricin, $P < 0.05$). G1, group1; G2, group2; and G3, group 3.

Cardiovascular Activity

Two dihydrotricin derivatives isolated from the stems of *Calamus quiquesetinervius* namely calquiquelignan A and dihydrotricin exhibited cardiovascular protective effect as they showed significant vasodilatory potencies, as indicated by 60.3% and 80.3% relaxations (Chang et al., 2010). The vasorelaxation assays were carried out *in vitro* on thoracic aorta sections of adult male Sprague–Dawley rats.

Antihistaminic Activity

Tricin, that was isolated from Malagasy plant (*Agelaea pentagyna),* showed a potent inhibitory activity toward exocytosis from antigen-stimulated rat leukemia basophiles (Kuwabara et al., 2003).

Antiviral activity

Tricin and tricin 7-*O*-*β*-D-glucopyranoside proved to possess inhibitory activity against hepatitis B virus (HBV) replication (Li et al., 2005).

Tricin showed to have a potential *in vitro* anti-human cytomegalovirus activity. The activity against human cytomegalovirus (HCMV) was detected in the hot water extract of *Sasa albo-marginata*, and in the isolated tricin as well. Western blot analysis demonstrated that the extract decreased the expression of IE antigen and late antigen of HCMV in the infected cells (Sakai et al., 2008).

Very recently, tricin was identified to have potential anti-influenza virus activity *in vitro* and *in vivo*, as it ameliorates body weight loss and survival rate of influenza-A-virus-infected mice. It significantly reduced seasonal A (H1N1), (H3N2) viruses, novel A (H1N1pdm) virus, as well as B virus in a dose-dependent manner (Yazawa K et al., 2011).

Antioxidant activity

Tricin was isolated and identified in the grains of Japanese Barnyard Millet (*Echinochloa utilis*), where it showed an antioxidative activity along with other phenolic compounds identified in the extract (Watanabe, 1999).

Likewise, tricin isolated from the stem of Sorghum bicolor revealed a strong free radical scavenging activity along with an anti-lipid peroxidation activity (Kwon and Kim, 2003).

The phenolic extract from sugar cane (*Saccharum officinarum L.*) juice containing a glycosylated derivative of tricin as one of the major constituents (10% of the total polyphenolic content), showed protective effect against *in vivo* MeHgCl intoxication and potent inhibition of lipoperoxidation of rat brain homogenates, suggesting its potential use for beneficial health effects and/or therapeutic applications (Maurício Duarte-Almeida et al., 2006).

Two other glycosylated derivatives of tricin; tricin-4′-*O*-β-D-glucopyranoside and tricin-5-*O*-β-D-glucopyranoside, were isolated from hot-water extracts of the leaves of *Sasa kurilensi*s, however they didn't exhibit any free radical scavenging activity (Hasegawa et al., 2008).

Another tricin derivative (tricin-7-*O*-*B*-(6"-methoxycinnamic)-glucoside) that was isolated from sugar cane juice (Duarte-Almeida et al., 2007) was shown to have antioxidant activity higher than Trolox$^{@}$ (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid, positive control) and an *in vitro* antiproliferative activity against several human cancer cell lines, especially those of the breast resistant NIC/ADR line.

Furthermore, the antiradical activity of tricin was shown in the extract of DRB (defatted rice bran).The study demonstrated that the various phytochemical constituents of DRB extracts including tricin exhibited excellent superoxide radical scavenging activity and thus directly supporting the superior antiradical efficacies of DRB extracts (Renuka Devi and Arumughan, 2007).

Tricin 7-O-*β*-D-glucoside isolated from the leaves of *Ginkgo biloba* showed *in vitro* antioxidant activities as it was examined for its scavenging activity on superoxide anion and its inhibitory effect on rats' polymorphonuclear neutrophil (PMN) respiratory burst by chemiluminescence (Jun-Ping and Ling-Li, 2008).

Recently, a group of tricin derivatives (calquiquelignan A–B, dihydrotricin and tricin) exhibited more potent hydroxyl radical ($^{\bullet}$OH) scavenging activity than trolox as characterized by the ultra- weak chemiluminescence assay (Chang et al., 2010).

2.5 **Pharmacokinetic and Bioavailability**

The two methoxyl groups present on the B-ring of tricin appear to be responsible for its lipophilicity that seems to play the key role in its biological activity, cellular uptake and its *in vivo* stability, especially in the intestine and colon. Together with the presence of three phenolic hydroxyl groups that catalyze the antioxidant activity of this molecule (Jiao et al., 2007). It is due to this dual characteristic that tricin possess a pharmacokinetic advantage *in vivo*, when consumed with the diet in mice, over its non-methylated analogue, apigenin, that exhibit a very rapid metabolism (Cai et al., 2007). Differences in their glucuronidation may account for their deferential availability, tricin seems to be more available than apigenin in blood and tissues

Exploration of the relationship between systemic and tissue concentration and cancer chemopreventive efficacy of tricin in animals for pre-clinical investigation purpose was conducted in mouse plasma, liver and small intestinal mucosa (Cai et al., 2005b; Cai et al., 2003).

Another *in vivo* study tested the bioavailability of tricin through its effect on the development of tumours grown in immune-compromised MF-1 mice (Cai et al., 2004) where it was confirmed to prevent colorectal carcinogenesis, by virtue of its high levels in the gastrointestinal tract after dietary intake. In nude mice consumption of tricin with the diet (0.2%, w w-1) from 1 week prior to MDA-MB-468 cell implantation failed to impede tumour development. Even though, the considerable *in vitro* growth-inhibitory potency of tricin in MDA-MB-468 breast cancer cells; its systemic bioavailability in plasma after dietary intake was low.

These results suggest that the potent breast tumor cell growth-inhibitory activity of tricin, *in vitro,* does not directly translate into activity in the nude mouse bearing the MDA MB-468 tumor**,** which implied optimization of the formulation of tricin as a suitable pharmaceutical prodrug development to increase its bioavailability for breast cancer therapy. The fact that tricin levels were relatively higher in liver and gastrointestinal tract than that measured in the plasma, suggests that it would be advisable to study the effect of dietary tricin on the prevention of hepatic and gastrointestinal malignancies in rodents.

A recent study to improve the bioavailability of tricin was conducted to synthesize tricin-amino acid derivative as a new tricin prodrug.The tricin-alanine-glutamic acid conjugate (Fig 1-7), exhibited enhanced permeability, stability in (Madin-Darby canine

kidney) MDCK cells *in vitro*, and excellent bioavailability after oral administration in Crl:CD (SD) male rats (Ninomiya et al., 2011)

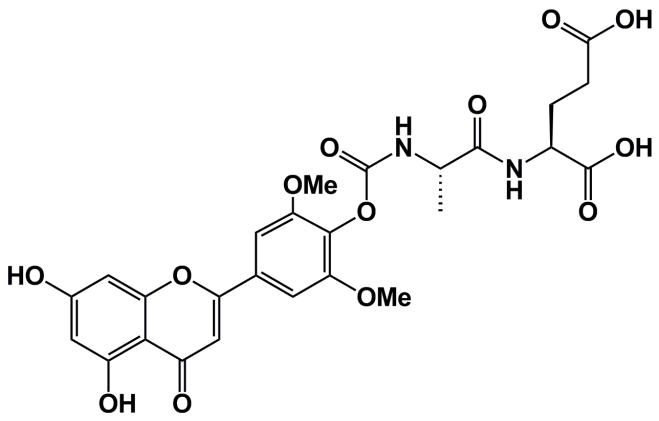
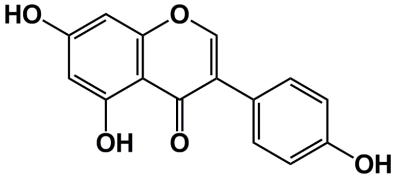


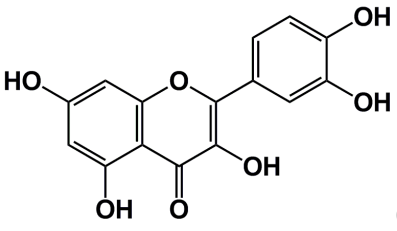
Figure 1-7: The tricin-alanine-glutamic acid conjugate

2.6 **Safety of Tricin**

The safety of tricin was assessed based on experiments carried out by (Verschoyle et al., 2006). It was suggested that tricin may be considered safe enough for clinical development as a cancer chemopreventive agent. It was compared with two other potential chemopreventive poly-hydroxylated flavonoids genistein (Fig. 1-8a) and quercetin (Figure 1-8b), whose adverse effects of mutagenicity compromised their clinical usefulness.



(a)



(b)

Figure 1-8: (a) Genistein, (b) Quercetin

Tricin didn't cause any pathological or morphological changes in liver, lung, heart, spleen, kidney, adrenal gland, pancreas or thymus tissues studied in mice receiving tricin, by oral route for five consecutive days. Tricin failed to cause MLL gene breakage in human leukaemia, and it didn't inhibit human topoisomerase II at concentration of 10, 50 or100 μM). Moreover, tricin lacked any genotoxic properties, as no mutagenicity or ability to chromosomal change was observed in the *Salmonella/Escherichia coli* assay and no clastogenicity in Chinese hamster ovary cells as tested by chromosomal aberrations. Accordingly, tricin was favored for its safety profile, as a suitable candidate for further clinical applications as a potent and safe chemopreventive agent.

2.7 **Tricin Biosynthesis**

The biosynthesis of tricin proceeds as described earlier (see section 1.1 and Fig. 1-3), involving the stepwise reaction of malonyl CoA and *p*-coumaroyl CoA, then the sequential action of chalcone synthase ***(CHS),*** and chalcone isomerase ***(CHI),*** to yield naringenin chalcone following by the flavanone, naringenin, respectively.

Subsequently, B-ring hydroxylation takes place via *flavonoid 3'5' hydroxylase* ***(F3'5'H)*** then, the action of flavone synthase ***(FNS)*** that introduces a double bond between C-2 and C-3, and gives rise to tricetin (Fig. 1-9).

The last step in tricin biosynthesis involves the stepwise *O*-methylation of tricetin to its 3'-monomethyl-(selgin) and 3',5'-dimethyl-(tricin), with small amounts of 3',4',5'-trimethyl ether derivatives (Fig1-10). These *O*-methylations are catalyzed by a flavone *O*-methyltransferase (TaOMT2) representing a single gene product. The latter was

previously cloned and characterized from wheat, *Triticum aestivum L.* (Zhou et al., 2006).

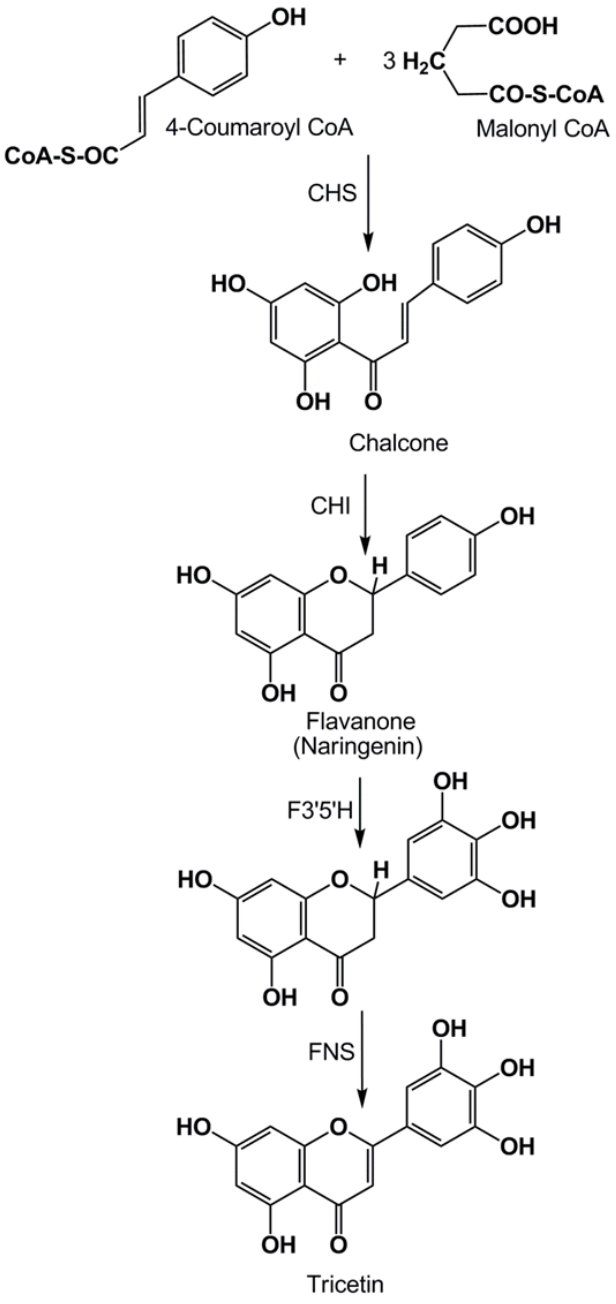


Figure 1-9-: Proposed pathways for tricetin biosynthesis

*Legends: chalcone synthase (**CHS**), chalcone isomerase (**CHI**), flavonoid 3'5' hydroxylase (**F3'5'H**), flavone synthase (**FNS**).*

2.8 **TaOMT2**

A full-length flavonoid OMT cDNA clone (*TaOMT2*) was first isolated and characterized from a wheat leaf cDNA library (Zhou et al., 2006). Its novel gene product catalyzes three sequential *O*-methylations of the flavone tricetin to its 3′-mono→3′,5′-di-→3′,4′,5′-trimethyl ether derivatives (Fig 1-10). The recombinant protein was affinity-purified to near homogeneity and tested against several potential methyl acceptor molecules substrates. Tricetin was found to be the preferred substrate and tricin the predominant product.

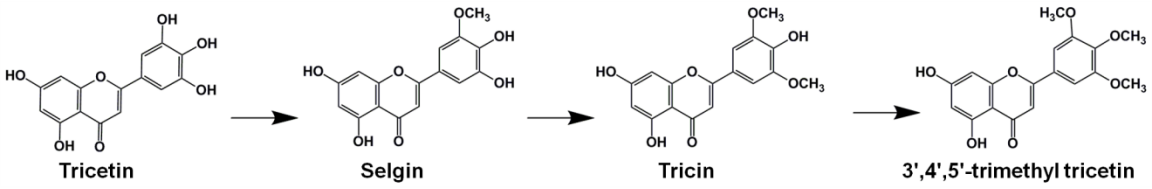


Figure 1-10: *O*-methylation of tricetin by TaOMT2

In plants, (OMTs) catalyze single methylation in a stepwise manner with *O*-methylation at position 3 being the first step in the process (Ibrahim et al., 2003). Sequential methylation is not really common. However, many cases demonstrate that the sequential synthesis in *Chrysosplenium americanum* (Saxifragaceae) of polymethylated flavonols are catalyzed, in a stepwise manner, from 3-methylquercetin (3-MeQ) to 3,7-MeQ to 3,7,4'-MeQ by a number of substrate-specific and position-oriented OMTs (Ibrahim et al., 1987). Another example for the sequential methylation was reported for the volatile phenolic derivatives in rose petals (Lavid et al., 2002).

Examples of multiple methylations catalyzed by single enzymes were also reported for the mammalian phosphatidylethanolamine (Walkey et al., 1996), plant phosphoethanolamine (Charron et al., 2002) and viral histone *N*-methyltransferases (Qian et al., 2006). The originality of TaOMT2 lies in the fact that it is capable of catalyzing a sequence of three methylations of tricetin resulting in three different products.

2.9 **Organic Synthesis of Tricin**

Tricin is very expensive, and it can only be isolated in small amounts from plant material at a relatively high cost. Although a number of methods are available for the synthesis of flavones in general, they are not ideal for the preparation of A-ring hydroxylated flavones due to derivatization of the phenolic groups of the intermediate esters or ethers. Only a few studies were reported for the synthesis of tricin. Of these, the target flavone was obtained using the lithium polyanions of trihydroxylated acetophenone (Nagarathnam and Cushman, 1991) (Fig 1-11), thus avoiding the laborious purification steps leading to its crystallization.

Another method involves direct condensation reaction of 2,4,6-trihydroxyacetophenone and 4-hydroxy-3,5-dimethoxybenzaldehyde to the corresponding flavanone, followed by dehydrogenation with iodine and NaOAc (Xiao-Hong et al., 1999) (Fig 1-12). However, this latter article did not include information on the steps or the conditions of synthesis. In fact, it was mainly based on a former study reporting the synthesis of polyhydroxyflavanones from hydroxyacetophenones and hydroxybenzaldehydes (Chan et al., 1996).

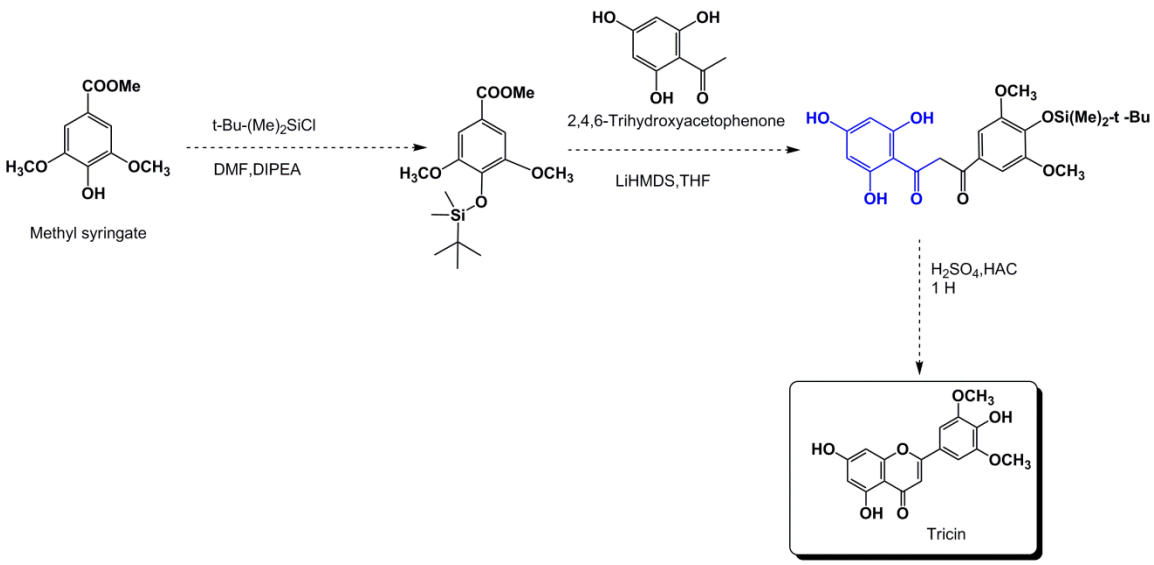


Figure 1-11: Organic synthesis of tricin using the lithium polyanions

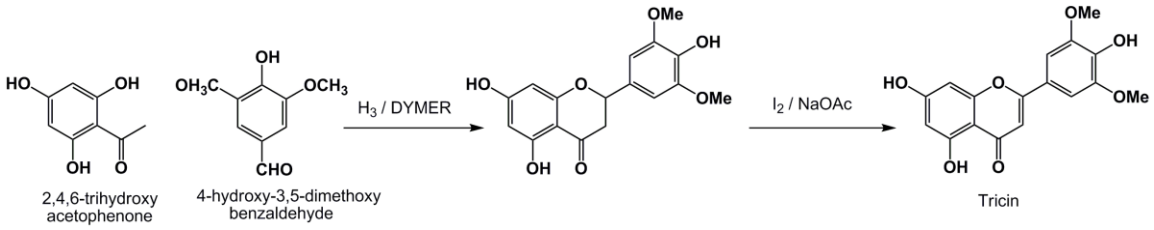


Figure 1-12: Synthesis of tricin via flavanones pathway

3. Wheat

Wheat is considered one of the most important crops in the world. According to the UN Food and Agriculture Organization (**FAO*)***, Canada is ranked sixth worldwide for the production of wheat and the third for its exportation, however, fluctuations in Canadian production due to weather can have a significant impact on world wheat supply.

Wheat is a good source of protein, minerals, B-group vitamins and dietary fiber, it provides nearly 55% of the carbohydrates and 20% of the food calories consumed globally (Kumar, 2011). It is easy to store and transport and can be processed into various types of food. It is cultivated over a wide range of climatic conditions.

There are two varieties of wheat: spring wheat planted in the spring season and harvested in late summer or early fall and a winter wheat variety sown in the fall and harvested in the next early summer.

3.1 Wheat's Phenolic Compounds

Wheat is not only considered as the main staple food in many countries, but also a potential source of natural products with nutraceutical and/or pharmaceutical importance. Its high content of phenolic compounds confers significant antioxidant, anticarcinogenic and health-promoting properties (Craig, 1999). In fact, the antioxidant and radical scavenging activities of wheat bran extract were reported to be higher than those of the synthetic compound, butylated hydroxytoluene (BHT) (L. Brindzova et al., 2009).

Investigations of phenolic compounds in wheat are limited (Asenstorfer et al., 2006; Cavalière et al., 2005; Feng and McDonald, 1989; Feng; and McDonald, 1989),

and a comprehensive profiling of wheat phenolic compounds, especially in relation to environmental stresses is lacking. (Estiarte et al., 1999; Nadeau et al., 1987; Olenichenko et al., 2008; Zagoskina et al., 2005).

3.2 Stress Factors and Phenolic Compounds in Wheat

Plants are exposed to various abiotic (fluctuations in temperature, light quality/intensity, wounding, etc) and biotic (infection by virus, bacteria, fungi, etc) stress factors. In response, plants synthesize and accumulate a variety of phenolic compounds especially phenylpropanoids, including flavonoids (Dixon and Paiva, 1995); (Fig. 1-13) that summarizes the types of phenylpropanoid compounds induced in plants by various biotic and abiotic stresses.

The results of several investigations indicated that such metabolites protect the plant against UV irradiation, oxidative stress and microbial attacks (Winkel-Shirley, 2002). Abiotic stress may lead either to irreversible injuries or to the induction of a chain of reactions resulting in the plant's adjustment to such stress conditions, i.e. plant acclimation. Wheat is a widely adapted crop to abiotic stresses (Tardif et al., 2007); however, studies related to wheat secondary metabolism are very rare.

3.3 Cold Acclimation in Wheat

Wheat and its relative grain plants have evolved a broad range of complex systems that are expressed in anticipitation of, and during exposure to, different stress conditions, especially low and freezing temperatures. These highly integrated systems of structural, regulatory and developmental genes that activate and control the low

temperature (LT) protective mechanisms rendered wheat as one of the most widely adapted crops in the world.

Cold stress is one of the limiting environmental factors in crop productivity (Fowler, 2008), exposure to sublethal, non-freezing temperatures allows plants to acquire freezing and chilling tolerance, a process known as cold acclimation (Thomashow, 1999). In fact, cold acclimation involves the expression of certain cold-induced genes in wheat that function to stabilize membranes against freeze-induced injury. Plants have the ability to sense changes in the environment that signal the up-coming of winter and as a result, they exhibit an increase in freezing tolerance (Thomashow, 1999). This process induces a number of morphological, physiological and biochemical changes that protect the plant against cell dehydration resulting from ice formation (Uemura and Steponkus, 1997), and against reactive oxygen species (McKersie et al., 1997).

The accumulation of total soluble phenolics content under low-temperature stress was reported in wheat leaves cv.*Mironovskaya* 808, (Zagoskina et al., 2005). This result was explained in terms of the role of soluble phenolic compounds in plant defence mechanism, as an antioxidant against stress factors, especially cold stress.

The total content of phenolic compounds almost doubled in response to cold treatment as compared with control leaves, although there was no change in the qualitative composition (Olenichenko et al., 2006). However, these studies concerned only the water-soluble fraction containing glycosides and polar compounds, but not the lipophilic flavonoids, especially tricin.

3.4 Salt and Drought Stresses

Salt stress (high soil sodium content) and drought stress are among the factors affecting osmotic balance of the plants and, consequently, could severely limit crop production (Boyer, 1982), by limiting absorption of water from the soil (ionic stress). To counteract the effect of these stresses, plants evolved protective mechanisms through synthesis and accumulation of low molecular weight metabolites called compatible solutes, such as amino acids, sugars (that contribute to the regulation of ROS signaling as well as osmotic adjustments during abiotic stresses (Seki et al., 2007), quaternary ammonium compounds that may accumulate with the aim to increase the ability of cells to retain water without affecting normal metabolism, betaine, and proline. Quaternary ammonium compounds, betaine (Rhodes et al., 1987) and proline (Miralles and Serrano, 1995) are the most common nitrogen-containing compatible compounds.

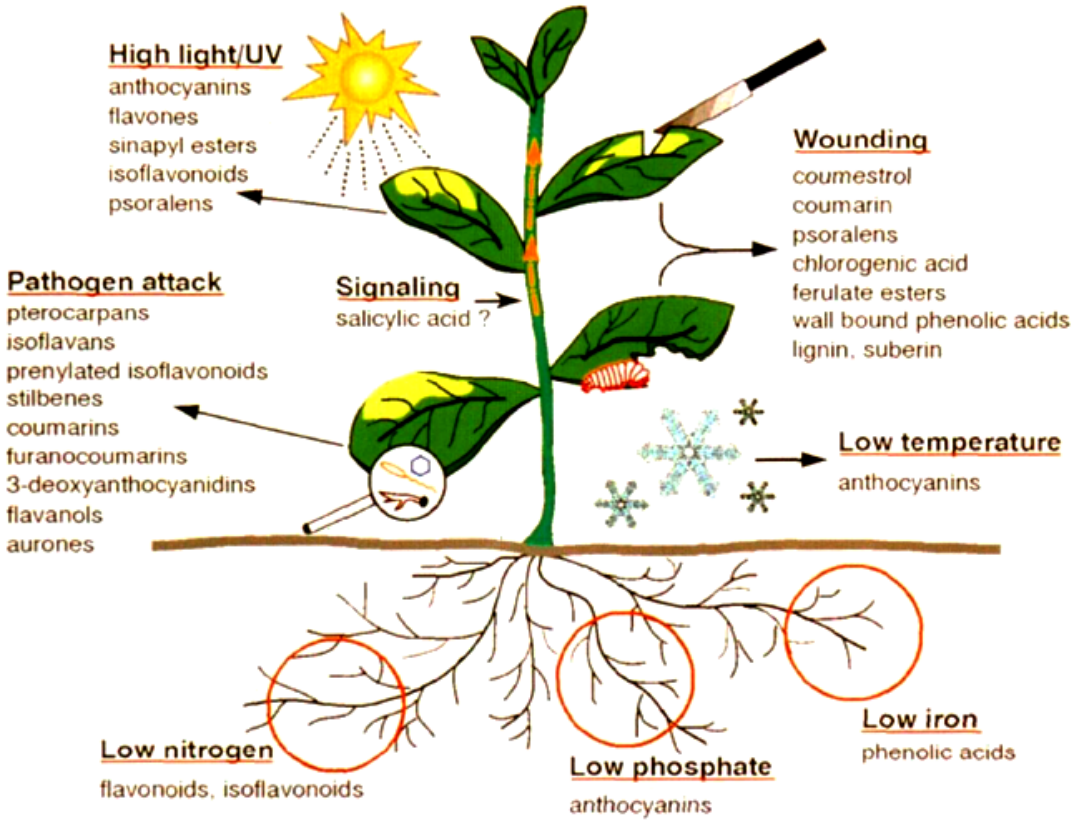


Figure 1-13: Examples of stress-induced phenylpropanoids

Figure from (Dixon and Paiva, 1995) "Copyright American Society of Plant Biologists"

4. Stress Factors and Tricin

Only a few investigations were reported on the effect of some stress factors on the level of tricin in plants, Among these, one recent study reported the effects of the herbicide 'safener' on wheat seedlings. This study showed the increase in tricin and ferulic acid and the reduction of apigenin, luteolin and isorhamnetin levels concomitant with an increase in *O*-methyltransferase activity toward these flavonoid substrates (Cummins et al., 2006). The results suggested that safeners, besides altering the capacity

of wheat to metabolise herbicides and other xenobiotics, could selectively shift the metabolism of endogenous phenolics.

Safeners are chemically diverse synthetic compounds that are added to protect crop plants from herbicide injury without reducing the herbicidal activity. They help the plants to metabolise faster the herbicidal substance, breaking it down into decomposition products that lack herbicidal activity. The structural similarity of several herbicide-safener combinations may suggest that safeners compete with herbicide molecules for binding sites on receptor or target proteins (Davies and Caseley, 1999).

It was also reported that, the flavonoid leaves of wheat (*Triticum aestivum* L. cv. Yecora Rojo), including tricin, increased by two orders of magnitude in a CO_2-enriched (Estiarte et al., 1999). This suggests that higher carbon availability provides the carbon source necessary for secondary metabolite synthesis, especially flavonoids.

5. Dietary Fibers

Dietary fibers constitute an important part of the plant material that is resistant to digestion by human enzymes. They are predominantly non-starch polysaccharides, lignins, and may include other associated substances (Dhingra et al., 2011). Dietary fibers are categorized according to their water solubility into two types: soluble, well fermented fibers (pectin, gums and mucilage) that are readily fermented in the colon into gases and active byproducts, and insoluble, less fermented fibers that are metabolically inert, absorbing water such as cellulose, hemicellulose, lignin, psylium husk and other roughage (Antia and Abraham, 1997). The insoluble dietary fibers class is also known as 'Novel fibers', its use is considered newborn and is responsible for most of the health benefits attributed to the use of whole grains.

The U.S. Department of Health and Human Services (HHS) and USDA, in its dietary guidelines for Americans, recommend that all adults eat half their grains as whole grains, which includes oats and whole wheat (HHS and USDA, 2005). USDA and HHS recommend the daily intake of dietary fiber to be 14 grams per 1000 calories which is 20-35 grams per day.

Whole grains such as wheat, oats and brown rice are cereals that are rich in dietary fibers and are known to provide healthy nutrients to humans (Anderson et al., 2009). Consumers of whole grains have a significantly better nutrient intake profile than non-consumers, with a higher intake of vitamins and minerals and a lower intake of fat and added sugars (Cleveland et al., 2000). Some of the reported benefits include

preventing disorders such as cardiovascular diseases, cancer and obesity (Verma et al., 2008) and refs. there in).

The direct effect of dietary fibers found in cereals on postprandial plasma glucose and insulin levels suggests a potential mechanism linking cereal fiber intake and reduced risk of type 2 diabetes (Weickert et al., 2006).

There are numerous publications highlighting the use of oat hull fiber in food products (K Kamaljit et al., 2011) and refs. there in), in addition to its use in some of the over-the-counter products, such as Metamucil®, the well-recognized laxative brand.

Taking the model of oat hulls and its applications, the enriching of daily food such as bread, cereal and bakery with wheat hull will result in raising the total dietary fiber content, reducing caloric content, and modifying the rheological properties of food systems. This in addition to the chemopreventive potential offered from increasing tricin intake.

Insoluble wheat fibers originate primarily from wheat hulls, which are parts of the whole wheat grain, that its safety is well-established based on its use as a food for decades. The composition of the wheat grain and its parts was discussed in many literature papers,

The difference between the bran layers and the hulls is found in the occurrence of different tissue components as strands of celluloses, hemicelluloses and pentosans (xylans in particular) that are unevenly distributed throughout the whole kernel, including the dehulled grains.

6. The Impact of Plant-based Foods on Human Health

Diets rich in phytonutrients, or plant-based foods, are mostly associated with reduced risks of major chronic diseases (Martin et al., 2011 and refs. there in). To be able to implement the role of these phytonutrients in human life, the collaboration between plant scientists, in plant biochemistry, plant genetics and metabolic engineering and researchers in nutrition and pathology of chronic diseases was recently highly recommended (Martin et al., 2011). Such collaboration and contribution will allow the designation of foods that reduce the risk of chronic disease and explain how these foods work to impact human health.

The development of dietary agents for cancer chemoprevention offers a safer and attractive alternative in comparison to the use of pharmaceuticals such as nonsteroidal anti-inflammatory drugs and tamoxifen as cancer chemopreventive agents. Recently, this approach was discussed in order to establish a protocol for development of chemopreventive phytochemicals and the selection of the optimal clinical dose required (Scott et al., 2009).

7. Techniques Used in this Work

7.1 LC-MS Techniques

Nowadays, LC-MS represents a well-established, rapid and powerful technique for the analysis of many natural products.

Mass spectrometry (MS) offers great selectivity and sensitivity as a detection technique, coupling it with high performance liquid chromatography (HPLC) enables effective analysis of complex matrices like plant extracts. This is mainly due to its ability to detect, within the same analysis, all metabolites encountered in the extract.

In addition, the use of tandem mass spectrometry (MS/MS) results in a full structural analysis of mixtures without using the tedious time-consuming isolation procedures.

Multiple Reaction Monitoring Technique (MRM)

(MRM) is a selective and sensitive LC-MS-MS tandem mass technique in which each ionized compound gives a distinct precursor-to-product ion transition indicative of a particular compound in an extract. Peaks containing co-eluting compounds are resolved by monitoring of specific precursor-to-product ion transitions (Chiwocha et al., 2003; Pang et al., 2009; Segarra et al., 2006).

Time-of-Flight (TOF)

(TOF) is a fast and precise ionization technique. A TOF instrument provides accurate mass measurement within a few parts-per-million (ppm) of a molecule's exact true mass (Cotter, 2004).

7.2 Viability Test

Two different methods were carried out during this work in order to determine the viability of different cell lines

Lactate dehydrogenase (LDH)

Lactate dehydrogenase **(LDH)** is an ubiquitous enzyme present in a wide variety of organisms, including plants and animals. It catalyzes the reversible reduction of pyruvate into lactate, with the ultimate interconversion of NADH into NAD^+ (Fig 1-14) (Henderson, 1984). In isolated organ systems, *in vitro* release of LDH by the cells into the culture fluid has been used to determine injury of the cells.

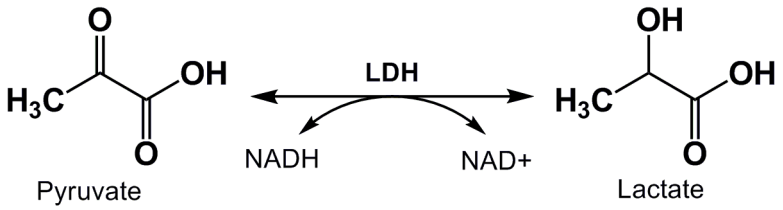


Figure 1-14: LDH reaction

The activity of lactate dehydrogenase is usually measured spectrophotometrically at 340 nm by one of the following two methods. Either the oxidation reaction of NADH with pyruvate and hence a decrease in the absorbance, or the reduction reaction of NAD^+ with lactate resulting in an increase in the absorbance. Under standard conditions, one unit of enzyme catalyses the oxidation of NADH or reduction of NAD^+ at 1 µmol per min (Markert, 1984).

In the current work the leakage of LDH was measured by monitoring the activity of lactate dehydrogenase with an aliquot of cell-free medium and compared to the total activity achieved after lysis of the cells (Moldéus et al., 1978).

Lysis of cells was done by treatment with Triton X-100 (0.5%). NADH (0.2 m*M* final concentration) and pyruvate (1.36 m*M* final concentration) were mixed with Krebs Henseleit buffer containing 2% albumin before being added to 25 µl of the cell-free medium once and to 25 µl of the cell-containing media after lysis. The rate of change in absorbance at 340 nm due to NADH oxidation was recorded.

FACScan (Fluorescence Activator Cells Sorter) Flow Cytometer

A flow cytometer is an instrument for detecting and measuring the amount of fluorescent dye on particles, and basically consists of one or more lasers for supplying excitation energy, and a series of filters and detectors for measuring the resultant fluorescent emissions. In this work a FACScan flow cytometer with CountBright™ absolute counting beads were used to measure viability of cells.

The CountBright™ absolute counting beads are a calibrated suspension of microspheres that are brightly fluorescent across a wide range of excitation and emission wavelengths and contain a known concentration of microspheres. (http://probes.invitrogen.com/media/pis/mp36950.pdf). For absolute counts, a specific volume of the microsphere suspension is added to a specific volume of sample, so that the ratio of sample volume to microsphere volume is known. The volume of sample analyzed can be calculated from the number of microsphere events, and can be used with

cell events to determine cell concentration. In general, at least 1,000 bead events should be acquired to assure a statistically significant determination of sample volume.

Cell concentration was calculated according to the following formula

A x C / B x D = concentration of sample as cells/μL; Where:
A = number of cell events
B = number of bead events
C = assigned bead count of the lot (beads/50 μL)
D = volume of sample (μL)

8. Scope of Thesis Research

The overall objective of the thesis focused on detailed studies of the biochemical, molecular and biological aspects of tricin, a promising chemopreventive agent. The research addresses the occurrence of tricin and other related phenolic compounds in wheat, how it is affected under different stress conditions, how to find a reliable source of tricin to incorporate it in our daily diet and how it does affect both cancer and normal cell lines.

Tricin is a rare and expensive compound; we studied its identification and quantification in extracts of different parts of wheat (leaves, bran, seed, etc.) in order to determine the best accumulator organ and variety. This was carried out by comparing two varieties of wheat: the winter variety, cv. Claire and the spring variety, cv. Bounty.

Investigations of phenolic compounds in wheat are limited (Asenstorfer et al., 2006; Cavalière et al., 2005; Feng and McDonald, 1989), and a comprehensive profiling of wheat phenolic compounds including tricin, especially in relation to environmental stresses is lacking (Estiarte et al., 1999; Nadeau et al., 1987; Olenichenko et al., 2008; Zagoskina et al., 2005). Thus, we studied in this work the effect of several abiotic stress factors, such as cold, drought and salt treatment, among others, on the biosynthesis and accumulation of tricin.

The accumulation of tricin in different parts of wheat was determined to identify the tissue that has the maximum accumulation. The analyses reveal that winter wheat hulls contain the highest content within the plant. In addition, an efficient method of isolation and purification of tricin was developed.

Thus, the research presented in this thesis provides an economical protocol for preparation of tricin from a part of the plant that is considered a waste by-product with low economic value. This will increase the value of wheat as a crop and improve the farmers' revenue.

Chapter 2 CHANGES IN WHEAT LEAF PHENOLOME IN RESPONSE TO COLD ACCLIMATION

Investigations of phenolic compounds in wheat are limited (Cavalière et al., 2005); (Asenstorfer et al., 2006; McDonald, 1989), and a comprehensive profiling of wheat phenolic compounds, especially in relation to environmental stresses is lacking (Estiarte et al., 1999; Nadeau et al., 1987; Olenichenko et al., 2008; Zagoskina et al., 2005). This prompted an investigation herein of the detectable wheat leaf phenolome during cold acclimation. This phenolic profile was established for both the winter (Claire) and spring (Bounty) wheat varieties with the aim of studying the function of these compounds in the adaptation of this important crop plant to environmental stresses.

This chapter of the thesis addresses the following research areas: (i) Wheat phenolic compounds common to the winter and spring varieties and the effect of cold acclimation on it, and (ii) Localization of phenolic derivatives in leaf apoplast fluid.

Techniques used in this chapter are: HPLC and LC-MS equipped with UV detector were the main analyses techniques used. For exact mass measurements, electrospray ionisation-time-of flight analyser (ESI-TOF) was used in positive ESI mode and for fragmentation reason tandem mass spectrometry using the triple quadrupole MS/MS system via MRM (multiple reaction monitoring) mode was applied.

The manuscript corresponding to this chapter was published in the Journal of *Phytochemistry*: "Amira Moheb, Ragai K. Ibrahim, René Roy and Fathey Sarhan (2011), Changes in wheat leaf phenolome in response to cold acclimation, *Phytochemistry* (2011), doi:10.1016/ j.phytochem.2011.08.021

Summary

A study of wheat (*Triticum aestivum* L.*)* leaves phenolome was carried out during cold acclimation of the winter (Claire) and spring (Bounty) varieties using a combination of HPLC-ESI-MS techniques. A total of 40 phenolic and flavonoid compounds were identified, and consisted mainly of two coumarin derivatives, eight simple phenolic derivatives, ten hydroxycinnamoyl amides and 20 flavonoid derivatives. Identification and quantification of individual compounds were performed using an HPLC system coupled with a photodiode array detector and two different ESI-MS systems, in combination with a multiple reaction monitoring (MRM) technique. The analyses indicated that, although there were no qualitative differences in their profiles, the winter variety exhibited a higher phenolic content compared to the spring variety when both were grown under non-acclimated (control) conditions. Cold acclimation, on the other hand, resulted in a significant differential accumulation of phenolic compounds in both varieties: mostly as luteolin *C*-glycosides and their *O*-methyl derivatives in the winter variety (Claire) and a derivative of hydroxycinnamoyl amide in the spring variety (Bounty). These compounds accumulated in large amounts in the apoplastic compartment. The accumulation of the *O*-methylated derivatives was associated with a marked increase in *O*-methyltransferase (OMT) enzyme activity. In addition, the trimethylated flavone, 3',4',5'-trimethyltricetin was identified for the first time in the native extracts of both control and cold-acclimated wheat leaves. The accumulation of a mixture of beneficial flavonoids as iso-orientin, vitexin and tricin in cold acclimated wheat leaves, attests for its potential as an inexpensive source of a health-promoting supplement to the human diet.

Introduction

Wheat is one of the most important crop plants worldwide. It is not only considered as the main staple food in many countries, but also as a potential source of natural products with nutraceutical and/or pharmaceutical importance. Its high content of phenolic compounds confers significant antioxidant, anticarcinogenic, and health-promoting properties (Craig, 1999). In fact, the antioxidant and radical scavenging activities of wheat bran extract were reported to be higher than those of the synthetic compound, butylated hydroxytoluene (BHT) (L. Brindzova and Rapta, 2009).

Wheat and its relatives have evolved a broad range of complex systems that are expressed in anticipitation of, and during exposure to, different stress conditions especially low and freezing temperatures. These highly integrated systems of structural, regulatory, and developmental genes that activate and control the low temperature (LT) protective mechanisms rendered wheat as one of the most widely adapted crops in the world. Recent studies have shown that exposing *Arabidopsis* plants to low temperature resulted in significant changes in the configuration of its metabolome. In response to cold treatment, 75% of *Arabidopsis* metabolites have increased in amount varying from 2- to 25-fold, of which 22% were identified as sugars or other carbohydrate derivatives, (Cook et al., 2004). Similar results were deduced from metabolic fingerprinting analysis of *Arabidopsis* leaves subjected to low temperature (Gray and Heath, 2005), thermal stress or cold shock (Kaplan et al., 2004). These metabolites may have a general or specific function that contributes to the plant survival. However, their exact role in plant defence is not well understood.

Flavonoids, as well as other phenolic derivatives, play important roles in the plant's interaction with its environment, especially protection against UV radiation, oxidative stress and microbial attacks, to mention only a few (Bohm, 1998 & refs. therein). In addition, they constitute an important component of the human diet and represent active ingredients in several medicinal plants.

The accumulation of soluble phenolic compounds in winter wheat leaves in response to cold acclimation has been explained in terms of their role in the plant defence mechanism as antioxidants against cold stress (Zagoskina et al., 2005). Treatment of wheat leaves with synthetic antioxidants under cold conditions increased their levels of sugars and flavonoids (Olenichenko et al., 2008). Other studies reported a significant accumulation of the polyamines, putrescine and spermidine, following exposure of wheat leaves to cold temperatures (Nadeau et al., 1987), which suggested the involvement of polyamines in the biochemical processes of cold acclimation. Hydroxycinnamic amides (HCAs) are of ubiquitous occurrence in the plant kingdom, including graminaceous plants, and several solanaceous species (Parr et al., 2005). They participate in various aspects of plant growth processes (Back, 2001a; Martin-Tanguy, 1985), and are induced in response to both biotic and abiotic stresses (Fixon-Owoo et al., 2003), including salt/osmotic stress (Gicquiaud et al., 2002), and were recently reported to play an important role in the plant defence mechanism (Hahlbrock and Scheel, 1989; Walters, 2003).

Investigations of phenolic compounds in wheat are limited (Cavalière et al., 2005); (Asenstorfer et al., 2006; McDonald, 1989), and a comprehensive profiling of wheat phenolic compounds, especially in relation to environmental stresses is lacking (Estiarte

et al., 1999; Nadeau et al., 1987; Olenichenko et al., 2008; Zagoskina et al., 2005). This prompted an investigation herein of the detectable wheat leaf phenolome during cold acclimation. This phenolic profile was established for both the winter (Claire) and spring (Bounty) wheat varieties with the aim of studying the function of these compounds in the adaptation of this important crop plant to environmental stresses.

Materials and Methods

Chemicals

Most phenolic and flavonoid compounds used in this study were from our laboratory collection, except for tricetin that was purchased from Indofine Chemical Company (Hillsborough, NJ) and tricin, from Dalton Chemical Company (Toronto, ON). Trimethyltricetin (40) was a gift from Dr. Y. Fukushi, Hokkaido University, Japan, and feruloylagmatine (**5)** was kindly provided by Drs. S. Jin and M. Yoshida, Agricultural Research Station, Sapporo, Japan. *S*-Adenosyl-L-[^{3}H] methionine (76.4 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO), and unlabeled *S*-Adenosyl-L-methionine from Sigma (Oakville, ON). Protein quantification reagents and 40% acrylamide/bis solution were purchased from Bio-Rad (Mississauga, ON). Unless otherwise specified, all other chemicals were of analytical grade.

Plant growth and acclimation conditions

Two wheat varieties (*Triticum aestivum*, 2nx6 = 42), a spring habit cultivar Bounty and a winter habit cultivar Claire, were grown in a controlled growth chamber as previously described (Danyluk et al., 2003). Plants were grown at 20 ºC under long days (LD), with 16-h photoperiod at a light intensity of 250 µmol m^{-2} s^{-1}. For cold acclimation (CA), 7-

day-old wheat plants were grown for 1, 10, 21 and 42 days at 4ºC under the same photoperiod and light conditions mentioned above.

Extraction of phenolic compounds

Fresh leaf samples (*ca.* 10 g) were ground in dry ice before extraction 3-times for 1hr with hot MeOH-H_2O (85:15, v/v). The extract was concentrated in vacuo, to remove methanol, and the resulting aq. suspension was defatted with hexanes, followed by 3-times liquid-liquid extraction with EtOAc. The combined organic layers were concentrated, and the residue dissolved in a minimum amount of MeOH for analysis. For acid hydrolysis, the defatted aq. extract was hydrolyzed with 2M HCl (30 min; 95 ºC) in order to release the phenolic aglycones from their parent *O*-glycosides, followed by liquid-liquid extraction with EtOAc. The combined organic layers were concentrated under vacuo, and the resulting residue dissolved in MeOH for analysis.

HPLC and LC/ MS analyses

For quantification of total phenolic compounds, samples were applied to chromatographed on a Varian HPLC system equipped with a UV detector and a Varian XDB-C18 column (4.6×150 mm; particle size, 5µm) using a linear gradient of MeOH-1% HOAC in H_2O (40:60, v/v) and a flow rate of 1 $ml.min^{-1}$ for 30 min and a column temperature of 25ºC. The process was repeated at least three times, and three injections were analysed for each sample. This system was used only for the quantification step of total phenolic compounds, but not for quantification of individual compounds where the MRM technique was better used for this purpose, since it allowed the determination of minor components. Quantification for total phenolic compounds (Fig. 2-1) was carried

out using the area under the curve method by calculating the summation of areas in relation to their UV absorbance, whereas quantification for individual compounds was performed using the MRM technique that allowed determination of minor compounds.

LC-MS analyses were carried using an Agilent 1200 HPLC system with binary pump, in-line degasser, high performance auto-sampler and thermo-stated column division, using a linear gradient of MeOH-0.1% HCOOH in H_2O (40:60, v/v) for 30 min with a flow rate of 0.35 $ml.min^{-1}$ on an Agilent SB-C18 column (2.1 ×30mm; particle size, 3.5µm), and a column temperature of 25ºC. The method was optimized with UV detection at 254 and 340 nm.

Two LC-ESI-MS systems were used: in System 1, the HPLC instrument was connected to an Agilent 6410 quadrupole (triple Q) mass spectrometer using electrospray ionisation in positive ESI mode with the following conditions capillary voltage: 3000 v, nebulizer pressure: 60 Psi, gas temperature 300ºC, drying gas: 5 L/min and a dwell time of 75 ms, and the data was processed using the Mass Hunter software. The same column was transferred to be used in system 2 where an identical HPLC instrument with the same conditions listed above was connected to another mass spectrometer consisting of an Agilent 6210 electrospray ionisation-time-of flight analyser (ESI-TOF) in positive ESI mode, at a capillary voltage of 4000V, nebulizer pressure of 35 Psi, gas temperature 350ºC, drying gas flow: 11.5 L/min and voltages of 125V and 60V for the fragmentor and the skimmer, respectively. The technical error and mass resolving power of the time-of-flight mass spectrometer in terms of mass accuracy was 2ppm, RMS, measured at the $[M+H]^+$ ion of reserpine (m/z 609.2807) was used as an internal mass reference. When

available, reference compounds were used to compare the corresponding retention times and mass spectral profiles of phenolic derivatives and flavonoids.

A quercetin calibration curve was constructed for quantification purposes, using different concentrations of 1.6µg.ml^{-1}, 5µg.ml^{-1}, 8.3µg.ml^{-1}, 16.6µg.ml^{-1}, 25µg.ml^{-1}, and 166µg.ml^{-1} and 266µg.ml^{-1} quercetin.

For MS/MS and MRM analyses in system 1, the collision energy (CE) was optimized for each individual compound; available standards were injected several times with different energies of collision: 5eV, 10eV, 20eV, 25eV, 35eV and comparing their MRM peak area resulted from the MS/MS analysis for each case. The (CE) with the highest and more intense area was selected as the optimum one for the analysis, then tried again in both negative and positive modes of ionization. A (CE) of 35eV was applied as the optimum energy of collision for the analysis of flavonoids and coumarins, and 10eV for simple phenolic compounds and 25eV for conjugated amines, and the scan range was from 200 to 900 m/z. For each compound, MRM acquisition was carried out by monitoring transitions of the combination of the parent ion mass and the fragment ions of highest abundance.

Protein extraction and quantification

Protein was extracted in Tris-HCl, pH 7.0 containing 0.1% PVPP, followed by centrifugation at 10,000 rpm. It was quantified by the method of (Bradford, 1976) using bovine serum albumin as the standard protein.

O-Methyltransferase assays

Protein was extracted from the plant material at 4°C. Wheat leaves were ground to a fine powder with dry ice and sand, before being homogenized with PBS buffer (pH 7.3). After centrifugation (14, 000xg) for 30 min, the supernatant was desalted on PD-10 and used directly for the assay of *O*-methyltransferase **(**OMT) activity against tricetin and 5-hydroxyferulic acid as external substrates. The enzyme assay consisted of 50µM S-adenosyl-L- methionine (AdoMet) containing 0.025 µCi of [^{3}H] label, and protein extract (100 µg) and 200 µM tricetin or 5-hydroxyferulic acid (in 1% DMSO[1]) in a total volume of 100 µl. The reaction was initiated by addition of enzyme, incubated for 30 min at 30°C, and terminated by the addition of 6M HCl (10 µl). The methylated products were extracted with ethyl acetate, and a fraction was counted for radioactivity using a toluene-based scintillation fluid. Enzyme assays were carried out in triplicate, and the experiment was repeated at least twice. Control reactions were carried out without addition of protein for background correction.

Extraction of soluble apoplastic metabolites

Soluble apoplastic metabolites were extracted as described by (Vanacker et al., 1998). Freshly cut leaves (*ca*.5 g) of both non-acclimated and acclimated winter wheat were washed with distilled H_2O, then immersed in Petri dishes containing 50 mM acetate buffer (50 ml) pH 4.5, 100 mM KCl and 2 mM $CaCl_2$. The dishes were transferred to a vacuum dessicator and a vaccum of 270 kPa was applied in order to extract the apoplastic content. After centrifugation for 10 min at 2900*x*g and 4°C, the extract was lyophilized

[1] DMSO: dimethylsulfoxide

and the resulting powder used for both HPLC-MS/MS analysis and gel electrophoresis. The apoplastic purity was assesed by its protein pattern on SDS-PAGE and the absence of any trace of Rubisco that is usually used as indicator of cell leakage (Diaz-Vivancos et al., 2006).

Results and discussion

Identification of wheat phenolic compounds common to the winter and spring varieties

By means of two LC-ESI-MS protocols, a number of phenolic compounds were identified in the leaves of both varieties. The LC-TOF system was equipped with Agilent software that allowed calculating and generating the molecular formula of each compound according to its mass spectrum obtained during analysis, whereas the triple quadrupole MS/MS system was used to confirm the product ions. A direct comparison of the MS spectra obtained from both systems made it possible to confirm compound identification. Moreover, the high sensitivity of the MS-MS detector used allowed for identification of minor constituents with a high degree of fidelity. The fact that several phenolic compounds exhibited almost similar polarities and retention times made it difficult to assign their corresponding peaks. However, the use of MS in combination with UV detection at 340 and 254 nm, in addition to the comparison of their spectral data with available reference compounds, allowed their identification with high degree of certainty. In addition, tandem mass spectrometry (MS-MS), exact mass measurements and fragmentation patterns were used together with literature data for the identification of compounds where reference samples were not available.

The application of several collision energies in the positive and negative modes to the sample was necessary to optimize a method with a consistent fragmentation pattern. The positive mode was compatible with almost all compounds. For optimum energy of collision, 10V and 25V were applied for simple phenolic derivatives and hydroxycinnamic acid conjugates, respectively, whereas 35V was chosen for other flavonoids and coumarin derivatives. This was performed through the application of tandem mass technique MS-MS in multiple reaction monitoring (MRM) mode and of exact mass measurement, both of these methods allowed the identification and quantification of most phenolic compounds.

MRM is a selective and sensitive LC-MS/MS technique in which each ionized compound gives a distinct precursor-to-product ion transition. Furthermore, peaks containing co-eluting compounds were resolved by monitoring for specific precursor-to-product ion transitions (Chiwocha et al., 2003; Pang et al., 2009; Segarra et al., 2006). However, closely related isomers could not be differentiated by this technique. Another analysis using the same conditions for HPLC was conducted on another instrument (ESI-TOF) in order to confirm the exact masses and empirical formulae of each of the identified structures.

Using these protocols allowed the separation and identification of 40 phenolic compounds in the extracts of both winter and spring wheat varieties. These consisted of two coumarin derivatives, eight simple phenolic compounds, ten hydroxycinnamoyl amides (HCAs) and 20 flavonoid derivatives (Table 2-1, Fig. 2-2). These results showed no qualitative differences observed in the phenolic profiles of both varieties under non-acclimated (control) conditions, (Figs. 2-1A and 2-1B).

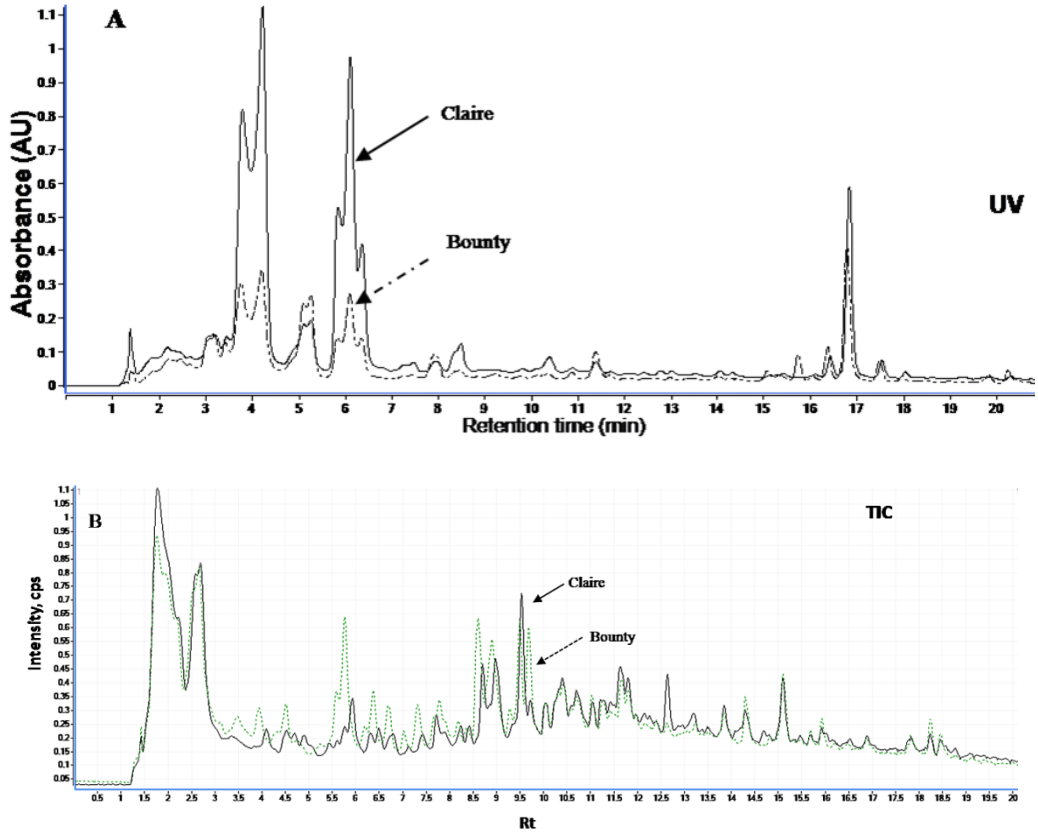


Figure 2-1: A, HPLC-UV; B, Total ion counts (TIC)-overlaid trace chromatograms of non-hydrolysable methanolic extract of leaves from 2-week-old Claire and Bounty

The UV and TIC chromatograms were obtained using an Agilent SB-C18 column (2.1 ×30mm; particle size, 3.5µm), and a column temperature of 25ºC; 40% to 90% MeOH in 0.1% HCOOH of linear gradient elution; flow rate 0.35 ml/min for 30 min.; injection volume, 10 µl; wavelength monitoring, 340 nm.

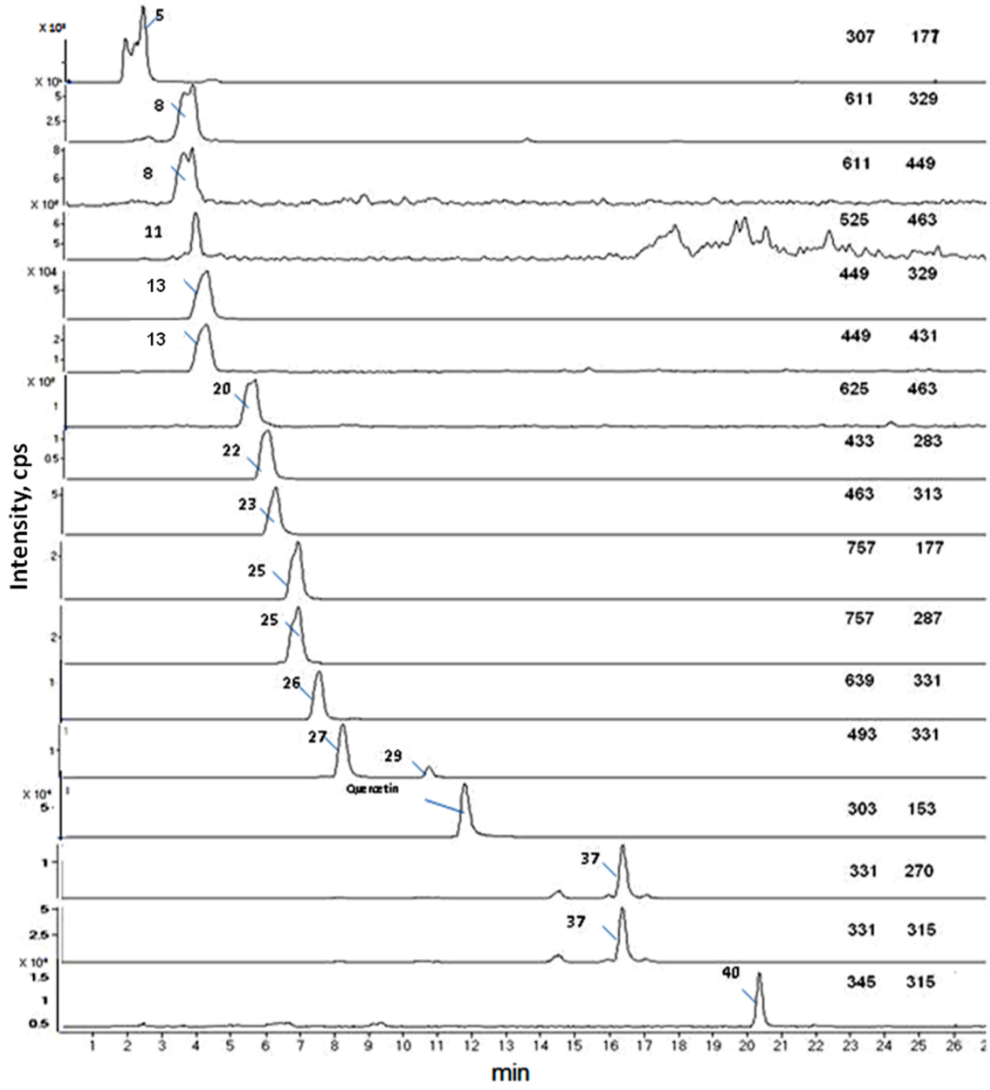


Figure 2-2: HPLC-UV-MS of methanol extract of leaves from 2-week-old winter wheat Claire

*Reconstructed MRM chromatogram [MS-MS] of most abundant protonated molecules (parent ion $[M+H]^+$ and its major ion peak fragments) in the positive mode resulting from LC-MS-MS analysis, showing MRM at: 307 -> 177 for compound (**5**), 611 -> 329, 611-> 449 for compound (**8**), 525 -> 463 for compound (**11**), 449 **->** 329 for compound (**13**), 625 **->** 463 for compound (**20**), 433 -> 283 for compound (**22**), 463 -> 313 for compound (**23**), 639 -> 331 for compound (**26**), 771 **->** 463**,** 771 **->** 625for compounds (**28**) and (**29**), 303 -> 153 for quercetin, 331 -> 315 for compound (**37**) and 345 -> 255 for compound (**40**)*

Hydroxycinnamoyl amides (HCAs)

The major HCAs identified in this study (Table 2-1) are *p*-coumaroylagmatine (**1**), caffeoylputrescine (paucine) (**2**), *p*-coumaroylputrescine (**3**), feruloylputrescine (**4**), *trans*-feruloylagmatine (**5**), *p*-coumaroylspermidine (**6**), *p*-coumaroyl-2-hydroxyputrescine (**7**), *bis*-dihydrocaffeoylspermine (**30**), dicaffeoylputrescine (**31**) and di-*p*-coumaroylputrescine (**34**).

Feruloylagmatine **(5),** was analysed using 25V collision energy, and its MRM transitions from 307 $[M+H]^+$ to 177 at Retention time of 2.01 min. This data was identical to those obtained for a reference sample of feruloylagmatine **(5)**. The identification of small amounts of dihydrocaffeoylspermine **(30)** in the spring variety (Table 1) is surprising, since dihydro derivatives of phenylpropanoid compounds are reported to be of rare occurrence in plants (Anterola and Lewis, 2002; Lewis, 1999). However, the occurrence of several dihydrocaffeoyl polyamines in potato tubers was reported during metabolite profiling of the plant (Parr et al., 2005), which provides an exemplary evidence for the efficient use of LC-EIS-MS-MS protocols in investigating wheat metabolites.

Flavonoids

Among the 20 flavonoids identified in this work, six represented the major constituents. These include (% of total and µM quercetin equivalent.g^{-1}): orientin (**12**) and iso-orientin (**13**) (42%, 0.47); vitexin (**21**) and iso-vitexin (**22**) (18.2%, 0.26); chrysoeriol 6-*C*-glucoside (**23**) (8.7%, 0.2) and tricin (**37**) (9 %, 0.21). Acid hydrolysis of methanolic extracts, that removed *O*-glycosidic linkages followed by LC-MS of the

hydrolysis products, confirmed the natural occurrence of these *C*-glycosides in wheat leaves and allowed the quantification of tricin (**37)** as a free aglycone. These results are consistent with the most recently published LC-MS analysis for wheat leaves (Cavalière et al., 2005), among others, which reported the occurrence of these flavonoids as *C*-glycosides, except tricin 37 which occurred as an *O*-glycoside.

In cereals, such as wheat, maize, barley and rice, both 6-*C*- and 8-*C*-glycosides of luteolin and /or apigenin are most abundant (Brazier-Hicks et al., 2009; Cummins et al., 2006). These compounds were suggested to act as antibiotics, antioxidants, feeding attractants or deterrents (Brazier-Hicks et al., 2009; Gould and Lister, 2005)**,** and as phytoalexins that were produced naturally in response to various stress conditions (Du et al., 2009; McNally et al., 2003). Furthermore, iso-orientin (**13)**, the major phenolic compound in wheat was reported to act as anti-nociceptive and anti-inflammatory agent in rats and mice at doses of 15 and 30 $mg.kg^{-1}$, without causing any apparent acute toxicity or gastric damage (Kupeli et al., 2004). In addition, there is ample evidence to suggest that luteolin **(36)** and its glycosides **(12)-(13)** might be used as cancer chemopreventive agents, or in chemotherapy (Lopez-Lazaro, 2009)& refs therein).

The relatively high abundance of iso-orientin (**13)** in wheat leaves, attests to its use as a potential source of active natural health-promoting compounds. Wheat leaves are considered as an edible part of the plant, and are used as a juice (wheatgrass juice) or added to several food products in North America and other parts of the world. These products are approved by the health authorities and sold by several established companies:

(http://www.greenhealthcanada.com/Benefits_of_Wheatgrass.html; http://www.wheatgrass.ca/; http://www.ble-de-vie.com/english.html), among others

Tricin (**37)** (5,7,4'-trihydroxy-3',5'-dimethoxyflavone) and its *O*-glycosides **26, 27**, **29** were found to constitute about 9% of the total phenolic fraction in wheat leaves after acid hydrolysis. It is known to occur mainly in the grass family, including cereal grain plants, and has been isolated from rice, oat, maize and wheat (Wollenweber, 2008). Apart from being considered as a powerful antioxidant, antimutagenic and anti-inflammatory agent (Zhou et al., 2006) and refs. therein)**.** In addition, tricin (**37)** has been reported to be an efficient chemopreventive agent in growth inhibition of human malignant breast tumour cells (Jeong et al., 2007) and colon cancer cells (Cai et al., 2004; Hudson et al., 2000), and has been considered safe enough for use in clinical studies (Verschoyle et al., 2006b).

Furthermore, 3',4',5'-trimethyltricetin (**40)** was identified, for the first time, as a wheat constituent using MS-MS analysis. It exhibited a parent ion peak in the positive mode at *m/z* 345 corresponding to $[M+H]^+$ and two major product ions at *m/z* 315 and 255 (Fig. 2-3). Its spectral data and Rt value were identical to those obtained with an authentic reference sample. 3',4',5'-Trimethyltricetin (**40)** has recently been reported as the final enzyme reaction product of a wheat recombinant OMT catalyzing the sequential methylation of the pentahydroxyflavone, tricetin as substrate (Zhou et al., 2006). A recent review of the occurrence and distribution of tricetin methyl ethers in plants (Wollenweber, 2008) indicates that 3′,4′,5′-trimethyltricetin (**40)** is a typical constituent of grasses, and has been identified as a natural constituent in 18 graminaceous species, but not including wheat (Kaneta, 1973).

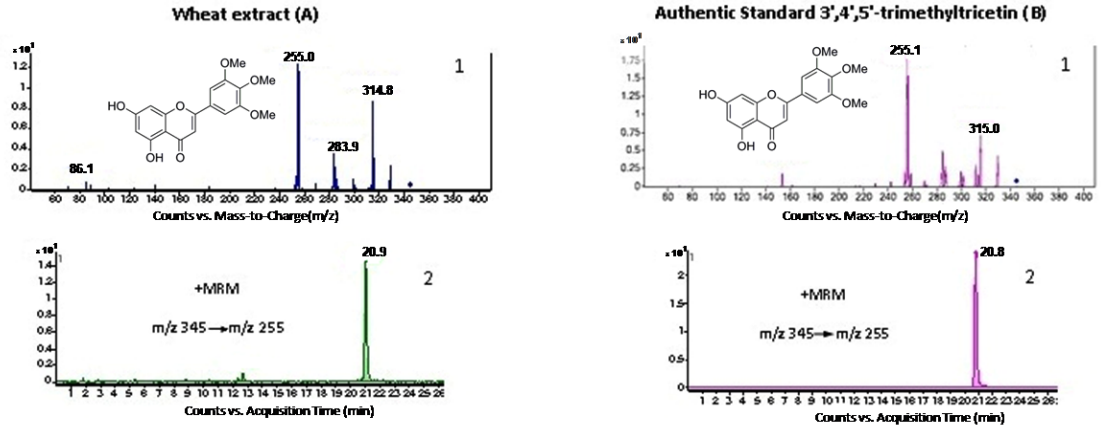


Figure 2-3: Identification of 3',4',5'-trimethyltricetin (40) in wheat leaves

A, HPLC-MS (1) MS-MS for m/z 345 [M+H]+ showing 255 and 315 as major product ions, (2) MRM signals at 345 and 255 ions at Rt 20.9 min.; B, corresponding MS-MS and MRM of standards (1)

Coumarins and Simple phenolic compounds

Esculetin **(9)** and its 7-methyl derivative, scopoletin **(17)** were identified in both wheat varieties (Table 1). The use of available reference compounds enabled us to compare their Rt and MRM profiles. Under non-acclimated conditions, the amount of scopoletin **(17)** is higher than esculetin, i.e. 0.06% compared to 0.01% of total phenolic compounds, respectively. This may be explained by the possible toxicity of the vicinal hydroxyl groups present at positions 6 and 7 of the coumarin structure, which may be reduced by methylation of esculetin **(9)** to scopoletin **(17)**.

The fact that trace amounts of simple hydroxycinnamic acids were observed in the extracts of both wheat varieties (Table 2-1) corroborates with the natural occurrence of their conjugated forms as esters or glycosides (Dixon and Paiva, 1995). In contrast, two

hydroxybenzoic acids, vanillic acid (**10**) and sinapic acid (**15**) were present in the free state as minor constituents.

Effect of cold acclimation on the phenolic profiles of winter and spring wheat

Wheat leaves (7-days-old) of both varieties were cold acclimated at 4°C for 0, 6, 12, 21and 42 days, and their phenolic profiles were determined by HPLC–UV methods, using quercetin as the internal standard, as described in the Experimental. During cold acclimation, there was no qualitative difference in the phenolic profiles of both varieties, but there was a significant accumulation of phenolic derivatives, reaching their highest level (2-fold increase) after 42 days of cold acclimation as compared to the corresponding non-acclimated plants (Fig. 2-4). The relative amount of phenolic compounds was consistently higher in the winter variety (Claire) than the spring variety (Bounty).

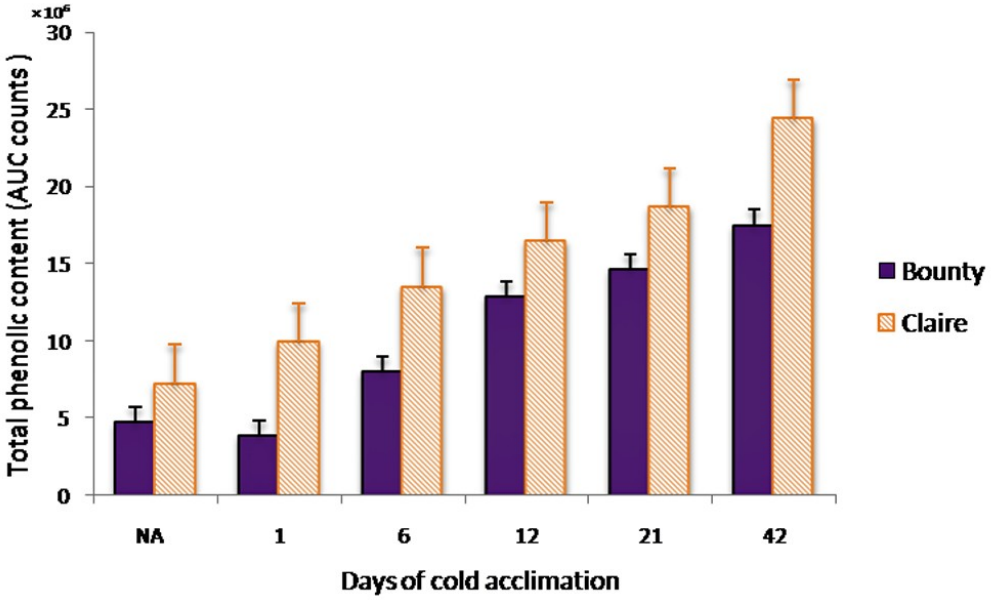


Figure 2-4: Total phenolic content during cold acclimation of both winter (Claire) and spring (Bounty) wheat.

(AUC): absorption unit counts. (NA): 7-day-old non-acclimated plants.

Both scopoletin (**17)** and esculetin (**9)** increased in their levels by 3- and 7-fold, respectively, after 21 days of cold acclimation of both varieties. However, their contribution to the phenolic pool is still limited due to their initial low relative abundance.

The increase in phenolic compounds in the winter variety is mostly represented by the *C*-glycosides of luteolin, iso-orientin **(13)** and orientin **(12)** (*ca* 3-fold) their methylated conjugates (1.3- to 2-fold) and of apigenin, vitexin **(21)** and isovitexin **(22)** (*ca* 3-fold).

Luteolin *C*-hexosyl-*O*-(*p*-coumaroyl) hexoside **25** (Table 2-1 and Fig 2-5), which accumulated in significant amounts (10- to 15-fold) in the cold acclimated winter variety, was identified based on its mass spectrum $[M+H]^+$ of 757.2 *m/z* and its product ions at *m/z* 757, 177, 463 and 287; where 177 is a characteristic fragment ion of *p*-coumaric acid. Moreover, its measured accurate mass was 756.1906 with only 0.6 ppm difference from the theoretical calculated value. Compound **25** reached its maximum concentration (0.2 to 0.3±0.14µM QE.g^{-1})[2] after 21 days of cold acclimation and represented one of the major phenolic constituents, amounting to approximately 9% of the total leaf phenolics. Although its role in wheat is not clear, this luteolin derivative **(25)** was reported to be associated with the protection of a UV-tolerant rice cultivar against UV-B radiation (Markham et al., 1998).

[2] QE: Quercetin equivalent.

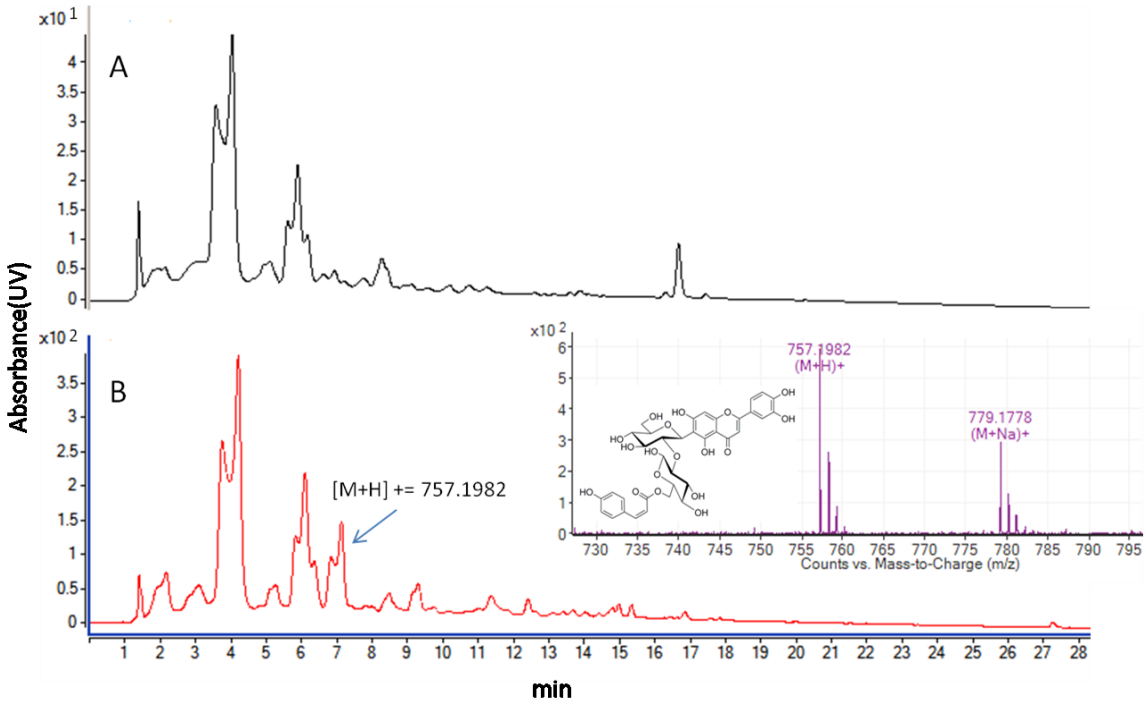


Figure 2-5: HPLC-UV-MS chromatograms of methanol extract of (A) non- acclimated and (B) 21-day cold acclimated Claire leaves.

Showing induction of levels of luteolin-C-hexosyl-O-(p-coumaroyl) hexoside (25) (Rt 7.2 min) corresponding to the measured accurate mass.

The fact that a number of HCAs especially compounds **4**, **5** and **31,** accumulated in wheat in significant amounts (*ca* 17- to 20-fold) in response to cold acclimation suggests a biological role in plant protection against low temperatures.

Feruloylagmatine **(5),** (Table 2-1 and Fig.2-6) was the most abundant HCA whose level was induced by cold treatment; it increased *ca* 20-fold after 21 days of cold acclimation of the winter variety. This result is in agreement with the recent finding of a 10-fold increase of feruloylagmatine **(5)** in wheat crowns exposed to low temperature (Jin and

Yoshida, 2000). These authors attributed its induction to its antifungal properties against the phytopathogenic fungus, *Microdochium nivale*. HCAs are synthesized in the cytosol and transported towards the cell wall, where they function as a resilient barrier against pathogen attacks (Hahlbrock and Scheel, 1989) and refs.therein), and act as stabilizers of cell membranes (Gicquiaud et al., 2002).

Therefore, it is reasonable to assume that their accumulation in wheat in significant amounts in response to low temperature functions to protect cell membranes during cold acclimation or fungal attack. This assumption deserves further study.

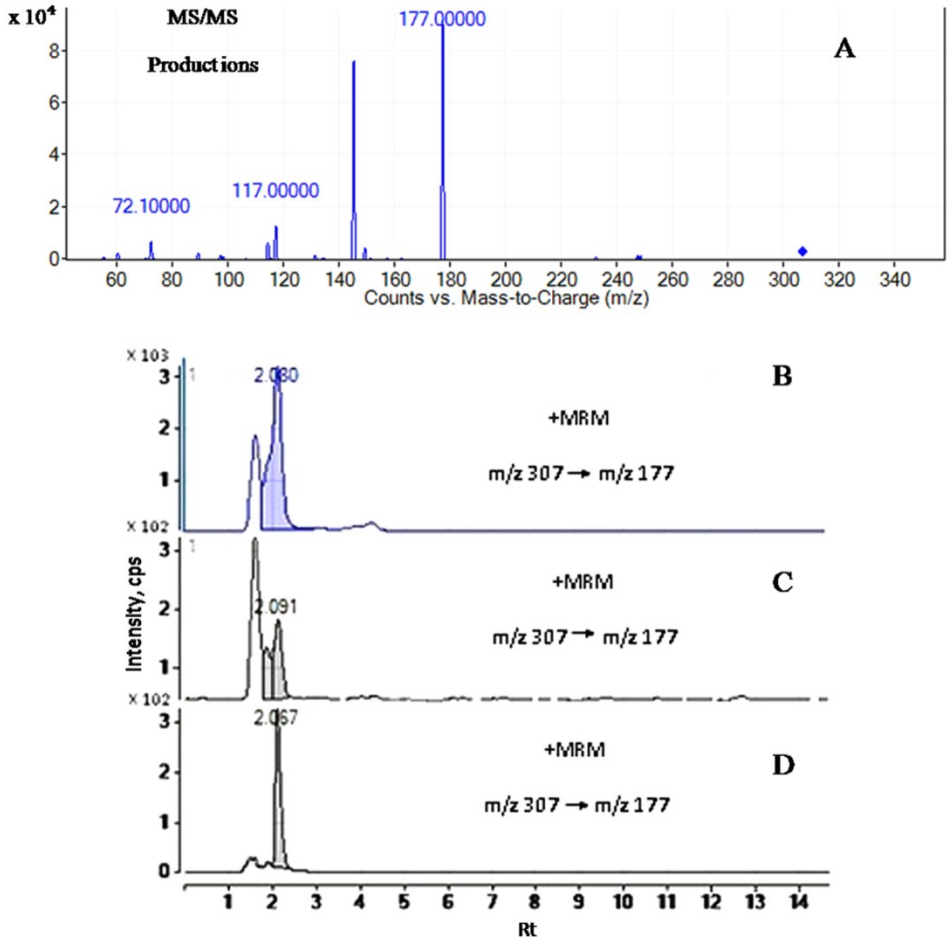


Figure 2-6: Identification of feruloylagmatine (5) in wheat leaves

A, MS-MS of an authentic standard at m/z 307 [M+H]+ showing 307 and 177 as major product ions; B, shows the MRM signals 307 and 177 ions at Rt 2.07 min of 21-day cold-acclimated winter wheat leaf extract; C, corresponding non-acclimated (control) extract; D, that of reference compound.

On the other hand, cold acclimation of the spring variety Bounty also resulted in an increase in iso-orientin derivatives **13**, **20**, **23**, **25**, and **28** (*ca*. 3-fold), similar to Claire. However, it exhibited an important increase in the HCA conjugate, dicaffeoylputrescine **31** (Table 2-1, Fig 2-7), which accounted to *ca* 15% of the total phenolic compounds after cold acclimation. The role of HCAs in plant defense against pathogens is well documented (Hahlbrock and Scheel, 1989), and was also recently reported for *Arabidopsis thaliana* (Muroi et al., 2009).

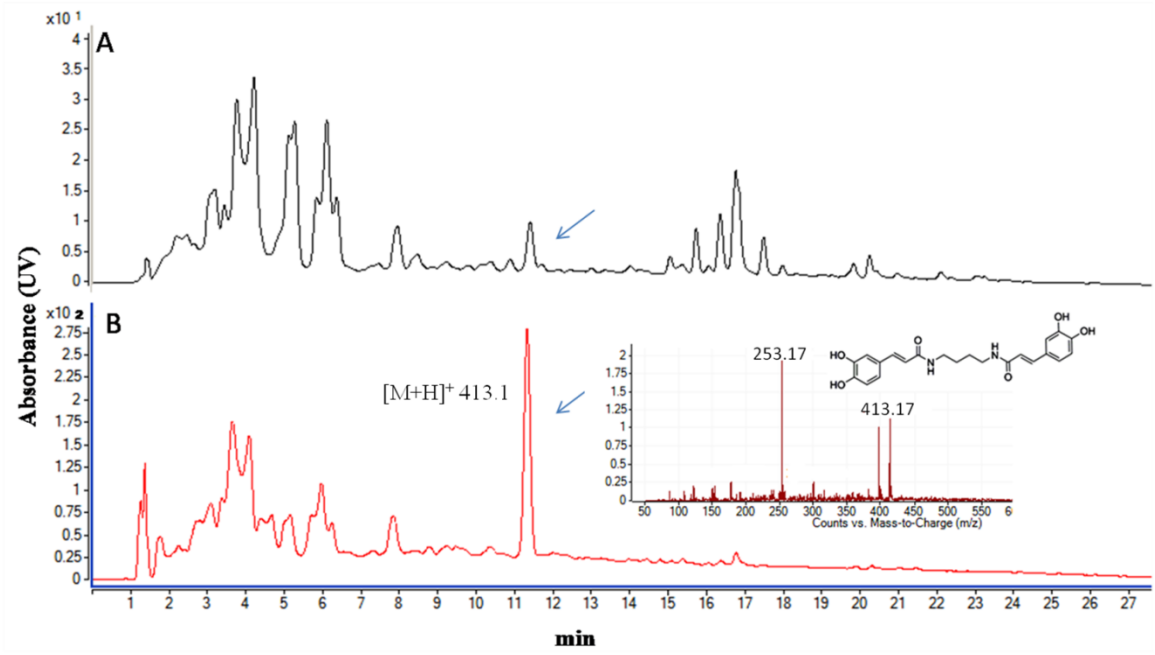


Figure 2-7: HPLC-UV-MS chromatogram of a methanolic extract of 6-day cold-acclimated Bounty leaves

Showing induction of levels of compound 31, dicaffeoyl-putrescine, (Rt 11.2 min) corresponding to [M+H]+ of 413.2

The differential accumulation of two classes of phenolic compounds during cold acclimation: *C*-glycoflavones and their methylated derivatives in the winter variety, and specific HCA conjugates in the spring variety, may provisionally be explained in terms of the differential regulation of expression of the structural genes encoding chalcone synthase (CHS) and hydoxycinnamoyl-CoA:amine-*N*-hydroxycinnamoyltransferase (AHT) that are involved in the biosynthesis of flavonoids and HCA derivatives, respectively (Fig.2-8).

AHTs for both aromatic (Back et al., 2001b); (Farmer et al., 1999) and aliphatic (Negrel, 1989; Negrel et al., 1992) amines have been characterized from several plant species. Such metabolic dimorphism may be considered a valuable agricultural trait that can be applied to the engineering of wheat for increasing its cold tolerance (flavonoids) and antimicrobial constituents (HCAs), or its health promoting flavonoids.

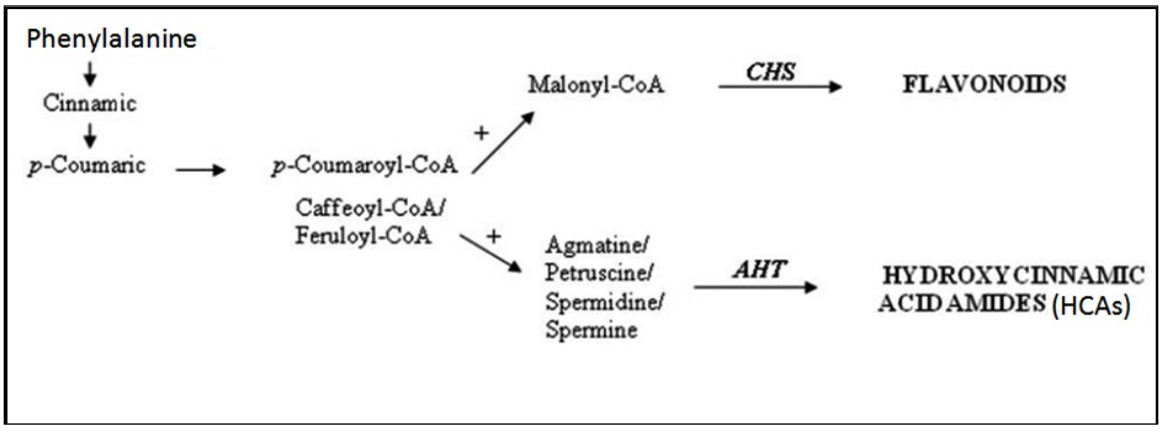


Figure 2-8: Proposed pathway for the regulation of flavonoid and HCAS biosynthesis

Legends: chalcone synthase (CHS), amine-N-hydroxycinnamoyltransferase (AHT)

O-Methyltransferase activity of cold-acclimated winter wheat

The fact that the methylated derivatives **20**, **23** and **28** of luteolin **(36)** are among the major flavonoid constituents of the cold-acclimated winter variety, prompted us to investigate the methylation process by measuring *O*-methyltransferase (OMT) enzyme activity of wheat leaves. Protein extracts of 6, 12, and 21-day, cold-acclimated Claire leaves were assayed for their OMT activities against tricetin and 5-hydroxyferulic acid, as substrates. The presence of internal phenolic substrates within the crude protein extract was accounted for, by subtracting the activity of the enzyme in the absence of added external substrates. The enzyme activity with internal substrates was used as a blank value of the reaction which varied between 5 and 10% of total activity, depending on variety and cold acclimation. The OMT activity against tricetin and 5-hydroxyferulic acid (Yamamoto et al., 1987) as substrates increased by 4- to 5-fold after 12-day and 21-day cold acclimation (Fig. 2-9). The increase in OMT activity is paralleled with the observed increase of methylated phenolic compounds during cold acclimation.

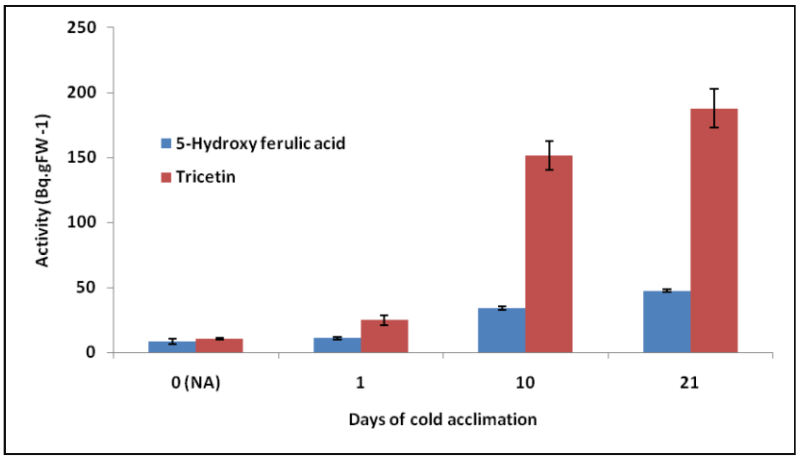


Figure 2-9: Changes in total methyltransferase (OMT) activities in winter wheat leaves during cold acclimation against tricetin and 5-hydroxyferulic acids as substrates.

Values represent mean ± SE from two independent experiments. (NA), 7-day-old non-acclimated plants

Localization of phenolic derivatives in leaf apoplast fluid

Leaf apoplast is not only considered a storage cellular compartment but also an internal physiological environment of the plant where important reactions, such as intercellular signaling and cellular response to many abiotic and biotic stress stimuli, take place (Fecht-Christoffers et al., 2003; Sakurai, 1998; Sattelmacher, 2001).

This prompted us to investigate the phenolic content and profile of the apoplast fluid of wheat leaves in relation to cold acclimation. The analysis of apoplastic fluid on SDS-PAGE, exhibited a typical pattern of apoplastic proteins, and the absence of any trace of Rubisco that is usually used as indicator of cell leakage. This indicated that the apoplastic extract was not contaminated with any of the intracellular metabolites**.**

The analysis of apoplastic phenolic content suggested the presence of 12 flavonoids and 5 HCAs (Figs. 2-10 and 2-11). These compounds were observed in quantities comparable to those obtained with the total methanol extracts, and their level of accumulation during cold acclimation was consistent with that observed in total extract; since the calculated % relative abundance of each compound in the apoplast extract and in the non-hydrolysed MeOH extract were identical, as well as their fold increase after cold acclimation (Table 2-1). In addition, the peaks obtained from LC-MS analyses were sharp and symmetric, with no background contaminants as those usually observed with crude plant extracts (Fig. 2-11).

p-Coumaroylagmatine (**1)**, *p*-coumaroylputrescine (**3)**, feruloylputrescine (**4)**, *trans*-feruloylagmatine (**5)** and *p*-coumaroyl-2-hydroxyputrescine (**7)** were identified in the apoplast fluid, together with the flavonoids 8, 13, 14, 20, 23, 25 and 28 (for iso-orientin (**13)**, its methylated form iso-scoparine (**23)** and their glycoside derivatives (**8,**

14, 20, 25, 28), in addition to isovitexin (**22)** and tricin derivatives (**26**, **27, 37**, and **40)**. These flavonoids were found as glycosides, containing at least one sugar moiety, except for tricin (**37)** and its methylated derivatives (**40)** that were present as aglycones. Glycosylation of phenolics increases their hydrophilicity and stability, and modifies their subcellular localization and binding properties**.** The sugar moieties are responsible for enhancing the water solubility of these compounds and thus facilitate their translocation within the cell from their site of biosynthesis to reach the apoplast (Kren and Martinkova, 2001; Wang and Hou, 2009). The hydrophobic flavonoids that represent most of the identified compounds in the apoplast extract are probably translocated to the apoplast by transmembrane protein carriers, such as ABC transporters. These transporters were found to play an important role in the translocation of isoflavone aglycone genistein into the soybean apoplast by ABC- transporter (Zhao and Dixon, 2010), an example among others.

The presence of such flavonoids and HCAs in the apoplast suggests its vital role as the first site of plant defense against abiotic stresses, such as low temperature. This finding is consitent with a recent proteomics analysis demonstrating the activation of pathogen defense enzymes (β-1,3-glucanase, peroxidase, PR4, and endochitinase) in the apoplast of rape seed (*Brassica napus* var. *napus*) infected with *Verticillium. longisporum.* (Floerl et al., 2008). It was also reported that these enzymes accumulate during cold acclimation in both wheat and rye (Griffith and Yaish, 2004).

The accumulation of both flavonoids and pathogen defense enzymes in the apoplast in response to pathogen attack and cold acclimation supports the hypothesis that the apoplast functions as the first line of defense against both biotic and abiotic stresses.

However, the nature of the interactions between the flavonoids and the defense enzymes in protecting the plant cell against these stresses deserves further investigation.

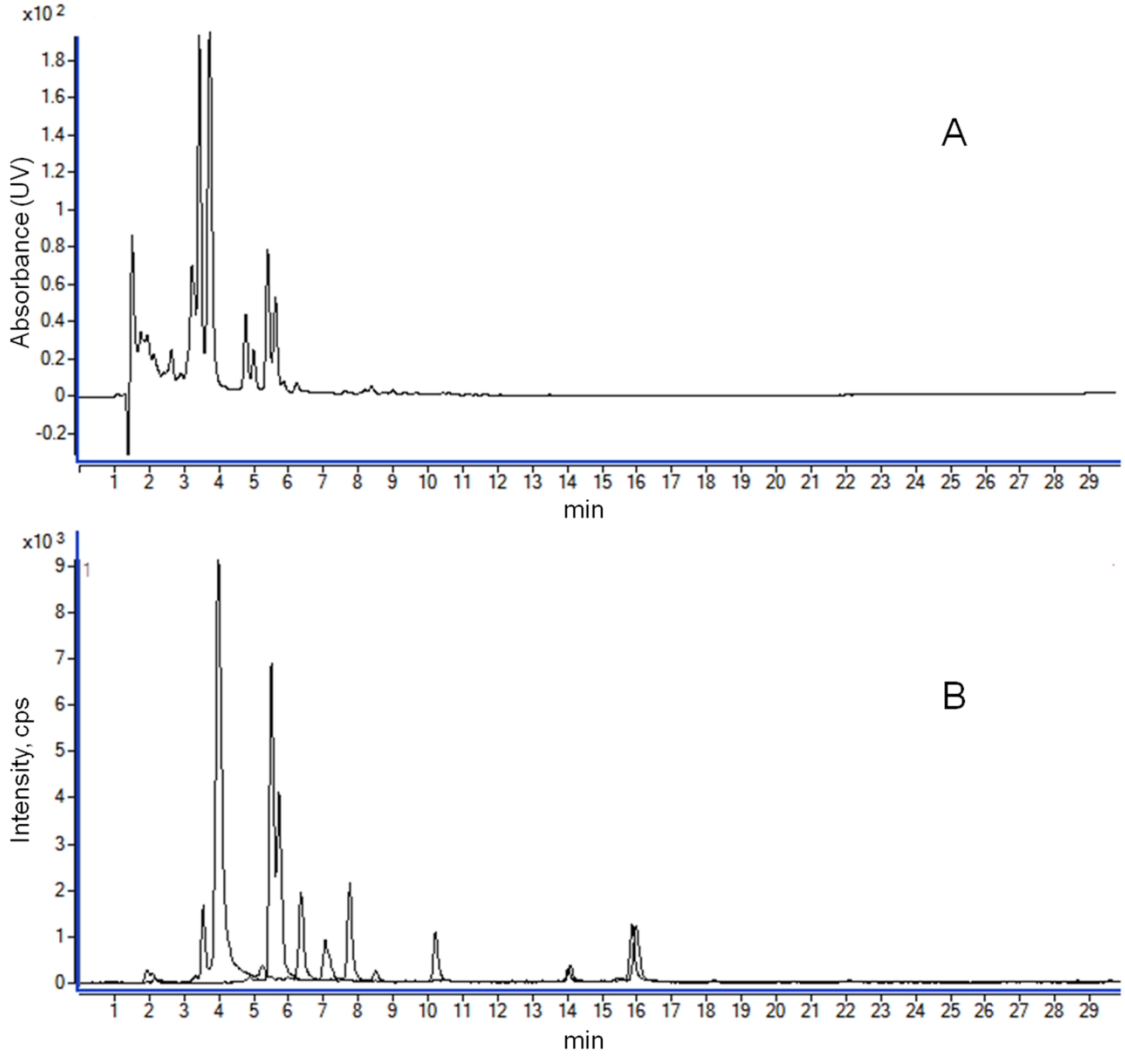
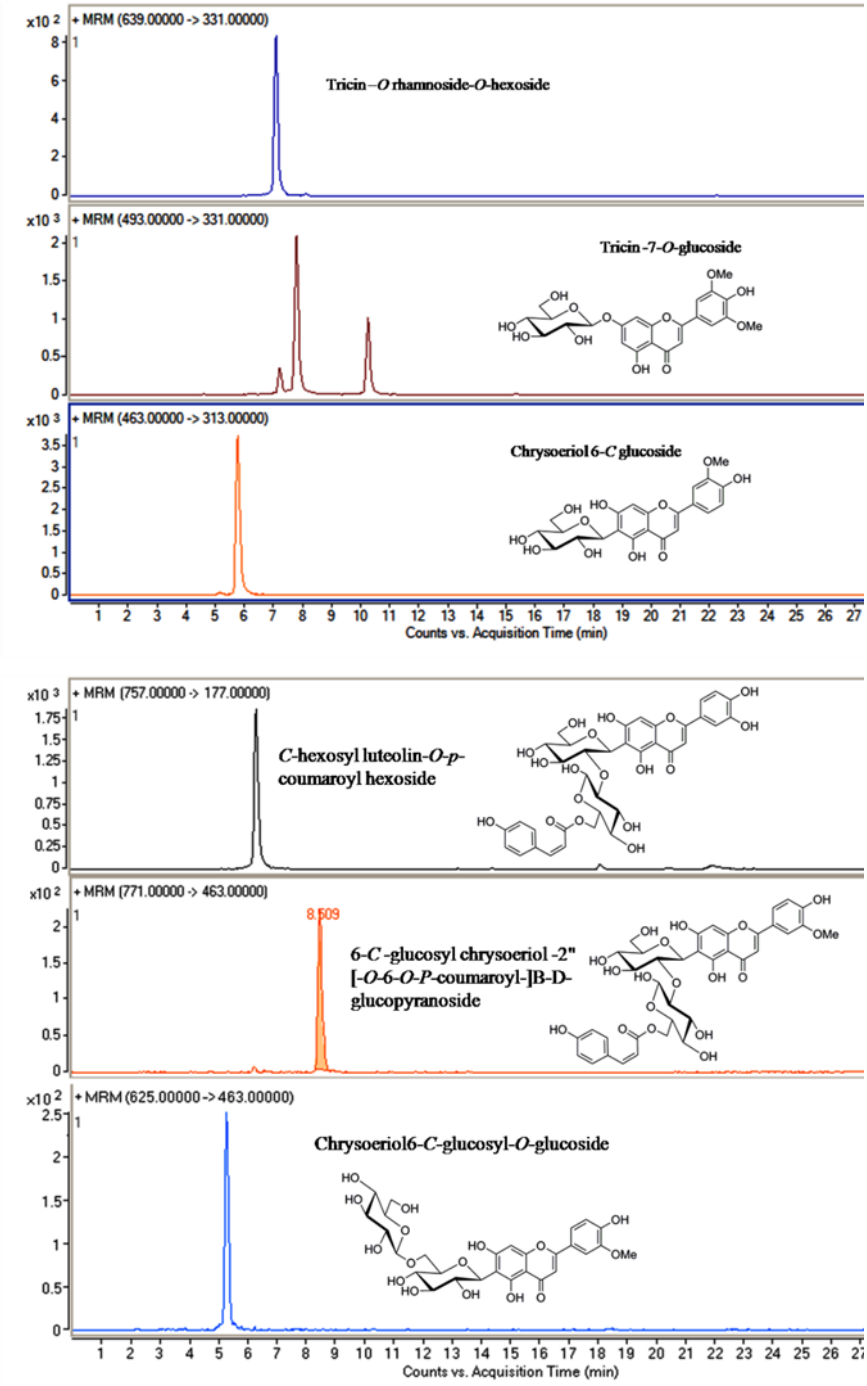


Figure 2-10: HPLC-UV-MS of apoplast extract from 21 day cold-acclimated winter leaves.

(A)UV-chromatogram monitored at 340nm; (B) Total ion counts (TIC)-overlaid trace chromatograms in MRM mode of the identified phenolic compounds.


+ MRM (639.00000 -> 331.00000)
Tricin –O rhamnoside-O-hexoside
+ MRM (493.00000 -> 331.00000)
Tricin -7-O-glucoside
+ MRM (463.00000 -> 313.00000)
Chrysoeriol 6-C glucoside
Counts vs. Acquisition Time (min)
+ MRM (757.00000 -> 177.00000)
C-hexosyl luteolin-O-p-coumaroyl hexoside
+ MRM (771.00000 -> 463.00000)
8.509
6-C -glucosyl chrysoeriol -2" [-O-6-O-P-coumaroyl-]B-D-glucopyranoside
+ MRM (625.00000 -> 463.00000)
Chrysoeriol6-C-glucosyl-O-glucoside
Counts vs. Acquisition Time (min)

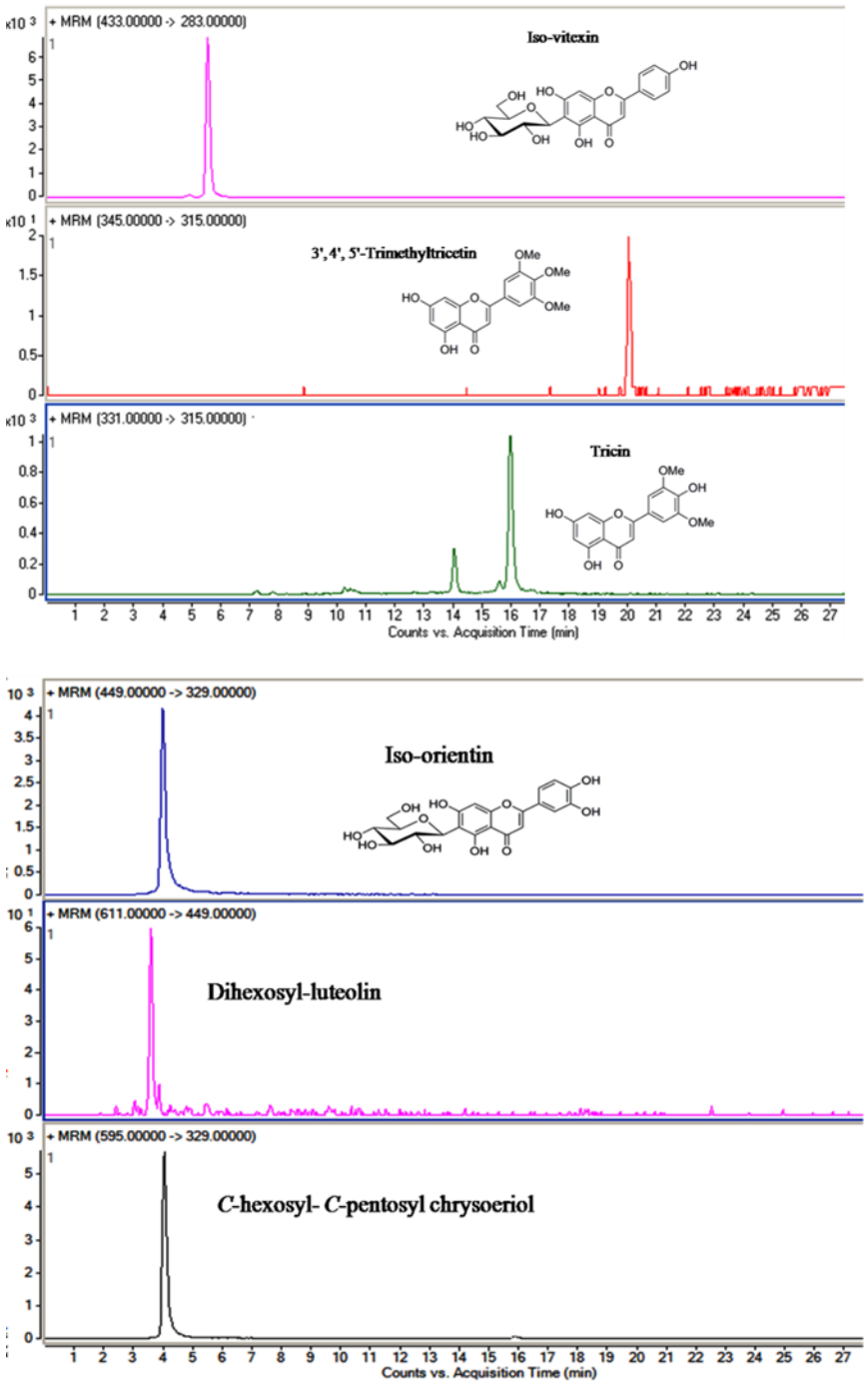


Figure 2-11: MRM chromatogram [MS-MS] of identified flavonoids identified in the apoplast fluid of the winter wheat Claire at 21 days of cold acclimation.

*Parent ion $[M+H]^+$ and its major ion peak fragments in the positive mode resulting from LC-MS-MS analysis, showing MRM at: 639 ->331 for tricin-O-rhamnoside-O-hexoside (**26**), 493-> 331 for tricin-O-malonyl hexoside (**27**), 463 ->313 for iso-scoparin (**23**), 757*

*->177 for luteolin-C-hexosyl-O-(p-coumaroyl) hexoside (**25**), 771 ->463, 771 ->625 for chrysoeriol-6-C -glucosyl -2"[-O-6-O-P-coumaroyl-]B-D-glucopyranoside (**28**), 625 -> 463 for chrysoeriol-6-C-glucosyl-O-glucoside (**20**), 433 ->283 for iso-vitexin (**22**), 345 ->255 for 3', 4', 5'-trimethyltricetin (**40**), 331 ->315 for tricin (**37**), 449 -> 329 for iso-orientin (**13**), 611->449, 611 ->329, for dihexosyl luteolin-(**8**), and595->329 for luteolin-O,C-rhamnosyl-glucosyl (**14**),*

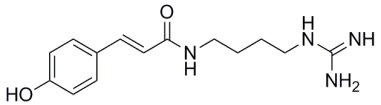
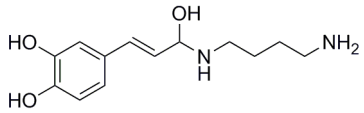
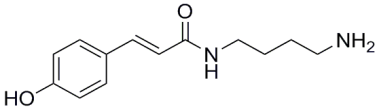
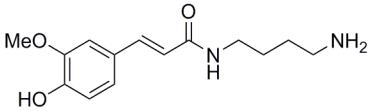
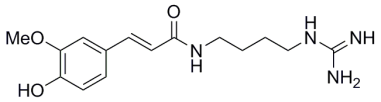
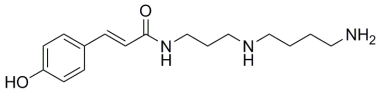
In this study the application of LC-ESI-MS protocols, coupled with the MRM technique described here, have shown to be powerful tools for the direct chemical screening of phenolic compounds in wheat leaves. They provided accurate, reproducible results, and allowed the characterization of some novel metabolites, and established the differential induction of levels of phenolic compounds in both winter and spring wheat when grown under cold acclimation conditions.

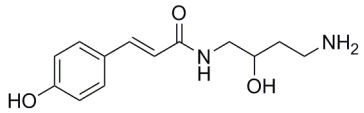
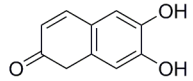
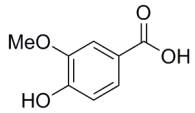
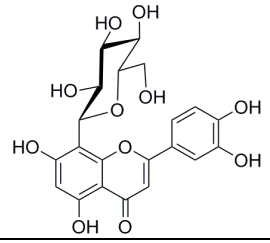
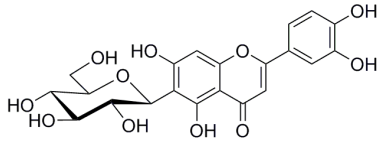
Iso-orientin (**13)** and its *C*-glycoside derivatives (**8**, **11**, **20**, **23**, **25**, **28)** followed by vitexin (**21)** and iso-vitexin (**22)** represented the most significant increase in phenolic derivatives during cold acclimation of the winter variety, whereas, the accumulation of dicaffeoylputrescine (**31)** was the predominant metabolite in the spring variety. Identification of the novel flavone, 3',4',5'-trimethyltricetin (**40)**, as well as feruloylagmatine (**5)**, by their characteristic product ion fragments will serve as future reference sources for easy detection of both compounds in plant extracts.

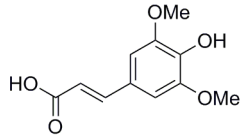
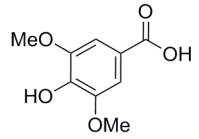
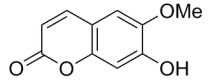
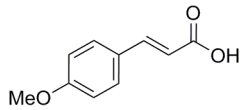
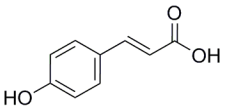
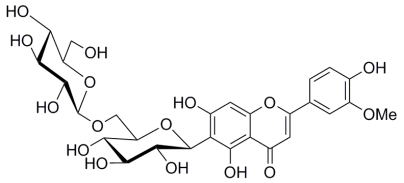
The fact that most of flavonoids and HCAs were identified in the apoplast compartment confirms its important role in plant defence mechanisms. On the other hand, the accumulation of a mixture of beneficial flavonoids as iso-orientin (**13)**, vitexin (**21)** and tricin (**37)** in cold acclimated wheat leaves, values its potential use as a source of an

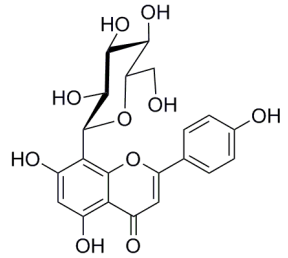
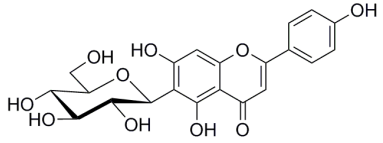
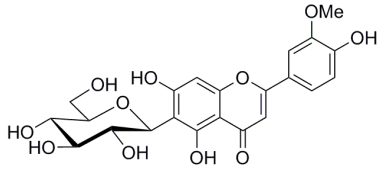
inexpensive and affordable supplement of a healthy diet, which may explain the popularity of wheat leaf juice that is gaining acceptance in North America and elsewhere as a beneficial healthy supplement.

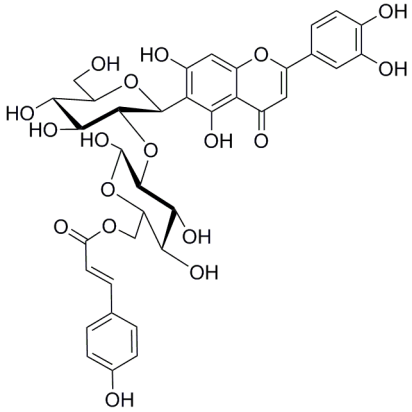
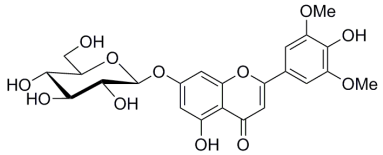
Table 2-1: Characterization of phenolic compounds common to the non-hydrolysable extracts of winter and spring wheat

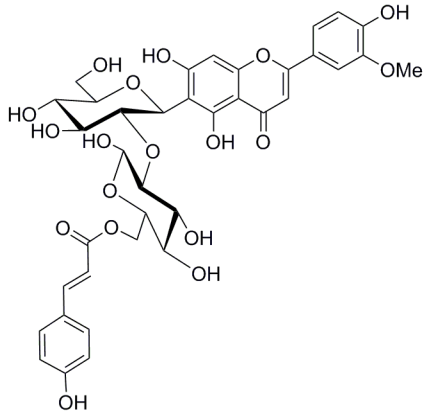
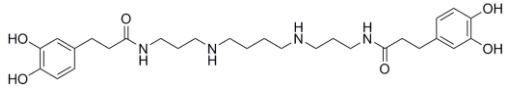
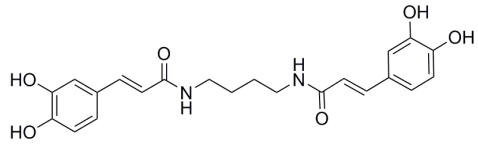
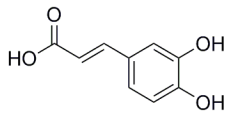
Peak[±]	Rt min	$[M+H]^+$	MRM (MS/MS) (Transition ions)	Measured accurate mass	Diff from theoretical target mass (ppm)	Identification	Structure	% Relative abundance[b]	Fold - increase after cold treatment
1	1.3	277	277 → 147	276.1599	4.5	*p*-Coumaroyl agmatine		0.24	2
2	1.7	253	253 → 163	252.1464	3.9	Caffeoyl putrescine (Paucine)		0.4	No change
3	1.9	235.2	235 → 147	234.138	5.1	*p*-Coumaroyl putrescine		0.2	2
4	2	265	265 → 177	264.147	-1.4	Feruloyl putrescine		0.06	17
5	2.06	307	307 → 177	306.1707	5.0	Trans-feruloyl-agmatine		0.03	20
6	2.1	292.3	292 → 147	291.1930	5.7	*p*-Coumaroyl-spermidine		0.1	2

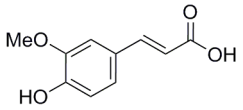
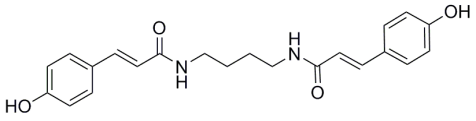
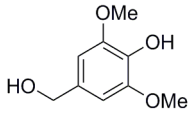
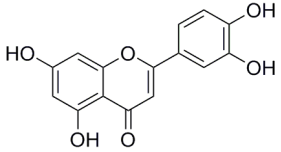
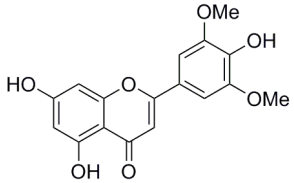
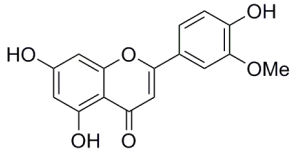
7	2.3	251.2	251 → 147	250.1311	-2.6	*p*-Coumaroyl-2-hydroxy putrescine		0.25	1.2
8	2.8	611	611 → 329 611 → 449	610.1512	-3.6	Luteolin-dihexosyl	-	0.1	3
9	2.9	179	179 → 133	178.0259	-3.2	Esculetin		0.01	7
10	3.5	169	169 → 153	168.0419	-4.1	Vanillic acid		1.5	No change
11	3.7	525	525 → 463			Chrysoeriol glycoside derivative	-	0.4	1.3
12	3.8	449	449 → 431	448.0999	-1.5	Luteolin 8-*C*-glycoside (**Orientin**)		33	3
13	4.4	449	449 → 329 449 → 299	448.0999	-1.5	Luteolin-6-*C* glucoside (**Iso-orientin**)			

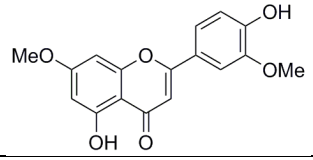
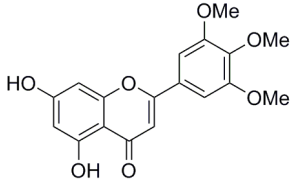
14	4.3	595.2	595 → 329	594.1549	- 6.0	Luteolin-*O,C*-rhamnosyl-glucosyl	-	0.74	1.5
15	4.7	225	225 → 207	224.0693	3.4	Sinapic acid		0.02	3
16	4.8	199	199 → 184	198.0537	4.4	Syringic acid		trace	No change
17	4.9	193	193 → 178 193 → 150	192.0430	3.96	Scopoletin		0.06	3
18	5.1	179.1	179 → 164	178.0622	-4.46	*p*-Coumaric acid methyl ether		trace	trace
19	5.2	165.1	165 → 121	164.0463	-6.3	*p*-Coumaric acid		trace	trace
20	5.3	625	625 → 463	624.1683	-1.2	Chrysoeriol 6-*C*-glucosyl-*O*-glucoside		0.42	2

21	5.8	433	433 → 283	432.1054	-0.4	Apigenin 8-*C* glycoside (**Vitexin**)		18	3
22	6.1	433	433 → 283	432.1054	-0.4	Apigenin 6-*C* glycoside (**Iso-vitexin**)			
23	6.3	463	463 → 313	462.1141	-4.4	Chrysoeriol 6-*C* glucoside (**Iso-scoparin**)		7.8	1.3
24	6.4	609	609 → 301 609 → 463	608.1500	-4.92	Chrysoeriol-*O*-*p*-coumaroyl hexoside	-	0.6	No change

25	7.1	757.2	757 → 177 757 → 287	756.1906	0.6	Luteolin-*C*-hexosyl-*O*-(*p*-coumaroyl) hexoside		0.4	10-to15-fold increase
26	7.5	639	639 → 331 639 → 493	638.1833	-2.1	Tricin –*O*-rhamnoside-*O*-hexoside	-	0.8	2
27	8.4	493	493 → 331	492.1261	-1.3	Tricin -7-*O*-glucoside		8.5	1.5

28	**8.9**	**771**	771 → 463 771 → 625	770.2033	-3.2	Chrysoeriol-6-*C*-glucosyl -2" [-*O*-6-*O*-*p*-coumaroyl-]B-D-glucopyranoside		0.15	3
29	10.3	579	493 → 331	578.1258	-2.3	Tricin –*O* malonyl hexoside	-	2.1	No change
30	11.0	531.6	531 → 166	530.3109	0.8	Bis-dihydro caffeoyl spermine		traces	Traces
31	11.1	413	413 → 253	412.1644	2.3	Dicaffeoyl-putrescine		4	ca 20 (in bounty only)
32	12.9	181.1	181 → 137	180.0427	2.4	Caffeic acid		trace	-

33	13.6	195	195 → 136	194.0589	5.1	Ferulic acid		trace	-
34	14.08	381.4	381 → 253 381 → 147	380.1717	-5.0	Di-*p*-coumaroyl-Putrescine		0.01	20
35	14.3	185.1	185 → 170	184.0724	-6.3	Syringyl alcohol		0.01	-
36	15.5	287	287 → 153	286.0464	-4.6	Luteolin		0.2	1.7- fold decrease
37	16.7	331	331 → 315 331 → 270	330.0748	2.5	Tricin		1.5	No change
38	18.1	301	301 → 285	300.0624	3.2	Chrysoeriol		0.05	1.4

39	18.9	315.2	315 → 299	314.0782	-2.5	Chrysoeriol-7-methyl ether		0.1	2-fold decrease
40	20.6	345	345 → 255 345 → 315	344.0880	4.6	3', 4', 5'-Trimethyltricetin		0.01	No change

[a] Identification of compounds: 5, 9, 10, 13, 15, 16, 17, 19, 21, 32, 33, 35, 36, 37 and 40 was confirmed by external reference compounds via comparison of their $[M+H]^+$, MS-MS and MRM. All other compounds were identified by comparison of their mass profiles $[M+H]^+$, MS-MS and MRM with those published (Cavalière et al., 2005; Cummins et al., 2006; Yannai, 2004), in addition to accurate measurement of their masses that confirm their molecular structures. UV wavelength was monitored at 340 and 254 nm for all compounds.

[b] % Relative abundance was calculated for each compound by dividing its area under the peak and summation of total phenolic peaks x 100

Chapter 3 WINTER WHEAT HULL (HUSK) IS A VALUABLE SOURCE OF TRICIN, A POTENTIAL SELECTIVE CYTOTOXIC AGENT

The previous chapter focused on qualitative and quantitative analysis of phenolic compounds including tricin in two different varieties of wheat leaves, under both normal and cold stress conditions. Its richness with a variety of beneficial phenolic compounds highlights its importance as a beneficial healthy supplement. In this chapter we study the occurrence and distribution of tricin in different parts of the wheat plant, with aim to find an affordable economic source. Furthermore, tricin activity was tested on the viability of two cancer cell lines of the liver and pancreas and one normal cell lines.

Techniques used in this chapter included HPLC, LC/MS, and flow cytometry (FACScan) for viability testing, and dissolution tester.

The manuscript of this chapter is planned to be submitted for publication in "*Phytomedicine*" under the title: "Winter wheat hull (husk) is a valuable source of the selective anticancer agent, tricin."

Summary

The flavone tricin (5,7,4'-trihydroxy-3',5'-dimethoxyflavone) has the greatest potential as anticancer and chemopreventive activity. In spite of these valuable biological benefits, its investigation in preclinical and clinical settingsis still limited. This is due to its rare availability and high production cost. Tricin is found mainly in cereal grains, such as wheat, rice, barley, oat and maize. The highest tricin concentration reported was in *Sasa albo-marginata*; and rice *Oryza sativa.* However this concentration is not sufficient for commercial use. To find another reliable rich source of tricin, we investigated its occurrence and distribution in different parts of wheat (*Triticum aestivum*), an important cereal and a staple diet for human and animal nutrition. The highest amount was found in the husk of winter wheat varieties and estimated to 770 ± 157 µg/g dry materials. This concentration is considered the highest in any plant materials suggesting the use of winter wheat husk as a good source of tricin. The purified wheat tricin was found to be selective potent inhibitor of two cancer cell lines of the liver and pancreas, while having no side effect on normal cells. This selectivity, makes tricin a potential candidate for anticancer agent. In addition, the tricin and fibers rich crude wheat husk powder could be used as chemopreventive agent against colon cancer.

Introduction

Flavonoids and polyphenols are ubiquitous in nature. They are naturally occurring compounds that constitute major classes of the plant natural products. They play important roles in plant growth and development, and its interaction with the environment. For humans, flavonoids are important diet constituents and are found as active ingredients in several medicinal plants.

Several studies reported their *in vitro* ability to inhibit the growth of, and in several cases kill, cancer cells (Jacquemin et al., 2010; Li-Weber, 2009; Neto, 2007). Thus, they are considered potential candidates for combating many forms of cancer due to their apparent chemoprevention effect. However, their *in vivo* efficacy in humans is still debatable. This is probably due to their poor bioavailability after oral ingestion (Ta and Walle, 2007; Walle et al., 2007). Their low bioavailability is probably due to their limited absorption and rapid metabolism in the intestine and liver through glucuronidation and/or sulfonation of their free hydroxyl groups. As a result, such glycoside and sulfonated forms lead to higher solubility and thus decreased bioavailability (Walle, 2007). In contrast, methylated flavonoids are less hydrophilic and have higher resistance to hepatic metabolism .Therefore, they exhibit a relatively higher intestinal absorption compared to their non-methylated forms (Wen and Walle, 2006).

Among flavonoids having anticancer activity, tricin (5,7,4'-trihydroxy-3',5'-dimethoxyflavone) has shown the greatest potential (Fig 3-1). It is a naturally occurring flavone of a relatively rare and sporadic occurrence (Wollenweber and Dorr, 2008). It is mainly found in cereal grains, such as wheat, rice, barley, oat and maize. It was first

isolated as a free aglycone from a rust-resistant variety of wheat leaves (*Triticum dicoccum L*. var.Khapli) (Anderson and Perkin, 1931) and identified as the first flavone detected in butterfly wings that feed on grasses (Harborne, 1967).

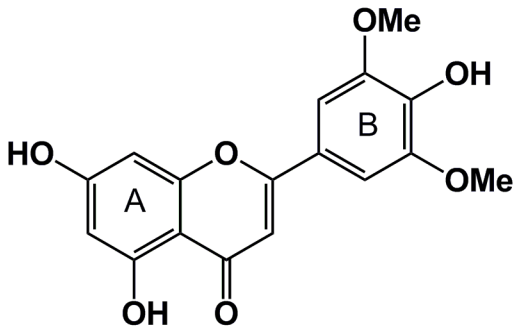


Figure 3-1: Tricin structure

Tricin has been reported as a valuable chemopreventive agent for its several beneficial pharmacological activities (Hudson et al., 2000). It was described as the most potent anti-clonogenic (colony-forming ability) agent of human-derived tumour breast cell lines (MDA MB 468) and human-derived colon carcinoma cell line SW 480 (Hudson et al., 2000). Such property may be attributed to its ability to inhibit cyclooxygenase activity and its interference with intestinal carcinogenesis in mice. This led to further pre-clinical trials to explore its suitability in the treatment of human intestinal polyps (Cai et al., 2005). In addition, tricin inhibited P-glycoprotein activity in adriamycin- resistant human breast cancer cells, thus delaying spontaneous mammary tumorigenesis and suppressing apoptosis oxidative stress-induced (Jeong et al., 2007).

A study of the bioavailability of tricin, conducted on nude mice *in vivo*, revealed its exclusive high level in the GIT (gastro-intestinal tract) after dietary intake, and its effect on slowing cancer cell growth. This result highlighted the potential of tricin in preventing colorectal cancer and led to study of its dietary effect on the prevention of hepatic and gastrointestinal malignancies (Baublis et al., 2000). Furthermore, dietary tricin effectively suppressed azoxymethane (AOM)/dextran sodium sulphate (DSS) - induced colon carcinogenesis in mouse model (Oyama et al., 2009). In addition, tricin was considered as a natural antioxidant and a cardiovascular drug (Chang et al., 2010). Recently, tricin was identified as a potential anti-influenza virus agent in vitro and *in vivo*, as it ameliorated body weight loss and survival rate of influenza-A-virus-infected mice (Yazawa K et al., 2011).

In spite of these valuable biological benefits on human health, the use of tricin is still limited. This is probably due to its rare availability and the relatively high cost. This prompted us to search for a reliable source of tricin at an affordable cost that will enable its use for further biological and pharmaceutical studies.

In Asia, where rice is the main staple food, the incidence of breast and colon cancer is markedly lower than that in the western world, that was attributed to the presence of tricin in rice bran (Hudson et al., 2000).This observation prompted us to evaluate the presence of tricin in other cereals with the aim of finding a valuable natural source of tricin. Our research revealed that wheat is a major source of the chemopreventive agent, tricin. It is present in significant amounts in the hulls of winter wheat, a part of the plant that is considered a waste by-product with low economic value. In this report we describe efficient methods of isolation and purification of tricin from

wheat tissues. We also demonstrate that tricin is a potent growth inhibitor of two cancer cell lines of liver and pancreas, while having no side effect on normal cells.

Materials and methods

Chemicals

Tricin was purchased from Dalton Chemical Company (Toronto, ON). Quercetin, which was used as an internal standard, was from our laboratory collection. Unless otherwise specified, all other chemicals were of analytical grade.

Plant growth conditions

Three wheat varieties (*Triticum aestivum*, 2nx6 = 42), a spring wheat cultivar (Bounty) and two winter wheat cultivars (Claire and Norstar) were grown in a controlled growth chamber at 20 ºC under long days (LD), with 16-h photoperiod at a light intensity of 250 μmol m^{-2} s^{-1}.

Extraction of tricin

Ab extraction procedure was generally carried out using *ca.* 10 g of plant material and 85% aq. MeOH (1:3, w/v) as solvent, and concentration of the methanolic extracts under vacuum to *ca* 2 ml before injecting 60 μl-aliquots for HPLC or LC-MS analyses.

For three month green inflorescence tissues and yellow straw samples, removal of the grains was carried out before extraction. Wheat bran was obtained after grinding the grains and passing through sieves (mesh size #12) to remove the endosperm and embryo layers. In case of seven-day-old fresh leaves and roots the remaining aq. portion, after

concentration, was defatted with a mixture of hexanes to remove chlorophyll and wax, and 60 µl of the defatted extract was injected to HPLC or LC-MS analyses.

HPLC and LC-MS analyses

For quantification of tricin, aliquots from the MeOH extract were chromatographed on a Varian HPLC system equipped with a UV detector, and a Varian Polaris 5 -C18-A column (4.6×150 mm; particle size, 5µm) using a linear gradient of 40% MeOH in 1% aq. HOAc at a flow rate of 1 $ml.min^{-1}$ for 30 min and a column temperature of 25ºC with monitoring at at 340 nm. The analysis was repeated with three biological replicates at least three times, and three injections were analyzed for each sample. Quantification was carried out using the 'area under the curve' method by calculating the areas in relation to their UV absorbance at 340 nm.

LC-MS analyses were performed using an Agilent 1200 HPLC system with a binary pump, in-line degasser, high performance auto-sampler and thermostated column division, using a linear gradient of 40% to 90% of MeOH: 0.1% aq. HCOOH for 30 min with a flow rate of 0.35 ml/min on an Agilent SB-C18 column (2.1×30mm; particle size, 3.5µm) and a column temperature of 25ºC. UV detection at 254 and 340 nm was used.

Two LC-ESI-MS systems were used: in System 1, the HPLC instrument was connected to an Agilent 6410 quadrupole (triple Q) mass spectrometer using electrospray ionisation in positive ESI mode with the following conditions: capillary voltage, 3000 v; nebulizer pressure 60º Psi; gas temperature, 300ºC; drying gas, 5 L/min; dwell time, 75 ms and the data was processed using the Agilent Mass Hunter software.

The same Agilent SB-C18 column was transferred to be used in system 2, where an identical HPLC instrument with the same conditions listed above was connected to another mass spectrometer consisting of an Agilent 6210 electrospray ionization-time-of flight analyzer (ESI-TOF) in the positive ESI mode at a capillary voltage of 4000V, nebulizer pressure of 35 Psi, gas temperature 350ºC, drying gas flow: 11.5 L/min and voltages of 125V and 60V for the fragmentor and the skimmer, respectively. The technical error and mass resolving power of the time-of-flight mass spectrometer in terms of mass accuracy was 2ppm RMS, measured at the $[M+H]^+$ ion of reserpine (m/z 609.2807) used as an internal mass reference. When available, reference compounds were used to compare the corresponding retention times and mass spectral profiles of phenolic derivatives and flavonoids.

A tricin calibration curve was constructed for quantification purposes, using different concentrations of 1, 5, 8.3, 16.6, 25, 166 and 272 $\mu g.ml^{-1}$ tricin, and quercetin was added as an internal standard. For MS/MS and multiple reaction monitoring (MRM) analyses in system 1, the collision energy (CE) was optimized; a CE of 35eV was applied as the optimum energy of collision for the analysis of tricin, and the scan range was from 200 to 900 m/z. MRM acquisition was carried out by monitoring transitions of the combination of the parent ion mass in positive mode, 331m/z and the fragment ions of highest abundance were 315 and 270 m/z.

INS-832/13, NIH 3T3 and HepG2 cell culture

Rat pancreatic cells (INS832/13), an insulin-secreting cell line derived from an X-ray-induced rat transplantable insulinoma cell line, were grown in monolayer cultures in a regular RPMI[3]-1640 (Sigma-Aldrich) medium supplemented with 10 mM HEPES, 10% heat-inactivated FBS, 2 mM ι-glutamine, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified (5% CO_2, 95% air) atmosphere.

Human hepatocellular carcinoma cells (HepG2) were grown in monolayer cultures in a regular MEM medium, supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin at 37 °C in a humidified (5% CO_2, 95% air) atmosphere (Grondin et al., 2008).

As the cells reached 80% confluence, after approximately 7 days, they were washed twice with PBS, and trypsin was added for 2-4 min. Trypsin was inactivated by addition of RPMI after centrifugation, the pellet was resuspended in a minimal volume.

Mouse Fibroblast cells (NIH 3T3) were grown in monolayer cultures in regular DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin at 37 °C in a humidified (5% CO_2, 95% air) atmosphere.

Cells were cultured in 6-well tissue culture plates in the specific media described above using a humidified atmosphere of 5% CO_2 and 95% air at 37˚C. One day after plating, tricin (7.5, 15, 30µM in DMSO) was added to the medium for 24 h. Following the addition of tricin, cells were trypsinized and counted by facs scan with CountBright absolute counting beads which are broadly fluorescent

[3] Roswell Park Memorial Institute

(http://probes.invitrogen.com/media/pis/mp36950.pdf). Fluorescence is excited by wavelengths from UV range to 635 nm, and its emission can be read between 385 nm and 800 nm.

Cell concentration was calculated according to the following formula

A x C / B x D = concentration of sample as cells/µL; Where:
A = number of cell events
B = number of bead events
C = assigned bead count of the lot (beads/50 µL)
D = volume of sample (µL)

Dissolution tests in vitro

The in vitro dissolution tests were carried out in a paddle system at 37º C and a speed of 70 rpm using an USP dissolution apparatus II (Distek 5100, North Brunswick, NJ, USA) .Wheat husks of the winter wheat Norstar were finely powdered to a mesh size # 30 before being used. The powder (3g) was added to 900 mL of two types of dissolution media: an acidic, 0.1 N HCl and an alkaline (pH 7.5 to 8) phosphate buffer. Samples were withdrawn at 10 min-intervals over a period of 0.5, 1, 2, 4, 6, 8, 12 and 24 h, and immediately injected into the Varian HPLC-UV system for analysis.

Tricin isolation from wheat husk

The crude MeOH extract of yellow husks was purified using a flash chromatography system CombiFlash Retrieve® (Teledyne Isco, Inc.USA) equipped with a RediSep® silica gel column and a UV/VIS detector. Elution of the column started with 100% CH_2Cl_2 to 50% MeOH in CH_2Cl_2.

Results and discussion

Identification of tricin in different parts of the wheat plant

Tricin was extracted from roots, leaves, bran, and straw (inflorescences and their stalks) as described in the Materials and Methods section. The extract was analysed by LC-MS or HPLC-UV systems and monitored at 340 and 254 nm for identification and quantification. Although, tricin was found mainly as a free aglycone, the presence of its glycosides could not be ignored since they contribute to the total amount of tricin that varies according to the plant age and the type of tissue. To determine the total content of tricin, acid hydrolysis was carried out on the extracts from leaves, stalks and bran in order to liberate the free aglycone.

Tricin was identified in all parts of the wheat plant except roots that lacked the presence of any significant flavonoids. Its identification was based on comparisons of retention time, MS and MS-MS profiles with those of a reference sample. Major ions products identified from the MS-MS were 315 and 270 in the positive mode (Fig 3-2), and the measured accurate $[M+H]^+$ mass was 331.0817 with a difference of 1.42 ppm from the theoretical calculated value.

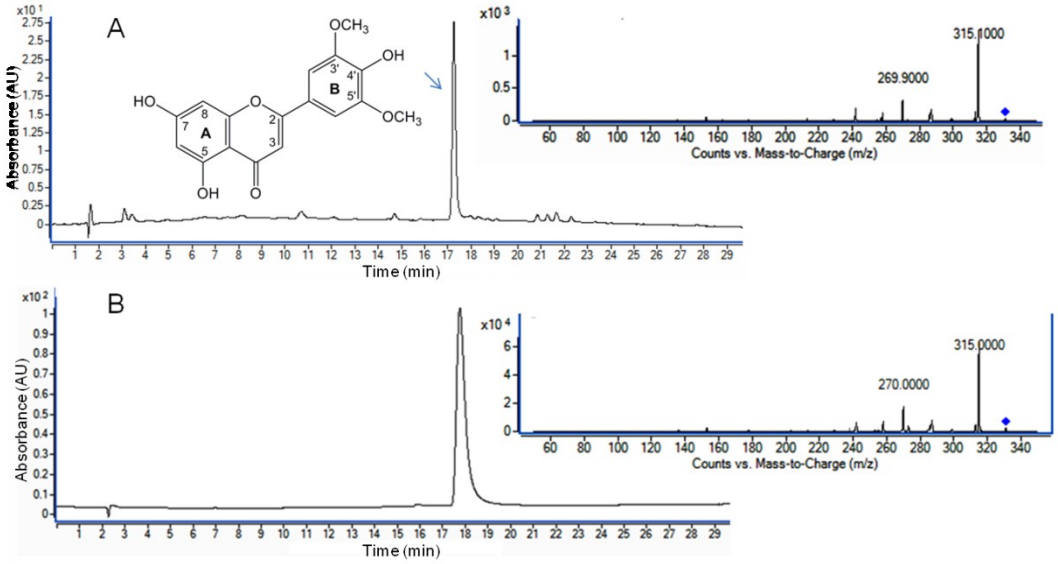


Figure 3-2 HPLC-UV chromatograms with the corresponding ions fragments obtained by MS/MS of **(A)** methanol extract of yellow dry inflorescences husk from Norstar variety of wheat and **(B)** tricin reference sample.

The UV and TIC chromatograms were obtained using an Agilent SB-C18 column (2.1 × 30mm; particle size, 3.5µm), and a column temperature of 25ºC; 40% to 90% Methanol in 0.1% HCOOH of linear gradient elution; flow rate 0.35 ml/min for 30 min.; injection volume, 10 µl; wavelength monitoring, 340 nm. The HPLC instrument was connected to an Agilent 6410 quadrupole (triple Q) mass spectrometer using electrospray ionisation in positive ESI mode with the following conditions collision energy of 30 eV capillary voltage: 3000 v, nebulizer pressure: 60º Psi, gas temperature 300ºC, drying gas: 5 L/min and a dwell time of 75 ms and the scan range was from 200 to 900 m/z.

Quantification and localization of tricin

Tricin accumulates primarily in the aerial part of the plant, including leaves and husk, and accumulates at higher amounts in the winter wheat variety (Claire) compared to the spring wheat Bounty (the spring phenotype) (Table 3-1). These results are in agreement with our previous study that showed a higher accumulation of phenolic compounds in the leaves of winter wheat than the spring variety (Moheb et al., 2011).

Leaf extracts from both cultivars are rich in other phenolic constituents and tricin content was estimated to range from 8-10% of the total phenolic compounds (see Table2-1).

However, the richness of wheat bran with several beneficial phenolic compounds and dietary fibers made it a valuable additive in several food products (Anderson et al., 2009; Dykes L., 2007; Verma et al., 2008). A recent review of the methods of analysis of dietary fiber in food was recently published (Dhingra et al., 2011).

Our analyses performed on different plant parts revealed that the highest amount of tricin was observed in 3-month old seedless mature inflorescences, also termed 'hull or husk' (Table 3-1). Such amount ranged from 750 to 795µg tricin/g dry weight of Claire hull, compared to 416 to 460 µg/g dry weight in Bounty, that corresponds to 40-45% and 30-35% of total phenolic compounds, respectively.

It is interesting to note that as the wheat plant matures and turns yellow, most of the phenolic and flavonoid compounds disappear, leaving tricin as the major constituent. The percentage of tricin in the green inflorescences of winter wheat Claire ranges from 25% to 27% compared to 45% of total phenolic content in the yellow straw. This suggests that during later stages of development, the plant modulates its metabolic pathway by

modifying its phenolic pattern towards the synthesis and accumulation of tricin, in order to protect the grains against biotic and abiotic stresses. This conclusion is in agreement with the latest report highlighting the allelochemical property of tricin in protecting rice seedling against rot disease (Kong et al., 2010).

Table 3-1: Tricin content in different parts of the two wheat cultivars (Claire and Bounty)

Part of the plant	µg Tricin/g dry weight	% Tricin of total phenolic compounds
Leaves:		
Bounty	235±21.2µg/g	7-8%
Claire	253±18.3 µg/g	9-10%
Bran:		
Bounty	45±8.6µg/g	1.2 -1.5%
Claire	33±15.9 µg/g	1.8 - 2.2%
Husk		
Bounty	408±11.3µg/g	30-35%
Claire	772±31.8µg/g	40-45%

Wheat husk is a good source of tricin

The fact that the winter wheat variety, Claire, accumulates a high level of tricin in the hulls, prompted us to investigate another winter variety (Norstar) for its tricin content. Dry seedless hulls from 3-month old (Norstar) were extracted with 85% aq. MeOH for HPLC analysis, which resulted in the presence of mainly one sharp peak at 17.2 min corresponding to tricin (Fig 3-2). In Norstar hulls, the concentration of tricin was significant, ranging from 770 ± 157 µg/g dry materials and representing 50 to 65% of the total phenolic compounds. In fact, this represents the highest concentration of tricin so far reported in any plant species. The highest tricin concentration so far reported amounted

to 200 µg/kg in *Sasa albo-marginata*; 66 µg/kg from *Oryza sativa* (Oyama et al., 2009), and 33-100 µg/kg obtained earlier from dried wheat leaf (Anderson and Perkin, 1931). This indicates that wheat hulls can be considered a rich source of tricin, particularly from winter wheat varieties. This prompted us to exploit this 'waste by-product' of wheat as a source of this rare chemopreventive flavonoid.

Wheat husk as a source of dietary-fibers

Dietary fibers are categorized according to their water solubility into two types: soluble and insoluble fibers. The insoluble form is also known as 'Novel fibers'(1993; Agency, 2010) and found to be responsible for most of the health benefits of whole grains. In addition to their valuable content of tricin, cereals such as wheat and rice are also rich in dietary fibers. Dietary fibers constitute part of the plant material that is resistant to digestion by human enzymes; they are predominantly non-starch polysaccharides and lignins and may include other associated substances.

The use of non-nutritive fibers like wheat straw, psylium husk and other roughage is considered newborn. It was suggested that this group of dietary fibers, called novel fibers, promotes several beneficial health effects. Some of the reported benefits include preventing disorders such as diabetes, cardiovascular diseases, cancer and obesity (Verma and Banerjee, 2010).

The use of whole cereal-based food, such as wheat bran, over refined grains is highly encouraged through a variety of studies and programs. Recently, in Europe, a new project "HEALTHGRAIN" was launched to study the metabolic benefits of whole grain products in diet which is markedly demonstrated to be protective against diet-related

disorders such as cardiovascular diseases and type 2 diabetes (http://www.healthgrain.eu/pub/).

Oat-hulls fibers which are also classified as 'novel fibers', were approved by FDA in 2008 and recognized as safe ("GRAS") substance for use and was exempted from the required pre-marketapproval (*www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000261.pdf*)

A similar exemption was granted for barley 'beta fiber' by FDA. *(www.fda.gov/.../06p-0393-cp00001-043-Tab-G-GRAS-Expert-Panel-vol2.pdf, 2003)*. Oat fibers were formulated into breads to add a further 6-7 g of oat fiber to a 50-g portion of cereal or a 60-g slice of white bread. The same could be recommended for wheat hulls which could be powdered to a certain particle mesh size.

Reduction in particle size is an important step that increases water absorption of raw ground wheat and breaks down the crystalline nature of the cell walls into a more texturally pleasant fiber.

The combined benefits of the insoluble fibers of wheat husk and the presence of tricin within its tissues render this 'waste by-product' a very promising and potent nutraceutical agent. This will target mainly the colon, and could be a useful chemopreventive agent against colon cancer. This strategy can find potential applications if used in other food products such as whole wheat bread.

Dissolution tests in vitro

The two methoxyl groups present on the B-ring of tricin more likely appear to be responsible for its lipophilicity that seems to play the key role in its biological activity, cellular uptake and its *in vivo* stability, especially in the intestine and colon. In addition, the presence of the three phenolic hydroxyl groups catalyzes the antioxidant activity of this molecule. This dual characteristic endows tricin the pharmacokinetic advantage *in vivo* over its non-methylated analogue; apigenin that exhibits a very rapid metabolism (Cai et al., 2007). Tricin is not readily soluble in aqueous solutions, the reason why we performed an *in vitro* dissolution test to mimic the effect of both stomach and *colon p*H media on the release of tricin from plant tissues.

Seedless husks were finely powdered and 3-g aliquots were added to each dissolution media: an acidic 0.1 N HCl *(p*H 1.1) and an alkaline phosphate buffer *(p*H 7.5 to 8) for simulating the stomach and colon pH media, respectively. Tricin release was monitored during a 24–h period. The highest tricin concentration was attained after 2h of dissolution and amounted to 60µg/g dry weight/L at acidic pH and 96µg/g dry weight/L at basic *p*H (7.5 to 8) media, followed by a steady state plateau until the end of the experimental period (Fig 3-4).

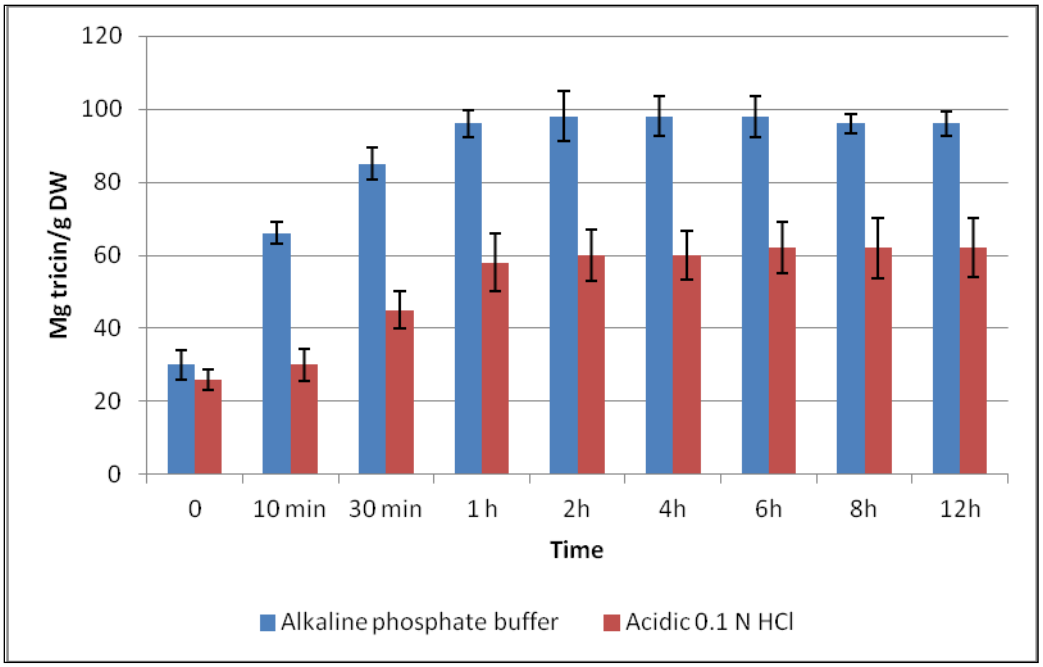


Figure 3-3: Solubility of tricin released from 1g dry weight Norstar husks in alkaline phosphate buffer and in acidic 0.1 N HCl, over 24h.

Since tricin is not readily soluble in water, the quantities released do not represent the real tricin content, as the actual content of tricin in the wheat hull powder is much higher than these amounts. Consequently, the amount of tricin released from plant tissues is cumulative; each person will receive at least the sum of both stomach and colon dissolution values, which is an average of 156µg/g or 156 ppm of dry powdered plant material. It was recently reported that dietary tricin resulted in a significant reduction of colonic adenomas and adenocarcinomas in mice administered 50 and 250 ppm tricin, respectively (Oyama et al., 2009). Consequently, the amount estimated to be released from the plant in both stomach and colon could be calculated to exert the desired effect.

Isolation of pure tricin

The possibility that the tricin aglycone could result from the hot extraction steps (Wollenweber, 2008), led us to conduct its extraction at room temperature and identify its presence (as the aglycone) before further processing steps .

The extraction procedure consisted of soaking the whole dried inflorescence in 85% aq/ MeOH for 24 h and the resulting extract was concentrated in vaccuo before loading into a Flash chromatography system equipped with a RediSep® silica gel column. Although this technique does not offer the ideal resolution of an HPLC system, it is considered a satisfactory tool for fast purification of organic molecules in a less complicated mixture, such as that obtained from wheat husks.

Tricin was eluted from the column once the concentration of MeOH reached 24.5 % in CH_2Cl_2, where the peak was monitored at both 254 and 340 nm (Fig 3-4). Its structure was confirmed by comparing its retention time, MS and NMR spectra to those obtained with a reference sample. The yield obtained was 1.5 mg of pure tricin from 3g dry plant material. On large scale, this procedure could be considered economic if compared to others synthetic methods, taking in consideration that it may depend on the degree of purity needed. Recently, tricin-amino acid derivatives as prodrugs were developed, that claimed to have superior pharmacokinetic properties over the tricin itself with enhanced permeability, stability, and excellent bioavailability after oral administration (Ninomiya et al., 2011).

This justifies the need for a reliable source of tricin to enable such type of research and experimentation.

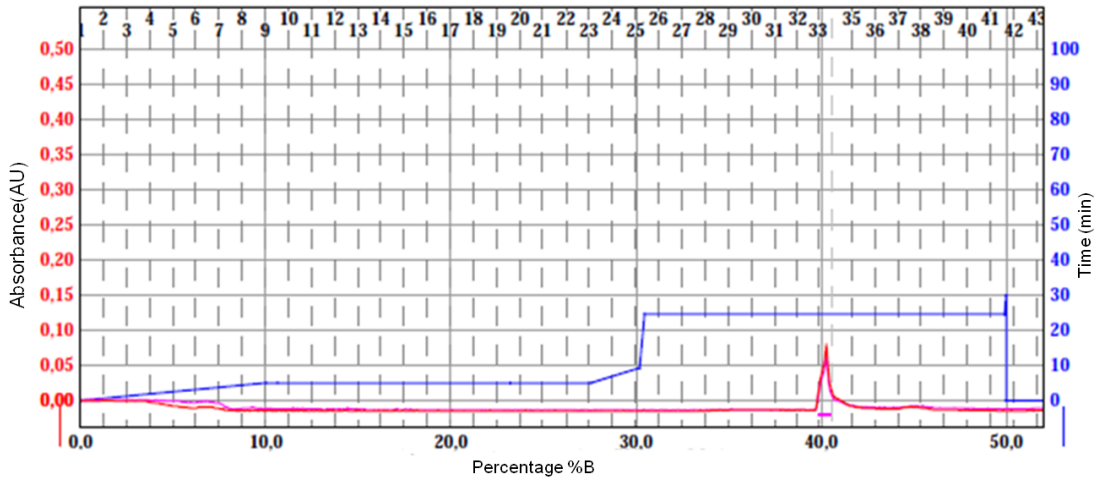


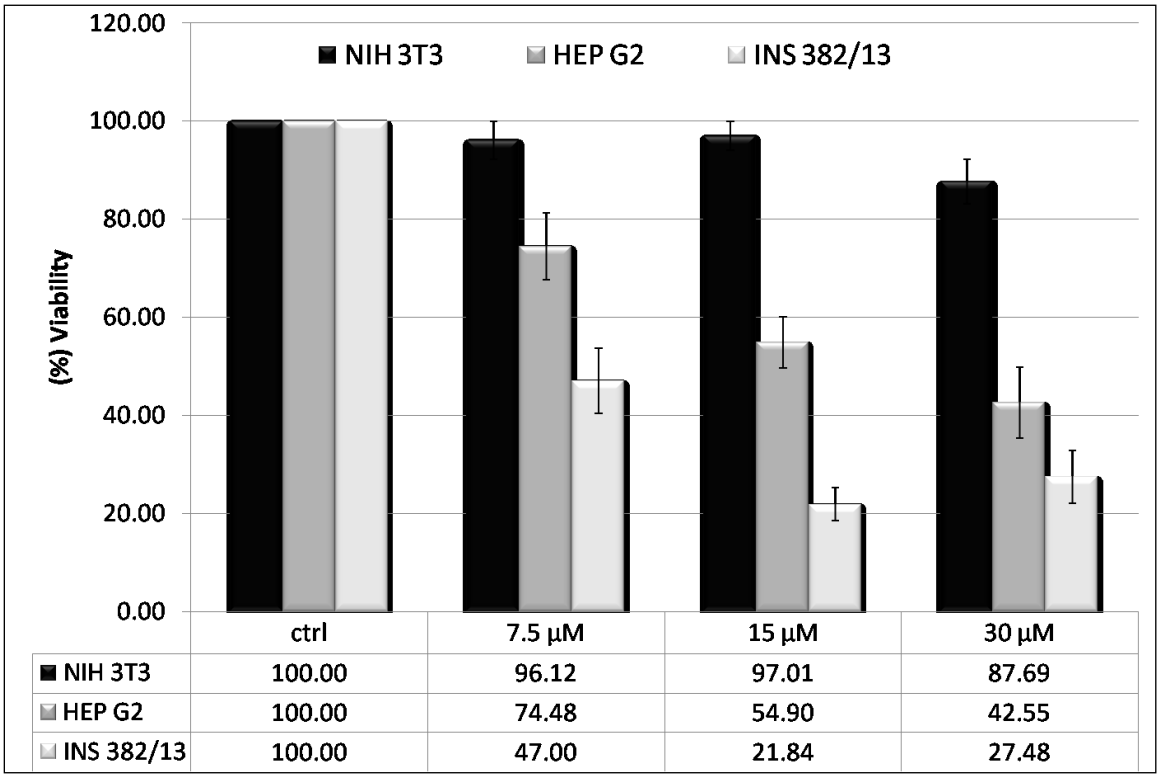
Figure 3-4: Isolation of tricin from wheat husks using a flash chromatography instrument.

The crude husk MeOH extract was purified on a RediSep® silica gel column and a UV/vis detector monitored at 340 and 254 nm. Elution started with CH_2Cl_2 100% up to 50% MeOH, and tricin was eluted from the column at 33 min with 24.5% MeOH/ CH_2Cl_2.

Effect of tricin on liver and pancreas cancer cell lines

Most cytotoxic drugs invoke a relatively high toxicity and narrow therapeutic index (ratio of toxic dose: effective dose (LD_{50}/ED_{50}), resulting in several side effects during the intended course of treatment (Bosanquet AG, 2004). This could be attributed to the fact that most of these drugs are not selective and cannot differentiate between cancer and normal cells. In the present study, we observed that low concentrations of tricin slowed the growth and killed cancer cells without any effect on normal cells. Two cancer cell lines: hepatic HepG2 and pancreatic INS383/12 were treated for 24-h with different tricin concentrations (7.5, 15 and 30µM) and their viability and toxicity profiles determined.

Tricin at 7.5 µM exhibits a strong selectivity towards killing cancerous cells where the viability of HepG2 and of INS383/12 decreased to 74.5% and 47%, respectively, without any effect on the normal cells NIH3T3. Tricin concentration needed to kill 50% of HepG2 cells was 15 µM compared to 7.5 µM for INS383/12. At these concentrations, the NIH3T3 normal cells were not affected and did not develop any cytotoxicity manifested by a high rate of viability equivalent to 97%. At a higher concentration (30µM), tricin seemed to have a little effect on normal cells NIH3T3 with a viability of 87.7% but, effectively killed tumor cells with a viability that decreased to 43% and 27.5% of HepG2 and of INS383/12, respectively (Fig 3-5).



	ctrl	7.5 µM	15 µM	30 µM
NIH 3T3	100.00	96.12	97.01	87.69
HEP G2	100.00	74.48	54.90	42.55
INS 382/13	100.00	47.00	21.84	27.48

Figure 3-5: Effect of tricin on different cells lines.

Cells (NIH 3T3, Hep G2 and INS 382/13) were cultured in tissue culture plates (6 wells) with their specific medium in a humidified atmosphere of 5% CO2 and 95% air at 37˚C. The following day, cells were pre-incubated with different concentrations of tricin (7.5, 15 and 30μM). After 24-h incubation, cells were counted by cytometry with CountBright absolute counting beads. Untreated cells were used as control.

This result is in agreement with a recent study that compared the cancer chemopreventive properties of methylated and non-methylated flavones, where 5,7-dimethoxyflavone, and 5,7,4′ trimethylflavone exhibited a selective effect towards cancer cells only, with a negligible effect on the other two normal cell lines (Walle et al., 2007). Until now, the mechanism underlying this selectivity is not well established and needs further investigation.

Several studies attempted to unravel the mechanism(s) of action of tricin; the National Cancer Institute of the U.S, through their 'War on Cancer' program, has recently considered tricin as one of the most promising new chemopreventive agents. Its target organ was identified as the colon, and its mechanism seems to be through the inhibition of both (cyclooxygenase-2) and phosphatidylinositol 3-kinase (PI3K) activities. Tricin affinity to COX and its ability to compromise PGE-2 generation were further elucidated and explained (Cai et al., 2009)

In addition, a recent *in vivo* study revealed that dietary tricin suppresses both TNF-α (a key element between inflammation and cancer) expression in the non lesional crypts and the proliferation of adenocarcinomas. The effect of tricin on TNF-α expression was referred to in the same study to be associated with chemopreventive activity of tricin in inflammation-associated colorectal carcinogenesis (Oyama et al., 2009). Taken together

these data suggest that, tricin activity, as an anticancer agent, is probably related to its effect on TNF-α, its affinity to COX, or a combination of both. Moreover, a very recent study (Seki et al., 2012), showed the effect of tricin on hepatic stellate cells (HSCs) in vitro, as it inhibited platelet-derived growth factor (PDGF)-BB-induced cell, thus, suggesting that tricin might be beneficial in therapeutic or chemopreventive applications for hepatic fibrosis.

Based on our data, we propose the following application of tricin: The first is to integrate the yellow hull powder of winter wheat, into bread and other bakery products in order to obtain a high quality food, that provide the combined benefits of richness in dietary fiber and tricin at the same time. We could also integrate the hull powder into a suitable pharmaceutical dosage form. Dissolution experiments carried out in this study could be used to predict the amount estimated to be released from the plant powder in the stomach and intestine and, thus help in determining the quantity of material needed.

The incidence of cancer can be substantially reduced by diet modification. The importance of phytonutrient-rich food consumption and their impact on human health were discussed recently (Martin et al., 2011), showing how vital is to have phytonutrient enrichment ways that could provide a daily phytonutrients at sufficient levels. Integrating, on a daily basis, a chemopreventive agent such as tricin in food will certainly have a positive impact on human health. The fact that low income people are more prone to attack by chronic diseases, including cancers (Martin et al., 2011), sheds light on the importance of a phytonutrient-rich food that could be used on daily basis. It should be cheap, affordable and meanwhile effective.

The second application is to isolate tricin and use it in pure form through a pharmaceutical approach, which is considered more economic. However, the degree of purity needed should be taken into consideration as it will reflect the overall cost. More purified products will need more elaborated processing steps.

In summary, we describe an affordable new rich source for the chemopreventive agent tricin from a wheat waste by-product and demonstrate its ability to selectively kill two cancer cell lines *in vitro* without harming the normal cell lines.

We propose also a natural strategy for the prevention of colon cancer through the consumption of the winter wheat-hull powder rich in both tricin and dietary fibers. This could be supplied in the form of phytonutrient- enriched food products, or in a suitably packaged pharmaceutical dosage form.

Chapter 4 SELECTIVE ANTI-CANCER POTENTIAL OF SEVERAL METHYLATED PHENOLIC COMPOUNDS

In the previous chapter we tested the potential anticancer effect of tricin against two different cancer cell lines, and we found that it possess a selective action towards that type of malignant tissues.

Believing that methylated phenolic compounds own more advantageous pharmacological and bioavailability profiles than the non-methylated derivatives, we decided to continue exploring some other methylated phenolic compounds that were chosen from our lab collection including a derivative of tricin, the (3',4',5' trimethyltricetin) to find out their *in vitro* potential to selectively inhibit cancer cell lines.

The viability of two cancer cell lines of the liver and lung and one normal cell lines were tested upon exposure to the selected methylated phenolic compounds for 24 h.

Techniques used in this chapter included, LDH method and plate microreader for viability testing.

The manuscript corresponding to this chapter is in preparation for submission as a short communication or letter to a peer-reviewed journal.

Summary

Most cytotoxic drugs cause a relatively high toxicity and a narrow therapeutic index (ratio of toxic dose: effective dose (LD_{50}/ED_{50}), thus resulting in several side effects during the course of treatment. In the present study, a number of naturally occurring methylated phenolic compounds were evaluated for their selective anticancer activity on two different cancer cell lines, alveolar A-549 and pancreatic INS383/12,and a normal mouse fibroblast cell lines (NIH 3T3). Compounds chosen for the study were among different classes of simple phenolics, phenylpropanoids, coumarins and flavonoids with previously known bioavailability and biological activities. Thirteen compounds showed anticancer activity with no noticeable toxicity against the normal cell lines. Ferulic acid (1) and trimethyltricetin (13) exhibited the highest selective anticancer activity against pancreatic INS383/12 and alveolar A-549, with mortality of 71% and 94%, respectively.

Overall, the present work provides a screening model that aims to enrich the natural products data platform with new promising leads compounds that could serve in the future as anticancer agents. These compounds are in need for further studies to enable their prospective use.

Introduction

Polyphenols and flavonoids are ubiquitous in human diet constituents and represent active ingredients in several medicinal plants. They are considered good candidates for fighting many forms of cancer as they act as potent shielding and chemopreventive agents. Several studies confirmed their in-vitro ability to inhibit the growth and kill diverse cancer cell lines (Jacquemin et al., 2010; Li-Weber, 2009; Neto, 2007). They are able to prevent DNA binding to carcinogens and to inhibit the carcinogenic process via inhibition of enzymes, such as Cytochrome protein 1A1 and Cytochrome protein 1B1, or induction of inactivating enzymes as UDP-glucuronosyltransferase (Walle, 2009 and refs. therein).

However, their *in vivo* efficacy in humans is still questionable, probably due to their poor bioavailability after oral ingestion (Ta and Walle, 2007; Walle et al., 2007). Their low bioavailability may be attributed to their excessive metabolism in the intestine and liver via glucuronidation and/or sulfonation of their free hydroxyl groups. In addition, their presences as glycosides, with several hydroxyl groups on their structure, further decrease their bioavailability (Walle, 2007). In contrast, their methylated forms are more resistant to hepatic metabolism and therefore, show relatively better intestinal absorption compared to their unmethylated forms (Wen and Walle, 2006), and consequently appear in higher concentration in blood and human tissues.

The anticancer activity of 5,7-dimethoxyflavone was reported to be greater than its non-methylated analog, 5,7-dihydroxyflavone (chrysin). Similar results were cited for 5,7,4'-trimethoxyflavone that was found to be eight times more potent than its non-methylated analog, 5,7,4'-trihydroxyflavone (apigenin) in SCC-9 human oral squamous carcinoma

cells (Walle, 2009). This was attributed to the higher uptake of the methylated polyphenolic compounds (Walle et al., 2007). Recently, the anticancer effect of a methylated apigenin (5,7,4-trimethoxyflavone) was found to be greater than that of the partially methylated flavones apigenin and tricin (Cai et al., 2007; Cai et al., 2009).

The presence of hydroxyl groups on polyphenolic compounds would be expected to enhance their solubility in aqueous media that mimic the environment of the gastrointestinal tract. However, it is interesting to note that the solubility of the methylated form of chrysin, 5,7-dimethylchrysin was surprisingly higher than that of chrysin (Walle, 2009). Although this finding may represent an advantage for these products, more work is required to compare the solubility and uptake of other phenolic compounds and their methylated forms.

The bioavailability of methylated phenolic compounds and their effectiveness as anticancer agents prompted us to conduct an *in vitro* screening platform of a number of methylated phenols for their anticancer activity on two different cancer cell lines (rat pancreas **INS-832** and human lung **A-549**) and a mouse fibroblast normal cell lines (**NIH 3T3**).

The presence of hydroxyl groups on polyphenolic compounds would be expected to enhance their solubility in aqueous media that mimic the environment of the gastrointestinal tract. However, it is interesting to note that the solubility of the methylated form of chrysin, 5,7-dimethylchrysin was surprisingly higher than that of chrysin (Walle, 2009).

This finding could add an advantage to these products, however it is still under investigation and more work is required to compare the solubility and uptake of other phenolic compounds and their methylated forms.

Materials and methods

Chemicals

Dimethyl sulfoxide (DMSO), sodium pyruvate and other chemicals were from Sigma Chemical Company (St. Louis, MO), phenolic compounds, were from our laboratory collection, and NADH from Bioshop Canada Inc (Burlington/Ontario).

Cell culture

INS-832/13, NIH 3T3 and A-549 cell culture

Rat pancreatic cells (**INS832/13**), an insulin-secreting cell line derived from an X-ray-induced rat transplantable insulinoma cell line, were grown in monolayer cultures in a regular RPMI-1640(Sigma-Aldrich) medium supplemented with 10 mM HEPES, 10% heat-inactivated FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified (5% CO_2, 95% air) atmosphere.

Mouse Fibroblast cells (**NIH 3T3**) were grown in monolayer cultures in regular DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin at 37 °C in a humidified (5% CO_2, 95% air) atmosphere.

Human adenocarcinomic alveolar basal epithelial cells **(A-549**) were grown in monolayer cultures in regular DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin at 37 °C in a humidified (5% CO_2, 95% air)

atmosphere. When cells reached 80% confluence after approximately 7 days, they were washed twice with PBS, and trypsin was added for 5 min. Trypsin was inactivated by addition of RPMI. After centrifugation, the pellet was resuspended in a minimal volume. Cells were cultured in tissue culture plates (6 wells) in the same specific medium as described above in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C. One day after plating, phenolic compounds at different concentrations (2.5, 5, 7.5, 15, 30, and 45µM) were added to the medium for 24 h.

Viability tests

Lactate dehydrogenase (LDH) method

The activity of lactate dehydrogenase was measured spectrophotometrically at 340 nm via the oxidation reaction of NADH with pyruvate resulting in a decrease in the absorbance (Markert, 1984). In the current work the leakage of (LDH) was measured according to (Moldéus et al., 1978), where the activity of lactate dehydrogenase was monitored in an aliquot of cell-free medium and compared to the total activity achieved after lysis of the cells.

Lysis of cells was performed by treatment with Triton X-100 (0.5%). NADH (0.2 mM final concentration) and pyruvate (1.36 mM final concentration) were mixed with Krebs Henseleit buffer containing 2% albumin before being added to 25 µl of the cell-free medium once and to25 µl of the cell-containing media after lysis. The rate of change in absorbance at 340 nm due to NADH oxidation was recorded.

Experiment was repeated at least three times and values represent the mean ± SE obtained from a triplicate of 3 independent experiments.

Results and discussion

The high toxicity and narrow therapeutic index (ratio of toxic dose: effective dose (LD_{50}/ED_{50}), of most cytotoxic drugs caused several side effects during the course of treatment (Bosanquet AG, 2004). This could be attributed to the fact that most of these drugs are not selective and cannot differentiate between cancer and normal cells.

In the present study, different methylated phenolic compounds were screened for their selective ability to inhibit cell growth and ultimately kill the cancer cells of two different cell lines: Alveolar A-549 and pancreatic INS383/12. Compounds were from different classes of polyphenols, such as phenylpropanoids C6-C3, simple phenolic, coumarins and flavonoids.

The tested compounds were selected based on their previously reported biological activity (Adams et al., 2006; Baccichetti, 1982; Butsat and Siriamornpun, 2010; Hua et al., 1999; Hudson et al., 2000; Jurd et al., 1971; Khattab et al., 2010) , their affordability and availability in market or as ingredients in our daily food (Harborne and Williams, 1969; Khattab et al., 2010; Uden et al., 1991).

The cell lines were treated with the selected compounds for 24-h at different concentrations varying between 2.5, 7.5, 15, 30, and 45μM, then their viability and toxicity profiles were determined.

Only optimum concentrations causing greater than 50% mortality of cancer cells and a minimal toxic effect on normal cells (less than 25% mortality) have been shown in this study (table1).

Among the phenylpropanoids C6-C3 and simple phenolic group, ferulic acid (**1**) (3-methoxy-4-hydroxycinnamic acid) showed the highest inhibition activity at 15 μM, by

killing 75.1% of the pancreatic cancer cells INS followed by, syringaldehyde (**2**) (4-hydroxy-3,5-dimethoxybenzaldehyde) 71% > orcinol (**3**) (5-methylresorcinol) 69%> vanillic acid (**4**) (4-hydroxy-3-methoxybenzoic acid) 67%> sinapoyl glucose (**5**) (1-O-sinapoyl-beta-D-glucose) > Coniferin 61% (**6**) (coniferyl alcohol-β-D-glucoside) 59%.

The effect on alveolar cancer cell lines A-549 was different. The analysis indicates that sinapoyl glucose (**5**) and vanillic acid (**4**) were the most effective compound in killing these cells with mortality rate of 87.6 % and 87.2% respectively, (Table 4-1).

All of the previously mentioned six phenolic compounds had very small toxic effect (12-18%) mortality on normal NIH cells thus highlighting their potential specificity and selectivity.

The three coumarin derivatives used in this study isosopoletin (**7**) (6-hydroxy-7-methoxycoumarin), herniarin (**8**) (7-O-methylumbelliferone), and xanthotoxin (**9**) (9-methoxy-7H-furo[3,2-g]chromen-7-one), exhibited comparable toxicity on both INS and A-549 cancer cell lines: (Table 4-1).

The flavonoids showed significant activity on both cancer cell lines, trimethyltricetin (**10**) (3',4',5'-*O*-dimethyltricetin) was the most effective in killing the A-549 lung cancer cells, followed by that of isorhamenetin (**11**) (3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl) chromen-4-one) >selgin (**12**) (3'-*O*-methyltricetin) > biochanin A (**13**) (5,7-dihydroxy-4'-methoxyisoflavone) (Table 4-1).

The strongest selectivity of trimethyltricetin towards killing cancerous cells A-549 was observed at 7.5 μM with 94% mortality and a 5% on the normal cells NIH3T3. This effect was the highest among all tested compounds. Increasing the concentration to 15 μM leaded to a decrease in efficacy to 81.2%, which demonstrate the importance of the

dose used to obtain the maximal effect. It is interesting to note that the trimethyltricetin (**10**) was recently identified as a constituent of wheat leaves (Moheb et al., 2011) and as an end product of the methylation reaction of TaOMT2 (Zhou et al., 2006).

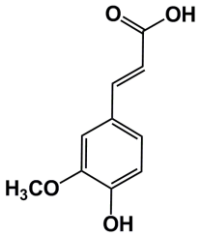
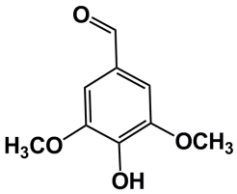
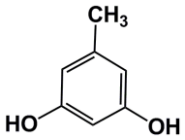
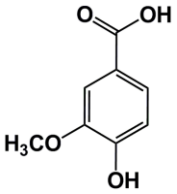
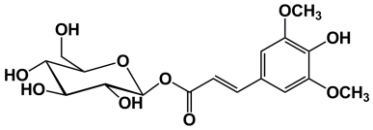
This suggests the possibility of using wheat constituent as a preventive cancer agent. This possibility requires further investigation.

In conclusion, thirteen, naturally occurring phenolic compounds with at least one methyl group exhibited selective anticancer activity towards two different types of cancer cell lines Alveolar A-549 and pancreatic INS383/12. The tested compounds were classified according to their chemical class into phenylpropanoids, simple phenolic, coumarins and flavonoids.

Among all classes, ferulic acid (**1**) and trimethyltricetin (**10**) exhibited superior selective activities against INS383/12 and A-549, respectively.

This study sheds light on the important potential of these methylated phenolic compounds as selective anticancer agents, and recommends further investigation regarding the effect of different methyl groups (type, position and number) on the structure activity relationship of these active compounds.

Table 4-1: The effect of selected methylated phenolic compounds on cell viability[4]

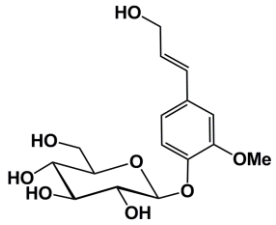
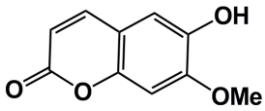
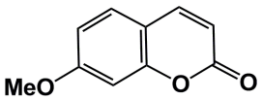
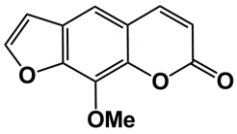
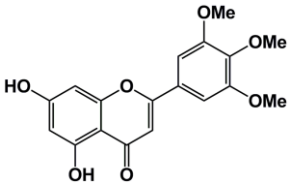
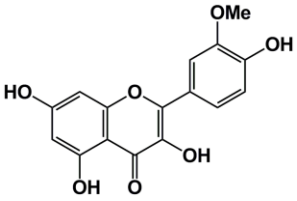
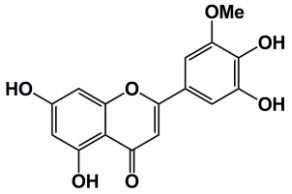
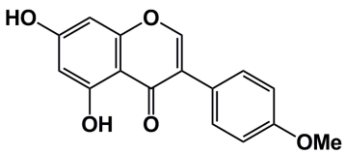
	Compound	Structure	Optimum Conc.	% Mortality		
				INS[5]	A-549[6]	NIH[7]
1	**Ferulic acid**		15 µM	75.1 ± 6.5 **(+++)**	84.9± 5.2 **(++++)**	17.4±9.2 **(±)**
2	**Syring-aldehyde**		5 µM	71.1±6.5 **(+++)**	81.7± 2.9 **(++++)**	13.9±4. **(±)**
3	**Orcinol**		5 µM	69.2±4.4 **(++)**	81.9±6.6 **(++++)**	15.9±6.4 **(±)**
4	**Vanillic acid**		15 µM	67.1±10.7 **(++)**	87.2±3.6 **(++++)**	18.9±5.8 **(±)**
5	**Sinapoyl glucose**		45µM	61±11.7 **(++)**	87.6±9.8 **(++++)**	14.6±7.6 **(±)**

[4] Phenylpropanoids C1-C3 and simple phenolic compounds from compound 1 to 7, coumarins 7 to 9 and flavonoids 10-13, values represent the mean ± SE obtained from a triplicate of 3 independent experiments. **(±)** 0–50%, **(+)** 51–60%, **(++)** 61–70%, **(+++)** 71–80%, **(++++)** 81–90%, **(+++++)** 91–100%

[5] Pancreatic cancer cell lines INS383/12

[6] Human adenocarcinomic alveolar basal epithelial cells

[7] Mouse fibroblast cells

6	**Coniferin**		30 µM	59.9± 9.1 **(+)**	81.4±6.8 **(++++)**	11.3± 7.8 **(±)**
7	**Isosopoletin**		5 µM	68.6±6.6 **(++)**	80.7±1.8 **(++++)**	15.9±5.4 **(±)**
8	**Herniarin**		30 µM	59.6±11.5 **(+)**	81.79±6.9 **(++++)**	18.9±3.3 **(±)**
9	**Xanthotoxin**		7.5 µM	53.4±9.2 **(+)**	82.8±10.9 **(++++)**	15.4±5.2 **(±)**
10	**Trimethyl tricetin**		7.5 µM	69.7±1.8 **(++)**	94±6 **(++++++)***	15.5±7.3 **(±)**
			15 µM	61.1±7.2 **(++)**	81.2±12. **(++++)**	17.8±2.2 **(±)**
11	**Isorhamnetin**		30 µM	63.5±5.02 **(++)**	89±1.11 **(++++)**	23.3±8.1 **(±)**
12	**Selgin**		7.5 µM	61.2±4.9 **(++)**	86.9±5.4 **(++++)**	15.1±5.5 **(±)**
13	**Biochanin A**		30 µM	61.2±12.1 (++)	84.9±3.9 (++++)	18.5±7.2 **(±)**

* Highest % mortality

Chapter 5 TRICIN BIOSYNTHESIS DURING GROWTH OF WHEAT UNDER DIFFERENT ABIOTIC STRESSES

Investigating the possible role and significance of tricin to the plant especially wheat, was the focus of this last chapter. The biological significance of TaOMT2, the enzyme that catalyzes the methylation of tricetin to tricin is discussed. The expression and the enzyme activity of TaOMT2, and the accumulation of its major product (tricin) were measured at different wheat developmental stages and in response to different abiotic stresses such as cold, salt and drought.

Techniques used to study TaOMT2 methylation reaction include: HPLC, LC/MS, fluorescence microscopy, enzyme activity and expression assays. The manuscript corresponding to this chapter is in preparation for submission to a peer-reviewed journal.

Summary

In plants, ***O*-methylation** is mediated by an enzyme family of *O*-methyltransferases (OMTs) that transfer the methyl groups from the methyl donor, *S*-adenosyl-L-methionine (AdoMet) to suitable phenolic acceptor molecules. In a previous study, a flavonoid OMT (*TaOMT2*) was isolated and characterized from wheat (*Triticum aestivum* L.) leaves. Its novel gene product catalyzes three sequential *O*-methylations of the flavone tricetin (5,7,3',4',5'-pentahydroxyflavone) to its 3′-monomethyl-→3′,5′-dimethyl-(tricin) →3′,4′,5′-trimethyl (TMT) ether derivatives, with tricin being the major product of the reaction.

The fact that tricin levels change in cold-acclimated wheat prompted an investigation of the biological significance of tricetin methylation, by measuring the OMT activity, its expression level, and the accumulation of its major product (tricin) at different developmental stages of wheat plants exposed to different abiotic stresses such as cold, salt and drought. The results show that tricin accumulates mostly in wheat inflorescences under normal conditions compared to others developmental stages. This accumulation is associated with increased TaOMT2 expression level and enzyme activity, suggesting a possible synthesis of the enzyme at this important developmental stage. This phenomenon may be attributed to the putative role of tricin in protecting seeds against biotic and abiotic stresses. The functions of tricin during growth and development of wheat and the importance of tricetin methylation during abiotic stresses are discussed.

Introduction

Enzymatic substitution reactions contribute to the structural and functional diversity of flavonoid compounds. These substitutions include glycosylation, acylation, hydroxylation, methylation and/or prenylation that take place mostly on the phenolic rings (Ibrahim and Anzellotti, 2003). Enzymatic *O*-methylation, which is catalyzed by a large family of *O*-methyltransferases (OMTs) plays an important role in reducing the toxicity and chemical reactivity of their phenolic hydroxyl groups and increasing their lipophilicity; and hence modulating their function and their antimicrobial activity (Middleton et al., 2000).

In a previous study, a flavonoid OMT (*TaOMT2*) was isolated and characterized from wheat (*Triticum aestivum* L.) leaves (Zhou et al., 2006). Its novel gene product exhibits a pronounced preference towards tricetin (5,7,3',4',5'-pentahydroxyflavone) as the substrate. It catalyzes three sequential *O*-methylations of the flavone tricetin to its 3'-monomethyl- (selgin), 3',5'-dimethyl- (tricin) and 3',4',5'-trimethyl- (TMT) ether derivatives. Tricin was found to be the predominant product of the reaction with only small amounts of the trimethyl ether derivative.

Both tricetin and tricin, have been reported to occur mostly in unrelated plant families and, more frequently, in cereals such as wheat, rice and barley (Wollenweber et al., 2002).

Tricin was reported to have potent chemopreventive properties, as it inhibits the growth of human malignant breast tumor cells and colon cancer cells (Hudson et al., 2000) and interferes with intestinal carcinogenesis in Apc mice by inhibiting cyclooxygenase activity (Cai et al., 2005), to mention only a few.

The reported accumulation of tricin in many cereals was explained in relation to its possible role in plant defence mechanism.

Tricin was also reported to be implicated in plant-insect interactions in rice (Bing et al., 2007) and possess a significant fungicidal activity against rice seedling rot disease (Kong et al., 2010). Moreover, tricin inhibited the growth of both weeds and fungal pathogens in rice, suggesting its possible function as an allelochemical, or a natural plant biopesticide (Kong et al., 2004). Tricin was also reported to possess a potential herbicidal activity in rice (*Oryza sativa*) hulls (Chung et al., 2005) as well as anti-feedant activity against the boll weevil, *Anthonomus grandis* (Miles et al., 1993), two aphid species, *Schizaphis graminum* and Myzus *persicae* in wheat (Dreyer and Jones, 1981).

To investigate the significance of tricetin methylation in wheat, a study was conducted with the aim to reveal the possible role of TaOMT2 and its major product, tricin in wheat plant under normal and stress conditions, particularly cold stress which is a predominant factor that affects various crop plants.

Materials and methods

Plant material

Wheat (*Triticum aestivum* L. cv. Claire (winter) and cv. Bounty (spring) seeds were germinated in a soil-vermiculite mixture (1:1, w/w) for 7 days. Leaves from plants of different developmental stages grown under normal and different stress conditions were used for enzyme activity assays, western blots and HPLC analyses

Plant treatment

For cold acclimation 7-day old plants were transferred to an environmental chamber at 4^{o}C (cold acclimated) and another batch at 20°C (control plants) for various periods of time. For drought treatment, plants were exposed to progressive water stress by withholding water for 3, 5, 7 and 12 days. For salt stress, plants in each pot were watered with 200mM of NaCl solution for 1, 3, 5, 7, 12 and 15 days respectively. As controls, untreated plants were kept for the same periods of time with normal watering. Aerial parts from control and treated plants were sampled at the same time for each time point and immediately frozen in liquid nitrogen and stored at -80°C for further analyses.

Chemicals

Most of the flavonoid compounds used were from our laboratory collection, except for tricetin that was purchased from Indofine Chemical Company (Hillsborough, NJ) and tricin from Dalton Chemical Company (Toronto, ON).). *S*-Adenosyl-L-[^{3}H] methionine (AdoMet; 76.4 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO), and the unlabeled AdoMet from Sigma (Oakville, ON). Protein quantification reagents and 40% acrylamide/bis solution (37.5:1) were obtained from

Bio-Rad (Mississauga, ON). All other chemicals were of analytical grade unless otherwise specified.

Protein extraction, enzyme assays, and immunoblot analyses

Extraction of wheat leaf protein was carried out at 4^{o}C. Leaves were ground to a fine powder with dry ice, before being homogenized with 50 mM Tris-HCl buffer pH 7.6 containing 14 mM ß-mercaptoethanol and 7 mM PMSF. The homogenate was filtered through mira cloth and centrifuged for 15 min at 14,000xg

The extracted proteins were analyzed by SDS-PAGE. Proteins extracted were resuspended in 2 X SDS electrophoresis sample buffer, (Laemmli, 1970), using Bench marker (Invitrogen) as a molecular mass ladder and bovine serum albumin as the standard protein.

After electrophoresis, proteins were stained with Coomassie Brilliant Blue (R-250). Protein concentration was determined using Quantity-one software from Bio-Rad with bovine serum albumin as standard protein.

TaOMT2 enzyme assays

After protein extraction and centrifugation (14,000x*g*, 30 min) the supernatant was assayed with tricetin as the substrate (50 µM, containing 0.025 µCi of [^{3}H]AdoMet, as the methyl donor, and up to 100 µg of protein in a total volume of 100 µl.. The reaction was started by addition of the enzyme, incubated at 30°C for 30 min and stopped by the addition of 10µl 6M HCl. The methylated products were extracted with ethyl acetate, and counted for radioactivity using a toluene-based scintillation fluid. For background correction, control incubations were performed in the absence of substrate or with boiled enzyme. All assays were conducted in duplicates.

Western blot analysis

Western blot analysis was performed to evaluate TaOMT2 levels at different stages of development and during growth under different abiotic stresses (cold, salinity and drought). Equal amounts of proteins were separated on a 12% (w/v) SDS-polyacrylamide gel and transferred electrophoretically for one h at 100 V on to a 0.45-µm nitrocellulose membrane (Hybond-C; Amersham Pharmacia Biotech) with no SDS in the transfer buffer.

After blocking with reconstituted skimmed milk (5% [w/v]) in phosphate-buffered saline containing 0.05% (w/v) Tween 20, the membrane was incubated with a1:250 (v/v) dilution of the purified antibody (rabbit anti-wheat TaOMT2 serum) TaOMT2-specific antibody was raised against a specific epitope in the protein sequence (J-M Zhou, unpublished data). After washing with phosphate-buffered saline-Tween, the proteins recognized by the primary antibody were revealed with peroxidase-coupled anti-rabbit IgG (Jackson Immunoresearch Inc., West Grove, PA) as a secondary antibody (1:10,000, v/v). The complex formed was revealed using the ECL chemiluminescence detection kit (Amersham PharmaciaBiotech, Uppsala) and X-OMAT-K film (Eastman-Kodak, Rochester, NY). For western-blot signal quantification, Coomassie stained proteins were first analyzed by densitometry usinga CCD camera and AlphaEase 3.3a software (Alpha Innotech Corp., San Leandro, CA). Immunoblot signals were subsequently scanned with a densitometer and analyzed with Image Quant 4.2 (Molecular Dynamics, Sunnyvale,CA). Immunoblot densitometric readings were adjusted against protein densitometric values and normalized by setting the maximum protein accumulation to

100%. For each antibody tested, the data represent the typical tendency curves from at least three independent immunoblots derived from two different extractions.

Inflorescence cross sections

Hand-cross sections in the spikelets of winter wheat (Norstar and Claire) were stained for 20 min with 5 mM diphenyl boric acid 2-aminoethyl ester (Murphy et al., 2000) before being examined with a Zeiss LSM 510 META (405 nm) diode laser confocal microscope using a post-processing software (LSM5 Macro) as described by the manufacturer.

Extraction of phenolic compounds

Fresh leaf samples (*ca.* 10 g) were ground in dry ice before extraction (3X, one h) with hot MeOH-H_2O (85:15, v/v). The extract was concentrated in vacuo, to remove MeOH, and the resulting aqueous suspension was defatted with a mixture of hexanes to remove lipids and chlorophyll pigments. The aqueous extract was hydrolysed with 2 M HCl (30 min; 95 ºC) to release the phenolic aglycones form their parent glycosides followed by 3X liquid-liquid extraction with EtOAc. The combined organic layers were concentrated under vacuo, and the resulting residue dissolved in MeOH for analysis.

HPLC and LC-MS analyses

For quantification of tricin, samples were chromatographed on an Agilent 1200 HPLC system with a binary pump, in-line degasser, high performance auto-sampler and thermo-stated column division, using a linear gradient of 40% up to 90% of methanol: 0.1% HCOOH-water for 30 min with a flow rate of 0.35 ml/min on an Agilent SB-C18 column (2.1×30mm; particle size, 3.5µm), and a column temperature of 25ºC. The method was optimized with UV detection at 254 and 340 nm. Quantification of tricin was carried out

using the area under the curve method by calculating the summation of areas in relation to their UV absorbance.

Two LC-ESI-MS systems were used. In System 1, the HPLC instrument was connected to an Agilent 6410 quadrupole (triple Q) mass spectrometer, yielding MS-MS spectra for confirming substructures. Measurements were performed using electrospray ionisation in the positive ESI mode under the following conditions: capillary voltage, 3000 v; nebulizer pressure, 60º Psi; gas temperature, 300ºC; drying gas, 5 L/min and a dwell time of 75 ms, and the data was processed using the Mass Hunter software. The same column was transferred to be used in System 2 where an identical HPLC instrument, under the same conditions mentioned above, was connected to another mass spectrometer consisting of an Agilent 6210 electrospray ionisation-time-of flight analyser (ESI-TOF) for measurement of exact masses, in the positive ESI mode, at a capillary voltage of 4000V, nebulizer pressure of 35 Psi, gas temperature 350ºC , drying gas flow: 11.5 L/min and voltages of 125V and 60V for the fragmentor and the skimmer, respectively. The technical error and mass resolving power of the time-of-flight mass spectrometer in terms of mass accuracy were 2ppm, RMS[8], measured at the $[M+H]^+$ ion of reserpine (m/z 609.2807) was used as an internal mass reference. Reference compounds for both tricin and tricetin were used to compare their corresponding retention times and mass spectral profiles.

A tricin calibration curve was constructed for quantification purposes, using different concentrations of 1.6µg.ml^{-1}, 5µg.ml^{-1}, 8.3µg.ml^{-1}, 16.6µg.ml^{-1}, 25µg.ml^{-1}, and 166µg.ml^{-1} and 266µg.ml^{-1} tricin.

[8] root mean square

For MS/MS in system 1, collision energy (CE) of 35eV was applied as the optimum energy of collision for the analysis of flavonoids, and the scan range was from 200 to 900 m/z.

Results and discussion

Identification of TaOMT2 *O*-methylation products in native wheat extracts

It was previously reported that the recombinant TaOMT2 catalyzes three sequential *O*-methylations of the substrate tricetin (Zhou et al., 2006), and demonstrated that the sequential order of B-ring methylation takes place through its 3'-monomethyl- (selgin) → 3',5'-dimethyl- (tricin) → 3′,4′,5′-trimethyl ether derivatives. In the present study, both tricin and trimethyl tricetin (TMT) were identified in the extracts of wheat leaves, but neither tricetin nor selgin.

The relative toxicity of the free reactive ortho OH groups of both tricetin, and selgin, may explain their undetectable level in the extract. In fact, the presence of an *ortho*-3',4'-dihydroxygroup in the B-ring of the polyphenols has been correlated with the relative toxicity of these compounds, due to formation of quinone-type metabolites responsible for their possible toxic effects (Duarte Silva et al., 2000; Macgregor and Jurd, 1978).

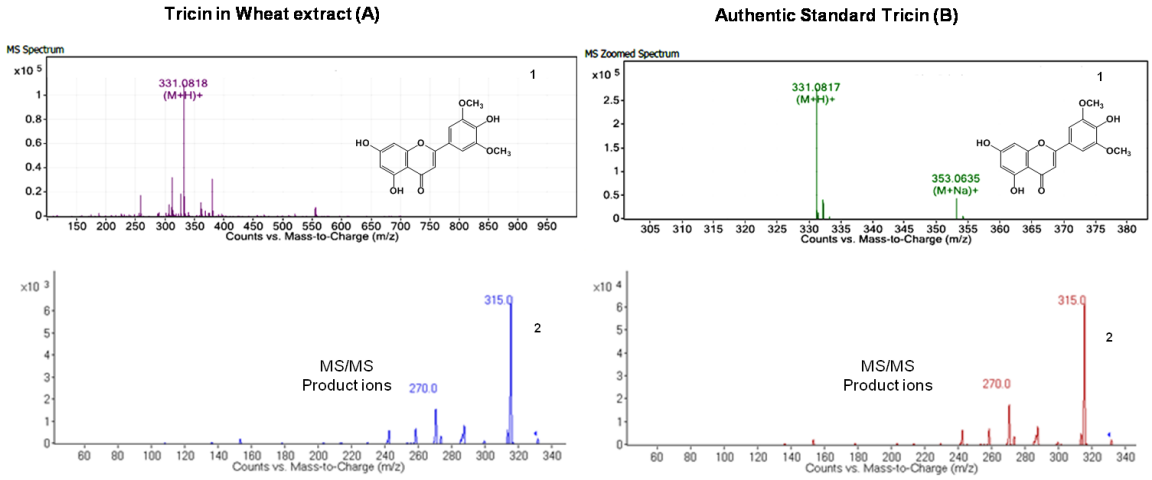


Figure 5-1: Identification of tricin in wheat seedlings.

(A) HPLC-MS of tricin in wheat extract , (1) Major $[M+H]^+$ ion at m/z 331 at 16.7 min, (2) MS-MS for m/z 331 showing 317 and 270 as major product ions. (B) The corresponding authentic standard $[M+H]^+$, MS-MS at (1), and (2) respectively.

TaOMT2 activity during different developmental stages

Enzyme activity assays of wheat leaf and influorescence tissues from different stages were measured with tricetin as the substrate. The result in Figure 5-2 indicates that TaOMT2 was highly expressed in the influorescence tissues. Its protein accumulation coincides with its increased enzyme activity and the intracellular content of tricin as the major product.

The expression of TaOMT2 protein and its activity increased by almost three-fold in the influorescence tissues, as compared with those in the leaves (Fig. 5-2). The accumulation kinetics of tricin at different developmental stages in winter wheat (Claire) shows that tricin increases during growth and development, reaching its maximum level of *ca.* 735±91 µg/g DW in the influorescence (Figs 5-2 and 5-5).

These results clearly demonstrate that tricin accumulation is the consequence of its synthesis in the inflorescence by the TaOMT2 and not by its transport from other parts of the plant. They also suggest an important biological function for tricin during this important stage of plant development.

Several studies have revealed the function of certain molecules produced by the plant that protect its floral organs from attack by microbes, pests and herbivores (e.g.David, 1992; Tregear et al., 2002 and refs therein). Wheat influorescences are exposed to different external stresses, such as uv radiation, herbivory, fungal, parasitic attack, etc. In order to produce viable high quality seeds with high germination rate, wheat seems to develop a specific strategy by which to protect the seeds during this critical stage of plant life cycle.

The accumulation of TaOMT2 and its product, tricin in the floral parts of wheat supports the possible role of this methylation in protecting seeds against possible fungal or parasitic attack. The proposed role of tricin in seed protection was anticipated previously in rice (Oryza sativa) (Kong et al., 2004; Chung et al., 2005) , and A. donax (Miles et al., 1993).

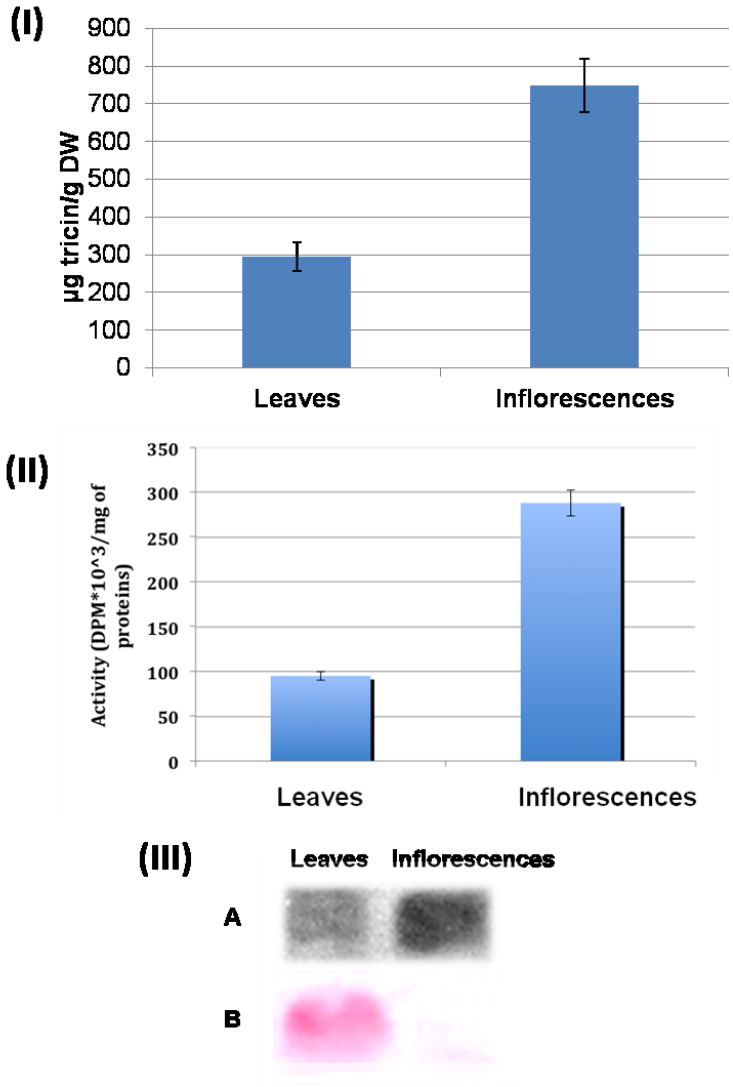


Figure 5-2:TaOMT2 assays in leaves and inflorescences of Claire (winter wheat variety)

Tricin level, TaOMT2 activity, and expression level in winter wheat (Claire) at different growth stages ***(I)*** *Tricin level µg/g DW in both leaves (one wk-old) and inflorescences. Estimation was carried out using HPLC-UV analysis at 340 nm; and calculating area under curve. Values are means of three determinations ± standard deviations.* ***(II)*** *Methyltransferase activity using tricetin as substrate in wheat leaves (1 week) and inflorescences; values are means of three determinations ± standard deviations.* ***(III)*** *Western blots of TaOMT2 in leaves and inflorescences A, Immunoblot of TaOMT2. B, PVDF membrane stained with Ponceau Red showing Rubisco as protein load.*

Localization of tricin within the inflorescence tissue

The occurrence of tricin in the outer/surface tissues of the inflorescence was investigated by shaking *ca*. 5g of whole inflorescence tissue with 50 ml acetone for 5 min, then injecting *ca.*60µl into HPLC. The analysis revealed the presence of a major tricin peak suggesting the presence of tricin in the outer layers of the wheat husk. This is consistent with the fact that in cereal grains most flavonoids and phenolic compounds are located in the outer layers of the kernel (the pericarp) (Dykes L., 2007).

To determine the location of tricin within the inflorescence tissue, cross sections of the spikelet, after grain removal were prepared and stained with 5 mM diphenylboric acid 2-aminoethyl ester, a general stain for polyphenolic compounds. The fact that tricin is the major flavonoid identified in wheat hulls (as seen under chapter 3); the intense greenish fluorescence observed in UV light is considered indicative of the presence of tricin in the outer layers of wheat (Fig. 5-3).

The accumulation of tricin in such a high concentration in the upper surface layers of wheat inflorescence strongly suggests a role for tricin in protecting the grains, the site of food storage of hulled wheat, against fungal/ pests attacks (Potts, 1997).

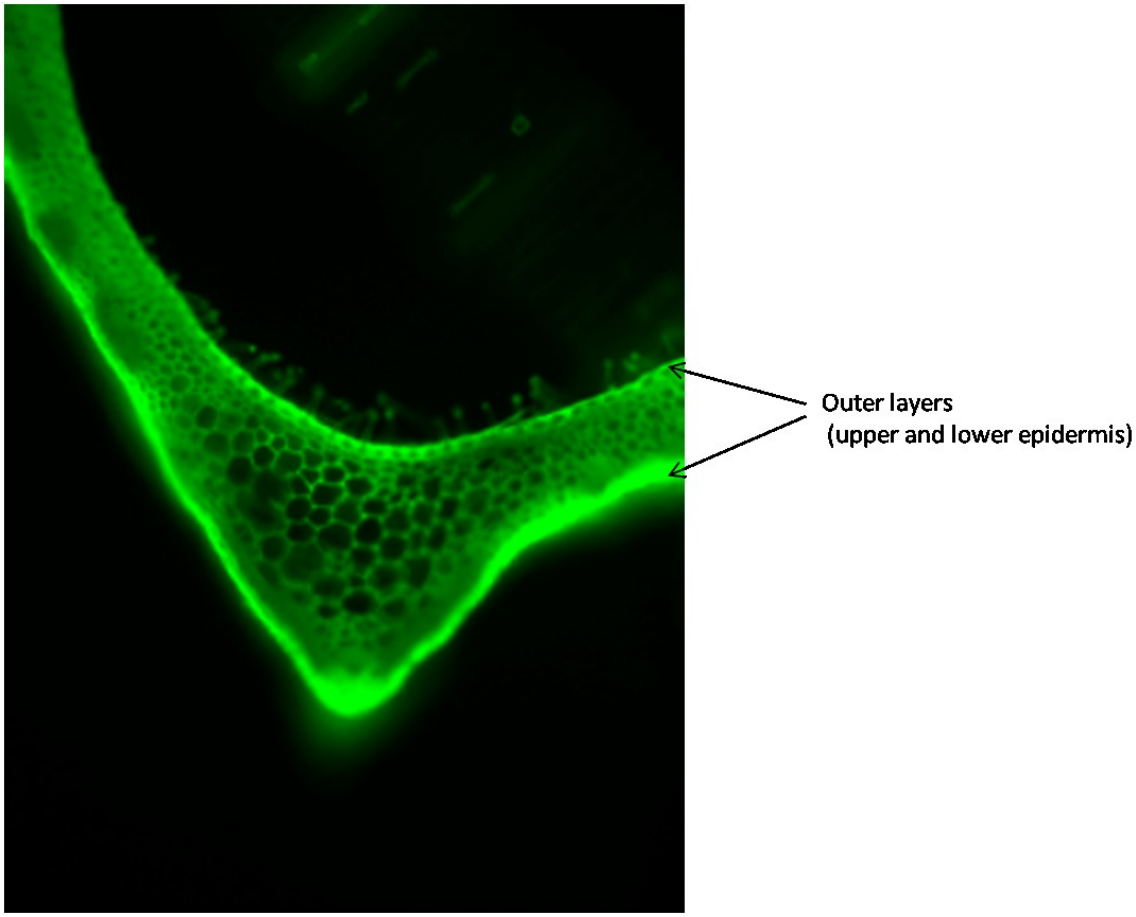


Figure 5-3:Cross-section in wheat outer/surface of the inflorescence of the winter variety

Section was stained with 5 mM diphenyl boric acid 2-aminoethyl ester for 20 min, viewed with a Zeiss LSM 510 META (405 nm) diode laser confocal microscope and post-processing software (LSM5 Macro) showing greenish fluorescence of tricin in the outer layers of inflorescence.

Effect of cold, salt and drought stresses on TaOMT2

Cold, drought, and salinity are among the major abiotic stresses that affect the plant growth and productivity. To assess the possible role of tricin in response to these stresses, tricin biosynthesis was determined by tricin quantification using HPLC, and measuring the content and activity of TaOMT2 during growth under different abiotic stresses.

During cold treatment (4°C) of winter wheat (Claire), the level of tricin remains relatively constant during 42 days of cold acclimation, but with a tendency to decrease when compared it to control plants (Fig. 5-4). At 12 days of cold treatment, tricin content amounted to 178 ±26.6µg tricin/g DW, compared to 338±36 µg tricin/g DW in control plants of corresponding age. Tricin % among total phenolic compounds was decreasing during cold acclimation in contrast to what was observed during the developmental stage as previously discussed (Fig. 5-5).

On the other hand the TaOMT2 content measured by immunoblot increased during low temperature acclimation (Fig. 5-4) while the specific activity of the enzyme remain constant, with a more pronounced tendency to decrease at the end of cold treatment. This phenomenon may be explained by a possible shift of the methylation reaction of TaOMT2 towards another substrate 5-hydroxyferulic acid (5HFA), a second most preferred substrate of TaOMT2 (Zhou et al., 2006), that is involved in lignin biosynthesis.

During cold periods, lignin tends to accumulate in the plant as it contributes to the strength of plant cell walls, facilitates water transport and impedes the degradation of wall polysaccharides (Griffith et al., 1985; Huner NPA, 1981; Wei et al., 2006 and refs. therein). In addition, it was reported that OMT involved in 5HFA methylation exhibited

the highest enzyme activity when rye plants were cold acclimated (Hatfield and Vermerris, 2001; NDong et al., 2002). Thus, an accumulation of TaOMT2 may suggest a role in lignin biosynthesis rather than in tricin biosynthesis during cold conditions.

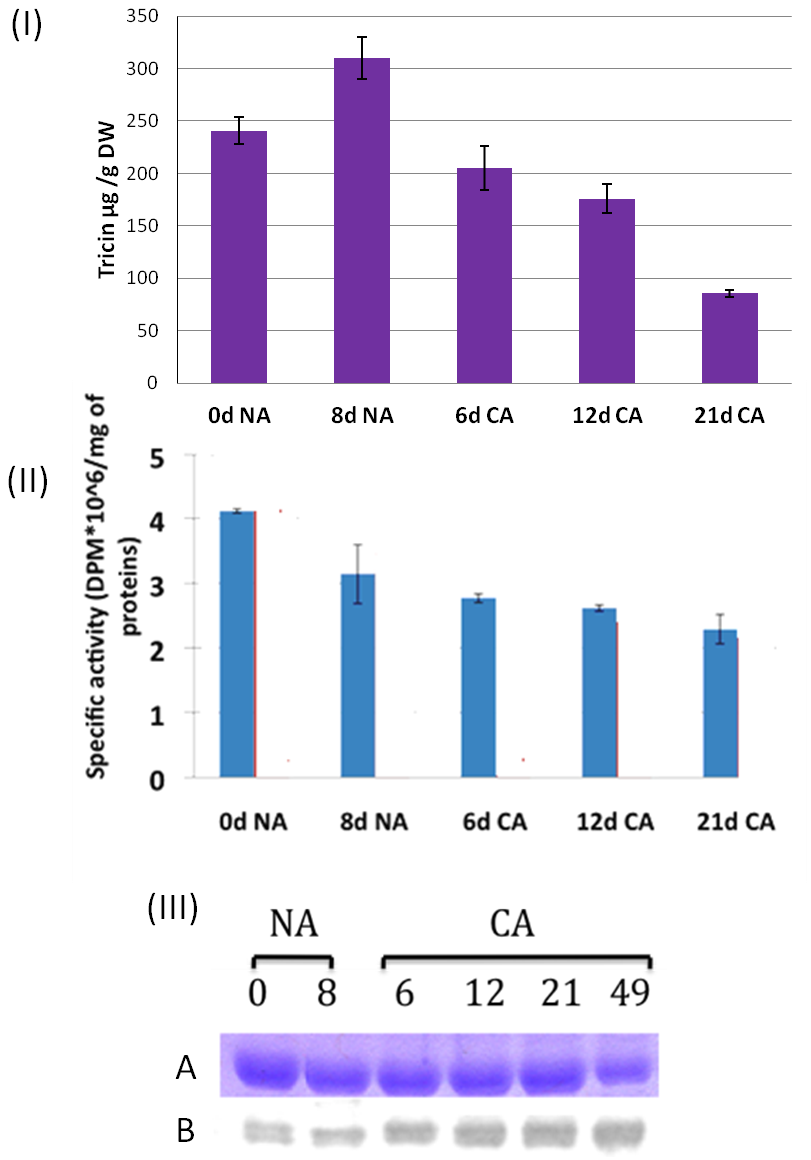


Figure 5-4: Effect of cold acclimation on tricin level, TaOMT2 activity and expression in wheat leaves (Claire)

(I) *Quantification of tricin in non-acclimated and cold-acclimated as determined by HPLC-UV analysis at 340 nm; values are means of three determinations ± standard deviations* ***(II)*** *Methyltransferase activity with tricetin as substrate and radiolabeled*

*[^{3}H S-adenosyl-L-methionine] as co-substrate per mg protein of wheat leaves during cold acclimation. Values represent the mean ± SE of two independent experiments. 0 d NA non-acclimated plants grown for 7 days representing the zero point control, 8 d NA non-acclimated plants grown for 2 weeks representing another control point; 6 d CA, 6 day cold-acclimated plants (**III**) Western blot analysis of TaOMT2: NA, non-acclimated; CA, cold-acclimated, A, SDS-PAGE gel stained with Coomassie blue showing Rubisco. B, Immunoblots of TaOMT2.*

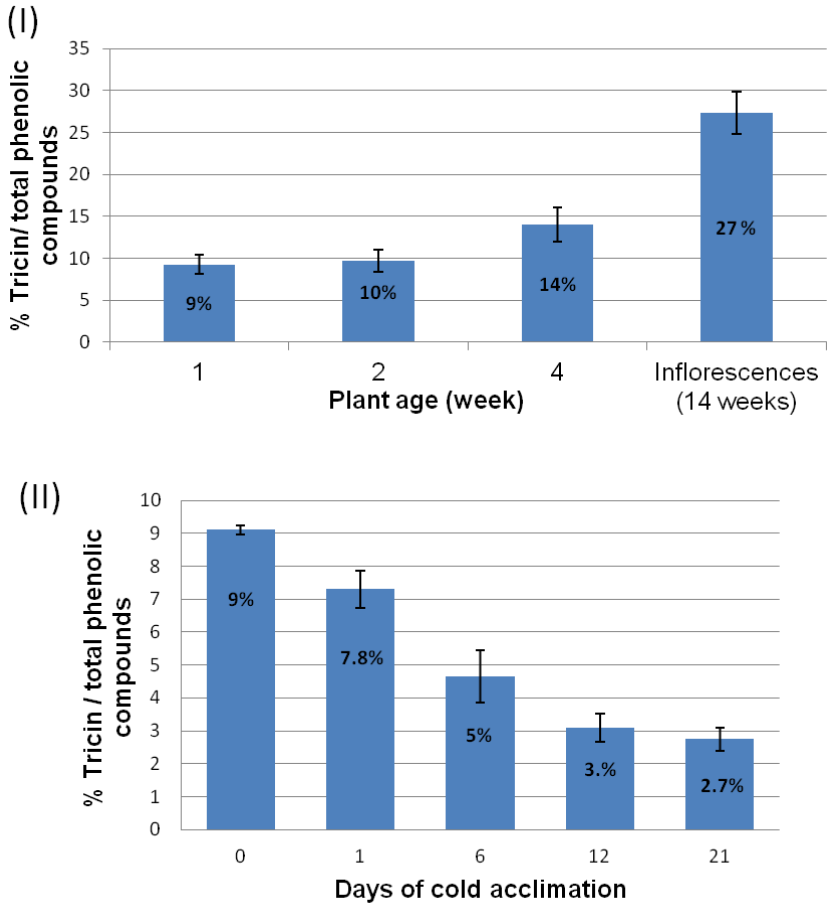


Figure 5-5: The % quantity of tricin per total phenolic compounds

***(I)** During the developmental stage of winter wheat (Claire) (**II**) during cold acclimation for 1,6,12 and 21 days of cold.*

Under salt and drought stresses (Fig. 5-6), tricin concentration tends to decrease (390± 21.2µg tricin/g DW) and (380±22.6µg tricin/g DW), respectively, when compared to the corresponding control at the same age (620±28µg tricin/g DW). These results are similar to those observed under cold acclimation. Immunoblot analyses and enzyme activity measurements of TaOMT2 show no increase in content accompanied by a decrease in activity. These results suggest that TaOMT2 does not participate in protecting the plant against salinity and/or drought stresses.

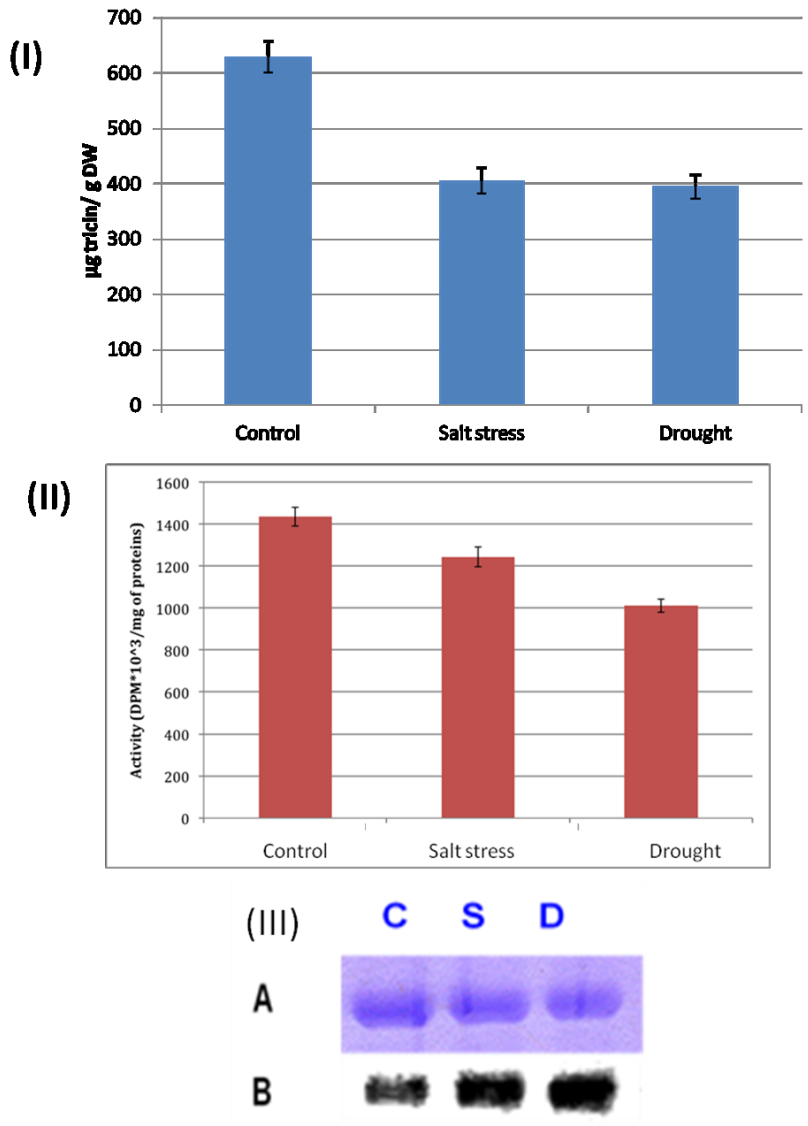


Figure 5-6: Effect of salt and drought stresses on tricin level, TaOMT2 activity and expression

*(**I**) Quantification of tricin in wheat leaves exposed to salt and drought stresses. Quantification was carried out using HPLC-UV analysis at 340 nm; values are means of three determinations ± standard deviations. (**II**) Methyltransferase activity with tricetin as substrate in salt and drought stressed wheat: Claire (winter variety), (**III**) Western blot of TaOMT2, C, control; S, salt stress; D, drought stress: A. SDS-PAGE gel after transfer stained with Coomassie blue showing Rubisco. B. Immunoblots of TaOMT2. Both control and stressed plants used were 1 month old winter wheat plants.*

The results reported in the present study shows that tricin does exponentially increase with wheat growth, until it reaches its maximum in the floral parts. This is associated with the increase of expression and activity of TaOMT2 suggesting an active biosynthesis of tricin in the influorescence, most probably to protect the developing seeds.

When plants are subjected to stresses that hinder that growth, such as cold, salt and drought, tricin stops to accumulate its pathway of biosynthesis may be diverted to form another product, such as lignin. This suggests that tricin accumulation is associated with normal growth rate that leads to its maximum accumulation during the sensitive flowering stage and formation of seeds that need protection against biotic stresses.

Chapter 6 GENERAL CONCLUSION

Throughout this work, we studied several aspects related to the flavone, tricin. Tricin (5,7,4'-trihyroxy-3',5'-dimethoxyflavone) is a naturally occurring flavone of relatively rare and sporadic occurrence. It is mainly found in cereal grain plants, such as rice, oat, maize, barley and wheat. Apart from being a powerful antioxidant, antimutagenic and anti-inflammatory agent, several studies have revealed the potential importance of this lipophilic flavone in cancer treatment and prevention. Tricin has been considered an efficient chemopreventive agent in growth inhibition of human malignant breast tumour cells and colon cancer cells. It is also considered safe enough for clinical studies. However, its commercial unavailability as a pure compound hinders its further experimentation

The presence in wheat leaves of a mixture of beneficial flavonoids as tricin, iso-orientin and vitexin, values its potential use as a source of an affordable supplement of a healthy diet, which may explain the popularity of wheat leaf juice.

The use of wheat leaves (grass) is gaining ground in North America and other parts of the world. They are considered as an edible part of the plant being used as a juice (wheatgrass juice) or added to several food products. However, only few reports offer a complete profile of its phenolic content including tricin. Therefore, in (chapter 2) we investigated the phenolic profile (Phenolome) of two varieties: the winter (Claire) and spring (Bounty) varieties of wheat (*Triticum aestivum* L*)* leaves with the aim to identify, quantify and compare the most important phenolic compounds in normal and under cold conditions.

The application of LC-ESI-MS protocols, coupled with the MRM technique used, have proven to be powerful tools for the direct chemical screening of phenolic compounds in wheat leaves. They provided accurate, reproducible results, and allowed the characterization of some novel metabolites, and established the differential induction of levels of phenolic compounds in both winter and spring wheat when grown under cold acclimation conditions.

During cold acclimation iso-orientin and its *C*-glycoside derivatives followed by vitexin and iso-vitexin represented the most significant increase in phenolic derivatives of the winter variety, whereas, the accumulation of dicaffeoylputrescine was the predominant metabolite in the spring variety. Identification of the novel flavone, 3',4',5'-trimethyltricetin, as well as feruloylagmatine, by their characteristic product ion fragments, will serve as future reference sources for easy detection of both compounds in plant extracts.

Moreover, the fact that most of flavonoids (including tricin) and HCAs were identified in the apoplast compartment confirms the important role of the latter in plant defense mechanisms.

In (chapter 3) we investigated the distribution of tricin in different parts of wheat (Triticum aestivum) with the aim to designate a reliable rich source for its production. The highest amount was found in the husk of winter wheat varieties and was estimated to be 770 ± 157 μg/g dry materials. This concentration is considered the highest in any plant materials suggesting the use of winter wheat husk as a good source of tricin.

The purified wheat tricin was found to be selective potent inhibitor of two cancer cell lines of the liver and pancreas, while having no side effect on normal cells. This

selectivity makes tricin a potential candidate for anticancer therapy. Thus, we describe an affordable new rich source for the chemopreventive agent tricin from a wheat waste by-product. Tricin was isolated from wheat husk that has long been considered as a waste product. The exploitation of this product for the production of tricin could change potentially its market applications.

We propose also a natural strategy for the prevention of colon cancer and liver cirrhosis through the consumption of the winter wheat-hull powder rich in both tricin and dietary fibers. This could be supplied in the form of phytonutrient-enriched food ingredient to be added to many food and bakery products, or in a suitably packaged pharmaceutical dosage form.

In (chapter 4), we wanted to further explore the selective anticancer effect of several methylated phenolic and flavonoid compounds using LDH-Spectrophotometric method to assess the viability of the cell lines. Several candidates were found to possess a remarkable antitumor activity on these malignant cell lines, such as trimethyltricetin, a tricin derivative, that exhibited a superior selective activity against human adenocarcinomic alveolar basal epithelial cells **(A-549)**.

In (chapter 5), we tested the effects of abiotic stress factors, such as cold, drought and salt treatments, among others, on the biosynthesis and accumulation of tricin in different parts of wheat (*Triticum aestivum* L). The results show that the levels of tricin increase exponentially with wheat growth, until it reaches its maximum in the floral parts. This is associated with the increase of expression and activity of TaOMT2 suggesting an active biosynthesis of tricin in the influorescence, most probably to protect the developing seeds against any invader.

When plants are subjected to stresses that hinder growth, such as cold, salt and drought, tricin stops to accumulate. Its pathway of biosynthesis seems to be diverted to form another product, such as lignin. This suggests that tricin accumulation is associated with normal growth rate that leads to its maximum accumulation during the sensitive flowering stage and formation of seeds that need protection against biotic stresses.

References

Adams, M., Efferth, T., Bauer, R., 2006. Activity-guided isolation of scopoletin and isoscopoletin, the inhibitory active principles towards CCRF-CEM leukaemia cells and multi-drug resistant CEM/ADR5000 C cells, from *Artemisia argyi.* Planta Med 72, 862-864.

Agency, C. F. I., 2010. The elements within the nutrition facts table

Andersen, M. M., K. R., 2006. Flavonoids: chemistry, biochemistry and applications. CRC Press, Boca Raton.

Anderson, J. A., Perkin, A. G., 1931. CCCLXV.-The yellow colouring matter of khapli wheat, Triticum dicoccum. J Chem Soc (Resumed), 2624-2625.

Anderson, J. W., Baird, P., Davis Jr, R. H., Ferreri, S., Knudtson, M., Koraym, A., Waters, V., Williams, C. L., 2009. Health benefits of dietary fiber. Nutr Rev 67, 188-205.

Antia, F. P., Abraham, P., 1997. Clinical Dietetics and Nutrition. Oxford University Press, Usa (1998-10-29), 73–77.

Asenstorfer, R. E., Wang, Y., Mares, D. J., 2006. Chemical structure of flavonoid compounds in wheat (*Triticum aestivum* L.) flour that contribute to the yellow colour of Asian alkaline noodles. J Cereal Sci 43, 108-119.

Baccichetti, F. P., IT), Bordin, Franco, Carlassare, Francesco, Dall'acqua, Francesco, Guiotto, Adriano, Pastorini, Giovanni, Rodighiero, Giovanni (Padua, IT), Rodighiero, Paolo, Vedaldi, Daniela, 1982. Furocoumarin for the

photochemotherapy of psoriasis and related skin diseases. Consiglio, Nazionale Delle Ricerche (Rome, IT), United States.

Back, K., 2001a. Hydroxycinnamic acid amides and their possible utilization for enhancing agronomic traits. Plant Pathol J. 17, 123-127.

Back, K., Jang, S. M., Lee, B.-C., Schmidt, A., Strack, D., Kim, K.-M., 2001b. Cloning and characterization of a hydroxycinnamoyl-CoA:Tyramine N-(Hydroxycinnamoyl) transferase induced in response to UV-C and wounding from Capsicum annuum. Plant Cell Physiol. 42, 475-481.

Baublis, A. J., Lu, C., Clydesdale, F. M., Decker, E. A., 2000. Potential of wheat-based breakfast cereals as a source of dietary antioxidants. J Am Coll Nutr. 19, 308S-311S.

Bing, L., Hongxia, D., Maoxin, Z., Di, X., Jingshu, W., 2007. Potential resistance of tricin in rice against brown planthopper *Nilaparvata lugens* (Stal). Acta Ecologica Sinica 27, 1300-1306.

Bohm, B. A., 1998. Introduction to flavonoids. Harwood Academic Publishers.

Bosanquet AG, B. P., 2004. Ex vivo therapeutic index by drug sensitivity assay using fresh human normal and tumor cells. J Exp Ther Oncol 4, 145-154.

Boyer, J. S., 1982. Plant productivity and environment. Science 218, 443-448.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-254.

Brazier-Hicks, M., Evans, K. M., Gershater, M. C., Puschmann, H., Steel, P. G., Edwards, R., 2009. The *C*-glycosylation of flavonoids in cereals. J. Biol. Chem. 284, 17926-17934.

Brouillard, R., Cheminat, A., 1988. Flavonoids and plant color. Prog Clin Biol Res 280, 93-106.

Butsat, S., Siriamornpun, S., 2010. Phenolic acids and antioxidant activities in husk of different thai rice varieties. Food Sci and Technol Internat 16, 329-336.

Cai, H., Al-Fayez, M., Tunstall, R. G., Platton, S., Greaves, P., Steward, W. P., Gescher, A. J., 2005a. The rice bran constituent tricin potently inhibits cyclooxygenase enzymes and interferes with intestinal carcinogenesis in ApcMin mice. Mol Cancer Ther. 4, 1287.

Cai, H., Boocock, D., Steward, W., Gescher, A., 2007. Tissue distribution in mice and metabolism in murine and human liver of apigenin and tricin, flavones with putative cancer chemopreventive properties. Cancer Chemother. Pharmacol 60, 257-266.

Cai, H., Hudson, E. A., Mann, P., Verschoyle, R. D., Greaves, P., Manson, M. M., Steward, W. P., Gescher, A. J., 2004. Growth-inhibitory and cell cycle-arresting properties of the rice bran constituent tricin in human-derived breast cancer cells in vitro and in nude mice in vivo. Br J Cancer 91, 1364-1371.

Cai, H., Sale, S., Schmid, R., Britton, R. G., Brown, K., Steward, W. P., Gescher, A. J., 2009. Flavones as colorectal cancer chemopreventive agents--phenol-*o*-methylation enhances efficacy. Cancer Prev Res 2, 743-750.

Cai, H., Steward, W. P., Gescher, A. J., 2005b. Determination of the putative cancer chemopreventive flavone tricin in plasma and tissues of mice by HPLC with UV–visible detection. Biomed. Chromatogr. 19, 518-522.

Cai, H., Verschoyle, R. D., Steward, W. P., Gescher, A. J., 2003. Determination of the flavone tricin in human plasma by high-performance liquid chromatography. Biomed. Chromatogr. 17, 435-439.

Cavalière, C., Foglia, P., Pastorini, E., Samperi, R., Lagana, A., 2005. Identification and mass spectrometric characterization of glycosylated flavonoids in Triticum durum plants by high-performance liquid chromatography with tandem mass spectrometry. Rapid Commun Mass Spectrom 19, 3143-3158.

Chan, W. L., Lin, Y. C., Zhang, W. H., Tang, P. L., Szeto, Y. S., 1996. One-step synthesis of polyhydroxyflavanones from hydroxyacetophenones and hydroxybenzaldehydes. ChemInform 27.

Chang, C.-L., Wang, G.-J., Zhang, L.-J., Tsai, W.-J., Chen, R.-Y., Wu, Y.-C., Kuo, Y.-H., 2010. Cardiovascular protective flavonolignans and flavonoids from *Calamus quiquesetinervius*. Phytochemistry 71, 271-279.

Chiwocha, S. D. S., Abrams, S. R., Ambrose, S. J., Cutler, A. J., Loewen, M., Ross, A. R. S., Kermode, A. R., 2003. A method for profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (*Lactuca sativa* L.) seeds. The Plant Journal 35, 405-417.

Chung, I.-M., Hahn, S.-J., Ahmad, A., 2005. Confirmation of potential herbicidal agents in hulls of rice, Oryza sativa. J Chem Ecol 31, 1339-1352.

Cleveland, L. E., Moshfegh, A. J., Albertson, A. M., Goldman, J. D., 2000. Dietary intake of whole grains. J Am Coll Nutr 19, 331S-338S.

Colombo, R., Yariwake, J. H., Queiroz, E. F., Ndjoko, K., Hostettmann, K., 2006. On-line identification of further flavone *C*- and *O*-glycosides from sugarcane (Saccharum officinarum L., Gramineae) by HPLC-UV-MS. Phytochem Anal 17, 337-343.

Cook, D., Fowler, S., Fiehn, O., Thomashow, M. F., 2004. A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. Proc. Natl. Acad. Sci. USA 101, 15243-15248.

Cotter, R. J., 2004. Time-of-flight mass spectrometry. Encyclopedia of Genetics, Genomics, Proteomics and Bioinformatics. John Wiley & Sons, Ltd.

Craig, W. J., 1999. Health-promoting properties of common herbs. Am J Clin Nutr 70, 491S-499S.

Cummins, I., Brazier-Hicks, M., Stobiecki, M., Franski, R., Edwards, R., 2006. Selective disruption of wheat secondary metabolism by herbicide safeners. Phytochemistry 67, 1722-1730.

Cushnie, T. P. T., Lamb, A. J., 2005. Antimicrobial activity of flavonoids. Int. J. Antimicrob. Agents 26, 343-356.

David W.M, L., 1992. Involvement of plant chitinase in sexual reproduction of higher plants. Phytochemistry 31, 1899-1900.

Davies, J., Caseley, J. C., 1999. Herbicide safeners: a review. Pestic Sci 55, 1043-1058.

Deng, D., Zhang, J., Cooney, J. M., Skinner, M. A., Adaim, A., Jensen, D. J., Stevenson, D. E., 2006. Methylated polyphenols are poor (chemical) antioxidants but can still

effectively protect cells from hydrogen peroxide-induced cytotoxicity. FEBS letters 580, 5247-5250.

Dhingra, D., Michael, M., Rajput, H., Patil, R., 2011. Dietary fibre in foods: a review. J Food Sci Technol 48,1-12.

Dixon, R. A., Paiva, N. L., 1995. Stress-Induced Phenylpropanoid Metabolism. Plant Cell 7, 1085-1097.

Dreyer, D. L., Jones, K. C., 1981. Feeding deterrency of flavonoids and related phenolics towards Schizaphis graminum and Myzus persicae: Aphid feeding deterrents in wheat. Phytochemistry 20, 2489-2493.

Du, Y., Chu, H., Wang, M., Chu, I. K., Lo, C., 2009. Identification of flavone phytoalexins and a pathogen-inducible flavone synthase II gene (SbFNSII) in sorghum. J Exp Bot 61, 983-994.

Duarte-Almeida, J. M., Negri, G., Salatino, A., de Carvalho, J. E., Lajolo, F. M., 2007. Antiproliferative and antioxidant activities of a tricin acylated glycoside from sugarcane (Saccharum officinarum) juice. Phytochemistry 68, 1165-1171.

Duarte Silva, I., Gaspar, J., Gomes da Costa, G., Rodrigues, A. S., Laires, A., Rueff, J., 2000. Chemical features of flavonols affecting their genotoxicity. Potential implications in their use as therapeutical agents. Chem. Biol. Interact. 124, 29-51.

Dykes L., R. L. W., 2007. Phenolic compounds in cereal grains and their health benefits Cereal Food World 52, 105-111

Estiarte, M., Peñuelas, J., Kimball, B. A., Hendrix, D. L., Pinter Jr, P. J., Wall, G. W., LaMorte, R. L., Hunsaker, D. J., 1999. Free-air CO2 enrichment of wheat: leaf

flavonoid concentration throughout the growth cycle. Physiologia Plantarum 105, 423-433.

Farmer, M. J., Czernic, P., Michael, A., Negrel, J., 1999. Identification and characterization of cDNA clones encoding hydroxycinnamoyl-CoA:tyramine N-hydroxycinnamoyltransferase from tobacco. Eur J Biochem 263, 686-694.

Fecht-Christoffers, M. M., Braun, H. P., Lemaitre-Guillier, C., VanDorsselaer, A., Horst, W. J., 2003. Effect of manganese toxicity on the proteome of the leaf apoplast in cowpea. Plant Physiol 133, 1935-1946.

Feng, Y., McDonald, C. E., 1989. Comparison of flavonoids in bran of four classes of wheat. Cereal Chem 66, 516-518.

Fixon-Owoo, S., Levasseur, F., Williams, K., Sabado, T. N., Lowe, M., Klose, M., Joffre Mercier, A., Fields, P., Atkinson, J., 2003. Preparation and biological assessment of hydroxycinnamic acid amides of polyamines. Phytochemistry 63, 315-334.

Floerl, S., Druebert, C., Majcherczyk, A., Karlovsky, P., Kues, U., Polle, A., 2008. Defence reactions in the apoplastic proteome of oilseed rape (Brassica napus var. napus) attenuate Verticillium longisporum growth but not disease symptoms. BMC Plant Biol 8, 129.

Food Directorate Guideline No. 9, Policy respecting dietary fibre in meal replacements In: Heatlth Canada, Nutrition, F. a. (Eds.) 1993.

Forkmann, G., Heller, W., 1999. Biosynthesis of flavonoids. In: Sankawa., U. (Ed.), Comprehensive Natural Products Chemistry, vol. 1. Elsevier, Amsterdam, 714-748

Fowler, D. B., 2008. Cold acclimation threshold induction temperatures in cereals. Crop Sci. 48, 1147-1154.

Galeotti, F., Barile, E., Curir, P., Dolci, M., Lanzotti, V., 2008. Flavonoids from carnation (Dianthus caryophyllus) and their antifungal activity. Phytochemistry Letters 1, 44-48.

García-Mediavilla, V., Crespo, I., Collado, P. S., Esteller, A., Sánchez-Campos, S., Tuñón, M. J., González-Gallego, J., 2007. The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappa B pathway in chang liver cells. Eur. J. Pharmacol. 557, 221-229.

Gicquiaud, L., Hennion, F., Esnault, M. A., 2002. Physiological comparisons among four related bromus species with varying ecological amplitude: polyamine and aromatic amine aomposition in response to salt spray and drought. Plant Biology 4, 746-753.

Gould, K., Lister, C., 2005. Flavonoid functions in plants. Flavonoids. CRC Press, 397-441.

Gray, G. R., Heath, D., 2005. A global reorganization of the metabolome in Arabidopsis during cold acclimation is revealed by metabolic fingerprinting. Physiologia Plantarum 124, 236-248.

Griffith, M., Huner, N. P. A., Espelie, K. E., Kolattukudy, P. E., 1985. Lipid polymers accumulate in the epidermis and mestome sheath cell walls during low temperature development of winter rye leaves. Protoplasma 125, 53-64.

Griffith, M., Yaish, M. W., 2004. Antifreeze proteins in overwintering plants: a tale of two activities. Trends Plant Sci. 9, 399-405.

Grondin, M., Hamel, F., Sarhan, F., Averill-Bates, D. A., 2008. Metabolic activity of cytochrome p450 isoforms in hepatocytes cryopreserved with wheat protein extract. Drug Metab Dispos 36, 2121-2129.

Hahlbrock, K., Scheel, D., 1989. Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol 40, 347-369.

Harborne, J. B., 1967. Comparative biochemistry of the flavonoids-VI. : Flavonoid patterns in the bignoniaceae and the gesneriaceae. Phytochemistry 6, 1643-1651.

Harborne, J. B., 1988. Plant flavonoids in biology and medicine II. Biochemical, cellular, and medicinal properties Alan R. Liss Publisher, pp. 17-27.

Harborne, J. B., Hall, E., 1964. Plant polyphenols--XII. : The occurrence of tricin and of glycoflavones in grasses. Phytochemistry 3, 421-428.

Harborne, J. B., Williams, C. A., 1969. The identification of orcinol in higher plants in the family ericaceae. Phytochemistry 8, 2223-2226.

Harborne, J. B., Williams, C. A., 1976. Flavonoid patterns in leaves of the Gramineae. Biochem. Syst. Ecol. 4, 267-280.

Hasegawa, T., Tanaka, A., Hosoda, A., Takano, F., Ohta, T., 2008. Antioxidant C-glycosyl flavones from the leaves of Sasa kurilensis var. gigantea. Phytochemistry 69, 1419-1424.

Hatfield, R., Vermerris, W., 2001. Lignin formation in plants. The dilemma of linkage specificity. Plant Physiol 126, 1351-1357

Henderson, B., 1984. Quantitative cytochemistry of lactate dehydrogenase activity.Cell Biochem. Funct. 2, 149-152.

Hertog, M. l. G. L., Hollman, P. C. H., Katan, M. B., Kromhout, D., 1993. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. Nutr and Cancer 20, 21-29.

HHS, USDA, 2005. Dietary Guidelines for Americans. http:www.health.gov/dietarvnuidelines/dga2005. The U.S. Department of Health and Human Services, 25-28.

Holden, J. M., Bhagwat, S. A., Haytowitz, D. B., Gebhardt, S. E., Dwyer, J. T., Peterson, J., Beecher, G. R., Eldridge, A. L., Balentine, D., 2005. Development of a database of critically evaluated flavonoids data: application of USDA's data quality evaluation system. J Food Comp Anal 18, 829-844.

http://probes.invitrogen.com/media/pis/mp36950.pdf.

http://www.healthgrain.eu/pub/.

Hua, S. S. T., Grosjean, O. K., Baker, J. L., 1999. Inhibition of aflatoxin biosynthesis by phenolic compounds. Lett Appl Microbiol 29, 289-291.

Hudson, E. A., Dinh, P. A., Kokubun, T., Simmonds, M. S., Gescher, A., 2000. Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. Cancer Epidemiol Biomarkers Prev 9, 1163-1170.

Huner NPA, P. J., Li PH, Carter JV., 1981. Anatomical changes in leaves of Puma rye in response to growth at cold-hardening temperatures. Bot. Gaz. 142, 55-62.

Ibrahim, R. K., 2001. Flavonoids. eLS. John Wiley & Sons, Ltd.

Ibrahim, R. K., Anzellotti, D., 2003. Chapter one: The enzymatic basis of flavonoid biodiversity. Recent Advances in Phytochemistry 37, 1-36.

Ibrahim, R. K., De Luca, V., Khouri, H., Latchinian, L., Brisson, L., Charest, P. M., 1987. Enzymology and compartmentation of polymethylated flavonol glucosides in chrysosplenium americanum. Phytochemistry 26, 1237-1245.

Ibrahim, R. K., Muzac, I., John T. Romeo, R. I. L. V., Vincenzo De, L., 2000. Chapter Eleven The methyltransferase gene superfamily: A tree with multiple branches. Recent Adv Phytochem 34 349-384.

Imin, N., Nizamidin, M., Wu, T., Rolfe, B. G., 2007. Factors involved in root formation in Medicago truncatula. J Exp Bot 58, 439-451.

Jacquemin, G., Shirley, S., Micheau, O., 2010. Combining naturally occurring polyphenols with TNF-related apoptosis-inducing ligand: a promising approach to kill resistant cancer cells? Cell Mol Life Sci 67, 3115-3130.

Jeong, Y. H., Chung, S. Y., Han, A. R., Sung, M. K., Jang, D. S., Lee, J., Kwon, Y., Lee, H. J., Seo, E. K., 2007. P-glycoprotein inhibitory activity of two phenolic compounds, (-)-syringaresinol and tricin from Sasa borealis. Chem Biodivers 4, 12-16.

Jiao, J., Zhang, Y., Liu, C., Liu, J. e., Wu, X., Zhang, Y., 2007. Separation and purification of tricin from an antioxidant product derived from Bamboo leaves. J Agric Food Chem 55, 10086-10092.

Jin, S., Yoshida, M., 2000. Antifungal compound, feruloylagmatine, induced in winter wheat exposed to a low temperature. Biosci Biotechnol Biochem 64, 1614-1617.

Jun-Ping, K., Ling-Li, M., 2008. Antioxidant activities of the chemical constituents isolated from the leaves of ginkgo biloba. Chinese Journal of Natural Medicines 6, 26.

Jurd, L., King Jr, A. D., Mihara, K., 1971. Antimicrobial properties of umbelliferone derivatives. Phytochemistry 10, 2965-2970.

K Kamaljit, K Amarjeet, Pal., S. T., 2011. Analysis of ingredients, functionality, formulation optimization and shelf life evaluation of high fiber bread Am. J. Food Technol. 4, 306-313

Kaneta, M. a. N. S., 1973. Identification of flavone compounds in eighteen Gramineae species. Agric Biol Chem 37, 2663-2665.

Kapetanovic, I. M., 2009. Rapid access to preventive intervention development program in the division of cancer prevention of the U.S. National Cancer Institute: an overview. Cancer Epidemiol Biomarkers Prev. 18, 698-700.

Kaplan, F., Kopka, J., Haskell, D. W., Zhao, W., Schiller, K. C., Gatzke, N., Sung, D. Y., Guy, C. L., 2004. Exploring the temperature-stress metabolome of Arabidopsis. Plant Physiol 136, 4159-4168.

Khattab, R., Eskin, M., Aliani, M., Thiyam, U., 2010. Determination of sinapic acid derivatives in canola extracts using High-Performance Liquid Chromatography. J Am Oil Chem Soc 87, 147-155.

Kleijnen, J., Knipschild, P., 1992. Ginkgo biloba. The Lancet 340, 1136-1139.

Kong, C.-H., Xu, X.-H., Zhang, M., Zhang, S.-Z., 2010. Allelochemical tricin in rice hull and its aurone isomer against rice seedling rot disease. Pest Manag Sci 66, 1018-1024.

Kong, C., Xu, X., Zhou, B., Hu, F., Zhang, C., Zhang, M., 2004. Two compounds from allelopathic rice accession and their inhibitory activity on weeds and fungal pathogens. Phytochemistry 65, 1123-1128.

Kren, V., Martinkova, L., 2001. Glycosides in medicine:the role of glycosidic residue in biological activity. Curr. Med. Chem. 8, 1303-1328.

Kupeli, E., Aslan, M., Gurbuz, I., Yesilada, E., 2004. Evaluation of in vivo biological activity profile of isoorientin. Z Naturforsch C 59, 787-790.

Kumar, P., 2011. Nutritional contents and medicinal properties of wheat: a review Life sciences and medicine research 2011, LSMR-22

Kuwabara, H., Mouri, K., Otsuka, H., Kasai, R., Yamasaki, K., 2003. Tricin from a Malagasy connaraceous plant with potent antihistaminic Activity. Journal of Natural Products 66, 1273-1275.

Kwon, Y., Kim, C., 2003. Antioxidant constituents from the stem of; Sorghum bicolor. Arch. Pharm. Res. 26, 535-539.

Kwon, Y., Kim, E., Kim, W., Kim, W., Kim, C., 2002. Antioxidant constituents from *Setaria viridis*. Archives of Pharmacal Research 25, 300-305.

L. Brindzova, M. Z., T. Jakubik, M. Mikulasova, M. Takacsova,, Rapta, S. M., P., 2009. Antimutagenic and radical scavenging activity of wheat bran. Cereal Res Commun 37, 45-55.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Laks, P. E., Pruner, M. S., 1989. Flavonoid biocides: Structure/activity relations of flavonoid phytoalexin analogues. Phytochemistry 28, 87-91.

Lavid, N., Wang, J., Shalit, M., Guterman, I., Bar, E., Beuerle, T., Menda, N., Shafir, S., Zamir, D., Adam, Z., Vainstein, A., Weiss, D., Pichersky, E., Lewinsohn, E., 2002.

O-Methyltransferases involved in the biosynthesis of volatile phenolic derivatives in rose petals. Plant Physiol 129, 1899-1907.

Li-Weber, M., 2009. New therapeutic aspects of flavones: the anticancer properties of Scutellaria and its main active constituents Wogonin, Baicalein and Baicalin. Cancer Treat Rev 35, 57-68.

Li, H., Zhou, C. X., Pan, Y., Gao, X., Wu, X., Bai, H., Zhou, L., Chen, Z., Zhang, S., Shi, S., Luo, J., Xu, J., Chen, L., Zheng, X., Zhao, Y., 2005. Evaluation of antiviral activity of compounds isolated from *ranunculus sieboldii* and *Ranunculus sceleratus*. Planta Med 71, 1128-1133.

Lin, Y.-P., Chen, T.-Y., Tseng, H.-W., Lee, M.-H., Chen, S.-T., 2009. Neural cell protective compounds isolated from Phoenix hanceana var. formosana. Phytochemistry 70, 1173-1181.

Long, S. R., 2001. Genes and signals in the rhizobium-legume symbiosis. Plant Physiol 125, 69-72.

Lopez-Lazaro, M., 2009. Distribution and biological activities of the flavonoid luteolin. Mini Rev Med Chem 9, 31-59.

Luszczki, J. J., Andres-Mach, M., Gleñsk, M., Skalicka-Woyniak, K., 2010. Anticonvulsant effects of four linear furanocoumarins, bergapten, imperatorin,oxypeucedanin, and xanthotoxin, in the mousemaximal electroshock-induced seizure model:a comparative study. Pharmacol Rep 62, 1231-1236.

Macgregor, J. T., Jurd, L., 1978. Mutagenicity of plant flavonoids: Structural requirements for mutagenic activity in *Salmonella typhimurium.* Mutat Res Envir Muta 54, 297-309.

Markert, C. L., 1984. Lactate dehydrogenase. Biochemistry and function of lactate dehydrogenase. Cell Biochem. Funct 2, 131-134.

Markham, K. R., Tanner, G. J., Caasi-Lit, M., Whitecross, M. I., Nayudu, M., Mitchell, K. A., 1998. Possible protective role for 3',4'-dihydroxyflavones induced by enhanced UV-B in a UV-tolerant rice cultivar. Phytochemistry 49, 1913-1919.

Martin-Tanguy, J., 1985. The occurrence and possible function of hydroxycinnamoyl acid amides in plants. Plant Growth Regul 3, 381-399.

Martin, C., Butelli, E., Petroni, K., Tonelli, C., 2011. How can research on plants contribute to promoting human health? The Plant Cell 23, 1685-1699.

Mathesius, U., Bayliss, C., Weinman, J. J., Schlaman, H. R. M., Spaink, H. P., Rolfe, B. G., McCully, M. E., Djordjevic, M. A., 1998. Flavonoids synthesized in cortical cells during nodule initiation are early developmental markers in white clover. Mol Plant Microbe Interact. 11, 1223-1232.

Maurício Duarte-Almeida, J., Novoa, A., Linares, A., Lajolo, F., Inés Genovese, M., 2006. Antioxidant activity of phenolics compounds from sugar cane (*Saccharum officinarum* L.) juice. Plant Foods Hum Nutr 61, 187-192.

McDonald, Y. F. a. C. E., 1989. Comparison of flavonoids in bran of four classes of wheat. Cereal Chem 66, 516-518.

McKersie, B., Murnaghan, J., Bowley, S., 1997. Manipulating freezing tolerance in transgenic plants. Acta Physiol Plant 19, 485-495.

McNally, D. J., Wurms, K. V., Labbe, C., Quideau, S., Belanger, R. R., 2003. Complex C-glycosyl flavonoid phytoalexins from *Cucumis sativus*. J Nat Prod 66, 1280-1283.

Middleton, E., Jr., Kandaswami, C., Theoharides, T. C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 52, 673-751.

Middleton, E. J., Kandaswami, C., 1994. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In: JB, H. (Ed.), The flavonoids advances in research since 1986. London: Chapman and Hall, 619-652.

Miles, D. H., Tunsuwan, K., Chittawong, V., Kokpol, U., Choudhary, M. I., Clardy, J., 1993. Boll weevil antifeedants from Arundo donax. Phytochemistry 34, 1277-1279.

Miralles, V. J., Serrano, R., 1995. A genomic locus in Saccharomyces cerevisiae with four genes up-regulated by osmotic stress. Mol. Microbiol. 17, 653-662.

Moheb, A., Ibrahim, R., Roy, R. and Sarhan, F. 2011. Changes in wheat leaf phenolome in response to cold acclimation. Phytochemistry 72, 2294-2307.

Moldéus, P., Högberg, J., Orrenius, S., Sidney, F., Lester, P., 1978. Isolation and use of liver cells. Methods Enzymol, 52, 60-71.

Morris, A. C., Djordjevic, M. A., 2006. The rhizobium leguminosarum biovar trifolii ANU794 induces novel developmental responses on the subterranean clover cultivar Woogenellup. Mol Plant Microbe Interact. 19, 471-479.

Muroi, A., Ishihara, A., Tanaka, C., Ishizuka, A., Takabayashi, J., Miyoshi, H., Nishioka, T., 2009. Accumulation of hydroxycinnamic acid amides induced by pathogen infection and identification of agmatine coumaroyltransferase in *Arabidopsis thaliana*. Planta 230, 517-527.

Murphy, A., Peer, W. A., Taiz, L., 2000. Regulation of auxin transport by aminopeptidases and endogenous flavonoids. Planta 211, 315-324.

N'Dong, C., Danyluk, J., Wilson, K., Pocock, T., Huner, N., Sarhan, F., 2002. Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analyses. Plant Physiol 129, 1368 - 1381.

Nadeau, P., Delaney, S., Chouinard, L., 1987. Effects of cold hardening on the regulation of polyamine levels in Wheat (*Triticum aestivum L.*) and Alfalfa (*Medicago sativa L.*). Plant Physiol 84, 73-77.

Nagarathnam, D., Cushman, M., 1991. A short and facile synthetic route to hydroxylated flavones. New syntheses of apigenin, tricin, and luteolin. ChemInform 22

Negrel, J., 1989. The biosynthesis of cinnamoyl putrescines in callus tissue cultures of *Nicotiana tabacum*. Phytochemistry 28, 477-481.

Negrel, J., Paynot, M., Javelle, F., 1992. Purification and properties of putrescine hydroxycinnamoyl transferase from Tobacco *(Nicotiana tabacum)* cell suspensions. Plant Physiol 98, 1264-1269.

Neto, C. C., 2007. Cranberry and its phytochemicals: a review of in vitro anticancer studies. J.Nutr 137, 186S-193S.

Ninomiya, M., Tanaka, K., Tsuchida, Y., Muto, Y., Koketsu, M., Watanabe, K., 2011 Increased bioavailability of tricin-amino acid derivatives via a prodrug approach. J Med Chem 54, 1529-1536.

Olenichenko, N., Ossipov, V., Zagoskina, N., 2006. Effect of cold hardening on the phenolic complex of winter wheat leaves. Russ J Plant Physl+ 53, 495-500.

Olenichenko, N. A., Zagoskina, N. V., Astakhova, N. V., Trunova, T. I., Kuznetsov Iu, V., 2008. Primary and secondary metabolism of winter wheat under cold hardening and treatment with antioxidants. Prikl Biokhim Mikrobiol 44, 589-594.

Oyama, T., Yasui, Y., Sugie, S., Koketsu, M., Watanabe, K., Tanaka, T., 2009. Dietary tricin suppresses inflammation-related colon carcinogenesis in male Crj: CD-1 mice. Cancer Prev Res (Phila) 2, 1031-1038.

Pang, W., Yang, H., Wu, Z., Huang, M., Hu, J., 2009. LC-MS–MS in MRM mode for detection and structural identification of synthetic hypoglycemic drugs added illegally to 'natural' anti-diabetic herbal products. Chromatographia 70, 1353-1359.

Park, H.-S., Lim, J., Kim, H., Choi, H., Lee, I.-S., 2007. Antioxidant flavone glycosides from the leaves of *Sasa borealis* Arch. Pharm. Res. 30, 161-166.

Parr, A. J., Mellon, F. A., Colquhoun, I. J., Davies, H. V., 2005. Dihydrocaffeoyl polyamines (kukoamine and allies) in potato (*Solanum tuberosum*) tubers detected during metabolite profiling. J Agric Food Chem 53, 5461-5466.

Passamonti, S., Terdoslavich, M., Franca, R., Vanzo, A., Tramer, F., Braidot, E., Petrussa, E., Vianello, A., 2009. Bioavailability of flavonoids: a review of their membrane transport and the function of bilitranslocase in animal and plant organisms. Curr Drug Metab 10, 369-394.

Peer, W. A., Murphy, A. S., 2007. Flavonoids and auxin transport: modulators or regulators? Trends Plant Sci. 12, 556-563.

Peters, N., Frost, J., Long, S., 1986. A plant flavone, luteolin, induces expression of Rhizobium meliloti nodulation genes. Science 233, 977-980.

Picman, A. K., Schneider, E. F., Picman, J., 1995. Effect of flavonoids on mycelial growth of *Verticillium albo-atrum*. Biochem. Syst. Ecol. 23, 683-693.

Polya, G., 2003. Biochemical targets of plant bioactive compounds. A pharmacological reference guide to sites of action and biological effects. Taylor& Francis Group.

Potts, D. T. (Ed.), 1997. Mesopotamian civilization : the material foundations Ithaca, N.Y. : Cornell Unviersity Press.

Redmond, J. W., Batley, M., Djordjevic, M. A., Innes, R. W., Kuempel, P. L., Rolfe, B. G., 1986. Flavones induce expression of nodulation genes in Rhizobium. Nature 323, 632-635.

Renuka Devi, R., Arumughan, C., 2007. Antiradical efficacy of phytochemical extracts from defatted rice bran. Food Chem. Toxicol. 45, 2014-2021.

Rhodes, D., Rich, P. J., Myers, A. C., Reuter, C. C., Jamieson, G. C., 1987. Determination of Betaines by Fast Atom Bombardment Mass Spectrometry: Identification of Glycine Betaine Deficient Genotypes of Zea mays. Plant Physiol 84, 781-788.

Sakai, A., Watanabe, K., Koketsu, M., Akuzawa, K., Yamada, R., Li, Z., Sadanari, H., Matsubara, K., Muroyama, T., 2008. Anti-human cytomegalovirus activity of constituents from Sasa albo-marginata (Kumazasa in Japan). Antivir Chem Chemother 19, 125-132.

Sakurai, N., 1998. Dynamic function and regulation of apoplast in the plant body. J. Plant Res. 111, 133-148.

Sattelmacher, B., 2001. The apoplast and its significance for plant mineral nutrition. New Phytol 149, 167-192.

Scott, E. N., Gescher, A. J., Steward, W. P., Brown, K., 2009. Development of dietary phytochemical chemopreventive agents: biomarkers and choice of dose for early clinical trials. Cancer Prev Res 2, 525-530.

Segarra, G., Jauregui, O., Casanova, E., Trillas, I., 2006. Simultaneous quantitative LC-ESI-MS/MS analyses of salicylic acid and jasmonic acid in crude extracts of Cucumis sativus under biotic stress. Phytochemistry 67, 395-401.

Seki, M., Umezawa, T., Urano, K., Shinozaki, K., 2007. Regulatory metabolic networks in drought stress responses. Curr Opin Plant Biol 10, 296-302.

Seki, N., Toh, U., Kawaguchi, K., Ninomiya, M., Koketsu, M., Watanabe, K., Aoki, M., Fujii, T., Nakamura, A., Akagi, Y., Kusukawa, J., Kage, M., Shirouzu, K., Yamana, H., 2012. Tricin inhibits proliferation of human hepatic stellate cells in vitro by blocking tyrosine phosphorylation of PDGF receptor and its signaling pathways. J Cell Biochem. DOI 10.1002/jcb.24107

Shang, M., Cai, S., Han, J., Li, J., Zhao, Y., Zheng, J., Namba, T., Kadota, S., Tezuka, Y., Fan, W., 1998. Studies on flavonoids from Fenugreek (*Trigonella foenumgraecum* L.). Zhongguo Zhong Yao Za Zhi 23, 614-616.

Stochmal, A., Simonet, A. M., Macias, F. A., Oleszek, W., 2001. Alfalfa (Medicago sativa L.) flavonoids. 2. Tricin and chrysoeriol glycosides from aerial parts. J Agric Food Chem 49, 5310-5314.

Swain, T., 1975. Evolution of flavonoids compounds. In: Harborne, J. B., Mabry, T. J., Mabry, H. (Eds.), The Flavonoids, Chapman & Hall, London, 1096-1129.

Ta, N., Walle, T., 2007. Aromatase inhibition by bioavailable methylated flavones. J Steroid Biochem Mol Biol 107, 127-129.

Tardif, G., Kane, N., Adam, H., Labrie, L., Major, G., Gulick, P., Sarhan, F., Laliberté, J.-F., 2007. Interaction network of proteins associated with abiotic stress response and development in wheat. Plant Mol Biol 63, 703-718.

Taylor, L. P., Grotewold, E., 2005. Flavonoids as developmental regulators. Curr Opin Plant Biol 8, 317-323.

Thomashow, M. F., 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50, 571-599.

Timberlake, C. F., Henry, B. S., 1986. Plant pigments as natural food colours. Endeavour 10, 31-36.

Tregear, J. W., Morcillo, F., Richaud, F. d. r., Berger, A. l., Singh, R., Cheah, S. C., Hartmann, C., Rival, A., Duval, Y., 2002. Characterization of a defensin gene expressed in oil palm inflorescences: induction during tissue culture and possible association with epigenetic somaclonal variation events. J Exp Bot 53, 1387-1396.

Uden, W., Pras, N., Batterman, S., Visser, J. F., Malingré, T. M., 1991. The accumulation and isolation of coniferin from a high-producing cell suspension of *Linum flavum* L. Planta 183, 25-30.

Uemura, M., Steponkus, P. L., 1997. Effect of cold acclimation on the lipid composition of the inner and outer membrane of the chloroplast envelope isolated from rye leaves. Plant Physiol 114, 1493-1500.

Vanacker, H., Carver, T. L., Foyer, C. H., 1998. Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves. Plant Physiol 117, 1103-1114.

Verma, A., Banerjee, R., 2010. Dietary fibre as functional ingredient in meat products: a novel approach for healthy living — a review. J. Food Sci. Technol. 47, 247-257.

Verma, B., Hucl, P., Chibbar, R. N., 2008. Phenolic content and antioxidant properties of bran in 51 wheat cultivars. Cereal Chem 85, 544-549.

Verschoyle, R. D., Greaves, P., Cai, H., Borkhardt, A., Broggini, M., D'Incalci, M., Riccio, E., Doppalapudi, R., Kapetanovic, I. M., Steward, W. P., Gescher, A. J., 2006. Preliminary safety evaluation of the putative cancer chemopreventive agent tricin, a naturally occurring flavone. Cancer Chemother Pharmacol 57, 1-6.

Ververidis, F., Trantas, E., Douglas, C., Vollmer, G., Kretzschmar, G., Panopoulos, N., 2007. Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I: Chemical diversity, impacts on plant biology and human health. Biotechnol. J. 2, 1214-1234.

Virgili, F., Marino, M., 2008. Regulation of cellular signals from nutritional molecules: a specific role for phytochemicals, beyond antioxidant activity. Free Radical Biol. Med. 45, 1205-1216.

Walle, T., 2007. Methoxylated flavones, a superior cancer chemopreventive flavonoid subclass? **S**emin. Cancer Biol. 17, 354-362.

Walle, T., 2009. Methylation of dietary flavones increases their metabolic stability and chemopreventive effects. Int. J. Mol. Sci. 10, 5002-5019.

Walle, T., Ta, N., Kawamori, T., Wen, X., Tsuji, P. A., Walle, U. K., 2007. Cancer chemopreventive properties of orally bioavailable flavonoids--Methylated versus unmethylated flavones. Biochem Pharmacol 73, 1288-1296.

Walters, D. R., 2003. Polyamines and plant disease. Phytochemistry 64, 97-107.

Wang, J., Hou, B., 2009. Glycosyltransferases: key players involved in the modification of plant secondary metabolites. Front. Biol. China 4, 39-46.

Wasson, A. P., Pellerone, F. I., Mathesius, U., 2006. Silencing the flavonoid pathway in *Medicago truncatula* inhibits root nodule formation and prevents auxin transport Regulation by Rhizobia. The Plant Cell 18, 1617-1629.

Watanabe, M., 1999. Antioxidative phenolic compounds from Japanese barnyard millet (*Echinochloa utilis*) grains. J Agric Food Chem 47, 4500-4505.

Wei, H. U. I., Dhanaraj, A. L., Arora, R., Rowland, L. J., Fu, Y. A. N., Sun, L. I., 2006. Identification of cold acclimation-responsive Rhododendron genes for lipid metabolism, membrane transport and lignin biosynthesis: importance of moderately abundant ESTs in genomic studies. Plant Cell Environ 29, 558-570.

Weickert, M. O., Mohlig, M., Schofl, C., Arafat, A. M., Otto, B. r., Viehoff, H., Koebnick, C., Kohl, A., Spranger, J., Pfeiffer, A. F. H., 2006. Cereal fiber improves whole-body insulin sensitivity in overweight and obese women. Diabetes Care 29, 775-780.

Weidenbörner, M., Jha, H. C., 1997. Antifungal spectrum of flavone and flavanone tested against 34 different fungi. Mycol Res 101, 733-736.

Wen, X., Walle, T., 2006. Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. Drug Metab and Dispos 34, 1786-1792.

Williams, R. J., Spencer, J. P. E., Rice-Evans, C., 2004. Flavonoids: antioxidants or signalling molecules? Free Radical Biol. Med. 36, 838-849.

Winkel-Shirley, B., 2001. Flavonoid Biosynthesis. A Colorful Model for Genetics, Biochemistry, Cell Biology, and Biotechnology. Plant Physiol 126, 485-493.

Winkel-Shirley, B., 2002. Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5, 218-223.

Wollenweber, E., Dorr, M., 2008. Occurrence and distribution of the flavone tricetin and its methyl derivatives as free aglycones. Nat. Prod. Commun. 3, 1293-1298.

Wollenweber, E., Wehde, R., Dorr, M., Stevens, J. F., 2002. On the occurrence of exudate flavonoids in the borage family (Boraginaceae). Z Naturforsch C 57, 445-448.

www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000261.pdf. GRAS Notification for Oat Fiber 2008 FDA.

www.fda.gov/.../06p-0393-cp00001-043-Tab-G-GRAS-Expert-Panel-vol2.pdf, 2003, FDA.

Xiao-Hong, S., Xiong-Yu, W., Yong-Cheng, L., C.Wing-Lai, , 1999. Zhongshan Daxue Xuebao, Ziran Kexueban 38, 127.

Yamamoto, E., Lewis, N. G., Ohashi, H., Towers, G. H. N., 1987. 5-hydroxyferulic acid in Zea mays and Hordeum vulgare cell walls. Phytochemistry 26, 1915-1916.

Yannai, S., 2004. Dictionary of food compounds with CD-ROM : additives, flavors, and ingredients. In: APA (Ed.). Chapmann & Hall / CRC.

Yazawa K, Kurokawa M, Obuchi M, L. Y., Yamada R, Sadanari H, Matsubara K, Watanabe K, Koketsu M, Tuchida Y, T, M., 2011. Anti-influenza virus activity of tricin, 4',5,7-trihydroxy-3',5'-dimethoxyflavone, Antivir Chem Chemother 22, 1-11.

Zagoskina, N. V., Olenichenko, N. A., Klimov, S. V., Astakhova, N. V., Zhivukhina, E. A., Trunova, T. I., 2005. The effects of cold acclimation of winter wheat plants on changes in CO_2 exchange and phenolic compound formation, Russ. J. Plant Physiol. 52, 320-325.

Zhang, J., Subramanian, S., Stacey, G., Yu, O., 2009. Flavones and flavonols play distinct critical roles during nodulation of *Medicago truncatula* by *Sinorhizobium meliloti*. The Plant Journal 57, 171-183.

Zhang, L. H., Fan, C. L., Ye, W. C., Li, Y. P., 2008. Study on flavonoids and phenolic acids from the herb of *Lygodium japonicum*. Zhong Yao Cai 31, 224-226.

Zhao, J., Dixon, R. A., 2010. The 'ins' and 'outs' of flavonoid transport. Trends Plant Sci. 15, 72-80.

Zhou, J. M., Gold, N. D., Martin, V. J., Wollenweber, E., Ibrahim, R. K., 2006a. Sequential *O*-methylation of tricetin by a single gene product in wheat. Biochim Biophys Acta 1760, 1115-1124.

Zhu, B. T., Ezell, E. L., Liehr, J. G., 1994. Catechol-*O*-methyltransferase-catalyzed rapid *O*-methylation of mutagenic flavonoids. Metabolic inactivation as a possible reason for their lack of carcinogenicity in vivo. J. Biol. Chem. 269, 292-299.

Appendix A: Image for Wheat Husk

Figure A-1: Winter wheat (Claire) yellow inflorescence (husk)

Appendix B: Image for Wheat (T. aestivum) Leaves

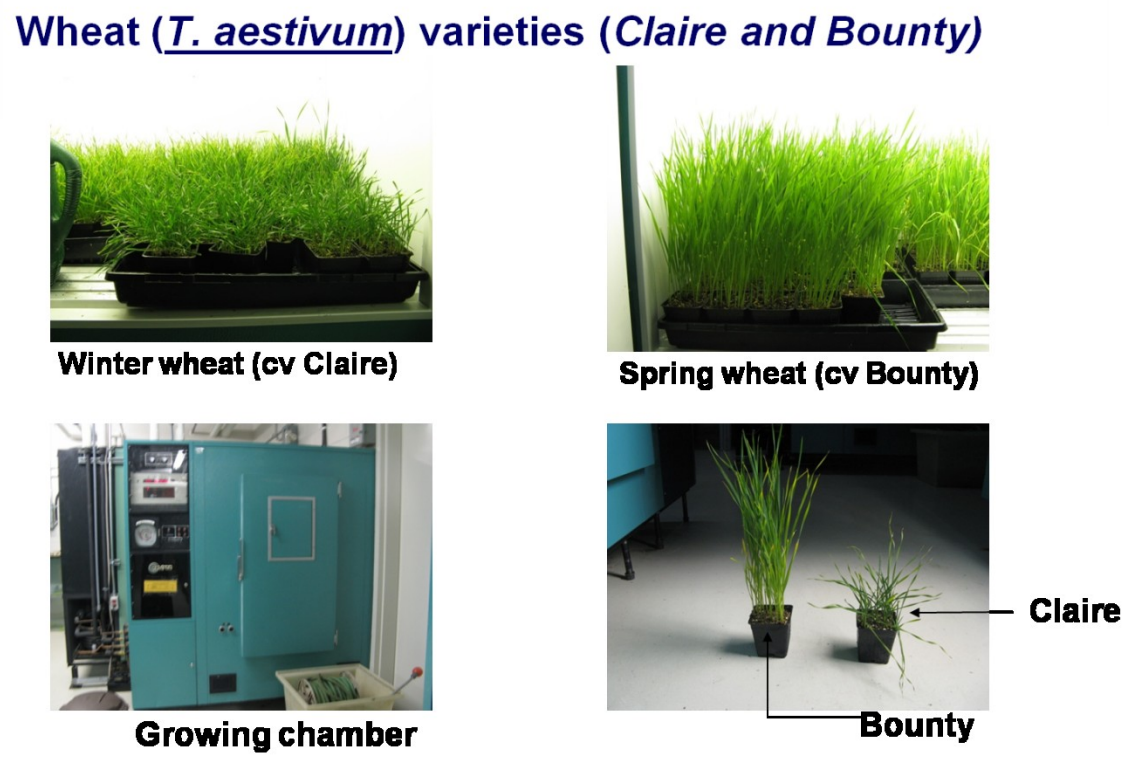


Figure B-1: Wheat (*T. aestivum*) leaves, winter wheat (Claire) and spring wheat (Bounty), and the growing chamber.

Appendix C: Image for the Dissolution Tester

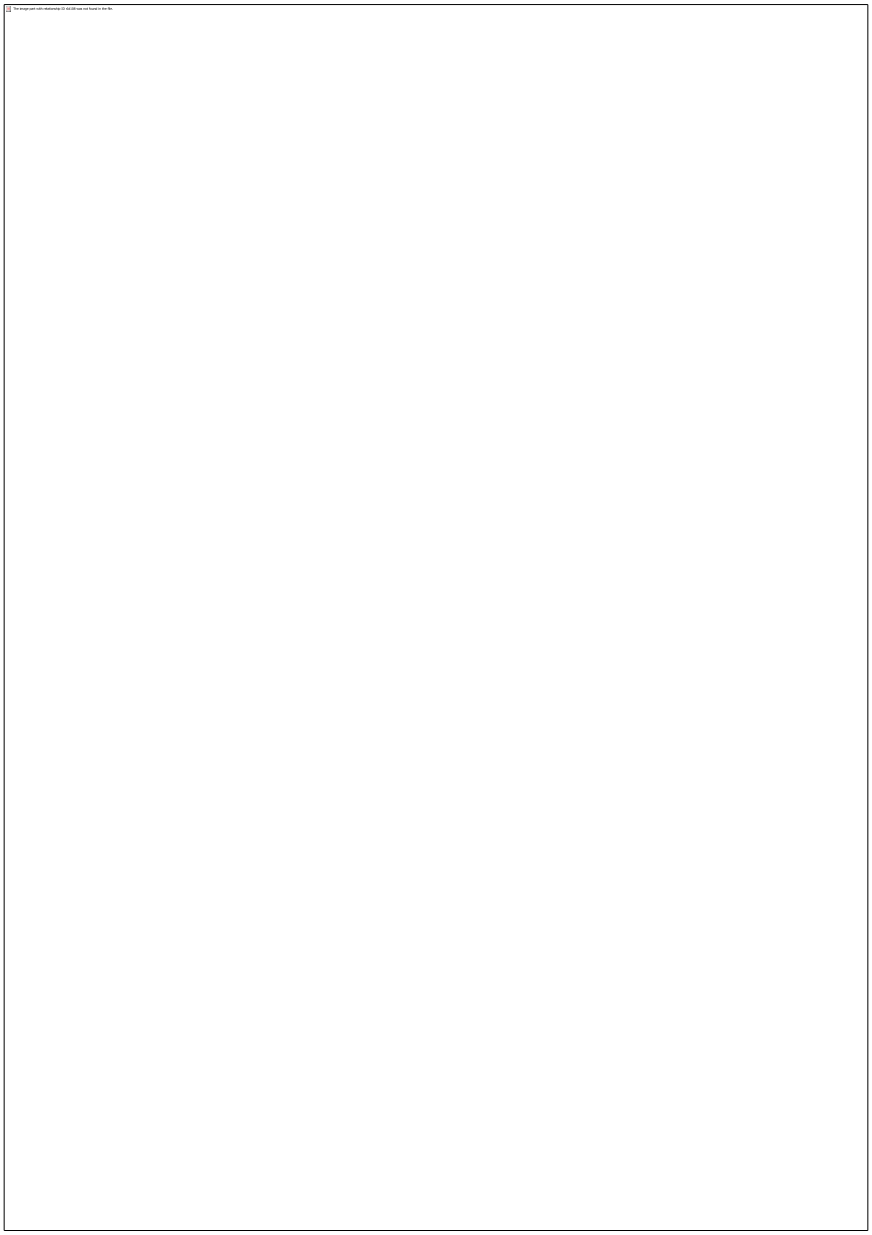


Figure C-1: Dissolution tester

Appendix D: Major Branch Pathways of Flavonoid Biosynthesis

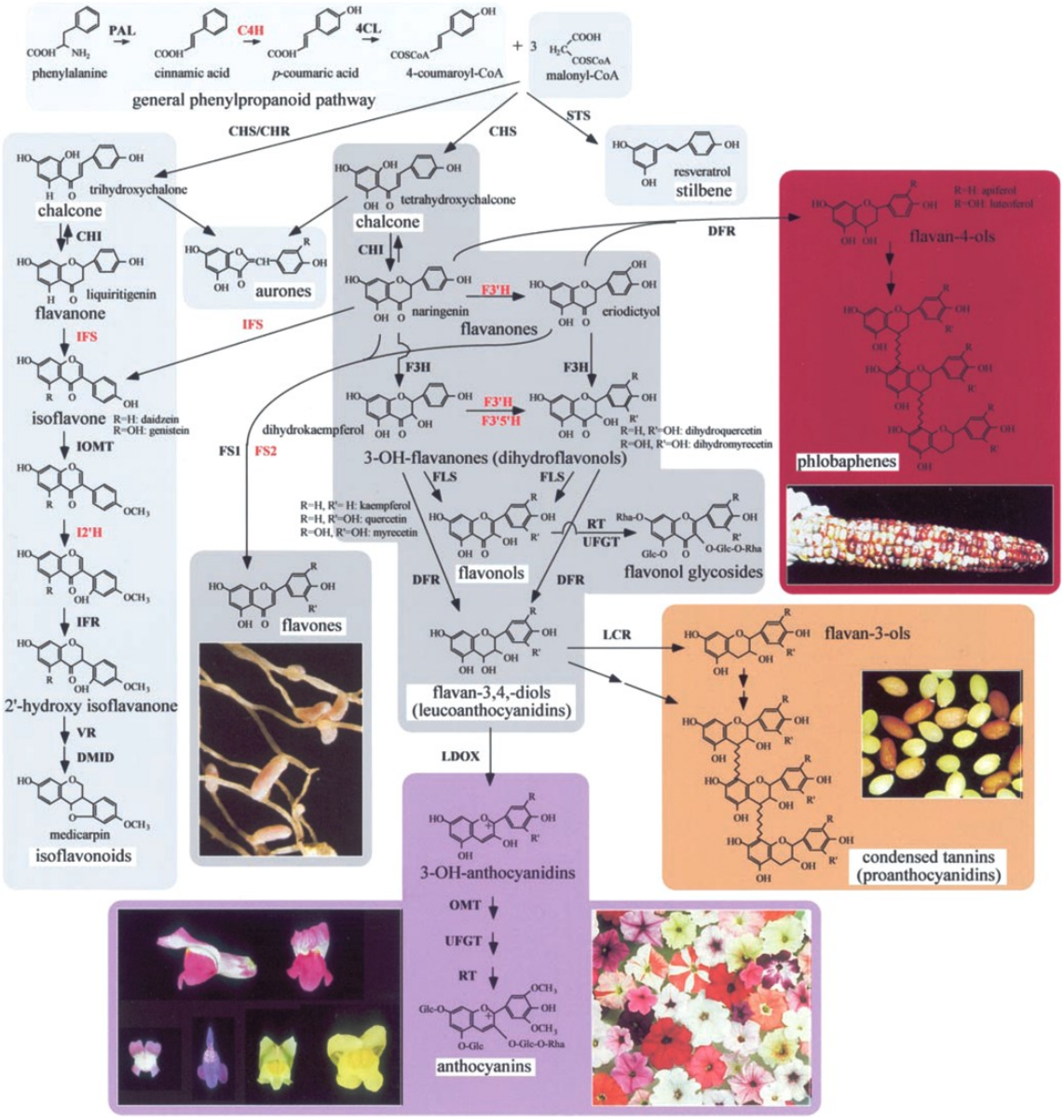


Figure D-1: Schematic of the major branch pathways of flavonoid biosynthesis

(Figure from (Winkel-Shirley, 2001)) "Copyright American Society of Plant Biologists, www.plantphysiol.org"

Schematic representation of the major branch pathways of flavonoid biosynthesis, starting with the general phenylpropanoid metabolism and leading to the nine major classes of flavonoids: the chalcones, aurones, isoflavonoids, flavones, flavonols, and

*flavandiols (gray boxes), and the anthocyanins, condensed tannins, and phlobaphene pigments (colored boxes). Enzyme names are abbreviated as follows: cinnamate-4-hydroxylase (**C4H**), chalcone isomerase (**CHI**), chalcone reductase (**CHR**), chalcone synthase (**CHS**), 4-coumaroyl:CoA-ligase (**4CL**), dihydroflavonol 4-reductase (**DFR**), 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase (**DMID**), flavanone 3-hydroxylase (**F3H**), flavone synthase (**FSI** and **FSII**), flavonoid 3' hydroxylase **(F3'H**) or flavonoid 3'5' hydroxylase (**F3'5'H**), isoflavone O-methyltransferase **(IOMT**), isoflavone reductase (**IFR**), isoflavone 2'-hydroxylase (**I2'H**), isoflavone synthase (**IFS**), leucoanthocyanidin dioxygenase (**LDOX**), leucoanthocyanidin reductase (LCR), O-methyltransferase (**OMT**), Phe ammonia-lyase (**PAL**), rhamnosyl transferase (**RT**), stilbene synthase (**STS**), UDPGflavonoid glucosyl transferase (**UFGT**), and vestitone reductase **(VR**).*