# Antioxidant Activities of *Sonchus oleraceus* L.

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## ABSTRACT

Supernumerary free radicals and other reactive species can cause oxidative damage in animal cells, potentially leading to non-infectious diseases. Diets rich in low molecular weight antioxidants (LMWAs) may prevent or arrest the pathogenesis of these diseases. Leaves of *Sonchus oleraceus* L. may be an excellent dietary LMWA source for humans given their apparent strong antioxidant activities *in vitro*. However, different *S. oleraceus* plants vary in their antioxidant capacity. Nothing is known of possible environmental effects on antioxidant potential. Equally, the effects of cooking and gastrointestinal digestion are unknown. The goals of this research were: (i) to study the effects of plant age, locality, and abiotic stressors on antioxidant potential; (ii) to study the effects of cooking and *in vitro* gastrointestinal digestion on antioxidant activity and uptake in human cells; and (iii) to study extractable antioxidant activities of *S. oleraceus* cell suspension cultures in relation to abiotic stressors.

Antioxidant activities and levels of total phenolics, hydroxycinnamic acids and ascorbate increased as plants aged. An ecotype from Acacia Bay had a higher phenolic content and antioxidant activities than one from Oamaru; these differences were maintained across generations as well as in calli from *in vitro* cultures. This indicates heritability and genetic fidelity of antioxidant potential.

Chilling and salinity had variable effects on concentrations of phenolics and antioxidant activities in plants, and the combination of the two stressors was not synergistic. This indicates that these two stressors share signalling and response pathways. Stressor-induced increases in antioxidant activities of leaf extracts correlated with improved cellular antioxidant activities (CAA) inside HepG2 cells. Antioxidants were released from leaves following *in vitro* gastrointestinal digestion, which were then subsequently uptaken by Caco2 and HepG2 cells wherein they displayed CAAs. Thus, elevated levels of antioxidants in stressor-imposed plants provide potentially more antioxidant protection to live human cells.

Caftaric, chlorogenic and chicoric acids accounted for 92% of the phenolic compounds in *S. oleraceus* leaves. Of these, only chlorogenic acid was inducible by stressors, both in

intact plants and in calli. In young stressor-applied plants, chlorogenic acid was enhanced to the levels achievable with plant ageing.

Boiling leaves prior to digestion did not diminish the caftaric and chlorogenic acid levels released through digestion, but chicoric acid levels were. Out of the nine phenolic compounds in leaves, only chicoric, chlorogenic and caftaric acids were released into the medium during *in vitro* gastrointestinal digestion. Digestion of leaves resulted in effective release of caftaric and chlorogenic acids from leaves but the levels of released chicoric acid were diminished by digestion.

This study offers insights into the factors that influence the antioxidant potential of *S. oleraceus* L. *in vivo, in vitro,* during cooking and *in vitro* gastrointestinal digestion. These results provide the foundation for: (1) encouraging the consumption of its fresh shoots as an antioxidant rich food; (2) further improving its antioxidant activities through manipulation of agronomy, ecotype and breeding; (3) developing its cell cultures as a commercial platform for phyto-antioxidant production aimed at formulating dietary supplements or food additives in biopharmaceutical industry.

## ACKNOWLEDGEMENTS

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# CHAPTER 1: REVIEW OF LITERATURE

## 1.1 REACTIVE SPECIES AND ANTIOXIDANT DEFENCES

A wide range of free radicals and other reactive species (Table 1.1) are capable of damaging the lipids, proteins, and DNA in plant and animal cells, and are associated with the development of various diseases. Of these reactive species the radical and non-radical derivatives of oxygen, collectively known as reactive oxygen species (ROS) are the most abundant type in plants and animals (Lushchak, 2011). ROS are either generated internally within the various tissues of organisms, or may be absorbed from external sources. ROS are formed internally by leakage of electrons to molecular oxygen in the photosynthetic and respiratory electron transport chains (Halliwell and Gutteridge, 2007). The formation of ROS in organisms can be induced externally by exposure to cigarette smoke, environmental pollutants, radiation, ultraviolet light, some chemicals and ozone (Halliwell and Gutteridge, 2007).

A system of antioxidant defences effectively neutralizes the ROS and counteracts their negative effects. However, when antioxidant defences are overwhelmed by the generation of supernumerary reactive species, tissues may be subjected to oxidative assault, resulting in damage to biomolecules. In humans, this can potentially lead to premature aging (Kregel and Zhang, 2007), inflammation (Federico *et al.*, 2007), cancer (Halliwell, 2007), atherosclerosis (Singh and Jialal, 2006), diabetes (Baynes and Thorpe, 1999; Mehta *et al.*, 2006), liver injury (Parola and Robino, 2001; Choi and James Ou, 2006), Alzheimer's disease (Mamelak, 2007), Parkinson's disease (Fahn, 1992), and coronary heart problems (Singal *et al.*, 1998; Madamanchi *et al.*, 2005).

The antioxidant defences in organisms comprise: (i) enzymes, which catalytically remove ROS; (ii) enzymes that regenerate oxidised antioxidants; (iii) proteins that minimise the availability of pro-oxidant ions such as iron and copper; (iv) proteins that protect biomolecules against oxidative damage; and (v) a variety of low molecular weight antioxidants (LMWAs), which scavenge ROS (Halliwell and Gutteridge, 2007). Humans synthesise some LMWAs *in vivo*; these include glutathione, α-keto acids, sex hormones, melatonin, lipoic acid, coenzyme Q10, uric acid, histidine, and melanins. Others are

derived from ingested food particularly derived from plants, and include tocopherols (vitamin E), ascorbic acid (vitamin C), carotenoids (vitamin A), and an assortment of phenolic and polyphenolic compounds (Halliwell and Gutteridge, 2007).

**Table 1.1 List of selected free radicals and other reactive species**

| Radicals | Non-radicals |
|---|---|
| Reactive oxygen species (ROS) | |
| • Superoxide ($O_2^{\bullet-}$) | • Hydrogen peroxide ($H_2O_2$) |
| • Hydroxyl ($OH^{\bullet}$) | • Hypochlorous (HOCl) |
| • Peroxyl ($RO_2^{\bullet}$) | • Ozone ($O_3$) |
| • Alkoxyl ($RO^{\bullet}$) | • Singlet oxygen ($^1O_2$) |
| • Hydroperoxyl ($HO_2^{\bullet}$) | • Peroxynitrite ($ONOO^-$) |
| Reactive nitrogen species | |
| • Nitric oxide ($NO^{\bullet}$) | • Nitrous acid ($HNO_2$) |
| • Nitrogen dioxide ($NO_2^{\bullet}$) | • Nitrosyl cation ($NO^+$) |
| | • Nitroxyl anion ($NO^-$) |
| | • Alkyl peroxynitriles (ROONO) |
| Reactive chlorine species | |
| • Atomic chlorine ($Cl^{\bullet}$) | • Chlorine gas ($Cl_2$) |
| | • Bromine chloride (BrCl) |
| | • Chlorine dioxide ($ClO_2$) |
| Sulphur centered radicals | |
| • Thiyl radicals ($RS^{\bullet}$) | |
| • Perthiyl ($RSS^{\bullet}$) | |
| Carbon centred radicals | |
| • Trichloromethyl ($CCl_3^{\bullet}$) | |
| Transition metal ions | |
| | • Iron ions ($Fe^{2+}$, $Fe^{3+}$) |
| | • Copper ions ($Cu^{2+}$) |
| | • Manganese ions ($Mn^{2+}$) |

Source: Halliwell and Gutteridge (2007)

## 1.2 LOW MOLECULAR WEIGHT ANTIOXIDANTS OF DIETARY ORIGIN

### 1.2.1 Ascorbic acid

Animals lack the capacity to synthesise ascorbic acid, and therefore obtain it exclusively from their diet (Halliwell and Gutteridge, 2007). Because plants are the only natural dietary source of ascorbic acid for humans, efforts are underway to enhance ascorbic acid levels in plants through breeding and manipulation of growth conditions (Smirnoff, 2011). Several putative pathways of ascorbic acid biosynthesis occur simultaneously in plants (Smirnoff, 2011). In biological systems ascorbic acid functions as an antioxidant by donating electrons to neutralise ROS (Halliwell and Gutteridge, 2007). Donation of one electron by ascorbate forms the ascorbyl radical, which then disproportionates to monodehydroascorbate (MDHA). Further oxidation of MDHA produces dehydroascorbate (DHA). MDHA and DHA can be reduced to form ascorbate by energy dependent reactions, each catalysed by MDHA reductase (MDHAR) and DHA reductase (DHAR) enzymes; these reactions comprise the ascorbate-glutathione cycle (Figure 1.1).

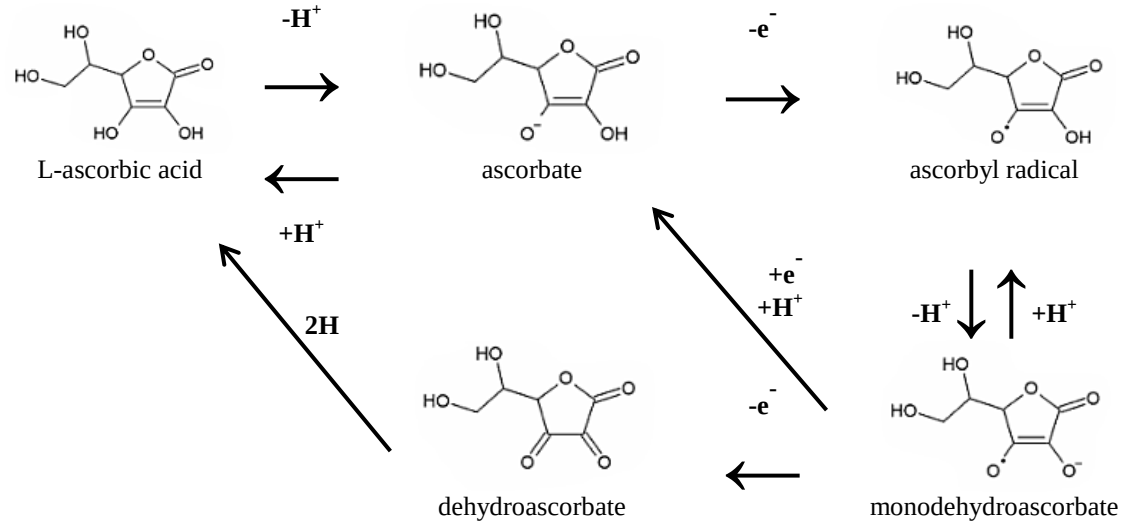



**Figure 1.1 L-ascorbic acid and its oxidation products**

**Source: Smirnoff (2011)**

Sufficient supply of vitamin C has been associated with reduced onset of diseases such as Alzheimer's (Zandi *et al.*, 2004), diabetes (Murthy *et al.*, 1992), cardiovascular diseases (Diaz *et al.*, 1997) and other oxidative stress disorders (Grassmann *et al.*, 2002) possibly due to its antioxidant activity *in vivo*.

### 1.2.2 Carotenoids

Carotenoids are a family of pigmented antioxidants such as α-carotene, β-carotene, lycopene, lutein and zeaxanthin. They have a characteristic long chain of conjugated double bonds (Figure 1.2), with bilateral symmetry around the central double bond (Britton, 1995). Modification of the basic structure at the end groups by hydrogenation, dehydrogenation, cyclization, oxidation or any combination of these processes results in formation of derivatives with specific colours and antioxidant properties (Raoa and Raob, 2007).

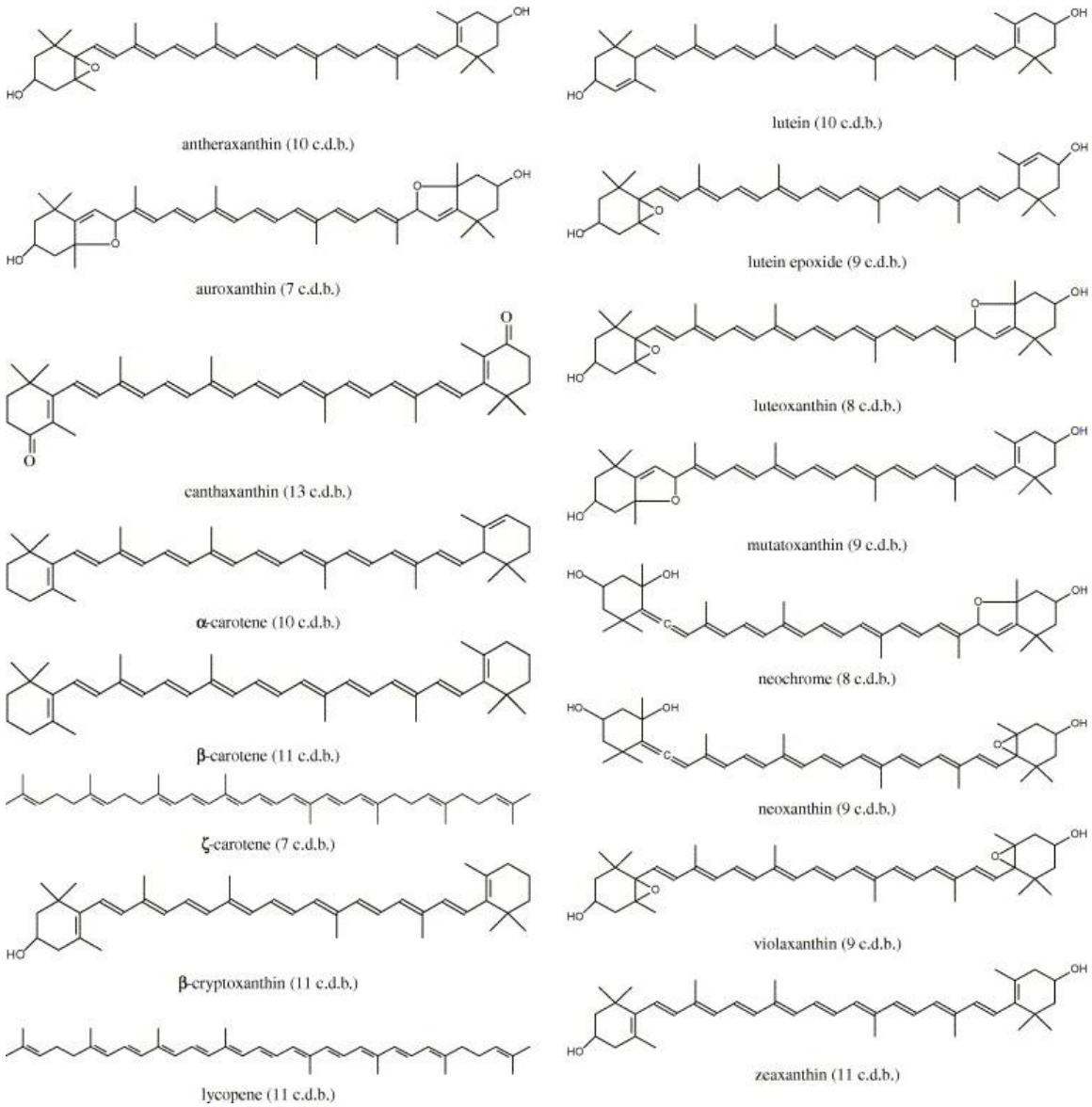



**Figure 1.2 Structures of several carotenoids found in plants and animals**

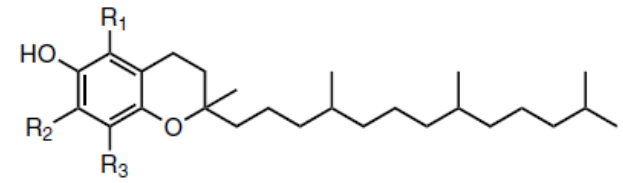
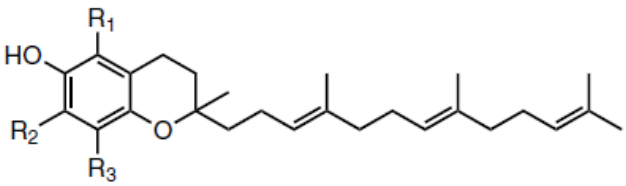
**Source: Meléndez-Martínez *et al.* (2007)**

Fruits and vegetables constitute the major sources of carotenoids in the adult human diet (Johnson, 2002). Some carotenoids such as β-carotene and β-cryptoxanthin serve as the precursor for fat-soluble vitamin A (Maiani *et al.*, 2009). Carotenoids are distributed in the membranes, lipoproteins, and adipocytes of the human body because of their lipophilic nature. Carotenoids display *in vivo* antioxidant properties by quenching ROS such as singlet oxygen (formed during lipid peroxidation). Quenching activity leaves the carotenoid structure intact, thus regeneration is not required (Sies and Stahl, 1995). Carotenoids were shown to have preventive activity against cardiovascular diseases (Ford and Giles, 2000) and UV-induced erythema (Stahl *et al.*, 2001).

### 1.2.3 Vitamin E

Vitamin E refers to a group of lipid-soluble, naturally occurring antioxidants comprising α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol (Table 1.2), which are capable of neutralising a variety of ROS (Halliwell and Gutteridge, 2007). Tocopherols and tocotrienols scavenge lipid peroxyl radicals and singlet oxygen, thereby preventing lipid peroxidation and subsequent oxidative injury in biological systems (Niki, 2013). Ingested tocopherols have been correlated with reduced indices of oxidative stress (Roberts II *et al.*, 2007) in patients with cardiovascular disease (Rimm *et al.*, 1993) and coronary artery disease (Devaraj *et al.*, 2007).

**Table 1.2 Structures of tocopherols and tocotrienols**

| Basic structure | Example | **R1** | **R2** | **R3** |
|---|---|---|---|---|
| Tocopherols | α-tocopherol | $CH_3$ | $CH_3$ | $CH_3$ |
|  | β-tocopherol | $CH_3$ | H | $CH_3$ |
| | γ-tocopherols | H | $CH_3$ | $CH_3$ |
| | δ-tocopherols | H | H | $CH_3$ |
| Tocotrienol | α-tocotrienol | $CH_3$ | $CH_3$ | $CH_3$ |
|  | β-tocotrienol | $CH_3$ | H | $CH_3$ |
| | γ-tocotrienol | H | $CH_3$ | $CH_3$ |
| | δ-tocotrienol | H | H | $CH_3$ |

**Source: Dörmann (2007)**

### 1.2.4 Phenolic compounds

Plants contain a large variety of phenolic compounds such as phenolic acids, flavonoids, stilbenes and their various derivatives. Phenolic compounds in plants are ubiquitous, diverse and are extremely important as compounds that affect pigmentation, astringency, antibacterial, anticancer, antioxidant and antiviral properties of plant derived products and foods (Jaganath and Crozier, 2010). Phenolic compounds in plants are synthesised from phenylalanine through the phenylpropanoid pathway (Halliwell and Gutteridge, 2007). Phenolic compounds are characterized by at least one hydroxyl group attached to a benzene ring (Halliwell and Gutteridge, 2007).

#### 1.2.4.1 Phenolic acids

There are two major groups of phenolic acids: hydroxybenzoic acids and hydroxycinnamic acids (Table 1.3) and their associated derivatives. Derivatives of phenolic acids occur when they bind with cellulose, lignin, sugars and proteins through ester bonds (Halliwell and Gutteridge, 2007). Examples of hydroxycinnamic acid derivatives include: chlorogenic acid, which is an ester of caffeic acid and quinic acid; caftaric acid, which is an ester of caffeic acid and tartaric acid; and chicoric acid, which comprises of two caffeic acid molecules (Table 1.4).

**Table 1.3 Classification, occurrence and structure of selected phenolic acids in plants**

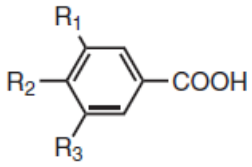
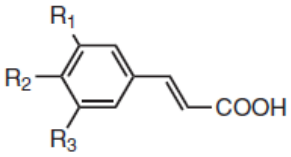
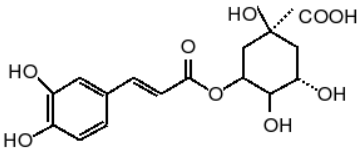
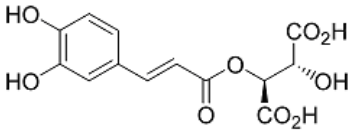
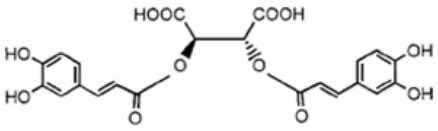
| Basic structure | Phenolic acid | R1 | R2 | R3 | Occurrence |
|---|---|---|---|---|---|
| Hydroxybenzoic acid   | Hydroxybenzoic | H | OH | H | Apples, cherries, grapefruit, grapes, kiwi fruit, olives, oranges, peaches, pears, potatoes, wine (Tomás-Barberán and Clifford, 2000) |
| | Protocatechuic | OH | OH | H | |
| | Vannilic | $OCH_3$ | OH | H | |
| | Syringic | $OCH_3$ | OH | $OCH_3$ | |
| | Gallic | OH | OH | OH | |
| Hydroxycinnamic acid   | Caffeic | H | OH | H | Apples, blueberries, cereal bran, cherries, citrus species, coffee, kiwi fruit, olives, peaches, pears, potatoes, tomatoes, white grapes, white wine (Clifford, 1999) |
| | Ferulic | OH | OH | H | |
| | Cinnamic | $OCH_3$ | OH | H | |
| | Sinapic | $OCH_3$ | OH | $OCH_3$ | |

**Table 1.4 Selected hydroxycinnamic acid derivatives and occurrence in plants**

| Basic structure | Occurrence |
|---|---|
| Chlorogenic acid (5-caffeoylquinic acid)  | Commercial coffee beverage (Mateos *et al.*, 2006)<br>*Echinacea* spp (Pellati *et al.*, 2004) |
| Caftaric acid  | *Ocimum basilicum* (Lee and Scagel, 2009)<br>*Echinacea* spp (Perry *et al.*, 2001; Pellati *et al.*, 2004) |
| Chicoric acid  | *Echinacea purpurea* (Pellati *et al.*, 2004)<br>*Taraxacum officinale* (Chkhikvishvili and Kharebava, 2001)<br>*Lactuca sativa* (Nicolle *et al.*, 2004)<br>*Ocimum basilicum* (Lee and Scagel, 2009)<br>*Syringodium filiforme* (Nuissier *et al.*, 2010) |

### 1.2.4.2 Flavonoids

Flavonoids (Figure 1.3) are characterized by two benzene rings (A and B), which are linked together by a γ-pyrone ring (C). Flavonoids are a broad class of LMWAs in plants through various modifications to the basic structure by the addition of hydroxyl, methyl or glycoside moieties (Tables 1.5 and 1.6). In plants, these compounds function as pigments, perform a number of regulatory roles in plant development and may provide protection against ultraviolet radiation, pathogens, and herbivores (Gould, 2004; Treutter, 2006).

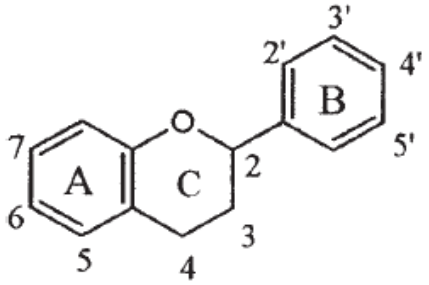
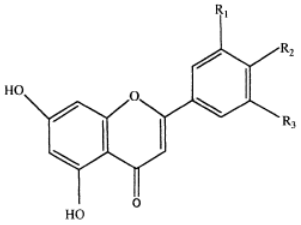
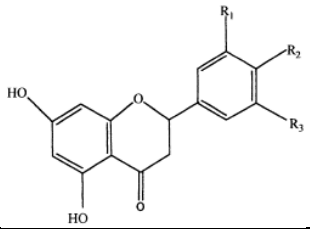
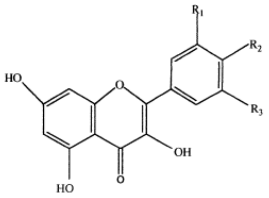
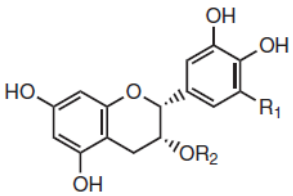
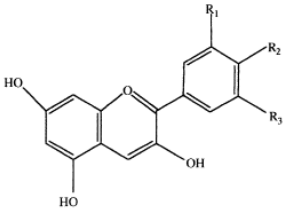
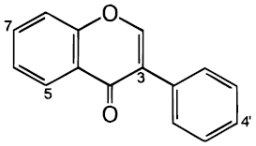


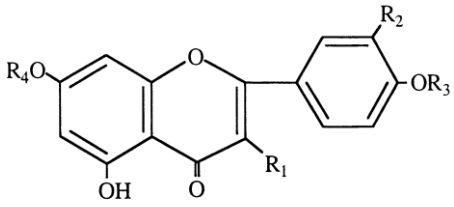

**Figure 1.3 Basic structure of flavonoid molecule**

**Table 1.5 Classification, occurrence and structure of selected flavonoids naturally occurring in plants**

| Classes and their basic structure | Examples | R1 | R2 | R3 | Occurrence |
|---|---|---|---|---|---|
| Flavones  | Apigenin | H | OH | H | Celery, citrus fruits, parsley, red pepper, red wine (Hollman and Arts, 2000) |
| | Luteolin | H | OH | OH | |
| Flavanones  | Hesperetin | H | $OCH_3$ | OH | Citrus fruits (Erlund, 2004; Peterson *et al.*, 2006a; Peterson *et al.*, 2006b) |
| | Naringenin | H | OH | H | |
| Flavonols  | Quercetin | H | OH | OH | Apples, berries, black tea, grapefruit, grapes, olives, onions, red wine (Hollman and Arts, 2000; Erlund, 2004) |
| | Kaempferol | H | OH | H | |
| | Myricetin | OH | OH | OH | |
| Flavan-3-ols  | Epicatechin | H | H | - | Apples, apricots, cherries, green tea, black tea, peaches, plums, red wine (Hollman and Arts, 2000; Monagas *et al.*, 2005) |
| | Epicatechin gallate | H | gallate | - | |
| | Epigallocatechin | OH | H | - | |
| | Epigallocatechin gallate | OH | gallate | - | |
| Anthocyanidins  | Peonidin | $OCH_3$ | OH | H | Berries, blood oranges, red cabbage, eggplant, grapes, plums, red wine, rhubarb (Clifford, 2000a) |
| | Malvidin | $OCH_3$ | OH | $OCH_3$ | |
| | Delphinidin | OH | OH | OH | |
| | Cyanidin | H | OH | OH | |
| Isoflavones  | Genistein | 5 OH | 7 OH | 4 OH | Bean, chick pea, cowpea, mung bean, peanut, soybean (Erlund, 2004) |
| | Genistin | OH | *O*-gluc | OH | |
| | Daidzein | | OH | OH | |

gluc: glucose

**Table 1.6 Examples for selected glucoside derivatives of flavonoids naturally occurring in plants**

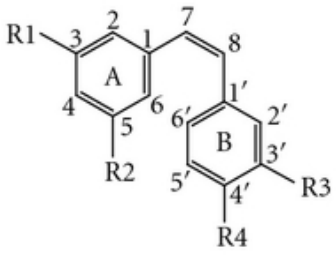
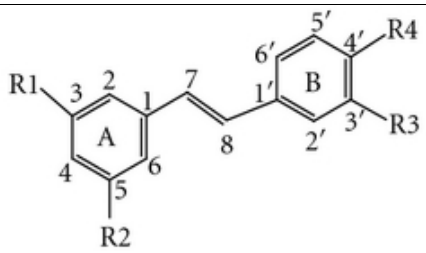
| Glucoside derivatives of flavonoids | Examples | R1 | R2 | R3 | R4 |
|---|---|---|---|---|---|
|  | Luteolin-7-*O*-β-D-glucoside | H | OH | H | -β-D-gluc |
| | Apigenin-7-*O*-β-D-glucoside | H | H | H | -β-D-gluc |
| | Kaempferol-3-*O*-β-D-glucoside | *O*-β-D-gluc | H | H | H |
| | Quercetin-3-*O*-β-D-glucoside | *O*-β-D-gluc | OH | H | H |

gluc: glucose

### 1.2.4.3 Stilbenes

Stilbenes comprise a 1,2-diphenyl-ethylene backbone with two phenyl groups arranged in either *cis* or *trans* configuration (Table 1.7). Stilbenes are widely distributed in plants and particularly abundant in grapevine and peanuts (Versari *et al.*, 2001; Hasan *et al.*, 2012). Multiple health benefits such as anti-inflammatory, antitumor, and antioxidant activities have been postulated for stilbenes, particularly for resveratrol (Paredes-López *et al.*, 2010). Stilbenes are also potent phytoalexins in plants.

**Table 1.7 Classification and structure of selected derivatives of stilbenes in plants**

| Basic structure | Examples | R1 | R2 | R3 | R4 |
|---|---|---|---|---|---|
|  | *cis*-Stilbene | H | H | H | H |
| | *cis*-Resveratrol | OH | OH | H | OH |
|  | *trans*-Resveratrol | OH | OH | H | OH |
| | *trans*-Resveratrol-3-*O*-glucoside | *O*-gluc | OH | H | OH |

gluc: glucose

1.2.4.4 Antioxidant activities of phenolic compounds

The strong antioxidant activity of phenolic compounds is attributable to (i) their ready ability to donate protons or electrons; (ii) the property of the resultant radical to stabilize and delocalize unpaired electrons; and (iii) their ability to chelate with transition metal ions (Rice-Evans *et al.*, 1996).

The antioxidant capacity of phenolic compounds varies according to the number and position of the hydroxyl groups (Rice-Evans *et al.*, 1996; Pietta, 2000; Akdemir *et al.*, 2001; Villaño *et al.*, 2005). Hydroxyl groups on the B-ring donate hydrogen and an electron to ROS radicals, stabilizing them and giving rise to a relatively stable flavonoid radical (Figure 1.4). Among structurally homologous flavones and flavanones, radical scavenging increases according to the total number of OH groups (Cao *et al.*, 1997).

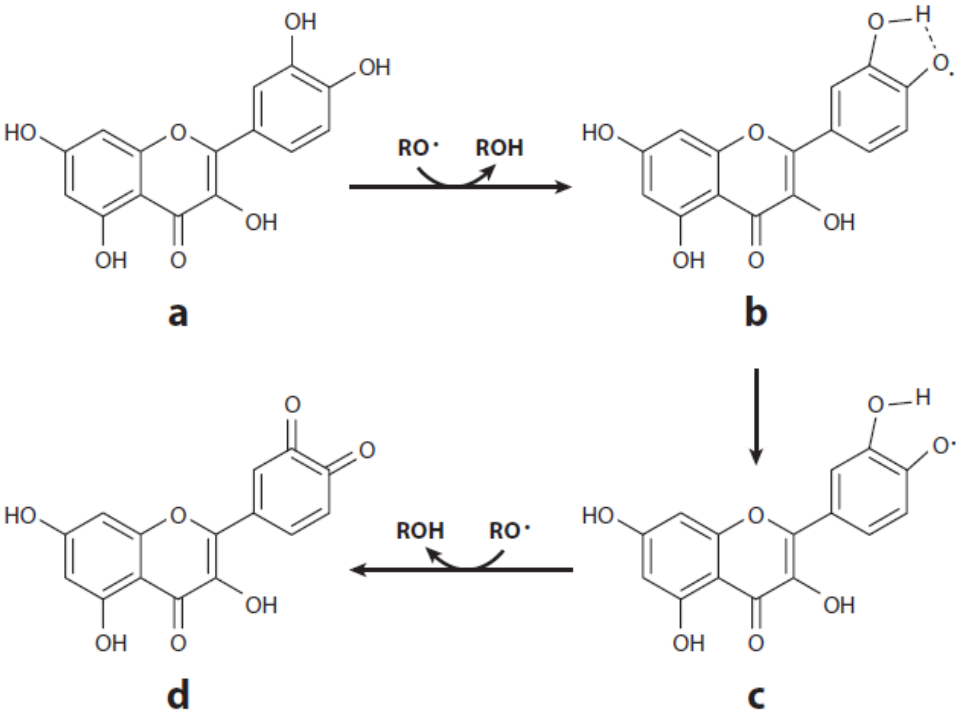



**Figure 1.4 Oxidation-dependent changes in the structure of flavonoids; (a) flavonoid, (b) flavonoid radical stabilized by a hydrogen bond, (c) flavonoid phenoxy radical and (d) further oxidation of the flavonoid radical leads to the formation of an orthodiquinone, which is stable.**

**Source: Jacob *et al.* (2012)**

The antioxidant activities of phenolic compounds are not only governed by their degree of hydroxylation; but ortho hydroxylation on the benzene ring also influences radical scavenging (Burda and Oleszek, 2001; Nenadis *et al.*, 2003). For example, ortho hydroxylated chlorogenic acid is 35% more potent than kaempferol in scavenging DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals (Tabart *et al.*, 2009).

Additionally, the antioxidant activities of flavonoids are influenced by the position and structural properties of the sugar moiety (Rice-Evans *et al.*, 1996). The antioxidant properties of flavonoids decreases as the number of glycosidic moieties increases (Plumb *et al.*, 1999b), and thus glycosylated flavonoids are lower in radical scavenging capacity than their corresponding aglycones (Shahidi *et al.*, 1992).

In addition to scavenging ROS, phenolic compounds can also prevent ROS injury to cells. Phenolic compounds can modify the lipid packing order of cell membranes, which decreases the fluidity of the membranes sterically hindering the diffusion of ROS thus restricting peroxidation reactions in lipid membranes (Arora *et al.*, 2000).

In addition to their function as antioxidants, phenolic compounds display various other properties, which are potentially beneficial for human health (Stevenson and Hurst, 2007). These include; antibacterial, anticarcenogenic, antimutagenic, antiallergic, anti-inflammatory, antiviral and antiproliferative activities (Rao *et al.*, 1995; Fernandez *et al.*, 1998; De Mejía *et al.*, 1999; Tuck and Hayball, 2002; Lee *et al.*, 2005; Miles *et al.*, 2005; Roy *et al.*, 2007; Medeiros *et al.*, 2008; Suárez *et al.*, 2010).

## 1.3 EVIDENCE FOR INFLUENCE OF DIETARY LMWAS ON OXIDATIVE STRESS-INDUCED DISEASES

Certain food sources and diets, which provide rich intakes of LMWAs correlate with low incidences of non-infectious diseases of the consumers, according to epidemiological evidence (Table 1.8) and clinical feeding trials (Tables 1.9 and 1.10). For example, epidemiological studies have shown that diets rich in phenolic compounds are associated with a reduction in the initiation and occurrence of some cancers (Table 1.8). However, caution is necessary in interpreting epidemiological data since such correlations do not necessarily imply causality. Clinical feeding trials are more appropriate than epidemiological studies, to draw specific conclusions regarding possible benefits of specific LMWAs. However, in clinical studies the individual effects of LMWAs are hard or impossible to isolate since in a whole organism there are many complicated effects, which occur simultaneously. Therefore mechanistic studies are required to understand specific cause and effect.

The majority of clinical feeding trials involving humans afflicted with oxidative stress-related hypertension, inflammation, cardiovascular disease, cancer and atherosclerosis have shown that dietary intake of phenolic compounds such as fresh fruit and vegetables or in their concentrated forms is associated with reduced disease indices compared to the placebo group (Tables 1.9 and 1.10). The benefit of phenolic rich fruit and vegetable intake was seen in clinical trials where participants were under heavy oxidative stress and when the measured parameters were cardiovascular, plasma or bowel related low-density lipoprotein (LDL) oxidation and DNA damage (Tables 1.9 and 1.10). However in the few studies where dietary LMWAs intake did not reduce indicators of oxidative stress, the measures were done in sites such as lungs where the bioavailability of phenolic compounds are low (Cerda *et al.*, 2005). Furthermore among relatively young and healthy participants the protective effects of dietary phenolic compounds were less observable and below the measurable lower limits since their cells were experiencing lower degree of oxidative stress (Chang *et al.*, 2010).

**Table 1.8 Selected epidemiological studies on dietary intake of phenolic compounds and cancer**

| Compound/s | Cancer | Result/ correlation | Number of human subjects | Reference |
|---|---|---|---|---|
| Flavones and flavanones | Squamous cell carcinoma | Sig. inverse association | 1061/1425 a | (Christensen *et al.*, 2012) |
| Epicatechin catechin, quercetin, and kaempferol | Lung | Sig. inverse association | 558/837 a | (Cui *et al.*, 2008) |
| Flavanones and proanthocyanidins | Lung | Sig. inverse association | 34,708 b | (Cutler *et al.*, 2008) |
| Flavonols, flavones, flavanones, flavan-3-ols and anthocyanidins | Lung | Sig. inverse association | 2,590 b | (Mursu *et al.*, 2008) |
| Cinnamic acids, secoisolariciresinol and coumestrol | Gastric | Sig. inverse association | 257/478 a | (Hernández-Ramírez *et al.*, 2009) |
| Flavonoids | Colorectal | Sig. inverse association | 424/401 a | (Wahle *et al.*, 2010) |
| Flavonol and catechin | Colorectal | Sig. inverse association | 120,852 b | (Simons *et al.*, 2009) |
| Catechin | Rectal | Sig. inverse association | 120,852 b | (Simons *et al.*, 2009) |
| Flavones and flavanones | Adenocarcinoma | ns | 1061/1425 a | (Christensen *et al.*, 2012) |
| Thearubigins, hesperetin, naringenin, and myricetin | Lung | ns | 558/837 a | (Cui *et al.*, 2008) |
| Flavones | Colorectal | ns | 120,852 b | (Simons *et al.*, 2009) |
| Flavonols, flavones, flavanones, flavan-3-ols and anthocyanidins | Prostate and colorectal | ns | 2,590 b | (Mursu *et al.*, 2008) |

Sig. : Significant

a: cohort study (disease cases /healthy cases)

b: population-based case-control studies

ns: not significant

**Table 1.9 Selected randomized, controlled, crossover clinical feeding trial on effects of food and LMWAs on oxidative stress related diseases and conditions among humans**

| Diseases/ conditions | Number of patients | Food or LMWA | Measures | Result | Reference |
|---|---|---|---|---|---|
| Atherosclerosis | 42 | Cocoa beverage | Biomarkers of atherosclerosis | Significant reduction | (Monagas *et al.*, 2009) |
| Cardiovascular disease (CVD) risk | 24 | Orange juice (hesperidin) | Blood pressure | Significant reduction | (Morand *et al.*, 2011) |
| Oxidation of human low-density lipoprotein (LDL) | 8 | Olive oil (phenolic compounds) | LDL oxidation | Significant reduction | (De La Torre-Carbot *et al.*, 2010) |
| DNA oxidation | 182 | Olive oil (phenolic compounds) | DNA oxidation | Significant reduction | (Machowetz *et al.*, 2007) |
| Inflammation and oxidative damage | 20 | Almond (α-tocopherol) | Inflammation and LDL oxidation | Significant reduction | (Liu *et al.*, 2012) |
| Coronary artery disease | 21 | Walnut (α-tocopherol and α-linolenic) | Total cholesterol and LDL cholesterol and endothelium vasodilation | Significant reduction | (Ros *et al.*, 2004) |
| DNA damage and repair | 28 | Fruits and vegetables | DNA damage in lymphocytes | ns | (Chang *et al.*, 2010) |

ns: not significant

**Table 1.10 Selected randomized, double-blind, placebo-controlled clinical feeding trial on influence of food and LMWAs on oxidative stress related diseases among humans**

| Diseases | Number of patients | Food or LMWA | Measures | Result | Reference |
|---|---|---|---|---|---|
| Chronic inflammation | 106 | Encapsulated fruit and vegetable powder concentrate | Chronic inflammation (DNA damage in blood lymphocytes) | Significant reduction | (Cui *et al.*, 2012) |
| Inflammatory bowel disease | 44 | *Aloe vera* | Clinical remission | More frequent among treated patients | (Langmead *et al.*, 2004) |
| | | | Histological disease activity | Significant reduction | |
| CVD | 32 | Cranberry juice | Lipid oxidation | Significant reduction | (Basu *et al.*, 2011) |
| | | | Plasma antioxidant capacity | Significant increase | |
| CVD risk in subjects with type 2 diabetes | 48 | Flavonoid rich dietary supplement | Diabetes control | Significantly improved | (Zibadi *et al.*, 2008) |
| | | | CVD risk factors | Significant reduction | |
| Chronic obstructive pulmonary disease | 30 | Pomegranate juice | Clinical symptoms of the disease | ns | (Cerda *et al.*, 2005) |
| Oxidative stress related hypertension and endothelial dysfunction | 69 | Grape-seed polyphenols | Blood pressure | ns | (Ward *et al.*, 2005) |

ns: not significant

## 1.4 THE BIOACCESSIBILITY, BIOAVAILABILITY, BIOACTIVITY AND STABILITY OF PHENOLIC COMPOUNDS

For a phenolic compound to exert any beneficial biological effect, it must reach target tissues in chemically active form and at appropriate concentrations. Thus bioaccessibility, bioavailability, bioactivity and stability of an antioxidant-rich food source should be considered in order to fully utilize the possible health benefit of antioxidants in food plants. Bioaccessibility can be defined as the fraction of the ingested antioxidant(s) that is released and solubilised from the food matrix by gastrointestinal digestion and, which has the potential to be absorbed from the gastrointestinal tract into the intestinal epithelium (Hedren *et al.*, 2002). In this context, the stability of an antioxidant refers to the ability of the compounds to retain their antioxidant properties following digestion (Dinnella *et al.*, 2007). Bioavailability can be defined as the proportion of an administered dose that reaches the systemic circulation intact, and bioactivity is the ability to induce a biological response in an organism that consumes it (Finley, 2005).

The bioaccessibility of various components from foods has been studied using *in vitro* simulations of gastrointestinal digestion and absorption (Table 1.11). Similarly, the stability of LMWAs in food has been studied using *in vitro* antioxidant activity measurements of food products before and after simulated gastrointestinal incubation (Table 1.11). Recently, techniques have been developed to accommodate the three aspects of bioaccessibility, bioactivity and stability of food components using *in vitro* gastrointestinal simulations followed by cell culture models, which mimic uptake of LMWAs from the lumen of the gastrointestinal tract and the subsequent bioactivity within the cellular environment (Table 1.11). However, bioavailability also requires consideration of the complex effects of hepatic metabolism and activity of plasma enzymes on LMWAs following absorption from gastrointestinal tract. Therefore, to assess bioavailability, studies that mimic complex physiological conditions need to be done on human subjects due to the difficulty of designing *in vitro* models (Funes *et al.*, 2009; Ranga Rao *et al.*, 2010).

**Table 1.11 Selected studies on bioaccessibility, bioactivity and stability of antioxidants from various food sources**

| Food | Technique[a] | Reference |
|---|---|---|
| *Solanum esculentum* | 1 | (Toor *et al.*, 2008) |
| *Camellia sinensis* and *Salvia officinalis* | 1 | (Vermaak *et al.*, 2009) |
| *Aronia melanocarpa* | 2 | (Bermúdez-Soto *et al.*, 2007) |
| *Punica granatum* | 2 | (Pérez-Vicente *et al.*, 2002) |
| *Rubus idaeus* | 2 | (McDougall *et al.*, 2005a) |
| *Prunus dulcis* | 3 | (Mandalari *et al.*, 2010) |
| *Malus domestica* | 3 | (Bouayed *et al.*, 2007) |
| *Prunus avium* | 3 | (Fazzari *et al.*, 2008) |
| Fruits | 3 | (Tarko *et al.*, 2009) |
| Fruit juices | 3 | (Cilla *et al.*, 2011) |
| *Vitis vinifera* | 3 | (Tagliazucchi *et al.*, 2010) |
| *Olea europaea* | 3 | (Dinnella *et al.*, 2007) |
| Red wine | 3 | (Noguer *et al.*, 2008) |
| Vegetable juices | 3 | (Wootton-Beard *et al.*, 2011) |
| *Rubus* sp. | 4 | (Tavares *et al.*, 2012) |
| "Feijoada" whole meal[b] | 4 | (Faller *et al.*, 2012) |
| *Agaricus bisporus*, *Lentinula edodes* and *Boletus edulis* | 5 | (Soler-Rivas *et al.*, 2009b) |

[a] List of techniques used

1. *In vitro* gastrointestinal simulation + *in vitro* chemical antioxidant activity measures
2. *In vitro* gastrointestinal simulation + diffusion across cellulose membrane (D)
3. *In vitro* gastrointestinal simulation + D + *in vitro* chemical antioxidant activity measures
4. *In vitro* gastrointestinal simulation + D + *in vitro* chemical antioxidant activity measures + cellular uptake and cellular antioxidant activity (CAA) using human cell culture models
5. *In vitro* gastrointestinal simulation + cellular uptake and CAA using human cell culture models

[b] A traditional Brazilian meal rich in plant food

Not all phenolic compounds survive *in vitro* gastrointestinal digestion and their release and survival during digestion depends on the type of food that contained the compound (Tables 1.12 – 1.14). Additionally, not all compounds released in the bioaccessible fraction following *in vitro* gastrointestinal digestion were able to permeate across cellulose membranes or animal cell membranes (Tables 1.12 – 1.14). Passive absorption of phenolic compounds across cell membranes depends on the partition coefficient of the compound in question, polarity, size of the molecule and the moieties attached to the compound (Karakaya, 2004; Zhao and Moghadasian, 2010). For active absorption, the presence of specific transporters in cell membranes will be required (Ader *et al.*, 1996).

**Table 1.12 Compounds detected following *in vitro* gastrointestinal digestion of selected phenolic acids and their derivatives in food**

| Compound | Detected [a] | Food | References |
|---|---|---|---|
| 1-caffeoylquinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3-caffeoylquinic acid, 4,5-dicaffeoylquinic acid and 5-feruloylquinic acid | Nd[1] | *Crithmum maritimum* | (Siracusa *et al.*, 2011) |
| Derivatives of caffeic, ferulic, sinapic and *p*-coumaric acid | Nd[2] | Commercial orange juice | (Gil-Izquierdo *et al.*, 2001) |
| 4-caffeoylquinic acid | Yes[1] | *Capparis spinosa* | (Siracusa *et al.*, 2011) |
| | Nd[1] | *Crithmum maritimum* | |
| 4-feruloylquinic acid | Yes[1] | *Capparis spinosa* | |
| | Nd[1] | *Crithmum maritimum* | |
| 5-*p*-coumaroylquinic acid | Yes[1] | *Crithmum maritimum*<br>*Capparis spinosa* | |
| Caffeic acid | Yes[2] | *Prunus avium* | (Fazzari *et al.*, 2008) |
| Chlorogenic acid | Yes[1 and 2] | *Brassica oleracea*<br>*Prunus avium*<br>*Capparis spinosa*<br>*Crithmum maritimum*<br>*Prunus dulcis*<br>*Malus domestica* | (Vallejo *et al.*, 2003b; Kahle *et al.*, 2007; Fazzari *et al.*, 2008; Mandalari *et al.*, 2010; Siracusa *et al.*, 2011) |
| Neochlorogenic acid | Yes[2] | *Aronia melanocarpa*<br>*Prunus avium* | (Bermúdez-Soto *et al.*, 2007; Fazzari *et al.*, 2008) |
| Neochlorogenic acid | Nd[2] | *Brassica oleracea* | (Vallejo *et al.*, 2003b) |
| Derivatives of ferulic and sinapic acid | Yes[2] | | |
| Gallic acid | Yes[1] | *Camellia sinensis* | (Record and Lane, 2001) |
| *p*-Hydroxybenzoic acid | Yes[2] | *Prunus dulcis* | (Mandalari *et al.*, 2010) |
| *trans p*-Coumaric acid | | | |
| *p*-Coumaroylquinic acid | Yes[2] | *Prunus avium* | (Fazzari *et al.*, 2008) |
| Carnosol | Yes[3] | *Rosmarinus officinalis* | (Soler-Rivas *et al.*, 2009a) |

[a] Compounds detected (Yes) or not detected (Nd) following *in vitro* gastrointestinal digestion;

1. in release buffer,
2. in cellulose dialysis tube or
3. in cytoplasm of human cell cultures treated with extracts obtained after *in vitro* gastrointestinal digestion.

**Table 1.13 Compounds detected following *in vitro* gastrointestinal digestion of selected flavonoids and their derivatives in food**

| Compound | Detected[a] | Food | References |
|---|---|---|---|
| Epicatechin | Yes[1 and 2] | *Camellia sinensis*<br>*Malus domestica*<br>*Prunus dulcis* | (Record and Lane, 2001; Kahle *et al.*, 2007; Mandalari *et al.*, 2010) |
| Catechin | Yes[2] | *Malus domestica*<br>*Prunus dulcis* | (Kahle *et al.*, 2007; Mandalari *et al.*, 2010) |
| Epicatechin gallate | Yes[1] | *Camellia sinensis* | (Record and Lane, 2001) |
| Epigallocatechin | Nd[1] | | |
| Epigallocatechin gallate | | | |
| Gallocatechin gallate | | | |
| Isorhamnetin | Yes[2] | *Prunus dulcis* | (Mandalari *et al.*, 2010) |
| Isorhamnetin-3-*O*-glucoside | | | |
| Isorhamnetin-3-*O*-rutinoside | Nd[1] | *Capparis spinosa* | (Siracusa *et al.*, 2011) |
| | Yes[2] | *Prunus dulcis* | (Mandalari *et al.*, 2010) |
| Kaempferol | Yes[2] | *Brassica oleracea*<br>*Prunus dulcis* | (Vallejo *et al.*, 2003b; Mandalari *et al.*, 2010) |
| Kaempferol-3-*O*-glucoside | Nd[1] | *Capparis spinosa* | (Siracusa *et al.*, 2011) |
| | Yes[2] | *Prunus dulcis* | (Mandalari *et al.*, 2010) |
| Kaempferol-3-*O*-rutinoside | Nd[1] | *Capparis spinosa* | (Siracusa *et al.*, 2011) |
| | Yes[2] | *Prunus dulcis* | (Mandalari *et al.*, 2010) |
| Quercetin | Yes[2] | *Aronia melanocarpa*<br>*Brassica oleracea*<br>*Prunus dulcis* | (Vallejo *et al.*, 2003b; Bermúdez-Soto *et al.*, 2007; Mandalari *et al.*, 2010) |
| Quercetin-3-*O*-glucoside | Yes[2] | *Prunus dulcis*<br>*Malus domestica*<br>*Aronia melanocarpa* | (Bermúdez-Soto *et al.*, 2007; Kahle *et al.*, 2007; Mandalari *et al.*, 2010) |
| | Nd[1] | *Capparis spinosa* | (Siracusa *et al.*, 2011) |
| Quercetin-3-*O*-galactoside | Yes[2] | *Malus domestica*<br>*Aronia melanocarpa*<br>*Prunus dulcis* | (Bermúdez-Soto *et al.*, 2007; Kahle *et al.*, 2007; Mandalari *et al.*, 2010) |
| Quercetin-3-*O*-rutinoside | Yes[2] | *Prunus dulcis*<br>*Aronia melanocarpa* | (Bermúdez-Soto *et al.*, 2007; Mandalari *et al.*, 2010) |
| Quercetin arabinoside | Yes[2] | *Malus domestica* | (Kahle *et al.*, 2007) |
| Quercetin rhamnoside | | | |
| Quercetin xyloside | | | |
| Rutin | Nd[1] | *Capparis spinosa* | (Siracusa *et al.*, 2011) |
| Flavan-3-ols | Yes[2] | *Aronia melanocarpa* | (Bermúdez-Soto *et al.*, 2007) |

[a] Compounds detected (yes) or not detected (Nd) following *in vitro* gastrointestinal digestion;

1. in release buffer or
2. in cellulose dialysis tube.

**Table 1.14 Compounds detected following *in vitro* gastrointestinal digestion of selected anthocyanins and their derivatives in food**

| Compound | Detected[a] | Food | References |
|---|---|---|---|
| Cyanidin | Yes | *Aronia melanocarpa* | (Bermúdez-Soto *et al.*, 2007) |
| Cyanidin-3-arabinoside | | | |
| Cyanidin-3-galactoside | | | |
| Cyanidin-3,5-diglucoside | | *Punica granatum* | (Pérez-Vicente *et al.*, 2002) |
| Cyanidin-3-glucoside | Yes | *Aronia melanocarpa*<br>*Punica granatum*<br>*Prunus avium* | (Pérez-Vicente *et al.*, 2002; Bermúdez-Soto *et al.*, 2007; Fazzari *et al.*, 2008) |
| | Nd | Red wine | (McDougall *et al.*, 2005b) |
| Cyanidin-3-rutinoside | Yes | *Prunus avium* | (Fazzari *et al.*, 2008) |
| Cyanidin-3-xyloside | Yes | *Aronia melanocarpa* | (Bermúdez-Soto *et al.*, 2007) |
| Delphidin-3-*O*-glucosides | Nd | Red wine<br>*Punica granatum* | (Pérez-Vicente *et al.*, 2002; McDougall *et al.*, 2005b) |
| Delphinidin-3,5-diglucoside | | *Punica granatum* | (Pérez-Vicente *et al.*, 2002) |
| Delphinidin-3-*O*-coumaroylglucoside | | Red wine | (McDougall *et al.*, 2005b) |
| Malvidin-3-*O*-acetylglucoside | Yes | Red wine | (McDougall *et al.*, 2005b) |
| Malvidin-3-*O*-coumaroylglucoside | | | |
| Malvidin-3-*O*-glucosides | | | |
| Pelargonidin-3,5-diglucoside | Yes | *Punica granatum* | (Pérez-Vicente *et al.*, 2002) |
| Pelargonidin-3-glucoside | | | |
| Peonidin-3-*O*-acetylglucoside | Nd | Red wine | (McDougall *et al.*, 2005b) |
| Peonidin-3-*O*-coumaroylglucoside | | | |
| Peonidin-3-*O*-glucosides | Yes | | |
| Peonidin-3-rutinoside | | *Prunus avium* | (Fazzari *et al.*, 2008) |
| Petundin-3-*O*-acetylglucoside | Nd | Red wine | (McDougall *et al.*, 2005b) |
| Petundin-3-*O*-coumaroylglucoside | | | |
| Petundin-3-*O*-glucosides | | | |
| Naringenin | Yes | *Prunus dulcis* | (Mandalari *et al.*, 2010) |
| Naringenin-7-*O*-glucoside | | | |
| Eryodictiol | | | |
| Eryodictiol-7-*O*-glucoside | | | |

[a] Compounds detected (Yes) or not detected (Nd) in cellulose dialysis tube following *in vitro* gastrointestinal digestion.

*In vitro* digestion by gastrointestinal enzymes and those produced by colonic microflora causes certain phenolic compounds to undergo chemical changes to form new compounds (Tables 1.15 and 1.16). *In vitro* digestion by colonic microbes resulted in the formation of wide variety of metabolites (Table 1.16) compared to digestion by gastrointestinal enzymes: pepsin, lipase and amylase (Table 1.15). Microflora in the gut can cause ring-fission, degycosylation, methylation, deconjugation of phenolic compounds (Aura, 2008), while gastrointestinal enzymes are incapable of ring-fission and methylation.

**Table 1.15 Changes in metabolites detected following *in vitro* gastrointestinal digestion of selected phenolic compounds in food**

| **Compounds** | **Compounds released following digestion[a]** | | **Food** | **References** |
|---|---|---|---|---|
| | | **Metabolites** | | |
| Chlorogenic aid | Yes[1] | Neochlorogenic acid | *Camellia sinensis* | (Record and Lane, 2001) |
| Protocatechuic acid | Yes[1] | 3-(2,4-dihydroxyphenyl)propionic acid<br>*o*-Hydroxyphenylacetic acid<br>*p*-hydroxybenzoic acid<br>Phenylacetic acid | *Prunus dulcis* | (Mandalari *et al.*, 2010) |
| Carnosic acid | Yes[2] | Carnosol | *Rosmarinus officinalis* | (Soler-Rivas *et al.*, 2009a) |
| Carnosol<br>Carnosic acid<br>Methyl carnosate | Yes[2] | Unidentified derivatives of abietanes | | |
| Anthocyanins | Yes[1] | Ionised chalcones of anthocyanins | *Punica granatum* | (Pérez-Vicente *et al.*, 2002) |
| Procyanidin | Yes[1] | Catechin | *Malus domestica* | (Kahle *et al.*, 2007) |
| Hesperetin | Nd[1] | Chalcones of hesperetin | *Crithmum maritimum* | (Siracusa *et al.*, 2011) |
| Hesperidin | Yes[1] | Chalcones of hesperidin | | |
| Narirutin | Nd[1] | Chalcones of narirutin | | |

[a] Compounds detected (Yes) or not detected (Nd) following *in vitro* gastrointestinal digestion;

1. in cellulose dialysis tube or
2. in cytoplasm of human cell cultures treated with extracts obtained after *in vitro* gastrointestinal digestion.

Phenolic compounds are largely absorbed through the jejunum and to a smaller extent, through the ileum and to a lesser degree in the stomach before reaching the microflora in the colon (Spencer *et al.*, 1999; Clifford, 2000b; Mandalari *et al.*, 2013). Furthermore the microbial conversion processes do not affect all compounds in a similar manner; for example, deglycosylation of flavonoids depends on the sugar moiety, while the chain length inversely influenced the conversion of proanthocyanidins into phenolic acids (Keppler and Humpf, 2005; Bazzocco *et al.*, 2008).

**Table 1.16 Changes in metabolites detected following *in vitro* digestion by colonic microflora or *in situ* digestion by rats of selected phenolic compounds in food**

| Compound | Compounds released following digestion[a] | | References |
|---|---|---|---|
| | Original compound | Metabolites | |
| Caffeic and Chicoric acid | Yes[1] | *m*-hydroxyphenylpropionic acid | *Echinacea purpurea* (Ye, 2009) |
| Caftaric acid | Nd[1] | *m*-hydroxyphenylpropionic acid | |
| Chlorogenic acid | Yes[2] | Caffeic acid<br>Quinic acid | Chlorogenic acid (Plumb *et al.*, 1999a) |
| Gallic acid | Yes[2] | 3-(4-Hydroxyphenyl)propionic acid<br>Phenylacetic acid<br>*p*-Hydroxyphenylacetic acid<br>Protocatechuic acid | Flavonoids and phenolic acids (Serra *et al.*, 2012) |
| Protocatechuic acid | Yes[2] | Unchanged | |
| Luteolin | Yes[2] | 3-(2,4-Dihydroxyphenyl)propionic acid | |
| Myricetin | Yes[2] | 3-(2,4-Dihydroxyphenyl)propionic acid<br>3,4-Dihydroxyphenylacetic acid<br>Phenylacetic acid | |
| Quercetin | Yes[2] | 3-(3,4-Dihydroxyphenyl)propionic acid<br>3,4-Dihydroxyphenylacetic acid<br>Homovainillic acid<br>*m*-Hydroxyphenylacetic acid<br>*o*-Hydroxyphenylacetic acid<br>*p*-Hydroxybenzoic acid<br>*p*-Hydroxyphenylacetic acid<br>Protocatechuic acid<br>Phenylacetic acid | |
| Rutin | Yes[3] | None | *Hypericum perforatum* (Ye, 2009) |
| Quercetin galactoside | Yes[3] | None | |

**Table 1.16 continued**

| | | | |
|---|---|---|---|
| Quercetin rhamnoside | Yes[2] | 3,4-Dihydroxyphenylacetic<br>Dihydroxyphenyl)propionic acid<br>m-Hydroxyphenylacetic acid<br>*o*-Hydroxyphenylacetic acid<br>Phenylacetic acid<br>*p*-Hydroxybenzoic acid<br>*p*-Hydroxyphenylacetic acid<br>Protocatechuic acid | Flavonoids and phenolic acids (Serra *et al.*, 2012) |
| Quercetin rutinoside | Yes[2] | 3-(3,4-Dihydroxyphenyl)propionic acid<br>3,4-Dihydroxyphenylacetic acid<br>*m*-Hydroxyphenylacetic acid<br>Phenylacetic acid | |
| Kaempferol rutinoside | Yes[2] | *o*-Hydroxyphenylacetic acid<br>Phenylacetic acid<br>*p*-Hydroxybenzioc acid | |
| Naringenin | Yes[2] | 3-(2,4-Dihydroxyphenyl)propionic acid<br>3-(4-Hydroxyphenyl)propionic acid<br>*o*-Hydroxyphenylacetic acid<br>Phenylacetic acid<br>*p*-Hydroxyphenylacetic acid<br>Protocatechuic acid | |

[a] Compounds detected (Yes) or not detected (Nd) following *in vitro* gastrointestinal digestion;

1. following *in vitro* fermentation using human, mouse or rat colonic microflora,
2. following *in vitro* digestion using extracts of human small intestine, liver, plasma and human colonic microflora, or
3. in plasma after *in situ* gastric administration to rats.

## 1.5 CHEMICAL ANTIOXIDANT ACTIVITY ASSAYS

A number of chemical assays and *in vitro* cellular techniques have been used to measure antioxidant capacities of food and metabolites in a number of physiological matrices including gastrointestinal fluids, urine and plasma (Prior *et al.*, 2005; Torres *et al.*, 2008). These antioxidant activity assays can be classified into two types based on their reaction mechanism and type of radical scavenged (Table 1.17).

**Table 1.17 Comparison of types of *in vitro* chemical antioxidant activity assays**

| **Characteristic** | **Antioxidant activity assays** | |
|---|---|---|
| | **Hydrogen atom transfer (HAT) measures** | **Single electron transfer (SET) measures** |
| Mechanism | Ability of an antioxidant to quench free radicals by proton donation | Ability of an antioxidant to transfer single electron, reducing the reactive species |
| Type of radical scavenged | The antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo-compounds | Assays measure the capacity of an antioxidant in the reduction of an oxidant, which is then correlated with the antioxidant concentrations |
| Examples | Oxygen radical absorbance capacity (ORAC)<br>Total radical trapping antioxidant parameter (TRAP)<br>Crocin bleaching assays | 2, 2'-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) assay<br>Ferric ion reducing antioxidant power (FRAP)<br>DPPH radical scavenging assay |

Further details on DPPH radical scavenging assay and ORAC (oxygen radical absorbance capacity) assay are detailed briefly (Sections 1.5.1 – 1.5.2) because they are most commonly used. They are also featured in the experimental sections of this thesis because the two techniques employ scavenging of two different radical species, at different temperature and pH conditions increasing the reliability of data (Sections 1.5.1 – 1.5.2).

### 1.5.1 DPPH radical scavenging assay

DPPH is a stable organic nitrogen radical with a non paired electron (Brand-Williams *et al.*, 1995). In the presence of antioxidants (AH), which are capable of donating H, the DPPH radical (DPPH$^{\bullet}$) reduces to the stable non-radical DPPH (Equation 1.1). This

reduction of the DPPH radical by an antioxidant causes the colour change (from violet to yellow), resulting in the loss of absorbance at 515 nm.

$$DPPH^{\bullet} + AH \rightarrow DPPH + A^{\bullet} \qquad \text{Equation 1.1}$$

The degree of absorbance loss is correlated with concentration of the antioxidant to calculate the $EC_{50}$, which is the concentration of antioxidant that causes 50% loss of initial DPPH concentration (Figure 1.5). The $EC_{50}$ of the sample can be expressed as equivalents of an appropriate standard such as ascorbic acid or trolox (commercial name for a vitamin E analogue).

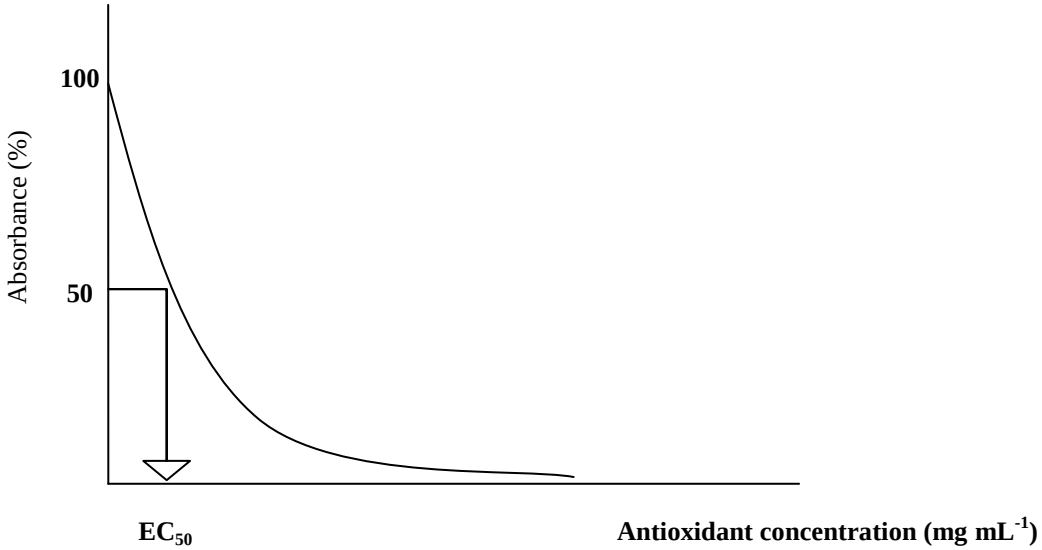



**Figure 1.5 Percentage decrease in absorbance ($A_{515nm}$) by DPPH (2,2-diphenyl-1-picrylhydrazyl) against concentration of antioxidant, where $EC_{50}$ denotes the concentration of antioxidant causing 50% loss of initial absorbance.**

There are a few drawbacks to the use of the DPPH method. Importantly, the DPPH radical differs from the highly reactive and transient peroxyl radicals involved in lipid peroxidation in biological systems (Huang *et al.*, 2005). Thus, antioxidants may display slower rate of scavenging towards the DPPH radical than towards peroxyl radicals (Ndhlala *et al.*, 2010). Furthermore, steric inaccessibility of radical site of DPPH may result in an overestimation of the antioxidant capacity of those smaller molecules, which have better access to the DPPH radical, and vice versa (Goupy *et al.*, 2002). For example, addition of two ($CH_2$) units to the molecular spacer of 3,5-Di-*tert*-butyl-4-hydroxybenzyl acid diminished its DPPH radical scavenging by 13-fold (Vol'eva *et al.*, 2011). Therefore,

efficient peroxyl radical scavengers can be inert in DPPH radical scavenging since they may have less access to the DPPH radical site (Goupy *et al.*, 2002). Nonetheless, the DPPH assay is a simple, rapid technique, which does not require special equipment and provides useful information on the capacity of phytochemical sources in scavenging free radicals, aiding screening of potent antioxidant sources. Furthermore, the technique is routinely used in assays of the antioxidant potential of foodstuffs (Huang *et al.*, 2005).

### 1.5.2 ORAC assay

ORAC measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects radical chain breaking antioxidant activity by $H^+$ transfer (Dávalos *et al.*, 2003). A synthetic free radical generator is thermally decomposed, supplying a continuous flux of peroxyl radicals ($ROO^{\bullet}$), which oxidises a fluorescent probe (PH) and reducing fluorescence intensity (Equation 1.2 and 1.3). The antioxidants (AH) inhibit the oxidation of the fluorescent probe by competing for peroxyl radicals, thus maintaining the fluorescence intensity (Equation 1.4 and 1.5).

$ROO^{\bullet} + PH \rightarrow ROOH + P^{\bullet}$ _____ Equation 1.2

$P^{\bullet} + ROO^{\bullet} \rightarrow ROOP$ _____ Equation 1.3

$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$ _____ Equation 1.4

$A^{\bullet} + ROO^{\bullet} \rightarrow AOOP$ _____ Equation 1.5

Fluorescence decay curves (Figure 1.6) reflect the protective effect of the antioxidants in the sample and can be numerically quantified as net integrated areas under the fluorescence decay curves (AUC). A standard curve is generated using the net AUC for concentrations gradient of trolox, and the trolox equivalents of the sample are calculated from the standard curve.

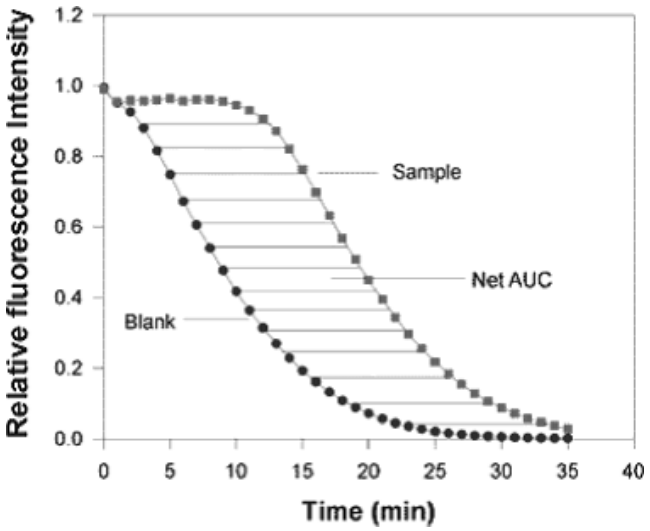



**Figure 1.6 Fluorescence decay curve for antioxidant sample, blank (without antioxidant) and shaded area showing net area under the curve (AUC)**

**Source: Gillespie *et al.* (2007)**

The ORAC assay is technically more complex compared to the DPPH radical scavenging assay, requiring temperature control throughout the assay period and a fluorometer to monitor fluorescence during the 1 h of analysis. The ORAC assay can be automated, reducing inter-assay variability by reducing errors and time spent in manual handling. In contrast to the DPPH assay, ORAC measures the scavenging of biologically relevant peroxyl radicals at physiologically relevant pH (7.4) and temperature (37 $^{o}$C), which simulates the antioxidant reactions involving lipids in biological systems (Ou *et al.*, 2001; Huang *et al.*, 2005). The ORAC values are more representative of total radical scavenging capacity since the ORAC values encompass the rate and time taken for scavenging the radicals by an antioxidant source into a single value. This is because the AUC of the florescence decay curve in ORAC is dependent on decaying rate and time taken for complete decay (Ou *et al.*, 2001; Huang *et al.*, 2005).

## 1.6 THE CELLULAR ANTIOXIDANT ACTIVITY (CAA) ASSAY

Extractable antioxidant activities measured by *in vitro* chemical assays (such as ORAC and DPPH) does not necessarily correlate positively with their *in vivo* antioxidant effects. The CAA assay is an *in vitro* technique that is used to evaluate the uptake, metabolism, and distribution of LMWAs in human hepatoma cells (Spencer *et al.*, 2004). Therefore, the CAA assay has several advantages over traditional chemical antioxidant activity

assays. The CAA assay has been used to quantify antioxidant activities of isolated antioxidant compounds (Wolfe and Liu, 2008), as well as those in fruits (Wolfe and Liu, 2007; Wolfe *et al.*, 2008), vegetables (Song *et al.*, 2010) and foliar extracts (McDowell *et al.*, 2011).

It is important when determining the health benefit of antioxidants in an ingested food matrix to evaluate their ability to be absorbed through the stomach and intestine into systemic circulation, to reach target tissues, and to exert physiological effects. However, monitoring the lumen of the human intestines and adjunct blood vessels and the target tissues *in vivo*, can be complex. Therefore, the development of suitable models that mimic human metabolism is useful for bioavailability and bioactivity studies (Grajek, 2005; Wolfe and Liu, 2007; Wolfe *et al.*, 2008; Wolfe and Liu, 2008; Song *et al.*, 2010; McDowell *et al.*, 2011). Cell lines such as L-02 (human normal hepatocytes) and HepG2 (human hepatoma cells) have most frequently been used for this work, since the liver is the central organ where the metabolism and detoxification takes place in humans. Human HepG2 cells are well differentiated experimental models that display biochemical responses comparable to the hepatocytes, and have been used successfully to evaluate cytotoxic and antioxidant potential of pure chemicals and plant extracts in the CAA assay (Wolfe and Liu, 2007; Wolfe *et al.*, 2008; Wolfe and Liu, 2008; Song *et al.*, 2010; McDowell *et al.*, 2011).

The CAA values positively correlated to ORAC antioxidant activities and total phenolic content in fruits such as *Vaccinium corymbosum, Rubus fruticosus, Fragaria ananassa, Rubus idaeus* and *Vaccinium macrocarpon* (Wolfe *et al.*, 2008) and in vegetables such as *Beta vulgaris, Capsicum annuum* and *Brassica oleracea* var. italica (Song *et al.*, 2010). The CAA assay has been conducted on foliar extracts of *Sonchus oleraceus*, for which the antioxidants were shown to be effectively absorbed into HepG2 cells and display antioxidant activity in the cytoplasm (McDowell *et al.*, 2011).

The low CAAs of certain LMWAs may be due to their poor or slower uptake into HepG2 cells. The CAA assay using pure compounds has shown that quercetin had the highest activity, while ascorbic acid was 100-fold lower in the CAA assay compared to quercetin (Table 1.18).

**Table 1.18 Cellular Antioxidant Activity (CAA) in human hepatoma cells by selected phytochemicals listed from highest to lowest CAA**

| Antioxidant | $EC_{50}$ (μM)[a] |
|---|---|
| Quercetin | 5.1 ± 0.2 |
| Kaempferol | 6.3 ± 0.2 |
| Epigallocatechin gallate | 15.8 ± 0.4 |
| Myricetin | 15.4 ± 0.5 |
| Luteolin | 23.1 ± 1.0 |
| Gallic acid | 335 ± 26 |
| Caffeic acid | 525 ± 38 |
| Ascorbic acid | >500 |

Source: Wolfe and Liu (2007)

[a] $EC_{50}$ reflects the median effective antioxidant concentration eliciting 50% CAA, obtained from dose-CAA response curves.

### 1.6.1 Cytotoxicity

High intakes of dietary LMWAs may have cytotoxic effects on human tissues. For example, the flavonoids luteolin, hydroxygenkwanin, and kaempferol possess significant *in vitro* cytotoxic effects on cultured L-02 and HepG2 cells (Li *et al.*, 2008). The cytotoxic effect of quercetin on HepG2 cells was dose- and time-dependant (Meyers *et al.*, 2003; Wolfe *et al.*, 2003). In contrast, chlorogenic acid and (-)-epicatechin were not toxic on HepG2 cells, within the tested upper limit of 1000 μM (Meyers *et al.*, 2003).

Measures of cytotoxicity of isolated phenolic compounds are higher than those for fruit and vegetable extracts, which naturally contain those compounds. For example, doses exceeding 6 μg $mL^{-1}$ of quercetin were cytotoxic on HepG2 cells, while most fruits and vegetables required doses of greater than 150 mg $mL^{-1}$ to be cytotoxic (Wolfe and Liu, 2007; Wolfe *et al.*, 2008; Song *et al.*, 2010; McDowell *et al.*, 2011; Faller *et al.*, 2012). The reason could be that the concentration of a specific LMWA in fruits and vegetables is lower, therefore to reach cytotoxic levels higher doses of a whole food is required compared to the pure compound alone.

## 1.7 TRADITIONAL DIET OF MĀORI AND INCIDENCE OF COLORECTAL CANCER

In New Zealand, a lower incidence of colorectal cancer has been reported for Māori than for non-Māori New Zealanders, according to colonoscopy records (from 2001 to 2005 involving 3000 participants) and statistics (from International Agency for Research on Cancer from 1980 to 1998 for nearly 4 x $10^6$ individuals). The prevalence of colorectal adenomas was 50% less in Māori than in non-Māori New Zealanders of European origin (Ferguson *et al.*, 1995; Thomson, 2002; Dickson *et al.*, 2010). These lower colorectal cancer rates in Māori occur, despite higher prevalence of oncogenic risk factors for colorectal cancers among Māori (Ferguson *et al.*, 1995; Thomson, 2002). Such oncogenic risk factors among Māori are: higher intakes of red meat, saturated fat and alcohol, higher food portion sizes, a higher prevalence of obesity and lower proportions of individuals who consume the recommended daily servings of fruit and vegetables. To explain this apparent paradox, it has been postulated that specific food plants, which are favoured especially by Māori might offer protection against the onset of colorectal cancers. Of the 51 food plants eaten by New Zealanders, four were eaten significantly more frequently by Māori than by European and Pacific Island descended New Zealanders (Ferguson *et al.*, 1995). These foods were sow thistle (*Sonchus oleraceus*), silverbeet (*Beta vulgaris* var. cicla), watercress (*Nasturtium offcinale*) and sweet potato (*Ipomoea batatas*); of these, methanolic extracts of sow thistle, silverbeet and watercress showed antimutagenic properties possibly leading to cancer prevention (Botting *et al.*, 1999). However sow thistle was indicated as the most frequently eaten plant food among Māori according to a survey of about 2000 Māori respondents, (Rush *et al.*, 2010).

## 1.8 *Sonchus oleraceus* L.

The *Sonchus oleraceus* (the smooth sow thistle known as “puha” by Māori) and possibly *S. asper* and *S. kirkii* were predominantly boiled or sometimes steamed before consumption in the traditional Māori diet (Whyte *et al.*, 2001; Cambie and Ferguson, 2003b). *S. oleraceus* has also been used in traditional medical practices of Hawaiian islands to treat a wide variety of disorders via both external application and internal administration (Leonard, 1998).

### 1.8.1 Biology of *S. oleraceus*

*S. oleraceus* L. (Compositae) is an annual herb (Holm and Center, 1977). It is native to Europe, North Africa, and Asia, and successfully grows and reproduces over a diverse range of habitats (Holm and Center, 1977). The older leaves form a basal rosette close to the ground, but later-formed leaves are on the flowering stem, which terminates in an inflorescence (Plate 1.1). The first leaves are orbicular with a slightly serrate margin. The mature leaves are pinnatifid with irregularly-toothed margins, and become increasingly lobed with maturity, reaching 10 – 25 cm in length. Bolting occurs when plants have 20 – 25 leaves (Cici *et al.*, 2009). After the appearance of the first floral buds, rapid elongation of internodes increases the plant height to 1 m or more (Holm and Center, 1977). The self-compatible flowers develop achenes one week after flowering. The average number of seeds per capitulum is around 140, and the mean number of capitula per plant is 4.4 (Cici *et al.*, 2009).

**Plate 1.1 *Sonchus oleraceus* whole plant and capitulum (inset)**

*S. oleraceus* seeds germinate approximately two weeks after sowing and require light for germination (Hutchinson *et al.*, 1984). Maximum germination rates are observed in trays filled with sand: potting mixture mixed in 1:1 ratio (Ellwood, 2007). Their germination is not greatly influenced by temperature (Chauhan *et al.*, 2006). The maximum germination occurred from seeds sown at the soil surface, while increasing osmotic potential and pH (by 160 mM NaCl and pH 10) resulted in low germination percentage (Chauhan *et al.*, 2006).

### 1.8.2 Nutrients in leaves of *S. oleraceus*

Leaves of *S. oleraceus* contain vitamin C, carotenoids, oxalic acid (Table 1.19), various elements (Table 1.20) and short chain fatty acids (Mercadante and Rodriguez-Amaya, 1990; Guil-Guerrero *et al.*, 1998; Liu *et al.*, 2002). The concentration of vitamin C and carotenoids in *S. oleraceus* leaves were 20- and three-fold lower respectively than those present in tomato on the same dry weight basis (Table 1.19).

**Table 1.19 Nutrient composition of *S. oleraceus* leaves collected from natural habitats in Spain**

| **Component** | **Content (g kg$^{-1}$ DW)[a] (Mean ± SD)** |
|---|---|
| Water[1] | 872.4 ± 14.0 |
| Protein | 31.7 ± 1.5 |
| Digestible carbohydrates | 18.2 ± 1.4 |
| Fibre | 32.5 ± 2.4 |
| Lipids | 7.5 ± 0.9 |
| Ash | 29.9 ± 1.8 |
| Oxalic acid[2] | 1.3 ± 0.3 |
| Vitamin C[3] | 0.8 ± 0.1 |
| Carotenoids[4] | 0.2 ± 0.0 |
| Energy[5] | 1098 ± 155 |

Source: Guil-Guerrero *et al*. (1998)

[a] except for water and energy

1. g kg$^{-1}$ FW
2. 4.4,
3. 16.4 and
4. 0.6 accordingly in *Solanum esculentum* (Guil-Guerrero and Rebolloso-Fuentes, 2009).
5. kJ kg$^{-1}$ DW

**Table 1.20 Mineral elements (mg $kg^{-1}$ DW) in *S. oleraceus* leaves collected from natural habitats in Spain**

| Mineral elements | Content (mean ± SD) |
|---|---|
| Na | 2582 ± 282 |
| K | 6225 ± 783 |
| Ca | 324 ± 102 |
| Mg | 759 ± 99 |
| P | 580 ± 96 |
| Fe | 38 ± 4 |
| Cu | 3 ± 1 |
| Zn | 8 ± 2 |
| Mn | 12 ± 2 |

Source: Guil-Guerrero *et al.* (1998)

### 1.8.3 LMWAs of *S. oleraceus*

Vegetative shoots of *S. oleraceus* are exceptionally rich in LMWAs; DPPH radical scavenging capacities of methanol-soluble extracts prepared from leaves of *S. oleraceus* were found to be four-fold greater than those for blueberry (*Vaccinium corymbosum*) extracts on dry weight basis (Gould *et al.*, 2006). Cauline leaves of *S. oleraceus* held higher levels of extractable antioxidants than did rosette leaves (Ellwood, 2007). DPPH radical scavenging capacities of methanolic extracts were greater than aqueous and ethanolic extracts of leaves (Yin *et al.*, 2007). *S. oleraceus* leaf extracts are more potent in LMWA activity than are related species, *S. asper* and *S. kirkii* (Ellwood, 2007).

Measurements of antioxidant activity in methanolic extracts of *S. oleraceus* leaves correlate to the presence of phenolic compounds (Simopoulos, 2004). In one study, caffeic acid derivatives were the major phenolic compounds present (0.8% DW), while luteolin-7-glucoside and apigenin-7-glucoside collectively accounted for 0.2% DW (Table 1.21). In addition, flavonoids (luteolin, apigenin, kaempferol and quercetin) and their glucoside derivatives were identified from whole plant extracts (Table 1.21). Recently caftaric acid has also been identified from leaf methanolic extracts (Ou *et al.*, 2012). Table 1.16 lists the DPPH radical scavenging activities of flavonoids and their derivatives in *S. oleraceus* extracts, where scavenging activities of glycosidic derivatives were lower than their original flavonoid aglycones (Yin *et al.*, 2007; Yin *et al.*, 2008).

The *S. oleraceus* foliar extracts were only cytotoxic on HepG2 cells at concentrations exceeding 100 mg DW $mL^{-1}$ (McDowell *et al.*, 2011). This indicates their safety for consumption as sources of dietary LMWAs.

**Table 1.21 Composition and antioxidant activities of phenolic compounds from leaf extracts of *S. oleraceus***

| **Phenolic compounds** | **Concentration[a]** | **DPPH[b] scavenging $EC_{50}$[c]** | |
|---|---|---|---|
| | **Mean ± SE (mg $g^{-1}$ leaf DW)** | **(µM)** | **(µg $mL^{-1}$)** |
| Caffeic acid derivatives | 2.6 ± 0.3 | nq | nq |
| Chlorogenic acid | 1.5 ± 0.2 | nq | nq |
| Chicoric acid | 12.5 ± 1.3 | nq | nq |
| Luteolin-7-*O*-β-D-glucoside | 3.6 ± 0.4 | 131 | 61 |
| Apigenin-7-*O*-β-D-glucoside | 1.7 ± 0.2 | 206 | 89 |
| Luteolin | nd | 42 | 12 |
| Apigenin | nd | 144 | 39 |
| Kaempferol | nd | 45 | 13 |
| Quercetin | nd | 36 | 11 |
| Kaempferol-3-*O*-β-D-glucoside | nd | 100 | 45 |
| Quercetin-3-*O*-β-D-glucoside | nd | 47 | 22 |

[a] *S. oleraceus* leaves collected from natural habitats in Italy (Gatto *et al.*, 2011)

[b] 2,2-diphenyl-1-picrylhydrazyl radical

[c] Antioxidant activities of isolated phenolic compounds from whole plants of *S. oleraceus* collected from natural habitats in South Korea (Yin *et al.*, 2008)

nq: not quantified

nd: not detected

### 1.8.4 Variation in antioxidant potential of *S. oleraceus*

*S. oleraceus* shows geographical variation in extractable antioxidant activity (Simopoulos, 2004; Schaffer, 2005; Ellwood, 2007). The antioxidant activity and polyphenol content of *S. oleraceus* leaves collected from wild populations in Spain, Greece and Italy varied two-fold (Schaffer, 2005). For plants naturalised in different parts of New Zealand, the antioxidant activity varied four-fold (Ellwood, 2007; Table 1.22). Plant growth conditions, too, may affect the antioxidant status of *S. oleraceus* leaves. In

one study, the antioxidant activity as measured by the DPPH assay was found to be higher in greenhouse grown plants than in those collected from the field, however, reasons for this effect were not identified (Ellwood, 2007). The antioxidant activity parent plants correlated to those of the F1 offspring for selfed plants, indicating heritability of antioxidant traits (Ellwood, 2007). Thus it is likely that combinations of genetic and environmental factors contribute to variability in LMWA in *S. oleraceus*. It is possible to enhance the LMWA activities in *S. oleraceus* by imposing abiotic stresses, since there are numerous examples of this in other species (Section 1.9).

**Table 1.22 Antioxidant activities of methanolic extracts from *S. oleraceus* leaves collected from natural habitats throughout New Zealand listed according to descending order of antioxidant capacity**

| Location | DPPH[a] radical scavenging capacity $EC_{50}$ (mg leaf DW $L^{-1}$) |
|---|---|
| Alexandra[1] | 42.4 ± 2.0 |
| Akaroa[2] | 42.7 ± 1.2 |
| Dunedin[3] | 47.6 ±3.3 |
| Acacia Bay[4] | 48.9 ± 4.9 |
| Otago Peninsula[3] | 50.2 ± 1.6 |
| Kuratau Dam[4] | 50.6 ± 2.0 |
| Arrowtown[1] | 52.1 ± 8.0 |
| Rotoaira Forest[4] | 59.9 ± 5.8 |
| Halfmoon Bay[5] | 60.1 ± 20.1 |
| Roxburg[1] | 61.3 ± 7.0 |
| Tokaanu[4] | 62.8 ± 4.6 |
| Oamaru[3] | 62.9 ± 2.5 |
| Mokai[4] | 63.4 ± 7.5 |

Source: Ellwood (2007)

[a]2,2-diphenyl-1-picrylhydrazyl

[1]Central Otago, [2]Cantebury, [3]Coastal Otago, [4]Central North Island and [5]Stewart Island.

## 1.9 EFFECT OF STRESS ON ANTIOXIDANT LEVELS IN PLANTS

ROS production in plants is an unavoidable by-product of aerobic metabolic processes such as photosynthesis and respiration. ROS serve a useful function as secondary messengers in signal transduction cascades (Mittler *et al.*, 2004; Foyer and Noctor, 2005; Miller *et al.*, 2008). However, in excess, they have the same capacity to adversely affect plant cell components as they do in humans. Many studies have shown that plants subjected to UV radiation, ozone, drought, salinity and temperature accumulate supernumerary free radicals and ROS (Mittler, 2002; Foyer and Noctor, 2003; Tausz *et al.*, 2004). In addition, pathogens and herbivore wounding are known to trigger the production of ROS (Orozco-Cardenas and Ryan, 1999); these apoplastic ROS mediate pathogen recognition by plants, which ultimately may lead to enhanced disease resistance (Lamb and Dixon, 1997; Grant *et al.*, 2000). ROS-yielding processes such as the Mehler reaction in chloroplasts, the glycolate oxidase reaction in peroxisomes and electron leakage in mitochondria are enhanced by such environmental conditions (Table 1.23).

Plants have evolved numerous mechanisms to limit the production of ROS during photosynthesis under excess excitation energy. For example, leaf anatomical adaptations (Mittler *et al.*, 2001), photoprotective pigments (Gould, 2004), and biochemical pathways such as water-water cycle can all retard rates of ROS production (Asada, 1999). However, when stressors generate ROS in excess of the plants' capacity to quench them, they bring about signal transduction cascades, which induce the production of both enzymatic (Table 1.24) and LMWAs (Sections 1.9.1 – 1.9.4), ultimately resulting in enhanced stress tolerance (Mano and Komatsuda, 2002).

Abiotic stresses upregulate key enzymes such as phenylalanine-ammonia-lyase (PAL), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), and flavonol synthase (FLS) of phenylpropanoid and flavonoid pathway; leading to elevated levels of phenolic compounds in the tissues under stress (Leyva *et al.*, 1995; Janas *et al.*, 2000; Niggeweg *et al.*, 2004; Løvdal *et al.*, 2010). Further details on enhancement of LMWAs in plants by abiotic stresses such as drought, chilling, salinity and light are detailed briefly (Sections 1.9.1 – 1.9.4) because these stresses are featured in the experimental sections of this thesis.

**Table 1.23 Examples of primary reactive species, which are induced by abiotic and biotic stressors and their possible mechanism for induction**

| Stressor | Reactive species | Mechanisms |
|---|---|---|
| Strong light | $O_2^{\bullet-}$, $H_2O_2$, $^1O_2$ | Enhanced Mehler activity; photorespiration; triplet chlorophyll excitation |
| Heat | $O_2^{\bullet-}$, $H_2O_2$, $NO^{\bullet}$ | Impairment of photosynthetic and mitochondrial electron transport; enzyme inhibition; increased membrane permeability |
| Cold | $O_2^{\bullet-}$, $H_2O_2$ | Enhanced Mehler activity; suppression of Calvin cycle enzymes; reduced antioxidant activity; decreased membrane fluidity |
| UV-B radiation | $OH^{\bullet}$, $O_2^{\bullet-}$, $H_2O_2$ | Inhibition of PSII reaction centre enzymes; possibly fission of $H_2O_2$ |
| Drought | $O_2^{\bullet-}$, $H_2O_2$, $NO^{\bullet}$ | Inhibition of rubisco; uncoupling of electron transport from ATP synthesis; enhanced Mehler activity; photoinhibition; inhibition of mitochondrial antioxidants; enhanced root respiration |
| Wounding | $O_2^{\bullet-}$, $H_2O_2$, $NO^{\bullet}$ | Elicitation by cell wall fragments; interference by with redox systems on plasma membrane |
| Salinity | $O_2^{\bullet-}$, $H_2O_2$, $NO^{\bullet}$ | Stomatal closure, causing $NADP^+$ deficit and $O_2$ reduction in mitochondria; suppression of Calvin cycle enzymes, by enhanced Mehler reaction and ion leakage through damaged membranes |
| Pathogens | $O_2^{\bullet-}$, $H_2O_2$, $NO^{\bullet}$ | Activation of membrane bound NADP oxidase or cell wall peroxidise |

Source: Reproduced after permission from Gould (2003)

**Table 1.24 Stress conditions that stimulate increased levels or activities of enzymatic antioxidants in plants**

| Antioxidant enzymes | Functions | Stress conditions |
|---|---|---|
| Ascorbate peroxidase | Convert $H_2O_2$ to water. | Drought, high $CO_2$, high light intensity, ozone, paraquat |
| Catalase | Convert $H_2O_2$ to water and oxygen | Chilling |
| Glutathione peroxidase and reductase | Glutathione peroxidase decomposes $H_2O_2$ and glutathione reductase regenerates glutathione. | Chilling, drought, high $CO_2$, ozone, paraquat |
| Superoxide dismutase | Converts superoxide anion radicals to $H_2O_2$. | Chilling, high $CO_2$, high light, increased $O_2$, ozone, paraquat, $SO_2$ |

Sources: Buchanan *et al*. (2000); Halliwell and Gutteridge (2007)

### 1.9.1 Drought stress on LMWA production in plants

There is a large body of evidence to indicate that LMWAs accumulate in plants in response to drought stress. Particularly phenolic compounds have the ability to mitigate effects of oxidative stress from water deficit either by scavenging ROS directly or by reducing light energy received by chloroplasts. This helps photosystems of drought stressed plants to process radiation without the formation of ROS (Bilger *et al.*, 2001). The response of the antioxidant system to water deficit varies among plant species and varieties, and may change with development of the plant, as well as the duration and intensity of the stress (Table 1.25).

### 1.9.2 Chilling stress on LMWA production in plants

The enhancement of antioxidant capacity and level of phenolic compounds in response to cold stress has been observed in many species (Solecka *et al.*, 1999; Janas *et al.*, 2000; Rivero *et al.*, 2001; Kirakosyan *et al.*, 2003; Pennycooke *et al.*, 2005; Olenichenko *et al.*, 2006; Liu *et al.*, 2007; Koc *et al.*, 2010). The hawthorn species (*Crataegus laevigata* and *C. monogyna*) upregulated different phenolics (such as vitexin-2"-*O*-rhamnoside, acetylvitexin-2"-*O*-rhamnoside, hyperoside, quercetin) at different magnitudes in response to identical conditions of cold stress (Kirakosyan *et al.*, 2003) indicating that species and varieties differ in their antioxidants defences facing the same stress.

### 1.9.3 Light stress on LMWA production in plants

Increasing light intensities enhanced the accumulation of phenolic compounds in species such as *Populus trichocarpata, Solanum esculentum, Vitis vinifera* and *Punica granatum* (Dumas *et al.*, 2003; Warren *et al.*, 2003; Pereira *et al.*, 2006; Gautier *et al.*, 2008; Schwartz *et al.*, 2009). Anthocyanins in particular, accumulate in vacuoles under high light, where they contribute to light screening, pigmentation and photoprotection among other functions (Gould, 2004; Hernández *et al.*, 2009). Increased ascorbic acid levels were associated with increasing light intensities in *Solanum esculentum* (Lee and Kader, 2000; Dumas *et al.*, 2003).

**Table 1.25 Selected examples for effects of drought on increased low molecular weight antioxidants (LMWAs) in plant species under experimental conditions**

| Enhanced LMWAs | Plant species | References |
|---|---|---|
| **Phenolic compounds** | | |
| Chlorogenic acid<br>Quercetin<br>Rutin<br>Quercetin glucoside<br>1,5-dihydroxyxanthone<br>Hypericin | *Hypericum perforatum*<br>*Hypericum brasiliense* | (Gray *et al.*, 2003; Nacif de Abreu and Mazzafera, 2005; Zobayed *et al.*, 2007) |
| Chlorogenic acid<br>Epicatechin<br>Catechin | *Crataegus* sp. | (Kirakosyan *et al.*, 2003) |
| **α-Tocopherol** | | |
| | *Arbutus unedo* | (Munné-Bosch and Peñuelas, 2004; Šircelj *et al.*, 2005) |
| | *Triticum aestivum*<br>*Festuca arundinacea*<br>*Agrostis palustris* | (Bartoli *et al.*, 1999; Herbinger *et al.*, 2002) |
| | *Rosmarinus officinalis* | (Munné-Bosch *et al.*, 1999) |
| | *Lavandula stoechas* | (Munné-Bosch *et al.*, 2001) |
| **Ascorbate** | | |
| | *Arbutus unedo* | (Munné-Bosch and Peñuelas, 2004; Šircelj *et al.*, 2005) |
| | *Rosmarinus officinalis*<br>*Salvia officinalis*<br>*Melissa officinalis* | (Munné-Bosch *et al.*, 1999; Munné-Bosch and Alegre, 2003) |
| **Carotenoids** | | |
| | *Rosmarinus officinalis* | (Munné-Bosch *et al.*, 1999) |
| | *Triticum aestivum* | (Bartoli *et al.*, 1999; Herbinger *et al.*, 2002) |

### 1.9.4 Salinity stress on LMWA production in plants

Numerous studies have indicated that plants subjected to salinity, in addition to regulating water and ionic relations; enhanced LMWAs in their tissues to remove excess ROS. The type of LMWAs accumulated and effective range of salinity is specific to plant species (Table 1.26).

**Table 1.26 Selected examples for effects of salinity on enhanced low molecular weight antioxidants (LMWAs) in plant species under experimental conditions**

| Enhanced LMWAs | Species | NaCl concentration (mM) | References |
|---|---|---|---|
| **Phenolic compounds** | | | |
| Polyphenolic content | *Cakile maritime* | 100 and 400 | (Ksouri *et al.*, 2007) |
| Isoorientin, orientin, rutin and vitexin | *Fagopyrum esculentum* | 10, 50, 100, and 200 | (Lim *et al.*, 2012) |
| Oleuropein | *Olea europaea* | 75 - 125 | (Petridis *et al.*, 2012) |
| **Ascorbate** | *Catharanthus roseus* | 50 - 100 | (Jaleel *et al.*, 2007) |
| | *Solanum esculentum* | Saline water | (Raffo *et al.*, 2006) |
| **Carotenoids** | | | |
| Lycopene | *Capsicum annuum* | 15 - 30 | (Navarro *et al.*, 2006) |
| Carotenoid | *Fagopyrum esculentum* | 50 and 100 | (Lim *et al.*, 2012) |
| **Tocopherols** | *Solanum esculentum* | 50 - 150 | (Incerti *et al.*, 2008) |

Enhancement of LMWAs due to abiotic stresses is not limited to intact plants but extends to *in vitro* cultured plant cells, as demonstrated for various plant species (Section 1.10; Table 1.27). Further, extraction of LMWAs from cell cultures have numerous advantages over extraction from whole plants (Section 1.10), thus *S. oleraceus* cell cultures may provide an effective and efficient means of LMWAs production.

## 1.10 PLANT CELL CULTURE FOR THE PRODUCTION OF LMWAS

Plant cell culture may be a viable option for the commercial scale extraction of antioxidants from *S. oleraceus*, avoiding the substantial variation of antioxidant content across plant material itself. Ideally, *S. oleraceus* cell cultures would continuously produce phenolic antioxidants, while the content could be controlled by manipulating culture conditions and imposing abiotic stressors. Abiotic stressors has been used successfully, to enhance LMWAs in experimental plant cell cultures of other species (Table 1.27), and the harvestable product has been manipulated by changing the source of explant, growth conditions, and nutrient medium composition. Many plant metabolites have been

produced via cell culture production at the industrial scale, such as anthocyanins, berberines, carthamin, ginsenosides, rosmarinic acid, scopolamine, shikonin and taxol (Sajc *et al.*, 2000; Verpoorte *et al.*, 2002; Kolewe *et al.*, 2008; Wilson and Roberts, 2012).

**Table 1.27 Selected examples for the effects of abiotic stresses on enhanced low molecular weight antioxidants (LMWAs) in plant cell cultures under experimental conditions**

| Enhanced LMWAs | Species | Abiotic elicitor | References |
|---|---|---|---|
| **Phenolic compounds** | | | |
| Flavonoids, flavonols and saponins | *Glycine max* | NaCl (10 mM) | (Radhakrishnan *et al.*, 2012) |
| *p*-coumaric acid and 2-3-*O*-glucosylresveratrol | *Vitis vinifera* | Hydrostatic pressure (40 MPa) | (Cai *et al.*, 2011) |
| Taxol | *Taxus yunnanensis* | $La^{3+}$ (5.8 µM) | (Wu *et al.*, 2001) |
| Saponins | *Panax ginseng* | Ultrasound (0.1 W $cm^{-3}$ at 38.5 kHz) | (Wu and Lin, 2002) |
| | *Panax notoginseng* | Hydrostatic pressure (670 kPa) | (Zhang *et al.*, 1995) |
| Anthocyanins | *Vitis vinifera* | Pulsed electric field (0.32 J $kg^{-1}$) | (Saw *et al.*, 2012) |
| **Ascorbate** | *Arabidopsis thaliana*<br>*Nicotiana tabacum* | Methyl jasmonate[1] (50 µM) | (Wolucka *et al.*, 2005) |
| **Carotenoids** | | | |
| β-carotene and violaxanthin | *Ipomoea batatas* | NaCl (100 – 200 mM) | (Kim *et al.*, 2012) |
| Zeaxanthin | *Scutellaria baicalensis* | NaCl (100 mM), ABA[2] (200 µM) | (Tuan *et al.*, 2013) |
| **Tocopherols** | | | |
| α-tocopherol | *Carthamus tinctorius* | NaCl (0.9 – 1.2 M) | (Chavan *et al.*, 2011) |

1. methyl jasmonate mediate biotic and abiotic stress responses in plant cells
2. Abscisic acid can simulate osmotic stress

Plant cell cultures are often preferred over whole plants for the extraction of phytochemicals at a commercial scale, since commercial extraction requires phytochemicals from sources, which are not limited by compound availability, compound variability, seasonal availability, species abundance and variable growth rate of the intact plants (Wilson and Roberts, 2012). Additionally, the extraction of phytochemicals from plant cell cultures may be a more economical option, since chemical synthesis is complex and costly (Zhang *et al.*, 1995; Sajc *et al.*, 2000). In a few cases, cell cultures have been found to produce higher levels of secondary metabolites than the differentiated mother plant itself (Rao and Ravishankar, 2002).

## 1.11 THESIS INTRODUCTION

*S. oleraceus* may be able to protect human cells from oxidative stress given their superb extractable antioxidant activities. However to substantiate this claim, further studies on the antioxidant properties of *S. oleraceus* are needed. Thus, the broad aim of this research was to study the antioxidant activities of *S. oleraceus* leaves based on following specific objectives:

### 1.11.1 Objectives

1. To study the effects of age of a plant, its original locality, and the imposition of abiotic stressors on the extractable antioxidant activities of *S. oleraceus* leaves; and the effects of stressor-induced leaf variations on cellular antioxidant activities of cultured human cells.

2. To study the effects of cooking and *in vitro* gastrointestinal digestion on extractable antioxidant activities and *in vitro* cellular antioxidant activities of *S. oleraceus* leaves.

3. To study extractable antioxidant activities of *S. oleraceus* cell suspension cultures in relation to abiotic stressors.

This dissertation is divided into three main chapters to test the above hypotheses, each corresponding to a stand-alone publication, and therefore a certain extent of overlap between background information is inevitable.

Chapter 2 outlines the general materials and methodology that were followed in Chapters 3 – 5. Chapter 3 identifies the variables that affect the extractable and cellular antioxidant activities of *S. oleraceus* leaves. Chapter 4 presents the effects of cooking and *in vitro* gastrointestinal digestion on extractable and cellular antioxidant activities of phenolic compounds in *S. oleraceus* leaves. Finally, Chapter 5 presents a protocol that allows continuous extraction of phenolic antioxidants from the *S. oleraceus* cell suspension cultures where the production varied with abiotic stressor applied. Chapter 6 presents general discussion based on findings from previous chapters and possible future directions.

# CHAPTER 2: GENERAL MATERIALS AND METHODS

## 2.1 CHEMICALS

2,2'-Azobis(2-amidino-propane) dihydrochloride (AAPH) and chlorogenic acid were purchased from Sapphire Bioscience (Hamilton, New Zealand). 2,2'-Azobis(2-methylpropionamididine) dihydrochloride (ABAP), 2',7'-dichlorofluorescin diacetate (DCFH-DA), 2,2-diphenyl-1-picrylhydrazyl **(**DPPH), caftaric acid, chicoric acid, Dulbecco's modified Eagle's medium (DMEM), fluorescein disodium, Folin–Ciocalteu reagent, Hank's balanced salt solution (HBSS), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, phosphate buffered saline solution (PBS) and quercetin dihydrate were purchased from Sigma-Aldrich (St Louis, MO). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and sodium carbonate ($Na_2CO_3$) were purchased from Thermo Fisher Scientific Australia (Scoresby, Australia). Tannic acid was purchased from Carl Roth Gmbh (Karlsruhe, Germany). Ascorbate assay kit (700420) from Cayman Chemical Company (Ann Arbor, MI, USA). Quercetin was kindly provided by Plant and Food Research, Chemistry Department, University of Otago, Dunedin, New Zealand. Solvents were of HPLC grade. Human hepatocellular carcinoma (HepG2) cells were gratefully obtained from the Pathology Department at the University of Otago, Dunedin, New Zealand. Human epithelial colorectal adenocarcinoma cells (Caco2) cells were kindly provided by Victoria University of Wellington, Wellington, New Zealand.

## 2.2 TOTAL PHENOLICS ASSAY

Total phenolics were measured using the Folin–Ciocalteu method, modified after Waterhouse (2001). Duplicate 1:2 serial dilutions of samples or a 0 – 60 μM tannic acid standard series were introduced into the wells of 96 well plates. Each well held a 25 μL sample, standard or blank ($ddH_2O$), plus 125 μL of 0.1 M Folin-Ciocalteu reagent. Plates were incubated in the dark on an orbital shaker (10 rpm) at room temperature for 3 min, and then 125 μL 0.6 M $Na_2CO_3$ added to each well and incubated for a further 30 min. Absorbance at 760 nm was read using an EnSpire 2300 multimode reader (PerkinElmer,

San Jose, CA). Total phenolic concentrations were expressed as mg tannic acid equivalents $g^{-1}$.

### 2.3 DPPH (2,2-diphenyl-1-picrylhydrazyl) RADICAL SCAVENGING ASSAY

DPPH radical scavenging capacity was measured for duplicate serial dilutions of the samples as described by Philpott *et al*. using 1mM Trolox as the standard (Philpott *et al.*, 2003). A 200 µL aliquot of 100 µM DPPH in methanol was added to 50 µL sample or standard, incubated in darkness on an orbital shaker for 30 min, and absorbance measured at 515 nm. Antioxidant activity was estimated as the reciprocal of $EC_{50}$, the concentration of sample or standard which resulted in a 50% reduction in $A_{515}$.

### 2.4 OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC) ASSAY

The ORAC-fluorescein assay, adapted for manual handling, was performed on serial dilutions of samples in black 96-well plates (Dávalos *et al.*, 2003). Into each well were dispensed 120 µL of 117 nM fluorescein disodium in 75 mM phosphate buffer at pH 7.4, and 20 µL of sample or Trolox in the same phosphate buffer. Plates were incubated at 37 °C for 5 min, and then 60 µL of 40 mM AAPH added to generate peroxyl radicals. Wells were excited at 485 nm, and fluorescence emission at 538 nm was read at minute intervals over 1 h using an EnSpire 2300 multimode reader, with 5 s shaking between readings. The areas under fluorescence decay curves (AUC) of samples were calculated, from which the AUC of the antioxidant-free blank (75 mM phosphate buffer) was subtracted. ORAC values for samples were expressed as µmol Trolox equivalents $g^{-1}$ using regression equations between net AUC and Trolox concentration.

### 2.5 ASCORBATE ASSAY

Ascorbate concentration was measured using the ascorbate assay kit (700420) from Cayman Chemical Company (Ann Arbor, MI, USA). The samples were resuspended in methanol: $ddH_2O$: diethylenetriaminepentaacetic acid DTPA (75: 22: 2.5, v/v/v) to obtain 0.05% (w/v) dilution. The concentration of ascorbate was measured using serial dilutions of samples in black 96-well plates. Into each well were dispensed 50 µL of DTPA, and 50 µL of sample or ascorbate standard. Then 100 µL of reconstituted ascorbate substrate was

added to all wells excluding sample background wells. Ascorbate assay buffer 100 µL was added to sample background wells. The plates were incubated in darkness at 25 °C for 10 min, and then 50 µL of ascorbate developer to all the wells. The plates were incubated in darkness at 25 °C for 5 min, and well contents were excited at 345 nm, and fluorescence emission at 425 nm was read using an EnSpire 2300 multimode reader. Ascorbate concentrations of samples were expressed as ascorbate mg $g^{-1}$.

## 2.6 HUMAN CELL CULTURES (HepG2 and Caco2)

Human HepG2 cells were grown in DMEM growth medium supplemented with 10% heat inactivated FBS, 0.2 M GlutaMAX and 0.1 mg $mL^{-1}$ penicillin-streptomycin. Human Caco2 cells were grown in complete DMEM medium (containing high glucose supplemented with 10% heat inactivated FBS, 10 mL $L^{-1}$ nonessential amino acids, 2 mmol $L^{-1}$ L-glutamine, 0.5 mg $L^{-1}$ amphotericin B, 50 mg $L^{-1}$ gentamicin, 15 mmol $L^{-1}$ HEPES and 44 mmol $L^{-1}$ NaHCO).

Both HepG2 and Caco2 cell cultures were maintained in 75 $cm^2$ flasks at 37 °C in a humidified atmosphere with 5% $CO_2$. Cells were routinely passaged every 2-3 d, upon reaching 90% confluency (when 90% of the growth surface of the flask is occupied by cells). The cells were passaged by removing media and incubating with trypsin for 15 min and adding fresh media. The HepG2 cells used for subsequent experiments were between passages 12 and 20 while Caco2 cells were between passages 21 and 30.

## 2.7 CELLULAR ANTIOXIDANT ACTIVITY (CAA) ASSAY

The CAA assay was performed according to Wolfe and Liu (2007). Human HepG2 and Caco2 cells were seeded at 6 x $10^4$ per well on a 96 well flat-bottom plate in 100 µL of DMEM, and incubated at 37 °C for 24 h. DMEM was removed, and the cells were washed with PBS. Samples, were (evaporated and) diluted with DMEM, and 50 µL of 25 mM DCFH-DA was added to 50 µL of the diluted extracts, to yield final concentrations of 1, 3, 10 and 30 g $L^{-1}$ of samples. Cells were treated for 1 h with samples or quercetin dihydrate standard (at 5, 10, 15 and 20 µM final concentration) in DCFH-DA. A 100 µL aliquot of 600 µM ABAP in HBSS was applied to the cells after PBS wash. The emission fluorescence at 538 nm (following excitation at 485 nm), was measured at 37 °C every 5

min for 1 h using a Fluoroskan Ascent microplate fluorometer (Thermo Electron, Franklin, MA).

CAA values were calculated as the integral of fluorescence emission using the following equation:

CAA unit = 100 – (∫SA / ∫CA) x 100,

where ∫SA and ∫CA is the integrated area under the curve of fluorescence versus time for sample and control curves, respectively (Wolfe and Liu, 2007).

## 2.8 ONLINE REVERSE PHASE HPLC-DPPH RADICAL SCAVENGING

The HPLC method reported by Yin *et al*. was modified and used to separate and quantify phenolics in samples (Yin *et al.*, 2008; Ou *et al.*, 2013). We used an Agilent Technologies 1200 series HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a quaternary pump and a diode array detector. Briefly, samples were injected at 4 °C into a reverse phase Alltima C18 column (3µm 150 x 2.1mm). Elution (0.2 mL $min^{-1}$) was performed using a solvent system comprising 1% formic acid (A) and 100% acetonitrile (B) using a gradient starting with 95% A, reducing to 85 % at 15 min; 76% at 27 min, 70% at 40 min, 20% from 41 to 45 min and 95% from 46 to 55 min. The absorption spectra at 320 nm were recorded. The HPLC-separated analytes were reacted postcolumn, with the DPPH reagent (250 µM) dissolved in 100% acetonitrile: 0.1 M sodium citrate buffer (50:50, v/v) at pH 7.6 and pumped at 0.2 mL $min^{-1}$. The induced bleaching was detected photometrically as a negative peak at 518 nm. Identification of main peaks in samples were confirmed by co-eluting with authentic compounds.

# CHAPTER 3: EFFECTS OF AGE, ECOTYPE, CHILLING AND SALINITY ON EXTRACTABLE ANTIOXIDANT ACTIVITIES OF *Sonchus oleraceus* L.

**Plate 3.1 Four week old *Sonchus oleraceus* L. plants growing in Victoria University of Wellington glasshouse.**

## 3.1 ABSTRACT

Leaves of *Sonchus oleraceus* L., a traditional component of the Māori diet in New Zealand, are rich in phenolics and show high antioxidant potential *in vitro*. Extractable antioxidant activities show considerable variation among plants, which may be due to variation in their growing environments and/or genotype, but the precise reasons for this variation are unknown. To study possible environmental effects on antioxidant activity, two ecotypes from Acacia Bay (ACB) and Oamaru (OAM) in New Zealand were exposed to chilling, salinity and their combination for two weeks commencing 8, 10 and 12 weeks after germination. Chlorophyll fluorescence parameters, extractable and cellular antioxidant activities, ascorbate and total phenolic contents were measured, and HPLC profiles obtained for methanolic extracts of leaves. None of the treatments significantly diminished the maximum quantum efficiency of photosystem II but rapid light response curves for chlorophyll fluorescence indicated acclimation to stressors after two weeks. Antioxidant activities and total phenolic content were higher in older plants compared to younger plants in both ecotypes, irrespective of the treatment. The ACB ecotype had a higher phenolic concentration and antioxidant activities compared to OAM, and these levels were maintained from F1 to F2 generation. Chlorogenic acid was enhanced in youngest plants by exposing plants to chilling, salinity and their combination. The cellular antioxidant activities in HepG2 cells correlated linearly with stressor-induced extractable antioxidant activities and LMWAs concentrations from the youngest plants. My data indicate that the imposition of stressors on already potent ecotypes can augment the extractable antioxidant potential in *S. oleraceus*. Further, these stressor-induced changes were transferable to human cells *in vitro*.

**KEYWORDS**: *Sonchus oleraceus*, antioxidant, phenolics, chilling, salinity

## 3.2 INTRODUCTION

Māori have long used the smooth sow thistle, *Sonchus oleraceus* L., both as a leafy vegetable and for medicinal purposes (Section 1.7). The leaves of *S. oleraceus* are rich in phenolic compounds and thus exceptionally high in extractable antioxidant activities (Section 1.8.3). *S. oleraceus* shoots showed variation in DPPH radical scavenging activities among plants collected from different locations in New Zealand (Section 1.8.4; Ellwood, 2007). In addition to genotypic variation, some of the variability in LMWA activity is likely attributable to differences in development stage, growth environment and the degree to which plants are subjected to abiotic stressors. However, the exact causes of this variation are unknown.

In other plant species, age-related increases in phenolic compounds and antioxidant activities are associated with increasing capacities of mature plants to invest resources in secondary metabolic processes, whereas in young plants, limited resources are demanded for primary metabolic processes required for growth (Fritz *et al.*, 2001; Barton, 2007). For example, the concentration of phenolics and antioxidant activities increased with age in species such as *Brassica oleracea*, *Cosmos caudatus* and *Lactuca sativa* (Vallejo *et al.*, 2003a; Oh *et al.*, 2011; Mediani *et al.*, 2012).

Intraspecific differences in extractable LMWA activities and phenolic concentration may be attributable to differences in concentration and activity of enzymes involved in the phenylpropanoid pathway. Differences in phenolic concentration and antioxidant activity among cultivars have been reported for species, such as *Fragaria ananassa*, *Rosa* sp., *Solanum tuberosum*, *Rubus idaeus*, *Vaccinium corymbosum* and *Solanum esculentum* (Ehlenfeldt and Prior, 2001; Vinokur *et al.*, 2006; Reddivari *et al.*, 2007; Tulipani *et al.*, 2008; Løvdal *et al.*, 2010; Krüger *et al.*, 2011).

There are numerous examples in which a single abiotic stressor (Section 1.9) was used to elevate phenolic levels and antioxidant activities in vegetative shoots of different species (Smirnoff, 1993; Bartoli *et al.*, 1999; Munné-Bosch *et al.*, 1999; Munné-Bosch and Alegre, 2000; Zhang and Schmidt, 2000; Munné-Bosch and Alegre, 2002; Kirakosyan *et al.*, 2003; Munné-Bosch and Peñuelas, 2004; Šircelj *et al.*, 2005). However, far fewer

studies have examined the effects of combinations of these stressors on antioxidant levels (Figure 3.1; Mittler, 2006).

| **Salinity** | **Heat** | **Chilling** | **Ozone** | **UV** | |
|---|---|---|---|---|---|
| Positive/ Negative No | Positive | No | Positive | Positive | **Drought** |
| | Negative | **Unknown** | No | Positive/ No | **Salinity** |
| | | | No | Negative | **Heat** |
| | | | **Unknown** | **Unknown** | **Chilling** |
| | | | | **Unknown** | **Ozone** |

No : no change by the combination compared to single stressor
Negative : negative interaction (decrease owing to the combination compared to single stressor)
Positive : positive interaction (increase owing to the combination compared to single stressor)
**Unknown** : effect of stressor combination unknown

**Figure 3.1 Effects of two combined abiotic stressors on extractable antioxidant activities of plants Sources: Pääkkönen *et al.* (1998); Aroca *et al.* (2003); Koti *et al.* (2007); Maggio *et al.* (2007); Shen *et al.* (2010); Silva *et al.* (2010); Hartikainen *et al.* (2012); Ahmed *et al.* (2013a); Ahmed *et al.*, (2013b)**

Plant responses to a combination of stressors may be complex, not simply the sum of effects of individual stressors. In laboratory experiments and field conditions involving different species, stressor combinations have been found to yield additive, antagonistic, synergistic effects on plant physiological, phytochemical or yield responses, and cannot be predicted through plants responses to the individual stressor. Additive effects are defined as when the two stressors together cause a response equal to the effects caused by

two stressors individually (i.e. simple addition of each individual response), therefore can be either positive or negative (Figure 3.2). Synergistic interaction occurs when two stressors together produce an effect greater than the sum of their individual effects (Figure 3.3A). Antagonistic interaction occurs when two stressors together produce an effect lower than the sum of their individual effects (Figure 3.3B). Additionally, the potential effects of stressor combination could also vary depending on the relative level of each of the different stressors combined (Mittler, 2006).

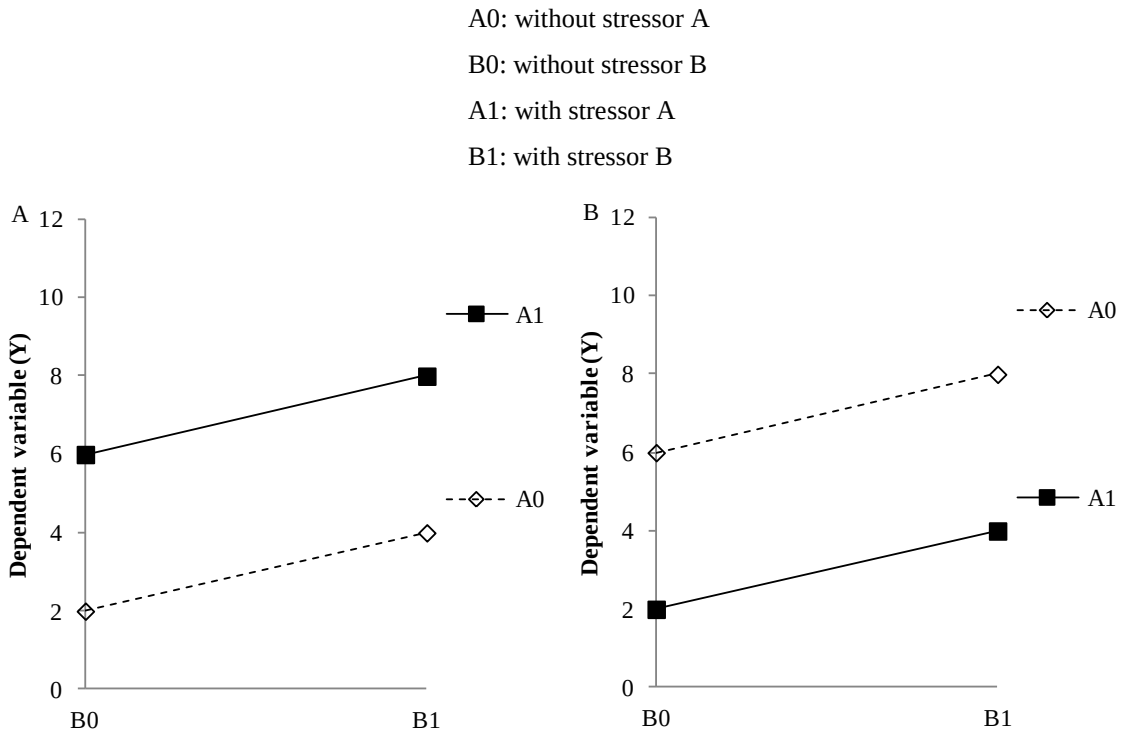



**Figure 3.2 ANOVA interaction plots for additive effect of two stressors (A1 and B1) together produce an effect equal to the sum of their individual stressor effects on the dependent variable (Y), which could be either (A) positive or (B) negative.**

**Source: Dunne (2010)**

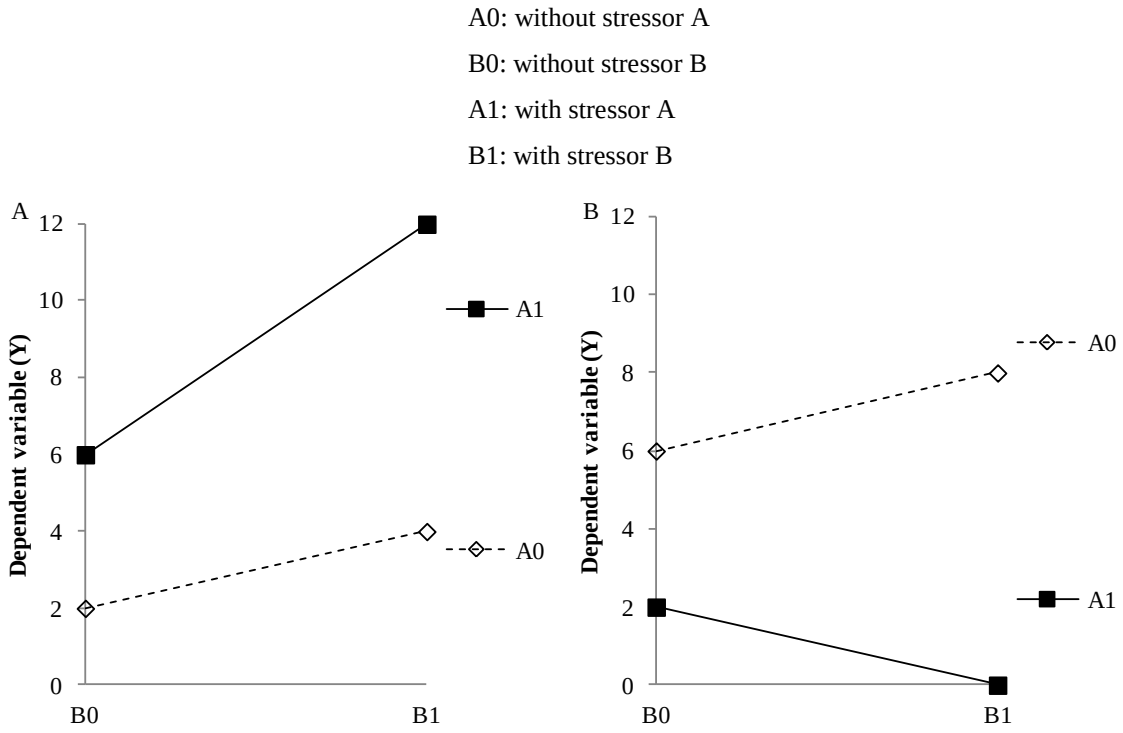



**Figure 3.3 ANOVA interaction plots when two stressors (A1 and B1) together produce either (A) synergistic or (B) antagonistic on the dependent variable (Y).**

**Source: Dunne (2010)**

Assays for extractable antioxidant activities alone are not themselves sufficient for us to conclude that those plants that possess the highest antioxidant levels would provide the greatest protection against effects of ROS in the cells of the consumer (Section 1.6). Nevertheless, associations between chemical measures of leaf antioxidant activities and CAA of *S. oleraceus* have not yet been studied. It is not known whether variation in the phenolic content and recorded antioxidant capacity in leaf extracts is accurately reflected in terms of the cellular antioxidant boost supplied to living human cells.

It was hypothesized that extractable LMWA activities of *S. oleraceus* leaves vary depending on age of a plant, its original locality, and the imposition of abiotic stressors; and stressor-induced variations correlates with cellular antioxidant activities in cultured human cells. Using ecotypes of *S. oleraceus* already known to possess potent LMWA activities, I describe here the effects of applying chilling and salinity treatments, both alone and in combination, on the levels of phenolic compounds in the leaves. In preliminary trials (Appendix A.1-A.2), these two stressors were shown to have the greatest potential for manipulating phenolic compound concentration.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Plant materials and treatments

Two ecotypes of *Sonchus oleraceus* were grown from seeds obtained from selfed F1 generation plants, which were themselves raised from seeds collected from wild populations at Acacia Bay and Oamaru in New Zealand (hereafter the ecotypes are abbreviated as ACB and OAM, respectively). These two ecotypes were selected due to their difference original growing environments (Table 3.1) and in antioxidant activities, where ACB was 1.6 times more efficient in scavenging DPPH radicals than OAM (Ellwood, 2007). OAM originated from a colder region than ACB (Table 3.1).

**Table 3.1 Comparison of the regions from which the two ecotypes of *Sonchus oleraceus* L. were collected**

| | **Ecotypes** | |
|---|---|---|
| | **ACB** | **OAM** |
| **Location** | Acacia Bay<br>Central North Island | Oamaru<br>Coastal South Island |
| **Coordinates** | S 38º 42', E 176º 02' | S 45º 05', E 170º 58' |
| **Temperature (ºC)[1]** | 12.4 ± 0.1 | 9.4 ± 0.1 |
| **Humidity (%)[1]** | 82.7 ± 0.2 | 78.4 ± 0.3 |

Source: NIWA (2012)

[1] Annual average for measurements at 3 h interval

The plants were grown in individual pots (12 x 12 x 15 cm) containing a 1:1 mixture of potting mix: sand inside an unheated glasshouse under natural light at Victoria University of Wellington campus during January to March 2011 (summer). Two ecotypes and four treatments (stressors) were combined to give eight treatments for which each had eight replicates. Treatments were imposed for 14 d on 8, 10 and 12 week old plants to test the effect of plant age on the responsiveness to abiotic stressors. Control plants were watered with tap water at 150 mL $day^{-1}$. For the chilling treatment, plants were repeatedly held at 5 $^{o}$C for 12 h overnight and returned to the glasshouse during the day. Salinity was imposed by irrigating with 150 mL $day^{-1}$ of 50 mM NaCl. The fourth group of plants was given both the chilling and salinity treatments.

The fully expanded youngest leaves from nodes 5 and 6 on each plant were selected on weeks 10, 12 and 14, for chlorophyll fluorescence measurements and then harvested for subsequent phytochemical analysis.

### 3.3.2 Chlorophyll fluorescence measurements

The maximum photosynthetic efficiency of PS II was measured in leaves dark-adapted for 8 h using a PAM 2500 chlorophyll fluorometer (Heinz Walz GmbH, 2008, Germany). The minimum level of fluorescence ($F_o$) was determined under low measuring-light irradiance (<0.05 µmol $m^{-2}$ $s^{-1}$), and then $F_m$ (maximum fluorescence) determined using a saturating pulse (12,000 µmol $m^{-2}$ $s^{-1}$ for 0.8 s). From those parameters, values of $F_v/F_m$ (the maximum quantum efficiency of PSII in the dark-adapted state) were approximated, where variable fluorescence ($F_v$) was calculated as $F_m–F_o$ (Table 3.2).

To obtain rapid light response curves, the leaves were irradiated with white actinic light supplied by the fluorometer. Steady state fluorescence ($F'_o$) was measured under a light ramp comprising 11 intensities from 0 to 3000 µmol $m^{-2}$ $s^{-1}$ at 30 s interval. The maximum fluorescence ($F'_m$) was recorded using saturating light pulses superimposed on the actinic light (Table 3.2). Accordingly, $\Delta F/F'_m$ (effective quantum yield) was obtained where $\Delta F$ (variable fluorescence) was calculated as $F'_m–F'_o$ (Table 3.2). Non photochemical quenching (NPQ) was calculated as $(F_m–F'_m)/F'_m$, where $F_m$ is the maximum fluorescence measured during light curve. $q_P$ (photochemical quenching) was calculated as $(F'_m–F_t)/(F'_m–F'_o)$, where $F_t$ defined as steady state yield of fluorescence in the light (Table 3.2 – 3.3). Apparent rate of photosynthetic electron transport of photosystem II (ETR) was calculated as $\Delta F_v/F'_m$ x 0.5 x 0.84 x PAR, (where PAR is photosynthetically active radiation, 400-700 nm; 0.84 is the ETR factor, the fraction of absorbed PAR by green leaves and 0.5 is the fraction of quanta distributed to PS II).

**Table 3.2 Definitions of fluorescence intensity parameters**

| Symbol | Fluorescence intensity parameter | Definition |
|---|---|---|
| $F_o$ | Minimum fluorescence (dark adapted leaf) | Fluorescence intensity with all PS II reaction centres open while the photosynthetic membrane is in the non-energized state. |
| $F_m$ | Maximum fluorescence (dark adapted leaf) | Fluorescence intensity with all PSII reaction centres closed all nonphotochemical quenching processes are at a minimum. |
| $F_v$ | Variable fluorescence (dark adapted leaf) | Maximum variable fluorescence in the state when all non-photochemical processes are at a minimum, calculated as ($F_m$–$F_o$). |
| $F'_o$ | Minimum fluorescence (light adapted leaf) | Fluorescence intensity with PS II reaction centres open in any light adapted state. |
| $F'_m$ | Maximum fluorescence (light adapted leaf) | Maximum fluorescence intensity with saturating pulse in any light adapted state. |
| $F_t$ | Steady state fluorescence (light adapted leaf) | Fluorescence immediately before a saturating pulse. |
| $\Delta F$ | Variable fluorescence (light adapted leaf) | Maximum variable fluorescence in any light adapted state, calculated as ($F'_m$–$F'_o$). |

Source: Maxwell and Johnson (2000)

**Table 3.3 Definitions of fluorescence quenching parameters**

| Fluorescence quenching parameters | Definition | Calculation |
|---|---|---|
| Maximum quantum efficiency (dark adapted leaf) | The maximum potential quantum efficiency of PSII in the dark adapted state. | $F_v/F_m$ |
| Effective quantum efficiency (light adapted leaf) | The proportion of the light absorbed by the chlorophyll associated with PSII that is used in photochemistry. | $\Delta F/F'_m$ |
| NPQ (Non photochemical quenching) | Quantum yield of regulated non-photochemical energy loss in PS II as heat. | $(F_m–F'_m)/F'_m$ |
| $q_P$ (Photochemical quenching) | The proportion of the PSII reaction centers that are open. | $(F'_m–F_t)/(F'_m–F'_o)$ |

Source: Genty *et al*. (1989)

The curve fit option in the PamWin-3 software, which has been derived by Eilers and Peeters' mechanistic model (Eilers and Peeters, 1988) was used to obtain three parameters from the ETR versus PAR curves:(i) efficiency of light capture in the light limiting region indicated by the initial slope of the curve (α); (ii) maximum

photosynthetic capacity indicated by the plateau in ETR at light saturation ($ETR_{max}$); and (iii) light saturation coefficient of the curve ($I_k$).

### 3.3.3 Phytochemical extraction

Leaves were removed and bisected longitudinally, one half to be used for phytochemical analysis and the other to determine dry matter percentage (all results presented on dry weight basis). The remainder of the plant was separated into shoot and root systems and oven dried at 70 $^{o}$C for 12 h to obtain dry biomass.

Material intended for phytochemical analysis was snap frozen in liquid nitrogen, ground to a fine powder, and dissolved in methanol: dd$H_2O$: acetic acid (70:23:7, v/v/v) to obtain a 10% (w/v) slurry. Aliquots were centrifuged at 24000 g for 5 min, and supernatants stored under nitrogen at −20 $^{o}$C. Phytochemical analyses were performed within 7 d of extraction.

The chemicals used are listed in Section 2.1. The analysis of total phenolic content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities, oxygen radical absorbance capacity (ORAC) assay, Cellular Antioxidant Activity (CAA) assay, ascorbate content and online reverse phase HPLC-DPPH radical scavenging were performed as described in Sections 2.2 – 2.8.

## 3.4 STATISTICAL ANALYSIS

A three-way analysis of variance (ANOVA) with Tukey's post hoc test was performed to identify effects of age, ecotype and abiotic stressors. Mixed repeated measures ANOVA was performed on light response curve data and Bonferroni post hoc tests. Probit analysis was performed for CAA dose-response data. Correlation analysis was performed between CAA and extractable antioxidant activities, total phenolic content, ascorbate content and hydroxycinnamic acid concentrations. Significances were defined at $P < 0.05$. All analyses were performed using SPSS 18.0 statistical software.

## 3.5 RESULTS

### 3.5.1 Hydroxycinnamic acids in *S. oleraceus* leaf extracts detected by HPLC

HPLC profiles revealed that caftaric, chicoric and chlorogenic acids were the most abundant antioxidants in methanolic leaf extracts of *S. oleraceus* (Table 3.4). These hydroxycinnamic acids collectively accounted for 84 – 93% of the phenolic compounds detected by HPLC-DPPH chromatograms (Table 3.4).

**Table 3.4 Retention times (RT) of phenolic compounds detected by HPLC at 320 nm and their content as a percentage of total peak area**

| Peak | Compound | RT (min)[1] | %total[2] |
|---|---|---|---|
| 1 | Caftaric acid | 11.4 ± 0.1 | 10.7 ± 1.3 |
| 2 | Chlorogenic acid | 14.4 ± 0.1 | 18.3 ± 1.6 |
| 3 | Unknown | 14.9 ± 0.1 | 0.5 ± 0.1 |
| 4 | Unknown | 18.3 ± 1.3 | 0.9 ± 0.2 |
| 5 | Unknown | 26.5 ± 0.1 | 0.2 ± 0.1 |
| 6 | Chicoric acid | 27.1 ± 0.1 | 59.7 ± 1.5 |
| 7 | Unknown | 28.4 ± 0.1 | 3.5 ± 0.2 |
| 8 | Unknown | 29.1 ± 0.1 | 1.9 ± 0.4 |
| 9 | Unknown | 31.0 ± 0.3 | 4.3 ± 0.6 |

[1] Retention time (mean ± SE, $n$ = 192)

[2] Means ± SE, $n$ = 192

### 3.5.2 Effect of age on extractable antioxidant activities, concentrations of total phenolic compounds and hydroxycinnamic acids

Of the three factors studied (age, ecotype and stressor), plant age had the largest effect on extractable antioxidant activities; a three-fold increase was seen at week 12 compared to week 10 as measured by both ORAC and DPPH assays ($P < 0.001$; Figure 3.4A,B). Plant age had the largest effect on total phenolic content; two-fold higher at week 12 compared to week 10 ($P < 0.001$; Figure 3.4C). These increases occurred irrespective of ecotype and across all treatments (Figure 3.4A-C).

The extractable concentrations of caftaric, chicoric and chlorogenic acids were 50% higher at week 12 compared to week 10 in both ecotypes irrespective of the stressor applied ($P < 0.001$; Figures 3.4D-F).

### 3.5.3 Effect of ecotype on extractable antioxidant activities, concentrations of total phenolic compounds and hydroxycinnamic acids

Differences between ecotypes were the next largest effect on ORAC activity and content of total phenolics and hydroxycinnamic acids in *S. oleraceus* leaves (Figure 3.4). For flowering individuals, the ORAC activities were between 40 and 50% higher in ACB than in OAM ($P = 0.01$; Figure 3.4A). Similarly, DPPH radical scavenging activities of ACB were 1.2-fold higher than those of OAM ($P = 0.06$; Figure 3.4B). Total phenolic contents were between 20% and 30% higher in ACB than OAM in older plants ($P < 0.001$; Figure 3.4C). In youngest plants, ACB and OAM were similar in antioxidant activities and total phenolic content (Figure 3.4A-C). ACB leaves contained 20 – 40 % more caftaric, chicoric and chlorogenic acids than OAM leaves specially when the plants were young ($P < 0.001$; Figure 3.4D-F).

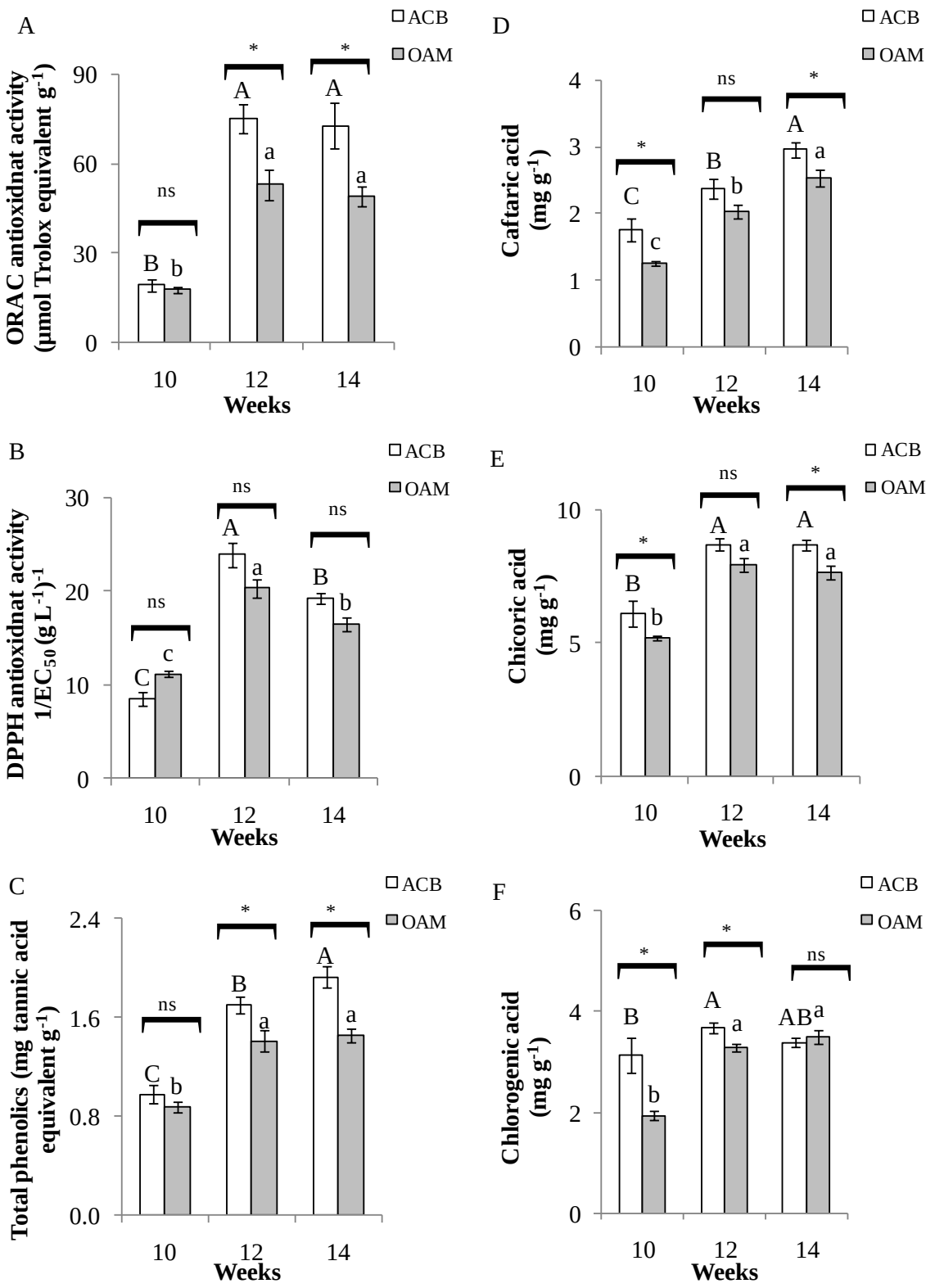



**Figure 3.4 Effects of age and ecotype on (A) ORAC activity, (B) DPPH radical scavenging, concentrations of (C) total phenolics, (D) caftaric, (E) chicoric and (F) chlorogenic acids in methanolic extracts of leaves of ACB and OAM ecotypes of *Sonchus oleraceus* L. Means ± SE (*n* = *32*). Bars with different letters of the same case indicate significant differences among plant ages within an ecotype (*P* < 0.05). *Significant difference between ecotypes (*P* < 0.05). ns: Ecotypes not significantly different (P > 0.05).**

The plants were vegetative at week 10, but were flowering in weeks 12 and 14 (Plate 3.2 A-C).

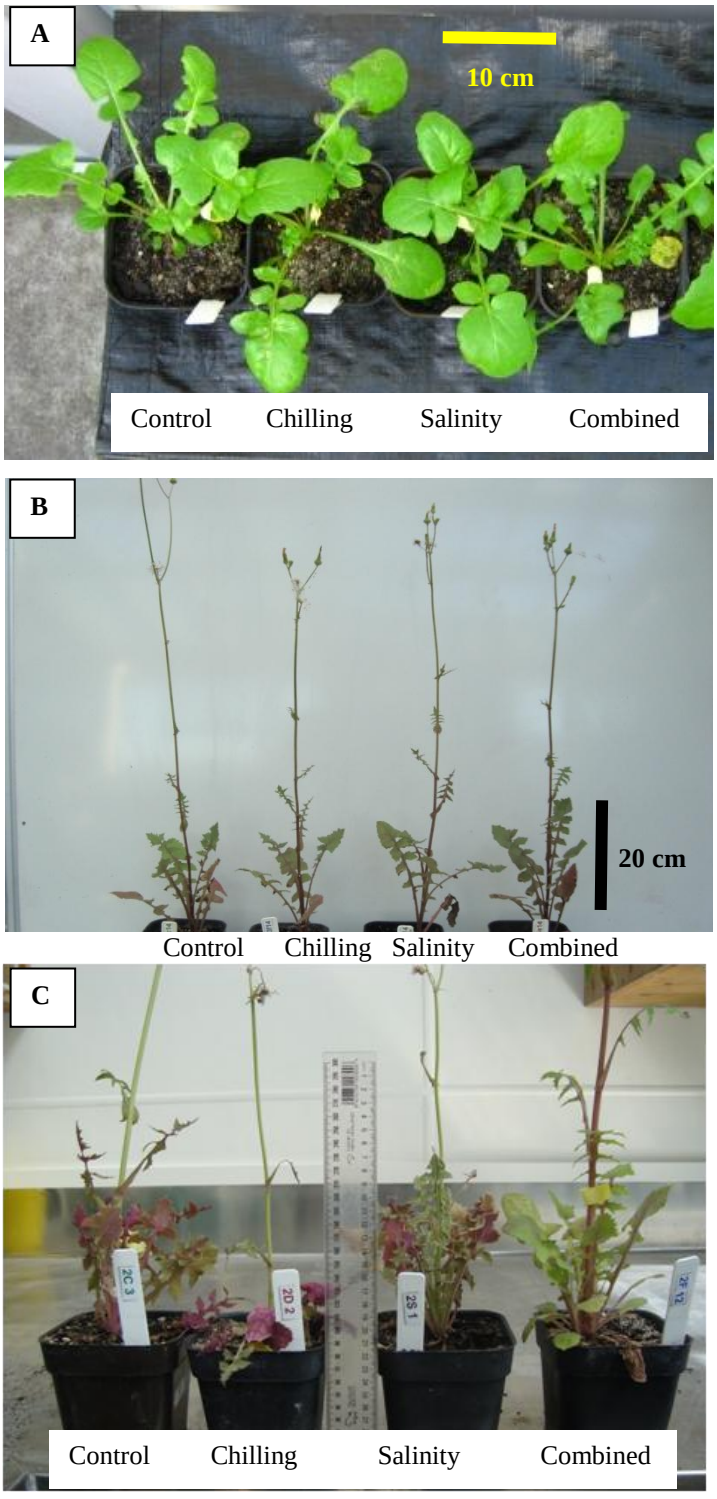



**Plate 3.2 Shoot morphology of *Sonchus oleraceus* L. at week (A) 10, (B) 12 and (C) 14, following two weeks of chilling, salinity and the combination of the two. Representative plants for each treatment are shown.**

### 3.5.4 Effects of stressors on extractable antioxidant activities, concentrations of total phenolic compounds and hydroxycinnamic acids

ORAC activities were not influenced by any of the stressor treatments ($P = 0.72$; Figure 3.5A) but DPPH radical scavenging activities were ($P < 0.05$; Figure 3.5B). Plants treated with chilling and salinity alone did not differ from the control plants in DPPH radical scavenging. However, when chilling and salinity were applied in combination to flowering plants, the DPPH radicals were scavenged 20% less efficiently compared to the control in both ecotypes (Figure 3.5B).

Concentrations of total phenolics were not affected by chilling and salinity alone compared to the control, across ecotypes and plant ages ($P = 0.01$; Figure 3.5C). However when chilling and salinity were applied in combination to older plants, the concentration of total phenolics was enhanced compared to the control in both ecotypes (Figure 3.5C).

Concentrations of caftaric ($P = 0.92$) and chicoric acids ($P = 0.47$) were not influenced by any of the treatments (Figure 3.6A,B). Stressors significantly increased chlorogenic acid concentration ($P < 0.001$; Figure 3.6C). Salinity and combined treatment doubled chlorogenic acid concentration compared to the control in both ecotypes (Figure 3.6C). Chilling increased chlorogenic acid concentration by three-fold in both ecotypes, but only in vegetative plants (Figure 3.6C). HPLC profiles of leaf methanolic extracts did not reveal any new peaks resulting from the stressor treatment, indicating that no new compounds had been formed (Figure 3.7).

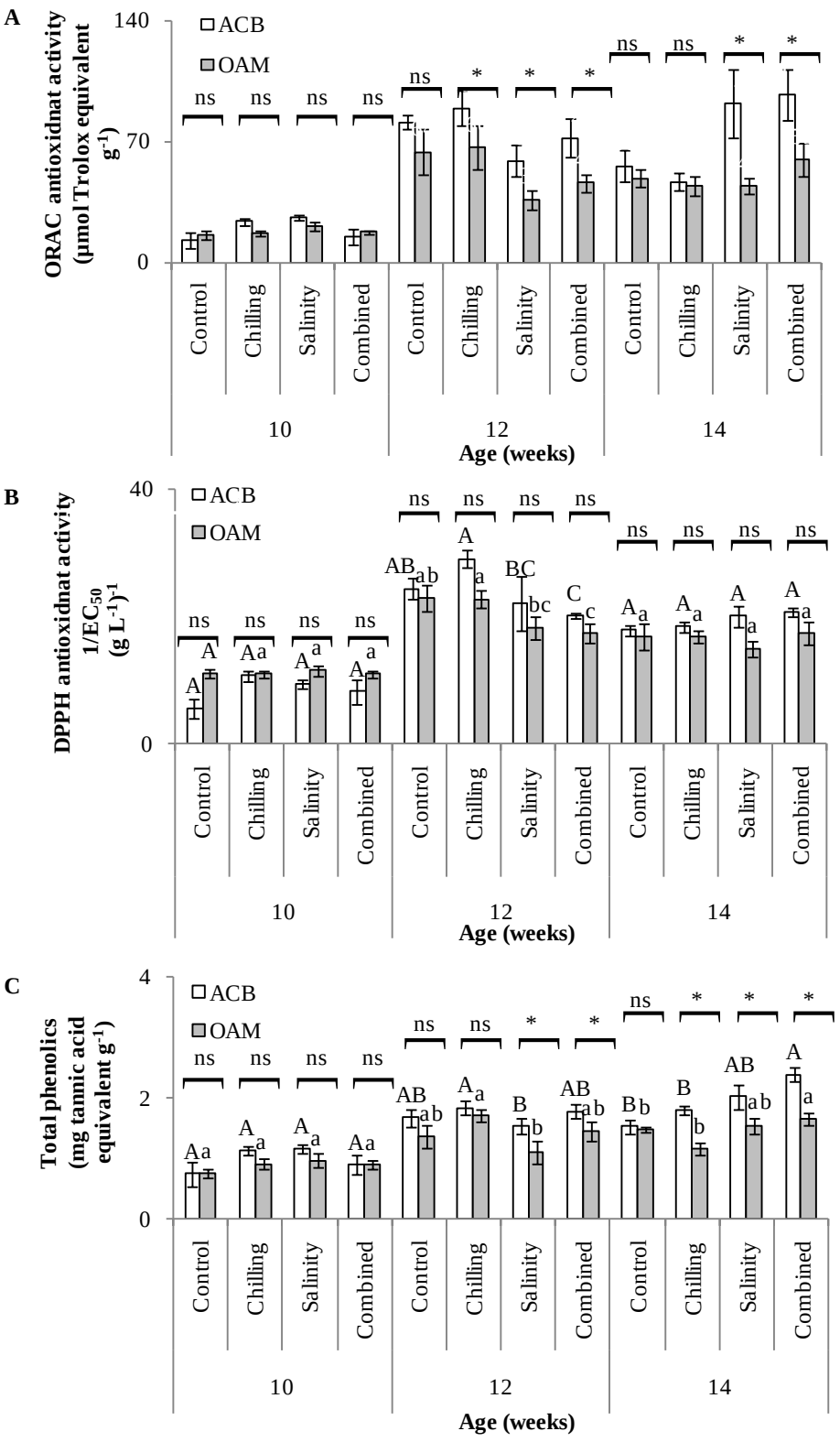



**Figure 3.5 Treatment effects on (A) ORAC, (B) DPPH radical scavenging and (C) concentration of total phenolic compounds in methanolic extracts of leaves of ACB and OAM ecotypes of *Sonchus oleraceus* L. at week 10, 12 and 14, which had been exposed to treatments for two weeks: control, chilling, salinity and the combination of the two. Means ± SE (*n* = *8*). Bars with different letters of the same case indicate significant differences across treatments within an ecotype at each week (*P* < 0.05). *Significant difference between ecotypes (*P* < 0.05). ns: Ecotypes not significantly different (P > 0.05).**

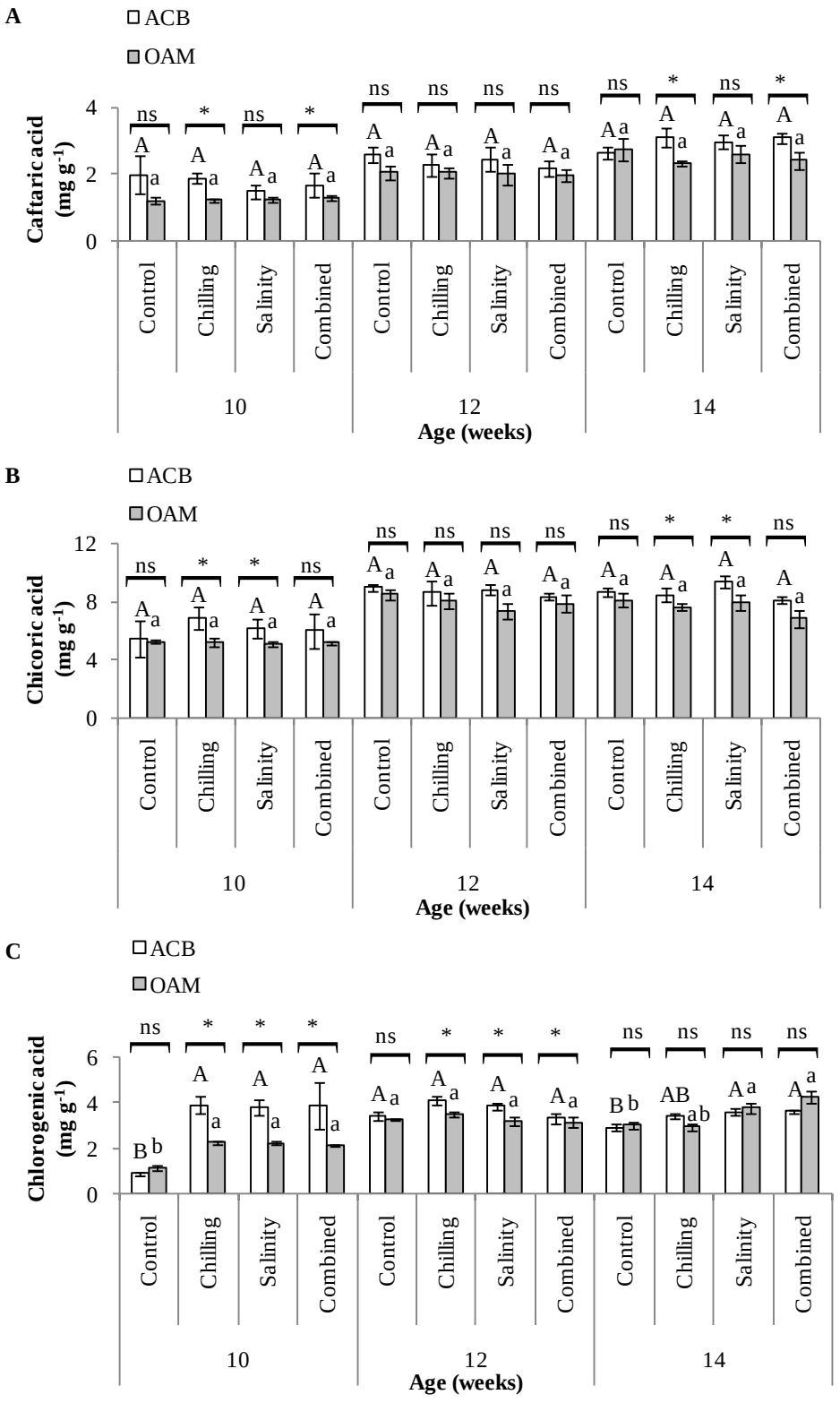



**Figure 3.6 Treatment effects on (A) caftaric (B) chicoric and (C) chlorogenic acid concentrations in methanolic extracts of leaves of ACB and OAM ecotypes of *Sonchus oleraceus* L. at week 10, 12 and 14, which had been exposed to treatments for two weeks: control, chilling, salinity and the combination of the two. Means ± SE (*n = 8*). Bars with different letters of the same case indicate significant differences across treatments within an ecotype at each week (*P* < 0.05). *Significant difference between ecotypes (*P* < 0.05). ns: Ecotypes not significantly different (P > 0.05).**

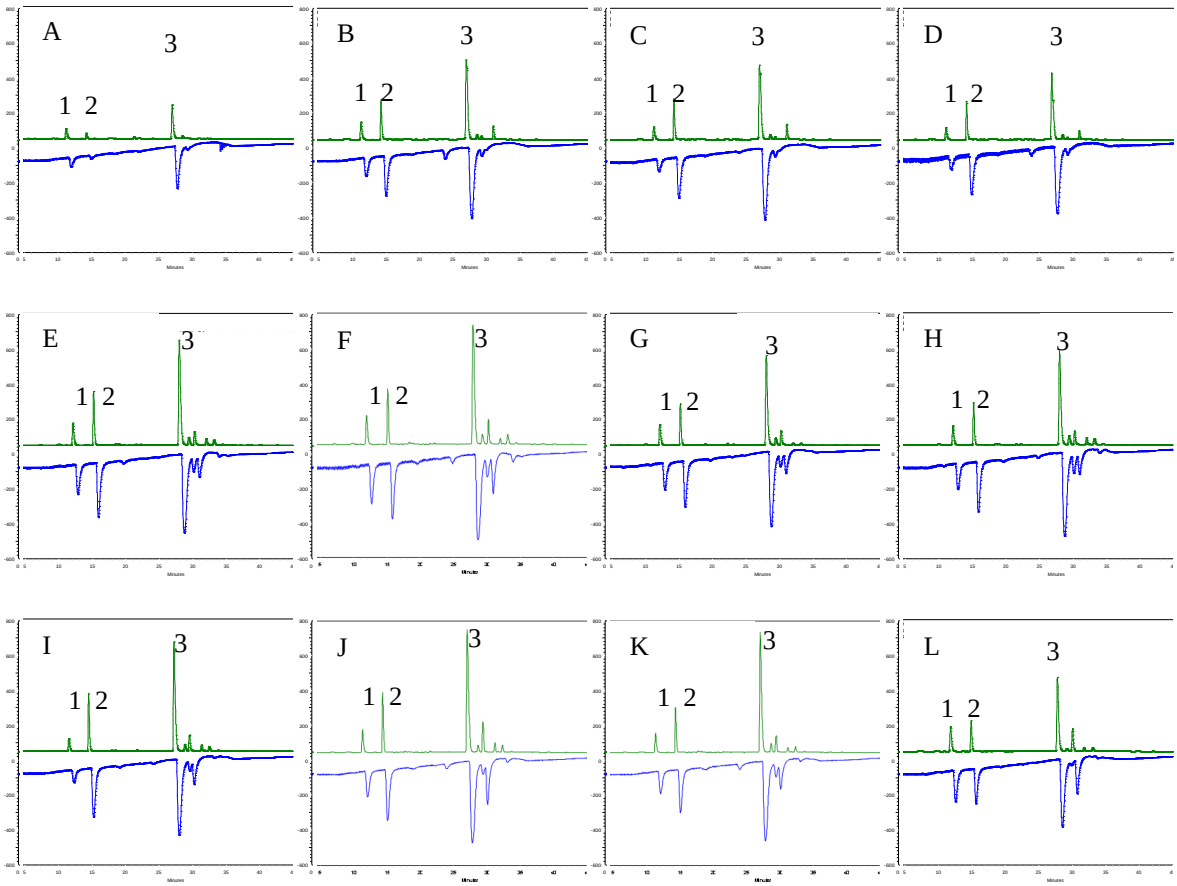



**Figure 3.7 RP-HPLC-DPPH chromatograms recorded for ACB methanolic extracts of leaves of *Sonchus oleraceus* L. at week 10 (A-D), 12 (E-H), and 14 (I-L), which had been exposed to treatments for two weeks: control (A,E,I), chilling (B,F,J), salinity (C,G,K) and the combination of the two (D,H,L). Phenolics (green line) measured at 320 nm; DPPH radical scavenging (blue line) at 518 nm. Peaks: 1, caftaric acid, 2, chlorogenic acid and 3, chicoric acid.**

### 3.5.5 Effects of age, ecotype and stressors on ascorbate concentration

Plant age had the largest effect on ascorbate concentration; a three-fold increase was observed at week 12 compared to week 10 in both ecotypes ($P < 0.001$; Figure 3.8A). The ACB had 30 – 60% higher leaf ascorbate concentration than OAM ($P < 0.001$; Figure 3.8). Stressors did not significantly affect leaf ascorbate level concentrations ($P = 0.65$; Figure 3.8).

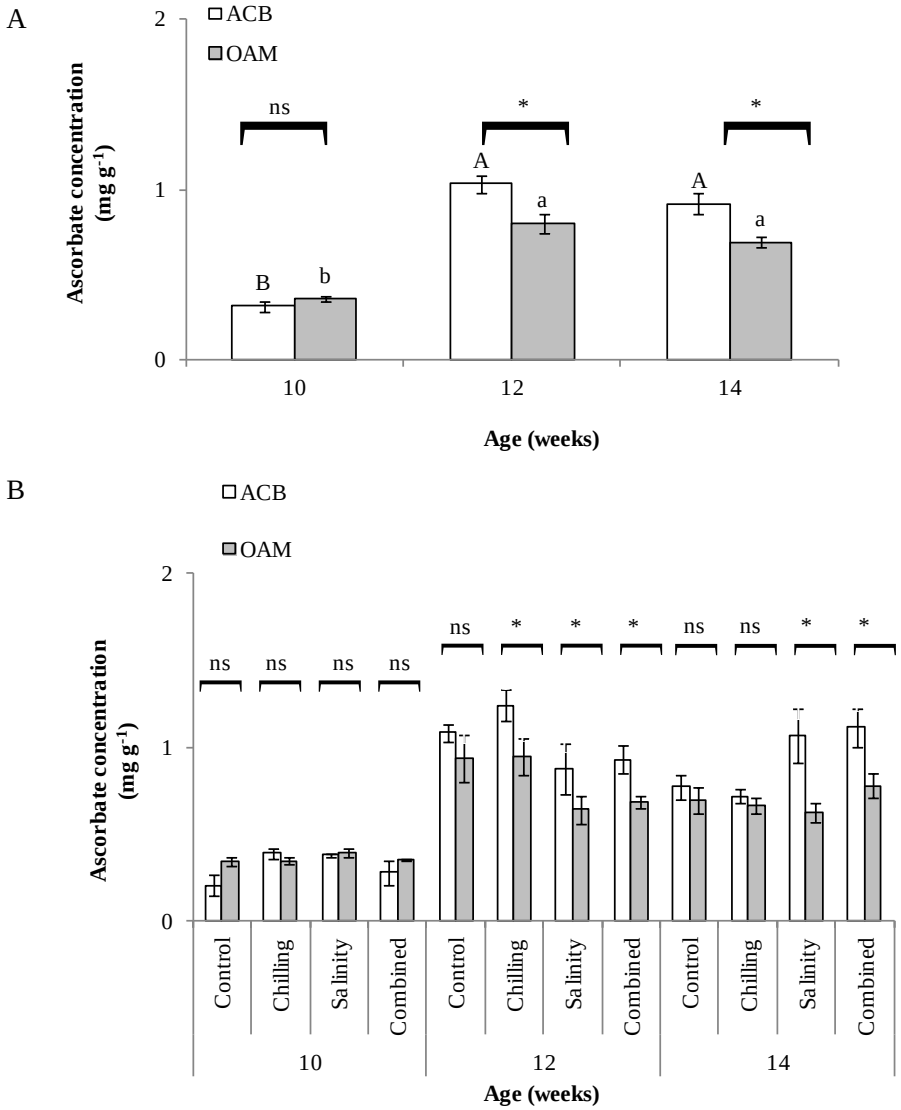



**Figure 3.8 Concentration of ascorbate in methanolic extracts of leaves of ACB and OAM ecotypes of *Sonchus oleraceus* L. with (A) plant age and (B) stressors at week 10, 12 and 14, which had been exposed to treatments for two weeks: control, chilling, salinity and the combination of the two. Means ± SE (*n = 32* and *n = 8*). Bars with different letters of same case indicate significant differences within an ecotype ($P < 0.05$).*Significant difference between ecotypes ($P < 0.05$). ns: Ecotypes not significantly different ($P > 0.05$). None of the treatments was statistically significant ($P > 0.05$).**

### 3.5.6 Effects of stressors on shoot, root and total dry biomass of plants.

None of the stressors significantly influenced the dry biomass of roots ($P$ = 0.86; Figure 3.9A), shoots ($P$ = 0.15; Figure 3.9B) or total plant ($P$ = 0.86; Figure 3.9C). However stressors created differences in plant morphology. Older plants subjected to stressors were shorter, had broader stems and more red leaves compared to the controls (Plate 3.2 B-C).

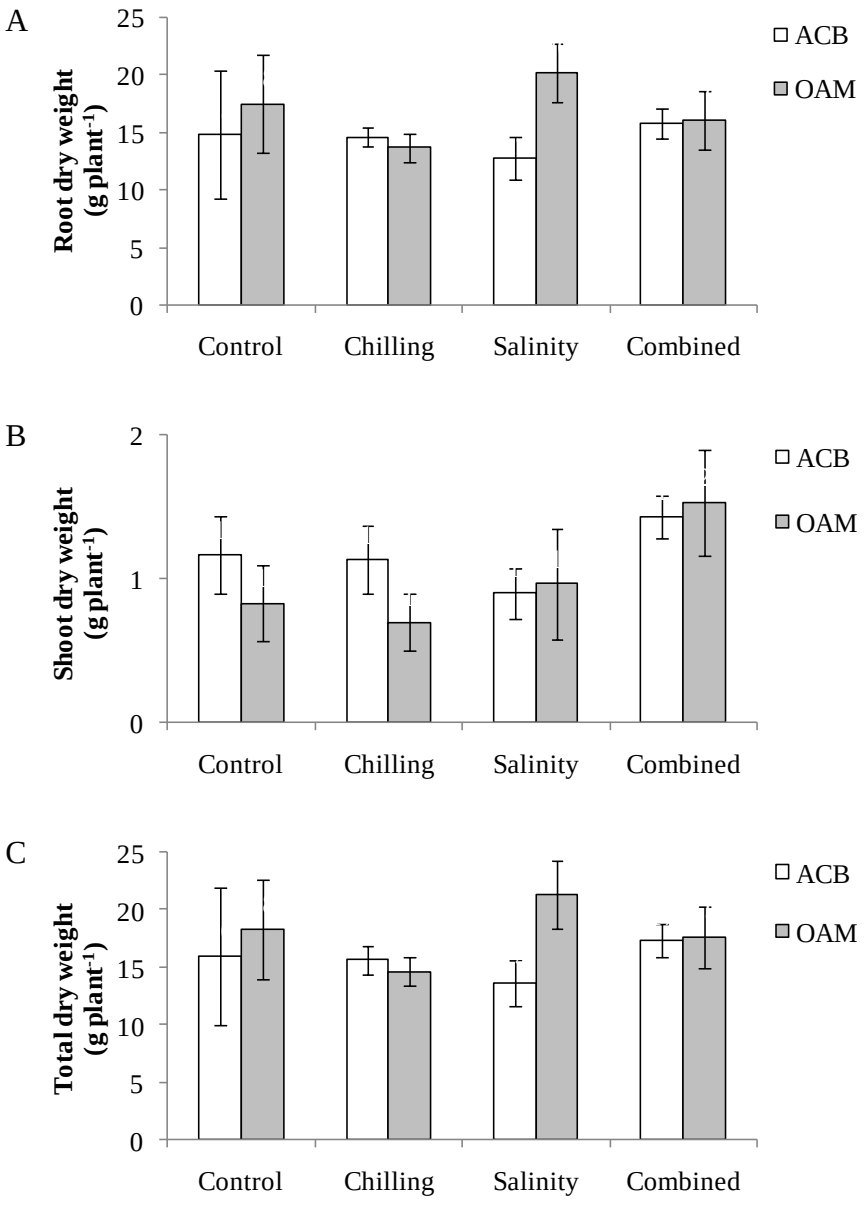



**Figure 3.9 Treatment effects on (A) root, (B) shoot and (C) total dry biomass of ACB and OAM ecotypes of *Sonchus oleraceus* L. at week 14. Means ± SE (*n* = *8*). None of the treatments significantly altered the dry biomass (*P* < 0.05).**

### 3.5.7 Chlorophyll fluorescence measurements

3.5.7.1 Maximum efficiency of photosystem II

Maximum efficiency of photosystem II ($F_v/F_m$) values of leaves averaged 0.81 ± 0.00 for all data and none was lower than 0.79 ± 0.00 (Figure 3.10).

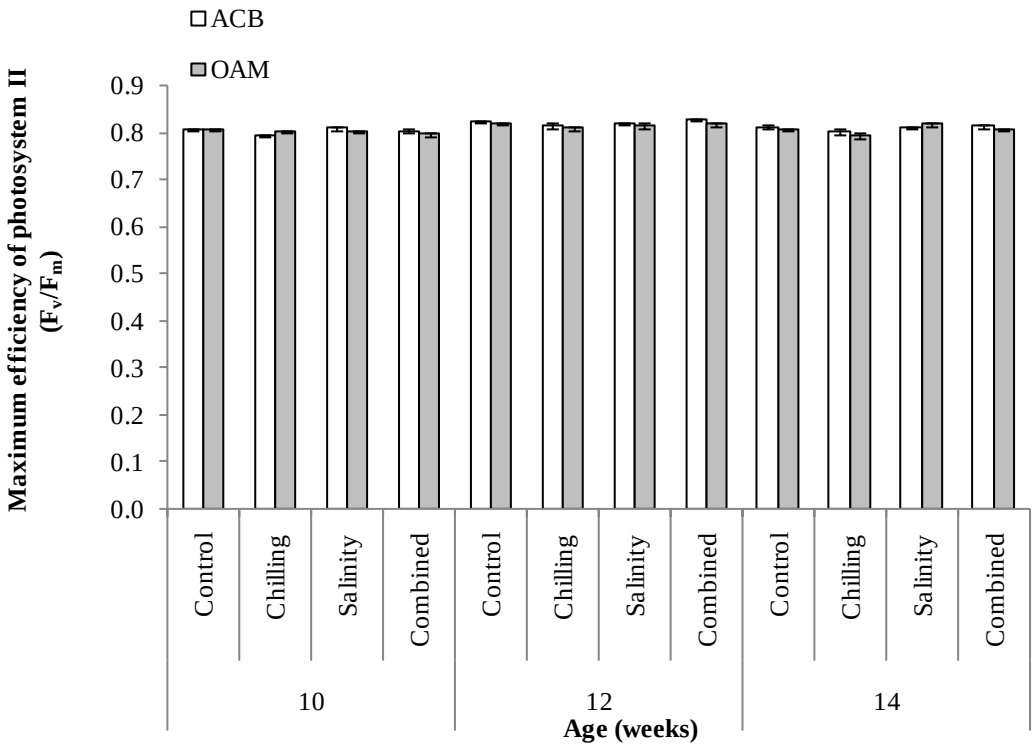



**Figure 3.10 Treatment effects on maximum efficiency of photosystem II ($F_v/F_m$) of leaves of ACB and OAM ecotypes of *Sonchus oleraceus* L. at week 10, 12 and 14, which had been exposed to treatments for two weeks: control, chilling, salinity and the combination of the two. Means ± SE (*n* = *8*). None of the treatments was statistically significant (*P* > 0.05).**

3.5.7.2 Light response curves for chlorophyll fluorescence

Ecotypes did not significantly influence the parameters $\Delta F_v/F'_m$, NPQ, $q_P$ and ETR as derived using rapid light response curves ($P > 0.5$; Figures 3.11 – 3.13).

**Light response curves for $\Delta F/F'_m$**: There were only small effects of stressors on the effective quantum yields of the plants, which became less pronounced as plants aged (*P* =

0.002; Figures 3.11A,B – 3.13A,B). The rate of decline of $\Delta F/F'_m$ with increasing light intensity was lower for plants subjected to stressors than for the respective controls in both ecotypes (Figure 3.11A,B).

**Light response curves for NPQ:** NPQ values were highest in flowering plants across ecotypes and all stressors ($P$ = 0.01; Figures 3.11C,D - 3.13C,D). NPQ curves did not differ significantly among stressors ($P$ = 0.11; Figures 3.11C,D - 3.13C,D).

**Light response curves for $q_P$:** For both ecotypes, the impact of stressors was greatest on the youngest plants, and became less prominent as plants aged ($P$ < 0.001; Figures 3.11E,F – 3.13E,F). Rate of decrease in qp with increasing light intensity was lower for stressor-imposed plants than the control in both ecotypes (Figures 3.11E,F – 3.12E,F). However, these effects due to stressors varied between ecotypes.

**Light response curves for ETR:** Influences of stressors on light response curves for ETR were more pronounced in younger than in older plants; in the oldest plants none of the stressors had any impact in both ecotypes ($P$ < 0.001; Figures 3.11G,H - 3.13G,H). Rates of increase in ETR with light were greater for stressor treated plants than for controls (Figure 3.11G,H). As plants aged, the rate of increase in ETR with increasing light diminished across both ecotypes and among all stressors (Figures 3.11G,H - 3.13G,H).

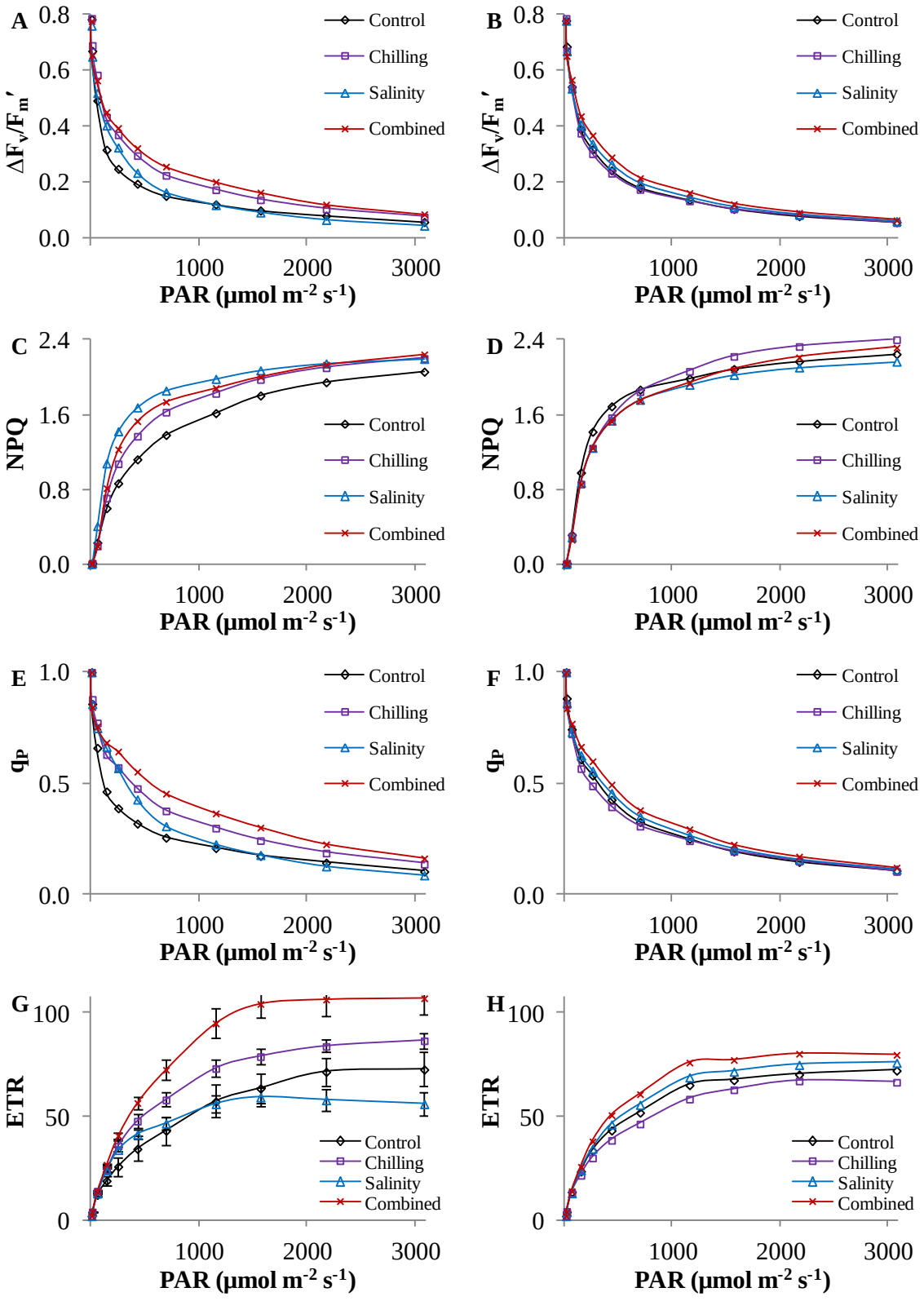



**Figure 3.11 Light response curves for quantum yield of PSII ($\Delta F/F'_m$; A,B), nonphotochemical quenching (NPQ; C,D) and photochemical quenching ($q_P$; E,F) and apparent electron transport rate (ETR; G,H) in leaves of ACB (A,C,E,G) and OAM (B,D,F,H) ecotypes of *Sonchus oleraceus* L. at week 10, which had been exposed to treatments for two weeks: control, chilling, salinity and the combination of the two. Means ± SE (*n* = 8).**

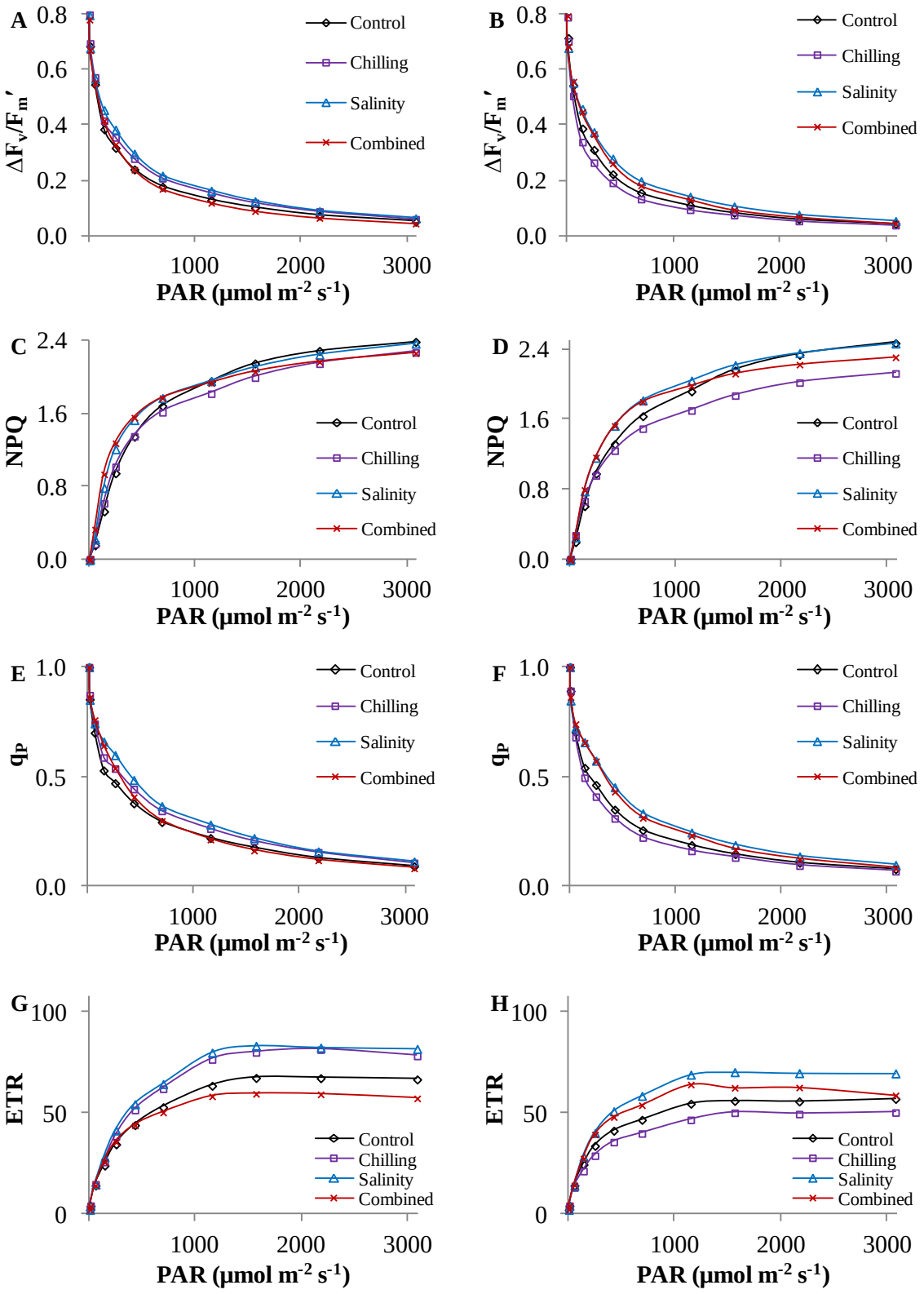



**Figure 3.12 Light response curves for quantum yield of PSII ($\Delta F/F'_m$; A,B), nonphotochemical quenching (NPQ; C,D) and photochemical quenching ($q_P$; E,F) and apparent electron transport rate (ETR; G,H) in leaves of ACB (A,C,E,G) and OAM (B,D,F,H) ecotypes of *Sonchus oleraceus* L. at week 12, which had been exposed to treatments for two weeks: control, chilling, salinity and the combination of the two. Means ± SE (*n* = 8).**

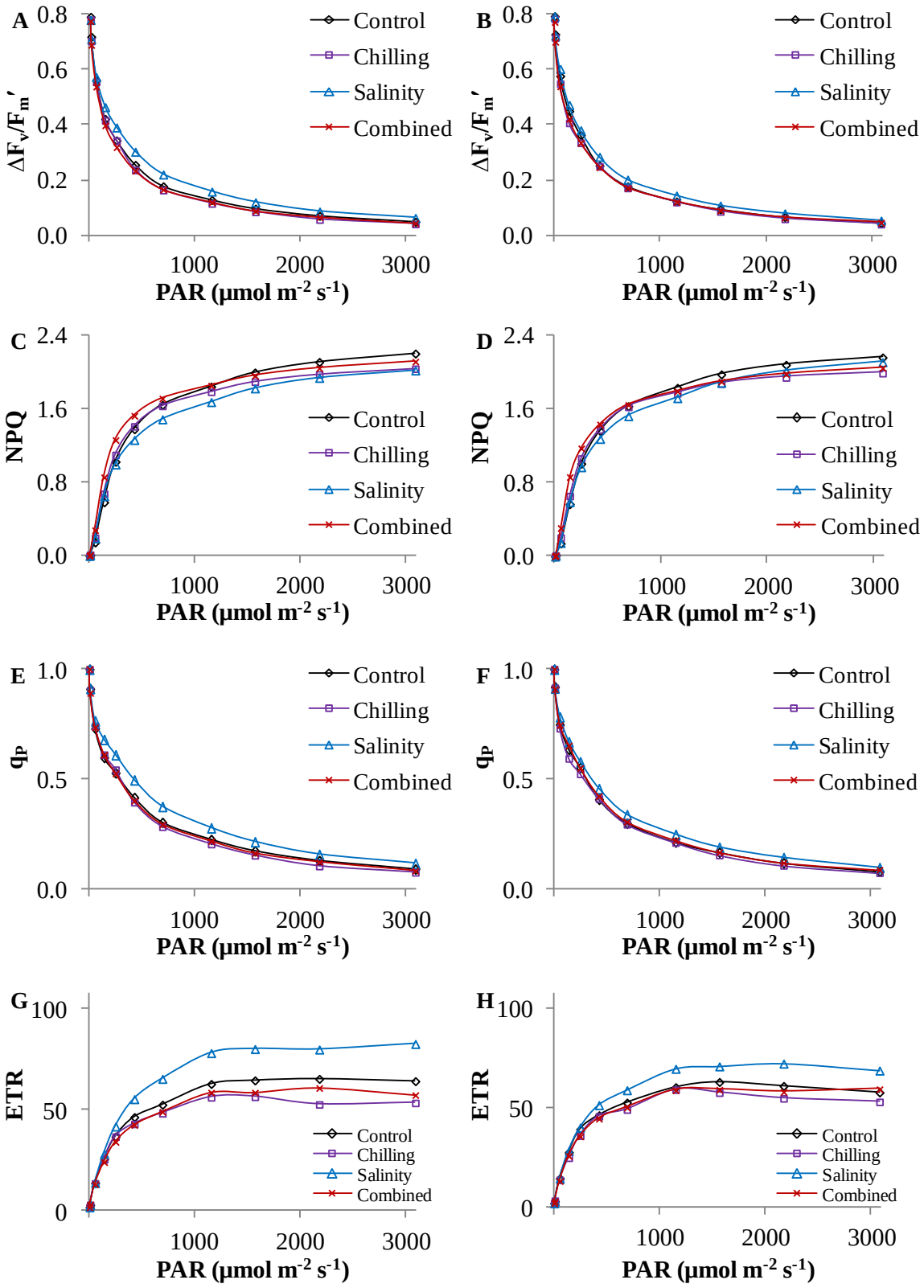



**Figure 3.13 Light response curves for quantum yield of PSII ($\Delta F/F'_m$; A,B), nonphotochemical quenching (NPQ; C,D) and photochemical quenching ($q_P$; E,F) and apparent electron transport rate (ETR; G,H) in leaves of ACB (A,C,E,G) and OAM (B,D,F,H) ecotypes of *Sonchus oleraceus* L. at week 14, which had been exposed to treatments for two weeks: control, chilling, salinity and the combination of the two. Means ± SE (*n* = 8).**

3.5.7.3 The parameters of light response curves for ETR

**Initial slope of light response curves for ETR (α)**: α was higher in older plants than young plants irrespective of ecotype or stressor ($P < 0.001$; Figure 3.14A). α between ecotypes were not different ($P = 0.94$; Figure 3.14). Stressors had variable impacts on α between ecotypes ($P < 0.01$). Chilling and the stressor combination on ACB resulted in higher α than control plants irrespective of their age (Figure 3.14B). Stressors did not significantly alter α in OAM (Figure 3.14B).

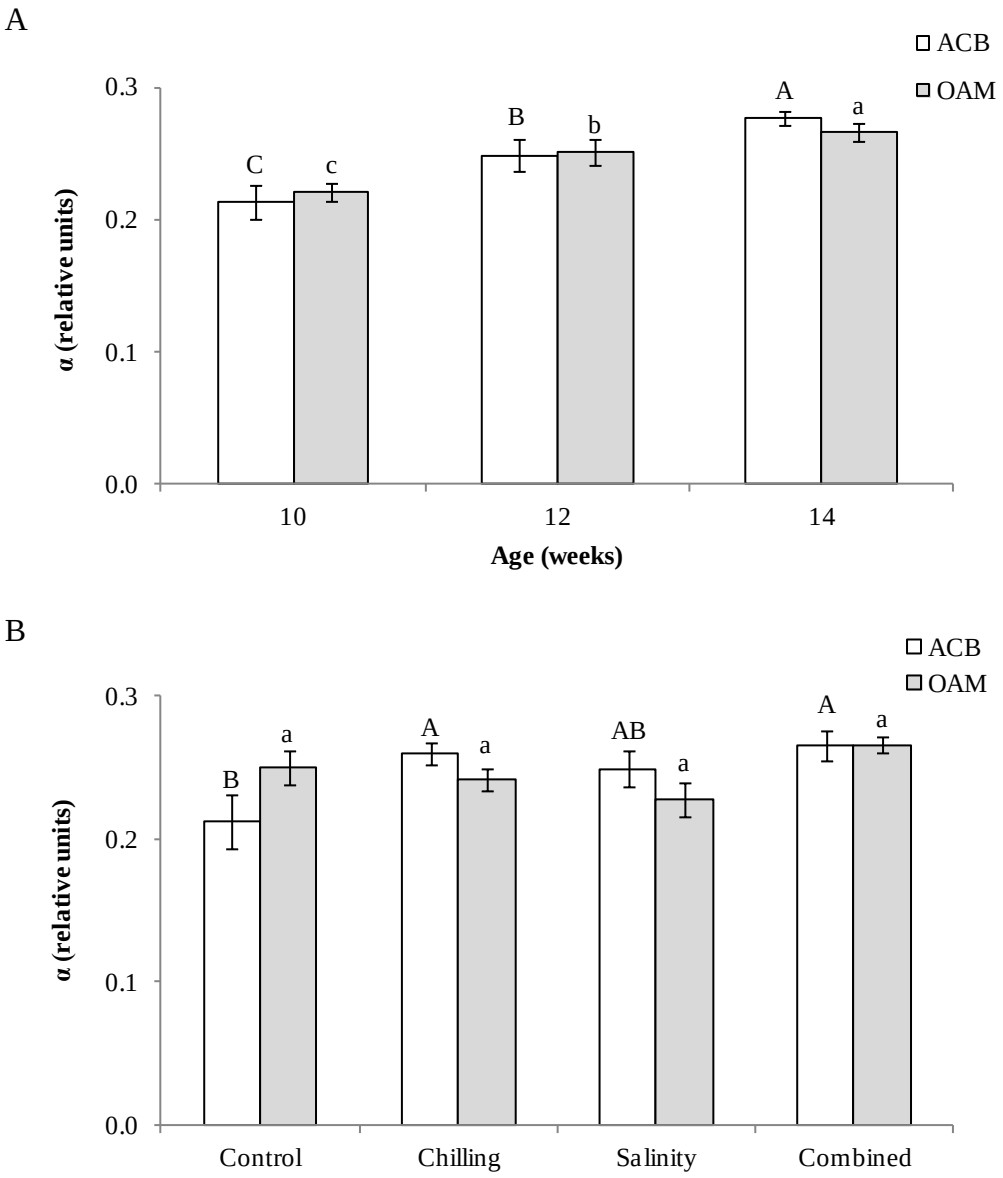



**Figure 3.14 Initial slope of light response curves for ETR (α) in leaves of ACB and OAM ecotypes of *Sonchus oleraceus* L. with (A) plant age and (B) stressors. Means ± SE ($n = 32$ and $n = 24$). Bars with different letters of same case indicate significant differences within an ecotype ($P < 0.05$).**

**Maximum electron transport rate ($ETR_{max}$)**: The $ETR_{max}$ of OAM diminished as plants aged for all the treatments, but not in ACB ($P < 0.001$; Figure 3.15A). The $ETR_{max}$ were not different between ecotypes ($P = 0.32$; Figure 3.15A). Stressors had variable impacts on $ETR_{max}$ between ecotypes ($P < 0.001$). Salinity treated OAM plants had lower $ETR_{max}$ than control, while in ACB stressor treated plants were not significantly different from the control (Figure 3.15B).

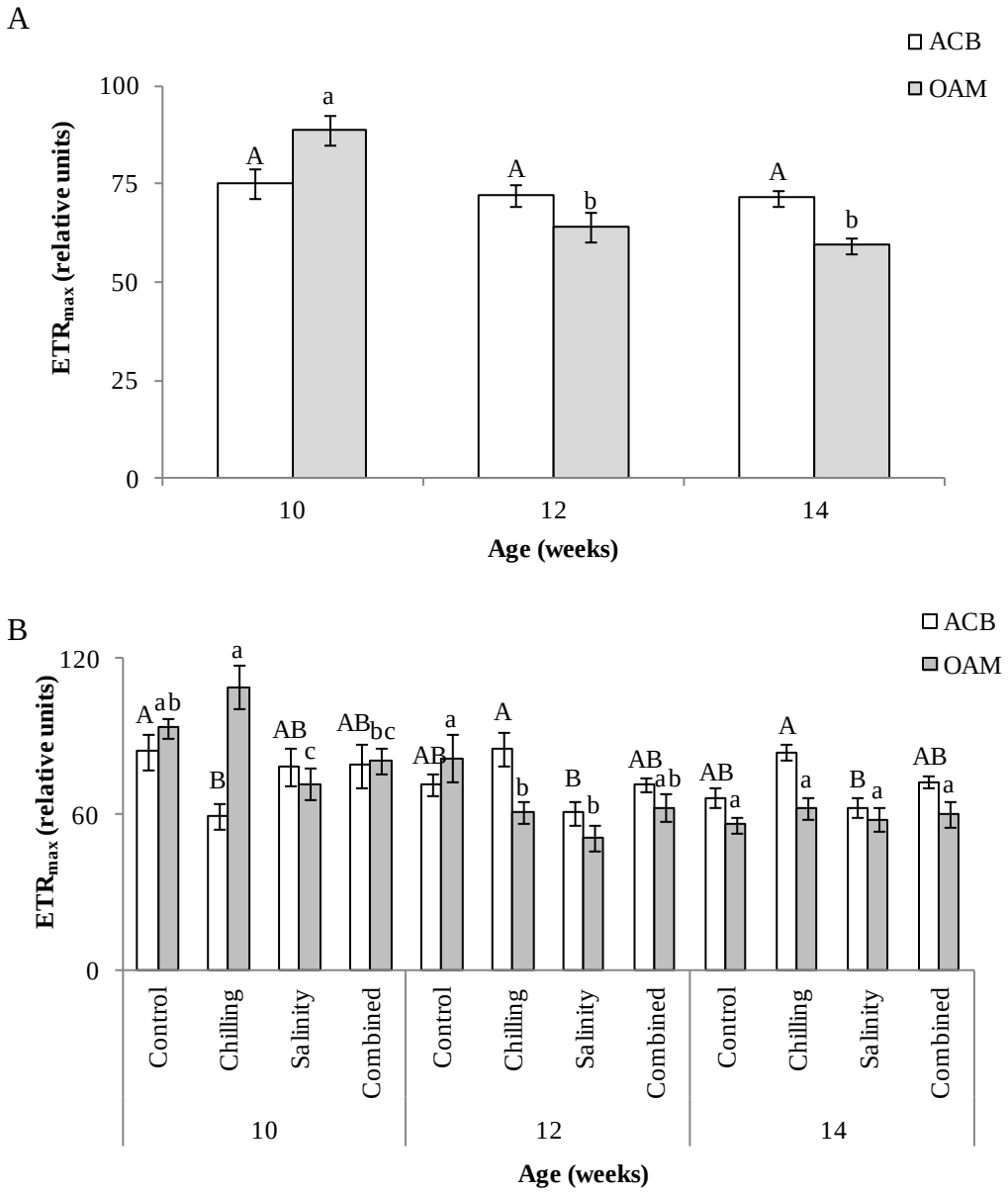



**Figure 3.15 Maximum electron transport rate ($ETR_{max}$) in leaves of ACB and OAM ecotypes of *Sonchus oleraceus* L. with (A) plant age and (B) stressors at week 10, 12 and 14, which had been exposed to treatments for two weeks: control, chilling, salinity and the combination of the two. Means ± SE ($n = 32$ and $n = 8$). Bars with different letters of same case indicate significant differences within an ecotype at each week ($P < 0.05$).**

**Minimum saturating irradiance ($l_k$)**: The $l_k$ declined as plants aged ($P < 0.001$; Figure 3.16A). The $l_k$ for two ecotypes differed in young plants ($P = 0.04$; Figure 3.16B). The $l_k$ of young ACB control plants was three-fold higher than that of the average recorded for rest of the data ($P < 0.001$; Figure 3.16B).

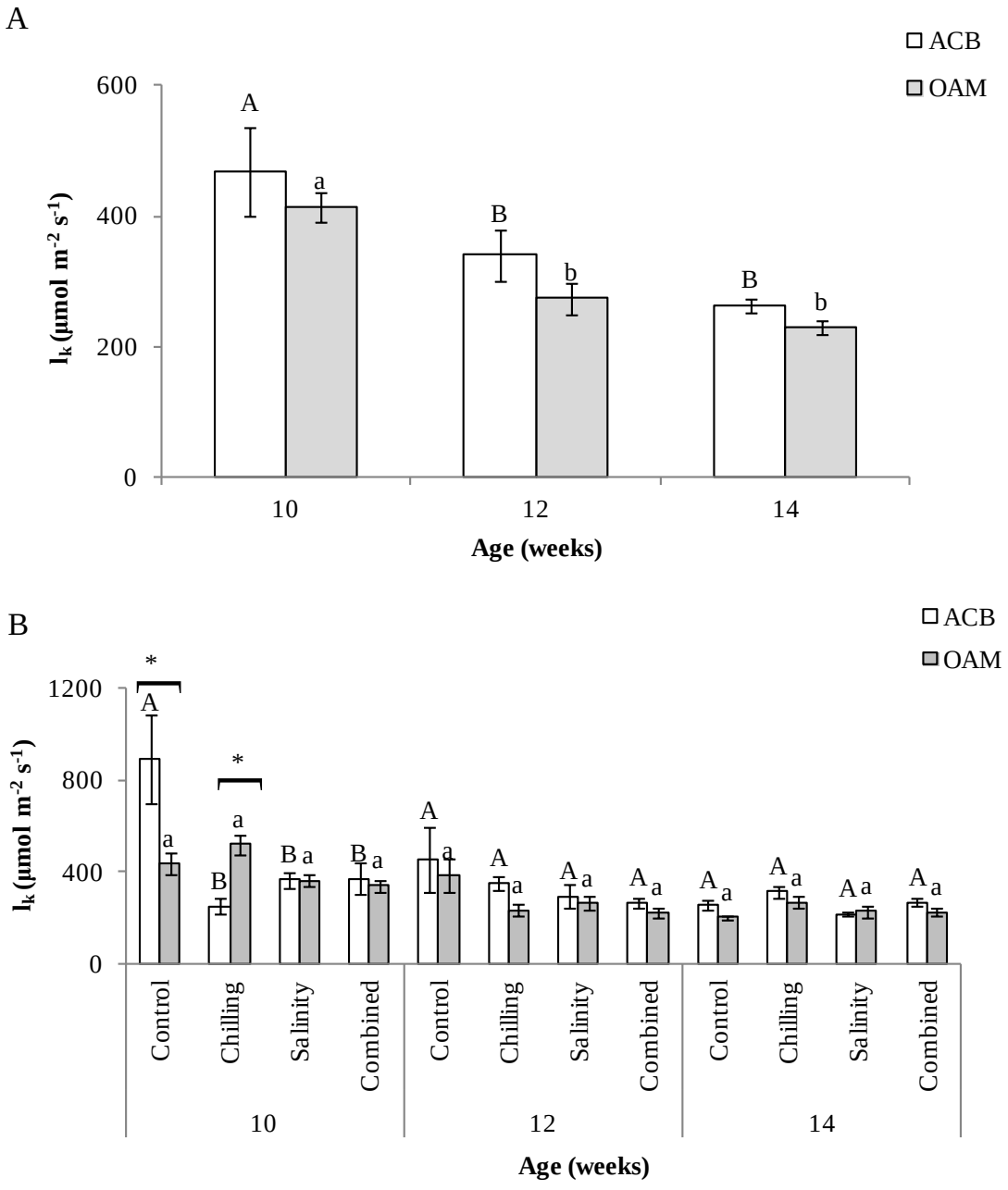



**Figure 3.16 Minimum saturating irradiance ($l_k$) in leaves of ACB and OAM ecotypes of *Sonchus oleraceus* L. with (A) plant age and (B) stressors at week 10, 12 and 14, which had been exposed to treatments for two weeks: control, chilling, salinity and the combination of the two. Means ± SE ($n$ = 32 and $n$ = 8). Bars with different letters of same case indicate significant differences within an ecotype at each week ($P < 0.05$). *Significant differences between ecotypes ($P < 0.05$).**

### 3.5.8 The stressor-induced variations in extractable antioxidant activities in leaves correlated with human cellular antioxidant activities

In order to see whether the differences in phenolic composition after stressors translated into antioxidant advantages inside human cells, HepG2 cells were infused with leaf extracts from youngest *S. oleraceus* plants that had been exposed to treatments for two weeks. Significant linear correlations between the CAA values in HepG2 cells and leaf extracted antioxidant activities and concentrations of total phenolics existed (Figure 3.17). Antioxidant activities measured by the ORAC assay better correlated with CAA values (Figure 3.17A) than did those obtained by the DPPH technique (Figure 3.17B). Concentrations of chlorogenic acid, chicoric acid and ascorbate of leaf methanolic extracts significantly linearly correlated with CAA values (Figure 3.18A-C). However, concentrations of caftaric acid did not significantly correlate with CAA (Figure 3.18D).

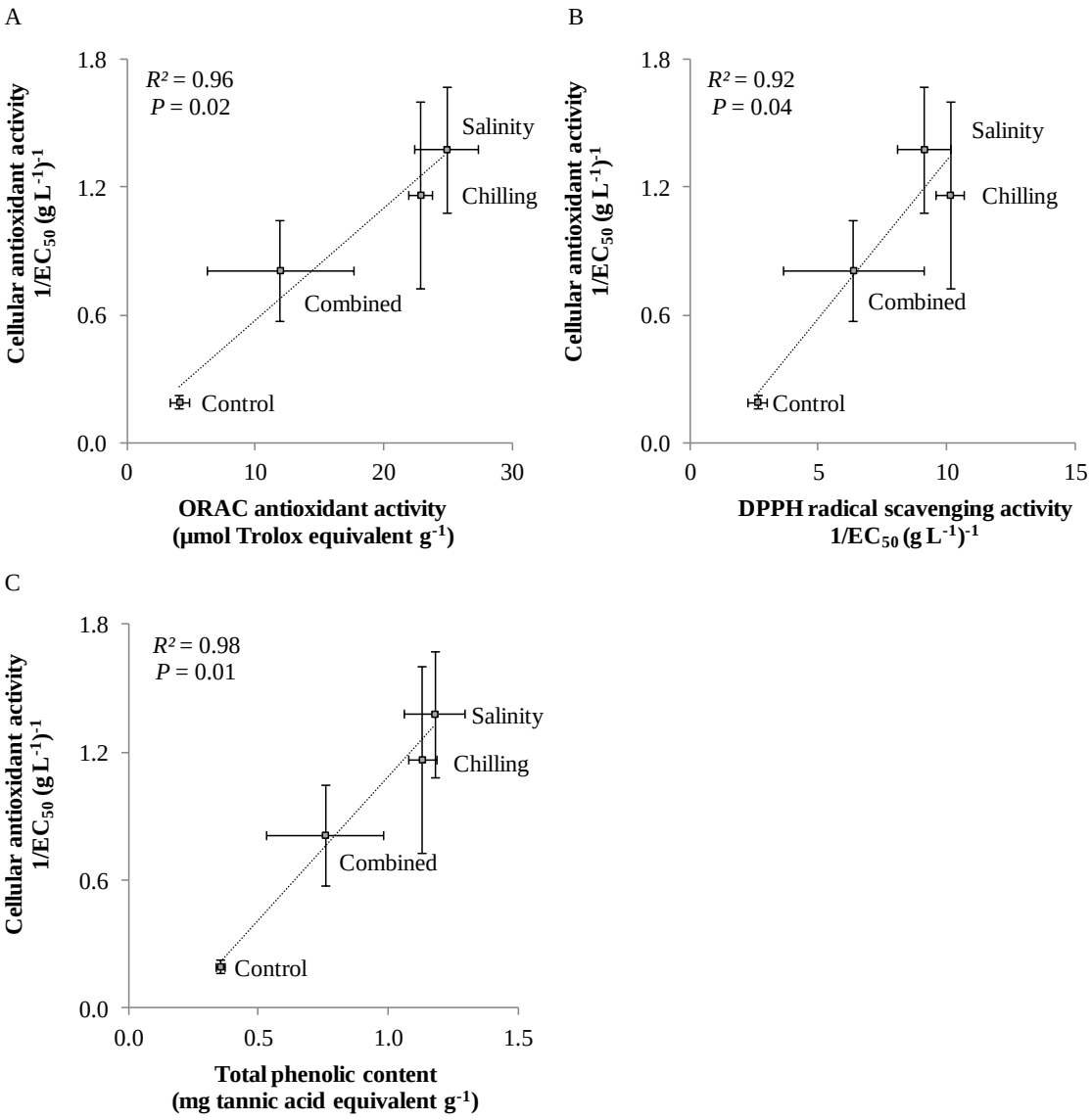



**Figure 3.17 Linear correlations between cellular antioxidant activities inside HepG2 cells infused with leaf extracts of ACB ecotype of *Sonchus oleraceus* L. at week 10, which had been exposed to two weeks of chilling, salinity and the combination of the two, with the extractable antioxidant activities measured by (A) ORAC and (B) DPPH techniques and (C) the concentration of total phenolic compounds. Means ± SE (*n* = 5).**

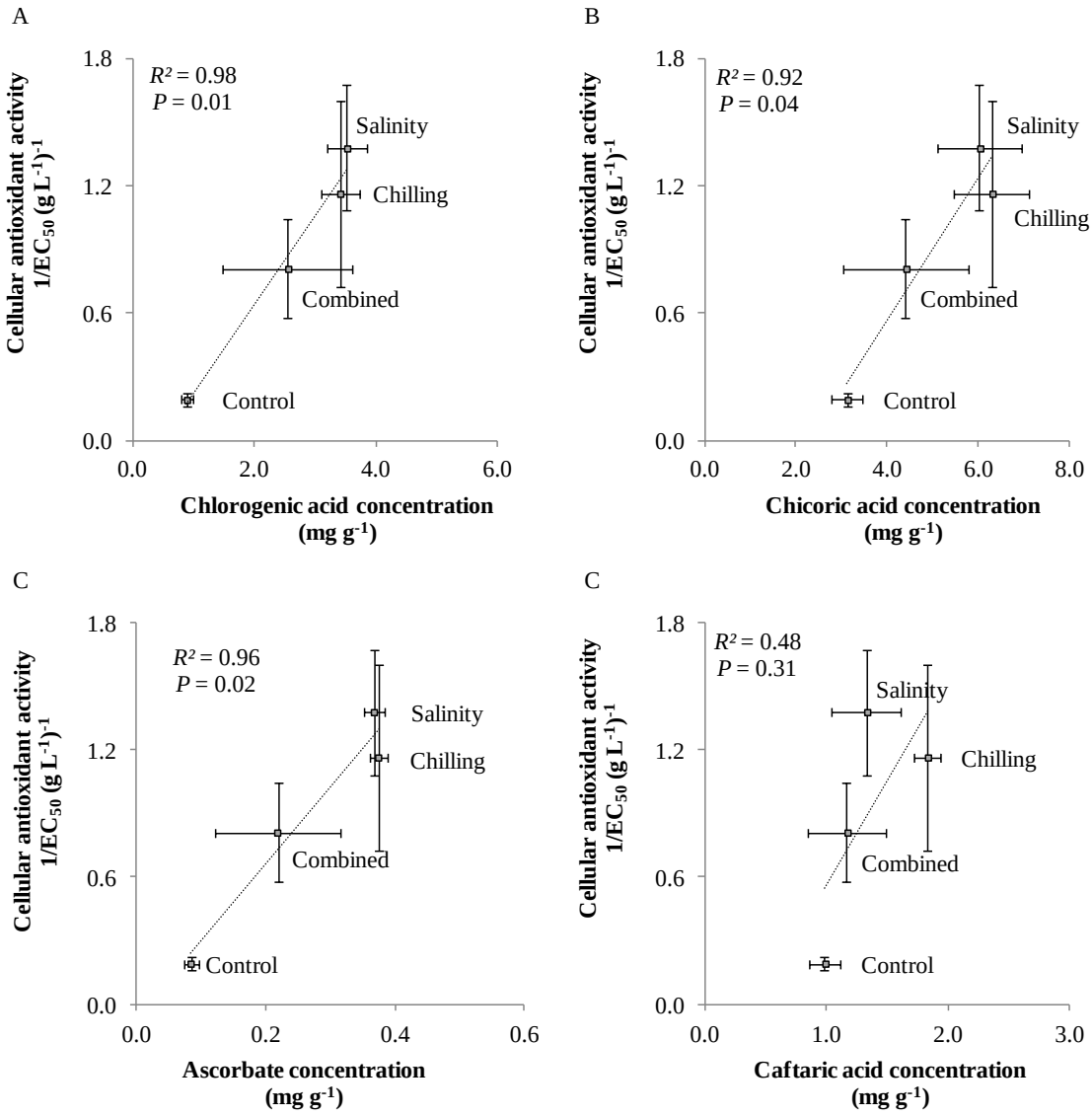



**Figure 3.18 Linear correlations between cellular antioxidant activities inside HepG2 cells infused with leaf extracts of ACB ecotype of *Sonchus oleraceus* L. at week 10, which had been exposed to two weeks of chilling, salinity and the combination of the two, with the concentrations of: (A) chlorogenic acid, (B) chicoric acid, (C) ascorbate and (D) caftaric acid. Means ± SE (*n* = 5).**

## 3.6 DISCUSSION

My study has confirmed that differences among plant age, ecotypes and growing conditions can lead to variation in extractable antioxidant activities of *S. oleraceus*. Of the three factors, plant maturation had the largest effect on content of hydroxycinnamic acids, total phenolics and on antioxidant activities, followed by ecotype, while stressors had minor effects. Older plants were richer in ascorbate, hydroxycinnamic acids and had higher antioxidant activities. Of the two ecotypes tested, ACB had the stronger antioxidant potential than OAM and was richer in ascorbate and phenolic acid concentration. Applications of chilling, salinity and their combination significantly increased concentrations of chlorogenic acid in leaves of young plants from both ecotypes. Furthermore this study confirmed that the concentration of chlorogenic acid, chicoric acid, total phenolics and chemical estimates of antioxidant capacity of the leaf extracts from the youngest plants, correlate well with cellular measures of antioxidant activities within cultured human HepG2 cells. These results indicate that it is indeed possible to manipulate antioxidant levels in *S. oleraceus* through the judicious use of high yielding ecotypes, the harvesting of mature plants, and the imposition of moderate stressors. Moreover, the data suggest that improvements in the antioxidant status of leaf cells may translate to higher antioxidant protection in cultured human cells.

The transition from vegetative to reproductive growth was associated with an increase in the leaf accumulated ascorbate, hydroxycinnamic acids and other phenolic compounds, and antioxidant activities. Such increases occurred irrespective of the treatment applied to the plants and the ecotype (Figures 3.4 and 3.8A). This may be explained by accelerated secondary metabolism during reproduction, which is often accompanied by an enhanced production of phenylpropanoid compounds (Acamovic and Brooker, 2005; Papageorgiou *et al.*, 2008; Franz *et al.*, 2011). In other species, too, phenolic compounds have been shown to increase to their maximum levels at flowering; for example, chlorogenic acid in *Hypericum origanifolium* (Çirak *et al.*, 2007), total phenolic content in *Mentha pulegium* (Karray-Bouraoui *et al.*, 2010), rutin, quercetin, isoquercetin, hypericin, and hyperforin in various *Hypericum* spp (Abreu *et al.*, 2004; Ayan *et al.*, 2006; Çirak *et al.*, 2006; Couceiro *et al.*, 2006).

That the combined chilling and salinity treatments did not show synergistic effects (Figures 3.5 and 3.6) suggests there may be a common mechanism for minimising damage that involves the activation and transcription of genes for phenolic biosynthesis by both chilling and salinity (Mittler, 2006; Tattersall *et al.*, 2007; Chattopadhyay *et al.*, 2011). Crosstalk between chilling- and salinity-stressor pathways has been reported previously, and is explainable since both stressors generate ROS due to osmotic stress (Bohnert and Sheveleva, 1998; Chinnusamy *et al.*, 2007), and they induce different but overlapping suites of genes (Narusaka *et al.*, 2004; Fujita *et al.*, 2006; Liu *et al.*, 2008; Fraire-Velázquez *et al.*, 2011). In *Helianthus annuus*, for example, of the genes that were activated by chilling or by salinity, 63% were similar for both stressors. In *A. thaliana* similar miRNA accumulated under both cold and salt stressors (Liu *et al.*, 2008). Thus, common responses stimulated by both stressors (Fernandez *et al.*, 2008) may have abolished possible additive or synergistic accumulations of phenolic acids as might have been predicted by the responses to each stressor individually.

Chilling and salinity, when applied separately, did not influence the concentration of total phenolics or ORAC antioxidant activities in *S. oleraceus* (Figure 3.5A,C). In other species chilling and salinity enhanced the biosynthesis of phenolic compounds; for example, chilling increased ferulic acid in *Glycine max* and caffeoyl derivatives in *Solanum esculentum*, while root zone NaCl salinity increased chlorogenic and chicoric acids in *Echinacea angustifolia*, and caffeic acid derivatives in *Olea europaea* (Janas *et al.*, 2000; Montanari *et al.*, 2008; Ben Ahmed *et al.*, 2009; Remorini *et al.*, 2009; Løvdal *et al.*, 2010). It is noteworthy that prolonged exposure to these stressors caused acclimatory responses, which resulted in polymerisation of synthesised phenolic compounds and incorporation into the cell wall (Janas *et al.*, 2000; López Pérez *et al.*, 2007). It is, therefore, possible that in *S. oleraceus*, too, prolonged exposure to stressors resulted in incorporation of phenolic compounds into cell wall phenylpropanoid compounds, which rendered them undetectable through the Folin-Ciocalteu method using methanolic leaf extracts (Pociecha *et al.*, 2008). Certainly, acclimation to stressors in *S. oleraceus* was indicated by higher rates for NPQ curves (Figures 3.11C,D – 3.13C,D) which indicated strong xanthophyll cycle activities, which presumably enabled the stressor-imposed plants to maintain lower declining rates for $\Delta F/F'_{m,}$ $q_P$ and ETR curves with increasing PAR compared to controls (Figures 3.11A,B,E-G – 3.13A,B,E-G). Similar observations were made in: cold acclimated *Triticum aestivum* and *Secale cereal*,

salinity treated *Sonneratia alba* and *Rhizophora stylosa* and fruiting stressed *Malus domestica* (Oquist *et al.*, 1993; DeEll and Toivonen, 2003; Kitao *et al.*, 2003; Wünsche *et al.*, 2005). In my experiments, none of the treatments significantly damaged the PS II complexes in *S. oleraceus*, as indicated by $F_v/F_m$ values, which were within 0.80–0.83 (Figure 3.10), a typical range for unstressed C3 plant species (Adams III *et al.*, 2006). Even if photosystems of *S. olearaceus* were initially damaged by chilling and salinity, they could have recovered prior to the measurements of $F_v/F_m$ through rapid replacement of damaged D1 protein (Kornyeyev *et al.*, 2002; Andersson and Aro, 2004; Strauss *et al.*, 2007). Alternatively, they may have been protected by the direct scavenging activities of accumulated antioxidants or through utilizing the excess energy from light harvesting complexes for phenylpropanoid biosynthesis (Grace and Logan, 2000; Niggeweg *et al.*, 2004; Mondolot *et al.*, 2006; Hernández and Van Breusegem, 2010).

Chilling, salinity and their combination upregulated chlorogenic acid production in *S. oleraceus* leaves, while caftaric and chicoric acids were not affected, indicating stressors had a variable effect on the accumulation of these hydroxycinnamic acids (Figure 3.6). Although chilling and salinity have been shown previously to induce the expression and activity of phenylalanine ammonia-lyase (PAL), the subsequent synthesis of each phenolic compound was not influenced to the same degree, as documented for *Echinacea angustifolia* (Montanari *et al*., 2008), *Lactuca sativa* (Oh *et al*., 2009), *Matricaria chamomilla* (Kováčik *et al*., 2007), *Glycine max* (Janas *et al*., 2000), *Olea europaea* (Ortega-García *et al*., 2008) transgenic *Nicotiana tabacum* (Howles *et al*., 1996), and numerous other species (Blount *et al.*, 2000; Blount *et al.*, 2002). This is because in addition to PAL, various other enzymes are involved in the phenylpropanoid pathway. After PAL, cinnamic acid 4-hydroxylase (C4H), hydroxylates cinnamic acid into 4-coumaric acid (Blount *et al.*, 2002), which is then esterified into different hydroxycinnamic acids through a number of biosynthetic steps by specific cytochrome P450 monooxygenases (Hahlbrock and Scheel, 1989; Ehlting *et al.*, 2006). Transcription and activity of genes for the cytochrome P450 monooxygenases are influenced by different stressors to variable degrees, as documented for *Arabidopsis thaliana* (Narusaka *et al*., 2004; Ehlting *et al*., 2006) resulting in the biosynthesis of different hydroxycinnamic acids in different quantities (Blount *et al.*, 2002).

Here, the effects of all three stressors on ascorbate concentration was not significantly different from the control (Figure 3.8B), which may be due to the null nett effect of stressor-enhanced transcription and activity of enzymes: ascorbate peroxidase (APX), L-galactono-1,4-lactone dehydrogenase (GLDH), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). GLDH is a key enzyme that regulates ascorbate biosynthesis, APX oxidises ascorbate while MDHAR and DHAR catalyses the regeneration of ascorbate from its oxidised forms in ascorbate-glutathione cycle (Wang and Frei, 2011). For example, salinity increased transcription and activity of enzymes catalysing the biosynthesis, recycling and oxidation of ascorbate in *Solanum esculentum* and *Fragaria ananassa* (Keutgen and Pawelzik, 2007; Sgherri *et al.*, 2007; Sgherri *et al.*, 2008).

It is clear, that the antioxidant potential of *S. oleraceus* ecotypes is heritable across generations; a strong genetic component contributes to the variation of antioxidant activities among *S. oleraceus* ecotypes overriding the effects of abiotic stressors. Leaf extracts from the ACB ecotype were on average 1.2 times more potent scavengers of DPPH radicals than were those of OAM irrespective of the treatment (Figure 3.4B). These plants were the F2 progeny of selfed plants, the parents of which showed 1.6-fold higher DPPH radical scavenging by ACB than by OAM leaf extracts (Ellwood, 2007). These higher antioxidant activities were associated with higher concentrations of phenolic compounds (Figures 3.4D-F). This may have arisen via differential expression of the PAL multigene family across plant accessions (Emmons and Peterson, 2001) and thus changing the concentrations of synthesised phenolics among members of the same species. Cultivar and ecotype differences in phenolic compound concentration have been reported for various species (Ehlenfeldt and Prior, 2001; Reddivari *et al.*, 2007; Løvdal *et al.*, 2010; Krüger *et al.*, 2011), including *S. oleraceus* (Schaffer, 2005; Ellwood, 2007).

ACB plants had more leaf ascorbate than OAM plants (Figure 3.8A). This may be explained by higher rates ascorbate biosynthesis and recycling in ACB compared to OAM. Reinforcing this idea, varieties of *Diospyros kaki*, *Solanum esculentum*, *Setaria italica* and *Triticum aestivum* that accumulated more ascorbate had higher transcription and activities of enzymes: GLDH, MDHAR, DHAR compared to low ascorbate-yield varieties (Sreenivasulu *et al.*, 2000; Bartoli *et al.*, 2005; Zushi and Matsuzoe, 2007). Discovery of ecotypes rich in ascorbate is important due to its potent antioxidant

activities and multiple health benefits for humans (Section 1.1.1). The DPPH radical scavenging capacity of ascorbate was 15 times more efficient than quercetin (LoNostro *et al.*, 2000).

Chlorogenic and chicoric acid concentrations in *S. oleraceus* leaves positively linearly correlated with CAA values in HepG2 cells (Figure 3.18A,B) while caftaric acid did not (Figure 3.18D). This may be explained by higher lipophilicity and lower polarity of chlorogenic and chicoric acid than caftaric acid, which better facilitates the absorbance into HepG2 cells and therefore their antioxidant activities within the cells. The lipophilicity estimated as the log of octanol-water partition coefficient (log $K_{ow}$) of chicoric acid (log $K_{ow}$=3), is three-fold higher than that of caftatric acid (log $K_{ow}$=1), thus lowering the ability of caftaric acid to cross cell membranes compared to chicoric acid (Iranshahi and Amanzadeh, 2008). Chlorogenic acid, too, is less polar and more lipophilic than caftaric acid since the polarity of the quinic acid moiety in chlorogenic acid is lower than the tartaric acid moiety in caftaric acid (Clifford *et al.*, 2003; Pellati *et al.*, 2004). Because higher lipophilicity ensures better transport of these across cell membranes (Burdette *et al.*, 2002; Wu *et al.*, 2007; Zhang *et al.*, 2008), these compounds are better antioxidants in cellular compartments compared to caftaric acid. Confocal laser scanning microscopy has confirmed that phenolic compounds in *S. oleraceus* leaf extracts are indeed absorbed into HepG2 cells (rather than being bound to external cell membranes) whereupon they exhibit antioxidant activities (McDowell *et al.*, 2011).

Total phenolic concentration and extractable antioxidant activities in *S. oleraceus* leaf extracts correlated well with CAA values in HepG2 cells (Figure 3.17). This indicates that phenolic compounds may have been largely responsible for the cellular antioxidant activities of *S. oleraceus*. Similarly, in other crops rich in phenolic compounds, strong correlations between ORAC values and CAA measures have been observed (Wolfe *et al.*, 2008). However in crops with lower phenolic antioxidant activities, the correlations between ORAC and CAA were not significant (Eberhardt *et al.*, 2005; Song *et al.*, 2010).

The DPPH radical scavenging activities of leaf extracts and CAAs showed a lesser degree of correlation than that between ORAC and CAAs values (Figure 3.17A,B). This may be because both ORAC and CAA assays measure the ability of antioxidants to scavenge peroxyl radicals generated by a free radical generator at physiologically relevant

conditions of 37 $^{o}$C and pH 7.4 (Section 4.5-4.6). In contrast, the DPPH assay measures the scavenging by antioxidants of the localised free electron on the DPPH molecule, which bears very little resemblance with biologically relevant conditions (Prior *et al.*, 2005).

In summary, the variation of LMWA activities in *S. oleraceus* is largely associated with plant ageing, followed by ecotype that appears to be heritable. There are also some beneficial effects of the abiotic environment. Furthermore, an extractable antioxidant activities of leaf extracts correlates well with the cellular antioxidant activities inside HepG2 cells. In conclusion, leaves with highest antioxidant activities and concentrations of LMWAs were obtained at week 12 from ACB, and exposure to cold night temperatures further augmented the levels. Agronomic recommendations that can be formulated from this study to obtain harvestable leaves rich in antioxidants are: use of selfed seeds from superior ecotypes across cropping cycles, harvesting leaves from flowering plants, and coinciding harvesting period with the incidences of low night temperatures.

# CHAPTER 4: EFFECTS OF COOKING AND *IN VITRO* GASTROINTESTINAL DIGESTION ON THE ANTIOXIDANT ACTIVITIES OF PHENOLIC COMPOUNDS IN *Sonchus oleraceus* L.

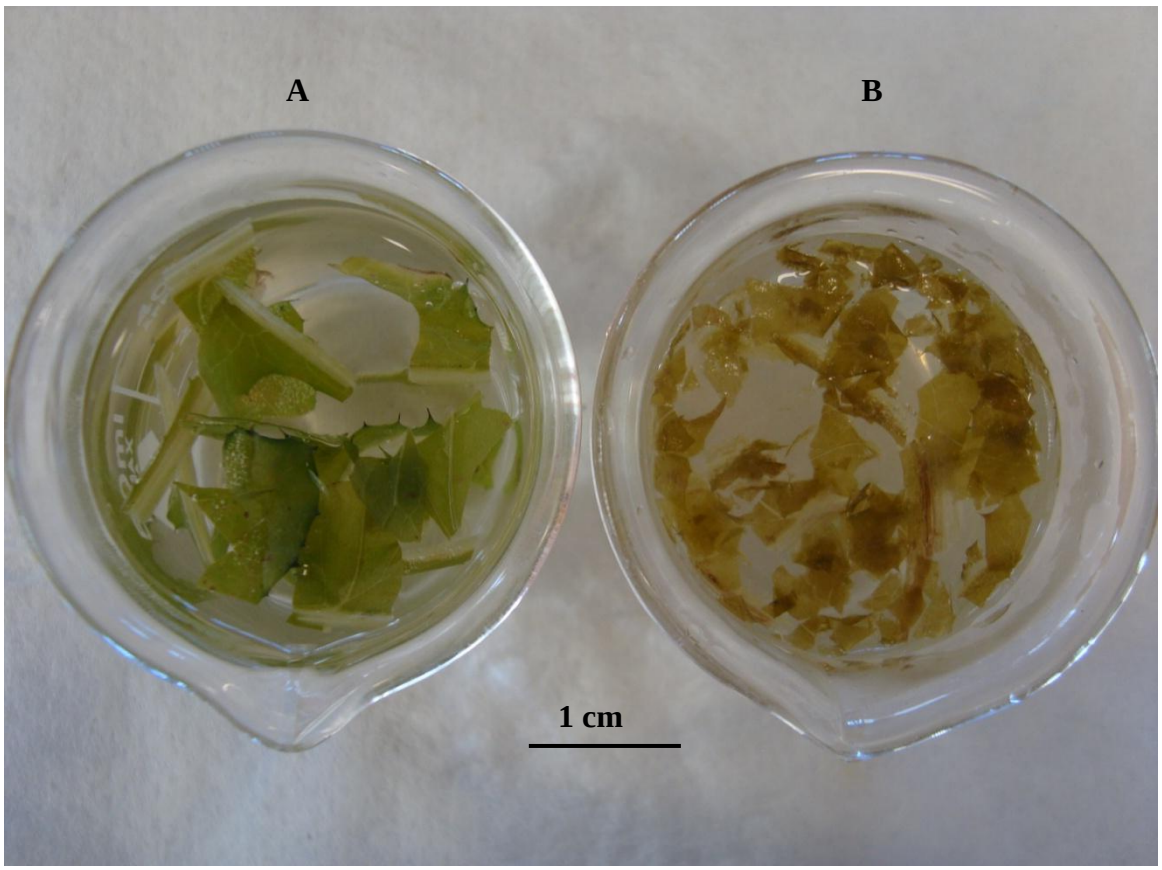



**Plate 4.1 *Sonchus oleraceus* L. leaves (A) boiled for 5 min and (B) digestate remaining after *in vitro* gastrointestinal digestion for 120 min.**

## 4.1 ABSTRACT

Leaves of *Sonchus oleraceus* L., a traditional component of the Māori diet in New Zealand, are rich in phenolics and show potent extractable antioxidant activities. However, the stability and antioxidant activities of these compounds after cooking and gastrointestinal digestion are unknown. Extractable antioxidant activities, HPLC profiles, the concentration of ascorbate and phenolic compounds, and cellular antioxidant activities in Caco2 and HepG2 cells were measured in raw and boiled *S. oleraceus* leaves and in their bioaccessible fraction obtained after gastric and intestinal digestion. Boiling significantly diminished the concentration of ascorbate, chicoric acid and oxygen radical absorbance capacity (ORAC), but did not affect 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity or concentrations of total phenolic compounds, caftaric and chlorogenic acids in the bioaccessible fraction compared to raw leaves. After 30 min gastric digestion, ORAC and DPPH activities, and total phenolic concentration in the bioaccessible fraction approached those measured from untreated leaves. Phenolics released from gastric digestion were absorbed into Caco2 and HepG2 cells and exerted antioxidant activity. Intestinal digestion of leaf residues after gastric digestion released further antioxidants. During gastrointestinal digestion only chicoric, chlorogenic, and caftaric acid were stable out of nine antioxidants present in untreated leaves. That these key phenolics are released from leaves by digestion, and are absorbed into human cells wherein they exert antioxidant activity, indicates that *S. oleraceus* leaves may be suitable as an excellent dietary antioxidant source.

**KEYWORDS**: *Sonchus oleraceus*, antioxidant stability, gastrointestinal digestion, phenolics, HepG2 cells

## 4.2 INTRODUCTION

The sow thistle, *Sonchus oleraceus* L. has traditionally been eaten by Māori as a leafy vegetable after boiling for 5 – 30 minutes (Cambie and Ferguson, 2003; Whyte *et al*, 2001). *S. oleraceus* produces leaves that contain exceptionally high concentrations of ascorbic acid and other phenolic compounds (Guil-Guerrero *et al*., 1998; Simopoulos, 2004). Indeed, *S. oleraceus* leaves held the highest concentration of phenolic compounds among 16 edible leafy plant species chosen from the Italian cuisine (Conforti *et al*., 2009). The *in vitro* antioxidant activities of *S. oleraceus* leaves were two times higher than those of spinach, which ranked first in antioxidant activity among 27 vegetables common in human diet (Song *et al*., 2009). However, the antioxidant potential measured by chemical assays (Chapter 3.0; Section 3.5) does not necessarily translate into antioxidant activity *in vivo*; the compounds need to retain their antioxidant activities through the processes of cooking, gastrointestinal digestion, absorption, and transport to the target tissues (Chapter 1.0; Section 1.4). Nothing has been documented about the fate of antioxidants in *S. oleraceus* leaves during the cooking and digestion processes. It is not known whether they are released from the leaf matrix under physiological conditions of the digestive tract, nor whether the antioxidant activities are retained.

In other leafy vegetables, cooking has been found either to increase or decrease the concentrations of available phenolic compounds. Some cell wall-bound phenolic compounds can be liberated during cooking because high temperatures dissociates the covalent, ester, ether, or acetal bonds that held them; thus, the concentrations of phenolic compounds and their associated antioxidant capacities may increase beyond the levels prior to cooking (Robbin, 2003). For example, cooking liberated more phenolic compounds increasing the antioxidant capacity of citrus (Seok-Moon *et al*., 2004). Alternatively, cooking might decrease the levels of some phenolic compounds because heat can either polymerise or decompose the aromatic rings, thereby denaturing the compounds.

Efforts to simulate gastrointestinal digestion have had variable effects on the apparent bioaccessibilty of phenolic compounds in diets (Section 1.4; Tables 1.12 – 1.14). *In vitro* gastrointestinal digestion released either more or lower levels of phenolic compounds than the levels that were extracted from the food prior to digestion (Rodríguez-Roque *et*

*al*., 2013; Tagliazucchi *et al*., 2010). The increases were attributed to the release of phenolic compounds bound to proteins and carbohydrates in the food matrix through acid hydrolysis in the gastric phase and enzymatic hydrolysis in gastric and intestinal phases (Rodríguez-Roque *et al*., 2013). For example, caffeic and chlorogenic acids, hesperidin, naringenin and rutin levels were higher following *in vitro* gastrointestinal digestion of fruit juices. However, following *in vitro* gastrointestinal digestion, sinapic acid levels from fruit juice diminished, while flavonoid levels remained constant for grapes (Laurent *et al*., 2007).

Monitoring gastrointestinal digestion and uptake of LMWAs into human cells *in vivo* can be complex, and therefore models that simulate human gastrointestinal digestion and uptake have often been employed (Section 1.4; Table 1.11 and Section 1.6). Consequently, dissolution testing can be used to evaluate the bioaccessibility of LMWAs. *In vivo* gastric and intestinal conditions can be reproduced in dissolution tests by employing a paddle type dissolution apparatus (Plate 4.2) and following the protocol for conventional release solid dosage forms (Anon,(1988; Hu, 1998; Mann and Pygall, 2012). Following *in vitro* gastrointestinal digestion, the bioaccessible fraction is obtained by either filtration or centrifugation of the simulated gastrointestinal solution (Moreda-Piñeiro *et al.*, 2011). After dissolution testing, the cell models can be employed to study the *in vivo* antioxidant activities of the antioxidant compounds in the bioaccessible fraction (Section 1.6).

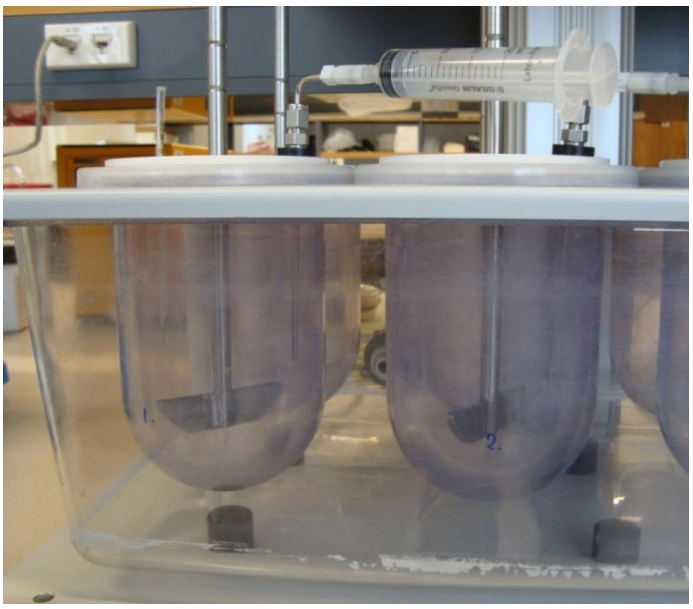


**Plate 4.2 Paddle type dissolution apparatus (six-station Erweka DT 600 Dissolution Tester)**

Here, we identify and quantify the ascorbate, hydroxycinnamic acids, total phenolics and antioxidant activities of raw and boiled *S. oleraceus* leaves during *in vitro* gastrointestinal digestion, and then employ the CAA assay to identify cellular uptake and antioxidant activity of the raw, digested leaves. It was hypothesized that *in vitro* cellular and chemical antioxidant activities of *S. oleraceus* leaves survive cooking and *in vitro* gastrointestinal digestion, and are absorbed into human cells wherein they exert antioxidant activities.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Chemicals

Pepsin from porcine gastric mucosa and pancreatin from porcine pancreas were purchased from Sigma-Aldrich (St Louis, MO). All other chemicals are listed at Section 2.1.

### 4.3.2 Plant Materials

*S. oleraceus* were grown as described in Section 3.3.1 from seeds collected from a natural population at Acacia Bay in North Island of New Zealand. The plants were grown for 84 days during January to April 2011. The leaves from nodes 5 and 6 were removed from 48 plants. Leaves were bisected longitudinally, one half (untreated control) was used for phytochemical analysis and the other for gastrointestinal digestion. For comparisons of antioxidant activities, fresh blueberry fruits (*Vaccinium corymbosum*) were purchased from a local supermarket (sourced from Blueberries Waikato, Ohaupo, New Zealand). Material intended for phytochemical analysis was prepared according to Section 3.3.3.

### 4.3.3 Boiling

Leaf portions were subdivided equally, and one half was boiled in water at 100 °C for 5 min to study the effects of cooking, and the other half used raw.

### 4.3.4 Gastric and Intestinal Digestion

Artificial gastric juice and intestinal fluid were prepared as described in the British Pharmacopoeia (1988) and dissolution test performed according to the protocol for conventional release solid dosage forms (Anon, 1988). Gastric juice contained 34.2 mM NaCl, 92.4 µM pepsin and 80 mM HCl at pH 1.2. Intestinal fluid was made using 50 mM $KH_2PO_4$, 15.4 mM NaOH, 1.1 g $L^{-1}$ of pancrease powder, and adjusted to pH 7.5. Digestion was performed in a six-station Erweka DT 600 Dissolution Tester (Plate 4.2; Erweka International AG, Basel, Switzerland) at 37 °C with a paddle speed of 50 rpm. Boiled and raw leaf portions cut into approximately 1 $cm^2$ pieces (9 g) were incubated in 900 mL artificial gastric juice for 1 h, and then the digestate was resuspended in 900 mL intestinal fluids for 1 h. Aliquots of the fluids were withdrawn 5, 15, 30, and 60 min into each digestion, centrifuged at 24000 g for 5 min, and supernatant stored at –20 °C under nitrogen. A blank prepared using the same chemicals but without the leaf material was treated identically.

The analysis of total phenolics, DPPH radical scavenging activities, ORAC Assay, Cellular Antioxidant Activity (CAA) assay and online reverse phase HPLC-DPPH radical scavenging were performed as described in Sections 2.2 – 2.8.

## 4.4 STATISTICAL ANALYSIS

Differences in antioxidant activities and antioxidant concentrations attributable to boiling and digestion were evaluated using repeated measures ANOVA with Bonferroni post hoc tests ($P< 0.05$). Correlations were established by simple linear regressions ($P< 0.05$). Comparisons of linear regression were performed by ANCOVA ($P< 0.05$). Probit analysis was performed for CAA dose-response data. All analysis was performed using SPSS 18.0 statistical software.

## 4.5 RESULTS

### 4.5.1 Antioxidant activities and concentrations of ascorbate and phenolic compounds from untreated raw leaf extracts

As measured by the DPPH assay, the antioxidant activities of methanolic extracts of fresh, untreated *S. oleraceus* leaves were on average four times greater than those of blueberry fruit on a dry weight comparison (ANOVA; $P < 0.001$; Table 4.1). In contrast, measurements of the same extracts using the ORAC assay were only 1.3-fold greater ($P = 0.5$; Table 4.1). Concentration of total phenolic compounds, expressed as tannic acid equivalents, were 1.3 times higher in the *S. oleraceus* leaf extracts than in blueberries ($P = 0.14$; Table 4.1). The extracts from blueberries were three times more concentrated in ascorbate than *S. oleraceus* leaves ($P < 0.001$; Table 4.1). Antioxidants from the methanolic extracts of fresh, untreated leaves entered the HepG2 and Caco2 cells and exhibited antioxidant activity *in situ* (Table 4.2).

**Table 4.1 Antioxidant activities as measured by DPPH and ORAC, and concentrations of ascorbate and total phenolic compounds for methanolic extracts of *Sonchus oleraceus* L. raw leaves and blueberry fruit[a]**

| Sample | DPPH[1] radical scavenging activity | | ORAC[2] | Total phenolics | Ascorbate |
|---|---|---|---|---|---|
| | $EC_{50}$ (mg $L^{-1}$) | µmol Trolox equiv.$g^{-1}$ | (µmol Trolox equiv.$g^{-1}$) | (mg Tannic acid equiv.$g^{-1}$) | (mg $g^{-1}$) |
| *S. oleraceus* | 38.6 ± 2.9 | 478.5 ± 34.3 | 24.9 ± 2.6 | 0.9 ± 0.1 | 0.8 ± 0.1 |
| Blueberry | 162.0 ± 19.1 | 120.9 ± 18.8 | 18.7 ± 8.4 | 0.7 ± 0.1 | 2.1 ± 0.1 |
| **Ratio** | | | | | |
| *S. oleraceus*: blueberry | 4.0 | | 1.3 | 1.3 | 0.4 |

[a]Values are means ± SE ($n = 6$).

[1]2,2-diphenyl-1-picrylhydrazyl

[2]Oxygen radical absorbance capacity

**Table 4.2 Cellular antioxidant activities (CAA) for methanolic extracts of *Sonchus oleraceus* L. raw leaves in HepG2 and Caco2 cells[a]**

| Cell line | CAA | |
|---|---|---|
| | $EC_{50}$ (g $L^{-1}$) | (µmol quercetin equivalent. $g^{-1}$) |
| HepG2 | 0.4 ± 0.1 | 30.2 ± 2.7 |
| Caco2 | 1.4 ± 0.1 | 3.9 ± 0.1 |

[a]Values are means ± SE ($n$ = 6).

HPLC profiles of the untreated leaf extracts revealed nine peaks at 320 nm that had corresponding DPPH radical scavenging activities (Figure 4.1). Of those, the hydroxycinnamic acids were present at the highest concentrations; caftaric, chlorogenic and chicoric acids eluted at 11.6, 14.5 and 27.0 min, respectively, at concentrations of 3.5 ± 0.5, 2.2 ± 0.2 and 9.5 ± 0.6 mg $g^{-1}$. These compounds were identified from their retention times in comparison with previous reports, and by co-elution with authentic standards (Ou *et al.*, 2013).

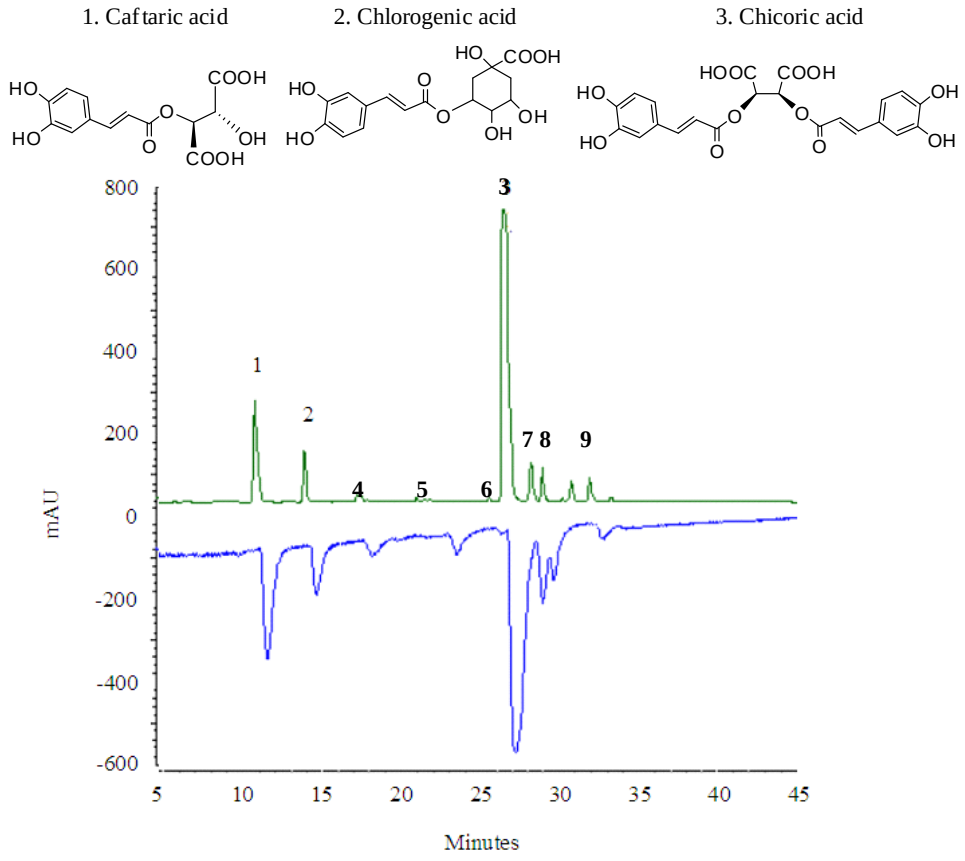



**Figure 4.1 Reverse phase HPLC-DPPH chromatograms for methanolic extracts of *Sonchus oleraceus* L. raw untreated leaves. Phenolic acids (green line) measured at 320 nm; DPPH radical scavenging (blue line) at 518 nm. Peaks: 1, caftaric acid; 2, chlorogenic acid; 3, chicoric acid and 4 – 9, unknown.**

### 4.5.2 Antioxidant activities and concentrations of ascorbate and phenolic compounds after boiling and gastrointestinal digestion

Boiling *S. oleraceus* leaves for 5 min at 100 °C did not significantly alter concentrations of total phenolics (ANOVA; $P = 0.14$) or antioxidant activities measured as DPPH ($P = 0.84$) or ORAC activities ($P = 0.99$) in their methanolic extracts of untreated leaves (Figure 4.2). Boiling diminished concentrations of ascorbate ($P = 0.03$) and total hydroxycinnamic acids ($P = 0.03$) in methanolic extracts compared to raw leaves (Figure 4.3).

The bioaccessible fraction from both boiled and raw leaves held comparable concentrations of total phenolics (Figure 4.2A). However, raw leaves released more ascorbate (Figure 4.3A) and total hydroxycinnamic acids (Figure 4.3B) than boiled leaves into the bioaccessible fraction obtained through gastrointestinal digestion.

ORAC values of the bioaccessible fraction were lower for boiled than for raw leaves following digestion in both gastric and intestinal conditions ($P < 0.001$; Figure 4.2C). In contrast, DPPH activities were higher for boiled than for raw leaves following gastric digestion ($P = 0.05$; Figure 4.2B), but were comparable following subsequent intestinal digestion.

Importantly, the antioxidants in the bioaccessible fraction from raw leaves were absorbed into HepG2 (Figure 4.4A) and Caco2 cells (Figure 4.4B). Their CAA in HepG2 ($P = 0.01$) and Caco2 cells ($P< 0.001$) were greater the longer leaves had been digested. The CAA values approached levels comparable to those from methanolic extracts of ground, untreated leaves just after 5 min of gastric digestion (Figure 4.4). Interestingly, the CAA values after 60 min of gastric digestion were three-fold greater than those of raw leaves in both cell types. Significant positive linear correlation exists for the CAA values obtained for the bioaccessible fraction between the two cell types (Figure 4.5). Increases in CAA in both cell types correlated linearly with the increases in hydroxycinnamic acid concentration in the bioaccessible fraction of raw leaves (Figure 4.6).

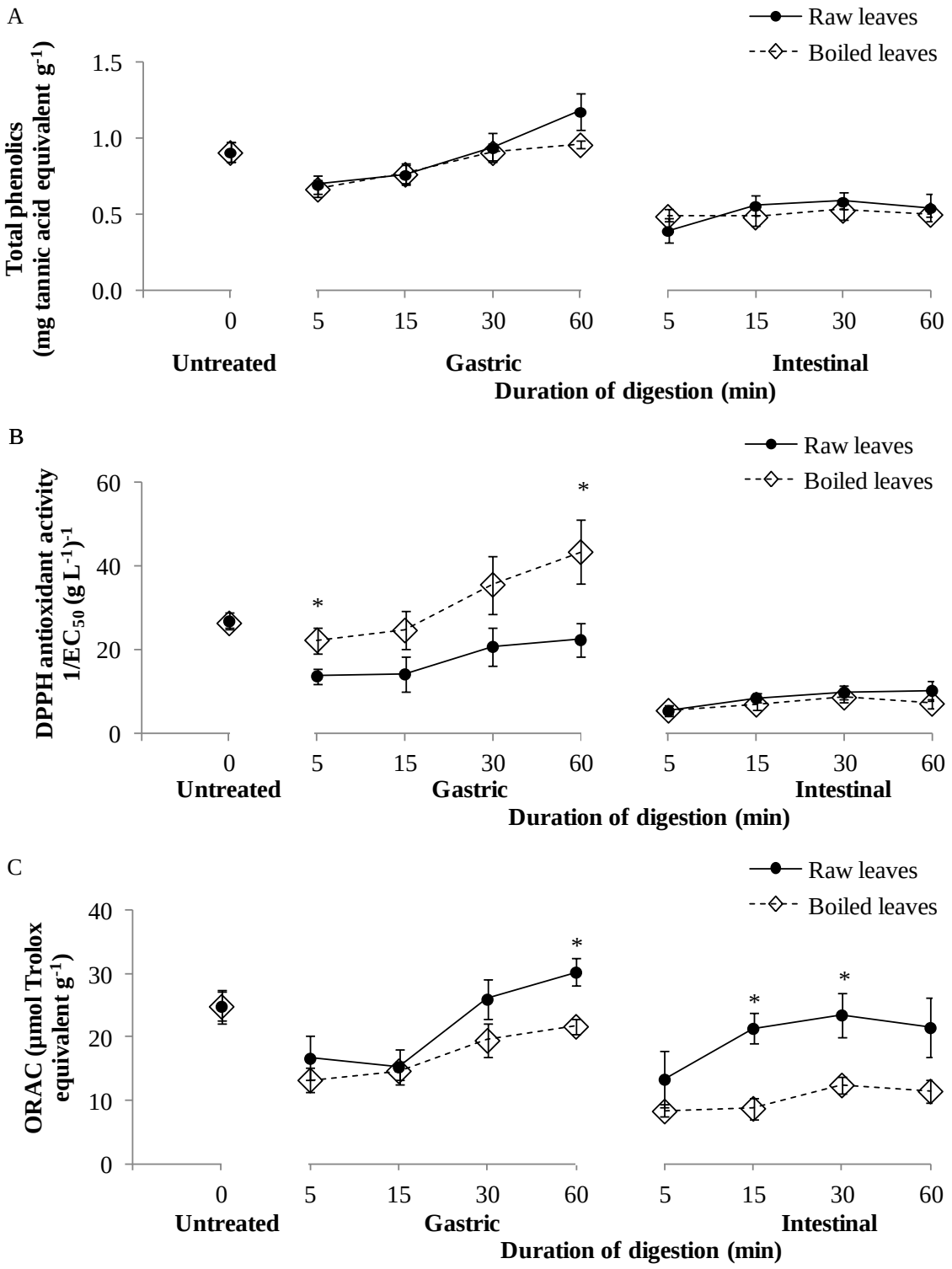



**Figure 4.2 Changes in (A) concentration of total phenolic compounds, (B) DPPH radical scavenging activity and (C) ORAC activity in the methanolic extracts of *Sonchus oleraceus* L. leaves and in the bioaccessible fraction during *in vitro* gastric and subsequent intestinal digestion. Means ± SE (*n*=6). *Significant difference between raw and boiled extracts at *P*< 0.05.**

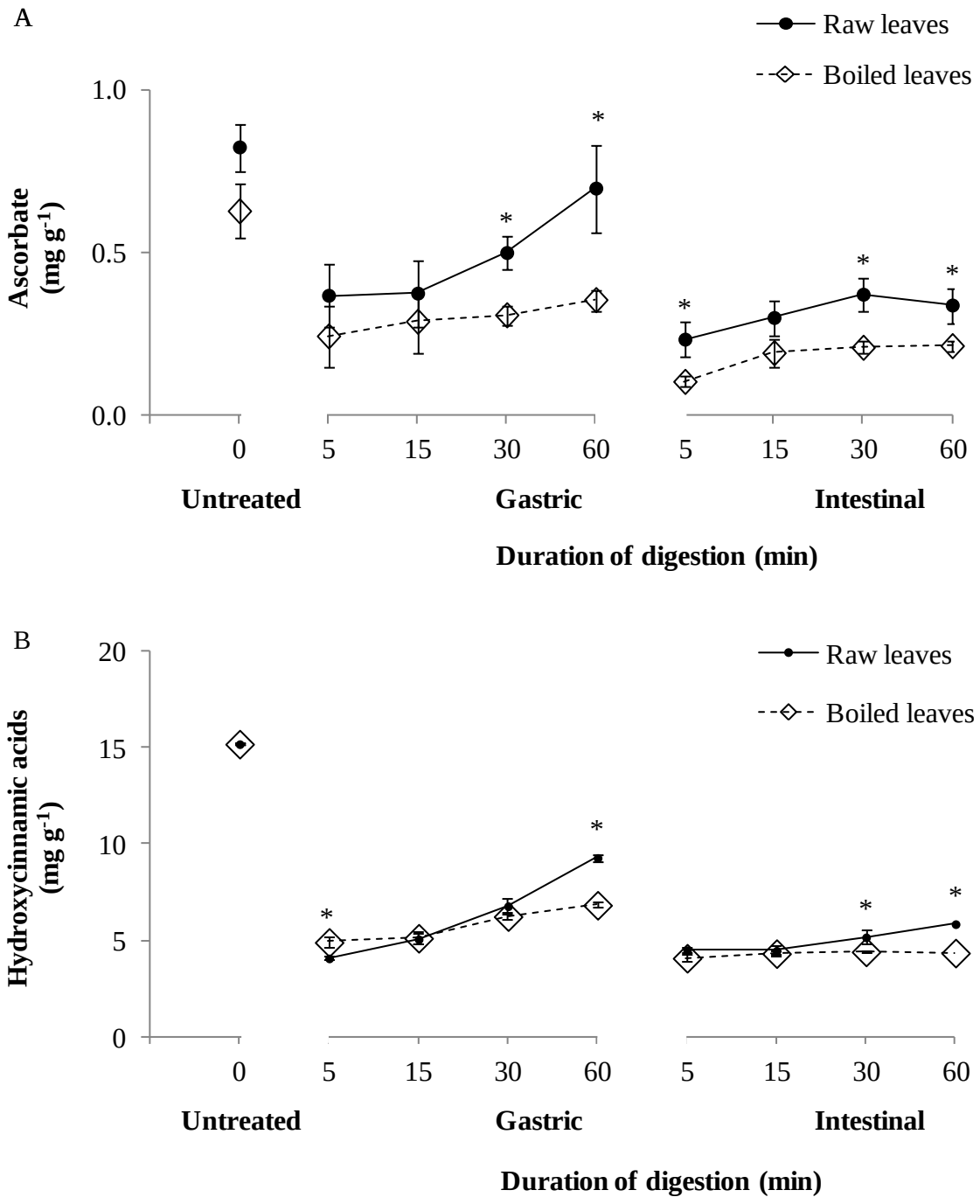



**Figure 4.3 Changes in concentrations of (A) ascorbate and (B) hydroxycinnamic acids in the methanolic extracts of *Sonchus oleraceus* L. leaves and in the bioaccessible fraction during *in vitro* gastric and subsequent intestinal digestion. Means ± SE (*n*=6). *Significant difference between raw and boiled extracts at *P*< 0.05.**

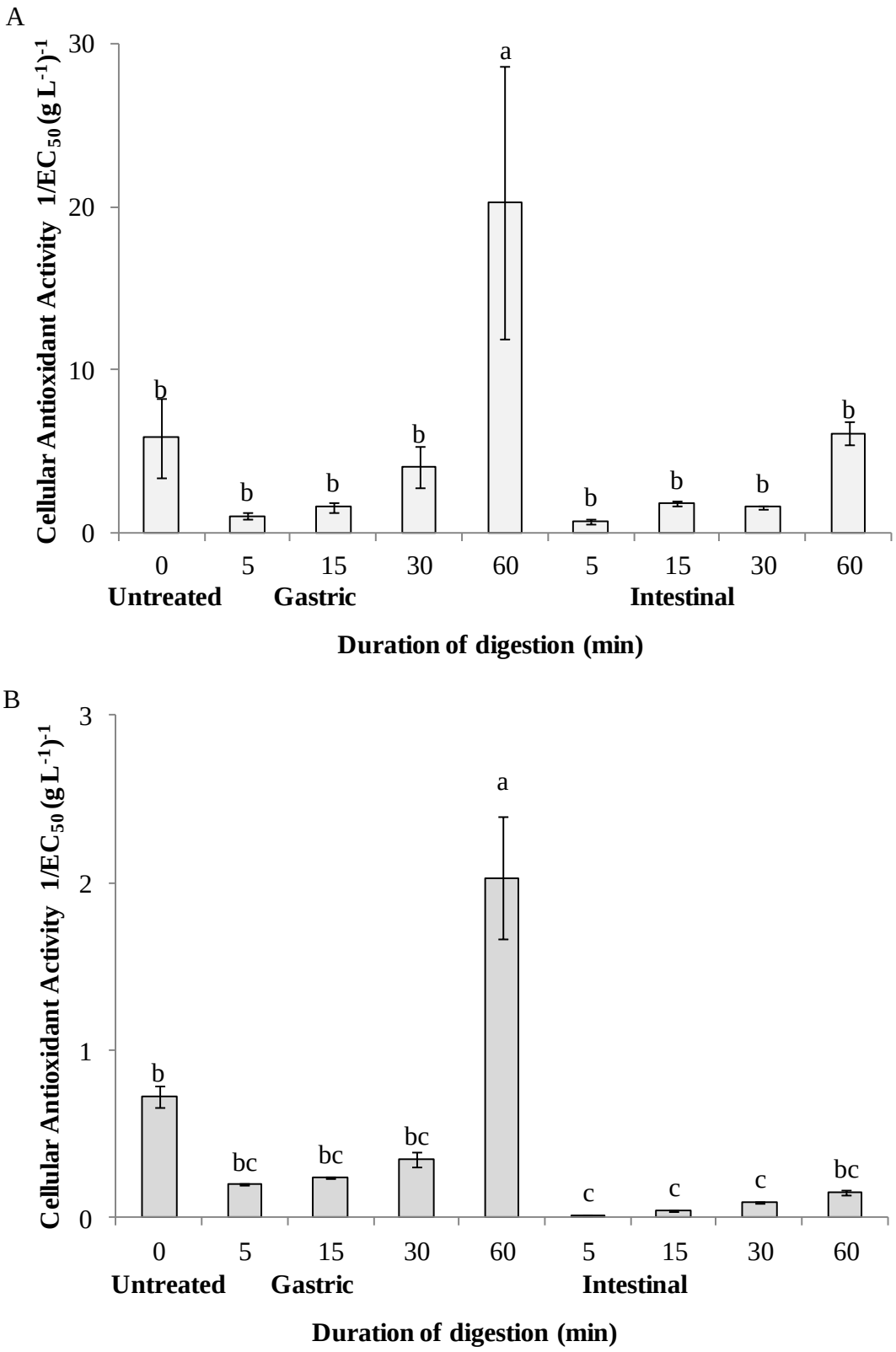



**Figure 4.4 Cellular antioxidant activity measured in (A) HepG2 and (B) Caco2 cells infused with the methanolic extracts of *Sonchus oleraceus* L. raw leaves and the bioaccessible fraction following *in vitro* gastric and subsequent intestinal digestion. Means ± SE (*n*=6). Bars with different letters are significantly different (*P*< 0.05).**

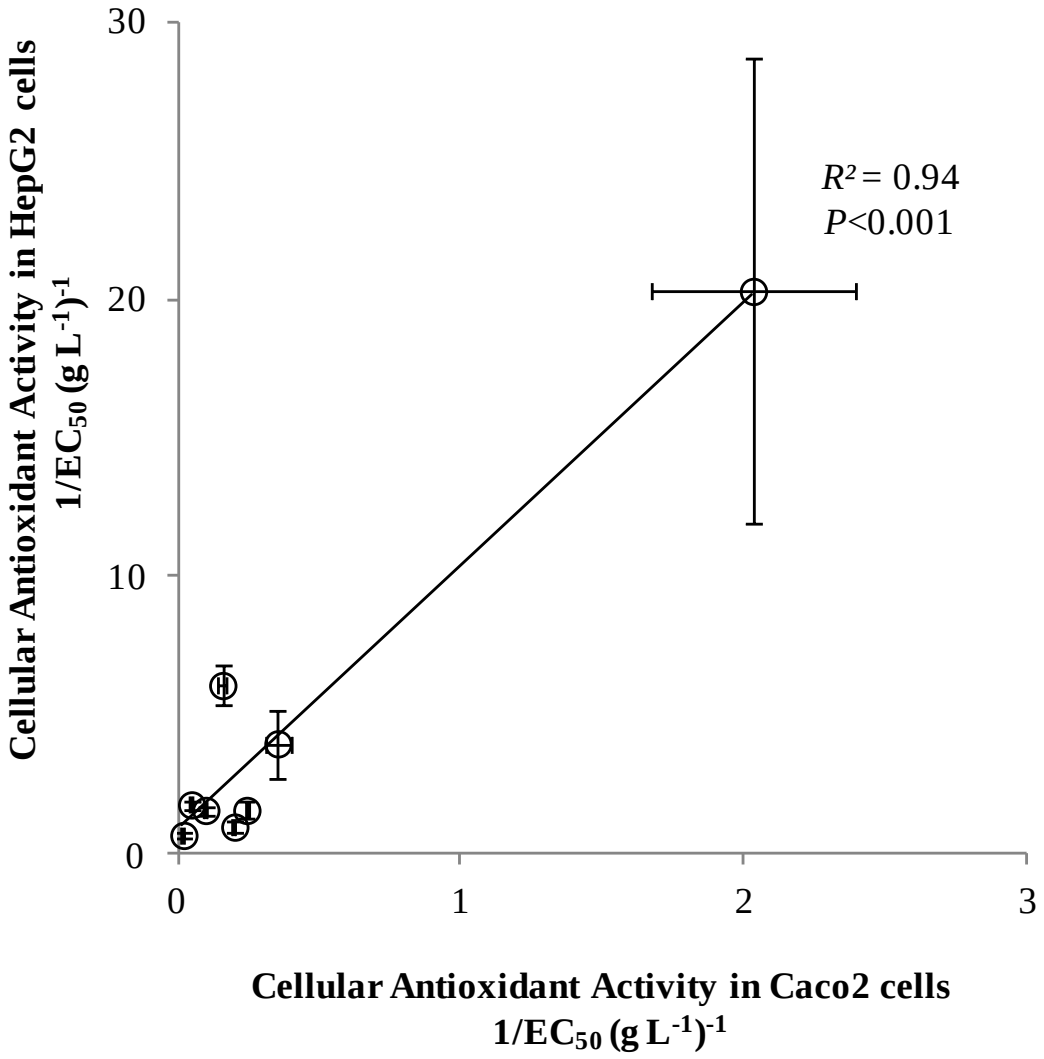



**Figure 4.5 Correlation of cellular antioxidant activities between Caco2 and HepG2 cells infused with the bioaccessible fraction following *in vitro* gastric and intestinal digestion of *Sonchus oleraceus* L. raw leaves. Data are means ± SE (*n*= 6) for 5, 15, 30, 60 min of gastric and 5, 15, 30 and 60 min intestinal digestion.**

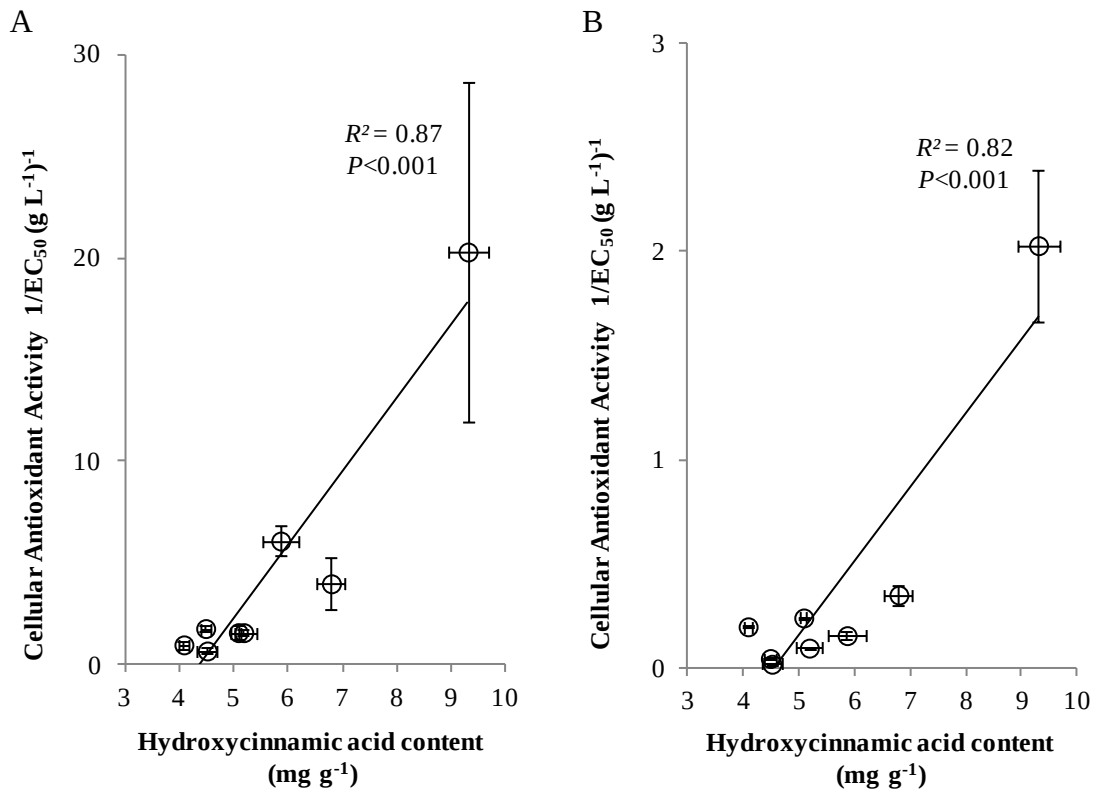



**Figure 4.6 Correlation between the concentration of hydroxycinnamic acids in the bioaccessible fraction and the cellular antioxidant activities measured in cells: (A) HepG2 and (B) Caco2, infused with the bioaccessible fraction following *in vitro* gastric and intestinal digestion of *Sonchus oleraceus* L. raw leaves. Data are means ± SE (*n*= 6) for 5, 15, 30 and 60 min of gastric and 5, 15, 30 and 60 min intestinal digestion.**

As evidenced by HPLC-DPPH chromatograms (Figure 4.1), gastrointestinal digestion apparently degraded six of the nine antioxidants present in methanolic extracts of untreated leaves and none remained in the digestate at the end of gastrointestinal digestion (Figure 4.7). Only caftaric, chlorogenic and chicoric acid were prominent in HPLC profiles collected from the bioaccessible fraction during gastric and intestinal digestion of leaf material (Figure 4.8 A-B). These three compounds had associated DPPH radical scavenging activity shown by the magnitude of the negative peak in the HPLC chromatograms.

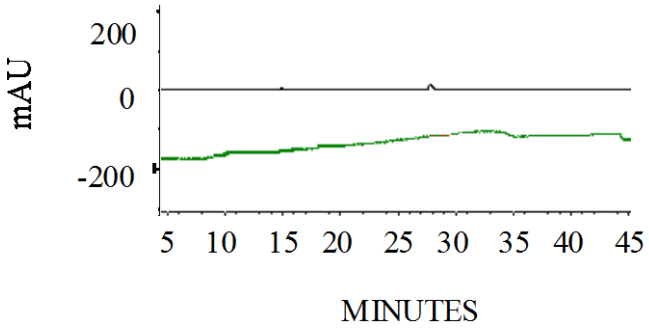



**Figure 4.7 Reverse phase HPLC-DPPH chromatogram for methanolic extract of leaf digestate remaining after *in vitro* gastric and subsequent intestinal digestion of *Sonchus oleraceus* L. leaves. Phenolic acids (black line) measured at 320 nm; DPPH radical scavenging (green line) at 518 nm.**

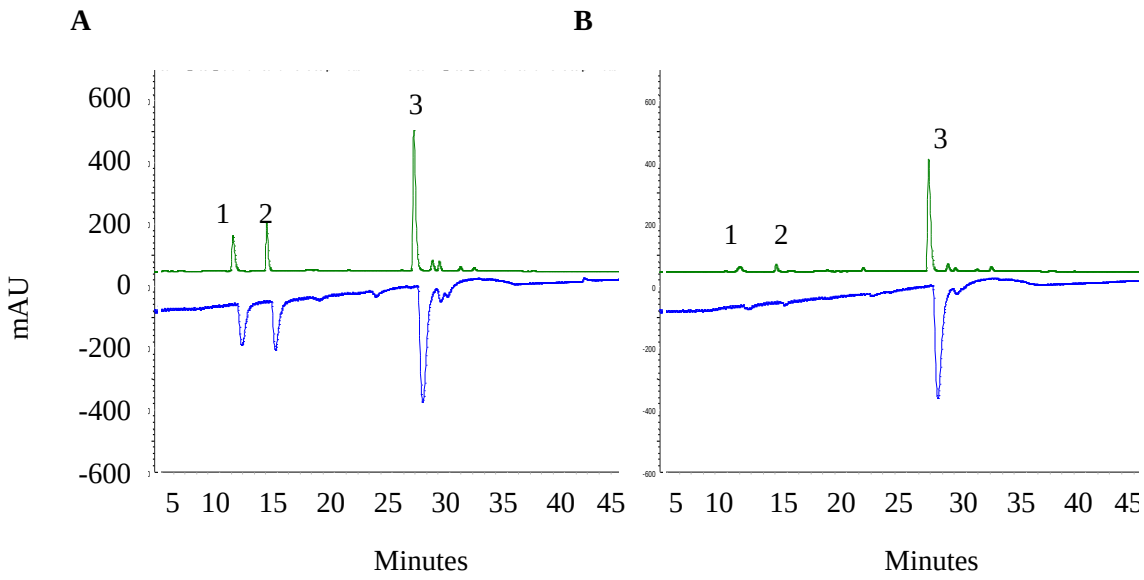



**Figure 4.8 Reverse phase HPLC-DPPH chromatograms recorded for bioaccessible fraction of *Sonchus oleraceus* L. raw leaves, 60 minutes after *in vitro* (A) gastric and (B) intestinal digestion. Phenolics (green line) measured at 320 nm; DPPH radical scavenging (blue line) at 518 nm. Peaks: 1, caftaric acid; 2, chlorogenic acid and 3, chicoric acid.**

Gastrointestinal digestion of leaves resulted in the release of all the chlorogenic acid (ANOVA; $P = 0.29$; Figure 4.9A) and ascorbate content ($P = 0.41$; Figure 4.9B), which were initially present in their leaves regardless of boiling. The level of chicoric acid released through gastrointestinal digestion was significantly lower than those extracted from untreated leaves ($P < 0.001$; Figure 4.9C).

The chicoric acid levels released through digestion by boiled leaves were lower than for raw leaves ($P = 0.004$; Figure 4.9C). However, the release of chlorogenic acid ($P = 0.14$; Figure 4.9A) and caftaric acid ($P = 0.24$; Figure 4.9D) by digestion was similar for boiled and raw leaves. The bioaccessible fraction held a higher proportion of caftaric and chlorogenic acids when using boiled rather than raw leaves (Figure 4.10).

The concentrations of total hydroxycinnamic acids in the bioaccessible fraction positively linearly correlated with the increases in: total phenolic concentrations (Figure 4.11A) and antioxidant capacities determined by ORAC (Figure 4.11B) and DPPH assays (Figure 4.11C), for both raw and boiled leaves. Linear regression coefficients for DPPH radical scavenging versus the hydroxycinnamic acids in the bioaccessible fractions differed between boiled and raw leaves; the gradient for boiled leaves was five-fold higher than for raw leaves (ANCOVA; $P<0.001$; Figure 4.11C).

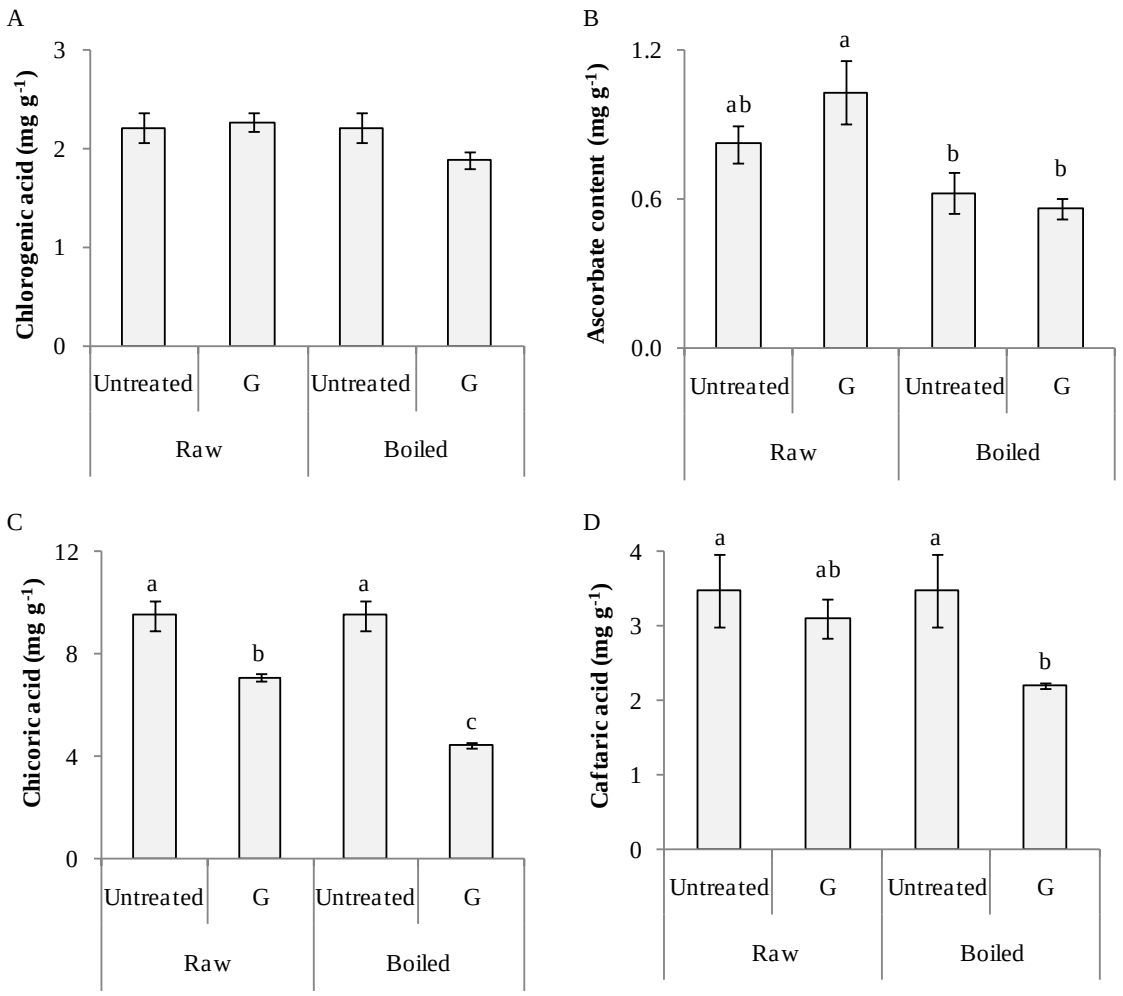



**Figure 4.9 Concentration of (A) chlorogenic, (B) ascorbic, (C) chicoric and (D) caftaric acids in the methanolic extracts of *Sonchus oleraceus* L. raw and boiled leaves, and in the bioaccessible fraction following *in vitro* gastrointestinal digestion (G). Means ± SE (*n*=6). Bars with different letters are significantly different (*P*< 0.05).**

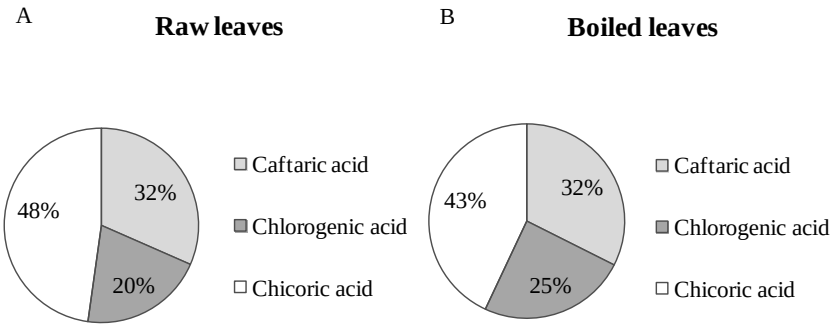



**Figure 4.10 Proportional contributions of three main hydroxycinnamic acids to the total phenolic acids in the bioaccessible fraction of *Sonchus oleraceus* L. raw and boiled leaves**

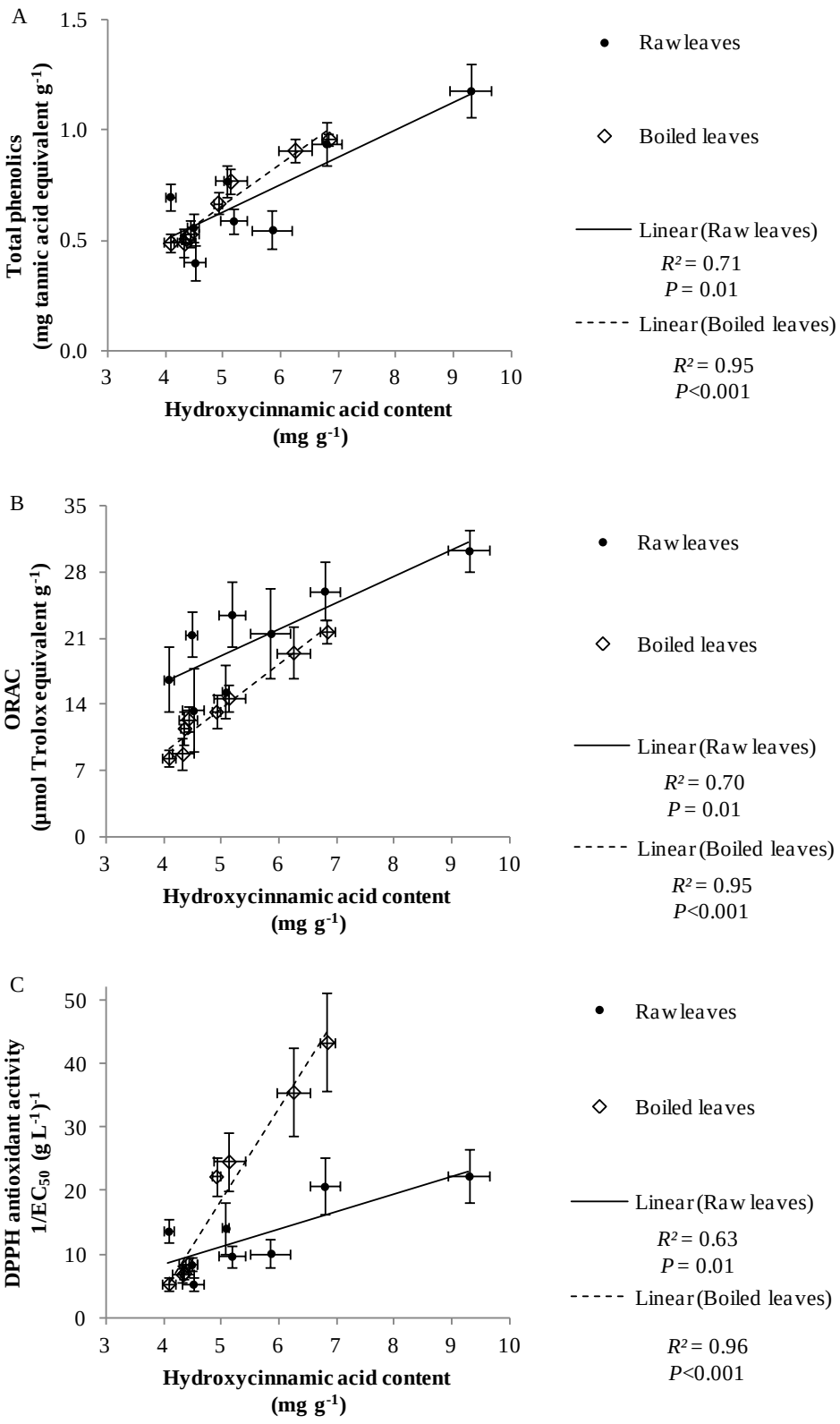



**Figure 4.11 Linear correlations between the concentration of hydroxycinnamic acids and (A) concentration of total phenolic compounds and antioxidant activities measured by (B) ORAC and (C) DPPH techniques for the bioaccessible fraction of *Sonchus oleraceus* L. raw and boiled leaves during *in vitro* gastric and intestinal digestion. Data are means ± SE (*n*=6) for 5, 15, 30 and 60 min of gastric and 5, 15, 30 and 60 min intestinal digestion.**

## 4.6 DISCUSSION

Our study confirmed previous reports that the leaves of *S. oleraceus* are a particularly rich source of phenolic antioxidants. We showed that cooking the leaves by boiling them for 5 min did not appreciably diminish these antioxidant activities prior to *in vitro* gastrointestinal digestion. Significantly, three of the major phenolic compounds (caftaric, chlorogenic, and chicoric acids) were released into solution during gastrointestinal digestion. These compounds were absorbed by human HepG2 cells, whereupon they exhibited apparent antioxidant activity. Collectively, these data argue a compelling case for *S. oleraceus* as an excellent dietary antioxidant supplement to promote human health.

The high antioxidant potential of untreated *S. oleraceus* leaf extracts was confirmed using both the DPPH and ORAC assays (Table 4.1). DPPH radical scavenging activities were comparable to previous reports for this species (Ellwood, 2007; Yin *et al.*, 2007), though four times weaker than values documented by McDowell *et al*. (2011) and three times more potent than reported by Simopoulos (2004). Differences are likely due to variation in growing environment and ecotypes on antioxidant activity of *S. oleraceus* (Section 3.5; Schaffer, 2005; Ellwood, 2007). ORAC values have not been reported previously for *S. oleraceus* leaves. On a dry weight basis, the leaves were richer sources of LMWAs than were blueberry fruit, though the magnitude of the difference varied substantially between the two assays (Table 4.1). Of the two, ORAC is likely to have provided the more reliable estimate of total antioxidant capacities, because steric restrictions to the radical site of DPPH can lead to overestimations of the capacities of the smaller antioxidant molecules (Prior *et al.*, 2005). Phenolic acids in *S. oleraceus* leaves are small molecules and their percentage in LMWAs pool was three-fold greater than that in blueberries, possibly leading to overestimated DPPH values. The reverse phase HPLC-DPPH profiles confirm that caftaric, chlorogenic and chicoric acid accounted for 89% of the LMWAs in *S. oleraceus* leaf extracts (Figure 4.1). These hydroxycinnamic acids have been previously documented in *S. oleraceus* leaves (Gatto *et al.*, 2011; Ou *et al.*, 2013). In contrast, in blueberries, large molecules such as anthocyanins and glycosylated flavonols accounted for 71% of the phenolic pool while chlorogenic acid accounted for only 29% (Zheng and Wang, 2002). Nevertheless, the DPPH assay proved useful when used in conjunction with HPLC to measure the activities of known phenolic compounds (Figures 4.1, 4.7, 4.8).

Boiling leaves for 5 min sufficiently cooks them (Whyte *et al.*, 2001; Cambie and Ferguson, 2003a), and did not significantly diminish their phenolic compound concentration (Figure 4.2A). Boiling decreased the levels of released chicoric acid (Figure 4.9C), but not chlorogenic (Figure 4.9A) or caftaric acids (Figure 4.9D), which are more resistant to heat degradation than chicoric acid (Chkhikvishvili and Kharebava, 2001; Stuart and Wills, 2003). In other crops, it has been reported that boiling causes either a decline (Chu *et al.*, 2000; Turkmen *et al.*, 2005) or an increase (Doğan *et al.*, 2005; Lee and Scagel, 2009) in the available phenolic compounds. Decreased levels of phenolics are often attributed to polymerisation or decomposition of aromatic rings, which denatures the compounds (Granito *et al.*, 2005), or else to their removal in the water used for boiling (Turkmen *et al.*, 2005). Alternatively, boiling may increase the release of phenolic compounds by enhancing the release of cell wall-bound compounds (Dewanto *et al.*, 2002), and/or by halting polyphenol oxidase driven enzymatic oxidation (Doğan *et al.*, 2005; Lee and Scagel, 2009).

Boiling did not significantly diminish the concentrations of ascorbate measured in methanolic extracts of leaves (Figure 4.3A) even though ascorbate is highly water soluble and heat labile. The ascorbate levels may have been stable because the oxidation of ascorbate into dehydroascorbate (DHA) in water at 100 $^{o}$C is reversible (Vieira *et al.*, 2000). Furthermore, the submerged leaves in boiling water may restrict contact between leaf ascorbate and oxygen thus limiting its oxidation; hot air drying of vegetables, for example, caused more ascorbate loss compared to boiling them at the same temperature (Mo *et al.*, 2006; Gupta *et al.*, 2013). Another possibility is that boiling for 5 min in water is insufficient to cause its loss through leaching into water. For example, vegetables that were boiled for long durations lost ascorbate through leaching but not when boiled for short durations (Lee and Kader, 2000; Oboh, 2005; Cruz *et al.*, 2008). However, irreversible hydrolysis of DHA into 2,3-L-diketo-L-gulonate (2,3-DKG) occurred if incubation was continued for 60 min at 37 $^{o}$C even without gastric or intestinal enzymes (Simpson and Ortwerth, 2000). This may have lowered the ascorbate levels detected in the bioaccessible fraction from boiled leaves compared to raw leaves (Figure 4.9B).

The bioaccessible fraction of the boiled leaves held less antioxidants than did raw leaves as detected by ORAC measurements (Figure 4.2C) and chicoric acid content (Figure 4.9C). However, this difference was not evident in the measurements of DPPH radical

scavenging (Figure 4.2B), total phenolics (Figure 4.2A), caftaric (Figure 4.9D) or chlorogenic acid content (Figure 4.9A). The boiled leaves released proportionately more caftaric and chlorogenic acids than did raw leaves in the processes of digestion (Figure 4.10). The DPPH assay would, therefore, likely overestimate the antioxidant capacity of boiled leaves (Figure 4.11C) because caftaric and chlorogenic acids are small molecules with coplanar structures than have better access to the radical of the DPPH molecule than chicoric acid (Silva *et al.*, 2000; Huang *et al.*, 2005). For example, the molar volumes of caftaric (184 $cm^3$ $mol^{-1}$) and chlorogenic acids (214 $cm^3$ $mol^{-1}$) are respectively 60% and 30% smaller than that of chicoric acid (289 $cm^3$ $mol^{-1}$) according to www.chemspider.com (accessed April 2012). This means that antioxidant activities of the bioaccessible fraction of *S. oleraceus* leaves depend largely on the type and proportion of the compounds, which remain in the bioaccessible fraction; similarly seen during *in vitro* digestion of *Solanum esculentum* (Toor *et al.*, 2008).

Temporal changes in antioxidant activities during gastrointestinal digestion recapitulated changes in concentrations of hydroxycinnamic acids, indicating that LMWA activities of *S. oleraceus* mainly depended on the release and stability of hydroxycinnamic acids during gastrointestinal digestion (Figure 4.11). The release of phenolics during *in vitro* gastric digestion has been attributed to acidity rather than to pepsin activity in a wide variety of fruits and vegetables (Bermúdez-Soto *et al.*, 2007; Tagliazucchi *et al.*, 2010). The glycosylated and esterified phenolic compounds are hydrolysed by the acidic conditions, which exist during gastric simulations (Liyana-Pathirana and Shahidi, 2005). Pepsin have lower activity or none towards phenolic compounds because pepsin prefer the hydrolysis of peptic bonds in amino acids (Beynon and Bond, 2001). The acid hydrolysis of bound phenolics in leaves (Liyana-Pathirana and Shahidi, 2005) would likely explain why levels of total phenolics released after combined gastric and intestinal digestions were approximately two-fold greater than those in methanolic extracts from untreated leaves (Figure 4.2C).

The reverse phase HPLC-DPPH profiles confirm that caftaric, chlorogenic and chicoric acids retain their stability (i.e. structure and antioxidant activity) following gastrointestinal digestion. There was no evidence that the three phenolics incurred structural transformations due to enzymatic action or pH variations that extinguished antioxidant activity (Figure 4.8). Gastrointestinal digestion facilitated the release of

caftaric, chlorogenic and chicoric acids from the leaves. Consistent with these results, chlorogenic acid has been shown to retain its structure in the stomachs of rats (Lafay *et al.*, 2006) and in ileostomized humans on a liquid chlorogenic acid supplement (Olthof *et al.*, 2001). Furthermore, chlorogenic acid is rapidly absorbed without structural transformations into the plasma in the stomachs of rats (Lafay *et al.*, 2006), in normal healthy humans (Monteiro *et al.*, 2007) and in ileostomized humans (Olthof *et al.*, 2001). Chlorogenic acid is not hydrolysed by intestinal enzymes during *in vitro* digestion (Plumb *et al.*, 1999a), though it was found to be transformed into neochlorogenic acid after 2 h of *in vitro* pancreatic digestion, attributable to the high pH (7.5) rather than to activities of pepsin or pancreatin (Bermúdez-Soto *et al.*, 2007). Similar to our results, the physiological pH (7.4) and temperature (37 °C) had no impact on structure and activity of chicoric acid during *in vitro* intestinal simulation (Rossetto *et al.*, 2008). Caftaric acid, too, maintains its structural integrity in the stomach of rats, and is rapidly absorbed into their plasma (Vanzo *et al.*, 2007).

The CAA of undigested and digested raw leaves were measured using Caco2 and HepG2 cells (Figure 4.4) since they are suitable models to study uptake and metabolism of antioxidants by cells, accordingly representing the intestinal epithelium and liver (Bornsek *et al.*, 2012). The *S. oleraceus* digested leaves were several-fold more effective in CAA (Figure 4.4A) than those values reported for fresh fruits and vegetables including fresh blueberries (Wolfe and Liu, 2007; Wolfe *et al.*, 2008; Song *et al.*, 2010). This indicates the potential of *S. oleraceus* leaves to protect human cells from oxidative stress is much higher than most plant food. Confocal laser scanning microscopy of HepG2 cells treated with *S. oleraceus* leaf extract and stained with Naturstoff reagent has confirmed that antioxidants entered the cells, rather than being bound to the cell membranes (McDowell *et al.*, 2011). Previous reports also verify that chlorogenic acid is readily taken up by human HepG2 cells (Mateos *et al.*, 2005), human Caco2 cells (Sato *et al.*, 2011), and protected human neuroblastoma SH-SY5Y cells from oxidative stress (Sato *et al.*, 2011). In addition, mouse erythrocytes (Ohnishi *et al.*, 1994) and granulocytes (Bouayed *et al.*, 2007) treated with chlorogenic acid were protected from $H_2O_2$ induced haemolysis and lipid peroxidation. Chicoric acid, too, was absorbed by neuron-like PC-12 cells extracted from rat pheochromocytoma, which were than protected from oxidative stress and maintained their viability (Heo *et al.*, 2010).

Chicoric, chlorogenic and caftaric acids are important dietary LMWAs (Clifford, 1999; Clifford, 2000b) with potent radical scavenging activities *in vitro* (Thygesen *et al.*, 2007; Rossetto *et al.*, 2008). Chicoric acid is comparable in activity to certain flavonoids and rosmarinic acid, which are efficient antioxidants (Thygesen *et al.*, 2007). It is several times more effective in scavenging peroxyl radicals than ascorbic acid in *in vitro* human intestinal conditions (Rossetto *et al.*, 2008). Chlorogenic acid was, respectively, three- and seven-fold more potent than ascorbic acid and trolox in simulated human intestinal conditions (Rossetto *et al.*, 2008). Despite their strong antioxidant activities, studies on bioavailability and stability of chicoric acid (Bailly and Cotelle, 2005) and caftaric acid (Nuissier *et al.*, 2010) are far less documented compared to chlorogenic acid. My data confirm that these hydroxycinnamic acids in *S. oleraceus* leaves were stable following gastrointestinal digestion as quantified by *in vitro* and cellular measures of antioxidant activity.

In conclusion, gastrointestinal digestion of *S. oleraceus* leaves resulted in a progressive extraction of hydroxycinnamic acids and ascorbate with corresponding antioxidant activities, where raw leaves were slightly superior to boiled leaves. The antioxidant activities of hydroxycinnamic acids were stable during gastrointestinal digestion, and displayed antioxidant activity inside HepG2 and Caco2 cells. Therefore these *in vitro* studies demonstrate that *S. oleraceus* raw leaves are an excellent dietary antioxidant source.

# CHAPTER 5: EXTRACTABLE ANTIOXIDANT ACTIVITIES OF CELL SUSPENSION CULTURES OF *Sonchus oleraceus* L. IN RELATION TO ABIOTIC STRESSORS

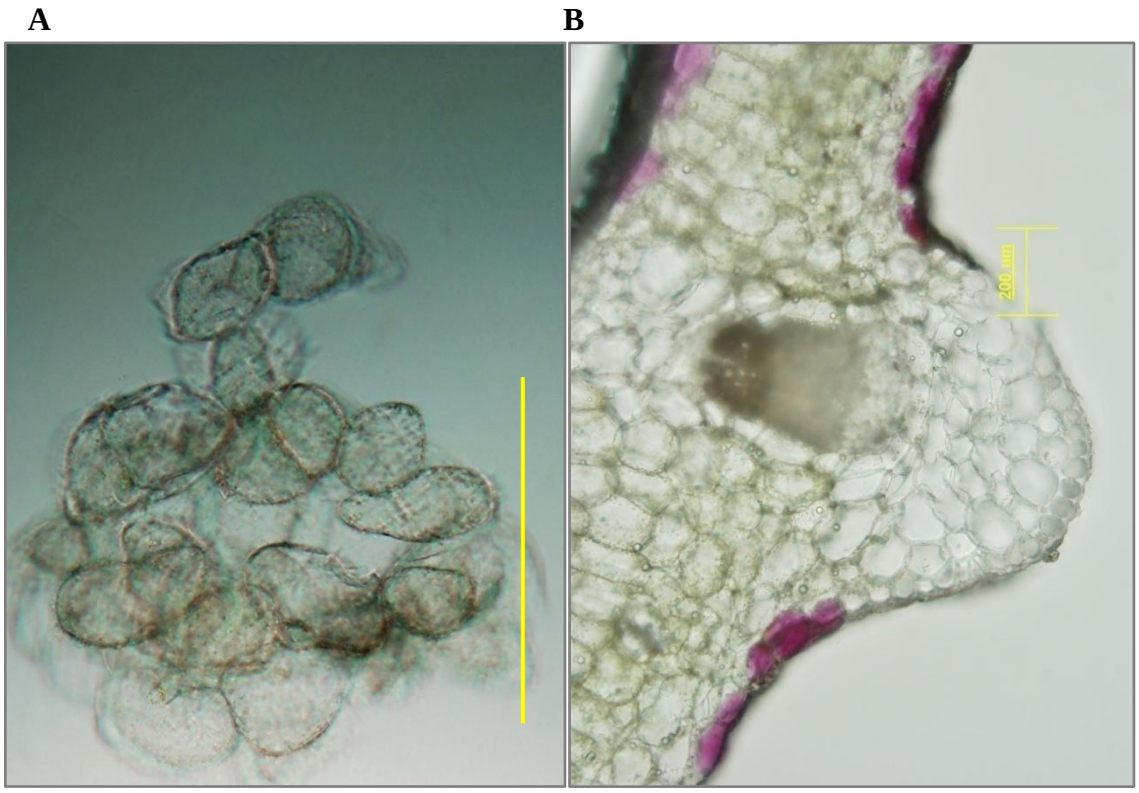



**Plate 5.1 Light micrographs of *Sonchus oleraceus* L. (A) cells in suspension cultures (bar = 50 µm) and (B) a transverse section of a leaf from a greenhouse grown plant (bar = 200 µm).**

## 5.1 ABSTRACT

Cell suspension cultures of *Sonchus oleraceus* L. may provide a continuous, efficient antioxidant production system unhindered by climatic and phenological effects associated with extraction from plants. Therefore the aim of this work was to examine the effects of abiotic stressors on extractable antioxidant activities, and concentrations of ascorbate and phenolic compounds in *S. oleraceus* cell suspension cultures of two ecotypes. Cell suspension cultures were initiated from *in vitro* shoots of two ecotypes originating from Acacia Bay (ACB) and Oamaru (OAM) in New Zealand. Cells were exposed to chilling, salinity and the combination of both for three weeks, and extractable antioxidant activities, concentrations of ascorbate and phenolic compounds were measured for calli and media. In both ecotypes all stressors increased antioxidant activities, and concentrations of total phenolics, ascorbate and chlorogenic acid compared to the control. The most effective stressor differed between ecotypes; the stressor combination for ACB, and chilling for OAM. Calli of ACB ecotype were more potent than OAM in antioxidant activities, and held higher concentrations of total phenolics, hydroxycinnamic acids and ascorbate irrespective of the stressor. At the stationary phase, calli and media of both ecotypes yielded highest extractable antioxidant activities, concentrations of ascorbate, hydroxycinnamic acids and total phenolics irrespective of the stressor. My data indicate that abiotic stressors can be used to augment antioxidant activities of *S. oleraceus* cells in suspension cultures.

**KEYWORDS**: *Sonchus oleraceus*, cell suspension culture, antioxidant, phenolics, chilling, salinity

## 5.2 INTRODUCTION

Twenty five percent of health-promoting chemicals in all prescribed pharmaceuticals in industrialized countries are still extracted from plants (Namdeo, 2007). Of firms that produced, distributed or sold bioactive compounds, 51% dealt in plant based LMWAs (Tebbens, 2002). This was because the consumer preference for a particular health-promoting biochemical was more if they were; plant based rather than of "industrial origin", extracted from a familiar food plant and possessed validated physiological benefits beyond basic nutritional functions (Marriott, 2000; Williams *et al*., 2004).

However, extraction of the chemicals directly from the plants is problematic since in most cases LMWAs were present at low levels, or accumulated only in a specific tissue and at a specific growth stage or upon certain growth or environmental conditions (Chapter 1; Section 1.10). In *Sonchus oleraceus* (Compositae) too, yields of LMWAs were variable due to climatic, phenological and genetic factors (Chapter 3). For these reasons cell cultures of *S. oleraceus* may be a more reliable commercial method for the production and extraction of antioxidants since they lack shortcomings associated with extracting from plants. *In vitro* cell cultures of number of other plant species have already been developed to extract LMWAs successfully (Table 5.1 – 5.2; Chapter 1; Section 1.10). Further, it may be possible to use abiotic stimuli such as chilling and salinity to further enhance LMWA production in *S. oleraceus* cell cultures. The application of biotic or abiotic stimuli, otherwise known as elicitors, is a proven strategy for increasing secondary metabolite production in some plant cell cultures (Chapter 1; Section 1.10; Table 1.27). An elicitor is defined as a substance which, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds (Radman *et al.*, 2003). Biotic elicitors are derived from live organisms, for example pectin, chitin and glucan of fungal and yeast origin, while inorganic salts (NaCl), heat shock, chilling, heavy metal ions, UV radiation and ultrasound are classified as abiotic elicitors (Zhou *et al.*, 2011).

**Table 5.1 Selected patents for production of phenolic compounds from plant cell suspension cultures**

| Phenolic compounds | Plant species | Patent number | References |
|---|---|---|---|
| Resveratrol | *Vitis vinifera* | US7309591B2 | (Martinez *et al*., 2007) |
| Rutin, Quercetin, Quercetin-3-glucoside, Kaempferol, Kaempferol-3-rutinoside, Naringenin, Naringenin-7-glucoside | *Solanum esculentum* | US20030101477A1 | (Colliver *et al*., 2003) |
| Catechin | *Taxus wallichiana* | US6620599B1 | (Chattopadhyay *et al*., 2003) |
| Isoflavone | *Glycine max* | US7354765B2 | (Federici *et al*., 2008) |
| Procyanidins | *Theobroma* spp. | US8568798B2 | (Venkatramesh *et al*., 2013) |
| Polyphenols | *Crocus sativus* | WO2013156862A1 | (Yoon *et al*., 2013) |

**Table 5.2 Selected commercial applications of plant cell suspension cultures for the production of LMWAs**

| LMWAs | Commercial product | Plant species | Manufacturer | Websites[1] |
|---|---|---|---|---|
| Procyanidins | Cocovanol | *Theobroma cacao* | DianaPlantSciences Inc., Portland, Oregon, USA | http://plantcellculture.com/aboutplantcellculture.html |
| Ginseng | Tissue cultured ginseng | *Panax ginseng* | Nitto Denko Medical Corporation, Osaka, Japan | http://www.nitto.com/about_us/corporate/history/ |
| Rosmarinic acid | Rosmarinic acid | *Coleus blumei* | A. Nattermann a Cie GmbH, Cologne, Germany | http://www.natterman.nl/index.html |

[1] accessed December 2013

Studies on effect of abiotic stressors on antioxidant activities of *S. oleraceus* cell suspension cultures have not yet been studied. Thus, it was hypothesized that antioxidant activities of calli and media obtained from *S. oleraceus* cell suspension cultures are promoted by abiotic stressors.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Chemicals

Murashige and Skoog (MS) medium, α-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), agar, sucrose, NaClO and glass vessels were purchased from Sigma-Aldrich (St Louis, MO). All other chemicals are listed at Section 2.1.

### 5.3.2 *In vitro* culture conditions

Axenically grown plants, callus cultures and cell suspension cultures were maintained in a sterile growth room at 25 $^{o}$C and 16 h photoperiod provided from cool white fluorescent tubes (94 µmol $m^{-2}$ $s^{-1}$). All cultures were maintained in cylindrical glass vessels (6 x 10 cm) secured with clear plastic caps.

### 5.3.3 Establishment of *in vitro* plants

Plants were raised from seeds obtained from selfed F1 generation plants, which were themselves raised from seeds collected from wild populations at Acacia Bay and Oamaru, New Zealand (Chapter 3; Section 3.3.1; Table 3.1). The seeds were surface sterilized by rinsing in 70% ethanol for 5 min followed by 10% NaClO for 15 min and then using sterile dd$H_2O$ for 5 min. The seeds were soaked in sterile dd$H_2O$ and kept in dark for 12 h. They were seeded at the rate of 10 – 15 seeds $vessel^{-1}$ in 50 mL MS medium supplemented with 6 g $L^{-1}$ agar and 87.7 mM sucrose, devoid of growth regulators.

### 5.3.4 Establishment of callus culture

Callus cultures of *Sonchus oleraceus* were initiated using leaf explants (1.0 x 1.0 cm) excised from 21 day old *in vitro* plants. The leaf explants were established on 50 mL MS medium supplemented with 6 g $L^{-1}$ agar, 87.7 mM sucrose, 5.4 mM NAA and 4.4 mM BAP. The growth regulator concentrations used here were determined through preliminary trials (Appendix B.1). Subculturing was performed at 21 day intervals.

### 5.3.5 Establishment of cell suspension culture and culture conditions

Suspension cultures were initiated using 21 day old callus. The cultures were maintained in 50 mL of MS liquid medium supplemented with 87.7 mM sucrose, 10.7 mM NAA and 4.4 mM BAP. The growth regulator concentrations used here were established through preliminary trials (Appendix B.2).

Cultures were maintained on a rotary shaker at 50 rpm. Suspension cultures were subcultured at 21 day interval. The sixth subculture was used to initiate suspension cultures used in Section 5.3.6, using 0.5 g cell FW $vessel^{-1}$.

### 5.3.6 Treatments

Seven day old suspension cultures were exposed to chilling, salinity or the combination of both for three weeks. Control cell suspensions were maintained continuously in the growth room. For the chilling treatment, cell suspensions were repeatedly held at 5 $^{o}$C for 8 h in dark and returned to the light in the growth room. Salinity was imposed by dissolving NaCl in the medium to obtain 50 mM. The fourth group of suspension cultures was given both the chilling and salinity treatments. Aliquots (10 mL $vessel^{-1}$) were withdrawn from 7, 14, 21 and 28 day old cell suspension cultures.

### 5.3.7 Phytochemical extraction

The aliquots were vacuum filtered to separate cells and extracellular medium. The cell fresh and dry biomass was measured. The relative growth rate (RGR) for cell dry biomass accumulation was calculated as:

$RGR = [(W_1 - W_0)/(t_1 - t_0)W_0]x100$

Where $W_0$ is the biomass at time $t_0$, $W_1$ is biomass at time $t_1$ and $t_1 - t_0$ is one week.

Calli intended for phytochemical analysis were prepared according to Section 3.3.3. The extracellular medium was vacuum dried and resuspended in 1.5 mL of methanol: $ddH_2O$: acetic acid (70:23:7, v/v/v). Calli and media extracts were stored under nitrogen at −20 $^{o}$C and phytochemical analyses were performed within 7 d of extraction.

The analysis of total phenolic content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities, oxygen radical absorbance capacity (ORAC) assay, ascorbate content for cells and medium, were performed as described in Sections 2.2 – 2.5. Online reverse phase HPLC was performed for cells and medium as described in Section 2.8 without post column reaction with DPPH reagent.

## 5.4 STATISTICAL ANALYSIS

Repeated measures ANOVA with Bonferroni post hoc tests ($P< 0.05$) were performed to identify significance of treatments. Analyses were performed using SPSS 18.0 statistical software.

## 5.5 RESULTS

### 5.5.1 *In vitro* plants, callus and suspension cultures of *S. oleraceus* L.

The seeds germinated rapidly and well developed cotyledons were present in seven day old cultures (Plate 5.2A). The *in vitro* plant had developed two to four true leaves at day 21 (Plate 5.2B). Callus cultures had rapid proliferation and appeared green and friable at day 21 (Plate 5.2C).

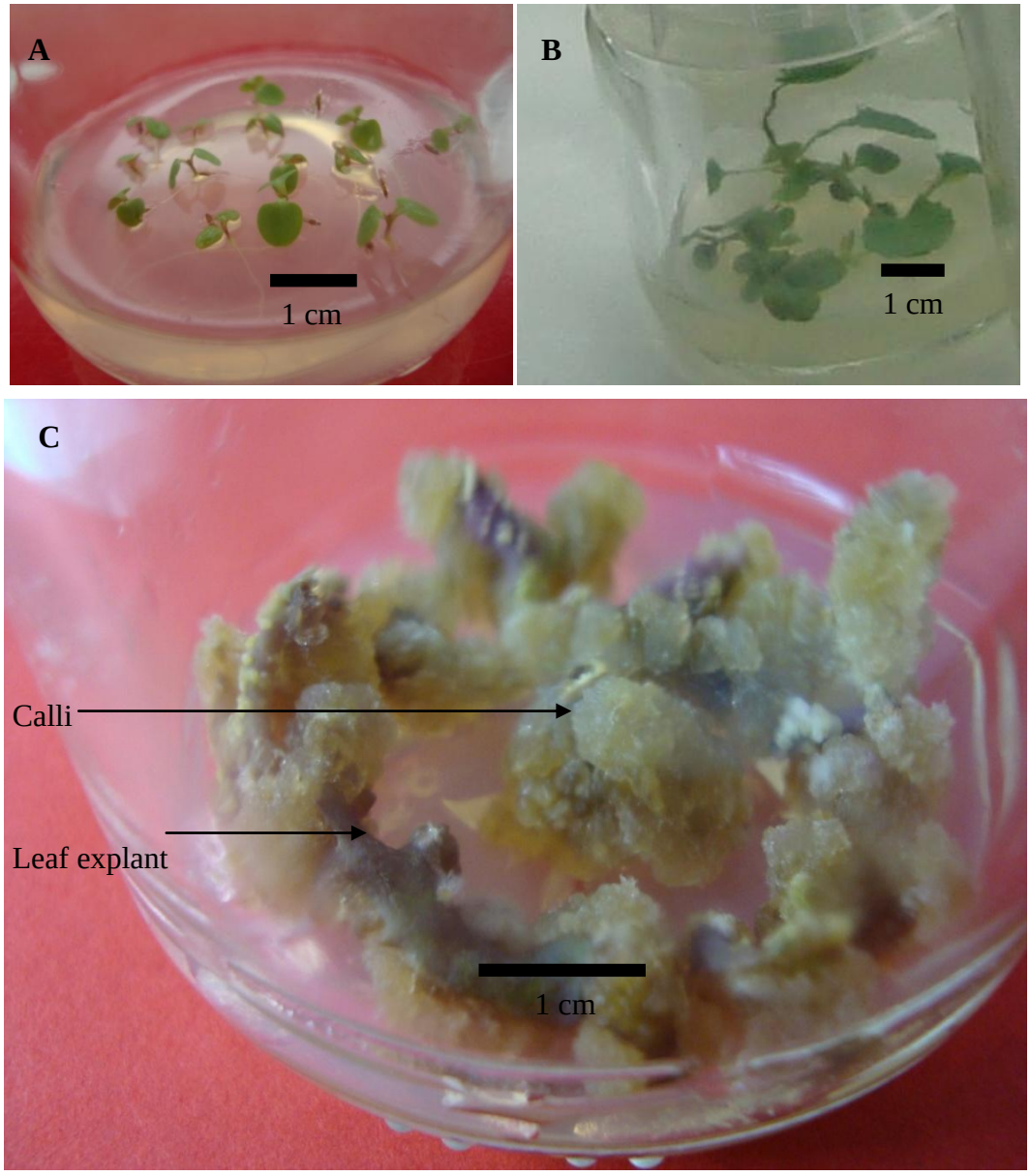



**Plate 5.2 *Sonchus oleraceus* L. (A) 7 day old, (B) 21 day old *in vitro* plants and (C) 21 day old callus cultures on Murashige and Skoog medium.**

Seven day old suspension cultures appeared turbid and yellowish green (Plate 5.3A) and 28 day old cultures were green and formed cell aggregates (Plate 5.3B).

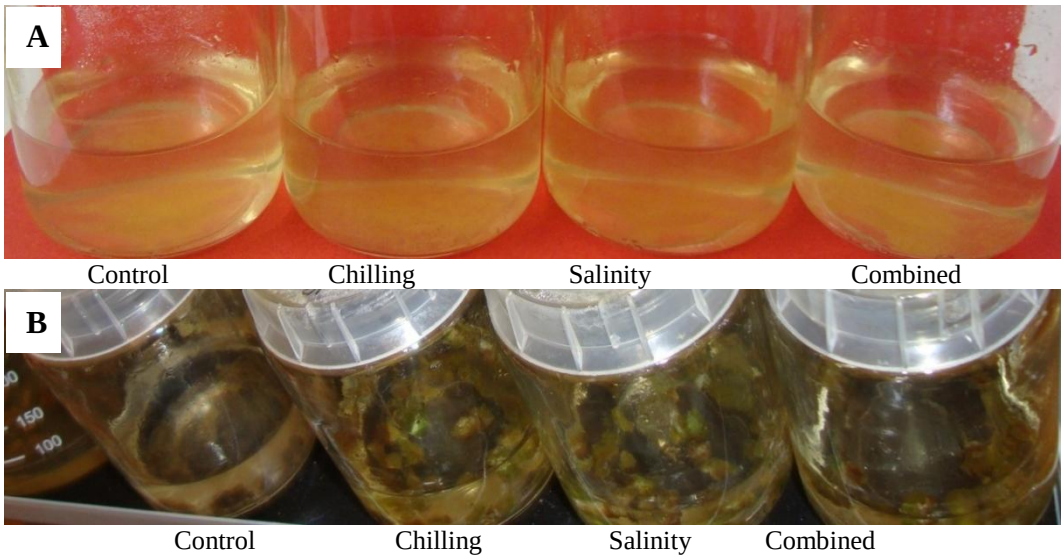



**Plate 5.3 Suspension cultures of *Sonchus oleraceus* L. at (A) 07 and (C) 28 days.**

### 5.5.2 Biomass of cells from suspension cultures

Accumulation of cell biomass did not vary significantly across ecotypes or stressor treatments (ANOVA; $P$ = 0.48; Figure 5.1). At day 21, the cell dry mass of cultures was maximal (0.6 ± 0.1 mg $mL^{-1}$), which was double the weight at day 7 ($P$< 0.001; Figure 5.1).

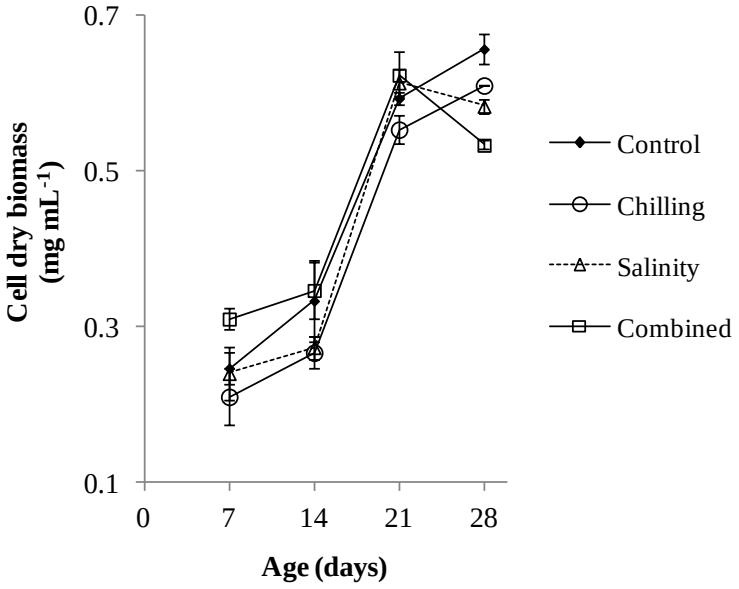



**Figure 5.1 Changes in dry biomass of cells from suspension cultures of *Sonchus oleraceus* L. Means ± SE ($n$ = 12).**

The growth rate of cultures indicated three distinct growth phases: lag, linear and stationary phases (Table 5.3). During the initial lag phase, cell dry biomass accumulation rate was low. The highest growth rate was during the linear phase. Onset of the stationary growth phase occurred following day 21. The stationary phase had the lowest growth rate.

**Table 5.3 Rates of cell dry biomass accumulation in suspension cultures of *Sonchus oleraceus* L.**

| Culture age (days) | Relative growth rate[a] (mg 100 mg$^{-1}$ week$^{-1}$) | Growth phase |
|---|---|---|
| 7 – 14 | 28 ± 7 | Lag phase |
| 14 – 21 | 110 ± 12 | Linear phase |
| 21 – 28 | 1 ± 3 | Stationary phase |

[a]Means ± SE ($n$ = 48).

### 5.5.3 Effects of abiotic stressors on extractable antioxidant activities, concentrations of total phenolics, hydroxycinnamic acids and ascorbate

Stressors increased the extractable antioxidant activities and concentrations of total phenolics, ascorbate and hydroxycinnamic acids excluding chicoric acid (Figures 5.2 – 5.8). These effects were seen in calli and media of both ecotypes.

In calli, all stressors enhanced the: extractable antioxidant activities measured as DPPH radical scavenging capacities ($P$< 0.001) and ORAC values ($P$< 0.001), concentrations of total phenolics ($P$< 0.001), chlorogenic acid ($P$< 0.001) and ascorbate ($P$< 0.001) compared to the controls (Figures 5.2A,B – 5.5A,B and 5.8A,B). Concentrations of caftaric acid were increased by the stressor combination and by salinity compared to the control, though only in ACB ($P$< 0.001; Figure 5.6A); these treatments did not significantly affect caftaric acid levels in OAM (Figure 5.6B). Treatments did not affect concentration of chicoric acid in both ecotypes ($P$ = 0.72; Figure 5.7A,B). The stressor which caused highest improvement in antioxidant activities and concentrations of LMWAs differed between ecotypes; stressor combination in ACB (Figures 5.2A – 5.6A and 5.8A), and chilling in OAM (Figures 5.2B – 5.5B and 5.8B). The stressor-induced increases compared to their respective controls were greater in ACB (Figures 5.2A – 5.6A and 5.8A) than in OAM (5.2B – 5.5B and 5.8B).

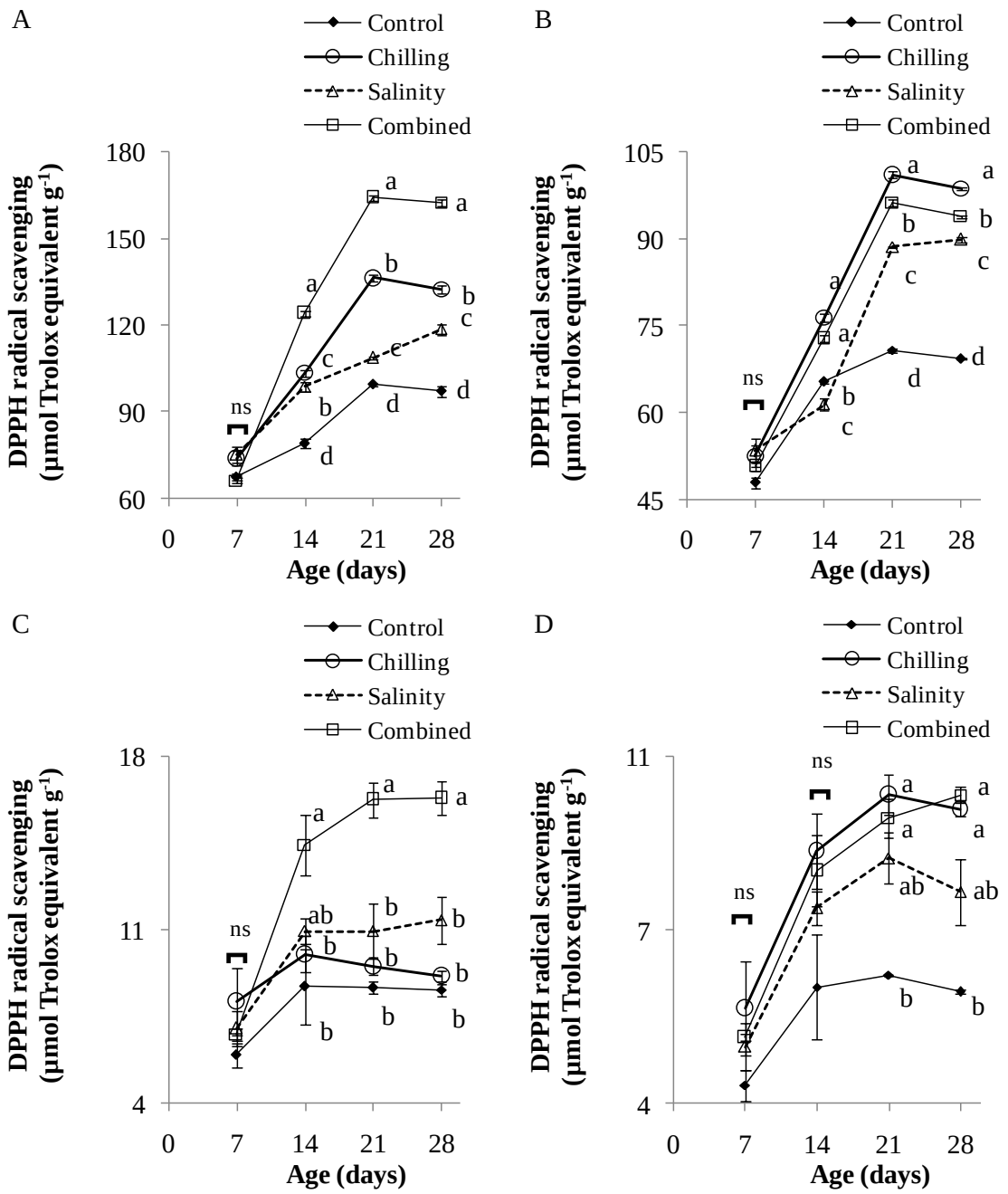



**Figure 5.2 Changes in DPPH radical scavenging capacity in calli (A,B) and media (C,D) of ACB (A,C) and OAM (B,D) ecotypes of *Sonchus oleraceus* L. suspension cultures. Means ± SE (*n* = 6). Data points with different letters indicate significant differences between treatments within the time point (*P*< 0.05). ns: Treatments are not significantly different within the time point (*P*> 0.05).**

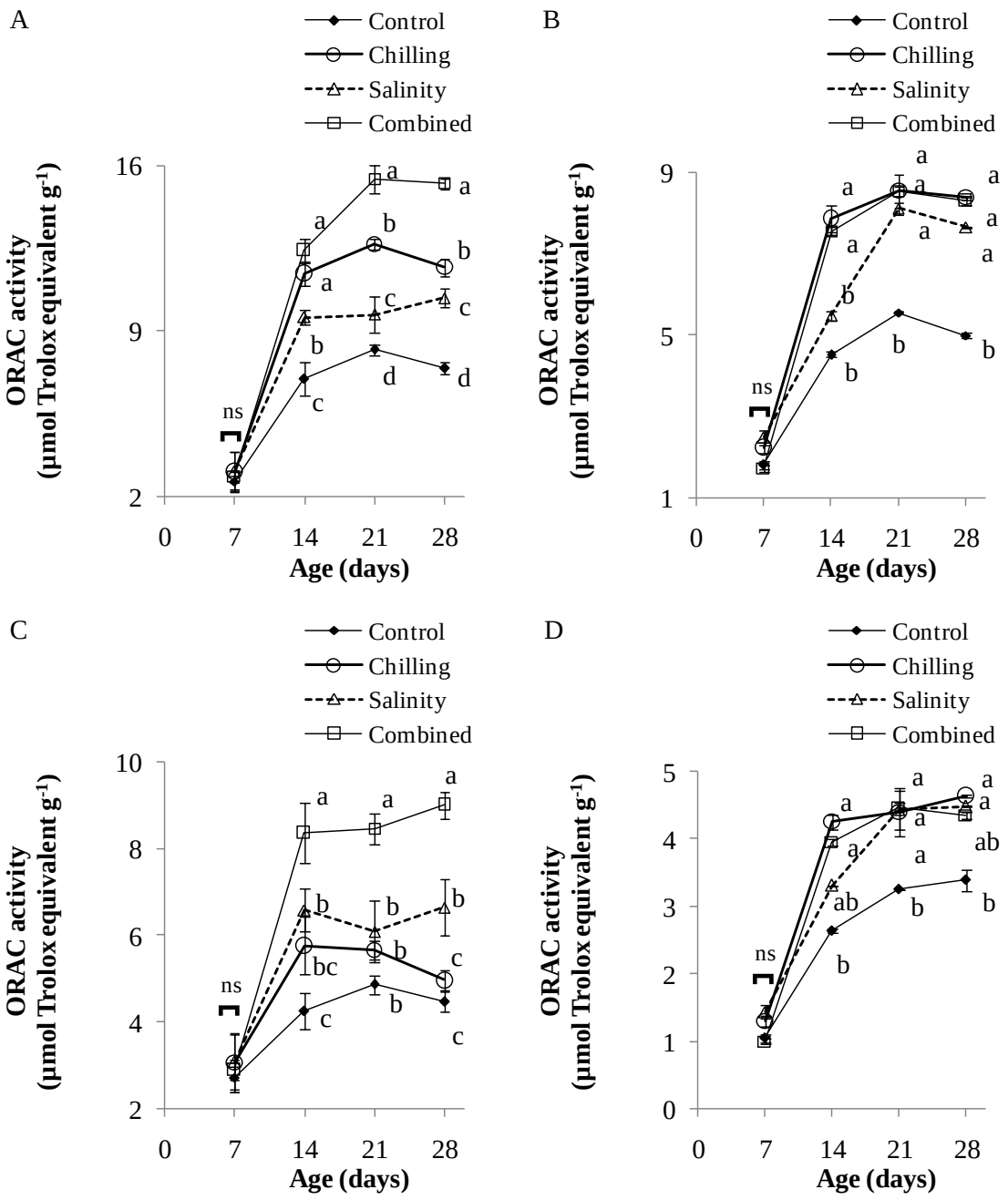



**Figure 5.3 Changes in ORAC activity in calli (A,B) and media (C,D) of ACB (A,C) and OAM (B,D) ecotypes of *Sonchus oleraceus* L. suspension cultures. Means ± SE (*n* = 6). Data points with different letters indicate significant differences between treatments within the time point (*P*< 0.05). ns: Treatments are not significantly different within the time point (*P*> 0.05).**

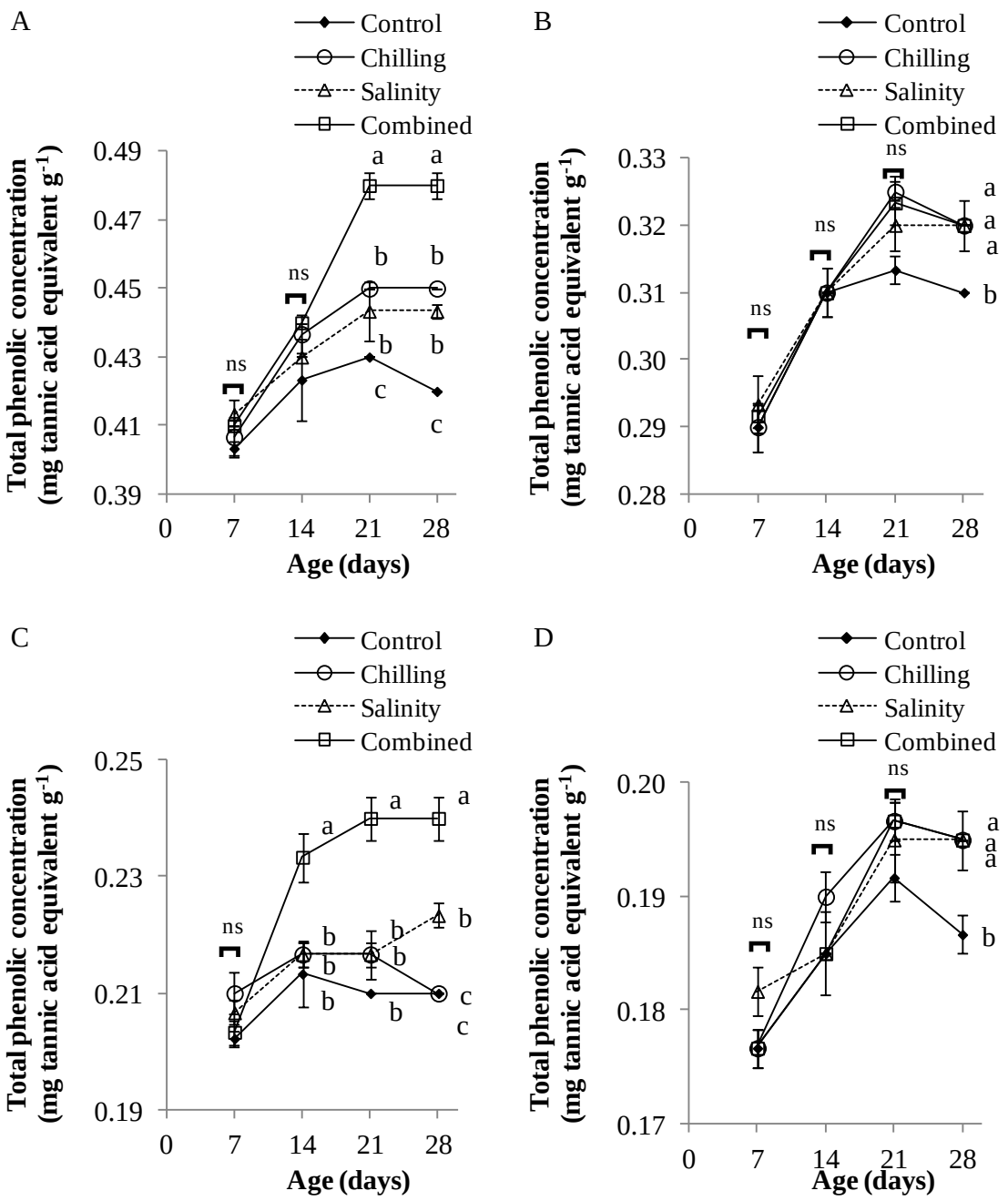



**Figure 5.4 Changes in concentration of total phenolics in calli (A,B) and media (C,D) of ACB (A,C) and OAM (B,D) ecotypes of *Sonchus oleraceus* L. suspension cultures. Means ± SE (*n* = 6). Data points with different letters indicate significant differences between treatments within the time point (*P*< 0.05). ns: Treatments are not significantly different within the time point (*P*> 0.05).**

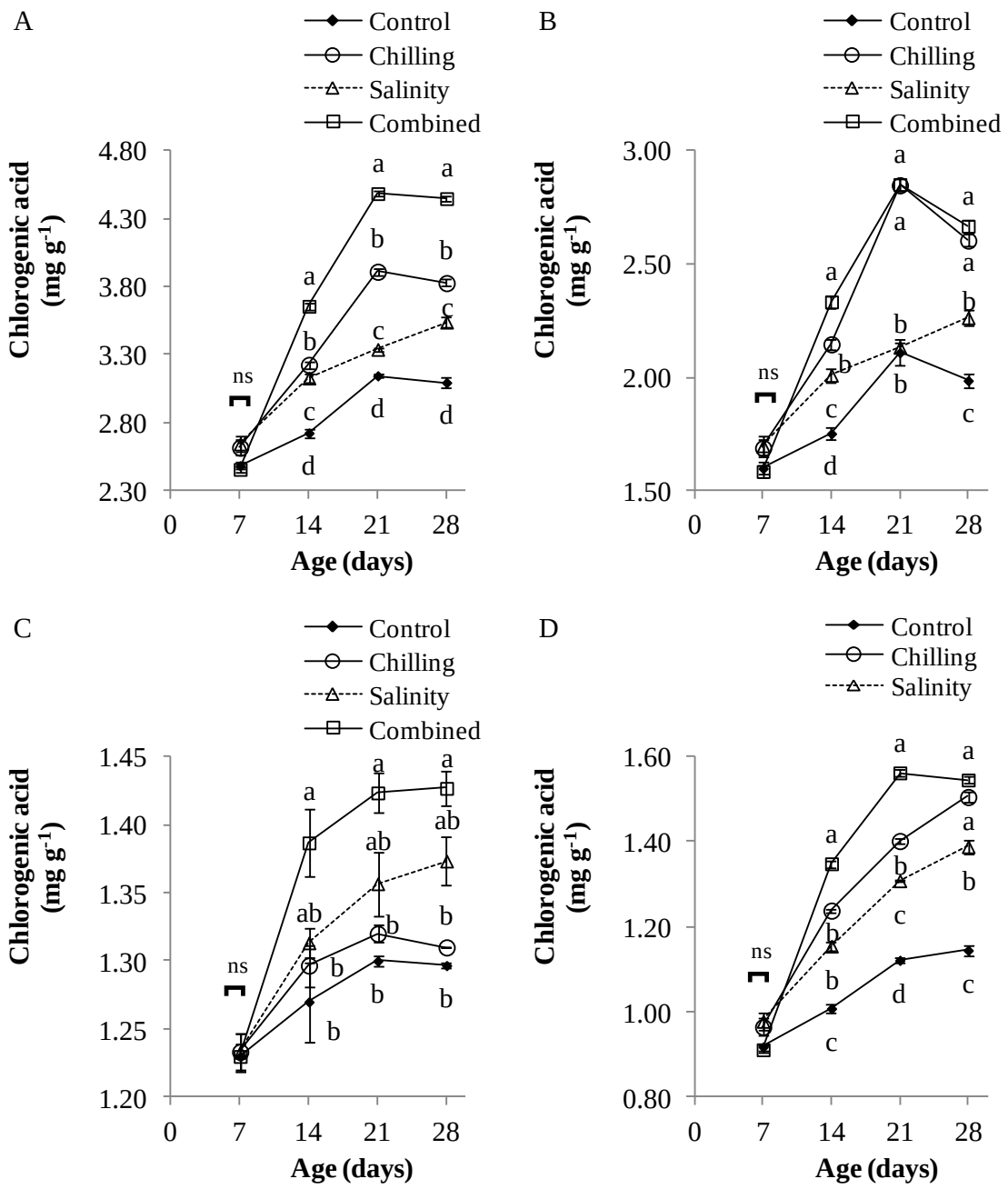



**Figure 5.5 Changes in concentration of chlorogenic acid in calli (A,B) and media (C,D) of ACB (A,C) and OAM (B,D) ecotypes of *Sonchus oleraceus* L. suspension cultures. Means ± SE (*n* = 6). Data points with different letters indicate significant differences between treatments within the time point (*P*< 0.05). ns: Treatments are not significantly different within the time point (*P*> 0.05).**

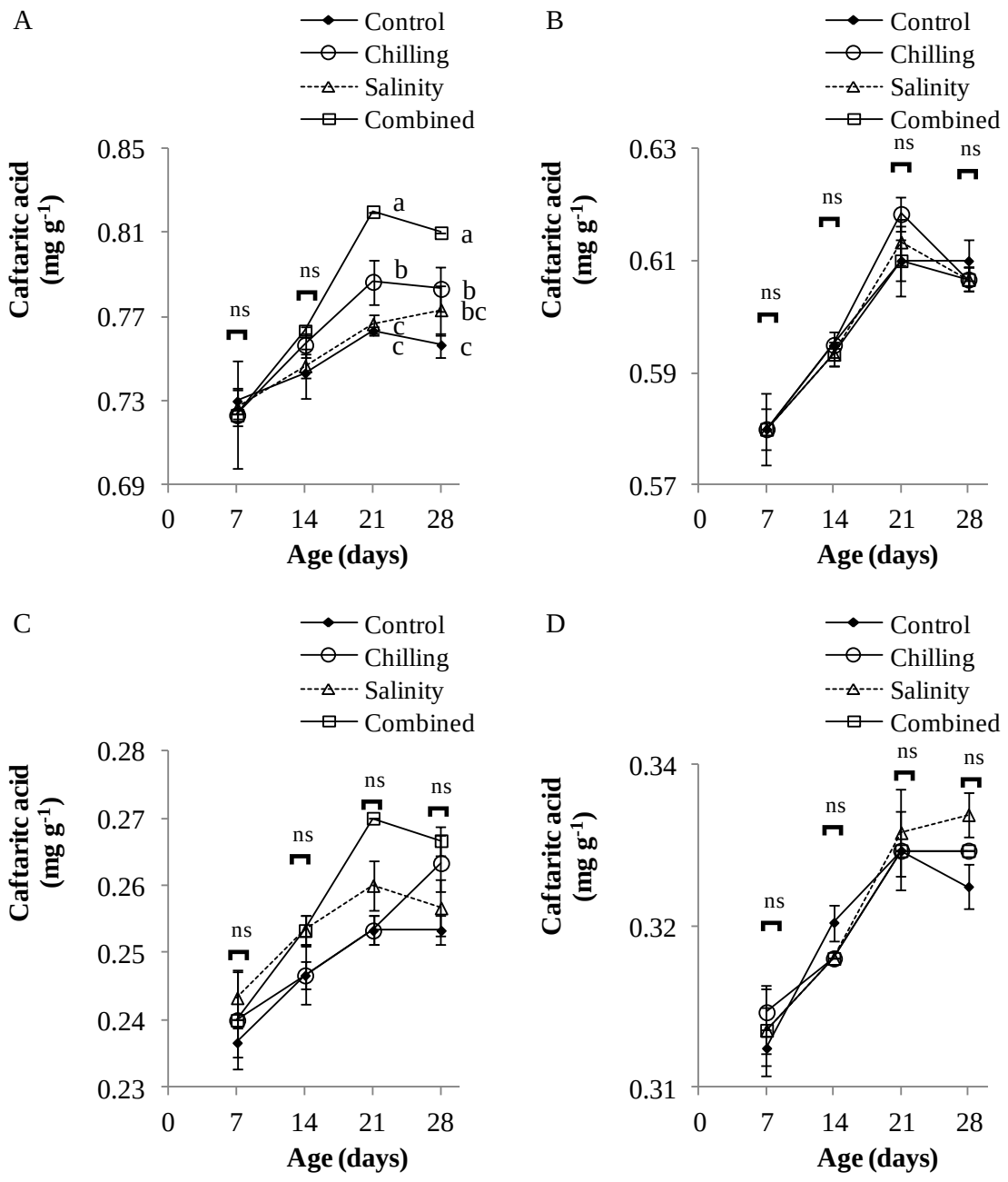



**Figure 5.6 Changes in concentration of caftaric acid in calli (A,B) and media (C,D) of ACB (A,C) and OAM (B,D) ecotypes of *Sonchus oleraceus* L. suspension cultures. Means ± SE (*n* = 6). Data points with different letters indicate significant differences between treatments within the time point (*P*< 0.05). ns: Treatments are not significantly different within the time point (*P*> 0.05).**

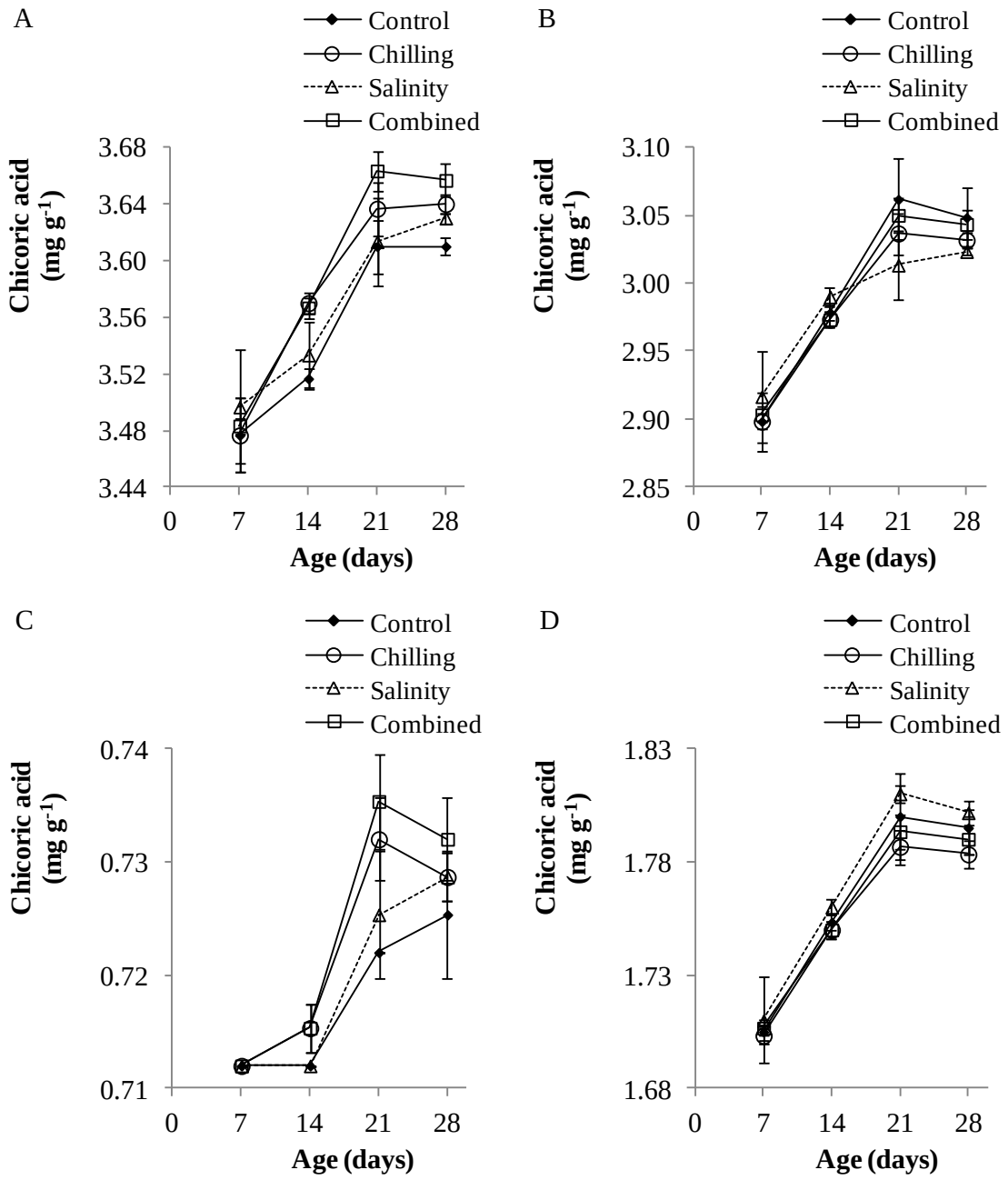



**Figure 5.7 Changes in concentration of chicoric acid in calli (A,B) and media (C,D) of ACB (A,C) and OAM (B,D) ecotypes of *Sonchus oleraceus* L. suspension cultures. Means ± SE (*n* = 6). Treatments are not significantly different (*P*> 0.05).**

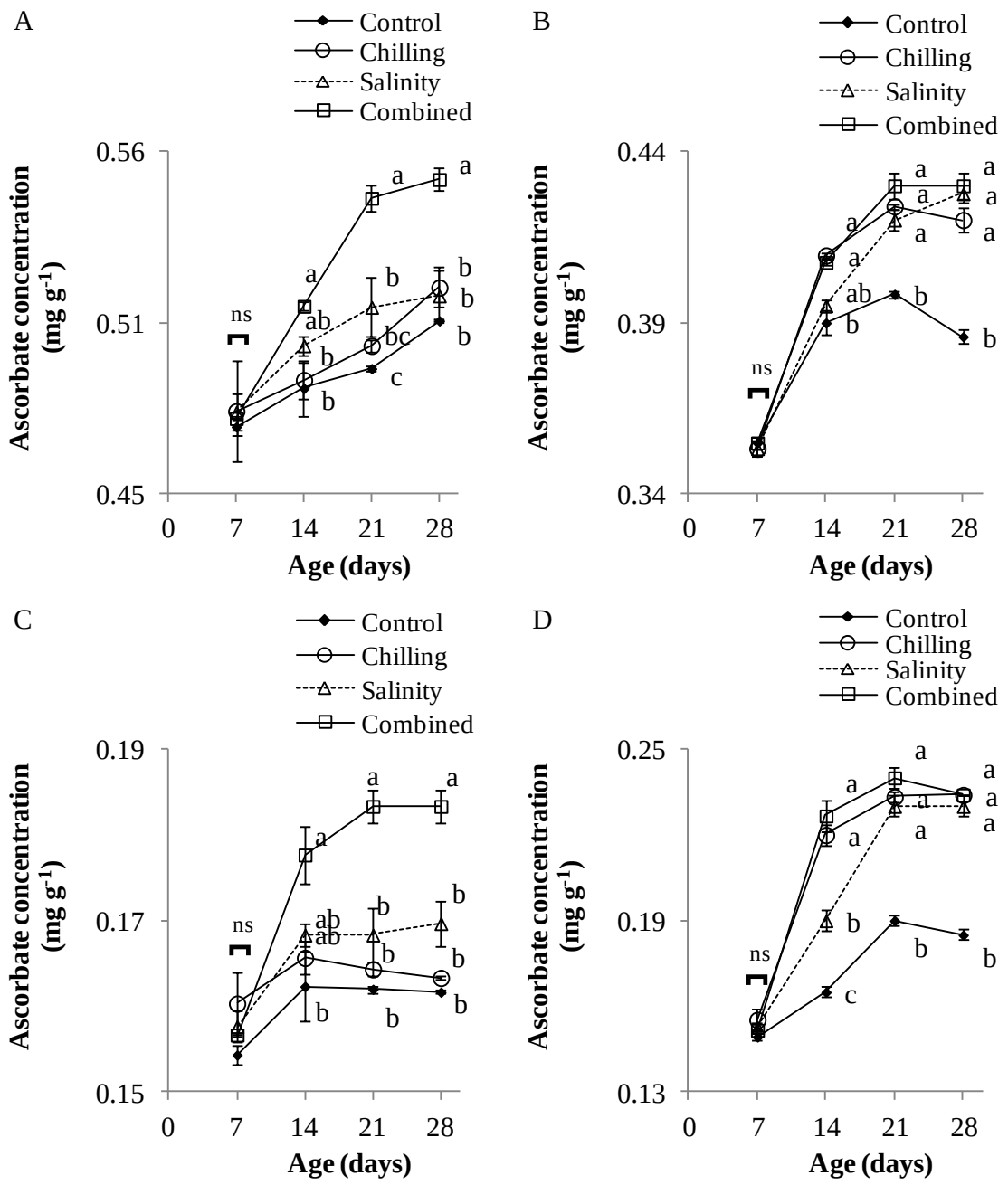



**Figure 5.8 Changes in concentration of ascorbate in calli (A,B) and media (C,D) of ACB (A,C) and OAM (B,D) ecotypes of *Sonchus oleraceus* L. suspension cultures. Means ± SE (*n* = 6). Data points with different letters indicate significant differences between treatments within the time point (*P*< 0.05). ns: Treatments are not significantly different within the time point (*P*> 0.05).**

The culture medium itself displayed higher antioxidant activities and held higher concentrations of total phenolics, chlorogenic acid and ascorbate after treatments of the plant cells (Figures 5.2C,D – 5.5C,D and 5.8C,D). However, these changes were less prominent than those that occurred in the calli (Figures 5.2A,B – 5.5A,B and 5.8A,B). Treatments did not significantly affect concentrations of caftaric and chicoric acids in the media (Figures 5.6C,D – 5.7C,D).

### 5.5.4 Ecotypes differ in extractable antioxidant activities, concentrations of total phenolics, hydroxycinnamic acids and ascorbate

Calli of ACB were 40 – 50% more potent than OAM in antioxidant activities as measured by DPPH radical scavenging ($P< 0.001$) and ORAC activities ($P< 0.001$). ACB calli also had 20 – 50% more concentrations of total phenolics ($P< 0.001$), chlorogenic acid ($P< 0.001$), caftaric acid ($P< 0.001$), chicoric acid ($P< 0.001$) and ascorbate ($P< 0.001$) irrespective of the stressor applied, and across all growth phases (Figures 5.2A,B – 5.8A,B).

The culture medium that had contained ACB calli held 20 – 50% of the LMWA load of calli whereas media of OAM cultures held 50 – 60% LMWA concentrations of its calli (Figures 5.2C,D – 5.8C,D). This was true for all the stressors and growth phases.

### 5.5.5 Growth phases differ in extractable antioxidant activities, concentrations of total phenolics, hydroxycinnamic acids and ascorbate

DPPH radical scavenging ($P< 0.001$), ORAC activities ($P< 0.001$), total phenolics ($P< 0.001$), and concentrations of chlorogenic ($P< 0.001$), caftaric ($P< 0.001$), chicoric ($P< 0.001$) and ascorbic acids ($P< 0.001$) were all greatest at the onset of the stationary phase, both in calli and the culture media for all ecotypes and treatments (Figures 5.2 – 5.8).

### 5.5.6 Extractable antioxidant activities, and concentrations of total phenolics, hydroxycinnamic acids and ascorbate are lower in media than in calli

The nutrient media from both ecotypes had consistently lower DPPH radical scavenging ($P< 0.001$) and ORAC activities ($P< 0.001$), as well as lower concentrations of total

phenolics ($P< 0.001$), hydroxycinnamic acids ($P< 0.001$) and ascorbate ($P< 0.001$) compared to the calli themselves (Figures 5.2 – 5.8).

### 5.5.7 Comparison of LMWA concentrations in extracts from calli of suspension cultures and from leaves of greenhouse grown plants

Stressor-induced concentrations of LMWAs extracted from calli were 30 – 80% lower than those from the leaves of similar stressor-imposed greenhouse grown plants (Table 5.4). However, in ACB the concentration of chlorogenic acid in calli extracts were three-fold higher than leaf extracts, when controls of youngest suspensions and plants were compared (Table 5.4). The LMWAs accumulated rapidly with lower variability in calli than in leaves (Table 5.4).

**Table 5.4 Comparison of phytochemical concentrations in extracts from calli of suspension cultures and from leaves of greenhouse grown plants of ACB and OAM ecotypes of *Sonchus oleraceus* L. following treatments: control, chilling, salinity and combination of the two.**

| Age (weeks) [Calli/ plant] | Treatment | Sample | Ascorbate concentration (mg $g^{-1}$) | | Hydroxycinnamic acid concentration (mg $g^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Caftaric | | Chlorogenic | | Chicoric | |
| | | | ACB | OAM | ACB | OAM | ACB | OAM | ACB | OAM |
| [2/10] | Control | Calli[1] | 0.5±0.0 | 0.4±0.0 | 0.7±0.0 | 0.6±0.0 | 2.7±0.0 | 1.8±0.0 | 3.5±0.1 | 3.0±0.1 |
| | | Leaf[2] | 0.2±0.1 | 0.3±0.0 | 2.0±0.6 | 1.2±0.1 | 0.9±0.1 | 1.1±0.1 | 5.4±1.2 | 5.2±0.1 |
| | | Ratio[3] | 2.3 | 1.1 | 0.4 | 0.5 | 3.0 | 1.5 | 0.6 | 0.6 |
| | Chilling | Calli | 0.5±0.0 | 0.4±0.0 | 0.8±0.0 | 0.6±0.0 | 3.2±0.0 | 2.1±0.0 | 3.6±0.0 | 3.0±0.0 |
| | | Leaf | 0.4±0.0 | 0.3±0.0 | 1.9±0.2 | 1.2±0.1 | 3.9±0.4 | 2.3±0.1 | 6.9±0.8 | 5.2±0.3 |
| | | Ratio | 1.2 | 1.2 | 0.4 | 0.5 | 0.8 | 1.0 | 0.5 | 0.6 |
| | Salinity | Calli | 0.5±0.0 | 0.4±0.0 | 0.7±0.0 | 0.6±0.0 | 3.1±0.0 | 2.0±0.0 | 3.5±0.0 | 3.0±0.0 |
| | | Leaf | 0.4±0.0 | 0.4±0.0 | 1.5±0.2 | 1.3±0.1 | 3.8±0.3 | 2.2±0.1 | 6.2±0.7 | 5.1±0.2 |
| | | Ratio | 1.3 | 1.0 | 0.5 | 0.5 | 0.8 | 0.9 | 0.6 | 0.6 |
| | Combined | Calli | 0.5±0.0 | 0.4±0.0 | 0.8±0.0 | 0.6±0.0 | 3.7±0.0 | 2.3±0.0 | 3.6±0.0 | 3.0±0.0 |
| | | Leaf | 0.3±0.1 | 0.4±0.0 | 1.7±0.4 | 1.3±0.1 | 3.9±1.0 | 2.1±0.0 | 6.0±1.2 | 5.2±0.2 |
| | | Ratio | 1.8 | 1.2 | 0.5 | 0.5 | 0.9 | 1.1 | 0.6 | 0.6 |
| [3/12] | Control | Calli | 0.5±0.0 | 0.4±0.0 | 0.8±0.0 | 0.6±0.0 | 3.1±0.0 | 2.1±0.1 | 3.6±0.0 | 3.1±0.0 |
| | | Leaf | 1.1±0.0 | 0.9±0.1 | 2.6±0.2 | 2.1±0.2 | 3.4±0.2 | 3.3±0.0 | 9.0±0.2 | 8.5±0.3 |
| | | Ratio | 0.5 | 0.4 | 0.3 | 0.3 | 0.9 | 0.6 | 0.4 | 0.4 |
| | Chilling | Calli | 0.5±0.0 | 0.4±0.0 | 0.8±0.0 | 0.6±0.0 | 3.9±0.0 | 2.8±0.0 | 3.6±0.0 | 3.8±0.0 |
| | | Leaf | 1.2±0.1 | 0.9±0.1 | 2.3±0.3 | 2.1±0.2 | 4.1±0.2 | 3.5±0.1 | 8.6±0.8 | 8.0±0.5 |
| | | Ratio | 0.4 | 0.4 | 0.3 | 0.3 | 1.0 | 0.8 | 0.4 | 0.4 |
| | Salinity | Calli | 0.5±0.0 | 0.4±0.0 | 0.8±0.0 | 0.6±0.0 | 3.3±0.0 | 2.1±0.0 | 3.6±0.0 | 3.0±0.0 |
| | | Leaf | 0.9±0.1 | 0.6±0.1 | 2.5±0.4 | 2.0±0.3 | 3.9±0.2 | 3.2±0.2 | 8.8±0.3 | 7.4±0.6 |
| | | Ratio | 0.6 | 0.7 | 0.3 | 0.3 | 0.9 | 0.7 | 0.4 | 0.4 |
| | Combined | Calli | 0.5±0.0 | 0.4±0.0 | 0.8±0.0 | 0.6±0.0 | 4.5±0.0 | 2.9±0.0 | 3.7±0.0 | 3.1±0.0 |
| | | Leaf | 0.9±0.1 | 0.7±0.0 | 2.2±0.2 | 2.0±0.2 | 3.3±0.2 | 3.1±0.2 | 8.3±0.2 | 7.9±0.6 |
| | | Ratio | 0.6 | 0.6 | 0.4 | 0.3 | 1.3 | 0.9 | 0.4 | 0.4 |
| [4/14] | Control | Calli | 0.5±0.0 | 0.4±0.0 | 0.8±0.0 | 0.6±0.0 | 3.1±0.0 | 2.0±0.0 | 3.6±0.0 | 3.0±0.0 |
| | | Leaf | 0.8±0.1 | 0.7±0.1 | 2.6±0.2 | 2.8±0.3 | 2.9±0.1 | 3.0±0.2 | 8.7±0.3 | 8.1±0.5 |
| | | Ratio | 0.7 | 0.6 | 0.3 | 0.2 | 1.1 | 0.7 | 0.4 | 0.4 |
| | Chilling | Calli | 0.5±0.0 | 0.4±0.0 | 0.8±0.0 | 0.6±0.0 | 3.8±0.0 | 2.6±0.0 | 3.6±0.0 | 3.0±0.0 |
| | | Leaf | 0.7±0.0 | 0.7±0.0 | 3.1±0.3 | 2.3±0.1 | 3.4±0.1 | 3.0±0.1 | 8.5±0.5 | 7.6±0.2 |
| | | Ratio | 0.7 | 0.6 | 0.3 | 0.3 | 1.1 | 0.9 | 0.4 | 0.4 |
| | Salinity | Calli | 0.5±0.0 | 0.4±0.0 | 0.8±0.0 | 0.6±0.0 | 3.5±0.0 | 2.3±0.0 | 3.6±0.0 | 3.0±0.0 |
| | | Leaf | 1.1±0.2 | 0.6±0.1 | 3.0±0.2 | 2.6±0.2 | 3.6±0.1 | 3.8±0.2 | 9.4±0.5 | 7.9±0.6 |
| | | Ratio | 0.5 | 0.7 | 0.3 | 0.2 | 1.0 | 0.6 | 0.4 | 0.4 |
| | Combined | Calli | 0.6±0.0 | 0.4±0.0 | 0.8±0.0 | 0.6±0.0 | 4.4±0.0 | 2.7±0.0 | 3.7±0.0 | 3.0±0.0 |
| | | Leaf | 1.1±0.1 | 0.8±0.1 | 3.1±0.2 | 2.4±0.3 | 3.6±0.1 | 4.3±0.3 | 8.1±0.2 | 6.8±0.6 |
| | | Ratio | 0.5 | 0.6 | 0.4 | 0.3 | 1.2 | 0.6 | 0.5 | 0.4 |

[1]Means ± SE (*n*=6) from Section 5.5.3 (Figures 5.5A,B – 5.8A,B)

[2]Means ± SE (*n*=8) from Section 3.5 (Figures 3.5B, 3.6 and 3.9B)

[3]Calli: Leaf

## 5.6 DISCUSSION

This study shows that antioxidant activities and concentrations of LMWAs in suspension cultures were promoted to different degrees depending on the type of stressor and the ecotype. Of the two ecotypes, ACB was the richer source of LMWAs, and was more potent in antioxidant activity. The stationary phase held the highest concentration of LMWAs and displayed most potent antioxidant activities. These results indicate there is considerable commercial potential of using abiotic stressors as elicitors to elevate antioxidant activities and concentrations of LMWAs of *S. oleraceus* cells in suspension cultures.

Here, *S. oleraceus in vitro* cultures showed promising traits that are conducive for commercial application of cell suspension cultures to produce LMWAs: they produced comparable levels of LMWAs with lower variability in a shorter duration compared to plant leaves (Table 5.4). This maybe due to the optimum concentrations of plant growth regulators in the *S. oleraceus* cell cultures favoured the production of LMWAs. However, in other plant species, the commercial application of plant cell cultures to produce LMWAs was constrained by low yield and variability (Kolewe *et al*., 2008; Lee *et al.*, 2010). The poor synthesis and variability of LMWAs in plant cell cultures were due to chromosomal aberrations caused by accelerated the rates of cellular-deprogramming and -reprogramming brought upon by supra-optimal plant growth regulator concentrations in the medium (Whitmer *et al.*, 2003; Morcillo *et al.*, 2006; Ekiert *et al.*, 2009; Dubrovina and Kiselev, 2012; Cheruvathur *et al.*, 2013; Szopa *et al.*, 2013).

All of the tested stressors resulted in higher concentrations and activities of LMWAs extractable from *S. oleraceus* calli in suspension cultures, than in control cultures and did not compromise cell growth (Figures 5.1 – 5.6 and 5.8). This is desirable if these abiotic stressors were to be used commercially to increase antioxidants. Usually, growth and secondary metabolite production are negatively correlated with one another, though it is possible to increase secondary metabolite production without compromising biomass accumulation by optimising elicitation conditions such as: type of elicitor, severity, the stage of application and the duration of application (Zhao *et al.*, 2010; Cai *et al.*, 2012a; Cai *et al.*, 2012b).

The combination of chilling and salinity were not synergistic on LMWA concentrations and activities in cell cultures (Figures 5.2 – 5.6 and 5.8). These results were similar to the effects of this stressor combination on intact plants (Section 3.5). Similarities in stressor-responses between whole plants and calli were reported when calli were initiated from leaf explants (Rus *et al.*, 2001).

The stressors increased the concentrations of chlorogenic acid to a greater or lesser extent, but did not affect chicoric acid (Figures 5.5 and 5.7). Differential chlorogenic acid accumulation across stressors is possible because different stressors induce differential transcription and activity of genes for the cytochrome P450 monooxygenases, as discussed in detail in Chapter 3 (Section 3.6).

The concentration of chlorogenic acid in calli extracts was three-fold higher compared to leaf extracts (Table 5.4). This indicates higher carbon: nitrogen ratio and near optimal plant growth regulator concentrations in the medium may have been conducive for chlorogenic acid synthesis. Similarly higher C:N ratio in *Cecropia obtusifolia in vitro* cultures enhanced chlorogenic acid accumulation by three-fold compared to leaves of trees (Fritz *et al.*, 2006; Nicasio-Torres *et al.*, 2012). Further, aloesin production was three times higher in *Aloe vera* callus cultures, than leaves from plants which was attributed to optimum plant growth regulator concentrations (Matos Acurero, 2008).

The extent to which ascorbate concentration varied between ecotypes after stressor treatments (Figure 5.8) may be explained by their different rates of ascorbate biosynthesis, recycling, oxidation and catabolism. This is because transcription of genes encoding enzymes, which regulate ascorbate biosynthesis, recycling and degradation are variably increased or decreased depending on the stressor and cultivar (Cruz-Rus *et al.*, 2011; Zhang *et al.*, 2011b; Mellidou *et al.*, 2012; Alós *et al.*, 2013; Li *et al.*, 2013). For example, accessions of *Fragaria ananassa* with higher stressor-induced ascorbate content had upregulated transcription of genes for ascorbate biosynthetic and recycling enzymes (Cruz-Rus *et al.*, 2011; Li *et al.*, 2013). In *Solanum esculentum* and *Capsicum annuum*, too, cultivars with higher stressor-induced fruit ascorbate content was attributed to enhanced transcription of ascorbate biosynthetic and recycling enzymes and lower transcription of ascorbate degradation enzymes (Mellidou *et al.*, 2012; Alós *et al.*, 2013).

The calli of two ecotypes differed in ability to increase caftaric acid accumulation in response to stressors (Figure 5.6A,B). This may imply differences in stressor-inducible genes encoding enzymes and transcription factors in phenylpropanoid pathway between ecotypes. Differences in founding genotypes and their geographic isolation between the two *S. oleraceus* populations from which the ecotypes originated may have lead to this (St John-Sweeting, 2011). For example, in certain accessions of *Zea mays*, complete or partial loss of either, one or both anthocyanin and flavonone biosynthetic pathways occurred (Zhang *et al.*, 2011b).

Calli of ACB were richer in LMWAs and had more potent antioxidant activities, than OAM (Figures 5.2A,B – 5.8A,B) possibly due to a strong genetic component controlling *S. oleraceus* antioxidant traits across ecotypes. The direction of the difference between ecotypes was consistent with that for greenhouse grown plants (Section 3.5; Figure 3.4), and with the unpublished results by Ellwood (2007). As discussed in detail in Section 3.6, this may be due to differential expression of PAL multigene family across plant accessions.

ACB is more tolerant to abiotic stressor in *in vitro* cultures than OAM. Of the two ecotypes, ACB had greater stressor-induced LMWAs levels and better cell membrane integrity indicated by proportionally lower LMWAs level in the media (Figures 5.2 – 5.8). Similarly, a lower efflux of cell contents from calli of a cadmium-tolerant *Populus nigra* ecotype, compared to that of a sensitive ecotype, was attributed to higher Cd-induced intracellular LMWAs accumulation in the tolerant ecotype aiding its cell membrane integrity by scavenging excess ROS efficiently (Iori *et al.*, 2012). In *Phoenix dactylifera* and *Triticum aestivum* too, these traits have been associated with the stress tolerant genotypes in *in vitro* cultures (Daayf *et al.*, 2003; Moheb *et al.*, 2011; Iori *et al.*, 2012).

The stationary phase held the highest ascorbate, hydroxycinnamic and total phenolic concentrations compared to any other growth phase (Figures 5.4-5.8), which may due to higher cell numbers at stationary phase compared to other growth stages. The onset of the stationary phase occurs once the cell cultures reach maximum carrying capacity (Naill and Roberts, 2005; Cacho *et al.*, 2010). Further, at stationary phase, the $G_0/G_1$cell ratio exceeds 90% and the $G_0$ cells have higher capacity and rates of secondary metabolism

than $G_1$ cells (Cheng *et al.*, 2006; Zhao *et al.*, 2010). In suspension cultures of other species, too, phenolic compound accumulation is maximal at the stationary phase (Jeong *et al.*, 2009; Cacho *et al.*, 2010; Cai *et al.*, 2012b; Karwasara and Dixit, 2012; Nicasio-Torres *et al.*, 2012; Yin *et al.*, 2012).

These results indicate the significant features of LMWAs production in *S. oleraceus* cell suspension cultures, which were: highly stressor-induced, lower in variability and accumulation within shorter culture duration compared to leaves of whole plants. Additionally, the growth of cell cultures was unchallenged by stressors. These basic traits are highly preferable in developing large scale commercial production and extraction systems of LMWAs from plant cell cultures.

# CHAPTER 6: GENERAL DISCUSSION

## 6.1 CONCLUSIONS

This study confirmed that vegetative shoots of *Sonchus oleraceus* L. are rich in LMWAs as shown by *in vitro* chemical and cellular antioxidant activity (CAA) measures (Chapter 4). The antioxidant activities and concentration of LMWAs of *S. oleraceus* leaves and calli significantly increased with maturation of plants and cell cultures (Chapters 3 and 5; Table 6.1). Furthermore, as shown in Chapters 3 and 5, the ecotype differences in antioxidant activities were largely maintained across progenies and in cell cultures indicating the heritability and genetic stability of antioxidant potential in *S. oleraceus* (Table 6.1).

Chapters 3 and 5 showed that, the degree of stressor-induced antioxidant activities compared to controls, were greater in plant cell cultures than in whole plants (Chapter 5: Table 5.4). Furthermore, of the two ecotypes tested here, the antioxidant activities and LMWAs content of the superior ecotype were enhanced to a greater degree by imposing stressors to plants and cell cultures (Chapters 3 and 5). Chapter 5 also demonstrated that for calli from suspension cultures, the most effective stressor differed between ecotypes; the stressor combination for ACB, and chilling for OAM. As shown in Chapter 3, leaves with highest antioxidant activities and concentrations of LMWAs were obtained from 14 week old plants originating from Acacia Bay (ACB) that had been exposed to two weeks of chilling night temperatures and salinity. Chapter 3 also showed that infusion of HepG2 cells with extracts from leaves with stressor-promoted antioxidant activities protected the cultured human cells from oxidative stress to a greater degree.

Furthermore, as shown in Chapter 4, cooking leaves, diminished the levels of some LMWAs in the bioaccessible fraction obtained through *in vitro* gastrointestinal digestion. Also, antioxidants from uncooked leaves that were digested in *in vitro* gastrointestinal conditions were absorbed into human cells *in vitro* and protected them from oxidative stress (Chapter 4).

**Table 6.1 Simplified summary of results from Chapters 3 – 5 showing factors affecting antioxidant activities in *Sonchus oleraceus* L. leaves and cell cultures**

| Comparison of factors | Sample | ORAC[1] | | DPPH[2] | | Total phenolics[3] | | Caftaric acid[3] | | Chlorogenic acid[3] | | Chicoric acid[3] | | Ascorbate[3] | | CAA[4] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ACB | OAM | ACB | OAM | ACB | OAM | ACB | OAM | ACB | OAM | ACB | OAM | ACB | OAM | ACB |
| Stressor : Control | **Calli** | ↑ | ↑ | ↑ | ↑ | ↑ | ^ | ^ | - | ↑ | ↑ | - | - | ^ | ↑ | |
| | Chilling | ↑ | ↑ | ↑ | ↑ | ↑ | ^ | ^ | - | ↑ | ^ | - | - | ^ | ↑ | |
| | Salinity | ↑ | ↑ | ↑ | ↑ | ↑ | ^ | ↑ | - | ↑ | ↑ | - | - | ↑ | ↑ | |
| | Combined | ↑ | ↑ | ↑ | - | ↑ | ↑ | - | - | ↑ | ↑ | - | - | - | - | α |
| | **Leaf** | ↑ | ↑ | ↑ | - | ↑ | ↑ | - | - | ↑ | ↑ | - | - | - | - | |
| | Chilling | ↑ | ↑ | ↑ | ^ | ↑ | ↑ | - | - | ↑ | ↑ | - | - | - | - | α |
| | Salinity | ↑ | ↑ | ↑ | ^ | ↑ | ↑ | - | - | ↑ | ↑ | - | - | - | - | |
| | Combined | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | |
| ACB: OAM | **Calli** | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | |
| | **Leaf** | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | |
| Mature: Young | **Calli**[5] | ↑ | ↑ | ↑ | ↑ | ^ | ^ | ^ | ^ | ↑ | ↑ | ^ | ^ | ^ | ^ | |
| | **Leaf**[6] | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ | |
| Calli: Leaf | | ↓ | ↓ | ↑ | ↑ | ↓ | ↓ | ↓ | ↓ | ↑ | ↓ | ↓ | ↓ | ↓ | ↓ | |
| Raw: Boiled | **Leaves** | - | | - | | - | | - | | - | | - | | - | | |
| | **Bioacc**[7] | ↑ | | ↓ | | - | | - | | - | | ↑ | | ↑ | | α[8] |

[1.] Oxygen Radical Absorbance Capacity
[2.] 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity
[3.] Concentration (mg $g^{-1}$ DW)
[4.] Cellular Antioxidant Activity (in HepG2 cells)
[5.] Stationary phase: Lag phase
[6.] Flowering stage: Vegetative stage
[7.] Bioaccessible fraction from *in vitro* gastrointestinal digestion
[8.] In HepG2 and Caco2 cells

**Key**

| Symbol | Change/result | Fold change |
|---|---|---|
| ↑ | Large increase | 1.50 – 6.00 |
| ↑ | Moderate increase | 1.10 – 1.49 |
| ^ | Very small increase | 1.00 – 1.09 |
| ↓ | Decrease | <0.99 |
| - | No change | 0.00 |
| α | Significant positive linear correlation | Not applicable |

## 6.2 DISCUSSION

There has been a recent drive to produce plants rich in LMWAs to promote consumer health. Consequently, increasingly more research is being done on the antioxidant properties of food plants (Kaur and Kapoor, 2001; García-Mier *et al.*, 2013). However, to commercialise a food plant as a antioxidant source, systematic research is required, focused on; (1) enhancing the levels of LMWAs in field grown plants; (2) developing commercially applicable *in vitro* culture techniques for efficient and effective LMWAs extraction; (3) the effects of food preparation on LMWAs; and (4) bioaccessibility, bioactivity, bioavailability and stability of LMWAs (Figure 6.1). My study on the antioxidant activities of *S. oleraceus* followed the scheme given in Figure 6.1 following recent recommendations on using plants as health promoting phytochemical sources (Finley, 2005; Rea *et al.*, 2011; Traka and Mithen, 2011; Tounekti and Munné-Bosch, 2012).

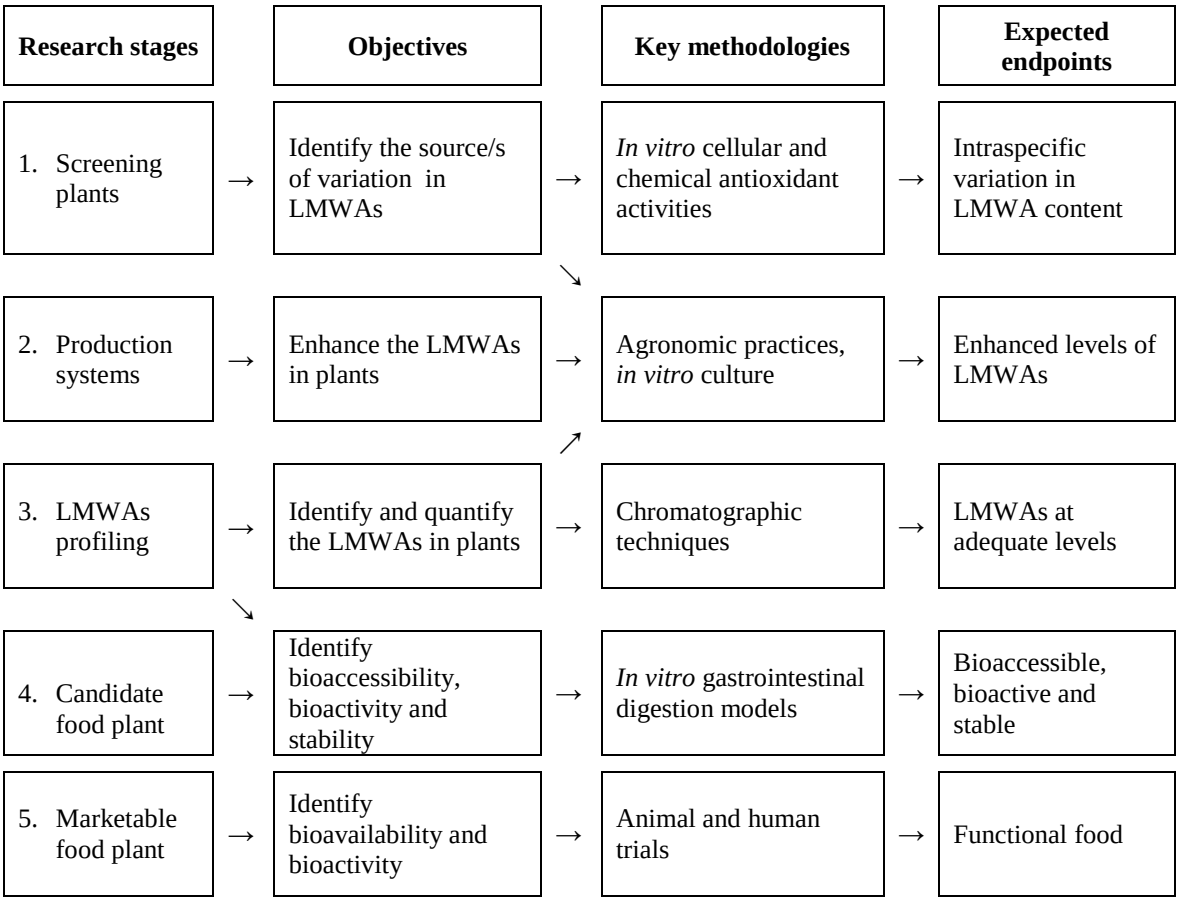



**Figure 6.1 Process for validating a food plant as a valuable source of dietary LMWAs with evidence of health benefits developed based on recommendations of Finley (2005); Rea *et al.* (2011); Traka and Mithen (2011); Tounekti and Munné-Bosch (2012).**

My work, demonstrated that extractable antioxidant activities of *S. oleraceus* leaves were superior to most commonly consumed food (Table 6.2). This was particularly true when leaves were selected from flowering plants of the more potent ecotype (ACB) that were exposed to stressors (Table 6.2). These results indicates the commercial potential of selecting potent ecotypes and practising correct agronomy to further improve extractable antioxidant activities of *S. oleraceus* leaves.

**Table 6.2 *Sonchus oleraceus* L. leaves and common food categorized into six groups ranked by their extractable antioxidant activities**

| **ORAC**[1] | ***S. oleraceus* leaves**[2] | **Fruits**[3] | **Vegetables**[3] | **Dried fruits and nuts**[3] | **Other food**[3] |
|---|---|---|---|---|---|
| **0 – 20** | Vegetative plants of both ecotypes | Watermelon<br>Cantaloupe<br>Nectarine<br>Pineapple<br>Banana<br>Grapes<br>Apricot<br>Avocado<br>Tangerine<br>Oranges<br>Peach<br>Pears | Cucumber<br>Beans<br>Green peas<br>Celery<br>Corn<br>Cauliflower<br>Onion<br>Potato<br>Pepper<br>Carrot<br>Broccoli<br>Lettuce | Brazil nut<br>Macadamia<br>Cashews | Bread<br>Ready-to-eat breakfast cereals |
| **21 – 40** | | Apple<br>Cherry<br>Strawberry | Cabbage<br>Spinach<br>Beet<br>Asparagus | Peanut<br>Dates<br>Raisin<br>Figs | |
| **41 – 60** | Flowering plants of OAM ecotype | Raspberry<br>Blackberry | | Almond | |
| **61 – 80** | Flowering, ACB without a stressor | Blueberry<br>Plum | | Pistachio | Milk chocolate |
| **81 – 90** | Flowering , ACB[4] | | | Prunes | |
| **91 – 96** | Flowering , ACB[5] | Cranberry | Artichoke | Hazelnut | |
| **97 - 100** | Flowering , ACB[6] | | | | |
| **1031** | | | | | Baking chocolate |

[1]Oxygen Radical Absorbance Capacity (µmol TE $g^{-1}$)

[2] Results from Chapter 3

[3]Source: Wu *et al*., 2004

Stressors; [4]chilling, [5]salinity and [6]combined

Here, extraction of LMWAs from *S. oleraceus in vitro* cultures showed advantages over leaves because produced LMWA levels were; (1) higher or comparable, (2) had low variability and (3) accumulated rapidly (Chapter 5; Table 5.4). These significant features displayed by *S. oleraceus in vitro* cultures have been considered essential first steps in commercial projects, such as the European Union project 'Nutra-Snack'. Nutra-Snack aimed to develop large scale pre-industrial production of antioxidants from cells and *in vitro* cultures of *Ocimum basilicum, Mentha piperita, Trifolium heldreichianum, Glycine max, Taraxacum officinale* and *Salvia officinalis*. The ultimate goal of the projects was to manufacture novel ready-to-eat snacks enriched with antioxidants of plant origin (Rea *et al.*, 2011). Further, plant cell cultures are gaining popularity as biofactories of bioactive phytochemicals (Lindsay, 2000; Jacobo-Velázquez *et al.*, 2011; Becerra-Moreno *et al.*, 2012; Jacobo-Velázquez and Cisneros-Zevallos, 2012; Tounekti and Munné-Bosch, 2012). Thus *S. oleraceus* cell cultures have the potential to be developed into commercial bioreactors to biosynthesise antioxidants that maybe developed into botanical antioxidant food products.

The *in vitro* cultures of *S. oleraceus* produced all three major hydroxycinnamic acids to lesser or comparable levels that were present in leaves harvested from plants (Chapter 5; Section 5.5.3; Table 5.4). This is of particular importance since some other plant species altogether lacked the capability to accumulate these hydroxycinnamic acids when they were grown as *in vitro* cell cultures than as whole plants (Table 6.3). Organ cultures too, can produce secondary metabolites at levels that are similar to those synthesized in whole plants. However, organ cultures unlike cell cultures are expensive and complex to maintain in bioreactors at large scale (Verpoorte *et al*., 2002). Therefore *S. oleraceus* cell suspension cultures have economic feasibility of scaling-up to produce hydroxycinnamic acids.

Further, the abiotic stressors that were used here have the potential to be used as elicitors particularly the combined stressor in commercial *in vitro* culture systems (Table 6.4). Fold increase in hydroxycinnamic acid levels due to elicitation of *in vitro* cultures of *S. oleraceus* was comparable with values obtained for other plant species that were in the experimental stages of developing into commercialised systems (Table 6.4).

**Table 6.3 Comparison of concentrations of hydroxycinnamic acids extracted from calli of suspension cultures and leaves of greenhouse grown plants of *Sonchus oleraceus* L. and other selected plant species**

| Species | | Hydroxycinnamic acid concentration (mg $g^{-1}$ DW) | | | References |
|---|---|---|---|---|---|
| | | Caftaric | Chlorogenic | Chicoric | |
| *Sonchus oleraceus* | Calli[1] | 0.8 | 3.5 | 3.6 | (Chapter 5; Section 5.5.3) |
| ACB ecotype | Leaf[2] | 2.4 | 3.4 | 7.8 | (Chapter 3; Section 3.5.2) |
| | **Ratio[3]** | **0.3** | **1.0** | **0.5** | |
| OAM ecotype | Calli | 0.6 | 2.3 | 3.0 | (Chapter 5; Section 5.5.3) |
| | Leaf | 1.9 | 2.9 | 6.9 | (Chapter 3; Section 3.5.2) |
| | **Ratio** | **0.3** | **0.8** | **0.4** | |
| *Lactuca virosa* | Calli | 0.1 | 0.1 | 0.6 | (Stojakowska *et al*., 2012) |
| | Leaf | 2.0 | 3.9 | 1.5 | |
| | **Ratio** | **0.1** | **0.0** | **0.4** | |
| *Eucommia ulmoides* | Calli | - | 2.2 | - | (Wang *et al.*, 2003) |
| | Leaf | - | 2.6 | - | |
| | **Ratio** | - | **0.8** | - | |
| *Lavandula viridis* | Calli | - | 1.8 | - | (Costa *et al.*, 2013). |
| | Leaf | - | 2.3 | - | |
| | **Ratio** | - | **0.8** | - | |
| *Echinacea angustifolia* | Calli | - | - | - | (Lucchesini *et al.*, 2009) |
| | Leaf | 4.3 | 1.2 | 1.5 | |
| | **Ratio** | - | - | - | |

[1] Means ($n$=72) pooled for ages and stressors
[2] Means ($n$=96) pooled for ages and stressors
[3] Calli: Leaf
- Compound is not detected

**Table 6.4 Effects of abiotic elicitors on concentrations of hydroxycinnamic acids extracted from calli of suspension cultures of *Sonchus oleraceus* L. and selected plant species**

| Species | Abiotic elicitors | Hydroxycinnamic acid concentration (mg $g^{-1}$ DW) | | | References |
|---|---|---|---|---|---|
| | | Caftaric | Chlorogenic | Chicoric | |
| *Sonchus oleraceus*[1] | Combined | 0.8 | 4.5 | 3.7 | (Chapter 5; Figure |
| | Control | 0.8 | 3.1 | 3.6 | 5.3A,C,E) |
| | **Ratio**[2] | **1.1** | **1.5** | **1.0** | |
| *Echinacea purpurea* | 20 $^{o}$C | 4.7 | 5.2 | 28.4 | (Wu *et al.*, 2007) |
| | 10 $^{o}$C | 4.4 | 4.9 | 29.1 | |
| | **Ratio** | **1.1** | **1.1** | **1.0** | |
| | Light | 6.1 | 2.3 | 27 | (Abbasi *et al.*, 2007) |
| | Dark | 4.1 | 1.0 | 20 | |
| | **Ratio** | **1.5** | **2.3** | **1.4** | |
| | Ultrasound | 10.2 | 1.4 | 23.4 | (Liu *et al*., 2012) |
| | Control | 7.7 | 1.0 | 16.6 | |
| | **Ratio** | **1.3** | **1.4** | **1.4** | |

[1] Means (*n*=6) calli at stationary phase

[2] Abiotic elicitor: Control

Combined stressor enhanced the extractable antioxidant activities of calli of suspension cultures to a greater degree whereas in leaves of greenhouse grown plants they were not improved, compared to their respective controls (Table 6.1). This indicates the possible differences between the responses at whole plant and cellular level to abiotic stressors. Cells in *in vitro* cultures may rely on elevated antioxidant activities to mitigate combined effects of chilling and salinity, more than whole plants. This is because once chilling injures the cell membranes; the increased cell membrane permeability will enhance the movement of inorganic ions into cells (Liu *et al*., 2013). The degree of ion movement into undifferentiated cells in suspension cultures would be more compared to whole plants in which roots exclude the salts. For example, in *Sesuvium portulacastrum* salinity-elevated antioxidant activities were higher in undifferentiated cells than in whole plants (Lokhande *et al*., 2010).

The discovery of heritable and genetically stable ecotype difference in LMWA activities may be significant for growers and breeders (Chapter 3 and 4). Growers may use selfed

seeds from superior accessions to produce LMWA-rich leaves across cropping cycles. Crop breeding and improvement techniques can be used to further augment their levels in leaves once their genetic basis for higher LMWAs content is understood. Presently, crop breeding programmes focus on identifying heritable differences in LMWA properties in their breeding stock and progenies, and on prioritizing genotypes with improved antioxidant properties beneficial to consumer health (Connor *et al.*, 2002; Stushnoff *et al.*, 2008; Cantín *et al.*, 2009; Yousef *et al.*, 2013). An example is *Solanum esculentum* for which hybrids with four-fold higher fruit lycopene content have been developed by the selective elimination of low lycopene-genes and by backcrossing which incorporated high lycopene-genes into progenies (Anon, 1998). In addition, the chlorogenic acid content in the fruit was doubled in *Solanum esculentum* through genetic engineering in which overexpression of hydroxycinnamoyl-CoA quinate: hydroxycinnamoyl transferase (HQT) enzyme was achieved (Niggeweg *et al.*, 2004).

My work has shown that stressor-enhanced LMWAs in *S. oleraceus* leaves provide improved antioxidant protection to human cells in cultures (Chapter 3; Figures 3.16-3.17). This finding is a significant first step because it demonstrates that agronomic efforts to increase LMWAs would likely translate into an improvement in the antioxidant status of live human cells if treated with LMWAs enriched plant extracts. Much research has focused on imposing stress on plants to improve *in vitro* antioxidant activities and to test the cellular antioxidant activities of plant foods, but few attempts have been made to correlate the two (Prior *et al.*, 1998; Wolfe *et al.*, 2008; Song *et al.*, 2010; Tsormpatsidis *et al.*, 2010; McDowell *et al.*, 2011; Zhang *et al.*, 2011a; Avena-Bustillos *et al.*, 2012; Bornsek *et al.*, 2012; Jacobo-Velázquez and Cisneros-Zevallos, 2012).

Here I identified how pre- and post-harvest factors: plant age, ecotype, abiotic stressors and cooking, contribute to variation in LMWAs and antioxidant activities of leaves (Chapter 3; Chapter 4; Table 6.1). This knowledge is the basis for developing good cropping and processing practices to obtain leaves enriched with LMWAs. These findings are particularly important, because modern day humans greatly prefer minimally processed plant food to fulfil their nutritional and health needs than the formulated merchandise such as capsules or tablets (Lindsay, 2000; Williams *et al.*, 2004; Herath *et al.*, 2008; Jacobo-Velázquez *et al.*, 2011; Becerra-Moreno *et al.*, 2012; Jacobo-Velázquez and Cisneros-Zevallos, 2012; Tounekti and Munné-Bosch, 2012).

Contemporary definition of food security by the Food and Agriculture Organization (FAO), recognises the importance of consuming food that would promote human health (Nestel *et al.*, 2006; FAO, 2008). However, even in European countries and USA, the average fruit and vegetable intake is below the World Health Organisation (WHO) recommendation (of ≥400 g $d^{-1}$ $person^{-1}$) for the prevention of diet-related chronic diseases (Anon, 2003; Ashfield-Watt *et al.*, 2004). To counteract the problem of low fruit and vegetable per capita intake, the Nutritional Enhancement of Plant Foods in European Trade (NEODIET) programme advocates a shift in public health strategy towards promoting the consumption of plants rich in LMWAs (Lindsay, 2000). In these contexts, there is a demand for plant produce that are highly concentrated in LMWAs. *S. oleraceus* can fulfill these expectations by following the basic guidelines provided in this study.

## 6.3 FUTURE DIRECTIONS

Elevated concentrations of LMWAs in *S. oleraceus* leaves do not automatically guarantee higher bioactivity in live organisms since the these compounds need to be stable through the interaction with plasma proteins and metabolism in the liver. Thus, bioactivity studies require animal and human trials (Schreiner *et al.*, 2012). The antioxidant capacity of plant food as measured by *in vitro* chemical assays and by cellular antioxidant activities following *in vitro* gastrointestinal simulation, may still differ from *in vivo* antioxidant activities, because of: (1) phenolic–microbiota interactions in the sigmoid colon; (2) phenolic-plasma protein interactions in the blood; and (3) hepatic metabolism (Rohn *et al.*, 2004; Silberberg *et al.*, 2006; Selma *et al.*, 2009). To understand the *in vivo* fate of phenolic compounds in *S. oleraceus* leaves would require animal and human intervention studies, because: (1) the colonic microbial diversity vary between individuals and continuously modified depending on the ingested phenolic compounds; (2) the diffusion of phenolic compounds from blood to target tissues depends on the degree of binding between phenolic compound and the plasma protein; and (3) hepatic metabolism of phenolic compounds depends on the location of the metabolizing enzymes in a liver's acini (Ballinger *et al.*, 1995; Requena *et al.*, 2010; Duarte and Farah, 2011; Xiao and Kai, 2011). For those reasons, bioactivity studies require intact live animal organs thus necessitating animal models prior to human clinical trials.

Studies of the *in vivo* transformation of phenolic compounds in *S. oleraceus* can done using leaves with $^{14}$C-labelled phenolic compounds obtained from plants grown in $^{14}CO_2$ supplied growth chambers. The leaves with radiolabeled phenolic compounds can be either administered orally or their leaf extracts can be perfused *in situ* to the colon and/or liver of rats. Time course monitoring of the quantitative and qualitative profiles of the radiolabeled compounds in target tissues (intestines, blood and liver) would provide information on *in vivo* transformation of phenolic compounds. The bioactivity can be quantified by measuring time course changes in cellular antioxidant activities in tissues and by antioxidant activities of blood and tissue lysates from sacrificed animals.

In conclusion, this study focused on the leaves of *Sonchus oleraceus* that are exceptionally rich in extractable antioxidants, since they have the potential to protect humans cells from oxidative stress. I identified the factors that caused significant variation in antioxidant properties of *S. oleraceus,* namely: maturation (of cell cultures and plants), genetic heredity, abiotic stressors, propagation method (whole plants versus cells), and cooking. Furthermore, it was observed that cultured human cells infused with leaf extracts were protected from oxidative stress even after the leaves had been digested in simulated gastrointestinal conditions. Importantly, human cells were more protected from oxidative damage if the leaves had stressor-augmented extractable antioxidant activities. Collectively, the data provide a compelling argument to explore the commercial potential of growing *S. oleraceus* as a functional food crop.

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# APPENDIX A

## A.1 Optimum concentration of NaCl that enhances the DPPH radical scavenging activities of leaf extracts

A pilot study was conducted (October – January 2009) to determine, which salinity levels are most likely to enhance the DPPH radical scavenging activities of plants. ACB and OAM were exposed to two salinity levels (50 and 200 mM NaCl) using eight week old plants. Leaf phytochemical analysis for DPPH radical scavenging was performed according to 3.3.1 and 3.3.3. Results (Figure A.1) indicated antioxidant activities were highest with 50 mM NaCl at 14 weeks in both ecotypes.

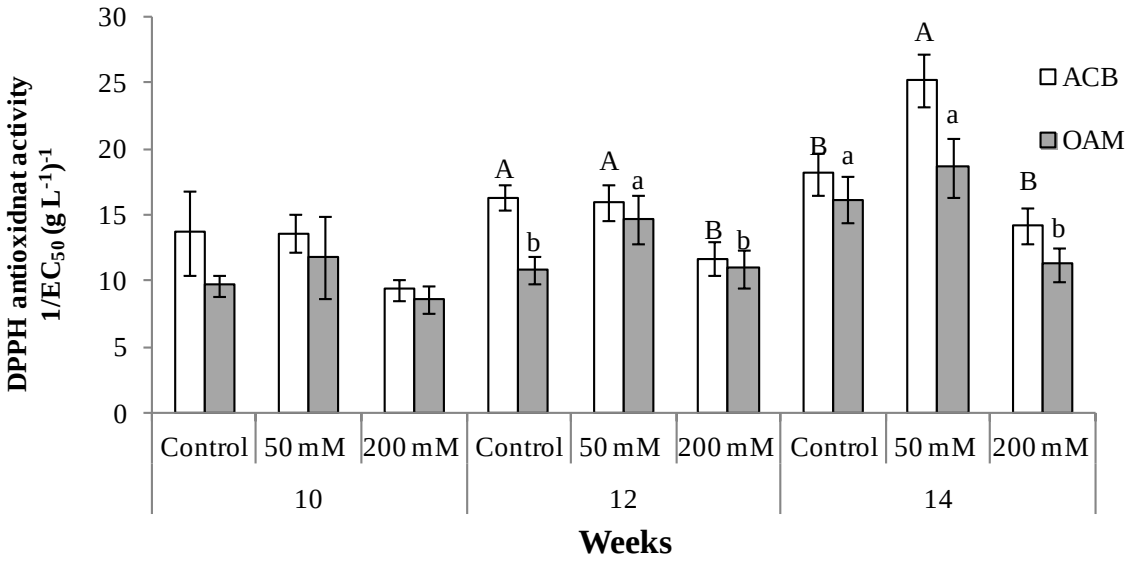


**Figure A.1 Treatment effects on DPPH scavenging in methanolic extracts of leaves from 10, 12 and 14 week old plants of ACB and OAM ecotypes of *Sonchus oleraceus* L. Means ± SE (*n* = 9). Bars with different letters of the same case indicate significant differences across treatments within an ecotype, and bars without letters indicate no significant effects of stressors within an ecotype (*P* < 0.05).**

## A.2 Stressors that enhances the DPPH radical scavenging activities of leaf extracts

A pilot study was conducted (April – July 2010) to determine, which stressors are most likely to enhance the extractable antioxidant activities of plants. ACB and OAM were exposed to chilling (5 $^{0}$C for 12 h overnight per week), salinity (50 mM NaCl), drought (withholding irrigation until wilting, followed by re-watering) and chilling in low light during daytime (5 $^{0}$C for 12 h at 96 µmol $m^{-2}$ $s^{-1}$ PAR) using eight week old plants. Leaf phytochemical analysis was performed according to Sections 2.2 – 2.4 and 3.3.1. Results (Figure A.2) indicated antioxidant activities and levels of total phenolics nearly doubled in week 14 compared to week 10 regardless of treatment or ecotype. Chilling doubled DPPH radical scavenging activities in week 14 compared to the control. Salinity doubled ORAC activities in week 14 compared to the control. Antioxidant activities and total phenolics content were reduced by 70% by chilling in low light during daytime compared to the control in week 14

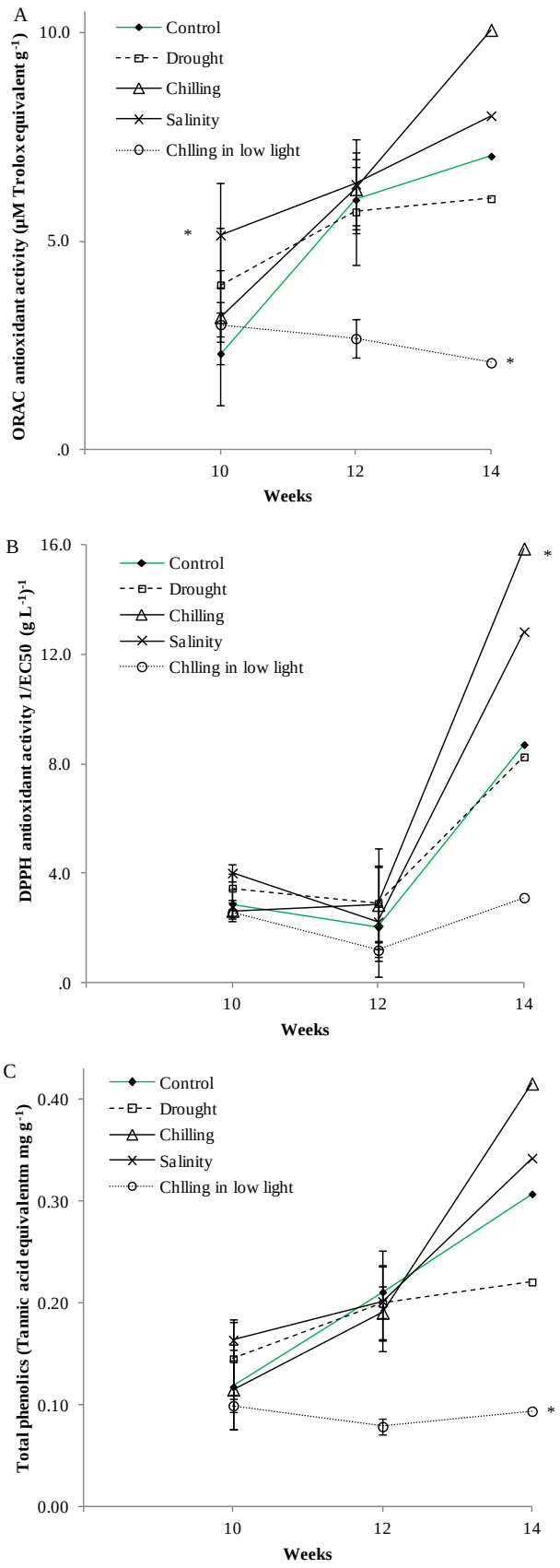


**Figure A.2 Effects of age and stressors on (A) ORAC, (B) DPPH scavenging, (C) total phenolic content, in methanolic extracts of *Sonchus oleraceus* L. leaves. Means ± SE (*n* = *5*). * Significant difference compared to control (*P* < 0.05).**

# APPENDIX B

## B.1 Effects of age, ecotype and stressor on antioxidant activity, total phenolic content, hydroxycinnamic acid content

**Table B.1 ANOVA results (F values) for effects of age, ecotype and stressor on antioxidant activity, total phenolic content, hydroxycinnamic acid content of *Sonchus oleraceus* L. leaf methanolic extracts[a]**

| Sources of variation | *df*[1] | F values | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Antioxidant activity | | | Hydroxycinnamic acids[2] | | | |
| | | ORAC technique[3] | DPPH technique[4] | Total phenolic content[5] | Caftaric acid | Chlorogenic acid | Chicoric acid | Total |
| Age (A) | 2 | 74* | 128* | 80* | 47* | 31* | 55* | 56* |
| Ecotype (E) | 1 | 22* | $4^{ns}$ | 30* | 17* | 19* | 15* | 23* |
| Stressor (S) | 3 | $0^{ns}$ | 3* | 4* | $0^{ns}$ | 18* | $1^{ns}$ | $1^{ns}$ |
| A*E | 2 | 4* | 9* | 4* | $0^{ns}$ | 12* | $0^{ns}$ | $0^{ns}$ |
| A*S | 6 | 6* | 4* | 5* | $0^{ns}$ | 8* | $1^{ns}$ | $1^{ns}$ |
| E*S | 3 | $2^{ns}$ | $2^{ns}$ | $1^{ns}$ | $0^{ns}$ | 3* | $1^{ns}$ | $1^{ns}$ |
| A*E*S | 6 | $1^{ns}$ | $0^{ns}$ | $1^{ns}$ | $1^{ns}$ | 2* | $0^{ns}$ | $0^{ns}$ |
| Error | 168 | $559^{b}$ | $20^{b}$ | $0.1^{b}$ | $1^{b}$ | $1^{b}$ | $3^{b}$ | $7^{b}$ |

[a] The factors were age (three levels), ecotype (two) and stressor (four) with eight plants for each combination of factors.

[b] Mean error squared

[1]Degrees of freedom

[2]Concentration (mg $g^{-1}$)

[3]Oxygen radical absorbance capacity expressed as µmol Trolox equivalent $g^{-1}$

[4]2,2-diphenyl-1-picrylhydrazyl radical scavenging activity expressed as $1/EC_{50}$ $(g\ L^{-1})^{-1}$

[5]mg tannic acid equivalent $g^{-1}$

* $P < 0.05$; $^{ns}$ $P > 0.05$

## B.2 Effects of age, ecotype and stressor on ascorbate concentration

**Table B.2 ANOVA results (F values) for effects of age, ecotype and stressor on ascorbate concentration of *Sonchus oleraceus* L. leaf methanolic extracts[a]**

| Sources of variation | *df*[1] | F values[2] |
|---|---|---|
| Age (A) | 2 | 126* |
| Ecotype (E) | 1 | 19* |
| Stressor (S) | 3 | 1 [ns] |
| A*E | 2 | 8* |
| A*S | 6 | 7* |
| E*S | 3 | 2 [ns] |
| A*E*S | 6 | 1[ns] |
| Error | 168 | 0.05[b] |

[a] The factors were age (three levels), ecotype (two) and stressor (four) with eight plants for each combination of factors.

[b] Mean error squared

[1]Degrees of freedom

[2]Ascorbate concentration (mg $g^{-1}$)

* $P < 0.05$; [ns] $P > 0.05$

# APPENDIX C

## C.1 Optimum concentrations of NAA and BAP for callus initiation and growth

Callus induction and dry biomass were measured using various concentrations of NAA (1, 2, 4) and BAP (0, 1, 2). Results (Figure B.1) indicated the best hormone combination as NAA 1.0 mg $L^{-1}$(5.4 mM) and BAP 1.0 mg $L^{-1}$(4.4 mM).

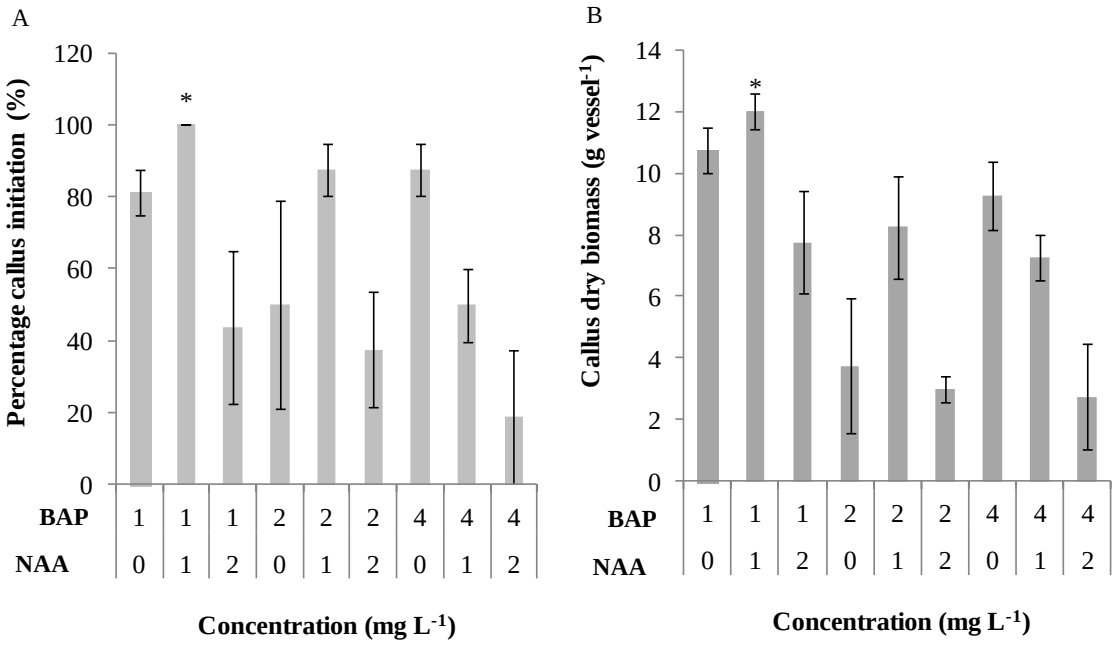


**Figure C.1 Different hormone concentrations on callus initiation and callus dry biomass of *Sonchus oleraceus* L. after 21 days of culture. Means ± SE (*n* = 8). *Selected hormone concentration.**

## C.2 Optimum concentrations of NAA and BAP for growth of suspension cultures

The dry biomass of calli was measured in suspension cultures maintained on variable concentrations of NAA (0.5, 1.0, 2.0) and BAP (0.5, 1.0). Results indicated best hormone combination as NAA 2 mg $L^{-1}$(10.7 mM) and BAP 1 mg $L^{-1}$(4.4mM) based on biomass accumulation (Figure B.2).

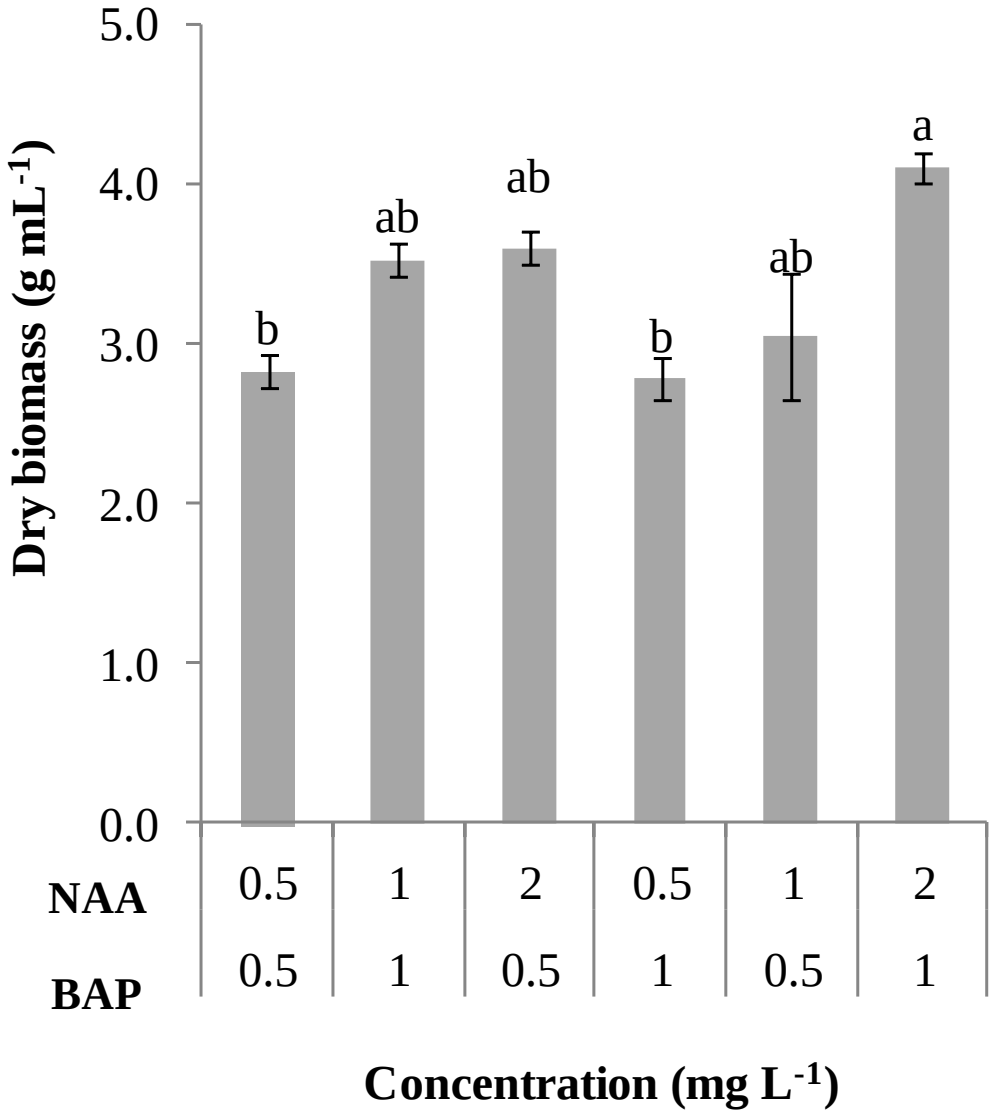


**Figure C.2 Different hormone concentrations on dry biomass of *Sonchus oleraceus* L. suspension cultures after 21 days of culture. Means ± SE (*n* = 10). Bars with different letters indicate significant differences of treatments (*P*< 0.05).**